Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

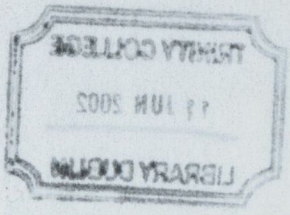
By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

On the role of Caspase-Recruitment Domain proteins in Apoptosis and NFκB activation

By

Lisa Bouchier-Hayes



A thesis submitted to Trinity College Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy

Submitted December 2001

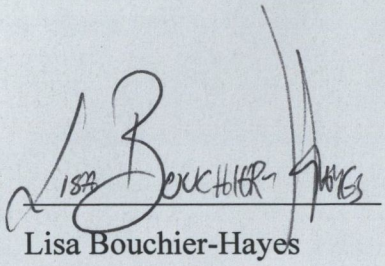
Research conducted in the Molecular Cell Biology Laboratory,
Department of Genetics, Trinity College Dublin, Dublin 2

Thesis Supervisor: Prof. Seamus J. Martin

DECLARATION

I certify that this thesis, submitted to Trinity College Dublin for the degree of Doctor of Philosophy, has not been submitted as an exercise for a degree at this or any other university. I certify that the work presented here is entirely my own, except where otherwise acknowledged.

I give permission for the Library to lend or copy this thesis upon request.



Lisa Bouchier-Hayes

December 2001

For Dad

ACKNOWLEDGEMENTS

I would sincerely like to thank the members of the lab who were involved in the CARDINAL project, Dr Helen Egan, Helen Conroy, Dr Emma Creagh and Dr Colin Adrain, for their help, their input and for allowing me to include some of their data in this thesis.

I wish to express my gratitude to my supervisor Prof. Seamus Martin for his dedicated supervision from the day I entered the lab. I have learnt so much more than I thought possible under your supervision and it will stay with me always, thank you.

Also to everyone else in the lab, those who have come and gone over the years, and those who are still there a big thank you, especially to Brona, Helen and Emma – I couldn't pick a better trio to share a lab with. To the girls, and the rest, Colin, Sean, Deirdre and Rebecca, you've all been so helpful and supportive over the last few weeks, I will be forever grateful. I would also like to say a special thank you to Helen Egan, for your friendship, for teaching me so much, and for making my first few months in the lab seem not so daunting.

To my parents, for their unconditional love and support throughout my life. I am especially indebted to my father, for inspiring my interest in this field and for his help and advice throughout this PhD, and in the writing of this thesis.

To John, I don't think this thesis could have been completed without your help. Thanks for helping me print it, for proof reading it, for keeping me sane and for being so supportive of everything over the last three years.

I would also like to thank my friends outside the lab, especially Emma Jane and Aoife, who I lived with for two years, giving me fond memories of the time I spent doing my PhD. Aoife thanks also for letting me pick your brain about all things of a bioinformatics nature, your advice was always very helpful and much appreciated. Thanks too to Shani, Emily and the rest of the gang for always being there.

I am also very grateful for the technical help I have received. To Prof. LukeO'Neill and the members of his lab in the Biochemistry Dept., for allowing me to monopolise their luminometer. To Orla and Prof. Voorheis, of the same department, for allowing me use of their confocal microscope. To Prof. Humpfries for use of their fluorescent microscope, especially to Denis for his recent help.

Finally I am grateful to the Wellcome Trust for their support both financial and otherwise. This work was funded by a Wellcome Trust prize studentship.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

TABLES

CHAPTER I

CHAPTER II

CHAPTER III

APPENDIX

Table I Origin of plasmids used for work undertaken in the thesis

Table II Primers used to generate some of the plasmids used for work undertaken in the thesis

Table III Primers used for automated sequencing applications

SUMMARY

Apoptosis and NFκB activation are two physiological processes that are essential for host mediated response to cellular injury. Apoptosis is a mechanism that removes damaged or unwanted cells. NFκB is a transcription factor that controls the expression of a number of cytokines and other mediators of inflammation. Recent studies have revealed that the molecular pathways that result in apoptosis or NFκB activation share a number of common elements. Pathways leading to apoptosis converge on the caspase cascade while pathways leading to NFκB activation converge on the IKK complex. Moreover, many of the steps in these pathways are controlled by a series of specific protein-protein interactions. A number of conserved protein modules such as the CARD and DED motifs mediate these interactions. To further characterise the role of CARD-containing proteins in apoptosis and NFκB activation this thesis focuses on the molecular characterisation of two CARD proteins CARDINAL and Nod1.

CHAPTER III

Caspase-10 is a long prodomain caspase, which contains two death effector domains. The role of this caspase in cell death pathways is unclear and a specific binding partner for caspase-10 is yet to be identified. We employed the yeast-two-hybrid library screen to obtain caspase-10 binding partners. Unfortunately the screen performed failed to find a strong caspase-10 interactor.

CHAPTER IV

This chapter describes the cloning and characterisation of CARDINAL (CARD inhibitor of NFκB activating ligands), a novel CARD containing protein. In contrast to the majority of CARD proteins described to date, CARDINAL failed to promote apoptosis or activate NFκB. Rather, CARDINAL potently suppressed NFκB activation associated with overexpression of DR4, DR5, RIP, RICK, Bcl-10 and TRADD, or through ligand-induce stimulation of the interleukin-1 or tumour necrosis factor receptors. Co-immunoprecipitation experiments revealed that CARDINAL interacts with the regulatory subunit of the IκB kinase complex IKKγ (NEMO), providing a molecular basis for CARDINAL function. Thus CARDINAL is a novel regulator of NFκB activation in the context of pro-inflammatory signals.

CHAPTER V

Nod1 is a member of the NBD-CARD subfamily of CARD proteins. Recent reports have proposed that this protein functions as an intracellular receptor for lipopolysaccharide (LPS). Using a subcellular localisation analysis approach we demonstrated that LPS treatment is associated with relocalisation of the NFκB p65 subunit from the cytoplasm to the nucleus, in cells that are transfected with Nod1. Reporter based assays confirmed that Nod1 sensitises cells to LPS-induced NFκB activation. Finally, CARDINAL suppressed Nod1 dependent LPS-induced NFκB activation.

ABBREVIATIONS

AD	Gal4 Activation Domain
Apaf-1	apoptotic protease activating factor 1
3-AT	3-Amino Triazole
Bcl	B-cell lymphoma
BCR	B-cell receptor
BH	Bcl-2 homology
BLAST	Basic local alignment search tool
BSA	Bovine Serum Albumin
CARD	Caspase recruitment domain
CARDINAL	CARD inhibitor of NFκB activating ligands
CASH	Caspase homologue
Caspase	Cysteine aspartate-specific protease
CC	Coiled coil domain
CED	Cell death defective
CFU	Colony forming unit
CIP	Calf Intestinal Phosphatase
CLN	Ceroid Lipofuscinosis
CrmA	Cytokine response modifier protein
DEFCAP	Death Effector Filament-forming Ced-4-like apoptosis protein
DB	Gal4 DNA binding domain
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
DEDAF	Death effector domain associated factor
DISC	Death-inducing signalling complex
DMSO	Dimethylsulphoxide
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNA-PK_{CS}	DNA-dependent protein kinase (catalytic subunit)
DR	Death receptor
DRADD	death-inducing Rb-associated death domain protein
DTT	Dithiotriethol
EDTA	Ethylendiaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
FADD	Fas associated protein with death domain
FasL	Fas Ligand
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
FL	Full length
FLASH	FLICE associated huge protein
FLICE	FADD-like ICE/CED-3 related protein
FLIP	FLICE-like inhibitory protein
GFP	Green Fluorescent Protein
Gld	generalised lymphoproliferative disease
GST	Glutathione Sepharose Transferase

HA	Haemagglutin
HBS	Hepes Buffered Saline
HEK	Human embryonic kidney
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein
ICE	Interleukin-1β Converting Enzyme
ICE-LAP	ICE-like apoptotic protease
Ig	Immunoglobulin
I-FLICE	Inhibitor of FLICE
IκB	Inhibitor of κB
IKBKAP	IκB Kinase Associated Protein
IKK	IκB Kinase
IL-1	Interleukin-1
IL-1R	Interleukin-1 receptor
IP	Immunoprecipitation
IPAF	ICE protease activating factor
IPTG	isopropylthio-β-D-galactoside
IRAK	IL-1R associated kinase
ISBP	Ich1S binding protein
ITT	*In vitro* Transcription and Translation
JNK	Jun N terminal Kinase
kDa	kilodaltons
LB	Luria Broth
LBP	LPS Binding Protein
LiAc	Lithium Acetate
Lpr	lymphoproliferation
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
MAGUK	Membrane Associated Guanylate Kinase
MALT	Mucosa associated lymphoid tissue
Mch	Mammalian ced-3 homologue
MCS	Multiple Cloning Site
MDM2	murine double minute-2
MyD88	Myeloid differentiation factor 88
NAC	NB domain and CARD protein
NBD	Nucleotide Binding Domain
NEMO	NFκB essential modulator
NFκB	Nuclear factor κB
NLS	Nuclear localisation signal
OD	Optical Density
ONPG	o-nitrophenyl β-D-galactopyranoside
PACAP	Pro-apoptotic caspase adaptor protein
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase Chain Reaction

PEG	Polyethylene Glycol
PIPES	Piperazine-N-N'-bis[2-ethanesulphonic acid]
PMSF	phenylmethylsulphonylfluoride
PTP	Permeability transition pore
PYD	Pyrin Domain
RAIDD	RIP-associated Ich-1/CED homologous protein with a DD
RICK	RIP-like interacting CLARP kinase
Rb	Retinoblastoma protein
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SD	Synthetic dropout medium
SDS	Sodium dodecyl sulphate
SMAC	Second mitochondrial activator of caspases
TAE	Tris acetate EDTA
TBST	Tris buffered saline with tween
TCR	T Cell Receptor
TE	Tris EDTA
TIR	Toll/IL-1R homology
TLR	Toll-like receptor
TMB	Tetramethyl benzidine
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
TRAP1	Tumour Necrosis Factor Receptor Associated Protein 1
TRADD	Tumour Necrosis Factor Receptor Associated protein with a DD
TRAIL	Tumour Necrosis Factor Related Apoptosis Inducing Ligand
TRAIL-R	Tumour Necrosis Factor Related Apoptosis Inducing Ligand Receptor
UBC9	ubiquitin conjugating enzyme
UV	ultraviolet irradiation
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
XIAP	X-linked inhibitor of apoptosis protein
YPD	Yeast Extract/Peptone/Dextrose
zVAD.fmk	benzyloxycarbonyl-Val-Ala-Asp-(Ome) fluoromethylketone

PUBLICATIONS

Bouchier-Hayes,L., Conroy,H., Egan,H., Adrain,C., Creagh,E.M., MacFarlane,M. and Martin,S.J. (2001) CARDINAL, a novel caspase recruitment domain protein, is an inhibitor of multiple NF-κB activation pathways. *J. Biol. Chem.* 47, 44069-44077.

Bouchier-Hayes,L. and Martin,S.J. (2002) CARD games in apoptosis and immunity *EMBO Reports* (in press).

Bouchier-Hayes,L. and Martin,S.J. (2002) Nod1/CARD4-dependent shuttling of NFκB p65 to the nucleus in response to bacterial lipopolysaccharides. Submitted to *Cell Death and Differentiation*

Keogh,S.A., Walczak,H., Bouchier-Hayes,L. and Martin,S.J. (2000) Failure of Bcl-2 to block cytochrome c redistribution during TRAIL-induced apoptosis. *FEBS Lett* 471:93-98.

CHAPTER I

Introduction

1.1 INTRODUCTION

On a daily basis multicellular organisms become exposed to noxious stimuli and infectious agents that provoke cellular injury or stress. Viral or bacterial infection, DNA damage associated with irradiation or chemotherapeutic drug treatment and competition for growth factors are a few examples of situations that may cause cellular stress. How a cell responds to such stimuli represents a decision-making process inherent to the makeup of all cell types. In essence, the choices available to the cell are to repair the damage, to die or to remove the threat by initiating an immune response. The outcome for the cell is thus a choice between death and survival and although these two endpoints seem to be diametrically opposed, the events leading to them are linked on a number of levels.

1.1.1 Apoptosis

How a cell chooses which of these two paths to take depends on a number of deciding factors. From the cell's perspective, if it becomes injured the most beneficial outcome is survival. However, in multicellular organisms, the presence of an injured cell can pose a significant threat to the host. For example, if a virally infected cell were to continue to divide this would serve to propagate the virus further throughout the host. Similarly in cancer, the persistance of transformed cells has deadly consequences for the organism. However, multicellular organisms have evolved a mechanism that allows the removal of defective, infected, transformed or senescent cells in a process known as programmed cell death (PCD) or apoptosis.

Apoptosis is not only restricted to the removal of damaged cells but is also fundamental to the normal development of higher organisms. For example, in the developing foetus the hands and feet are initially webbed but are then sculpted into mature digits as the interdigital cells are removed by apoptosis (Jacobson *et al.*, 1997). Similarly, in the developing brain excess neurons undergo apoptosis as the synaptic pathways are sculpted and laid out (Barde, 1989). Thus most cells appear to trigger a default death pathway which initiates apoptosis when the life cycle of the cell comes to an end.

Consequently, in the adult, apoptosis has a general role in ensuring that the number of cells in the body remains constant by balancing the rate of cell death with cell

division (Jacobson *et al.*, 1997). If this balance is disturbed the consequences are serious. An excess of cell death can be seen in degenerative diseases such as Alzheimer's disease, where too many nerve cells in the brain die, with devastating consequences for the organ's host (Yuan and Yankner, 2000). In contrast, when cells fail to die on cue, this can lead to cancer, as the cells become immortalised (Martin and Green, 1995).

The morphological changes seen when cells undergo apoptosis nicely demonstrate how programmed cell death is a damage limiting process containing toxic elements remain within the cell (Figure 1.1; Kerr *et al.*, 1972). First the cells shrink and pull away from their neighbouring cells. As the cells shrink, numerous blebs develop on the membrane surface. The chromatin also compacts and is degraded into smaller fragments. The cell is ultimately broken into many membrane enclosed apoptotic bodies, which are easily engulfed by resident macrophages. It is these morphological changes which demonstrate how unwanted cells can be removed without rupture of the cell membrane, hence they pose no threat to surrounding cells and tissues.

1.1.2 Cell Death: A social decision beneficial or detrimental to the host

A number of factors contribute to the decision making process required to make the choice between cell death and cell survival. The cell type or tissue involved is important. Certain areas such as the lining of the gut or the lung are exposed to pathogens in the form of food and airborne particles on a daily basis. Large-scale inflammation at such sites would be detrimental to the function of the organ and therefore is down-regulated (Pugin *et al.*, 1993). In the lining of the gut, for example, where there is a rapid cell turn over, it is much more beneficial for an injured cell to remove itself rather than have the organ continuously attacked by the immune system. Disturbance or distortion of this process is seen in conditions such as Crohn's disease where persistent and chronic inflammation in the bowel results in debilitating and occasionally lethal consequences for the patient (Truelove and Pena, 1976).

Cell type is also a critical factor in how a cell responds to DNA damage. If the DNA of a cell is damaged the cell can choose to repair the damage or to sacrifice

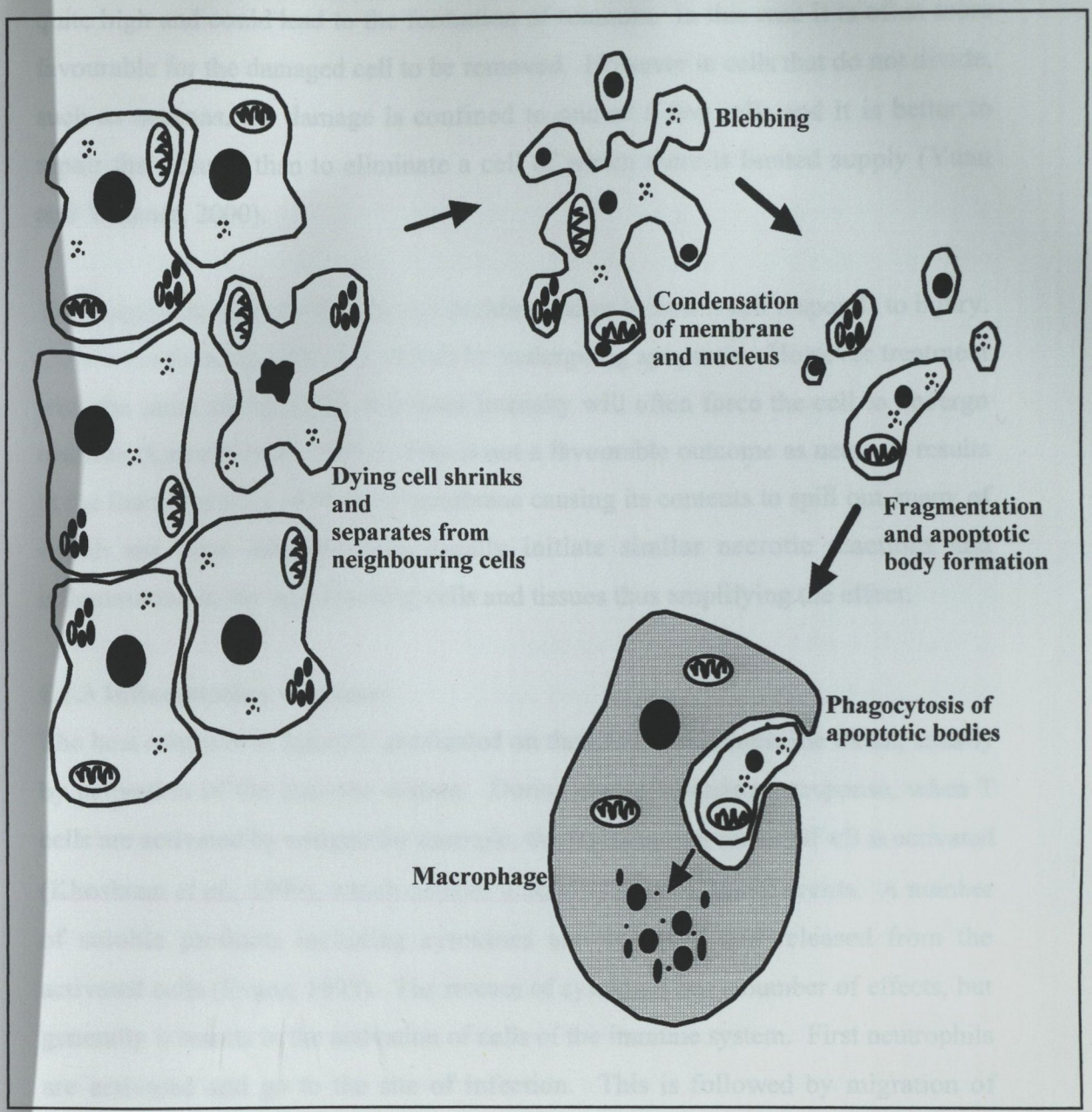


Figure 1.1:
Schematic representation of morphological changes exhibited by cells undergoing apoptosis
The cell targeted to undergo apoptosis shrinks and pulls away from neighbouring cells. As the cell shrinks, membrane blebbing occurs and the cell begins to fragment into membrane enclosed apoptotic bodies. The nucleus also condenses and fragments. The apoptotic bodies can then be removed by resident macrophages (Kerr *et al.* 1972).

itself (Levine, 1997). In proliferating cells, the risk of propagating a mutation is quite high and could lead to the formation of tumours. In this case it is often more favourable for the damaged cell to be removed. However in cells that do not divide, such as neurons, the damage is confined to one or a few cells and it is better to repair the damage than to eliminate a cell of which there is limited supply (Yuan and Yankner, 2000).

The magnitude of insult is a second deciding factor in how a cell responds to injury. Cells react to many injurious stimuli by undergoing apoptosis. However treatment with the same stimuli at an increased intensity will often force the cell to undergo necrosis (Lennon *et al.*, 1991). This is not a favourable outcome as necrosis results in the literal bursting of the cell membrane causing its contents to spill out, many of which are toxic and this will usually initiate similar necrotic reactions and inflammation in the neighbouring cells and tissues thus amplifying the effect.

1.1.3 Inflammatory response

The host response to injury is predicated on the need to eliminate the threat, usually by activation of the immune system. During the inflammatory response, when T cells are activated by antigen for example, the transcription factor NFκB is activated (Khoshnan *et al.*, 1999), which decides a number of subsequent events. A number of soluble products including cytokines are produced and released from the activated cells (Evans, 1993). The release of cytokines has a number of effects, but generally it results in the activation of cells of the immune system. First neutrophils are activated and go to the site of infection. This is followed by migration of monocytes and lymphocytes to the site of injury where they fight the infection. The result is thus a massive influx of cells to a specific area. This process can often lead to death of 'innocent bystanders' as dying cells rupture spilling out their toxic contents to the surrounding area. The orderly elimination of the activated cells (neutrophils, monocytes, lymphocytes and eosinophils) is also essential for health (Haslett, 1992).

Just as for apoptosis, the pathways that control and initiate the inflammatory response are complex and intricate. Once again, if the sequence of events are altered or interrupted in some way this can lead to serious consequences. Many

diseases, such as arthritis and asthma are due to the inflammatory response being deregulated (Dinarello, 1996). Often this can be due to a deregulation of apoptosis. For the inflammatory response to be completed successfully the activated immune cells have to be removed by apoptosis otherwise persistent inflammation occurs (Haslett, 1992). This is one example of the interplay that occurs between the two pathways that are involved in host defence. As higher organisms continue to develop defence strategies such as these, infecting organisms concurrently find means to evade the immune response. In HIV-infected cells, not only is the virus able to maintain survival of the infected cell by blocking apoptosis (Geleziunas *et al.*, 2001; Wolf *et al.*, 2001), it may also force the cell to kill surrounding immune cells by activating apoptosis (Zauli *et al.*, 1999).

1.1.4 Apoptosis and Inflammation: Medical implications

A number of examples of diseases and disorders have been mentioned where a causative factor is a failure of either the apoptosis or the inflammatory machinery to function correctly. This underscores the fact that the correct execution of the apoptosis and inflammatory processes are essential to the survival of the host. A number of genetic disorders occur due to the disruption of genes involved in one of these pathways. Physiological injury, caused by stroke or effects of alcoholism for example, may result in excess apoptosis occurring, contributing to long-term tissue damage (Thompson, 1995). Autoimmune diseases are thought to be the result of the failure of developing T-cells that recognise 'self' antigens to be removed by apoptosis in the developing thymus (Nagata, 1997). Thus dissecting the pathways that result in the initiation of the inflammatory response and apoptosis is invaluable to the understanding of the pathologies of diseases in which these processes are disrupted. This knowledge will contribute greatly to the development of treatments and therapies for such diseases.

1.1.5 Pathways Leading To Apoptosis and Immune Cell Activation

The processes of apoptosis and immune cell activation are co-dependant. For the immune system to mount an inflammatory response, the life spans of many of the cells, such as neutrophils, that migrate to the site of infection is extended, therefore apoptosis is transiently inhibited (reviewed in Watson, 1999). Once the infection or threat is cleared, activated cells need to be removed by apoptosis to avoid persistent

inflammation in the area (Haslett, 1992; Savill *et al.*, 1989). The signalling pathways that are intrinsically involved in the process that decides a cell's fate are complex and intertwined. Furthermore, the pathways that lead to apoptosis and those that result in NFκB activation share a number of features. Many stimuli, such as TNF, that stimulate apoptosis can also activate NFκB leading to inflammation (Hsu *et al.*, 1995). Furthermore, many of the proteins involved in these two pathways are closely related (Martin, 2001). Many advances have been made, in recent years, to dissect the molecular events that initiate apoptosis or the activation of the immune response, however the story is still incomplete. Although a large number of proteins have been identified and characterised that are involved in apoptosis and NFκB activation there is a sense that we have only scratched the surface. As a result there are a number of questions that remain unanswered as to how these pathways are mapped out. The identification of novel proteins involved in these pathways is crucial to completing our understanding of how these processes are controlled.

1.2 THE CASPASE FAMILY OF PROTEASES

Central to the apoptosis pathway in mammals is a family of proteases called caspases. There are currently 14 members of the caspase family (caspase-1, -2 etc), 12 of which are of human origin (Table 1.1). The family is classified by a highly conserved pentapeptide motif (QACXG), which contains the active cysteine central to it and hence, is termed the protease active site. Caspases are also categorised as a family by virtue of their characteristic cleavage specificity as they all cleave their substrates after an aspartate residue (Alnemri *et al.*, 1996).

1.2.1 Caspase Function

Currently, there is a long list of caspase substrates and many more which are likely to exist. Unsurprisingly, many of the substrates cleaved during apoptosis are structural proteins such as fodrin (Martin *et al.*, 1995), gelsolin (Kothakota *et al.*, 1997) and nuclear lamins A and B (Neamati *et al.*, 1995, Orth *et al.*, 1996). The cleavage of such proteins by caspases suggests that they contribute to the break down of the cell membrane and other cellular structures, resulting in the characteristic appearance of membrane blebbing (Figure 1.1).

Table 1.1: Summary of mammalian family of caspases, their functions and phenotypes of mice nullizygous for the indicated caspases

Caspase	Other Name	Knockout phenotype	Function	Ref.
CARD-Caspases				
Caspase-1	ICE	Defects in IL-1β processing Resistance to LPS induced septic shock IL-1α production defect	IL-1β processing, inflammation,	(Kuida *et al.*, 1995; Li *et al.*, 1995; Thornberry *et al.*, 1992)
Caspase-2	ICH-1 (human) Nedd2 (mouse)	Viable Excess oocytes	Apoptosis initiator/ effector?	(Bergeron *et al.*, 1998; Wang *et al.*, 1994)
Caspase-4	TX/ICH-2/ ICE_{rel}-II	Not done	Inflammation?	(Munday *et al.*, 1995)
Caspase-5	ICE_{rel}-III/TY	Not done	Inflammation?	(Munday *et al.*, 1995)
Caspase-9	ICE-LAP6/ Mch6	Embryonic Lethal Neuronal hyperplasia	Apoptosis Initiator in Apaf-1 pathway	(Duan *et al.*, 1996b; Hakem *et al.*, 1998; Kuida *et al.*, 1998)
Caspase-11 (mouse)	Ich3 (mouse)	Resistance to LPS induced septic shock	Inflammation	(Wang *et al.*, 1996; Wang *et al.*, 1998)
Caspase-12 (mouse)		Partially resistant to ER stress inducers	Implicated in ER stress-induced apoptosis	(Nakagawa *et al.*, 2000)
Caspase-13	ERICE	Not done	Unknown	(Humke *et al.*, 1998)
DED-Caspases				
Caspase-8	MACH/FLICE/ Mch5	Embryonic lethal Resistance to DR-induced apoptosis	Apoptosis initiator in death receptor pathways	(Muzio *et al.*, 1996; Varfolomeev *et al.*, 1998)
Caspase-10	Mch4/FLICE2	No murine equivalent	Initiator?	(Fernandes-Alnemri *et al.*, 1996; Vincenz and Dixit, 1997)
Short prodomain Caspases				
Caspase-3	CPP32/Yama/ Apopain	Embryonic lethal Resistance to variety of death inducers	Apoptosis Effector caspase	(Kuida *et al.*, 1996; Nicholson *et al.*, 1995; Tewari et al., 1995)
Caspase-6	Mch2	Viable (unpublished)	Apoptosis Effector caspase	(Fernandes-Alnemri *et al.*, 1995)
Caspase-7	Mch3/ICE-LAP3/CMH-1	Embryonic lethal (unpublished)	Apoptosis Effector caspase	(Duan *et al.*, 1996a)
Caspase-14	mICE	Not done	Role in development ?	(Hu *et al.*, 1998)

A second group of substrates are regulatory or signalling proteins such as the tumour suppressor, retinoblastoma (Janicke *et al*, 1996) and the p53 regulator, MDM2 (Erhardt *et al*., 1997). Cleavage of such regulatory proteins may function to either upregulate or ablate their function in some way that contributes to the demise of the cell.

In the third category of substrates are proteins that are involved in DNA repair or gene expression. These include poly(ADP-ribose) polymerase (PARP) (Lazebnik *et al*., 1994) and the catalytic subunit of DNA-dependant protein kinase (DNA-PK_{CS}) (McConnell *et al*., 1997).

1.2.2 *C.elegans*: a model organism for the study of apoptosis

The discovery of the caspase family of proteins was greatly facilitated by studies carried out on the nematode *C.elegans*. During the course of *C.elegans* development, 1090 somatic cells are produced, 131 of which die in a programmed manner.

The *C.elegans* cell death machinery is comprised of at least fourteen genes that control and regulate PCD. Among them are the cell death effectors *ced-3* and *ced-4* and the cell death repressor *ced-9*. If any of the 131 cells in *C.elegans* that are expected to undergo PCD carry mutations in either of the *ced-3* or *ced-4* genes the cell will survive and differentiate as normal (Ellis and Horvitz, 1996). Overexpression of the *ced-9* gene causes cells that usually undergo programmed cell death to survive (Hengartner and Horvitz, 1994).

Studies indicate that the *ced-9* gene product inhibits cell death by antagonising the functions of CED-3 and CED-4. The interplay between these three proteins is controlled by specific protein-protein interactions. CED-3 binds to CED-4 and in this conformation CED-4 can, in the presence of ATP, activate CED-3 to carry out its protease function (Chinnaiyan *et al*., 1997a). CED-9, which has been shown to localise to mitochondrial membranes, can also bind CED-4 and recruit the protein to mitochondria (Chinnaiyan *et al*., 1997b; Spector *et al*., 1997).

1.2.3 Caspase-1: a mammalian homologue of CED-3

A mammalian homologue of CED-3 was found to be interleukin-1β-converting enzyme (ICE; Yuan *et al.*, 1993). Caspase-1/ICE was the first cysteine-aspartate protease to be discovered. Caspase-1 cleaves interleukin-1β (IL-1β) from its inactive 31kD precursor form to the active proinflammatory 17kD form (Thornberry *et al.*, 1992). IL-1β is an important protein in mediating the inflammatory response. This cytokine is involved in the pathogenesis of many conditions and diseases including septic shock, wound healing, bone resorption and myelogenous leukaemia (Dinarello, 1996). The pivotal role of IL-1β in inflammation suggests that caspase-1 inhibition may be a powerful means of modulating the inflammatory response. The IL-1β cleavage activity of caspase-1 can be specifically inhibited by the cowpox serpin CrmA (cytokine response modifier protein; Ray *et al.*, 1992).

That caspase-1 is homologous to the *C.elegans* protein CED-3 is considered to be one of the key early discoveries in the field of cell death. The sequence similarity between CED-3 and caspase-1 suggested not only that CED-3 is a cysteine protease, but that caspase-1 may also promote cell death (Yuan *et al.*, 1993). Indeed, when murine ICE was over-expressed in Rat-1 fibroblasts they underwent apoptosis (Miura *et al.*, 1993). This activity was prevented by the expression of both CrmA and Bcl-2, the human homologue of CED-9 and a general inhibitor of apoptosis (Miura *et al.*, 1993; Hengartner and Horvitz 1994). CrmA was also shown to block nerve growth factor withdrawal-induced apoptosis when it was microinjected into chicken dorsal root ganglion cells, further suggesting a role for caspase-1 in cell death promotion (Gagliardini *et al.*, 1994).

Thus ICE/caspase-1 became the first member of the growing family of mammalian caspases. However, it is now generally established that caspase-1 does not play a central role in programmed cell death pathways. A number of lines of evidence suggest this. Firstly, overexpression of caspase-1 in cell lines causes apoptosis (Miura *et al.*, 1993), but it has been shown that almost any protease, when overexpressed will have a cytotoxic effect regardless of its role in a particular cell death pathway (Williams and Henkart, 1994). Secondly the discovery of further homologues to caspase-1 and CED-3 diminished the significance of some of the

early experiments that claimed that cell death was specifically associated with caspase-1. For example, the experiments described above in chicken dorsal root ganglion cells where the ability of CrmA to block apoptosis was used as evidence for caspase-1 function (Gagliardini *et al.*, 1994) can, be instead taken to suggest that CrmA also inhibits certain caspase-1 related proteins. This has since shown to be the case as CrmA can act as a general caspase inhibitor when overexpressed at high levels (Zhou *et al.*, 1997).

The strongest evidence to suggest that caspase-1 is not centrally involved in apoptosis is shown by the phenotype of the *CASP-1* knockout mouse (Kuida *et al.*, 1995; Li *et al.*, 1995). This mouse displayed no overt phenotype, which would be caused by a disruption of the death pathway, compared to the gross abnormalities resulting from the loss of *CASP-8*, *CASP-3* or *CASP-9* (increased brain size, embryonic lethality etc; Varfolomeev *et al.*, 1998; Kuida *et al.*, 1996; Hakem *et al.*, 1998; Kuida *et al.*, 1998; see below).

In the *CASP-1* knockout mouse, the loss of caspase-1 results in serious defects in IL-1 processing (Li *et al.*, 1995). There is no IL-1β exported from the mutant cells and also there is a significant decrease in the amount of IL-1α release. The loss of caspase-1 also results in resistance to LPS-induced septic shock, suggesting a more prominent role in inflammation and associated processes, than in cell death. LPS (lipopolysaccharide) is a bacterial endotoxin produced by gram-negative bacteria, that induces monocytes to activate several cytokines including IL-1β. When monocytes from wild type mice are stimulated with LPS, active IL-1β is produced and exported, but when the same treatment is applied to *CASP-1* (-/-) monocytes IL-1β is not processed. When a high dose of LPS is administered to wild type mice it usually results in death of the animal. However, similar doses do not have any effect on the *CASP-1* knockout mouse (Li *et al.*, 1995).

Thus caspase-1 is considered to have a primary role in inflammation. Caspases -1, -4, -5 (Munday *et al.*, 1995), -11 (Wang *et al.*, 1996b) and -13 (Humke *et al.*, 1998) are all members of the caspase-1 subfamily of caspases. This grouping is based on sequence homology between these proteins and they also share similar substrate

specificity (Garcia-Calvo *et al.*, 1998). Little is known about the function of the caspase-1 related caspases, but the available evidence suggests that they function primarily in the regulation of inflammation, rather than apoptosis. Caspase-11 is a murine caspase and has much in common with caspase-1 (Wang *et al.*, 1996b). The phenotype of the *CASP-11* knockout mouse is similar to that of *CASP-1*, displaying resistance to LPS induced septic shock and defects in IL-1 processing (Wang *et al.*, 1998b). Unlike caspase-1, however, caspase-11 protein expression is induced by LPS (Wang *et al.*, 1998b). The human homologue of caspase-11 is not known but is likely to be either caspase-4 or caspase-5. Caspase-4, but not caspase-5, can also cleave pIL-1β (Lin *et al.*, 2000). Interestingly, expression of caspase-5 is LPS inducible, suggesting that caspase-5 may be the human homologue of caspase-11 (Lin *et al.*, 2000). Thus it is clear that a subset of the caspases function in the regulation of the inflammatory response.

1.2.4 Caspase Structure

Each member of the caspase family has a similar domain structure, comprising a prodomain, a long subunit and a short subunit. The caspases can be loosely divided into two categories based on the length of their prodomains. Among the caspases that have been implicated in apoptosis, it is generally regarded that the caspases with long prodomains, such as caspase-9 and caspase-8, are upstream caspases, while short prodomain caspases, such as caspase-3, -6, and –7, are downstream or effector caspases (Table 1.1; Slee *et al.*, 1999). However this rule is not set in stone, because the full functions of all the different caspases, regardless of prodomain length, have not been fully elucidated. In order to become activated the prodomain of a caspase is usually removed. This either occurs in a self-cleaving or auto-processing event where a particular caspase can promote its own cleavage, or where one caspase is specifically cleaved by a different caspase.

The long prodomain caspases can be further divided into two groups, CARD-motif-containing caspases and DED-motif-containing caspases. The CARD and DED motifs are conserved domains found in the prodomains of some caspases and related proteins; the caspase recruitment domain (CARD) and the death effector domain (DED) respectively (Hofmann *et al.*, 1997). Both of these domains have a similar six-helical tertiary structure. CARD and DED motifs allow caspases to bind to

other proteins, which contain similar domains, thus regulating their activity. For example, caspase-8 (Muzio *et al.*, 1996) is a DED-containing caspase and its adaptor, called FADD also contains a DED in its sequence (Chinnaiyan *et al.*, 1995). Activation of caspase-8 by FADD occurs via an interaction between the DED domains of both proteins thus initiating apoptosis. A third protein called FLIP also contains two DED motifs and has been suggested to inhibit apoptosis by competing with FADD for the binding of caspase-8 (Irmler *et al.*, 1997). CARD containing caspases behave in a similar way. Caspase-9 (Duan *et al.*, 1996) is activated, by binding its adaptor Apaf-1, via a CARD-CARD interaction (Li *et al.*, 1997).

Protein-protein interactions such as the ones just described are crucial to the propagation of cell death signals. Consequently, the study of these interactions has been fundamental to the discovery of novel caspase regulators and in the dissection of the different stages of the apoptosis pathway, some of which are discussed below.

1.2.5 Routes to Caspase activation in apoptosis

Numerous forms of cell stress and cell damage, as well as signalling molecules secreted by other cells, can result in caspase activation and apoptosis. Although in many of these cases the upstream signalling events remain obscure, evidence suggests that all of these stimuli engage the caspases in one of two main ways; through death receptor stimulation or release of mitochondrial cytochrome *c* (Figure 1.2). These two routes to caspase activation will now be discussed.

1.3 DEATH RECEPTOR PATHWAYS

A number of different stimuli induce cell death by engagement of one or more of a family of trans-membrane proteins, collectively known as death receptors. There are currently a number of death receptors characterised and all are members of the tumour necrosis factor receptor (TNFR) family. The most fully characterised of the death receptors is a molecule known as Fas (APO-I/CD95) (Itoh *et al.*, 1991; Trauth *et al.*, 1989; Yonehara *et al.*, 1989). Fas, like other death receptors, contains an extracellular signal sequence at its amino-terminal end, a single transmembrane domain and the carboxyl terminus is located inside the cell (Itoh *et al.*, 1991). A region towards the carboxyl terminus is conserved between Fas and TNFR1, but is

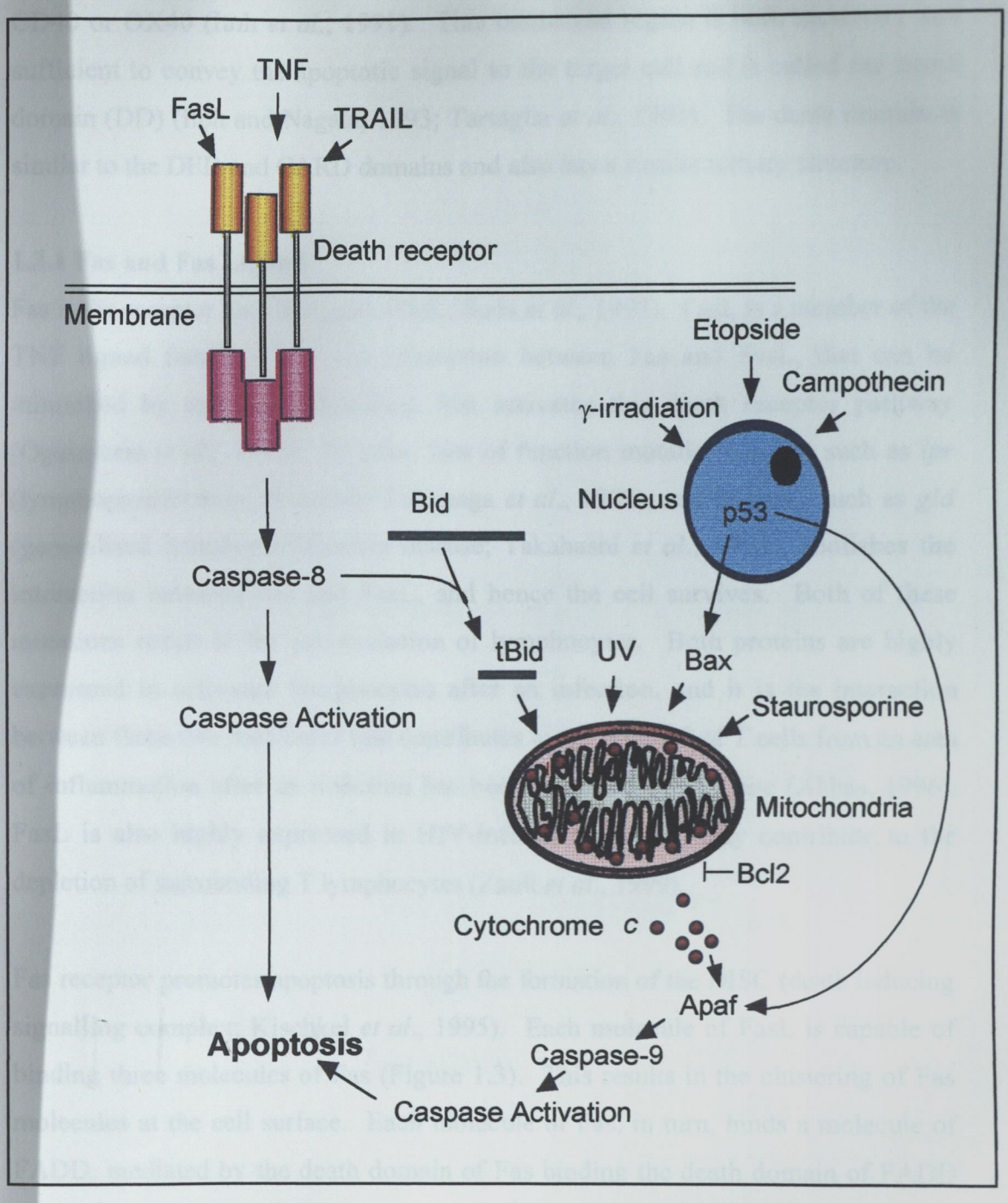


Figure 1.2:

Diverse stimuli initiate apoptosis through death receptor stimulation or release of mitochondrial cytochrome *c*

Ligands such as FasL, TNF and TRAIL initiate apoptosis via the death receptor pathway. Activation of caspase-8 directly activates downstream pathways and also leads to the cleavage of Bid. Cleaved Bid (tBid) can initiate cytochrome *c* release from the mitochondria. Other stimuli (UV etc.) directly activate the mitochondrial pathway. DNA damaging agents activate p53 which can induce the expression of the proapoptotic proteins Bax and Apaf-1.

not present in all other members of the TNF receptor family such as TNFR-II, CD40 or OX40 (Itoh *et al.*, 1991). This conserved region is both necessary and sufficient to convey the apoptotic signal to the target cell and is called the death domain (DD) (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). The death domain is similar to the DED and CARD domains and also has a similar tertiary structure.

1.3.1 Fas and Fas Ligand

Fas is the receptor for Fas ligand (FasL; Suda *et al.*, 1993). FasL is a member of the TNF ligand family. It is the interaction between Fas and FasL, that can be mimicked by anti-Fas antibodies, that activates this death receptor pathway (Ogasawara *et al.*, 1993). In mice, loss of function mutations in Fas such as *lpr* (lymphoproliferation; Watanabe-Fukunaga *et al.*, 1992), and in FasL, such as *gld* (generalised lymphoproliferative disease; Takahashi *et al.*, 1994), abolishes the interaction between Fas and FasL, and hence the cell survives. Both of these mutations result in the accumulation of lymphocytes. Both proteins are highly expressed in activated lymphocytes after an infection, and it is the interaction between these two molecules that contributes to the removal of T cells from an area of inflammation after an infection has been cleared from the site (Abbas, 1996). FasL is also highly expressed in HIV-infected cells and may contribute to the depletion of surrounding T lymphocytes (Zauli *et al.*, 1999).

Fas receptor promotes apoptosis through the formation of the DISC (death inducing signalling complex; Kischkel *et al.*, 1995). Each molecule of FasL is capable of binding three molecules of Fas (Figure 1.3). This results in the clustering of Fas molecules at the cell surface. Each molecule of Fas, in turn, binds a molecule of FADD, mediated by the death domain of Fas binding the death domain of FADD (Chinnaiyan *et al.*, 1995). Then, via DED-DED interactions, FADD binds to caspase-8. Once in close proximity the caspase-8 molecules activate each other leading to activation of the caspase cascade and consequently death of the cell (Medema *et al.*, 1997).

1.3.2 TNF and the TNF receptor

TNF (tumour necrosis factor) can promote apoptosis but, unlike FasL, it can also activate the NFκB survival pathway. As in Fas-induced cell death, TNF-induced

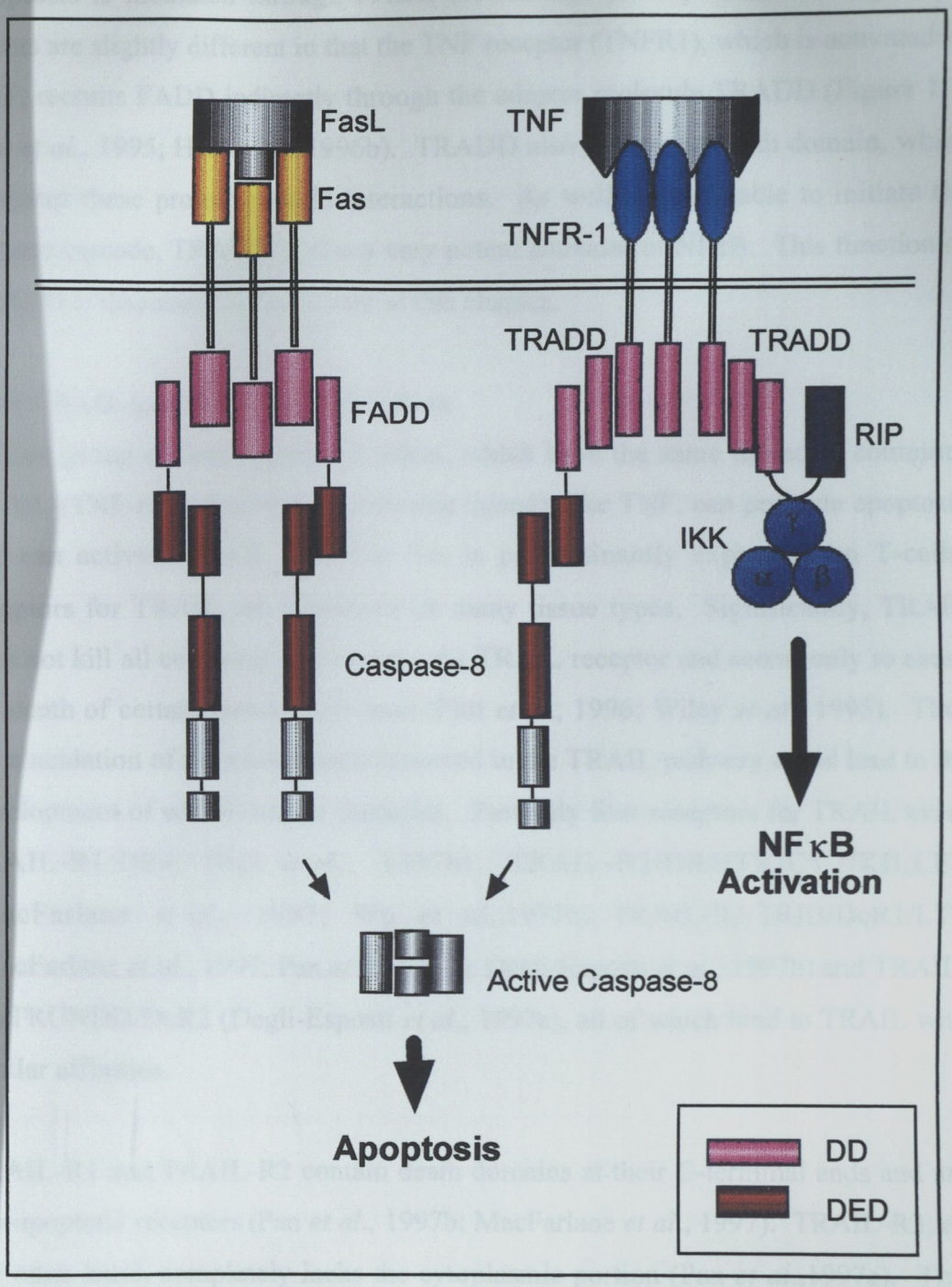


Figure 1.3:

Death receptor mediated activation of caspases and NFκB

Schematic representation of the molecular events that occur upon ligation of the death receptors Fas and TNFR with their respective ligands. Binding of the ligands FasL or TNF to the extracellular region of Fas and TNFR respectively induces aggregation of the intracellular death domain-containing portions. This enables recruitment and clustering of the adaptor molecules FADD and TRADD. FADD recruits caspase-8 increasing the local concentration of the caspase, enabling autoactivation of the caspase. TRADD promotes apoptosis via FADD, but alternatively TRADD recruits RIP which promotes assembly of the IKK complex by binding IKKγ resulting in NFκB activation.

apoptosis is mediated through FADD recruitment of caspase-8. The upstream events are slightly different in that the TNF receptor (TNFR1), which is activated by TNF, recruits FADD indirectly through the adaptor molecule TRADD (Figure 1.3; Hsu *et al.*, 1995; Hsu *et al.,* 1996b). TRADD also contains a death domain, which mediates these protein-protein interactions. As well as being able to initiate the caspase cascade, TRADD is also a very potent activator of NFκB. This function of TRADD is discussed in detail later in this chapter.

1.3.3 TRAIL and the TRAIL receptors

A third group of death receptors exists, which have the same ligand in common. TRAIL (TNF-related apoptosis inducing ligand), like TNF, can promote apoptosis and can activate NFκB. Whereas Fas is predominantly expressed on T-cells, receptors for TRAIL are expressed on many tissue types. Significantly, TRAIL does not kill all cell types that express the TRAIL receptor and seems only to cause the death of certain tumour cell lines (Pitti *et al.*, 1996; Wiley *et al.*, 1995). Thus the elucidation of the components involved in the TRAIL pathway could lead to the development of useful tumour therapies. Presently four receptors for TRAIL exist, TRAIL-R1/DR4, (Pan *et al.*, 1997b), TRAIL-R2/DR5/TRICK2/KILLER (MacFarlane *et al.*, 1997; Wu *et al.*,1997b), TRAIL-R3/TRID/DcR1/LTT (MacFarlane *et al.*, 1997; Pan *et al.*,1997a; Degli-Esposti *et al.*, 1997b) and TRAIL-R4/TRUNDD/DcR2 (Degli-Esposti *et al.*, 1997a), all of which bind to TRAIL with similar affinities.

TRAIL-R1 and TRAIL-R2 contain death domains at their C-terminal ends and are pro-apoptotic receptors (Pan *et al.*, 1997b; MacFarlane *et al.*, 1997). TRAIL-R3, on the other hand, completely lacks the cytoplasmic portion (Pan *et al.*,1997a). This receptor does not cause apoptosis and thus is widely assumed to act as a decoy receptor. This is thought to explain how TRAIL does not cause apoptosis of untransformed cells because it is sequestered by the TRAIL-R3 receptor, which does not promote cell death. This would be expected to be the case if the expression of TRAIL-R3 was highly expressed on most untransformed tissue types, but the expression of this receptor seems to be restricted to peripheral blood lymphocytes, spleen and lung (Pan *et al.*,1997a). TRAIL-R4 is also considered a decoy receptor. Unlike TRAIL-R3 it possesses a cytoplasmic region, yet this region

contains a truncated death domain (Degli-Esposti *et al.*, 1997a). This truncation is sufficient to abolish any pro-apoptotic ability. The expression pattern of TRAIL-R4 shows a wide distribution in comparison to TRAIL-R3 and is also expressed to a high level in a number of cancer cell lines (Degli-Esposti *et al.*, 1997a). This would suggest that that the selective cytotoxic properties of TRAIL are more complex than being solely due to the existence of these two decoy receptors. Because TRAIL-R4 expression is not restricted to untransformed cells, another mechanism must exist that prevents these cells from being protected from TRAIL-mediated killing. Recently it was shown that TRAIL could in fact cause apoptosis in untransformed cells, specifically human hepatocytes. If this is the case then the idea that TRAIL can be used as a cancer therapy still requires much investigation (Jo *et al.*, 2000).

1.4 THE MITOCHONDRIAL PATHWAY

Certain death inducing stimuli do not lead to the engagement of death receptor pathways. Stimuli such as UV irradiation or treatment with drugs such as staurosporine initiate the mitochondrial pathway of apoptosis (Figure 1.2). These stimuli lead to the rapid release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol. The presence of cytochrome *c* in the cytosol of a cell is a certain indication of the imminent death of the cell. Cytochrome *c*, in the presence of ATP, initiates the caspase-cascade through activation of Apaf-1 (Figure 1.4; Li *et al.*, 1997; Slee *et al.*, 1999).

1.4.1 Apaf-1 and cytochrome *c* release

One of the key recent discoveries in the field of apoptosis has been the discovery of the human CED4 homologue, Apaf-1 (Zou *et al.*, 1997). Apaf-1 is a molecule that contains a CARD domain, a nucleotide binding domain (NBD) and a series of WD40 repeats. The WD40 repeats are thought to be responsible for maintaining Apaf-1 in an inactive state, since removal of this domain serves to produce an active protein (Adrain *et al.*, 1999). When cytochrome *c* is released from mitochondria a number of events ensue. Cytochrome *c* binds to the WD40 repeats of Apaf-1, causing the molecule to be activated, presumably by inducing a conformational change in the molecule (Figure 1.4). Upon activation a number of Apaf-1 molecules aggregate to form part of a high molecular weight complex, known as the apoptosome (Cain *et al.*, 1999; Zou *et al.*, 1999). Each molecule of Apaf-1 binds to

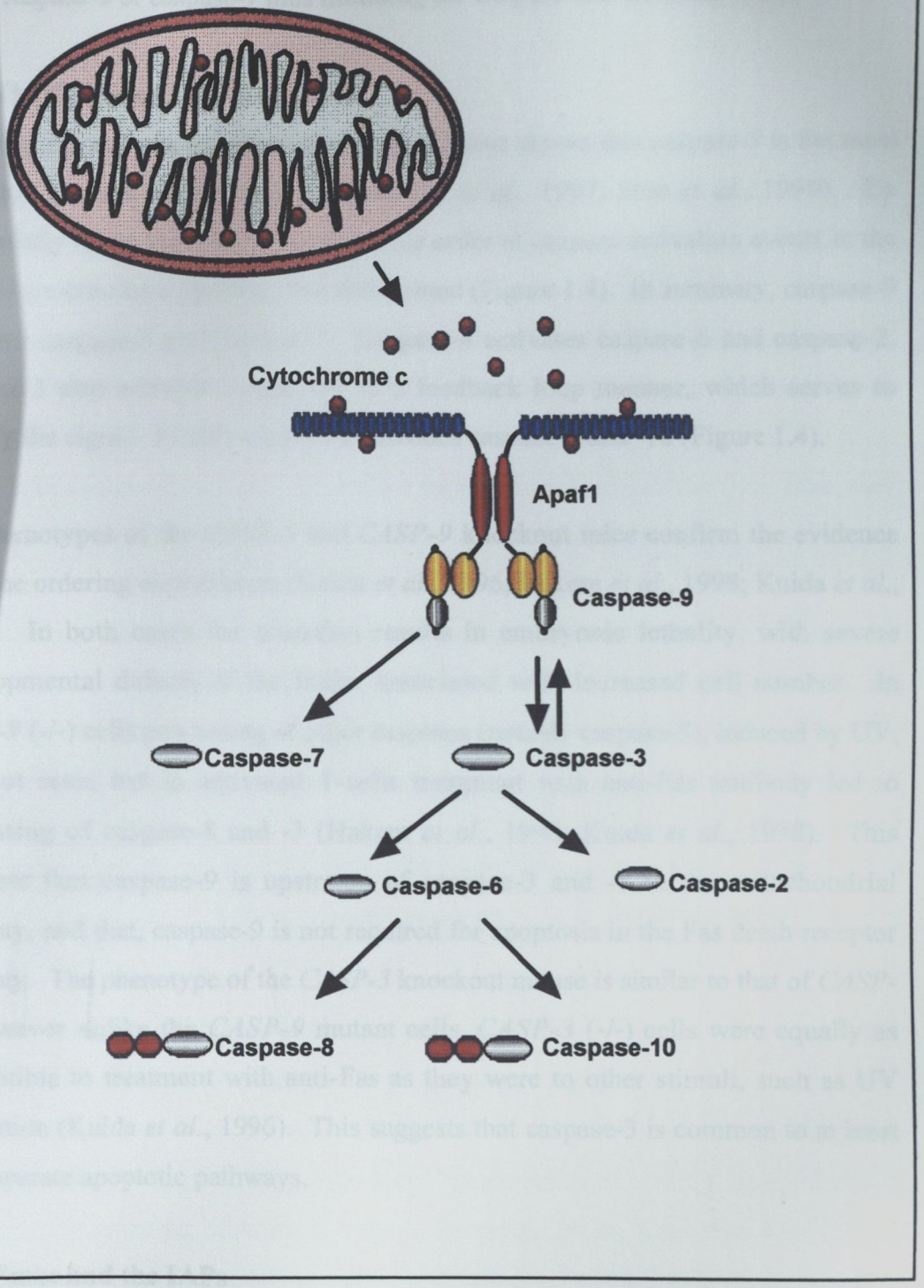


Figure 1.4:

Schematic representation of events that take place distal to the entry of cytochrome *c* into the cytoplasm.

The mitochondrial apoptosome is formed upon exit of cytochrome *c* from the mitochondrial intermembrane space. Upon activation, caspase-9 activates downstream caspases initiating the caspase cascade (Slee *et al.*, 1999).

a molecule of caspase-9 through a CARD-CARD interaction (Srinivasula *et al.*, 1998; Slee *et al.*, 1999). Each caspase-9 molecule, in turn, recruits and processes either caspase-3 or caspase-7 thus initiating the caspase cascade (Slee *et al.*, 1999).

1.4.2 The Caspase Cascade

Ordering experiments using cell free systems have shown that caspase-9 is the most upstream caspase in the Apaf pathway (Li *et al.*, 1997; Slee *et al.*, 1999). By sequentially removing specific caspases the order of caspase activation events in the Apaf-1/cytochrome *c* pathway was determined (Figure 1.4). In summary, caspase-9 activates caspase-3 and caspase-7. Caspase-3 activates caspase-6 and caspase-2. Caspase-3 also activates caspase-9 in a feedback loop manner, which serves to amplify the signal. Finally caspase-6 activates caspase-8 and -10 (Figure 1.4).

The phenotypes of the *CASP-3* and *CASP-9* knockout mice confirm the evidence from the ordering experiments (Kuida *et al.*, 1996; Hakem *et al.*, 1998; Kuida *et al.*, 1998). In both cases the mutation results in embryonic lethality, with severe developmental defects of the brain, associated with increased cell number. In *CASP-9* (-/-) cells processing of other caspases (notably caspase-3), induced by UV, was not seen, but in activated T-cells treatment with anti-Fas antibody led to processing of caspase-8 and -3 (Hakem *et al.*, 1998; Kuida *et al.*, 1998). This suggests that caspase-9 is upstream of caspase-3 and -8 in the mitochondrial pathway, and that, caspase-9 is not required for apoptosis in the Fas death receptor pathway. The phenotype of the *CASP-3* knockout mouse is similar to that of *CASP-9*, however unlike the *CASP-9* mutant cells, *CASP-3* (-/-) cells were equally as susceptible to treatment with anti-Fas as they were to other stimuli, such as UV irradiation (Kuida *et al.*, 1996). This suggests that caspase-3 is common to at least two separate apoptotic pathways.

1.4.3 Smac and the IAPs

Smac/DIABLO is a second protein that is released from the mitochondria (Du *et al.*, 2000; Verhagen *et al.*, 2000). Smac is released after cytochrome *c* and has been implicated in the neutralisation of IAPs namely XIAP. The IAPs are inhibitors of apoptosis that bind to caspases 3, 7 and 9 thereby neutralising their activity (Bratton *et al.*, 2001; Chai *et al.*, 2001; Deveraux *et al.*, 1998). However Smac can compete

out the IAP binding to caspases, by directly binding to the IAP proteins and displacing them from active caspases, thereby facilitating apoptosis (Srinivasula *et al.*, 2001).

1.4.4 The Bcl-2 family of proteins

The Bcl-2 family are an important family of apoptosis regulatory proteins. These proteins are characterised by the presence of BH1, BH2, or BH3 domains in their sequence (Yin *et al.*, 1994). The Bcl-2 family can be divided into two groups, promoters of apoptosis, such as Bax (Oltvai *et al.*, 1993) and inhibitors of apoptosis, such as Bcl-2, which is the mammalian homologue of CED-9 (Hengartner and Horvitz, 1994). The anti-apoptotic Bcl-2 family members generally tend to be anchored at the mitochondrial membrane (Wolter *et al.*, 1997), which is reminiscent of the subcellular localisation of CED-9 in *C.elegans*. Proteins, like Bax, that promote apoptosis, are usually found free in the cytoplasm and migrate to the mitochondria when they receive a death signal (Figure 1.2; Hsu *et al.*, 1997b; Wolter *et al.*, 1997). The mechanism that underlies the pro-apoptotic function of these proteins is not fully understood and a number of conflicting views exist concerning how such proteins cause cytochrome *c* release from the mitochondria and the mode in which cytochrome *c* is released.

The prevailing view is that members of the Bax subfamily oligomerise to form channels in the outer membrane of the mitochondrion that facilitates release of cytochrome *c* (Antonsson *et al.*, 1997; Wei *et al.*, 2001). The channels formed by Bax and related molecules either transport cytochrome *c* themselves or they may promote the transport of small molecules and ions (such as calcium) across the mitochondrial membrane leading to disruption of transmembrane potential, which could allow for the release of cytochrome *c* (Gogvadze *et al.*, 2001). The latter case results in the opening of what is termed the permeability transition pore (PTP), which causes mitochondrial swelling and rupture of the outer mitochondrial membrane, releasing cytochrome *c* and other mitochondrial proteins (Crompton, 1999). Usually the damage to mitochondria caused by PTP opening is irreversible leading to necrosis, since activation of the apoptosome requires ATP and hence functional mitochondria. However, under conditions where the stimulus is mild it is thought that enough mitochondria survive to produce ATP sufficient to activate

Apaf-1 resulting in apoptosis. The role of Bax in this process is unclear as certain reports show interaction of Bax with components of the PTP, while others state that PTP opening is independent of Bax or other Bax sub-family members (Marzo *et al.*, 1998; Eskes *et al.*, 2000). Bcl-2 has been shown to block the release of cytochrome *c* (Kluck *et al.*, 1997; Yang *et al.*, 1997), possibly through the neutralisation of Bax by binding to it, preventing its insertion into the mitochondrial membrane.

The two pathways just described, death receptor and mitochondrial are not mutually exclusive. Bid, which is a BH3-only protein, is the link between the two (Figure 1.2). When caspase-8 is activated by FADD it directly activates downstream caspases. However caspase-8 also cleaves Bid, the cleaved form of which promotes cytochrome *c* release and caspase-9 activation (Li *et al.*, 1998b). Investigations into the significance of caspase-8 induced cleavage of Bid has led to the proposal of the controversial theory that there are two types of cells which respond differently to Fas-induced cell death (Scaffidi *et al.*, 1998; Scaffidi *et al.*, 1999). In type I cells, where there is a high concentration of Fas, caspases are directly activated by caspase-8. Cytochrome *c* release occurs later and the cells have already been committed to death at this stage. In type II cells death receptor induced apoptosis induces less DISC formation than in type I cells. Therefore apoptosis occurs primarily via the mitochondria in response to caspase-8 mediated cleavage of Bid. Consequently the majority of caspase-8 activation occurs downstream of caspase-3 in these cells (Scaffidi *et al.*, 1998; Scaffidi *et al.*, 1999).

1.5 THE CARD FAMILY OF PROTEINS

One key point that becomes obvious through the description of the above pathways that lead to apoptosis, is that protein-protein interactions play an indispensable role. These interactions allow the death signal to be transmitted down through a cascade of proteins. A number of defined protein domains have been described that mediate these interactions: the death domain (DD) (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993), the death effector domain (DED), the caspase recruitment domain (CARD) (Hofmann *et al.*, 1997) and the recently described pyrin domain (PYD), (Bertin and DiStefano, 2000; Staub *et al.*, 2001). All of these modules form a six or seven antiparallel α-helical bundle that has been termed the death domain fold.

In the last two years a large number of proteins with CARD domains have been characterised (Figure 1.5). The CARD domain was first identified in certain caspases and their adaptor molecules. As the name suggests, CARD-containing proteins were originally classified as proapoptotic molecules that act as docking platforms for caspases. This action allows a number of caspase molecules to come into close proximity enabling their activation. As the family of CARD containing proteins expands, however, it has become apparent that the original nomenclature is too restrictive. Many of the recently characterised CARDs, rather than binding caspases, recruit other CARD proteins. Moreover, many CARD proteins do not promote caspase activation but participate in NFκB signalling pathways associated with innate or adaptive immune responses. At first glance it appears difficult to reconcile the multifunctional properties of the proteins that contain CARD motifs. Therefore, in order to simplify the following description of the CARD family of proteins, they have been sub-divided into four sub-families based on their overall domain structures as follows (Figure 1.5).

(1) The NBD-CARDs. These proteins contain a nucleotide-binding domain (NBD) in addition to the CARD motif and also contain either leucine-rich repeats (LRR) or WD40 repeats within their C-terminal regions. The NBD of Apaf-1 has been shown to act as an oligomerisation module (Adrain *et al.*, 1999) and presumably functions in a similar way in other members of this group. Overall this group of proteins probably act as molecular scaffolds around which protein complexes are assembled.

(2) The Coiled-Coil CARDs. Proteins within this group are broadly similar to the NBD-CARDs but the central NBD is replaced by a coiled-coil (CC) motif. Again these proteins are likely to be scaffold proteins for protein complex formation.

(3) The bipartite-CARDs. This group of proteins is less complex as members contain a CARD and a second domain (a kinase domain, death domain, or PYD domain). These tend to function as downstream adaptor or effector molecules, and are recruited to the scaffold proteins of the first two groups, leading to their activation, or enabling the to mediate th recruitment of additional effector molecules to the complex.

(4) The CARD-only CARDs. These proteins contain a CARD domain and little else and generally act as decoy molecules.

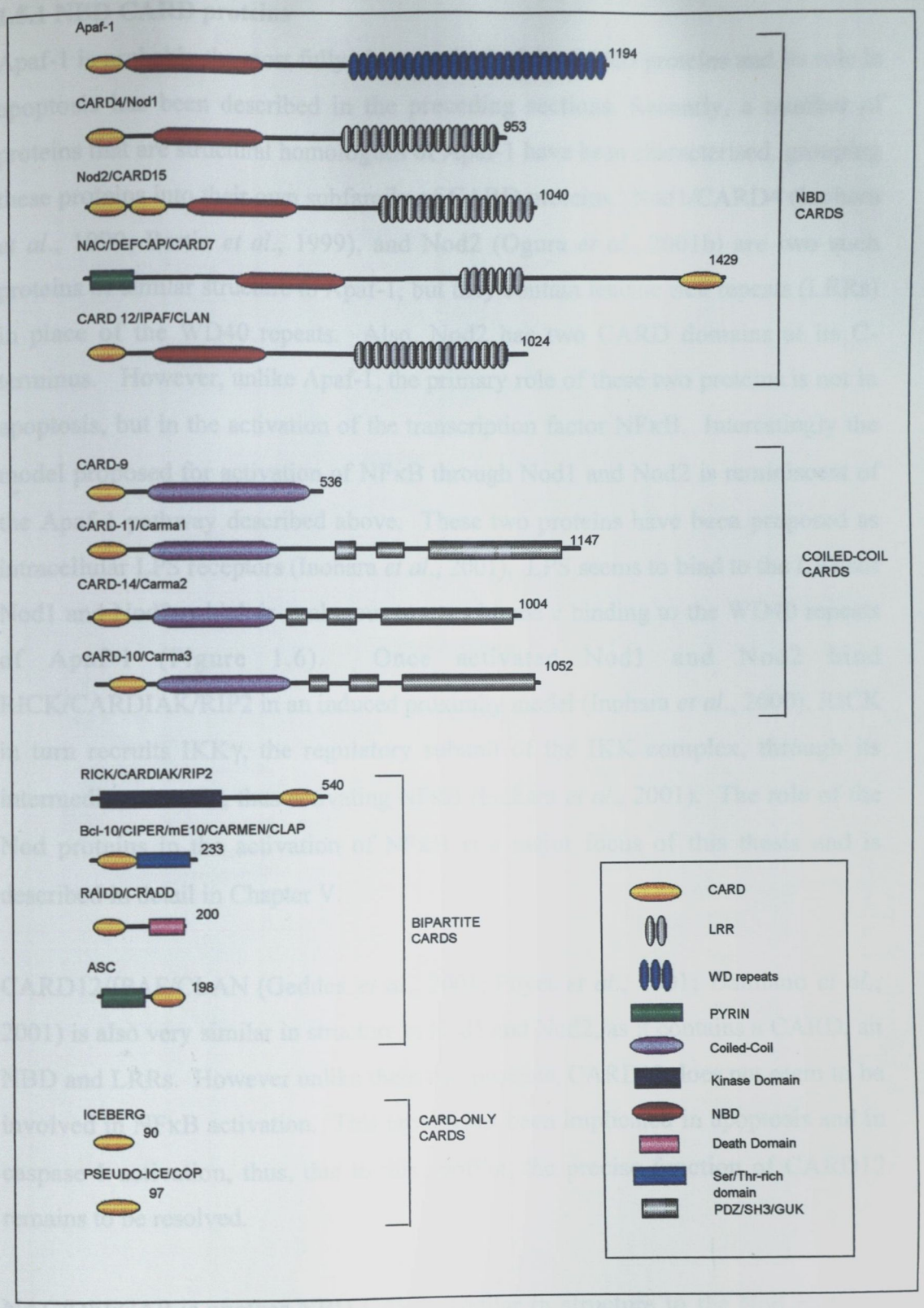


Figure 1.5:

Schematic representation of CARD family of proteins

Illustrated are a list of known CARD containing proteins. The four subgroups of CARD proteins are NBD-CARDs, Coiled-coil-CARDS, Bi-partite CARDs and CARD-only CARDs as discussed in the text.

1.5.1 NBD CARD proteins

Apaf-1 is probably the most fully characterised of the CARD proteins and its role in apoptosis has been described in the preceding sections. Recently, a number of proteins that are structural homologues of Apaf-1 have been characterised, grouping these proteins into their own subfamily of CARD proteins. Nod1/CARD4 (Inohara *et al.*, 1999; Bertin *et al.*, 1999), and Nod2 (Ogura *et al.*, 2001b) are two such proteins of similar structure to Apaf-1, but they contain leucine rich repeats (LRRs) in place of the WD40 repeats. Also, Nod2 has two CARD domains at its C-terminus. However, unlike Apaf-1, the primary role of these two proteins is not in apoptosis, but in the activation of the transcription factor NFκB. Interestingly the model proposed for activation of NFκB through Nod1 and Nod2 is reminiscent of the Apaf-1 pathway described above. These two proteins have been proposed as intracellular LPS receptors (Inohara *et al.*, 2001). LPS seems to bind to the LRRsof Nod1 and Nod2, which is analogous to cytochrome *c* binding to the WD40 repeats of Apaf-1 (Figure 1.6). Once activated Nod1 and Nod2 bind RICK/CARDIAK/RIP2 in an induced proximity model (Inohara *et al.*, 2000). RICK in turn recruits IKKγ, the regulatory subunit of the IKK complex, through its intermediate domain, thus activating NFκB (Inohara *et al.*, 2001). The role of the Nod proteins in the activation of NFκB is a major focus of this thesis and is described in detail in Chapter V.

CARD12/IPAF/CLAN (Geddes *et al.*, 2001; Poyet *et al.*, 2001; Damiano *et al.*, 2001) is also very similar in structure to Nod1 and Nod2, as it contains a CARD, an NBD and LRRs. However unlike these two proteins, CARD12 does not seem to be involved in NFκB activation. This protein has been implicated in apoptosis and in caspase-1 activation, thus, due to this conflict, the precise function of CARD12 remains to be resolved.

NAC/DEFCAP is another NBD-CARD, similar in structure to the Nod proteins, although with a few notable differences, (Chu *et al.*, 2001; Hliang *et al.*, 2001). Firstly the CARD domain is uniquely located at the C-terminus and it has a PYD domain at the N-terminus. The PYD motif is a recent finding in apoptosis related proteins (Bertin and DiStefano, 2000; Staub *et al.*, 2001). It is so called because it

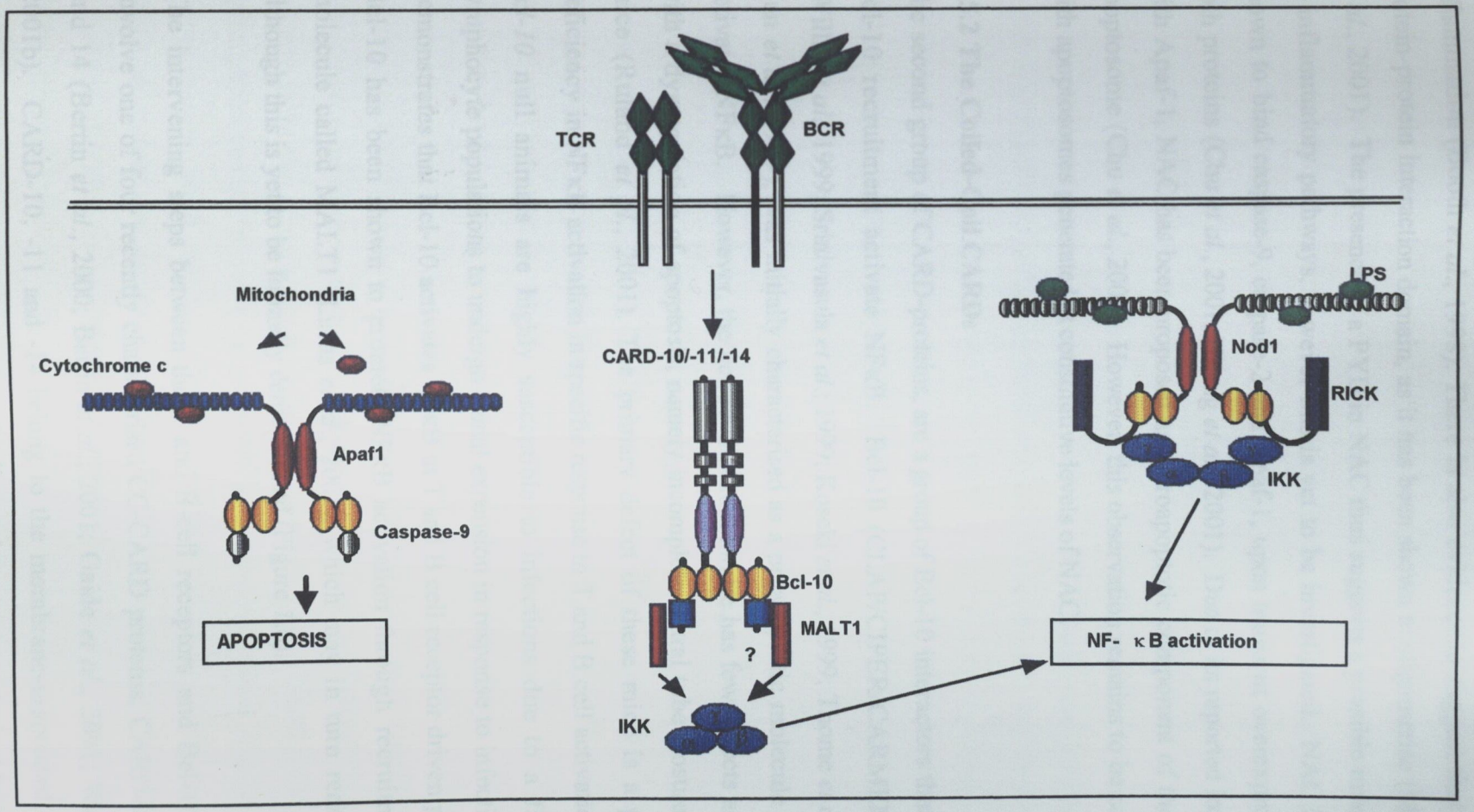


Figure 1.6:

CARD proteins are involved in pathways that lead to apoptosis and NFκB activation

Schematic representation of some of the pathways that involve CARD proteins. Cytochrome *c* release from the mitochondria activates Apaf-1, which recruits and activates caspase-9 leading to apoptosis. CARD-10,-11 and -14, in response to TCR and BCR activation recruit Bcl-10 which leads to the activation of NFκB. LPS binds to the LRRs of Nod1, Nod1 recruits and activates RICK allowing IKKγ recruitment and NFκB activation.

is found in a protein called pyrin, which is mutated in familial Mediterranean fever, an inherited disorder characterised by frequent occurrences of fever and inflammation (Booth *et al.*, 1998). There is also evidence to suggest that this is a protein-protein interaction domain, as it has been shown to oligomerise (Masumoto *et al.*, 2001). The presence of a PYD in NAC thus suggests a possible involvement in inflammatory pathways, however this is yet to be investigated. NAC has been shown to bind caspase-9, caspase-2 and Apaf-1, upon transient overexpression of both proteins (Chu *et al.*, 2001; Hliang *et al.*, 2001). Due to its reported interaction with Apaf-1, NAC has been proposed as a proapoptotic component of the Apaf-1 apoptosome (Chu *et al.*, 2001). However, this observation remains to be confirmed with apoptosomes generated at constitutive levels of NAC.

1.5.2 The Coiled-Coil CARDs

The second group of CARD-proteins, are a group of Bcl-10 interactors that through Bcl-10 recruitment activate NFκB. Bcl-10 (CLAP/CIPER/CARMEN/mE10) (Willis *et al.*, 1999; Srinivasula *et al.*; 1999; Koseki *et al.*, 1999; Thome *et al.*, 1999; Yan *et al.*, 1999), was initially characterised as a proapoptotic molecule that also activates NFκB. However, the *bcl-10* knockout mouse has few defects associated with a dysregulation of apoptosis, namely incomplete neural tube closure in some mice (Ruland *et al.*, 2001). The primary defect of these mice is a profound deficiency in NFκB activation in specific response to T and B cell activation. Thus *bcl-10* null animals are highly susceptible to infections due to a failure of lymphocyte populations to undergo clonal expansion in response to infection. This demonstrates that Bcl-10 activates NFκB in T and B cell receptor driven pathways. Bcl-10 has been shown to promote NFκB activation through recruitment of a molecule called MALT1 (Lucas *et al.*, 2001), which may in turn recruit IKKγ although this is yet to be formally demonstrated (Figure 1.6).

The intervening steps between the T and B-cell receptors and Bcl-10 seem to involve one of four recently characterised CC-CARD proteins, CARD-9, -10, -11 and 14 (Bertin *et al.*, 2000; Bertin *et al.*, 2001; Gaide *et al.*, 2001; Wang *et al.*, 2001b). CARD-10, -11 and -14 belong to the membrane-associated guanylate kinase (MAGUK) family of proteins. Such proteins are characterised by a tripartite

PDZ/SH3/GUK structure that acts as a molecular scaffold, co-ordinating protein complexes at the cell membrane (Figure 1.6; Fanning and Anderson, 1999). CARD-9 is related to the three other proteins but is truncated in comparison, lacking the MAGUK structure. It is not clear how these proteins result in the activation of Bcl-10 in response to T cell activation. CARD-9 has been shown to oligomerise through the coiled-coil domain (Bertin *et al.*, 2000), and it is likely that the other three behave in a similar manner.

1.5.3 Bipartite-CARDs

The proteins mentioned thus far are multi-domain proteins. The bipartate-CARD proteins are less complex, comprising a CARD and one additional domain. These proteins generally act as downstream adaptors for many of the aforementioned proteins. This is the case, as has been described above, for Bcl-10 and RICK in NFκB activating pathways. RICK (CARDIAK/RIP2) (Inohara *et al.*, 1998; McCarthy *et al.*, 1998; Thome *et al.*, 1998) has also been implicated as a caspase-1 binding protein, promoting caspase-1 activation. Whether the true primary function of RICK is in caspase-1 regulation or NFκB activation, or if it does, in fact, function in both pathways remains to be determined.

Similar to NAC, ASC is a CARD protein that also contains a PYD domain (Masumoto *et al.*, 1999). ASC has been shown to form aggregates in apoptotic cells but it is not clear what its role in apoptosis is. However this protein has been shown to bind pyrin suggesting a role in an inflammatory process (Richards *et al.*, 2001).

Finally, RAIDD/CRADD (Duan and Dixit, 1997; Ahmad *et al.,* 1997), one of the first CARDs to be described, has a death domain and a CARD domain and is thought to be the adaptor molecule linking RIP to caspase-2 leading to apoptosis, although the context in which this occurs remains obscure. The caspase-2 knockout mouse has no overt apoptotic phenotype (Bergeron *et al.*, 1998) and the RIP knockout mouse has demonstrated that the primary role for RIP is in NFκB activation (Kelliher *et al.*, 1998). Therefore the exact role of RAIDD in this pathway remains unclear.

1.5.4 CARD-only proteins

ICEBERG and pseudoICE/COP are two proteins that contain only a CARD domain (Druilhe *et al.*, 2001; Humke *et al.*, 2000; Lee *et al.,* 2001). They both function to inhibit capsase-1 activation and IL-1β processing. Both bind caspase-1 but pseudoICE also binds RICK and it is proposed that the interruption of the RICK-caspase-1 interaction leads to the inhibition. It seems reasonable to predict that if similar proteins are identified that contain a CARD domain and little else, that they would also function as decoy molecules as the two existing CARD-only proteins do.

From this list of CARD proteins and their functions it becomes apparent that there are three major pathways in which these proteins are involved: caspase-1 regulation, NFκB regulation and apoptosis. Broadly speaking, they function in cell death and in the inflammatory response. One key point to note is that structural similarities between these proteins does not always correlate with functional redundancy. Although all the CC-CARDS seem to operate in the same way leading to Bcl-10 activation, the NBD-CARDs have more diverse roles, Apaf-1 induces apoptosis, Nod1 and Nod2 activate NFκB and CARD12 is potentially a caspase-1 regulator. In order to reconcile how such similar molecules can operate in the seemingly diverse processes of caspase activation and NFκB regulation it is necessary to compare the different pathways. In doing so it becomes apparent that there are many common themes running between the two.

1.6 NFκB

Many of the proteins mentioned so far in the Introduction, although related to apoptosis regulating proteins, have been described as regulators of NFκB. NFκB is a transcription factor that activates a number of genes involved in the inflammatory and innate immune responses, including many cytokines such as IL-1 and IL-8 (Hiscott *et al.*, 1993; Ott *et al.*, 1998). NFκB also activates the transcription of genes encoding chemokines, adhesion molecules, cell surface receptors, and enzymes that produce secondary inflammatory mediators (Baeuerle and Baltimore, 1996). The NFκB protein is made up of two subunits, p65/RelA and p50/NFκB1 (Ghosh *et al.*, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991). Both can form homodimers as well as the more natural p65/p50 heterodimer (Urban *et al.*, 1991).

Signal transduction pathways that lead to the activation of NFκB display many similarities with the apoptosis pathways. Similar to apoptosis, a wide array of stimuli can lead to the activation of NFκB, but as apoptosis pathways all converge on the caspases, NFκB activation pathways all seem to converge at the point of the IKK complex (Figure 1.7).

1.6.1 The IKK signalosome

The IκB Kinase (IKK) complex or signalosome is made up of three proteins IKKα (DiDonato *et al.*, 1997), IKKβ (Faucheu *et al.*, 1996; Van Antwerp *et al.*, 1996), and IKKγ/NEMO (Rothwarf *et al.*, 1998; Yamaoka *et al.*, 1998). IKKα and IKKβ contain identical structural domains: a helix-loop-helix domain, a leucine zipper domain and a kinase domain. IKKγ does not contain a kinase domain and is regarded as the regulatory subunit of the complex (Figure 1.7, Rothwarf *et al.*, 1998). If one of these genes is knocked out in mouse, severe phenotypes are observed. The IKKα null mouse, surprisingly, does not have impaired responses to NFκB inducers such as IL-1 and TNF (Takeda *et al.*, 1999; Hu *et al.*, 1999). However, these animals have severe defects in skin formation. Failure of the skin to differentiate correctly results in it being ten times thicker than normal and does not allow emergence of the limbs. This phenomenon has been shown to be independent of the NFκB activating activity of IKKα, suggesting a second role for this enzyme (Hu *et al.*, 2001). IKKβ knockouts, on the other hand, have lost their ability to activate NFκB, resulting in embryonic lethality (Li *et al.*, 1999). The lethality is the result of severe liver degeneration. Similarly IKKγ mutant mice die *in utero* due to liver degeneration, and show no detectable NFκB DNA binding activity in response to a variety of stimuli (Rudolph *et al.*, 2000). The phenotype of the IKKγ (-/-) mouse is almost indistinguishable from that of mice where p65 is mutated (Beg *et al.*, 1995). The phenotype of these mice is in fact more severe than for IKKβ knockout mice suggesting that IKKγ is indispensable for NFκB activation.

Evidence suggests that IKKγ acts as a molecular scaffold that once activated recruits IKKα and IKKβ (Figure 1.7; Poyet *et al.*, 2000). This leads to phosphorylation of IKKβ, which results in activation of the IKK complex (Delhase *et al.*, 1999). The activation of the complex is amplified by the ability of IKKβ to phosphorylate

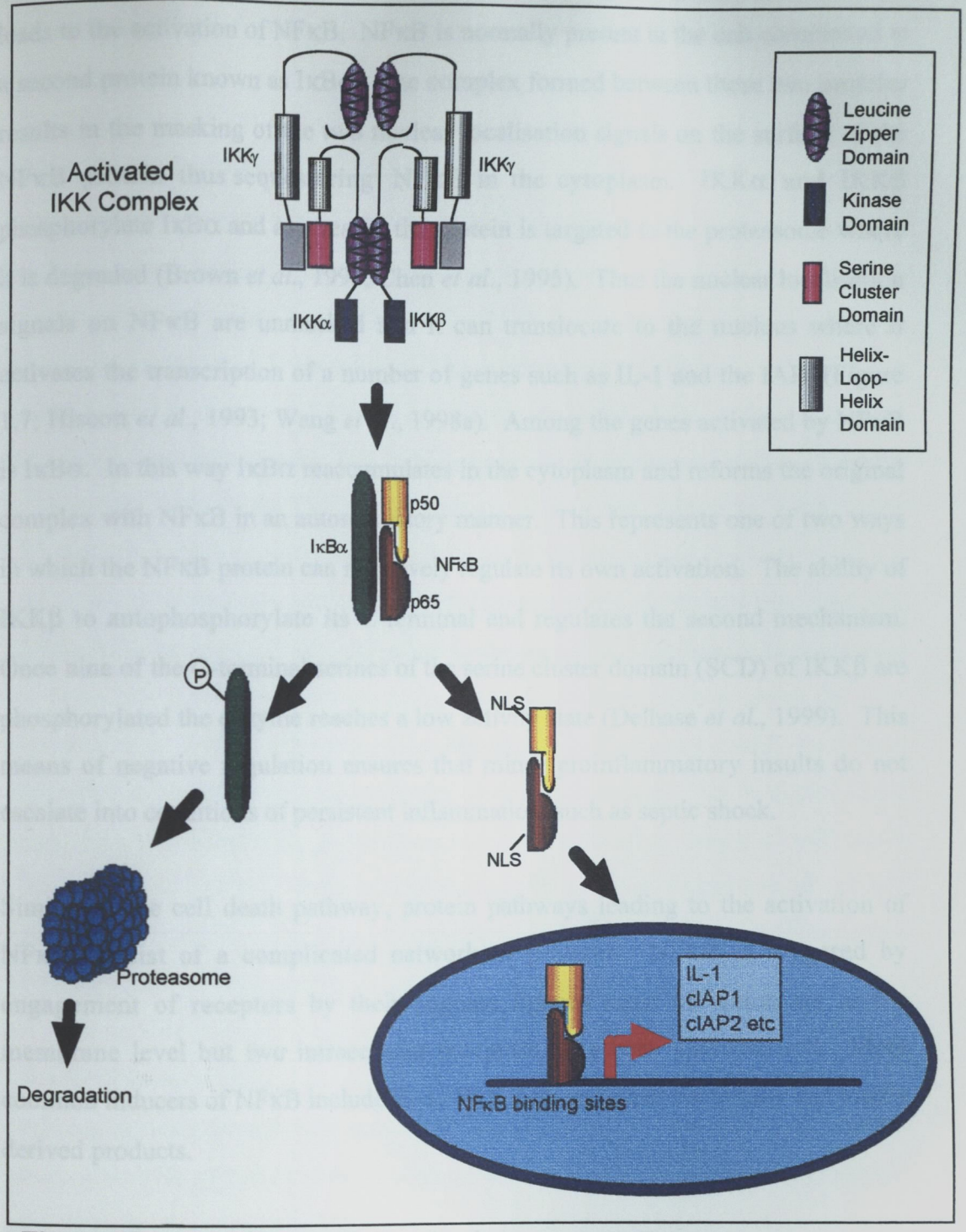


Figure 1.7:

Ihe IKK signalosome

Schematic diagram illustrating the composition of the activated IKK complex. IKKγ oligomerisation allows recruitment and subsequent activation of the kinases IKKα and IKKβ. Once activated the IKK complex phosphorylates IκB. Phosphorylated IκB dissociates from NFκB and is targeted to the proteasome where it is degraded. When separated from IκB, the nuclear localisation signals (NLS) on NFκB are unmasked and NFκB is targeted to the nucleus where it activates trascription of a number of genes.

IKKα and adjacent inactive complexes. Once activated the IKK complex, in turn, leads to the activation of NFκB. NFκB is normally present in the cell complexed to a second protein known as IκBα. The complex formed between these two proteins results in the masking of the two nuclear localisation signals on the surface of the NFκB protein thus sequestering NFκB in the cytoplasm. IKKα and IKKβ phosphorylate IκBα and as a result, the protein is targeted to the proteasome where it is degraded (Brown *et al.*, 1995; Chen *et al.*, 1995). Thus the nuclear localisation signals on NFκB are unmasked and it can translocate to the nucleus where it activates the transcription of a number of genes such as IL-1 and the IAPs (Figure 1.7; Hiscott *et al.*, 1993; Wang *et al.*, 1998a). Among the genes activated by NFκB is IκBα. In this way IκBα reaccumulates in the cytoplasm and reforms the original complex with NFκB in an autoregulatory manner. This represents one of two ways in which the NFκB protein can negatively regulate its own activation. The ability of IKKβ to autophosphorylate its C-terminal end regulates the second mechanism. Once nine of the C-terminal serines of the serine cluster domain (SCD) of IKKβ are phosphorylated the enzyme reaches a low activity state (Delhase *et al.*, 1999). This means of negative regulation ensures that minor proinflammatory insults do not escalate into conditions of persistent inflammation, such as septic shock.

Similar to the cell death pathway, protein pathways leading to the activation of NFκB consist of a complicated network of proteins. NFκB is triggered by engagement of receptors by their cognate ligands most of which are at the membrane level but two intracellular receptors have been characterised. More common inducers of NFκB include IL-1, TNF, LPS and other bacterially and virally derived products.

1.6.2 Interleukin-1 induced NFκB activation

Interleukin-1 (IL-1) initiates an NFκB activating pathway by engaging its receptor Interleukin-1 receptor (IL-1R) at the cell surface. The intracellular portion of the receptor then initiates a cascade of protein-protein interactions starting with the recruitment of MyD88 (Burns *et al.*, 1998; Wesche *et al.*, 1997). MyD88 through its death domain binds the death domain-containing protein IRAK (Burns *et al.*, 1998; Wesche *et al.*, 1997). The events that occur downstream of IRAK are unclear,

but it may directly phosphorylate the IKK complex or, by analogy with pathways that activate NFκB through RIP or RICK, it is plausible that IRAK also recruits IKKγ.

1.6.3 LPS induced NFκB activation

LPS has been shown to activate NFκB by two distinct pathways, at the membrane level and intracellularly. The intracellular receptors for LPS are Nod1 and Nod2 as has been discussed elsewhere. Otherwise, LPS acts as an extracellular pathogen through activation of a Toll receptor. The mammalian Toll-like receptor family is comprised of ten members (TLR1, 2 etc). They are so called due to their homology with the *Drosophila* protein Toll, which is involved in the establishment of the dorso-ventral axis in the *Drosophila* embryo (Belvin and Anderson, 1996). The LPS receptor was discovered to be TLR4 through studies on the *Lps* mutant mouse. *Lps* mice are completely resistant to LPS-induced septic shock and they also lack TLR4 (Poltorak *et al.*, 1998). All other members of the TLR family in these mice are intact and functional demonstrating that TLR4 is the sole receptor for LPS (Poltorak *et al.*, 1998). The other members of the Toll receptor family have been implicated as receptors of other pathogen derived substances. For example, TLR2 recognises peptidoglycan, a component of gram-positive bacterial cell walls (Takeuchi *et al.*, 1999). Once activated by LPS, TLR4 is thought to signal in a similar way to the IL-1 receptor by recruiting MyD88 and through IRAK activates NFκB (Medzhitov *et al.*, 1998).

1.6.4 TNF induced NFκB

TNF can activate both NFκB and apoptosis. Following engagement of the TNF receptor by TNF, TRADD either recruits RIP or FADD (Hsu *et al.*, 1996a; Stanger *et al.*, 1995). Recruitment of RIP leads to NFκB activation, while FADD activation results in apoptosis. What dictates the path that is taken upon engagement of the TNF receptor, ultimately resulting in prolonging the life of the cell or death, is unknown. It has been shown in a number of studies that if the NFκB response is blocked, cells become increasingly susceptible to apoptosis. Conversely, if apoptosis is blocked the amount of NFκB activation in response to TNF is increased (Van Antwerp *et al.*, 1996b; Beg and Baltimore, 1996). This is especially seen in

the knockout mouse studies of IKKγ and IKKβ (Li *et al.*, 1999; Rudolph *et al.*, 2000). These mice die of liver degeneration due to increased apoptosis of these cells. Furthermore cells from these mice are much more susceptible to apoptosis inducing stimuli.

1.6.5 The role of 'apoptosis' proteins in NFκB activation

Many proteins activate NFκB and, as has been described, a number of them were originally characterised as promoters of apoptosis. More recent research has shown that in most cases the primary role of these proteins is in NFκB signalling. However, there are a number of links and similarities between the two processes even though they result in opposing outcomes. For example the protein RIP was originally characterised as a very potent inducer of apoptosis (Stanger *et al.*, 1995). When overexpressed, it kills cells at a comparable level to that of FADD and it has a death domain, which suggests a role in PCD. However, RIP also induces NFκB. The apparent paradox was resolved by the phenotype of the RIP knockout mouse (Kelliher *et al.*, 1998). The phenotype of these mice is more similar to the phenotype that occurs when IKKβ, IKKγ or NF-κB p65 are knocked out (Beg *et al.*, 1995; Li *et al.*, 1999; Rothwarf *et al.*, 1998) than to knockouts of pro-apoptotic genes such as *CASP-8* or *APAF-1*, which are characterised by accumulation of excess cells (Cecconi *et al.*, 1998; Varfolomeev *et al.*, 1998; Yoshida *et al.*, 1998). *RIP*(-/-) mice are viable at birth but die within a few days due to increased apoptosis in the lymphoid and adipose tissue (Kelliher *et al.*, 1998). These mice are incapable of activating NFκB suggesting that this is the primary function of RIP (Kelliher *et al.*, 1998). As well as the death domain, RIP also has a kinase domain. Intriguingly, it is neither of these domains that is responsible for its ability to induce NFκB. The intermediate portion of the molecule between the two domains is the region that binds IKKγ thus triggering the NFκB response (Inohara *et al.*, 2000; Zhang *et al.*, 2000; Poyet *et al.*, 2000). The RIP-related protein, RICK, has been shown to function in a similar manner, also recruiting IKKγ through its intermediate domain (Inohara *et al.*, 2000).

1.7 CROSSTALK BETWEEN APOPTOSIS AND NFκB ACTIVATION PATHWAYS

As mentioned above there is cross talk between the two pathways that result in apoptosis and NFκB activation. This is exemplified by the role played by TNF described above. In short, TNF, depending on the context will activate either apoptosis or NFκB and activation of one process results in a shut down of the other. A certain amount of communication between the two processes allows for this level of control. When NFκB is activated the transcription of a number of genes is induced. Among these are certain inhibitors of apoptosis including Bcl-xL, cIAP-1, cIAP-2, FLIP and XIAP (Khoshnan *et al.*, 2000; Micheau *et al.*, 2001; Stehlik *et al.*, 1998; Wang *et al.*, 1998a). TNF also activates the Jun-N-terminal kinase pathway. Activation of JNK transcriptionally activates the proto-oncogene c-Jun. c-Jun by an unknown mechanism activates one or a number of caspases leading to apoptosis (Davis, 2000). Activation of NFκB by TNF has been shown to inhibit JNK induced apoptosis through induction of gadd45β (De Smaele *et al.*, 2001), a protein previously associated with cell cycle control and DNA repair (Vairapandi *et al.*, 1996). NFκB transcriptional activation of XIAP has also been shown to suppress TNF-induced JNK activation (De Smaele *et al.*, 2001; Tang *et al.*, 2001).

Further upstream, RIP has been implicated as a link between the two pathways. RIP is cleaved by caspase-8, which abolishes its NFκB inducing activity (Lin *et al.*, 1999). This suggests that in cells that are susceptible to TNF induced apoptosis, caspase-8 mediated cleavage of RIP is an early event that ensures the commitment of the cell to the apoptosis pathway, rather than NFκB activation. It is thus possible that other caspase substrates exist, the cleavage of which serves to interrupt a pathway that otherwise would lead to NFκB activation.

1.8 PROJECT AIMS

From the description of the above the pathways it seems apparent that the two physiological processes of apoptosis and immunity are linked both at the protein sequence/structure level and at the functional level, with common signalling proteins appearing in both contexts. It is the aim of this thesis to further explore and dissect these pathways through the characterisation of novel proteins that may

regulate signalling pathways leading to apoptosis, the activation of NFκB or both. In doing so it is envisioned that such discoveries could shed light on some of the questions that still remain concerning these protein pathways.

The introduction has highlighted the important role of protein-protein interactions and conserved protein interaction domains, such as the CARD domain, in both apoptosis pathways and pathways that lead to NFκB activation. Therefore it was the aim of this study to find binding partners for known proteins involved in these pathways, namely the caspases. To achieve this goal two separate strategies were employed. The first strategy was to use the yeast-two-hybrid assay to find novel binding partners for the DED containing caspase, caspase-10. The second strategy was to search the public gene databases for novel proteins with homology to the CARD containing caspase, caspase-1. Using the latter approach we identified a novel CARD-containing protein, called CARDINAL and investigated its role in caspase activation and NFκB activation.

CHAPTER II

Materials and Methods

2.1 MATERIALS

2.1.1 Antibodies

Antibodies used to produce data included in this thesis were obtained from the following sources

Anti mouse HRP κ light chain specific	Zymed labs inc., UK
T7 Tag antibody	Novagen, UK
T7 Tag antibody HRP conjugate	Novagen, UK
Anti-GFP monoclonal antibody	Clontech, UK
Anti-HA polyclonal antibody	Santa Cruz, UK
Anti-HA monoclonal antibody	Roche, UK
Anti-Gal-4 (DBD)	Santa Cruz, UK
Anti actin	ICN, UK
Anti-ICE (A-19) (Caspase-1)	Santa Cruz, UK
Anti-human IL-1β	R&D systems, UK
Anti-p65 (NF-κB RelA) monoclonal antibody	Translabs, UK
Anti-p65 (NF-κB RelA) polyclonal antibody	Santa Cruz, UK
Anti –RIP monoclonal antibody	Translabs, UK

2.1.2 Reagents

Restriction and modification enzymes were obtained from New England Biolabs (NEB, UK) unless otherwise stated. Chemicals and general reagents were purchased from Sigma-Aldrich, Ireland unless otherwise stated.

2.2 PURIFICATION AND MANIPULATION OF DNA

2.2.1 Plasmid Preparation

Generation of Ultra Competent DH5α (Inoue Method)

DH5α was grown at 18°C with rotation (250rpm) in SOB (2% (w/v) tryptone-peptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, pH6.7 (50ml)) to OD_{600} of 0.6, representing stationary phase of bacterial cell growth. The cell culture was placed on ice for 10mins. Cells were pelleted at 2500g for 10mins at 4°C in a bench top centrifuge (Sorvall). The cell pellet was resuspended in Inoue Transformation Buffer (10mM Pipes pH7.4, 55mM $MnCl_2$,

15mM $CaCl_2$, 250mM KCl, pH6.7, 16ml per 50ml of starter culture) to wash the cells and incubated on ice for 10mins. Cells were pelleted as before and resuspended in transformation buffer (4ml per 50ml starter culture). DMSO was added to a final concentration of 7% followed by a 10-minute incubation on ice. Cells were frozen in liquid nitrogen and stored in aliquots at –70°C.

Transformation into competent DH5α

Plasmid DNA was transformed into ultra competent *E.coli* DH5α by the following protocol. 50µl ultra competent DH5α cells were used per reaction, to which 3µl of a dilute stock of plasmid was added. Samples were incubated on ice for 30mins to allow the plasmid to coat the cells. Cells were heat shocked at 42°C for 40 seconds to induce plasmid uptake, followed by a 2min recovery on ice. 450µl of SOC (SOB, 20mM glucose) was added and cells were incubated at 37°C with 200rpm rotation for 1hr to allow expression of the Ampicillin resistance gene. Cells (25-50µl) were plated on LB plates with ampicillin (1% (w/v) tryptone-peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar, 100µg/ml ampicillin).

Plasmid Miniprep

LB containing ampicillin (3ml) was inoculated with a single colony of plasmid transformed DH5α and incubated overnight at 37°C, 300rpm. Bacterial culture (1ml) was pelleted at top speed in a bench top centrifuge for 1min. Cells were resuspended in P1 buffer (100µl, 50mM Tris-HCl pH8, 10mM EDTA) followed by lysis in P2 buffer (200µl, 0.2M NaOH, 1% SDS) on ice for 2mins. Genomic DNA was precipitated in P3 buffer (200µl, 3M KAc pH 5.5) on ice for 5mins. The precipitated DNA was then pelleted at top speed for 15mins in a microfuge. The supernatant was retained and plasmid DNA was precipitated with 0.7 volumes of isopropanol for 20mins. Plasmid DNA was pelleted at top speed in a microfuge for 10mins followed by washing with 1ml 70% ethanol. Pellets were dried and the DNA was resuspended in 10µl ddH_20 followed by 10µl 1X TE (10mM Tris-HCl pH8, 1mM EDTA).

Plasmid Midiprep Procedure

For large scale preps a 50ml culture was inoculated with the overnight starter culture (100µl). The culture was pelleted at 5000g in a bench top centrifuge for 10min. Bacterial cell pellets were resuspended in P1 buffer (4ml) containing 100µg/ml Rnase A (Qiagen, UK). Cells were lysed in P2 (4ml) buffer on ice for 2mins. Genomic DNA was precipitated in P3 buffer (4ml) on ice for 5mins. The precipitated DNA was then pelleted at 15,000g for 30mins in a Sorvall SS-34 rotor at 4°C. The supernatant was retained and plasmid DNA was precipitated with 0.7 volumes of isopropanol for 20mins. Plasmid DNA was pelleted at 18,000g in a Sorvall SS-34 rotor for 30mins. The DNA pellet was washed with 1ml 70% ethanol. Pellets were dried and the DNA was resuspended in 50µl ddH_20 followed by 50µl 1X TE (10mM Tris-HCl pH8, 1mM EDTA).

Plasmid Midiprep – Qiagen Procedure

To isolate pure DNA for use in transient transfections and in vitro transcription and translation (ITT) reactions the Qiagen procedure (Qiagen, UK) was followed. Following the P1/P2/P3 procedure detailed above, the plasmid supernatant was loaded onto a Qiagen tip-100 column, that had been equilibrated with 4ml QBT buffer (750mM NaCl, 50mM MOPS pH 7, 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100). The column was washed twice with 10ml QC buffer (1M NaCl, 50mM MOPS, pH7, 15% (v/v) isopropanol) and the DNA was eluted into 5ml QF buffer (1.25M NaCl, 50mM Tris-HCl, pH8.5, 15% (v/v) isopropanol). DNA was recovered by isopropanol precipitation as outlined in the previous section.

Restriction digest

Plasmids were subjected to restriction digest to verify the insert size or to digest the DNA for cloning. For a restriction digest the appropriate amount of DNA was typically digested for 18hrs with 1unit of the relevant enzyme(s) per µg of DNA in presence of the buffer supplied and recommended by the manufacturers (NEB, UK).

2.2.2 Plasmid Generation

Amplification and ligation of insert DNA

Generation of the plasmids used in this study was typically carried out using the following procedure. The coding sequence of interest was generated by PCR using an existing cDNA or the pACT2-Jurkat library (see expansion of Jurkat library) as a template. The primers for each plasmid generated are listed in Appendix Table II and each incorporates restriction sites which place the insert into the corresponding vector in the correct frame. PCR fragments were amplified using parameters listed in Table 2.1. The PCR fragment and the parent vector were digested with the relevant restriction enzymes for 18hrs at 37°C. The parent vector was treated with Calf Intestinal Phosphatase (CIP, 1 unit per 100pmol DNA ends) in the presence of the recommended CIP buffer (NEB, UK). CIP treatment was carried out for 1hr at 37°C to dephosphorylate the ends of the vector thus preventing self-ligation of the vector. The CIP enzyme was heat inactivated at 75°C for 10mins. The insert and vector were ligated together using T4 DNA ligase at 16°C for 18hrs.

Selection of positive clones

Plasmids were resolved on an agarose gel (0.6% agarose (w/v) in TAE buffer (40mM Tris adjusted to pH8 with acetic acid, 1mM EDTA)) alongside the empty parent vector. Plasmids that were upshifted on the gel compared to the empty vector were subjected to digestion with the restriction enzymes used to create the plasmid to ensure that they contain the insert of correct size.

Table 2.1: DNA cycle parameters for PCR amplification and other applications

Parameters (Temp, Time)	Method		
	Sequencing	Library screen PCR	PCR for cloning
Initial denaturation	NA	94°C, 3min	94°C, 3min
Denaturation	96°C , 10sec	94°C , 30sec	94°C , 30sec
Annealing	50°C, 5sec	57°C, 30sec	(Tm of primer-10)°C, 1 min
Elongation	60°C, 4min	72°C, 4min	72°C, 1 min/kb of PCR fragment
Cycles	25	35	35
Final Elongation	NA	72°C, 10min	72°C, 10min

Silica Cleaning of DNA

To purify a PCR product or a digested plasmid fragment, DNA was resolved on an agarose gel (1% (w/v) in TAE buffer). The fragment of interest was visualised by staining the agarose gel in ethidium bromide solution (10µg/ml) followed by UV trans-illumination. The area of the gel containing the DNA fragment of interest was excised and the gel band was melted by incubation with 3ml 6M NaI per gram of gel at 45-55°C for 5-10 minutes. This was followed by incubation with 1µl glass milk per µg of DNA, under rotation for 30mins, to allow the silica to bind the DNA. The silica was pelleted at top speed in a microfuge for 30 seconds and the pellets were washed twice in wash buffer (10mM Tris-HCl pH7.5, 50mM NaCl, 2.5mM EDTA, 50% ethanol). The wash buffer was removed after the last centrifugation and the silica pellet was allowed to air dry for 5mins. The DNA was eluted in 10mM Tris pH8, or ddH_2O as required. DNA could also be cleaned directly from solution. In this case the DNA solution was incubated with 3 volumes of 6M NaI and 10µl glass milk per 3µg of DNA and the rest of the procedure was followed as above.

Verification of Plasmids by sequencing

Plasmids and PCR products were sequenced using the ABI PRISM 310 Genetic Analyser (Perkin Elmer, UK). For PCR products approximately 100ng of gene-cleaned DNA was used as template for sequencing and for plasmids 400ng of Qiagen-purified or gene-cleaned plasmid DNA was used. The sequencing reaction was carried out using 200nM of the chosen forward or reverse primer (see Appendix for primer sequences) and 8µl of dye terminator ready reaction mix (Perkin Elmer, UK) in a 20µl reaction. The sequencing reaction was carried out using a DNA Engine (Perkin Elmer, UK) using recommended parameters (Table 2.1). Sequences were then precipitated in 50µl ethanol, 2µl NaOAc pH4.6 on ice for 10mins. Precipitated DNA was pelleted at top speed in a microfuge for 30mins. Pellets were washed with 70% ethanol (250µl). DNA was allowed to dry at 90°C for 10mins and then resuspended by vortexing in template suppressor reagent (25µl, Perkin Elmer, UK), followed by denaturation at 95°C for 5mins. Sequences were

analysed on the ABI PRISM 310 Genetic Analyser on a 50cm capillary detector according to the manufacturer's instructions.

2.3 PROTEIN PURIFICATION AND DETECTION

2.3.1 Protein Purification

Purification of GST fusion proteins

GST fusion proteins were induced and purified in *E.coli* DH5α using the pGEX system. *E.coli* DH5α was transformed with the pGEX4T2 plasmid of interest as previously described. A single colony was used to inoculate a 3ml culture in LB containing ampicillin. The overnight culture was diluted to OD_{600} of 0.2 and grown to OD_{600} of 0.4-0.6. The culture was induced with 100μM IPTG at 37°C for 3-4hrs. Cells were pelleted by centrifugation at 10,000g for 10mins. The cell pellet was lysed in NETN lysis buffer (20mM Tris-HCl pH8, 100mM NaCl, 1mM EDTA, 0.5% NP-40, 100μM PMSF, 10μg/ml leupeptin, 2μg/ml aprotinin, 2μg/ml pepstatin, 2ml per 100ml of original culture volume). Cells were further disrupted by sonication on ice. Cells were subjected to five 30 second pulses at 50% output on the sonicator, allowing the cells to recover on ice for 30 seconds between each pulse. The lysates were centrifuged at 15,000g for 30mins at 4°C to pellet inclusion bodies and debris. The supernatant was incubated with 50% Sepharose-Glutathione slurry (100μl/ml of bacterial lysate) at 4°C for 4hrs under constant rotation to allow capture of the GST fusion protein. The glutathione beads were collected by centrifugation at 800g and washed three times in cold PBS pH7.2 and resuspended to the original bead volume in PBS. Purified protein was resolved on SDS-PAGE gels stained with Coomassie blue (45% methanol, 10% glacial acetic acid, 0.25% brilliant blue) overnight and destained in destain (45% methanol, 10% glacial acetic acid).

Elution of GST fusion proteins

GST fusion proteins were eluted in elution buffer (10mM reduced glutathione, 150mM NaCl, 50mM Tris-HCl pH8) at 1ml per 100μl beads. Each elution was carried out at 37°C for 1hr. Again, eluted proteins were resolved on SDS-PAGE gels where they were run side by side with the beads prior to and after the elution to establish elution efficiency. Eluted proteins were concentrated using spin

concentrators (Sartorious, UK) of the appropriate molecular weight cut off (half the size of the protein). Proteins were spun through the concentrators at 5000g in a microfuge until the protein had been concentrated by the desired amount. The concentrated proteins were then washed to remove the elution buffer, with NEMN (20mM MOPS-HCl pH8, 100mM NaCl, 1mM EDTA, 0.5% NP-40). Known amounts of the concentrated protein were run on SDS-PAGE gels against a titration of BSA to verify the protein yield. Where required, proteins were dialysed in 3L 100mM MOPS to replace the Tris with MOPS, overnight at 4°C.

Estimation of protein concentration

The concentration of proteins was measured using the Biorad Protein Assay (Biorad, UK). A series of dilutions of BSA were made in ddH_2O within the concentration range of 0.2-0.9mg/ml (this represents the linear range of BSA). The protein sample was also diluted, usually by one in 50. The dye reagent was prepared by making a one in five dilution of the Dye Reagent Concentrate with ddH_2O. 20µl of each protein sample and standard was made up to 1ml with the dye reagent and the solution was mixed well. Samples were incubated at room temperature for 30-60mins. The absorbance of each standard and sample was read at 595nm.

2.3.2 Affinity purification of CARDINAL antibody

The CARDINAL antibody was affinity purified using an Affigel column (Biorad, UK) by Dr. Emma Creagh. The Affigel column was prepared by mixing equal portions of Affigel-10 and Affige1-15 (0.5ml of each). The gel was pelleted by centrifugation at 1000g for 30sec and the solvent supernatant was removed. The beads were washed twice with ice-cold ddH_2O (10ml). The gel was resuspended in 100mM MOPS pH7.5 (1ml). To couple the antigen to the gel (in this case GST-CARDINAL$^{321-431}$), 700µg of protein diluted in 100mM MOPS pH7.5 was added to the gel, and mixed under constant rotation at 4°C for 2-4hrs. Ethanolamine-HCl pH8 (100µl) was added to the gel and agitated for 1hr at 4°C to block unreacted esters binding to the column. At this stage the gel was transferred to an econocolumn (Biorad, UK). All the following steps were performed at 4°C. The column was prewashed once with 10ml of each of the following solutions: (1) 100mM MOPS, pH 7.5, (2) PBS pH7.2, (3) 100mM Glycine-HCl

pH2.4/150mMNaCl, (4) PBS pH 7.2. The serum was prepared by incubating at 56°C for 30min to inactivate complement. Following cooling on ice the serum was filtered through a 0.2µm filter and then spun in a microfuge at top speed for 10min. The serum was applied to the column and passed through four times. The serum was collected and stored at 4°C. The column was washed with PBS pH7.2 (15ml). The antibody was eluted in 15 x 1ml aliquots with 100mM Glycine HCl pH2.4/150mM NaCl. Each aliquot was collected into eppendorfs that contained 200µl 1M Tris pH8 to neutralise the elution buffer. Finally the column was washed with 10mls of (1) PBS pH7.2, (2) 100mM Glycine-HCl pH2.4/150mMNaCl and (3) PBS pH 7.2 and was stored at 4°C in PBS, 0.2% NaN_3. Each fraction of the purified antibody was assessed for its ability to detect the antigen by western blot. The purified antibody was concentrated using a protein-concentrator (Sartorious, UK) with a cut off of 10,000kD.

2.3.3 In vitro transcription and translation

^{35}S labelled protein was made using the TNT Coupled Rabbit Reticulocyte Lysate Transcription/Translation System (TNT, Promega, UK). Proteins were transcribed and translated from plasmids with a T7, T3 or SP6 promoter (generally pcDNA3 based plasmids). The reaction consisted of TNT Rabbit Reticulocyte Lysate (25µl), TNT buffer (2µl), template plasmid DNA (1µg approx.), RNasin Ribonuclease Inhibitor (1µl), amino acid mix minus methionine (1µl), translation grade ^{35}S radiolabeled Methionine (20-40µCi, Amersham, UK), RNA polymerase (T7, T3 or SP6 as required, 1µl) made up to 50µl with nuclease free ddH_2O. Reactions were incubated at 30°C for 2hrs and proteins were resolved on SDS-PAGE gels under standard conditions. Gels were fixed in 2-3 changes of fixer (45% methanol, 10% acetic acid) for several hours. This was followed by a 20min incubation in Amplify solution (Amersham, UK). The gels were rehydrated in water and then dried onto filter paper using a gel drier (Biorad, UK). Gels were exposed onto autoradiographic film for 12-24hrs at -70°C.

2.3.4 Protein Detection

SDS-PAGE

Standard SDS-polyacrylamide gel electropohoresis (SDS-PAGE) was carried out as detailed by Sambrook and Maniatis (Sambrook and Maniatis, 1989). 8-15% polyacrylamide gels were typically run at 50V to drive protein samples through the stacking gel, and then 70-80V through the resolving gel in the presence of SDS-PAGE running buffer (5mM Tris-HCl pH8.3, 50mM Glycine, 0.02% (w/v) SDS). Protein samples were prepared for electrophoresis by denaturation at 90°C for 7 minutes in the presence of SDS loading buffer (50mM Tris-HCl (pH6.8), 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol).

Western Blotting

Proteins were separated under standard reducing conditions on SDS-polyacrylamide gels. Separated proteins were blotted onto nitrocellulose membrane at 30mA for 14hrs. Blots were blocked for 1h in TBST (1mM Tris-HCl pH8, 15mM NaCl, 0.005% Tween.) containing 5% (w/v) non-fat dried milk and then probed for 2h with the primary antibody in the same buffer. Blots were then washed for 40min in several changes of TBST followed by probing for a further 1h with the appropriate horse-radish peroxidase coupled secondary antibodies (Amersham, UK). Bound antibody was detected by enhanced chemiluminesence using the Supersignal reagent (Pierce, UK).

Western blot analysis of protein expression on human tissues and tumour cell lines.

Total protein lysates (100μg/lane) prepared from a range of normal human tissues (Clontech, UK) were electrophoresed on 12% polyacrylamide gels under standard SDS-PAGE conditions. Total protein lysates were also prepared from a number of human tumour cell lines by lysing cells at 10^7/ml in SDS-PAGE loading buffer. These lysates were also resolved on 12% polyacrylamide gels. Proteins were transferred onto 0.45μm nitrocellulose membrane and were probed for protein expression using specific antibodies.

Enzme Linked Immunosorbent Assay (ELISA)

Detection of IL-1β in the supernatant of cells was carried out using the human IL-1β cytosets antibody pair assay system (Biosource, UK). 96-well ELISA plates (Nunc immulon maxisorp, Nunc, UK) were incubated with 4μg/ml coating antibody (100μl/well in PBS pH7.4) for 12-18hrs. Plates were blocked with PBS 0.5% BSA fraction V (300μl/well) for 2 hours. The wells were washed three times with PBS pH7.4 0.1% (v/v) Tween. The supernatant (100μl) was added to each well, compared against the relevant IL-1β standards, followed by 0.4μg/ml detection antibody (50μl/well, in PBS 0.5% (w/v) BSA, 0.1% (v/v) Tween) and allowed to incubate for 2 hrs. Wells were washed as before and incubated with streptavidin-HRP (100μl/well in PBS (1:2500 dilution)) for 30mins. The wells were washed again followed by incubation with tetramethylbenzidine (TMB, 100μl/well, PIERCE, UK). Upon colour change the reaction was stopped by addition of 1.8N H_2SO_4 (50μl/well). The plate was read at OD_{450} using OD_{595} as a correction on a plate reader.

2.4 MAMMALIAN CELL CULTURE BASED METHODS

2.4.1 Cell culture and transfections

Calcium-phosphate precipitation transfection

Human Embryonic Kidney 293T cells were cultured in DMEM containing 10% FCS. Transfection of cells was carried out by the calcium-phosphate precipitation method. Cells were seeded at the appropriate density as shown in Table 2.2. Precipitates were prepared by mixing the appropriate amount of DNA made up to 100μl (or 200μl for large scale) with sterile 0.5XTE (5mM Tris-HCl pH8, 0.5mM EDTA) with 25μl (or 50μl) 2.5M $CaCl_2$. To this 125μl (or 250μl) 2XHBS buffer (280mM NaCl, 10mM KCl, 1.5mM Na_2HPO_4, 12mM dextrose, 50mM HEPES, pH7.1) was added dropwise. Following a 30-minute incubation the complexes were added to the cells. After 6hrs the complexes were removed from the cells and replaced with fresh medium.

Table 2.2: Plating cells for calcium-phosphate transfection

Plating Format	Number of cells plated	Transfection scale
6-well plate	2×10^5/well	Small (250µl)
6cm dish	5×10^5	Small (250µl)
10cm dish	2×10^6	Large (500µl)

Fugene transfection

MCF-7 and HeLa cells were cultured in RPMI containing 5% FCS. Transfection of MCF-7 and HeLa cells was carried out using Fugene-6 (Roche, UK). Cells were plated at 1×10^5 per well of a 6-well plate and after approximately 18hrs they were transfected with the indicated amounts of plasmid. For plate based β-galactosidase reporter assays, cells were also transfected with 50ng pcMVβgal. 2µl of Fugene-6 were used per well of a six well plate, diluted to 100µl with serum free RPMI. The Fugene/RPMI mixture was allowed to incubate for 10mins, to allow for the lipid to disperse, prior to dropwise addition of the mixture to the DNA. The resulting mixture was incubated for 15mins to allow complexes to form and then the complexes were added to the cells. After 6hrs the complexes were removed from the cells and replaced with fresh medium.

Plate based β-galactosidase reporter assay to measure apoptosis

24-48h post transfection cells were fixed by replacing the medium with 1ml of PF fixative (2% paraformaldehyde, 0.2% gluteraldehyde in PBS pH7.2) per well and incubated for 5-10mins. Cells were washed with 2mls PBS pH7.2 for 5mins, followed by addition of 0.8ml β-gal staining buffer (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$, 0.02% (v/v) NP-40, 0.01% (w/v) SDS, 1mg/ml X-gal in PBS pH7.2) per well. Cells were incubated at 37°C until a sufficient number of the cells had developed a blue colour (1-12 hrs). The reaction was terminated by addition of PBS pH7.2, 0.5% (w/v) sodium azide (2ml/well). For apoptosis assays blue cells were counted under light microscopy and were assessed for features of apoptosis such as membrane blebbing and nuclear condensation. The number of apoptotic blue cells and live blue cells observed were recorded. At least 300 cells were counted per treatment.

Generation of stably transfected cell lines

Plasmids were linearised by digestion with a single cutting restriction enzyme. Each linearised plasmid (10μg) was incubated with 800μl of THP.1 cells at 10^7 cells/ml on ice for 10 minutes. Plasmids were transfected into THP.1 cells by electroporation (Biorad Gene Pulser II, Biorad, UK) at 0.25kV, 950μF with resistance = ∞. The cells were allowed to recover on ice for 10mins prior to resuspension in 10ml RPMI 10% FCS. After 48 hours incubation at 37°C, cells were washed and transferred into medium containing 1.4mg/ml G418 neomycin (Melford Labs, UK). This concentration of G418 neomycin was determined as optimal by incubating THP.1 cells with different concentrations of G418 neomycin over seven days. Cell viability was assessed by Trypan Blue exclusion assay and Hoechst staining. 1.4mg/ml was considered a concentration that would not be too high as to prevent transfected cells from proliferating, but high enough, that non-transfected cells would be killed. Cells were selected for 3 weeks at which stage G418 resistant cells were isolated and further selected in 1mg/ml G418. Expression of transfected proteins was confirmed by western blot on lysates from these cells using specific antibodies.

Trypan blue assay

Cell viability was determined using the trypan blue exclusion assay. 50μl of cell culture was incubated with 50μl Trypan blue reagent for 5mins. The cells were mounted on a haemocytometer and counted using a light microscope. Blue cells were characterised as non-viable while cells that had not taken up the blue stain were considered alive.

Hoescht staining

Hoescht staining was also used to determine cell viability. Cells were incubated in 50μl of a10μM Hoescht solution in 4% paraformaldehyde at 37°C for 30mins. The cells were mounted on microscope slides and viewed under the UV filter of a microscope. Nuclei were scored as apoptotic if they exhibited margination and condensation of the chromatin.

2.4.2 Luciferase reporter assays

Luciferase reporter assays were carried out using the Luciferase Reporter Gene Assay, constant light signal (Roche, UK) or using in-house prepared luciferase reagents (see below). Both methods gave comparable results.

For NFκB assays 2 x 10^5 HEK 293T cells were transfected with 100ng pGL3.5xκB-luc, 50ng pcMVβgal and the required amounts of the relevant expression plasmids. Total plasmid amounts per well were equalised with pcDNA3 empty vector. The p53 reporter assays were set up in the same way using 100ng p53-luc in place of the NFκB reporter. After 24hrs - 48 hrs transfection the media was removed from cells and they were washed with PBS pH7.2. Cells were incubated with PBS (150µl) and luciferase reagent (150µl, Roche, UK) for 5mins to allow lysis of the cells and for the light reaction to begin. The light emitted from triplicate 50µl aliquots of lysate was measured in black 96 well plates by luminometry. Transfection efficiencies were normalised by measuring β-gal activities. 50µl of cell lysate was incubated with 0.66mg/ml ONPG in 1ml Z buffer (60M Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH 7) at 37°C until a yellow colour developed (typically 30min). The reaction was stopped by addition of 1M Na_2CO_3 (300µl) followed by measurement of β-galactosidase activity at OD_{420}.

Luciferase reporter assay (in-house method)

HEK293T cells were transfected as described above. Each well of cells of a 6-well plate was lysed in 200µl of Reporter Lysis Solution (100mM Hepes pH8, 2mM $MgCl_2$, 2% (v/v) Triton-X100, 5µM DTT). Lysates were freeze-thawed to aid lysis of cells. 40µl of Luciferase assay reagent (20mM Glycylglycine, 1mM MgCl2, 100µM EDTA, 278µg/ml ATP, 213µg/ml Co Enzyme A, 2.5µM DTT, 50µl/ml beetle luciferin (Promega, UK)) was added to each of 20µl aliquots of cell lysate. The luminesence emitted from each sample was measured in a black 96 well plate as described above.

2.4.3 Subcellular localisation by immunofluoresence staining

HeLa cells were plated at 1 x 10^5 cells per well of a 6-well plate, on sterile coverslips. Where required cells were transfected with the relevant expression

plasmids. 24hrs after transfection the media was aspirated from the wells. Each monolayer was washed in 3 x 2ml PBS pH 7.2. The cells were fixed in 3% paraformaldehyde in PBS pH7.2 for 10mins. After 3 x 10min washes in PBS, the cells were permeabilised in PBS, 0.15% (v/v) Triton for 10mins. Cells were blocked in 2% (w/v) BSA for 30mins followed by staining with 100µl of a mixture of the relevant antibodies, each at 1:100 dilution in 2% (w/v) BSA, for 1hr. Where two antibodies were used, one was a rabbit polyclonal and the other was a mouse monoclonal antibody to prevent cross reactivity of the secondary antibodies. Cells were washed in 3 x 10min of PBS, 2% (w/v) BSA. The coverslips were incubated with 100µl mixture of the appropriate secondary antibodies (Molecular Probes, UK) at a 1:250 dilution in 2% (w/v) BSA for 45mins. Generally one of the secondary antibodies would be FITC conjugated to give a green immunostaining pattern and the other would be Rhodamine conjugated to give a red staining pattern. Cells were washed x 3 in PBS and the coverslips were mounted in antifade reagent (Molecular Probes, UK). Immunofluoresence was visualised using a confocal microscope (Biorad, UK) under the FITC and Rhodamine channel, or using a fluorescent microscope (Zeiss, UK) using a FITC and Rhodamine filter as required.

2.4.4 Co-immunoprecipitation assays.

HEK293T cells were plated on 10cm plates as described the day before transfection. Cells were transfected with 5-10µg of the appropriate plasmids. Cells were lysed 24-48hrs after transfection in 800µl IP lysis buffer (50mM Tris-HCl, pH8.0, 150mM NaCl, 1% (v/v) NP-40, 100µM PMSF, 10µg/ml leupeptin, 1µg/ml aprotinin) on ice for 15mins. The lysates were cleared by centrifugation at 10,000g for 10mins and pre-cleared with 20µl agarose-coupled protein A/G (Santa Cruz) for 1hr at 4°C under rotation. Immunoprecipitation was carried out using 30µl protein A/G beads and 1µg of the appropriate antibody (or 7µl of the anti-CARDINAL antiserum). Samples were incubated with rotation for 3-6hrs at 4°C. Complexes were pelleted at 1000g and washed 3-4 timed in IP lysis buffer containing 0.1% (v/v) NP-40. Immunoprecipitates were then analysed by immunoblotting using appropriate primary and secondary antibodies.

2.5 YEAST TWO HYBRID METHODS

2.5.1 Expansion of pACT2 human leukaemia Jurkat cDNA library

Human leukaemia cDNA library in pACT2 (Clontech, UK) was expanded in DH5α *E.coli*. The titre of the library was calculated by plating different concentrations of the library on LB plates containing ampicillin. After 18hrs incubation at 37°C the number of colonies on each plate was counted and the titre was calculated to be 6.8 x 10^9cfu/ml. The size of the library is 1 x 10^6 clones. Three times the complexity of the library (i.e. 3 x 10^6 clones) is required for the yeast-two-hybrid screen. To expand the library to obtain at least 3 x 10^6cfu, the library needed to be plated on 120 plates (at 50,000cfu/plate). Therefore 1µl (equivalent to 6.8 x 10^6cfu) of library was resuspended in 30ml of LB and 250µl of this culture was plated on each of 125 15cm LB plates containing ampicillin. Plates were incubated at 37°C for 18hrs. Colonies were harvested by addition of 5ml LB broth to each plate and using a cell scraper to collect the cells into a GSA centrifuge tube. Bacterial cells were pelleted at 6000rpm in a Sorvall centrifuge at 4°C. Bacterial cell pellets were resuspended and pooled into 30ml P1 buffer, 100µl/ml RnaseA. P2 buffer (30ml) was added and after 2min incubation on ice, P3 (30ml) was added. Genomic DNA was allowed to precipitate on ice for 5mins and the lysates were centrifuged at 10,000g for 40mins. The supernatant was decanted into fresh tubes and due to the high viscosity of the liquid the lysates were centrifuged again at 10,000g for 30mins. Plasmid DNA was purified using two Qiagen tip-500 columns according to manufacturer's instructions. Library plasmid DNA was resolved on a 0.6% agarose gel. The plasmid concentration was measured by UV spectroscopy and by comparison of linearised plasmid DNA (*XhoI* restriction digest) against a λ*HindIII* ladder (NEB, UK).

2.5.2 Yeast Transformation

Saccharomyces cerevisiae strain Y190 (Clontech, UK) was transformed by the lithium acetate method as described by (Gietz *et al.*, 1995) using the pAS2-1/pACT2 Matchmaker Two-Hybrid System 2 kit (Clontech, UK). Three general protocols for yeast transformation were used depending on the application. These protocols are modifications of the same general protocol. The three protocols are small-scale co-transformation; where two different plasmids are transformed

together, small-scale sequential transformation; where one plasmid is singly transformed into yeast followed by transformation of the second plasmid, and large-scale sequential transformation, which was used for the library screen.

The general object of the yeast transformation protocol is to induce yeast to take up either a pAS2-1 based expression plasmid which contains the GAL-4 DNA binding domain (BD), or a pACT2 based expression plasmid which contains the GAL-4 activation domain (AD), or both plasmids together. pAS2-1 plasmids contain a tryptophan synthetase gene allowing yeast transformed with this plasmid to grow on media lacking tryptophan (Figure 2.1A). Similarly pACT2 plasmids contain a leucine synthetase gene enabling pACT2-containing yeast to grow on leucine free medium (Figure 2.1B). Likewise if both plasmids are present in a yeast cell it can grow on media lacking both amino acids. Finally if the plasmids are engineered to encode a pair of proteins that interact, transcription from the histidine reporter gene will be activated and consequently growth on media lacking histidine, tryptophan and leucine will be observed (Table 2.3).

Yeast Media

The following types of media were prepared for use in the yeast transformation protocols. Yeast Extract/Peptone/Dextrose (YPD, 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, pH5.8 (2% (w/v) agar for plates)) was used for non-selective growth of yeast cultures. For growth of yeast transformed with a plasmid or pair of plasmids, yeast were grown on selective media as detailed above. To prepare the selective media a 10X amino acid stock solution was prepared (300mg/ml L-isoleucine, 1500mg/ml L-valine, 200mg/ml L-adenine hemisulphate salt, 200mg/ml L-arginine HCl, 300mg/ml L-lysine HCl, 200mg/ml L-methionine, 500mg/ml L-phenylalanine, 2000mg/ml L-threonine, 300mg/ml L-tyrosine, 200mg/ml L-uracil) which contained Histidine (200mg/ml) Leucine (1000mg/ml), or Tryptophan (200mg/ml) as required. The appropriate amino acid stock solution (Leu-, Trp-, Leu/Trp- or His/Leu/Trp-) was used to prepare the required synthetic dropout medium (6.7g/L yeast nitrogen base with ammonium sulphate, without amino acids, 1X amino acid stock solution, 2% (w/v) dextrose, pH5.8 (2% (w/v) agar for plates)). His/Leu/Trp- media also contained 25mM 3-aminotriazole (3-AT).

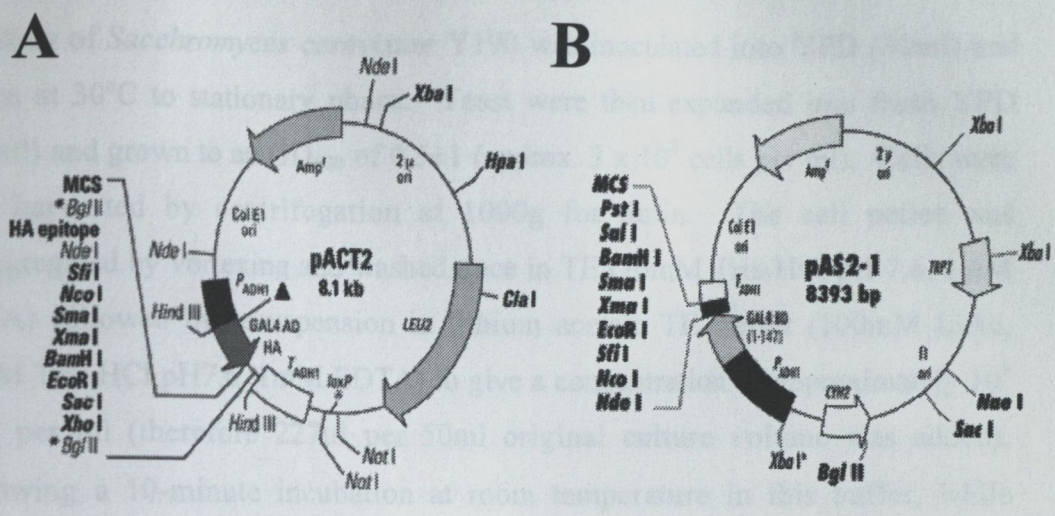


Figure 2.1:

Schematic diagram of yeast-two-hybrid plasmids

(A) pACT2 vector map, pACT2 generates a fusion of the GAL-4 activation domain (GAL4 AD), a HA epitope tag and a protein of interest into the MCS. This plasmid also encodes a leucine synthetase gene(LEU2) (This figure was reproduced from the Clontech catalogue) **(B)** pAS2-1 vector map, pAS2-1 generates a fusion of the GAL-4 DNA binding domain (GAL4 BD) and a protein of interest cloned into the multiple cloning site (MCS). This plasmid also encodes a tryptophan synthetase gene (TRP1)

Table 2.3: Growth properties of *Sacchromyces cerevisiae* Y190 transformed with yeast-two-hybrid plasmids. (+) indicates ability to grow (-) indicates lack of ability to grow

Transformed plasmid (s)	Leu-	Trp-	Leu/Trp-	His/Leu/Trp-
pAS2-1	-	+	-	-
pACT2	+	-	-	-
pAS2-1(X)/pACT2(Y) (X and Y are not interacting proteins)	+	+	+	-
pAS2-1(X)/pACT2(Y) (X and Y are interacting proteins)	+	+	+	+

Small scale transformation

A colony of *Sacchromyces cerevisiae* Y190 was inoculated into YPD (30ml) and grown at 30°C to stationary phase. Yeast were then expanded into fresh YPD (500ml) and grown to an OD_{600} of 0.5±1 (approx. 3 x 10^8 cells per ml). Cells were then harvested by centrifugation at 1000g for 5min. The cell pellet was disaggregated by vortexing and washed once in TE (10mM Tris-HCl pH 7.6, 1mM EDTA) followed by resuspension in lithium acetate TE buffer (100mM LiAc, 10mM Tris-HCl pH7.6, 1mM EDTA) to give a concentration of approximately 10^9 cells per ml (therefore 227µl per 50ml original culture volume was added). Following a 10-minute incubation at room temperature in this buffer, while vortexing every 2mins, 100µl aliquots (10^8cells) of the competent yeast were added to tubes each containing herring sperm (9.4µl), and plasmid (up to 5µg). For single transformation only one plasmid is transformed, but for co-transformation both the bait and prey plasmids are transformed together. After a brief vortex to mix, 40% PEG /LiAc /TE (40% (w/v) polyethylene glycol (PEG), 100mM LiAc, 10mM Tris-HCl pH7.6, 1M EDTA, 600µl) was added to each sample. Samples were vortexed vigorously for 10 seconds followed by incubation at 30°C with 200rpm rotation for 30min. DMSO was added to a final concentration of 10% to disrupt the yeast cell wall. Cells were heat shocked at 42°C for 15min, followed by a 2min recovery time on ice. 700µl ice-cold ddH_2O was added to each sample and the sample was mixed by inversion. Yeast cells were pelleted at 1000g for 5min in a microfuge and were then resuspended in 1ml 1xTE. 100µl of each sample was plated on the respective selective medium. Plates were incubated at 30°C for 4-7 days.

For transformations where lithium sorbitol was used cells were incubated in lithium sorbitol buffer (100mM LiAc, 10mM Tris-HCl pH7.6, 1mM EDTA, 1M Sorbitol) at 100µl per ml of starter culture, instead of the lithium acetate TE buffer, for 30mins at 30°C. Cells were pelleted by centrifugation at 1000g for 5mins followed by resuspension in lithium sorbitol buffer at 4.5µl/ml of starter culture on ice. 100µl aliquots (10^8cells) of the competent yeast were added to tubes each containing herring sperm (9.4µl), and plasmid as before and the above protocol was followed from this point.

Where YPD recovery was used to improve transformation efficiency, cells were resuspended in 10ml YPD after the heat shock step and incubated for 1-2hrs at 30°C. Cells were harvested by centrifugation at 1000g for 5mins and plated as before.

Library Screen Transformation

The two-hybrid protein-protein interaction library screen was conducted using a protocol that is a scaled up version of the small-scale transformation detailed above. pAS2-1-caspase-10^{1-372} was singly transformed into Y190 cells as described above. Trp- dropout media (150ml) was inoculated with pAS2-1-caspase-10^{1-372} colonies. The resulting culture was incubated at 30°C for 16-18hrs at 250rpm until the cells had reached stationary phase (OD_{600} of 2.0). A portion of the starter culture was diluted in YPD to produce an OD_{600} of 0.2 in 500ml YPD and expanded as for the small-scale transformation. Upon harvesting, the cells were washed in 125ml TE. 2.27ml 1xLiAc/TE was used to resuspend the cell pellet. The competent yeast cells were added as before to each of 20 reaction tubes. Thus each transformation reaction consisted of 5µg pACT2-library plasmid; 20µl denatured herring sperm carrier DNA, 100µl pAS2-1-caspase-10^{1-372} transformed yeast competent cells and 600µl 40% PEG/LiAc solution. Cells were vortexed and allowed to recover at 30°C for 30mins as before. DMSO (70µl) was added to each reaction prior to heat shock. Samples were then pooled in 1L YPD and incubated at 30°C, 200rpm for 1hr. Cells were harvested in 50ml aliquots by centrifugation at 1000g and each cell pellet was resuspended in 1ml TE.

For selection of positive interacting clones, 250µl yeast were plated on each of 80 15cm petri dishes containing synthetic dropout (SD) medium lacking tryptophan, leucine and histidine and supplemented with 25mM 3-aminotriazole (3-AT). 10µl was plated on each of three LT- plates to assess transformation efficiency. Plates were incubated at 30°C for 10 days. Putative positive colonies were picked after 10 days of incubation and restreaked onto selective medium.

2.5.3 Expression of yeast proteins

To assess that the GAL4 fusion proteins were being expressed in yeast, protein lysates were made. A single colony of transformed Y190 (e.g. pAS2.1 or pAS2.1-caspase10$^{1-372}$) was inoculated into the appropriate selective medium and grown to an OD_{600} of 2.0. Cultures were diluted to an OD_{600} of 0.2 and expanded in YPD to stationary phase (OD_{600} = 0.6). To chill the cells they were poured into, pre-chilled, GSA centrifuge tubes, half filled with ice. Cells were pelleted in a GSA centrifuge at 1000g for 5mins at 4°C. The supernatant was discarded and the yeast cell pellet was resuspended in ice cold ddH_2O (50mls). The cell pellet was recovered by centrifugation at 1000g for 5min at 4°C. The cell pellet was frozen in liquid nitrogen and stored at –70°C.

To extract the protein from the yeast cells, 100ml of warm cracking buffer (8M urea, 5% (w/v) SDS, 40mM Tris-HCl pH 6.8, 0.1mM EDTA, 0.4mg/ml bromophenol blue, 0.3mM leupeptin, 145mM benzamidine, 0.37mg/ml aprotinin, 60°C) per OD unit was added. Cells were added to 80mg glass beads per 7.5 OD_{600} unit of cells and heated at 70°C for 10min. Samples were vortexed for 1 min. Cells were pelleted by centrifugation at 14000 rpm for 5mins at 4°C. The supernatant was removed and denatured at 95°C for 7 minutes. Proteins were resolved on SDS-PAGE gels under standard reducing conditions. Expression of the fusion proteins was determined by immunoblot with the anti-GAL4 antibody.

2.5.4 Plasmid rescue from yeast

Plasmids were isolated from transformed yeast by the following protocol. A single colony of Y190 transformed with a particular plasmid was inoculated into 5ml of the appropriate selective medium. After three days incubation at 30°C with 230rpm shaking, yeast cells were pelleted at 1000g for 5mins. Pellets were resuspended in rescue buffer (10mM Tris, pH 8, 0.1M NaCl, 1mM EDTA, 2% (v/v) Triton X-100, 1% (w/v) SDS, 200µl). Cells were lysed and plasmids were extracted following vortexing for 2.5mins with glass beads (300mg) in the presence of phenol/chloroform/isoamyl alcohol (200µl). The aqueous phase was removed and the debris pelleted. Plasmids were precipitated from the supernatant with two volumes of ethanol and 0.1 volume 3M NaOAc at –70°C for 10mins. DNA was pelleted in a microfuge at top speed for 15mins, washed in 70% ethanol and

resuspended in TE (5µl). This DNA was used as a template for PCR amplification of the plasmid insert (Table 2.1). Alternatively, the DNA was transformed into bacteria to rescue the plasmid. For doubly transformed yeast where the selection of the pACT2 plasmid is required (as is necessary in a yeast-2-hybrid screen) DNA was transformed into *E.coli* HB101 cells and grown on media containing ampicillin and lacking leucine. Plasmid DNA was extracted from these cells by the usual protocol.

CHAPTER III

Use of the Yeast-Two-Hybrid Interaction Trap Assay to seek novel binding partners for caspase-10.

3.1 INTRODUCTION

As described in the introduction, the cysteine protease family of caspases can be divided into two broad categories: initiator caspases and effector caspases. The initiator caspases, which include caspase-9, caspase-8 and caspase-10 are thought to act at the apex of cell death signalling pathways. The functions of caspases-9 and -8 are fairly well characterised, being the apical proteases in the Apaf-1 and Fas pathways respectively. In contrast, little is known concerning the function of caspase-10 (FLICE 2/Mch4) in apoptosis. Due to the extensive homology between caspase-10 and caspase-8, caspase-10 has been defined as an initiator caspase. However, it remains unclear whether caspase-8 and -10 are functionally redundant. Therefore the identification of a caspase-10-specific binding partner could reveal much about the function of caspase-10 in apoptosis.

3.1.1 Caspase-10

Caspase-10 is very similar in structure to caspase-8, the prodomain of caspase-10 also contains two death effector domain (DED) modules (Fernandes-Alnemri *et al.*, 1996). Caspase-10 has been shown to activate caspase-3 and to provoke apoptosis when overexpressed (Fernandes-Alnemri *et al.,* 1996; Vincenz and Dixit, 1997). This is similar to properties exhibited by caspase-8 and, thus it has been broadly assumed that caspase-10 functions in a similar mode to casapase-8, as an apical caspase in death receptor contexts (Muzio *et al.*, 1996). This assumption suggests that caspase-10 and caspase-8 are functionally redundant.

The role played by caspase-8 as the apical caspase in the Fas death receptor pathway is well characterised (Muzio *et al.*, 1997). Deletion of *CASP-8* in mice results in a lethal embryonic phenotype. Moreover, cells in the *CASP-8* knockout mouse are completely resistant to TNF, DR3 and Fas-induced cell death (Varfolomeev *et al.*, 1998), although they remain sensitive to other death inducing stimuli, such as UV irradiation or staurosporine, which activate the mitochondrial cell death pathway (Figure 1.2; Introduction; Varfolomeev *et al.*, 1998). This total blockage of death receptor-induced apoptosis demonstrates that functional caspase-8 is absolutely required in the death receptor pathway. If caspase-10 and caspase-8 were functionally redundant, it would be expected that caspase-10 would compensate for the loss of functional caspase-8 in these mice and the apoptotic

phenotype of the *CASP-8* null mouse would be less severe. On the other hand, the murine version of caspase-10 has not yet been found, so it is likely that caspase-10 arose from a recent gene duplication event of caspase-8, and does not exist in mice. Indeed, caspase-8 deficient human cells, unlike their murine counterparts, remain susceptible to TRAIL and Fas, but the cell death response is significantly delayed in comparison to wild type cells (Kischkel *et al.*, 2001).

In human cells, the evidence supporting a role for caspase-10 in death receptor induced apoptosis is conflicting. TRAIL, TNF and FasL use similar mechanisms to induce apoptosis, through assembly of a death inducing signalling complex at the cell membrane (DISC; Figure 1.3 in Introduction). Isolation of the Fas DISC from cells by co-immunoprecipitaion with anti-Fas antibodies show that the complex is comprised of Fas, FADD and caspase-8 molecules (Kischkel *et al.*, 1995). Caspase-10 has been shown to interact with components of the Fas, TNF and TRAIL DISCs when overexpressed (MacFarlane *et al.*, 1997; Pan *et al.*, 1997b; Vincenz and Dixit, 1997). However, since all these studies rely on overexpressed caspase-10, they do not prove that the physiological role of caspase-10 is in Fas, TNF or TRAIL induced apoptosis. In such experiments caspase-10 may mimic caspase-8 when present at non-physiological levels. Isolation of the TRAIL DISC from a variety of cell lines by co-immunoprecipitation has shown that FADD and capsase-8 are always present in the complex (Bodmer *et al.*, 2000; Sprick *et al.*, 2000). However certain studies have shown caspase-10 to be present (Kischkel *et al.*, 2001) while others find no evidence that caspase-10 is a compontent of the TRAIL DISC (Bodmer *et al.*, 2000).

Thus, the true function of caspase-10 remains something of a grey area. It may be the case that caspase-10 functions in a caspase-8 like manner in tissues where caspase-8 is absent, or of low abundance, in a compensatory mechanism. This would suggest that caspase-10 does not have a global role in death receptor pathways, but is quite cell and context specific. Given the fact that caspase-10 has been assumed to have a similar function to caspase-8 based on the homology between the two genes, it is entirely plausible that the primary function of caspase-10 has been overlooked. Thus the approach of using a two-hybrid screen could be

very useful in elucidating the function of caspase-10 through the characterisation of a novel binding-partner for this protein.

3.1.2 The Principle of the Yeast-Two-Hybrid System

The yeast two-hybrid system is a method enabling the identification and cloning of proteins that interact with a protein of interest. Originally developed by Stanley Fields and co-workers, the method demonstrates whether a pair of proteins can interact through exploitation of the Gal4 promoter system (Fields and Song 1989). The Gal4 transcription factor has two domains, the activation domain and the DNA binding domain (Keegan *et al.*, 1986). Only when the two domains are juxtaposed, can a reporter gene (usually histidine or β-galactosidase), driven by the Gal4 transcription factor, be expressed. In the yeast-two-hybrid system one protein is fused to the activation domain (AD) while the other is fused to the DNA binding domain (BD). Only if the two proteins physically interact will the two Gal4 domains come in contact and the strength of the interaction is measured by the relative expression of the reporter gene (Chien *et al.*, 1991).

This strategy can be extended to search for novel proteins that interact with a protein of interest. If a cDNA library is fused to the Gal4 activation domain then the library can be searched using the yeast-two-hybrid method with a specific protein attached to the Gal4 DNA binding domain as bait. Only if the "bait" protein finds a binding partner in the library will the reporter gene be expressed. This method has proven very useful for discovering proteins involved in the cell death signalling pathways, since many of the key steps involve protein-protein interactions. Thus many proteins have been discovered functionally and physically linked to proteins already implicated in apoptosis through use of this method (Table 3.1).

3.1.3 Caspase-10 as a Candidate for a Yeast Two Hybrid Screen

We performed a yeast-two-hybrid screen to seek novel binding partners for caspase-10 that may be involved in regulating the activation or activity of this caspase. Caspase-10 is a suitable choice as a candidate for a yeast-two-hybrid screen for a number of reasons. Firstly, as discussed above, little is known concerning the function of caspase-10, therefore substantial insights on the role of this protease in

Table 3.1: Examples of proteins cloned through use of the yeast-two-hybrid screen protocol.

Bait protein	Cloned protein	Function	Ref
TNFR-1	TRADD	Apoptosis, NFκB activation	(Hsu *et al.*, 1995)
	WSL-1	Apoptosis, binds TRADD	(Kitson *et al.*, 1996)
Fas	FADD	Apoptosis	(Chinnaiyan *et al.*, 1995)
	UBC9	Ubiquitin conjugating enzyme (involved in protein degradation)	(Becker *et al.*, 1997)
	F1Aα	Induces apoptosis	(Chan *et al.*, 1999)
TRADD	RIP	NFκB activation	(Hsu *et al.*, 1996)
RIP	RIP3	Apoptosis, NFκB activation	(Sun *et al.*, 1999)
Caspase-2	ISBP	Ich1S binding protein, inhibits caspase-2 processing	(Ito *et al.*, 2000)
	PACAP	Apoptosis, also binds caspase-9	(Bonfoco *et al.*, 2001)
Caspase-3	Gelsolin	Substrate (inactive mutant of caspase-3 was used as bait)	(Kamada *et al.*, 1998)
Caspase-8	FLASH	Apoptosis (?)	(Imai *et al.*, 1999)
Caspase-9	DRADD	Apoptosis, binds Rb	(Adrain, 2001)
Caspase-10	CASH/FLIP	Inhibitor of apoptosis	(Goltsev *et al.*, 1997)
	DEDAF	DED binding protein	(Zheng *et al.*, 2001)
Bax	MAP1	Proapoptotic Bcl-2 family member	(Tan *et al.*, 2001)
	Bif1	Proapoptotic Bcl-2 family member	(Cuddeback *et al.*, 2001)
Bcl-xL	Aven	Anti-apoptotic, binds Apaf-1	(Chau *et al.*, 2000)
Bcl-2	Bad	Proapoptotic Bcl-2 family member	(Yang *et al.*, 1995)
Mcl-1	Bok	Proapoptotic Bcl-2 family member	(Hsu *et al.*, 1997)

apoptosis could be made through identification of its binding partners. Secondly, the structure of the caspase-10 prodomain would suggest that it functions through protein-protein interactions, by virtue of the presence of the DED protein-protein interaction motifs.

Finally, at the time that this screen was initiated only one yeast-two-hybrid screen had been performed using the caspase-10 prodomain as the bait. The study in question identified a protein named CASH (Goltsev *et al.*, 1997). CASH has also been found independently as FLIP, Casper, I-FLICE, and CLARP (Irmler, 1997; Shu *et al.*, 1997; Hu *et al.*, 1997, Inohara *et al.*, 1997). This protein is thought to act as a decoy molecule leading to inhibition of the caspase-8 cell death pathway.

Thus, in using the prodomain of caspase-10 as the bait in a yeast-two-hybrid screen the aim would be to identify novel binding partners for caspase-10, which would allow us to further dissect the function of this caspase in apoptosis pathways.

3.2 RESULTS

3.2.1 Design of Caspase-10 Two-Hybrid Bait Protein

For the purpose of the yeast-two-hybrid screen, a bait plasmid comprising the N-terminal prodomain and the large subunit (amino acids 1-372) of caspase-10, fused to the DNA binding domain of the Gal-4 transcription factor was constructed (Figure 3.1A). This region contains the DEDs of caspase-10. This plasmid was transformed into *Sacchromyces cerevisiae* Y190 yeast cells and used to screen a human Jurkat cDNA library of Gal4 activation domain fusion proteins.

3.2.2 Expression of Caspase-10-GAL4BD

Expression of the bait protein from the pAS2-1-caspase-10^{1-372} plasmid was verified by western blotting of yeast lysates (Figure 3.1B). This confirmed that the GAL4 DNA binding domain (BD) was expressed as a 17kD protein as expected, and that the GAL4BD-caspase-10^{1-372} fusion was expressed as a 65kD protein.

3.2.3 Caspase-10^{1-372} does not interact with FADD

In overexpression experiments caspase-10 has been reported to interact with FADD through its DED motifs. To test this interaction, pAS2-1-caspase-10^{1-372} was co-transformed into Y190 yeast with pACT2-FADD (Figure 3.2). This was tested alongside a number of positive controls (Caspase-8pro/FADD and Caspase-9pro/Apaf(1-601)) as well as negative controls (pAS2-1/pACT2, Caspase-10^{1-372}/pACT2). Compared to the positive controls that showed strong growth on media lacking histidine, caspase-10^{1-372} exhibited no interaction with FADD indicated by its inability to grow on the selective medium. This result also demonstrated that pAS2-1-caspase10^{1-372} bait did not spontaneously activate the His reporter, as there was no growth observed when co-transformed with pACT2 empty vector.

3.2.4 Assessment of the transformation efficiency of the caspase-10 bait plasmid

In order to determine the suitability of caspase-10^{1-372} as a bait plasmid in a yeast-two-hybrid screen, the transformation efficiency was first tested. For a successful library screen the transformation efficiency of the yeast for a particular plasmid should be as high as possible and preferably greater than 5×10^5cfu/μg. This is to

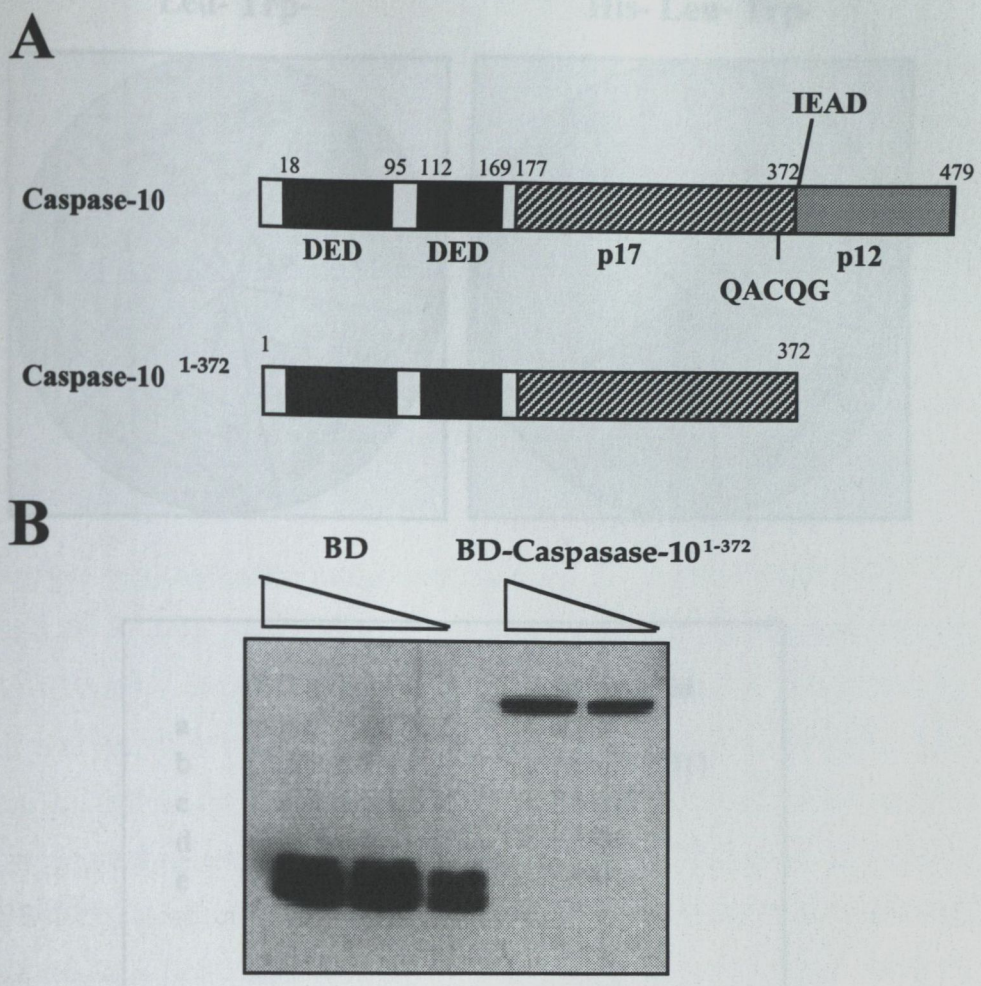
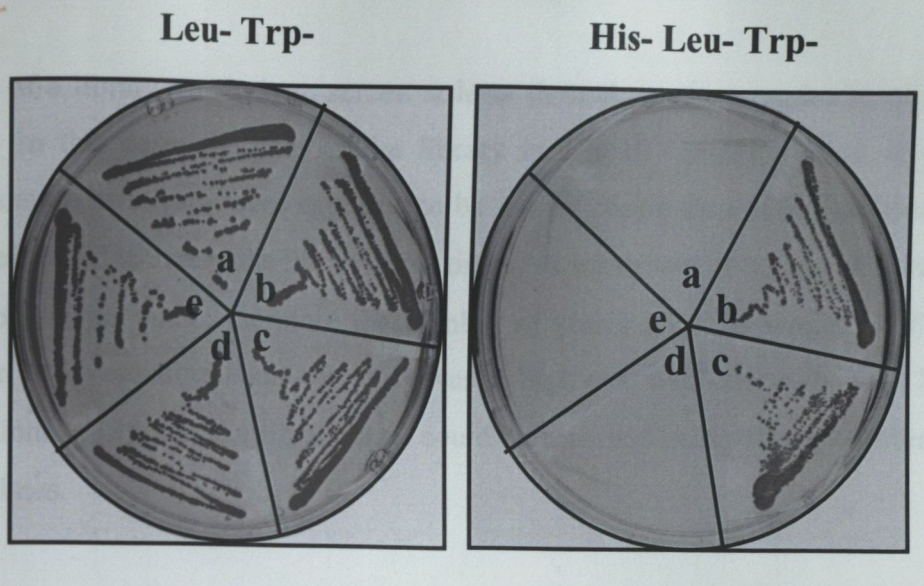


Figure 3.1:

Generation and expression of caspase-10^{1-372} yeast-two-hybrid bait.

(A) Schematic representation of caspase-10 structure. The Death Effector Domain (DED) regions, the large subunit and the small subunit are indicated by black, hatched and grey shading respectively. Numbers represent amino acid positions and the caspase-cleavage site (IEAD) and the active site (QACQG) are indicated. A deletion mutant of caspase-10 (Casp-10^{1-372}) was made to include the prodomain (the two DEDs) and the large subunit. This truncation was cloned into the pAS2-1 vector to be used as bait in the yeast-two-hybrid assay.

(B) Western blot analysis of protein lysates from Y190 yeast transformed with either pAS2-1 empty vector (lanes 1-3) or pAS2-1-Caspase-10^{1-372} (lanes 4-5), demonstrating expression of GAL4-BD and the GAL4-BD- Caspase-10^{1-372} fusion respectively.



	BD hybrid	AD hybrid
a	none	none
b	Caspase-9pro	Apaf(1-601)
c	Caspase-8pro	FADD
d	Caspase-10(1-372)	FADD
e	Caspase-10(1-372)	none

Figure 3.2

Caspase-10^{1-372} does not interact with FADD in yeast.

The yeast-two-hybrid assay was used to determine if there is an interaction between a Gal4 DNA binding domain (BD) fusion of Caspase-10^{1-372} and an activation domain fusion (AD) of FADD. The presence of interacting pairs was indicated by the transactivation of the HIS3 reporter gene in yeast, which confers ability to grow on medium lacking histidine. *Left*, control plates showing equal loading of yeast; *right*, the same transformants growing on plates lacking histidine where colony growth indicates an interaction. The Caspase-10/FADD interaction was tested in parallel with the positive controls (Caspase-8pro/FADD and Caspase-9pro/Apaf(1-601)) and negative controls (pAS2-1 empty vector/pACT2 empty vector and Caspase-10^{1-372}/pACT2 empty vector).

maximise the number of library clones screened and also to ensure that the number of plates that need to be plated for the screen is not prohibitive.

The aim of a library screen is to screen at least three times the complexity of the library. In this case, the size of the library is 3×10^6 clones. Thus, if the transformation efficiency were expected to be 1×10^4cfu/μg then 900μg of library would be needed to screen the library three times. A maximum of 1.25μg of library can be plated per plate; therefore the number of plates required would be 720. However, if the transformation efficiency was as high as 1×10^6cfu/μg then 1×10^8 library clones (33 times the library size) could be screened using 100μg of library and 80 plates.

To assess the transformation efficiency, pAS2-1 or pAS2-1-caspase-10^{1-372} transformed Y190 cells were sequentially transformed with varying amounts of either pACT2 empty vector or pACT2-library plasmid. The average transformation efficiency was between 4 and 9×10^4cfu/μg DNA after 5 days incubation. The transformation efficiency for pAS2-1-caspase-10^{1-372} transformed cells was the same as for pAS2-1 transformed cells (Figure 3.3A). This indicates that expressing caspase-10^{1-372} in yeast cells did not cause any overt toxicity compared to cells expressing the Gal4 DNA binding domain alone. During the screen, yeast colonies are incubated for up to 10 days, so to investigate if longer incubation time had an effect on the transformation efficiency, colony counts were made at 5 and 7 days (Figure 3.3B). The longer incubation time only marginally increased the transformation efficiency, by around 5000cfu/μg DNA.

In order to further optimise the transformation protocol, two modifications were made to the original protocol. In the first method, cells were incubated in Lithium Sorbitol instead of the Lithium Acetate buffer usually used. The use of sorbitol was to further permeate the yeast cells to allow increased uptake of DNA into the cells. The drawback of using this method is that it is quite an aggressive treatment and may kill more cells than the standard protocol. The second modification of the protocol was to incubate the cells in YPD for 2hrs after the transformation phase to allow for recovery of the transformed yeast cells. Figure 3.3C shows that when

A

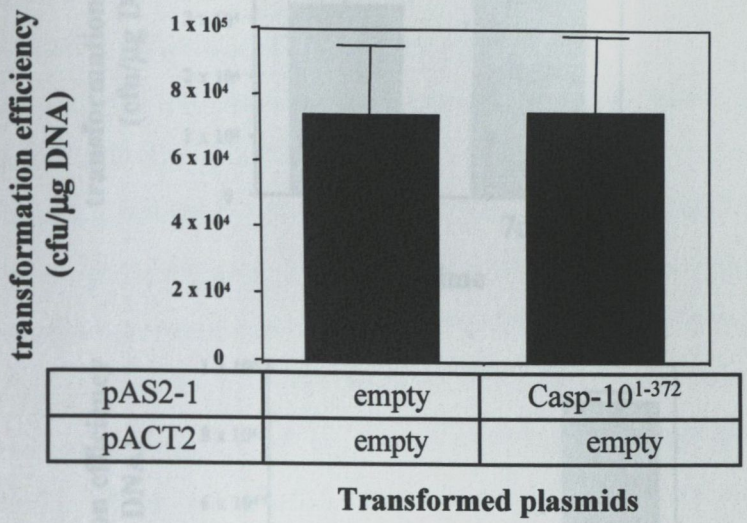
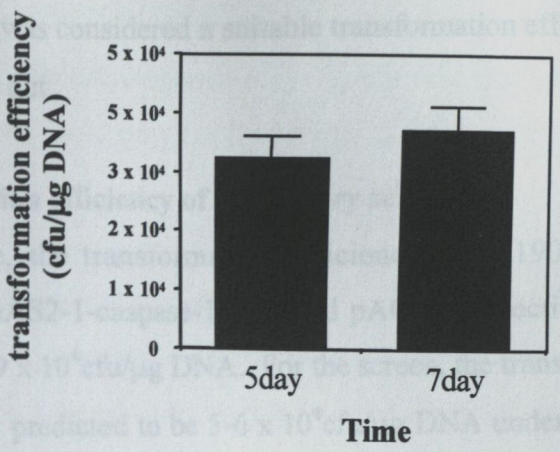


Figure 3.3:

Assessment of transformation efficiency of pAS2-1-Caspase-10^{1-372} transformed yeast.

(A) Comparison of the transformation efficiency of pAS2-1 empty vector and pAS2-1-Caspase-10^{1-372} transformed Y190, sequentially transformed with pACT2 empty vector. Y190 yeast were transformed with either pAS2-1 empty vector or pAS2-1-Caspase-10^{1-372} followed by transformation with known amounts of pACT2 empty vector. Transformed Y190 yeast were plated on media lacking leucine and tryptophan and incubated for 5 days. The emerging colonies were counted and the transformation efficiency in cfu/µg DNA was calculated.

B



C

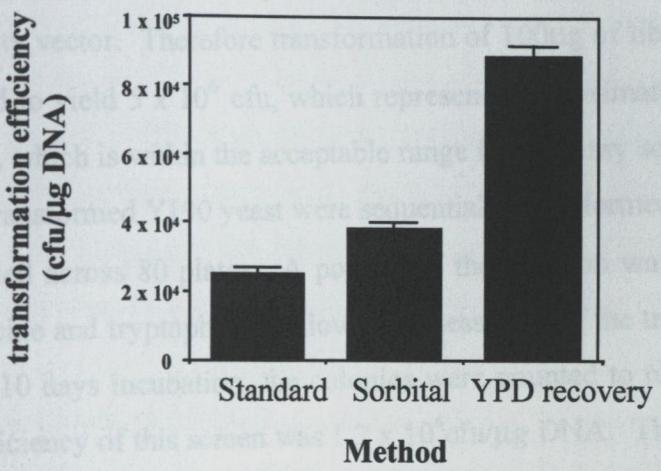


Figure 3.3:

Assessment of transformation efficiency of pAS2-1-Caspase-10^{1-372} transformed yeast.

(B) The effect of duration of incubation on transformation efficiency. pAS2-1-Caspase-10^{1-372} Y190 was transformed as in Figure 3.3A with pACT2 empty vector. Transformation efficiencies were determined at 5 days and 7 days incubation.

(C) Comparison of yeast transformation efficiencies using three different protocols. To optimise the transformation efficiency, variations of the original protocol (Standard) were used. Sorbitol implies that an incubation step with Lithium Sorbitol was included as described in the Materials and Methods. The second variation (YPD recovery) included a 1-2 hour incubation with YPD after the yeast cells were transformed. Each of these results are representative of at least three separate experiments.

compared to the normal procedure used, the use of sorbitol did not significantly increase the transformation efficiency. However inclusion of the YPD recovery period more than doubled the transformation efficiency, bringing it up to 9 x 10^4 cfu/μg DNA. This was considered a suitable transformation efficiency to enable the screen to be carried out.

3.2.5 Transformation efficiency of the library screen

As detailed above, the transformation efficiency for Y190 yeast sequentially transformed with pAS2-1-caspase-10^{1-372} and pACT2 respectively using the YPD recovery step was 9 x 10^4cfu/μg DNA. For the screen, the transformation efficiency was conservatively predicted to be 5-6 x 10^4cfu/μg DNA under the assumption that the efficiency might decrease for the larger pACT2-library plasmid compared to that of the pACT2 empty vector. Therefore transformation of 100μg of library plasmid would be predicted to yield 5 x 10^6 cfu, which represents approximately twice the library complexity, which is within the acceptable range for a library screen. pAS2-1-caspase-10^{1-372} transformed Y190 yeast were sequentially transformed with 100μg of library and plated across 80 plates. A portion of the reaction was plated onto media lacking leucine and tryptophan to allow for assessment of the transformation efficiency. After 10 days incubation, the colonies were counted to reveal that the transformation efficiency of this screen was 1.2 x 10^4cfu/μg DNA. Therefore 1.2 x 10^6 library clones were screened, which represents just over one third of the complexity of the library.

3.2.6 Appearance of putative positive colonies

After 3 days incubation at 30°C there was only background growth observed on the selective media and there was no appearance of any positive colonies. By day 5 there were indications of a number of positive clones emerging, with around 12 plates out of the 80 being identified as containing these colonies. On day 7 a number of colonies growing above background levels were noted, many of which were identified as potential positive clones. By day 10 colonies growing above background were more obvious and numerous than on day 7. A number of the colonies identified as potential positive colonies had grown larger in size and some had turned from a white to a pink colour. However, some of these colonies had

regressed back to background growth characteristics (small flat and white). Many additional colonies had emerged since day 7. More than 400 potential positive clones were initially identified but only 307 of these (usually the larger or pinker colonies) were selected to be restreaked. 307 colonies were restreaked onto HLT- plates with 25mM 3-aminotriazole (3-AT). The media is supplemented with 3-AT to suppress growth of background colonies due to leaky expression of the histidine reporter gene.

3.2.7 Emergence of restreaked colonies

After two days of incubation, 163 of the restreaked colonies showed signs of growth; 13 of which seemed to be strong growers and 56, medium growers. After three days, 203 colonies showed signs of growth; 51 were strong and 94 intermediate. On day 4, 210 had grown; 76 were strong and 98 intermediate. Therefore, from a screen of 1 x 10^6 library clones, two hundred colonies were identified that activated the HIS3 reporter gene (indicating a possible interaction with the bait plasmid) and that regrew on a second round of selection on plates lacking histidine. Prey plasmids were rescued from these clones and 75 random clones were chosen for identification by DNA sequencing.

3.2.8 Identification of positive colonies

BLASTx and BLASTn searches against the non-redundant public sequence databases revealed that each of the 75 sequences were unique (Table 3.2 – 3.3) except for 3 copies of the ribosomal protein HL23 which is a common false positive in yeast-two-hybrid screens. Some of the sequences were revealed to be nonsense DNA with little or no homology to any published sequence.

Although the majority of the clones were unique, some common elements can be identified within the group. Almost a third of the clones were DNA binding and ribosomal proteins. A number of the clones were identified as mitotic spindle associated proteins or displayed homology with such proteins. These include p126 (clone 54) and chromatin assembly factor p48 (clone 10). p48 is also an Rb binding protein. Two other Rb associated proteins featured in the group, p84 (clone 96) and E1A-associated protein shared some homology with clone 207. Three of the clones (158, 163 and 210) shared a high degree of homology with proteins that are

Table 3.2: BLASTx of sequenced clones isolated from caspase-10 yeast-two-hybrid screen against the non-redundant (*Homo sapiens*) database

Clone#	Homologue	Comment	Score	E-value
5	ALU subfamily		76	3.00E-14
10	chromatin assembly factor 1 p48	Rb binding WD repeat protein	40	2.00E-06
	Apaf-1	WD repeat region	27	23
11	DNA binding protein	DNA binding	25	66
15	homology to plant EST		50	4.00E-06
24	aggrecan core protein receptor		26	33
28	htra-2 alpha	Pro apoptotic mitochondrial protein, binds xIAP	25	79
	regulator of mitotic spindle assembly		26	46
32	no hit		NA	NA
38	Myogenic factor 4		32	0.29
46	K1AA0807	Similar to mouse microtubule associated testis specific serine/threonine protein kinase	31	1.3
47	zinc finger protein		39	5.00E-08
48	S164	Ribosomal protein	31	1.3
54	p126	Mitotic spindle associated protein	29	5.2
	NAIP		25	98
59	ATPase Achain		155	6.00E-72
55	EF-1 beta	Elongation factor (DNA binding protein)	27	16
60	Hsp90 beta		194	8.00E-50
64	potassium channel protein		27	15
67	oxytocin receptor		22	764
71	his rich Ca binding protein		27	26
72	IKBKAP	component of IKK signalosome	59	4.00E-09
83	trichohyalin	Ca binding structural protein	36	0.04
95	sodium channel alpha subunit		28	9.1
96	p84	Rb binding protein	122	3.00E-38
97	tyrosine kinase		178	6.00E-45
103	dynamin		36	0.064
111	tyrosine protein kinase ABL2		32	300
112	KIAA0346	Ubiquitously transcribed tetracore repeat gene	30	2.4
	5HTT	Serotonin receptor	29	7.1

Table 3.2: BLASTx of sequenced clones isolated from caspase-10 yeast-two-hybrid screen against the non-redundant (Homo sapiens) database

Clone#	Homologue	Comment	Score	E-value
116	NO synthase		31	0.048
118	ribosomal protein S18	DNA binding	251	6.00E-67
119	mannosyl-oligosaccharide alpha	Calcium binding protein	53	3.00E-07
	harahkiri	Bcl-2 family member	28	9
122	TF SOX4	Transcription factor	23	363
131	HL23 ribosomal protein	DNA binding	212	4.00E-55
133	no hit		NA	NA
141	aspartoacetylase		29	3.9
142	ribosomal protein L13A	DNA binding	274	6.00E-74
145	carboxyl methyltransferase		109	4.00E-24
147	no hit		NA	NA
148	actin		229	2.00E-60
149	unknown		36	0.032
150	60S Ribosomal protein L44	DNA binding	152	2.00E-48
152	orf2		88	9.00E-18
158	CLN5 putative transmembrane protein	Ceroid lipofuscinosis neuronal protein (involved in neurodegenerative disorder)	236	2.00E-62
160	unknown protein CIT987SK		23	521
163	atrophin-1	Dentatorubal pallidduysian atrophy protein	27	20
164	HL23 ribosomal protein	DNA binding	149	9.00E-51
169	DEAD-box protein	DNA helicase	159	3.00E-73
170	HL23 ribosomal protein	DNA binding	139	1.00E-59
173	cote1	Implicated in Gaucher's disease	32	1
176	HL23 ribosomal protein	DNA binding	122	2.00E-26
177	acyl coenzymeA dehydrogenase		129	2.00E-46
178	ribosomal protein S3	DNA binding	66	3.00E-11
182	telomeric repeat binding factor 1	DNA binding	27	31
187	ribosomal protein L8	DNA binding	225	4.00E-59

Table 3.2: BLASTx of sequenced clones isolated from caspase-10 yeast-two-hybrid screen against the non-redundant (*Homo sapiens*) database

Clone#	Homologue	Comment	Score	E-value
188	KI-67	Cell proliferation associated antigen (nuclear protein)	160	2.00E-39
189	hnRNP C	DNA binding	81	1.00E-15
193	zinc finger protein GLI1	DNA binding	27	25
207	ERF-2 protein	EGF response factor, transcriptional adaptor	31	1.4
	E1A-associated protein	Involved in Rb regulation	27	16
210	ataxin 2	Spinocerebellar ataxia protein	33	0.39
214	no hit		NA	NA
222	ribosomal protein S6	DNA binding	779	0
225	similar to U28928		29	4.8
227	AF-4 protein	Proto-oncogene, FEL protein	27	26
228	no hit		NA	NA
237	HL23 ribosomal protein	DNA binding	127	9.00E-53
241	NADH-ubiquinone oxidoreductase chain 4L		36	0.002
248	meningioma expressed antigen		24	145
251	myh-1c (myosin)	ATPase	25	55
252	ribosomal protein S6	DNA binding	31	0.9
254	40S ribosomal protein S9	DNA binding	29	4.9
283	5-hydroxytryptamine 1F receptor		27	23
	c-CRK		26	30
288	monoclonal Ab heavy chain variable region		29	4.7
294	40S ribosomal protein S15A	DNA binding	237	7.00E-63
304	ankyrin	Death domain containing protein	25	98
305	unknown		104	1.00E-22

Table 3.3: BLASTn of sequenced clones isolated from caspase-10 yeast-two-hybrid screen against the non-redundant (*Homo sapiens*) database

Clone#	Homologue	Score	E-value
5	PAC 166H1	194	5.00E-48
10	BAC GSHB-227L	34	10
11	Human thyroid transcription factor	52	3.00E-05
15	mRNA expressed in thyroid	272	3.00E-71
24	ArgBIP	84	1.00E-14
28	htra-2 alpha	174	5.00E-42
32	BAC GSHB	34	12
38	PAC 313L4	406	1.00E-112
46	12q13.1	40	0.14
47	chromosome 19 cosmid	38	0.78
48	cosmid Q7A10	42	0.037
54	PAC 970D1	42	0.046
59	Mitochondrial genome	997	0
55	C23	42	0.046
60	Hsp90beta	896	0.0
64	profillinII	952	0
67	PAC RPCIP704A	32	62
71	RG181H17	710	0
72	IKBKAP	618	1.00E-175
83	Chromosome-19 fosmid	272	3.00E-71
	atm gene	38	0.71
	int-2 proto-oncogene	38	0.71
95	PS alpha gene	40	0.19
96	p84	387	1.00E-105
97	tyrosine kinase	888	0
103	PAC chromosome Xp21	40	0.21
	MAP kinase activating death domain protein (MADD)	34	13
111	BAC RG315L10	38	0.59
112	Xp22 BAC	42	0.047
116	chromosome 19 cosmid	868	0
118	ribosomal protein S18	850	0
119	873P14	40	0.18
122	PAC 717L17	34	13
131	HL23 ribosomal protein	775	0
133	L23 ribosomal protein	775	0
141	B331M8	48	7.00E-04
142	23kD basic protein	793	0
145	carboxyl methyltransferase	480	1.00E-134
147	serine protease gene	38	0.66
148	actin	858	0
149	chromosome 5 BAC	54	1.00E-05
150	PAC DJ0620P06	597	1.00E-169
152	Tigger1	448	1.00E-124
158	CLN5 putative transmembrane protein	850	0
160	BAC GS117010	38	0.91

Table 3.3: BLASTn of sequenced clones isolated from caspase-10 yeast-two-hybrid screen against the non-redundant (*Homo sapiens*) database

Clone#	Homologue	Score	E-value
163	BAC 326584	46	3.00E-03
164	ribosomal protein L17	652	0
169	DDX1	914	0
170	HL23 ribosomal protein	801	0
173	PAC pDJ457	337	7.00E-91
176	HL23 ribosomal protein	468	1.00E-130
177	acyl coenzymeA dehydrogenase	751	0
178	ribosomal protein S3	297	6.00E-79
182	cig5mRNA	42	0.053
187	ribosomal protein L8	825	0
188	mki67a	813	0
189	hnRNP C	819	0
193	BAC119j3	751	0
207	CpG island	40	0.19
210	PAC 79C4	38	0.79
214	cycA gene	32	51
222	ribosomal protein S6	177	7.00E-68
225	22q13 cosmid	739	0
227	Mitochondrial genome	821	0
228	T-cell antigen receptor gene	32	67
237	HL23 ribosomal protein	793	0
241	Mitochondrial genome	125	4.00E-27
248	326L12	36	3.1
251	chromosome 17 clone	38	0.67
252	hmd3b09	174	4.00E-42
254	40S ribosomal protein S9	58	9.00E-07
283	PAC 426I6	38	0.64
288	Mitochondrial genome	694	0
294	40S ribosomal protein S15A	789	0
304	59B16	36	3.5
305	BAC286B10	837	0

associated with neurodegenerative disorders and they all contain trinucleotide repeats in their sequence. Two of the clones (83 and 71) showed a low level of homology with calcium binding proteins. Other clones that were of interest that did not fall into any of the above groups were clone-60 (Hsp90β), clone 28 (Htra-2) and clone 72 (IKBKAP). Clones that were identified as being potential binding partners for caspase-10 were transformed into bacteria in order to rescue the plasmid DNA. Overall the results of the screen were disappointing as only single copies of each 'hit' were found. Previous experience from yeast-two-hybrid screens carried out in the laboratory strongly suggested that multiple copies of true interactors (greater than ten) would be found in a successful yeast-two-hybrid screen.

3.2.9 Hsp90 is a not a caspase-10 interactor

One of the potential positive clones from the screen was identified as a heat shock protein Hsp90β (Figure 3.4A). Due to the reported role of heat shock proteins in apoptosis (Jaattela *et al.*, 1998), and the fact that Hsp90 has been reported to be present in the Apaf-1 apoptosome (Pandey *et al.*, 2000), Hsp90 seemed a good candidate as a caspase-10 interactor. This clone was retransformed into Y190 with a panel of bait plasmids to assess specificity of binding. Thus the Hsp90 clone (clone-60) was co-transformed into Y190 with the N-terminal pro-domain regions of caspase-7, caspase-8, caspase-9, caspase-10 (Figure 3.4B). However, the apparent interaction between caspase-10 and Hsp90 was not repeated and Hsp90 did not bind to any of the other caspase prodomains listed above. Therefore it was concluded that this was a non-specific interaction.

3.2.10 Remaking of Caspase-10 pro bait plasmid

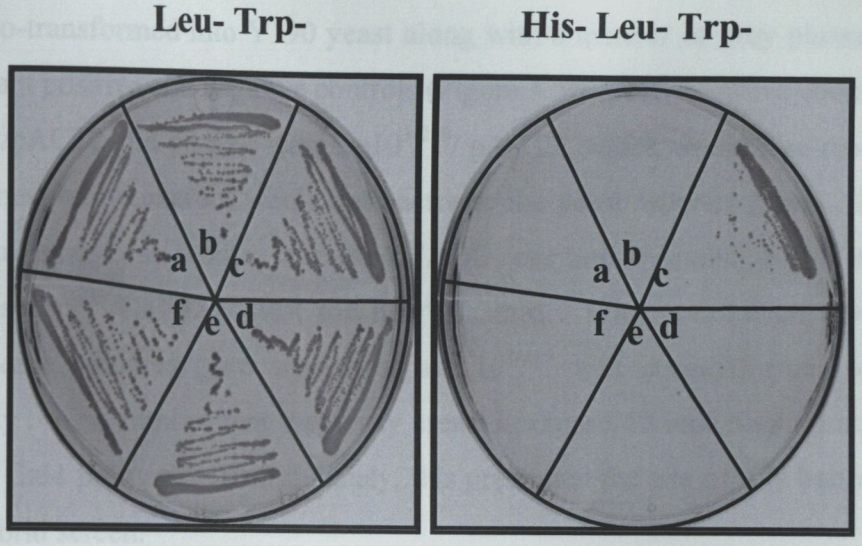
Due to the apparent failure of the caspase-10 screen to identify a strong binding partner for capase-10, it was concluded that the fault might lie in the construction of the bait plasmid. The plasmid used in the screen included the prodomain and the large subunit of caspase-10. The choice of this construct was guided by observations made in our lab, as well as others, that this represented the processed (i.e. active) form of capsase-10 (Slee *et al.*, 1999). At the time the screen was performed there were no positive controls to validate the plasmid. Thus it could be concluded that the region of caspase-10 chosen as bait was too large and that the

A

MPEEVHHGEEEVETFAFQAEIAQLMSLIINTFYSNKEIFLR
ELISNASDALDKIRYESLTDPSKLDSGKELKIDIIPNPQERT
LTLVDTGIGMTKADLINNLGTIAKSGTKAFMEALQAGADI
SMIGQFGVGFYSAYLVAEKVVVIRKHNDDEQYAWESSAG
GSFTVRADHGEPIGMGTKVILHLKEDQTEYLEERRVKEV
VKKHSQFIGYPITLYLEKEREKEISDDEAEEEKGEKEEED
KDDEEKPKIEDVGSDEEDDSGKDKKKKTKKIKEKYIDQE
ELNKTKPIWTRNPDDITQEEYGEFYKSLTNDWEDHLAVK
HFSVEGQLEFRALLFIPRRAPFDLFENKKKKNNIKLYVRR
VFIMDSCDELIPEYLNFIRGVVDSEDLPLNISREMLQQSKI
LKVIRKNIVKKCLELFSELAEDKENYKKFYEAFSKNLKLGI
HEDSTNRRRLSELLRYHTSQSGDEMTSLSEYVSRMKET
QKSIYYITGESKEQVANSAFVERVRKRGFEVVYMTEPIDE
YCVQQLKEFDGKSLVSVTKEGLELPEDEEEKKKMEESK
AKFENLCKLMKEILDKKVEKVTISNRLVSSPCCIVTSTYG
WTANMERIMKAQALRDNSTMGYMMAKKHLEINPDHPIVE
TLRQKAEADKNDKAVKDLVVLLFETALLSGFSLEDPQTH
SNRIYRMIKLGLGIDEDEVAAEEPNAAVPDEIPPLEGDED
ASRMEEVD

Figure 3.4:
Hsp90β is a potential caspase-10 interactor.
(A) Published amino acid sequence of human Hsp90β. The sequence of clone-60 that was pulled out by caspase-10^{1-372} in the yeast-two-hybrid screen is underlined.

B



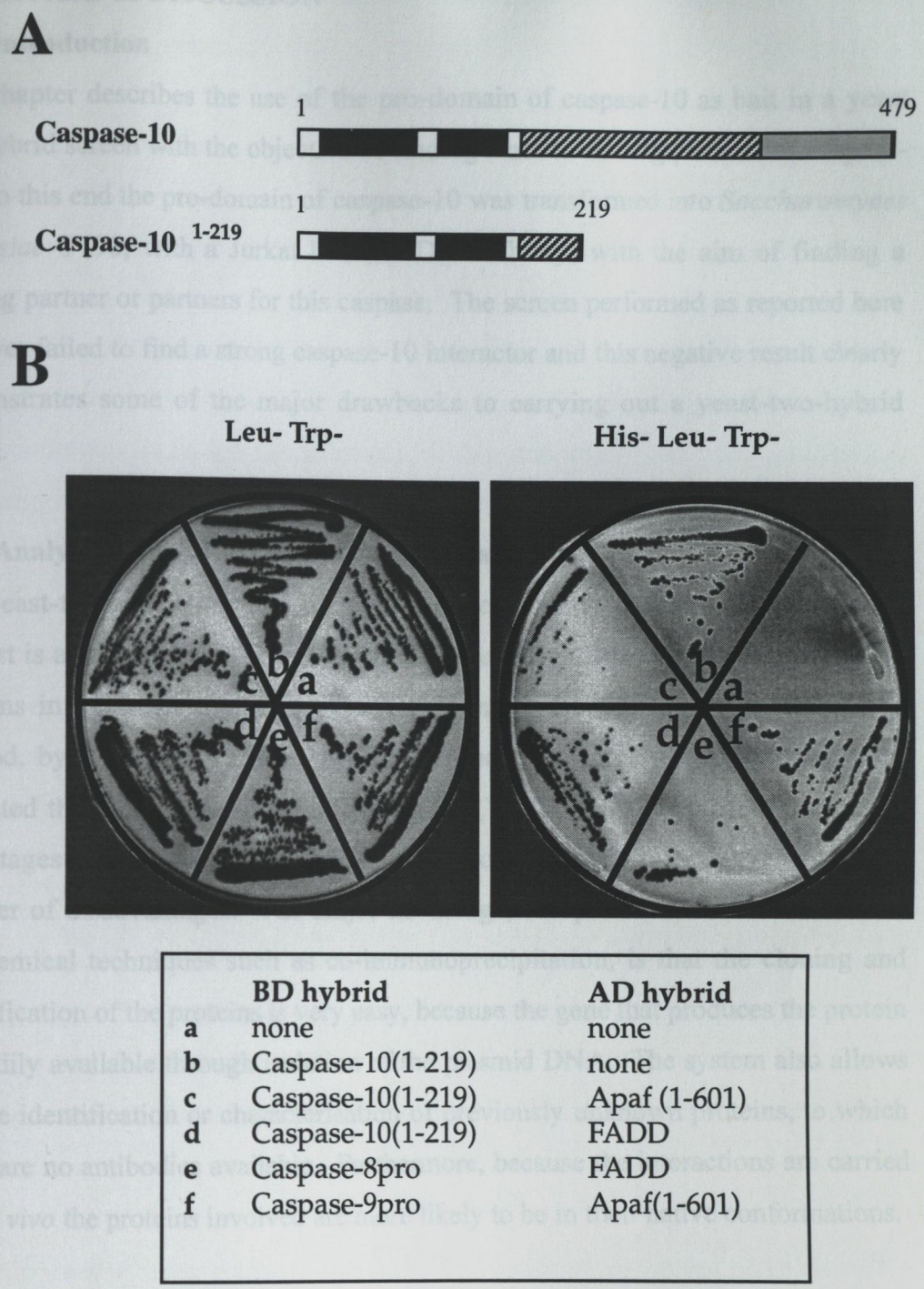
	BD hybrid	AD hybrid
a	caspase-10 (1-372)	clone 60
b	none	clone 60
c	caspase-8 pro	FADD
d	caspase-7 pro	clone 60
e	caspase-8 pro	clone 60
f	caspase-9 pro	clone 60

Figure 3.4:
Hsp90β does not interact with caspase-10^{1-372}
(B) Yeast two-hybrid assay illustrating interaction between Gal4-AD-clone-60 (Hsp90β) and the indicated Gal4-BD-caspase prodomain fusions. *Left*, control plates showing equal loading of yeast. *Right*, the same transformants growing on plates lacking histidine where colony growth indicates an interaction. The interactions were tested in parallel with the positive control Caspase-8pro/FADD

expressed protein could be misfolded. Thus the bait plasmid was remade to include just the prodomain of caspase-10 (Figure 3.5A).

3.2.11 Caspase-10^{1-219} in the pAS2-1 plasmid autoactivates the histidine reporter gene.

The pAS2-1 bait plasmid containing the shorter version of caspase-10 (amino acids 1-219) was co-transformed into Y190 yeast along with a number of prey plasmids and the relevant positive and negative controls (Figure 3.5B). The negative controls were pAS2-1/pACT2 and pAS2-1-caspase10^{1-219}/ pACT2, which would also reveal whether the new bait construct would auto-activate the yeast reporter genes. The positive controls were as before, caspase-8/FADD, caspase-9/Apaf-1(1-601) and finally caspase10^{1-219}/FADD to test the new plasmid. When plated on media lacking histidine colonies grew where caspase-10^{1-219} was cotransformed with pACT2 empty . This implies that the newly created caspase-10 bait plasmid auto-activated the Gal4 promoter. Unfortunately, this precluded the use of this bait in a yeast-two-hybrid screen.



	BD hybrid	AD hybrid
a	none	none
b	Caspase-10(1-219)	none
c	Caspase-10(1-219)	Apaf (1-601)
d	Caspase-10(1-219)	FADD
e	Caspase-8pro	FADD
f	Caspase-9pro	Apaf(1-601)

Figure 3.5:
A shorter version of caspase-10 autoactivates the histidine reporter gene.
(A) Schematic representation of caspase-10^{1-219} truncation made to include the prodomain (DEDs are shaded in black). Numbers indicate amino acid positions.
(B) Yeast two hybrid assay to assess suitability of the caspase-10 construct for a screen. Interactions between Gal4-BD-caspase-10^{1-219} and the indicated Gal4-AD-caspase fusions were compared with the positive controls (caspase-8pro/FADD and casapase-9pro/Apaf (1-601). *Left*, control plates showing equal loading of yeast. *Right*, the same transformants growing on plates lacking histidine where colony growth indicates an interaction. This result is representative of three separate experiments.

3.3 CASPASE-10 DISCUSSION

3.3.1 Introduction

This chapter describes the use of the pro-domain of caspase-10 as bait in a yeast two-hybrid screen with the objective of finding a novel binding partner for caspase-10. To this end the pro-domain of caspase-10 was transformed into *Saccharomyces cerevisiae* Y190, with a Jurkat human cDNA library, with the aim of finding a binding partner or partners for this caspase. The screen performed as reported here however failed to find a strong caspase-10 interactor and this negative result clearly demonstrates some of the major drawbacks to carrying out a yeast-two-hybrid screen.

3.3.2 Analysis of the yeast two hybrid screen approach

The yeast-two-hybrid method of finding novel binding partners for proteins of interest is a very useful tool. As detailed in the introduction of this chapter, many proteins involved in apoptosis have been identified using the yeast-two-hybrid method, by virtue of the fact that many of the steps in cell death pathways are mediated through protein-protein interactions (Table 3.1). There are a number of advantages to this strategy of finding novel protein-protein interactions but also a number of disadvantages. The major advantage, compared to more conventional biochemical techniques such as co-immunoprecipitation, is that the cloning and identification of the proteins is very easy, because the gene that produces the protein is readily available through isolation of the plasmid DNA. The system also allows for the identification or characterisation of previously unknown proteins, to which there are no antibodies available. Furthermore, because the interactions are carried out *in vivo* the proteins involved are more likely to be in their native conformations.

However, there are also a number of drawbacks inherent to this method. The main disadvantage is the appearance of false positive colonies on the selective medium. Certain proteins (such as transcription factors or other DNA binding proteins) have the ability to bind yeast DNA and can act as transcriptional activators. This effect leads to the non-specific expression of the reporter genes in what is termed auto-activation. False negative results can be another problem. The interacting proteins may require accessory elements, such as chaperones, to facilitate their binding, which are not present in yeast. Finally some mammalian proteins are toxic in yeast

and thus will not yield any interaction, regardless of the existence of binding partners for such a protein.

Another practical problem with a yeast two-hybrid library screen is the scale of the experiment. For a screen to be successful the largest possible number of library clones need to be searched. This objective can be seriously hampered if the bait construct being used does not allow efficient transformation of the library plasmid. The lowering of the transformation efficiency effectively increases the scale of the assay. Consequently, to screen a sufficient number of library clones, the number of plates required increases considerably. This can easily become prohibitive, especially if incubator space or other necessary elements are restricting.

For a screen to be successful a number of criteria need to be met. Firstly the transformation efficiency should be sufficient so that at least 9 million library clones (in this case 3 times the library size) are screened. The appearance of multiple copies of a particular protein is also indicative of a profitable screen, because it suggests that such proteins are real interactors and worthy of further investigation. Also if the protein of interest already has a known binding partner and such a protein is isolated in the screen, this would be an indication that the screen worked and that the other isolated positive clones represent real interactors. Conversely, if the majority of the positive clones isolated from a screen represent proteins that are known to be false positives in this system, such as transcription factors and DNA binding proteins, this would diminish chances of the screen having identified a meaningful interaction, as was the case here.

In the caspase-10 screen carried out here, the 75 clones sequenced were identified all to be independent of each other except three copies representing different regions of the ORF for the ribosomal protein HL23. This was a screen of low transformation efficiency being only 1.2×10^4cfu/μg DNA. Therefore 1.2×10^6 library clones were screened, which represents just over 0.3 times the complexity of the library. Thus by the criteria outlined above this was not a very successful yeast-two-hybrid screen.

The two main problems with the screen are associated. It may have been that the low efficiency of the screen prevented the emergence of multiple copies of any one interactor. The significance of three copies of the HL23 ribosomal protein was considered negligible since ribosomal proteins in general and this one in particular, tend to be false positives in the yeast two-hybrid system by virtue of their nucleic acid binding capabilities. Only 10^6 clones were screened which represents only one copy of the library complexity. For a successful screen the transformation efficiency needs to be as high as possible, in reality it is difficult to achieve a transformation efficiency of higher than 5×10^4 in test transformations using the standard protocol. This efficiency generally tends to increase when the actual screen is performed, possibly due to the increase in the scale of the experiment. In this screen, however, this was not the case. In the test transformations the efficiency was variable but reached a consistency of 5×10^4 (Figure 3.3) but in the actual screen it was much lower. Therefore, due to the low efficiency of the screen, fewer copies of potential interactors would be expected. However, this does not alleviate the problem of deciding which clones to choose for further investigation. Obviously, out of 75 individual clones, it is not possible to characterise each of them. In this case, the only approach that could be taken, in the absence of any other clues, was to choose the clones that have previously been reported to have links with cell death pathways.

3.3.3 Putative Caspase-10 interactors

Although this screen was not very successful according to the above criteria a number of interesting molecules were identified as putative positive interactors, which could be speculated to have a role in a caspase-10 pathway (Table 3.2).

Mitotic spindle associated proteins

Three proteins were identified that showed homology to mitotic spindle associated proteins. This would suggest a role for caspase-10 in cell cycle regulation. The IAP protein survivin is a protein that is associated with microtubules and is considered to be a mitotic spindle checkpoint protein, controlling cell division (Li *et al.*, 1998a). Immunoprecipitation experiments have suggested an interaction between caspase-9 and survivin, although it is unclear whether this is a direct or indirect interaction (O'Connor *et al.*, 2000). The significance of such an interaction

is also vague. One hypothesis is that survivin monitors mitotic spindle integrity and promotes apoptosis of cells with incorrectly formed spindles. Could caspase-10 act in a similar way by interacting with a related spindle associated protein? This idea of course is highly speculative considering the evidence for a survivin/caspase-9 functional interaction is not entirely persuasive.

Proteins associated with neurodegenerative disorders

A second group of proteins isolated from the screen are related to each other because they are all associated with neurodegenerative diseases. Clone 158 is present in the database as CLN5, a putative transmembrane protein. Ceroid Lipofuscinosis Neuronal Protein 5 (CLN5) is a protein involved in the neurodegenerative disease Ceroid Lipofuscinosis (Savukoski *et al.*, 1998). Cotel (clone 173) is a protein implicated in the neurodegenerative disorder Gaucher's disease (Winfield *et al.*, 1997). Atrophin (clone 163) and ataxin (clone 210) are both triplet repeat proteins involved in Dentatorubal Pallidoluysian Atrophy (Nagafuchi *et al.*, 1994) and spinocerebellar ataxia (Pulst *et al.*, 1996) respectively. The expansion of trinucleotide repeats is a common causitive factor of a number of degenerative disorders including muscular dystrophy and Huntington's disease. Huntingtin, which is mutated in Huntingtin's disease, and other triplet repeat proteins have been shown to be substrates for caspase cleavage (Goldberg *et al.*, 1996; Wellington *et al.*, 1998). It is possible that the proteins described above represent caspase-10 substrates that transiently bind to caspase-10 in order to undergo proteolytic processing.

Heat Shock Protein 90β

Of the clones that were identified in the screen one showed significant homology to heat shock protein 90β (HSP90β). Heat shock proteins have been reported to play a role in apoptosis and Hsp90 and Hsp70 have been suggested to be part of the apoptosome (Jaattela *et al.*, 1998; Pandey *et al.*, 2000; Saleh *et al.*, 2000). Hsp90 shows a high degree of homology to a protein called TRAP1. Very little is known about this protein but has been suggested to interact with TNFR1 (Song *et al.*, 1995). The TNFR pathway is analogous to the Fas pathway in that TNFR1 interacts with TRADD, which recruits FADD, which binds to Caspase-8 via the death

effector domain. Thus Hsp90 or TRAP1 could be hypothesised to, by virtue of this yeast-two-hybrid result, in a separate pathway, recruit caspase-10 to TNFR1 and initiate a caspase cascade in that way.

Despite the interesting potential of Hsp90 as a caspase-10 interactor, or even a caspase interactor, the interaction could not be verified in subsequent co-transformations with caspase-10 (Figure 3.4B). Furthermore this clone which represents the amino acids 520-691 of Hsp90β could not interact with the pro-domains of caspase-7, -8, or -9. In the apoptosome model Hsp90 is reported to be recruited to the apoptosome to bind caspase-9. This result however suggests that either Hsp90 does not bind to caspase-9, that the binding does not occur through the pro-domain of caspase-9, or alternatively, that the binding is mediated by a portion of Hsp90 that is not included in the clone isolated from the screen. Co-immunoprecipitation assays of caspase-9 and Hsp90β showed no binding, which would argue against a caspase-9/Hsp90 interaction (personal communication Dr. Colin Adrian). However, Hsp90 may require the caspase-9 complexed with other members of the apoptosome, such as Apaf-1, to enable it be recruited to caspase-9.

p84

A second protein of interest that was pulled out with the caspase-10 screen was p84. p84 is an Rb-interacting protein (Dufree *et al.,* 1994), a role for which has been implicated in apoptosis (Adrain *et al.*, 2001). Interestingly, this protein has been found in a separate screen carried out in the lab as a caspase-9 interactor, and it has a death domain as well as a CARD-like domain (Adrain *et al.*, 2001). In the study that characterised p84 (DRADD) as a caspase-9 interacting protein, a possible interaction between p84 and caspase-10 was tested using the same caspase-10^{1-372} plasmid as was used in this study. In this case no interaction was observed. Thus it can be concluded that this was also a false positive interaction.

IKBKAP

Two proteins in particular were pulled out from the screen where the significance of any role they may have in apoptosis was not apparent at the time. The first is IKBKAP (clone- 72, IκB Kinase Activating Protein). This protein is part of the

IKK complex that is required for NFκB activation, although its function is not clear (Cohen *et al.*, 1998). Although a role for caspase-10 in NFκB activation has never been shown, the related inhibitor of apoptosis FLIP has been shown by a number of groups to activate NFκB (Hu *et al.*, 2000; Yeh *et al.*, 2000). The bait construct used in this screen is quite similar to the structure of FLIP comprising two death effector domains. Thus, it is reasonable to suggest that this may represent an interaction between FLIP and IKBKAP and, in an analogous way to the mechanism by which RIP or RICK mediates recruitment of the IKK complex through IKKγ, is thus able to activate NFκB.

HtrA2

A number of reports have recently identified the protein HtrA2 as having a role in apoptosis (Hegde *et al.*, 2001, Martins *et al.*, 2001; Suzuki *et al.*, 2001; Verhagen *et al.*, 2001). HtrA2/Omi is a serine protease that is released from the mitochondria during apoptosis and, like Smac, binds to XIAP (Hegde *et al.*, 2001). Not enough is known about Omi to allow for speculation as to how caspase-10 may interact with an IAP binding mitochondrial protein and what the significance of this may be, since the assumption is that caspase-10 functions upstream of the mitochondria. However, it has been shown that caspase-10 is also activated downstream of cytochrome c release by caspase-6 (Slee *et al.*, 1999). Therefore it is feasible that caspase-10 may interact with Omi at this point, modulating its function in some way. It must be noted, however, that the clone (clone-28) that was isolated in the screen shared quite a low level of homology with Omi, therefore it is unlikely that caspase-10 interacts with this protein.

3.3.4 Suitability of the bait

In conclusion, the caspase-10 screen performed did not yield any proteins that could be verified as true caspase-10 interactors. Aside from the fact that this was a low efficiency screen, the presence of a number of false positive clones (ribosomal proteins, transcription factors) and the lack of multiple copies of any one protein, suggests that the failure of the screen may have been contributed to by another factor.

The choice of the correct bait construct is vital to ensure the success of a screen. When a truncation of a protein is made, as was in this case, there is the risk that it will not fold in its natural conformation, thus preventing it from recruiting its natural binding partners. In this case, it was required to truncate the protein because generally it is not feasible to carry out screens with large proteins, as they do not allow for high yeast transformation efficiencies. Moreover, because caspase-10 is a protease, expression of the full-length caspase-10 protein in yeast would almost certainly be toxic. The caspase-10^{1-372} plasmid used included, not only the pro-domain of caspase-10, but also the large subunit (Figure 3.1A). It is quite possible that the DNA binding domain fused to this large gene segment may have induced mis-folding of the protein thus shielding the DEDs or another binding domain from potential interaction.

Due to the fact that at the time of the screen there was no binding partner reported for caspase-10, there was no positive control with which to test the plasmid. The ability of FADD to bind caspase-10 was only reported in one paper at that time of embarking upon this screen (Vincenz and Dixit, 1997). Since then, the ability of FADD to recruit capase-10 under physiological conditions has been widely debated. It has recently been shown that the controversy that has arisen over the function of caspase-10 has been contributed to by the fact that many of the commercially available antibodies to caspase-10 are actually cross-reactive with the abundant protein Hsp60, which is the same size (Kischkel *et al.*, 2001).

Since the time of carrying out the screen the positive interaction between caspase-10 and FADD has been verified, at least between the overexpressed proteins, (Wang *et al.*, 1999; Wang *et al.*, 2001a). In the preparation for the yeast two-hybrid screen, a possible interaction between caspase-10 and FADD was tested giving a negative result (Figure 3.2). At the time, due to the controversy that existed over the role of caspase-10 in death receptor pathways, this result was taken to be a true representation of the binding properties of caspase-10. However, now this result could also be interpreted to suggest that the plasmid used for the yeast-two-hybrid screen does not represent caspase-10 in its true native state.

Therefore an alternative version of the caspase-10 plasmid was made to include just the pro-domain of this protease (Figure 3.5A). This was done under the assumption that the smaller truncation would have a greater probability of folding correctly. However when this plasmid was tested it was shown to auto-activate the yeast reporter genes and based on this result this construct could not be used in a subsequent screen (Figure 3.5B). Certain proteins tend to auto-activate in the yeast-two-hybrid system. This usually occurs if the protein contains a transcriptional activation domain, which once fused to the Gal4-binding-domain mimics the activation-domain/binding-domain complex that represents a pair of interacting proteins, leading to non-specific activation of the reporter gene. Other proteins that are not normally involved in transcription can have the same effect for unknown reasons.

Once this effect of autonomous activation occurs it is impossible to obtain meaningful results with the bait construct. The only solution to the problem of auto-activation is to choose a different portion of the protein to use as bait in the screen. However, this was not feasible, as the second caspase-10 truncation made represented the smallest part of caspase-10 that could be used. If it was truncated any further the likelihood of it being a true representation of caspase-10 (as opposed to being a caspase-8 or FLIP-like molecule) would have been significantly diminished.

3.3.5 Summary and Conclusions

In summary the screen performed here demonstrates a number of the pitfalls that can be involved in using the yeast two-hybrid assay as a method of finding novel proteins involved in apoptosis pathways. Recently a report of a caspase-10 screen was published, where a protein named DEDAF was isolated as a caspase-8 and caspase-10 interactor (Zheng *et al.*, 2001). Unfortunately, even such a finding did not contribute much to unlocking the function of caspase-10. DEDAF is a DED interactor that binds caspase-8, -10 and FLIP, rather than a specific caspase-10 binding protein. Further work will have to be done on DEDAF to determine whether it has a role that is specific to caspase-10 function.

In conclusion, the yeast-two-hybrid assay, although a powerful tool in analysing protein-protein interactions, can often, as in this case, yield negative results. However, in order to find novel proteins involved in the apoptosis pathway, alternative methods are available. One way which can very effectively identify such proteins, as is shown in the next chapter, is by searching the human genome for proteins that show homology to a specific domain present in known apoptosis proteins such as the DED or the CARD motifs. Advantages of this method over the yeast-two-hybrid approach, is that it is quick and inexpensive. However, once a protein is found it can be much more difficult to assign a function to it. In comparison, isolation of a protein in the yeast-two-hybrid system implies that this protein operates in the same pathway as the bait protein used, thus giving an obvious starting point for further investigation.

CHAPTER IV

Cloning and functional characterisation of CARDINAL, a Novel CARD Protein

4.1 INTRODUCTION

The previous chapter described the yeast-two-hybrid method of identifying novel proteins involved in particular pathway using the caspase-10 prodomain as bait. Proteins likely to participate in particular pathways can also be identified based on the presence of conserved protein-protein interaction domains, such as the DED motif, in the sequence of the protein. Thus, based on homologies between two proteins, or between domains common to two proteins, one can infer a plausible function for novel proteins. The yeast-two-hybrid screen described in Chapter III failed to identify a novel DED-containing protein or a novel caspase-10 interactor, therefore we used an alternative strategy to find such proteins.

Database searching for proteins that contain conserved protein motifs has proven to be an invaluable tool in the field of apoptosis. Due to the presence of conserved protein domains in many apoptosis-signalling proteins such as the Death Domain (DD), the Death Effector Domain (DED) and the Caspase Recruitment Domain (CARD), bioinformatics-based approaches have been used effectively to identify many key proteins involved in apoptosis pathways. Thus, as an alternative to the yeast-two-hybrid approach, we used the prodomain of caspase-10 to search the public sequence databases for novel DED-containing proteins. These searches failed to find a novel DED-containing protein. Therefore we switched our focus to a second conserved protein motif, the CARD domain, that is found in many proteins involved in apoptosis. The CARD domain of caspase-1 was used for these searches, as only one CARD protein had been implicated as a caspase-1 interactor (RICK/CARDIAK) when these searches were performed. Moreover, relatively little is known about the pathway in which caspase-1 becomes activated to effect IL-1β processing. These searches identified a novel CARD-containing protein we have called CARDINAL.

At present, there is a wide variety of CARD proteins and these have been described in detail in the Introduction. In summary, the CARD proteins fall into three main functional categories, those involved in caspase activation leading to apoptosis, those involved in caspase activation leading to cytokine processing and inflammation, and those involved in pathways leading to NFκB activation.

NFκB mediated transcription is central to the inflammatory response, leading to the expression of a number of pro-inflammatory genes, such as IL-1. Among the many proteins that lead to the activation of NFκB, many of them, such as RICK, Nod-1 and Bcl-10, contain CARD domains (Inohara *et al.*, 1999; McCarthy *et al.*, 1998; Willis *et al.*, 1999). RICK has been shown to bind IKKγ, the regulatory subunit of the IKK kinase complex (Inohara *et al.*, 2000). The activation of the IKK complex is essential for NFκB activation, being the point of convergence of all known NFκB pathways. RIP, a death domain containing kinase, also triggers NFκB through recruitment of IKKγ (Inohara *et al.*, 2000).

Here we describe a novel CARD-containing protein CARDINAL, which does not appear to be involved in the activation of either caspases or NFκB. Instead, CARDINAL was found to inhibit divergent NFκB activation signals and to interact with the regulatory subunit of the IκB kinase complex, IKKγ. Thus CARDINAL may play a role in setting a threshold for NFκB activation, or in limiting the duration of the NFκB response in the context of pro-inflammatory signals.

4.2 RESULTS

4.2.1 Identification of CARDINAL

To identify novel CARD-containing proteins, we performed BLASTp and tBLASTn searches of the public databases, using the prodomain of caspase-1, which includes a CARD motif, as the query sequence. These searches identified a hypothetical protein denoted KIAA0955 (Genbank™ accession no. BAA76799). Sequence analysis of this clone revealed an open reading frame of 431 amino acids (Figure 4.1, A and B). The C-terminus of this protein shows significant homology with the CARD domain of caspase-1. Alignments showed that the CARD domain of this protein shares homology with the CARD domains of many other CARD-containing proteins, including Caspase-2, RAIDD, ASC, CARD4, ICEBERG and NAC (Figure 4.1C).

The KIAA0955 protein molecule also exhibits a high degree of homology with C–terminus of the recently identified molecule NAC/DEFCAP (Figure 4.1D). The aligned portion of the molecules displayed here represents not only the CARD domain of NAC (located at the C-terminal end) but extends for approximately 300 amino acids into the NAC protein from the beginning of the NAC CARD motif. The latter region represents the portion between the leucine rich repeats of NAC and the CARD domain and does not contain any characterised sequence domains or motifs. Due to the functional properties of this protein we have termed it CARDINAL for CARD inhibitor of NFκB activating ligands (see below).

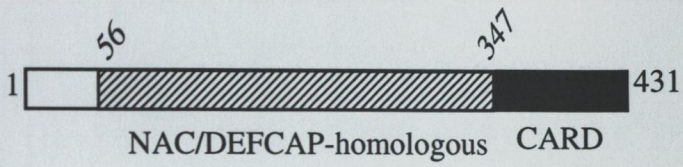
4.2.2 Cloning of CARDINAL

The coding sequence of full length CARDINAL was supplied as pbluescript-KIAA0955 by the Kazusa institute (Japan). The KIAA0955 plasmid was used as a template for all subsequent cloning manipulations of this gene except for the cDNA used to make the pcDNA3-CARDINAL$^{345\text{-}431}$ deletion mutant, which was cloned by amplification of the fragment from a Jurkat cDNA library using gene specific primers (Table II in Appendix). The pcDNA3-CARDINAL sequence was verified by automated sequencing (Figure 4.1A).

A

MMRQRQSHYCSVLFLSVNYLGGTFPGDICSEENQIVSSYA
SKVCFEIEEDYKNRQFLGPGGNVDVELIDKSTNRYSVWFP
TAGWYLWSATGLGFLVRDEVTVTIAFGSWSQHLALDLQHH
EQWLVGGPLFDVTAEPEEAVAEIHLPHFISLQGEVDVSWF
LVAHFKNEGMVLEHPARVEPFYAVLESPSFSLMGILLRIA
SGTRLSIPITSNTLIYYHPHPEDIKFHLYLVPSDALLTKA
IDDEEDRFHGVRLQTSPPMEPLNFGSSYIVSNSANLKVMP
KELKLSYRSPGEIQHFSKFYAGQMKEPIQLEITEKRHGTL
VWDTEMKPVDLQLVAASAPPPFSGAAFVKENHRQLQARMG
DLKGVLDDLQDNEVLTENEKELVEQEKTRQSKNEALLSMV
EKKGDLALDVLFRSISERDPYPVSYLRQQNL

B



C

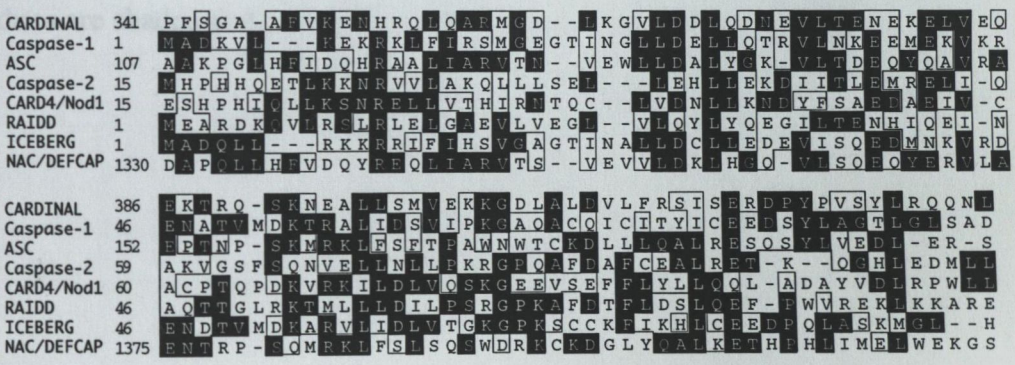


Figure 4.1:

Sequence analysis of CARDINAL and alignment with proteins possessing similar motifs.

(A) The CARDINAL open reading frame encodes a predicted protein of 431 amino acids. The region encompassing the CARD motif (amino acids 347-431) is underlined.

(B) Schematic representation of the domain structure of CARDINAL. The region showing homology with NAC and the CARD domain are indicated by hatched and black shading respectively. Numbers indicate amino acid positions.

(C) Alignment of the CARD domain of CARDINAL (amino acids 341-435) with the CARD domains of other known proteins. Identical residues are shaded and conservative substitutions are boxed.

D

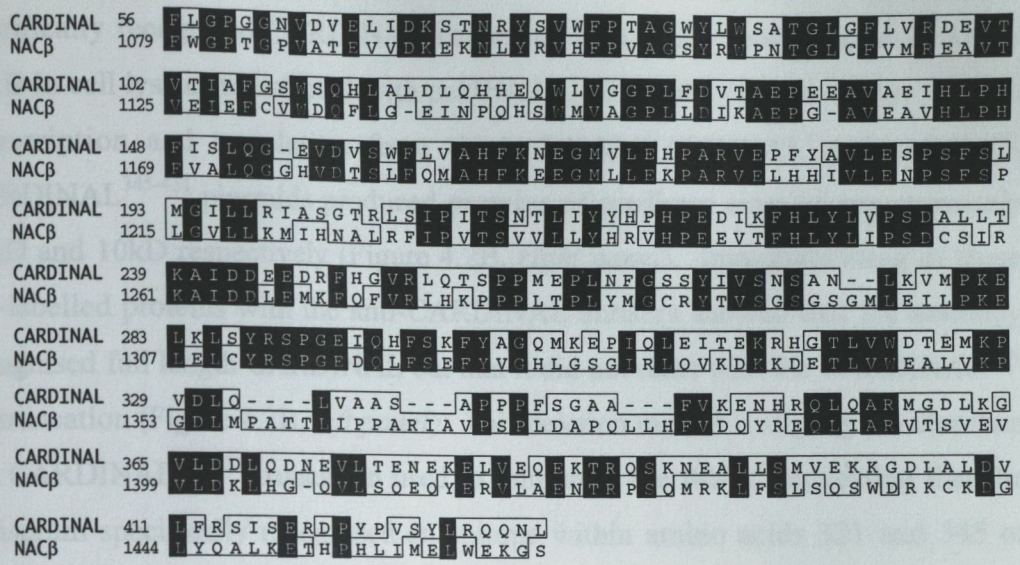


Figure 4.1:

Sequence analysis of CARDINAL and alignment with proteins possessing similar motifs.

(D) Alignment of the NAC homologous domain of CARDINAL with NAC. Identical residues are shaded and conservative substitutions are boxed.

4.2.3 Generation of anti-CARDINAL antibody

To verify expression of CARDINAL, we generated a polyclonal antibody by immunising rabbits with a GST-CARDINAL$^{321-431}$ fusion protein. This antibody specifically recognised CARDINAL migrating as a single band of approximately 50kD in cell lysates transfected with pcDNA3-CARDINAL (Figure 4.2A). *In vitro* transcription and translation from the pcDNA3-CARDINAL and pcDNA3-CARDINAL$^{345-431}$ plasmids produced proteins of predicted sizes of approximately 50kD and 10kD respectively (Figure 4.2B, *right panel*). Immunoblotting of these ^{35}S-labelled proteins with the anti-CARDINAL antisera showed that the antibody recognised full length CARDINAL but that it did not react with the CARDINAL$^{345-431}$ truncation (Figure 4.2B, *left panel*). This deletion mutant is slightly smaller than the CARDINAL$^{321-431}$ truncation used to immunise the rabbit, suggesting that the antiserum specifically recognised an epitope within amino acids 321 and 345 of CARDINAL.

The antibody in its crude form was not very efficient at detecting endogenous CARDINAL. Therefore the antibody was affinity purified and concentrated using Affigel-coupled CARDINAL$^{321-345}$. Each of the first five eluate fractions were assessed, for CARDINAL reactivity by probing cell lysates that had been transfected with CARDINAL (Figure 4.2C). Each fraction recognised full length CARDINAL and did not display any cross reactivity with untransfected HEK 293T cell lysates (Figure 4.2C). Each fraction was subsequently concentrated to increase the titre of antibody. Affinity-purified CARDINAL antibody was then used to probe multi-tissue and multi-cell line western blots to determine the pattern of CARDINAL expression (see below).

4.2.4 Tissue Expression of CARDINAL

Analysis of CARDINAL expression in tissues suggested that CARDINAL is expressed highly in certain immune privileged sites, the testis, the placenta, the ovaries and the lung (Figure 4.3A). CARDINAL is also expressed to a lesser extent in heart, kidney and liver. A number of tumour cell lines were analysed for CARDINAL expression and these revealed that CARDINAL was expressed very highly in the MCF-7 breast carcinoma cell line and was expressed at moderate levels in THP-1 and U937 cells, which are monocytic cell lines (Figure 4.3B). This

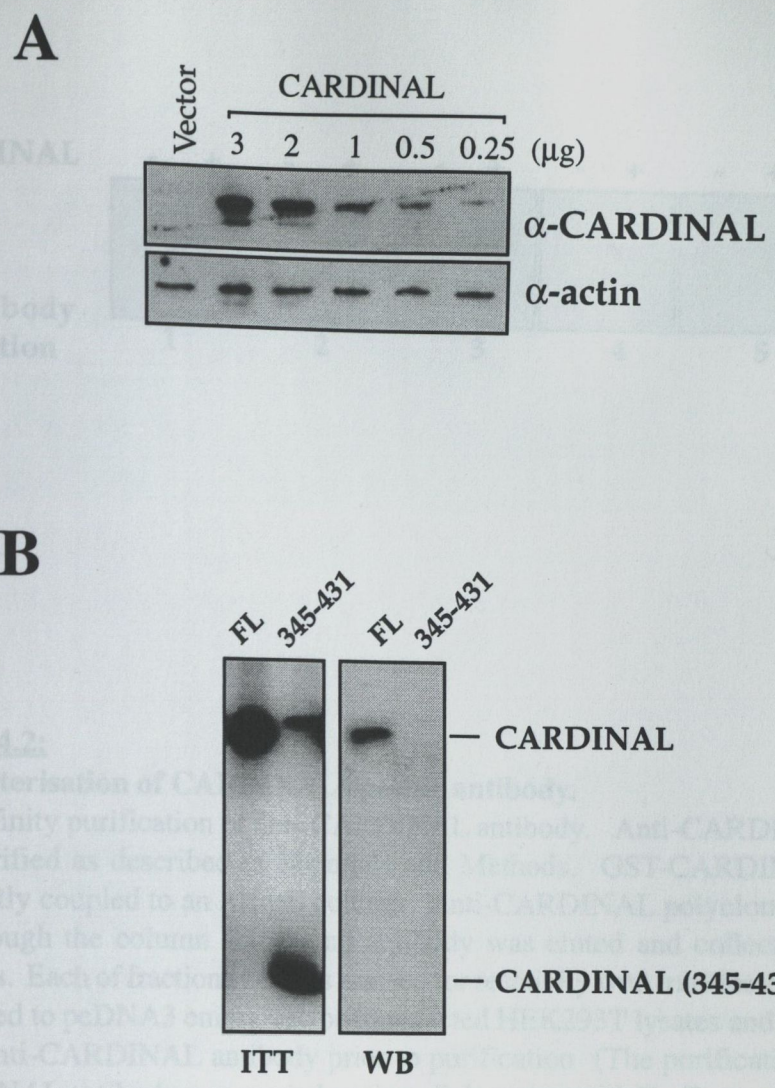


Figure 4.2:

Characterisation of CARDINAL-specific antibody.

(A) A rabbit polyclonal antibody was raised against GST-CARDINAL $^{321-431}$ (amino acids 321-431) which specifically recognises CARDINAL. The indicated amounts of pcDNA3-CARDINAL or 3μg of pcDNA3 empty vector were transfected into HEK293T cells and lysates made 24hrs later. Protein expression was detected by immunoblot with anti-CARDINAL antibody or anti-actin antibody as indicated.

(B) *In vitro* transcription and translation (ITT) of CARDINAL and CARDINAL $^{321-431}$. *Left panel*, ITT ^{35}S-labelled CARDINAL and CARDINAL $^{321-431}$ were generated as described in Materials and Methods, and labelled proteins were detected by autoradiography. *Right panel*, ITT ^{35}S-labelled CARDINAL and CARDINAL $^{321-431}$ were transferred onto nitrocellulose and probed with anti-CARDINAL antibody to detect protein expression.

C

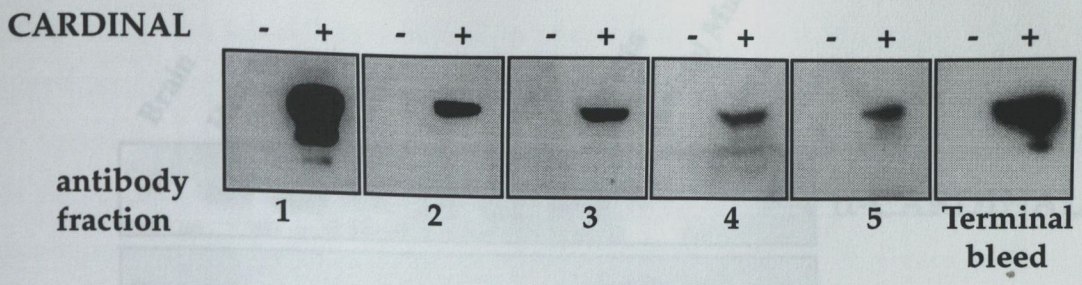


Figure 4.2:

Characterisation of CARDINAL-specific antibody.

(C) Affinity purification of anti-CARDINAL antibody. Anti-CARDINAL antibody was purified as described in Materials and Methods. GST-CARDINAL $^{321-431}$ was covalently coupled to an Affigel column. Anti-CARDINAL polyclonal antisera was run through the column and bound antibody was eluted and collected in separate fractions. Each of fractions 1-5 was assesed for reactivity with transfected CARDINAL compared to pcDNA3 empty vector-transfected HEK293T lysates and was compared to the anti-CARDINAL antibody prior to purification (The purification of the anti-CARDINAL antibody was carried out in collaboration with Dr. Emma Creagh and Dr. Helen Egan).

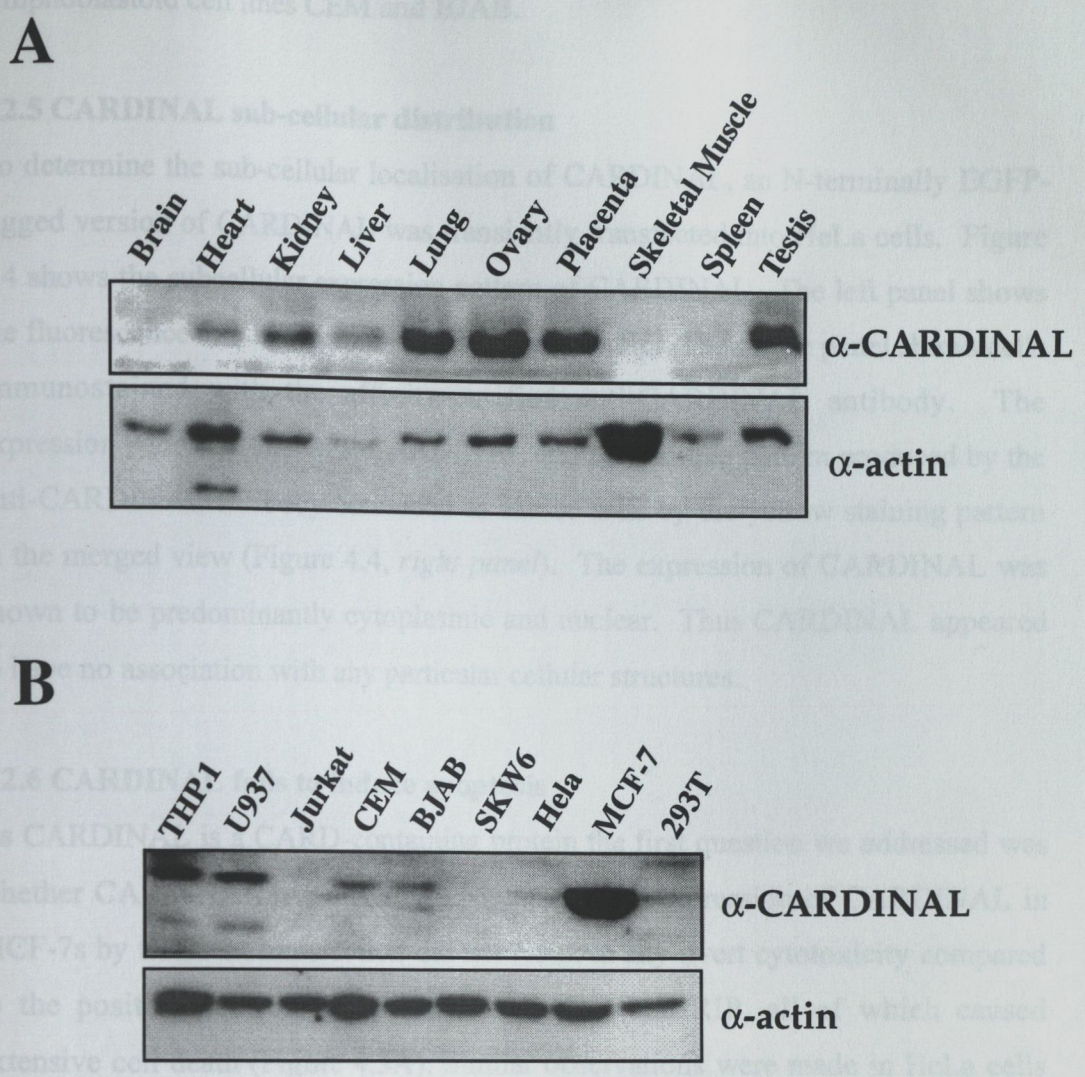


Figure 4.3:

CARDINAL protein expression in normal human tissues and transformed cell lines.

(A) CARDINAL protein expression in human tissues. Whole cell lysates (100μg per lane) from various human tissues were analysed for CARDINAL expression by immunoblotting with affinity purified anti-CARDINAL antibody.

(B) CARDINAL protein expression in human tumour cell lines. Total protein lysates (50μg per lane) from the indicated cell lines were assessed for CARDINAL expression by immunoblotting with affinity purified anti-CARDINAL antibody (This experiment was done in collaboration with Dr. Colin Adrain).

analysis also revealed a low level of CARIDNAL expression in the T and B lymphoblastoid cell lines CEM and BJAB.

4.2.5 CARDINAL sub-cellular distribution

To determine the sub-cellular localisation of CARDINAL, an N-terminally EGFP-tagged version of CARDINAL was transiently transfected into HeLa cells. Figure 4.4 shows the subcellular expression pattern of CARDINAL. The left panel shows the fluorescence due to the GFP-CARDINAL protein. The centre panel shows cells immunostained with the affinity-purified anti-CARDINAL antibody. The expression pattern of the GFP-CARDINAL and the staining pattern produced by the anti-CARDINAL antibody coincided as can be seen by the yellow staining pattern in the merged view (Figure 4.4, *right panel*). The expression of CARDINAL was shown to be predominantly cytoplasmic and nuclear. Thus CARDINAL appeared to have no association with any particular cellular structures.

4.2.6 CARDINAL fails to induce apoptosis

As CARDINAL is a CARD-containing protein the first question we addressed was whether CARDINAL has a role in apoptosis. Overexpression of CARDINAL in MCF-7s by transient transfection did not result in any overt cytotoxicity compared to the positive controls Bax, FADD, TRADD and RIP, all of which caused extensive cell death (Figure 4.5A). Similar observations were made in HeLa cells (data not shown).

To explore whether CARDINAL could modulate other death-promoting stimuli, CARDINAL was transiently transfected into MCF-7 cells along with Bax, and also with the alternative apoptosis inducers TRADD or FADD. After 24hrs CARDINAL did not substantially augment or inhibit apoptosis provoked by FADD, TRADD or Bax (Figure 4.5B). CARDINAL did appear to modestly augment Bax induced cell death in certain experiments however (Figure 4.5C).

4.2.7 CARDINAL fails to induce NFκB

As described above, our initial investigations failed to confirm a role for CARDINAL in promoting apoptosis. However, many CARD containing proteins described to date are not involved in caspase activation or apoptosis. Rather, these

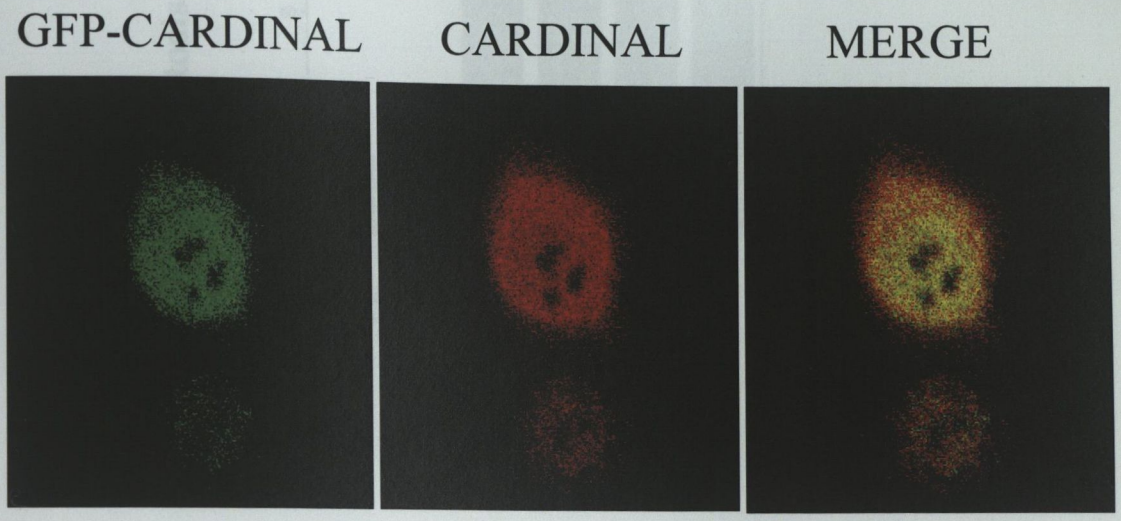


Figure 4.4:
CARDINAL subcellular distribution
HeLa cells were transfected with 800ng of EGFP-CARDINAL. 24hrs after transfection, cells were fixed and assessed for GFP-CARDINAL expression using the affinity purified anti-CARDINAL polyclonal antisera. Cells were examined under confocal microscopy for GFP fluorescence (Green, *left panel*) or GFP-CARDINAL expression detected by staining with the affinity purified anti-CARDINAL antibody (Red, *centre panel*). *Right panel* (merge), shows co-localisation of the two staining patterns (Yellow). These images are representative of a number of different fields of cells.

A

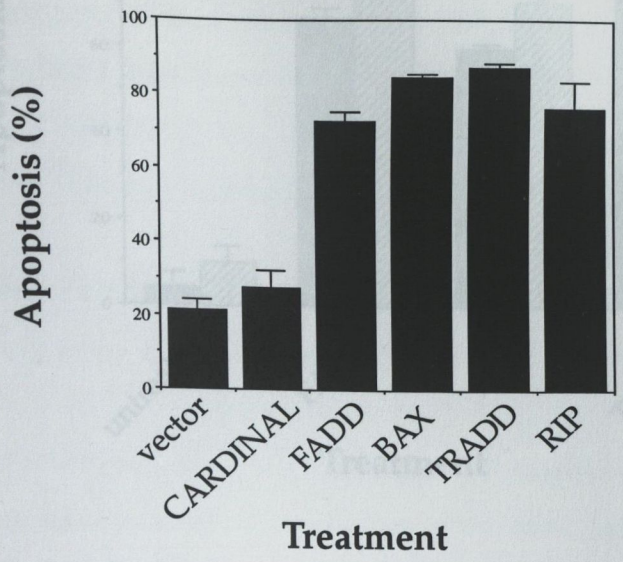
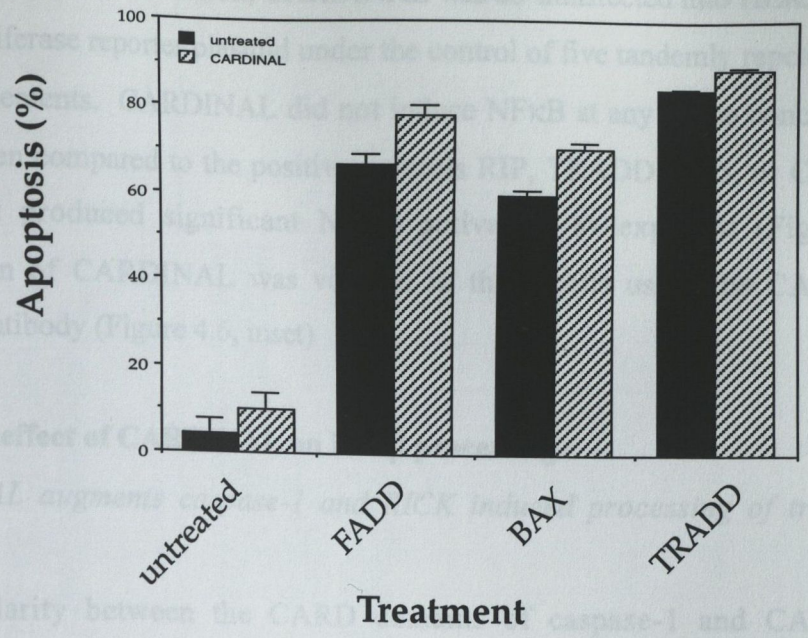


Figure 4.5

Assessment of apoptosis associated with CARDINAL overexpression.

(A) MCF-7 cells were transfected with pcDNA3 empty vector (800ng) or the same amount of expression plasmids encoding CARDINAL, FADD, Bax, TRADD, or RIP as indicated, along with 50ng of β-galactosidase reporter plasmid (pcMVβ). 48hrs after transfection, the percentage of β-galactosidase positive (blue) cells exhibiting apoptotic features was determined from a minimum of 300 cells/well. Results are representative of three separate experiments.

B



C

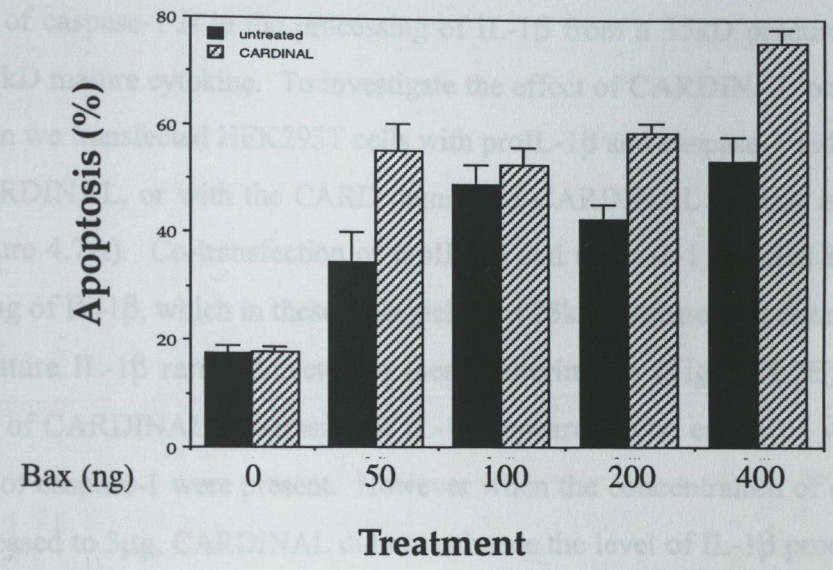


Figure 4.5:

Assessment of apoptosis associated with CARDINAL overexpression.

(C) MCF-7 cells were transfected with 400ng of pcDNA3 empty vector or expression plasmids encoding FADD, Bax or TRADD along with 400ng of either empty vector (black shading) or 400ng of pcDNA3-CARDINAL (hatched shading). Each well also received 50ng of β-galactosidase reporter plasmid (pcMVβ). 24hrs after transfection, the percentage of β-galactosidase positive (blue) cells exhibiting apoptotic features was determined as before. Results are representative of two separate experiments.

(D) HeLa cells were transfected with the indicated amounts of pcDNA3-Bax along with 400ng of pcDNA3-CARDINAL, or the same amount of empty vector. 24hrs after transfection, the percentage of β-galactosidase positive (blue) cells exhibiting apoptotic features was determined as before. Results are representative of three separate experiments.

CARDs participate in pathways leading to NFκB activation. To test if CARDINAL could induce NFκB activation, CARDINAL was co-transfected into HEK293T cells with a luciferase reporter plasmid under the control of five tandemly repeated NFκB binding elements. CARDINAL did not induce NFκB at any of the concentrations tested when compared to the positive controls RIP, TRADD, DR5, or CARDIAK which all produced significant NFκB activation as expected (Figure 4.6). Expression of CARDINAL was verified in these cells using the CARDINAL specific antibody (Figure 4.6, inset)

4.2.8 The effect of CARDINAL on IL-1β processing

CARDINAL augments caspase-1 and RICK induced processing of transfected proIL-1β

The similarity between the CARD domains of caspase-1 and CARDINAL suggested that these proteins might function in the same pathway. The primary function of caspase-1 is in the processing of IL-1β from a 35kD precursor to the active 17kD mature cytokine. To investigate the effect of CARDINAL on caspase-1 function we transfected HEK293T cells with proIL-1β and caspase-1 either alone, with CARDINAL, or with the CARD domain of CARDINAL (amino acids 345-431, Figure 4.7A). Co-transfection of proIL-1β and caspase-1 resulted in modest processing of IL-1β, which in these cells yielded a 25kD intermediate form with the 17kD mature IL-1β rarely detected in these experiments (Figure 4.7B). In the presence of CARDINAL, processing of IL-1β appeared to be enhanced when 2μg and 1μg of caspase-1 were present. However when the concentration of caspase-1 was increased to 3μg, CARDINAL did not enhance the level of IL-1β processing to the same extent as seen with the lower concentrations. When the CARDINAL$^{345-431}$ deletion mutant was present there was a profound inhibition of caspase-1 induced processing of proIL-1β at all concentrations of caspase-1 tested (Figure 4.7B).

In the experiments described above, the 17kD mature fragment of IL-1β could not be detected by western blotting, therefore we considered that the mature cytokine may be released into the surrounding media. Thus, ELISA assays were performed on the supernatants from the transfection assays to measure the amount of mature IL-1β released under the various conditions to verify that this corresponded to the

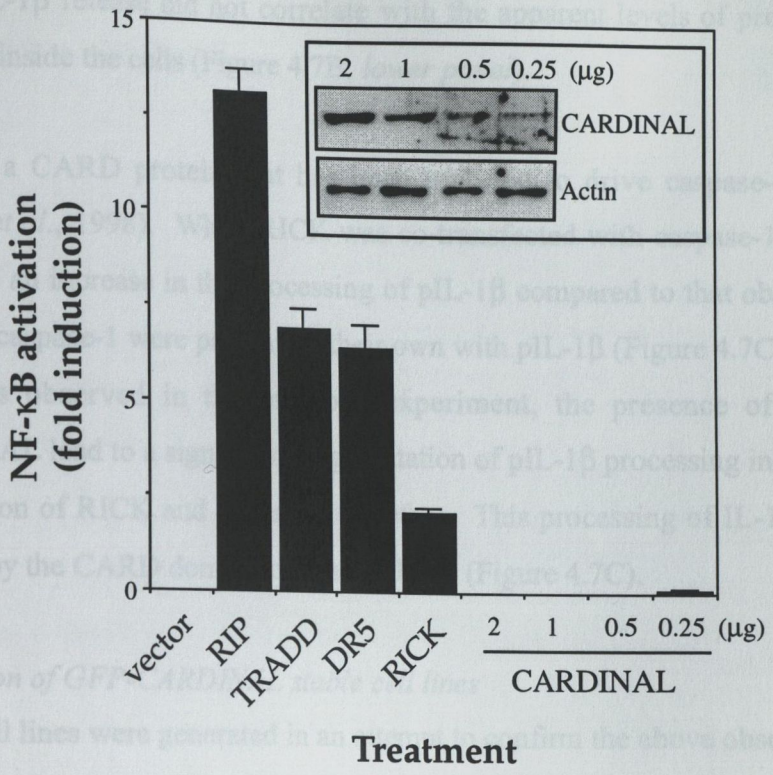


Figure 4.6:

Assessment of NF-κB activation associated with CARDINAL overexpression. Using standard calcium phosphate precipitation, HEK293T cells were transfected with either 1μg of empty vector or with 1μg of plasmids encoding RIP, TRADD, DR5 or RICK, alongside the indicated amounts of pcDNA3-CARDINAL plasmid. All wells also received 200ng of pGL35XκB-luc and 100ng of pcMVβ reporter plasmids and were normalised to the same amount of total DNA using pcDNA3 empty vector. 24hrs after transfection, cell lysates were prepared and luciferase activities associated with activation of the NFκB-driven luciferase reporter was measured in triplicate, as described in Materials and Methods. Luciferase activity values were normalised to β-galactosidase activity values to correct for variability in transfection efficiency between wells. NFκB values are expressed as fold-induction of the luciferase activity associated with the vector control. The NFκB value associated with the vector transfected cells was set at zero and all other values were corrected with respect to the vector sample. Cell lysates prepared from CARDINAL-transfected cells were also assessed for CARDINAL expression by immunoblotting (inset). Results are representative of at least 6 separate experiments.

level of IL-1β processing detected by western analysis (Figure 4.7B, *lower panel*). However, only very small amounts of IL-1β (a maximum of 25pg/ml) could be detected in the cell supernatants, thus any changes observed in the levels of IL-1β released from the cells were unlikely to be significant. In addition, the pattern of mature IL-1β release did not correlate with the apparent levels of processing that was seen inside the cells (Figure 4.7B, *lower panel*).

RICK is a CARD protein that has been reported to drive caspase-1 activation (Thome *et al.*, 1998). When RICK was co-transfected with caspase-1 and pIL-1β there was an increase in the processing of pIL-1β compared to that observed when RICK or caspase-1 were present on their own with pIL-1β (Figure 4.7C). Similar to what was observed in the previous experiment, the presence of full length CARDINAL lead to a significant augmentation of pIL-1β processing induced by co-transfection of RICK and caspase-1 together. This processing of IL-1β was again blocked by the CARD domain of CARDINAL (Figure 4.7C).

Generation of GFP-CARDINAL stable cell lines

Stable cell lines were generated in an attempt to confirm the above observations in a more natural context of IL-1β maturation. THP.1 cells were chosen to create these cell lines, because they release IL-1β in response to LPS stimulation and contain high levels of endogenous caspase-1. To assess the normal response of THP.1 cells to LPS, cells were either left untreated or treated with LPS over a 24hr time course (Figure 4.8). THP.1 cells did not exhibit constitutive expression of proIL-1β, but upregulated this cytokine upon LPS treatment. Upregulation of pIL-1β in THP.1 cells was accompanied by an increase in the release of the mature IL-1β protein into the surrounding media as measured by ELISA (Figure 4.8 *lower panel*). Note that the mature form of IL-1β did not remain in the cell and was released rapidly. Interestingly, the induction and activation of IL-1β was not accompanied by any detectable activation of capase-1, which was detected as the full-length molecule in THP.1 cells throughout the duration of the experiment.

N-terminally EGFP-tagged CARDINAL, EGFP-CARDINAL$^{321\text{-}431}$ or EGFP control plasmid were transfected into THP.1 cells and cultured in G418 over several weeks

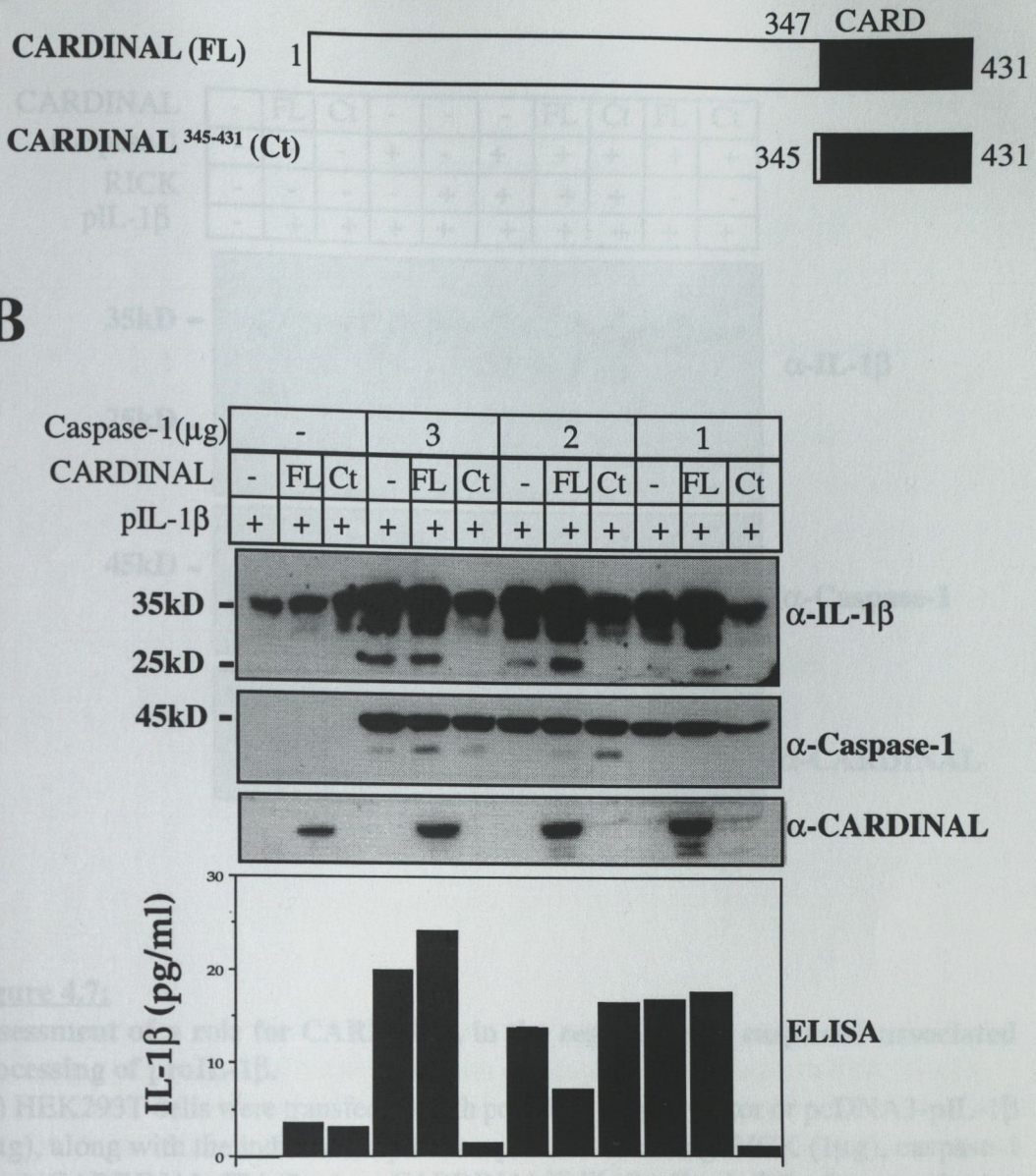


Figure 4.7:

Assessment of a role for CARDINAL in the regulation of caspase-1 associated processing of proIL-1β.

(A) Schematic representation of CARDINAL deletion mutants: Full length CARDINAL (FL) and the c-term truncation (Ct) representing amino acids 345-431. The CARD region is shaded in black.

(B) HEK293T cells were transfected with pcDNA3-pIL-1β (1μg) and the indicated amounts of pcDNA3- caspase-1, along with either 1μg of pcDNA3 empty vector, pcDNA3-CARDINAL (FL) or pcDNA3-CARDINAL$^{345-431}$ (Ct). 24h after transfection cells were harvested and total cell lysates (50μg/lane) were analysed by immunoblot with the indicated antibodies. The cell culture supernatants were collected and the levels of mature IL-1β present was measured by ELISA (lower panel).

C

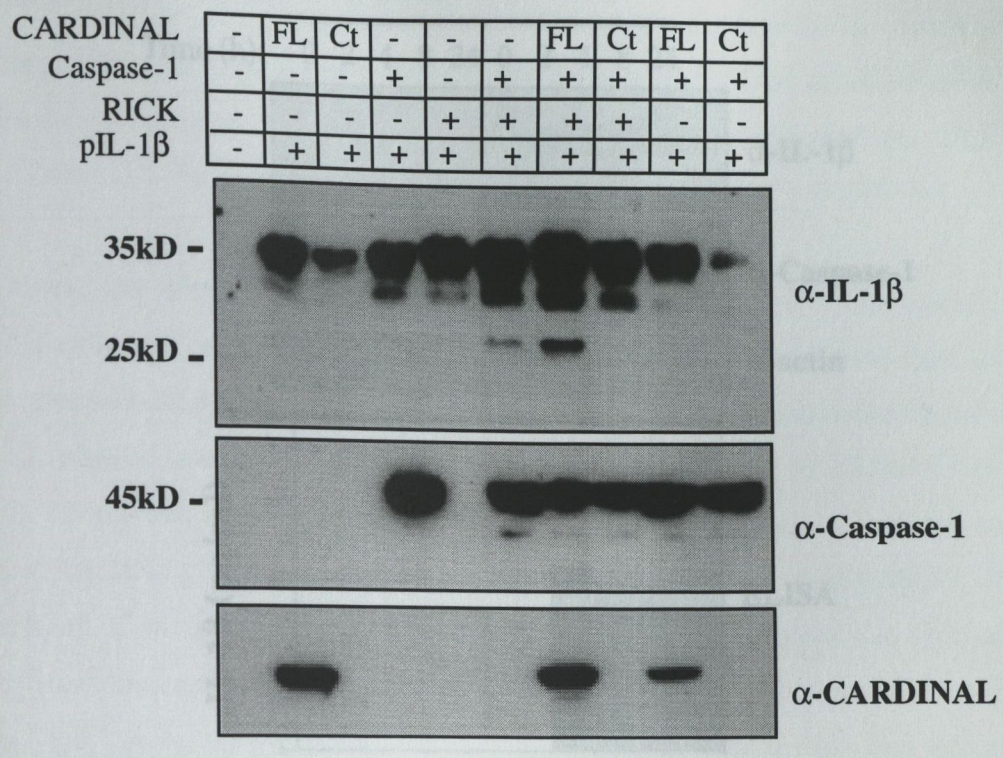


Figure 4.7:

Assessment of a role for CARDINAL in the regulation of caspase-1 associated processing of proIL-1β.

(C) HEK293T cells were transfected with pcDNA3 empty vector or pcDNA3-pIL-1β (1μg), along with the indicated expression plamids encoding RICK (1μg), caspase-1 (2μg), CARDINAL (FL) (2μg) or CARDINAL$^{345-431}$ (Ct) (2μg). 24h after transfection cells were harvested and total cell lysates (50μg/lane) were analysed by immunoblot with the indicated antibodies.

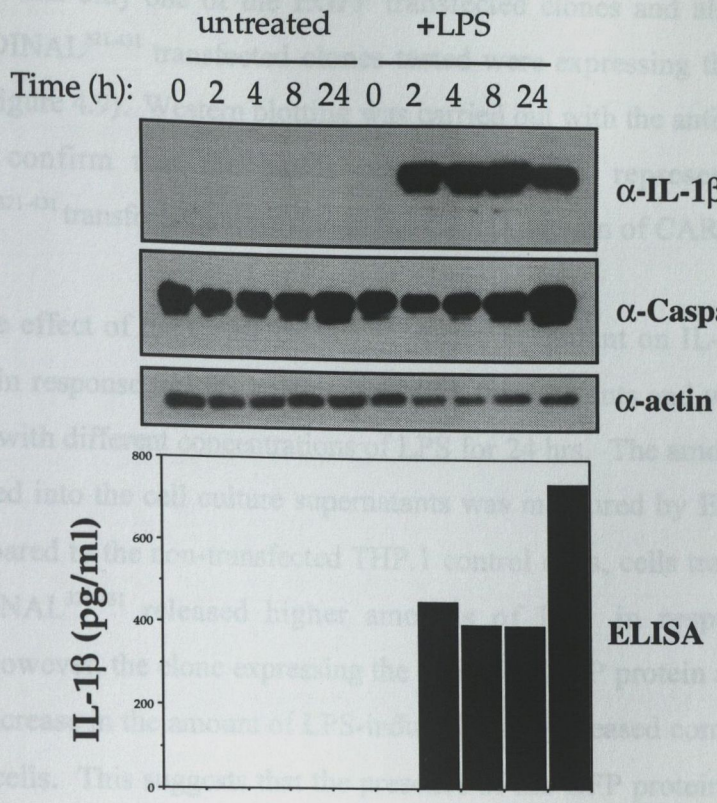


Figure 4.8:
LPS upregulates pIL-1β and promotes the release of mIL-1β in THP.1 cells. THP.1 cells were either left untreated or treated with LPS (1μg/ml) for the indicated times. Total cell lysates (50μg/lane) were analysed by immunoblot with the indicated antibodies. The cell culture supernatants were collected and the levels of mature IL-1β present were measured by ELISA (*lower panel*). These results are representative of three separate experiments.

to select for stably transfected clones. A number of clones transfected with EGFP, EGFP-CARDINAL and EGFP-CARDINAL$^{321-431}$ formed viable colonies that were cultured into cell populations. However, western blot analysis on lysates from these cells showed that only one of the EGFP transfected clones and all three of the EGFP-CARDINAL$^{321-431}$ transfected clones tested were expressing the transfected constructs (Figure 4.9). Western blotting was carried out with the anti-CARDINAL antisera to confirm that the bands present in lanes representing EGFP-CARDINAL$^{321-431}$ transfectants were due to the CARD domain of CARDINAL .

To assess the effect of the CARDINAL$^{321-431}$-deletion mutant on IL-1β release in THP.1 cells in response to LPS treatment, THP.1 transfectants and wild type cells were treated with different concentrations of LPS for 24 hrs. The amount of mature IL-1β released into the cell culture supernatants was measured by ELISA (Figure 4.10). Compared to the non-transfected THP.1 control cells, cells transfected with GFP-CARDINAL$^{321-431}$ released higher amounts of IL-1 in response to LPS treatment. However, the clone expressing the wild-type GFP protein also showed a significant increase in the amount of LPS-induced IL-1β released compared to wild type THP.1 cells. This suggests that the presence of the GFP protein was in some way distorting the normal reaction of these cells to LPS or that the drug selection in G418 had randomly selected highly LPS responsive THP.1 clones. Thus when the CARDINAL$^{321-431}$ transfected clones were compared to the GFP transfected control cells, clones (1) and (3) showed a decrease in the amount of mIL-1β release in response to LPS. In contrast, clone (2) exhibited a higher level of mIL-1β release in response to lower concentrations of LPS. However at the highest concentration of LPS used, the amount of mIL-1β released was slightly decreased compared to the amount released by the GFP-transfected control. Attempts were made to create stable THP.1 cell lines expressing the untagged version of CARDINAL but no viable clones expressing the protein could be isolated.

Thus the enhancing effect of CARDINAL on IL-1β processing could not be confirmed in the THP.1 system. Moreover, the enhancing effect of CARDINAL$^{321-431}$ on IL-1β processing observed was at odds with the data generated in the transient overexpression system using HEK293T cells where it appeared to antagonise

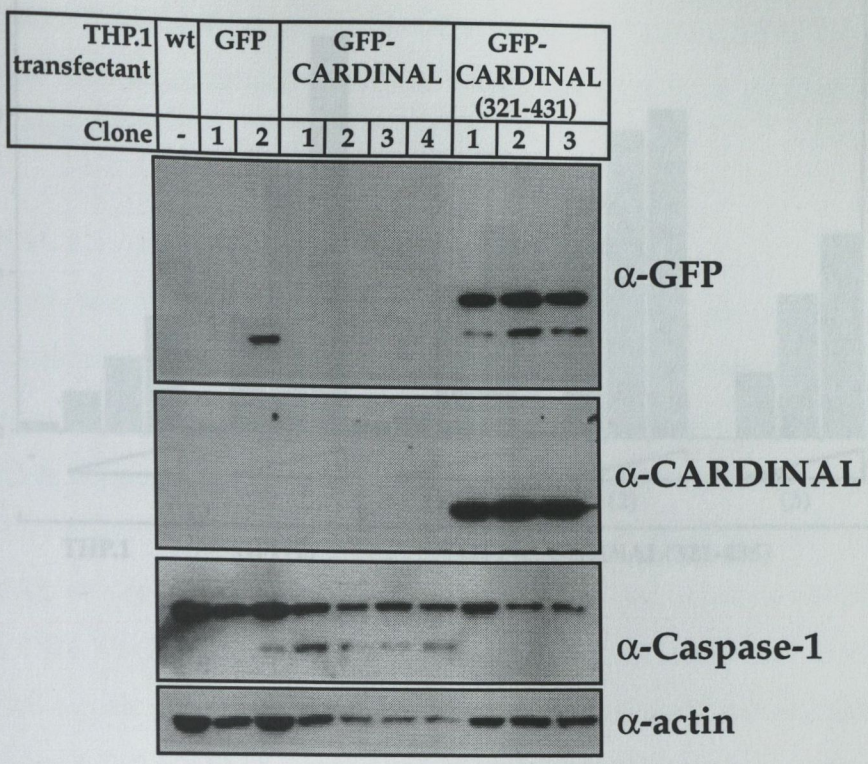


Figure 4.9:

Expression of EGFP, EGFP-CARDINAL and EGFP-CARDINAL$^{321-431}$ proteins in stably transfected THP.1 cells.

pEGFP, pEGFP-CARDINAL and pEGFP-CARDINAL$^{321-435}$ were transfected into THP1. cells by electroporation. Cells were selected for G418 resistance over 6 weeks in 1.4mg/ml G418/neomycin. Total cell lysates (50μg/lane) were analysed by immunoblot for expression of GFP, GFP-CARDINAL or GFP-CARDINAL$^{321-435}$ fusion proteins alongside non transfected wild type (wt) THP.1 cells with the indicated antibodies.

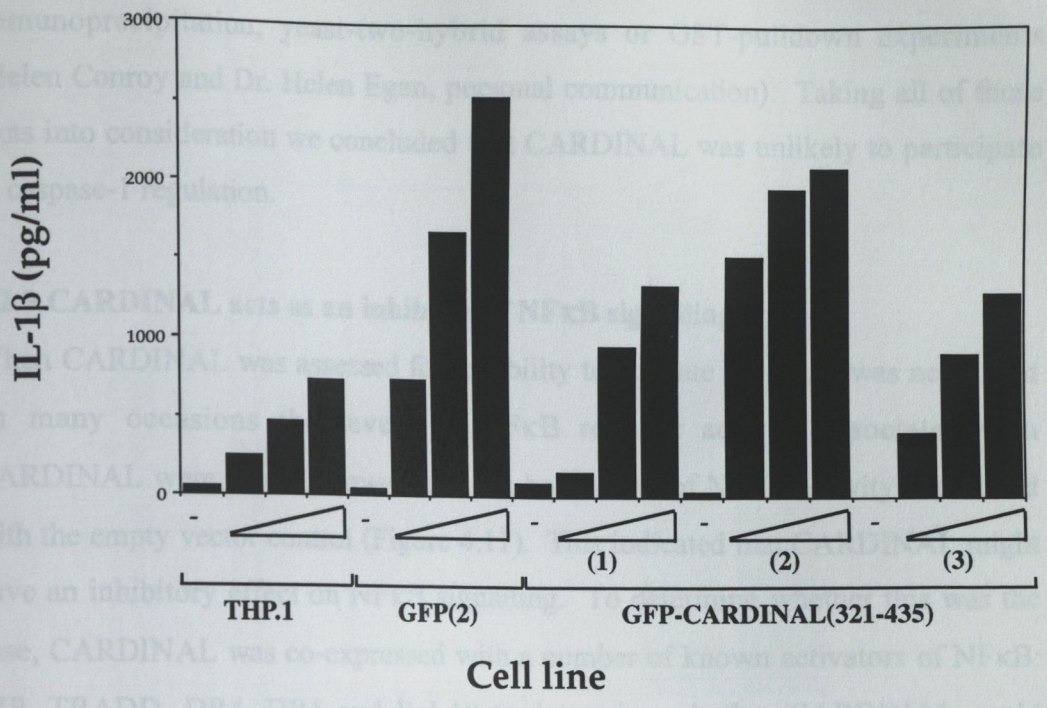


Figure 4.10:

Assessment of LPS-induced IL-1β production in THP.1 wild type cells and in transfectants.

Wild type THP.1 cells or THP.1 cell stably tranfected with either pEGFP or pEGFP-CARDINAL$^{321-435}$ were either left untreated or treated with 100ng, 1μg or 10μg LPS for 24hrs. Numbers in parenthesis represent clone numbers as they are in Figure 4.9. Cell culture supernatants were clarified and analysed for IL-1β release by ELISA. Results are representative of three separate experiments.

caspase-1 activation and IL-1β processing. Additionally, we could not find evidence for an interaction between CARDINAL and caspase-1 by co-immunoprecipitation, yeast-two-hybrid assays or GST-pulldown experiments (Helen Conroy and Dr. Helen Egan, personal communication). Taking all of these facts into consideration we concluded that CARDINAL was unlikely to participate in caspase-1 regulation.

4.2.9 CARDINAL acts as an inhibitor of NFκB signalling

When CARDINAL was assessed for its ability to activate NFκB, it was noted that on many occasions the levels of NFκB reporter activity associated with CARDINAL were actually lower than the basal level of NFκB activity associated with the empty vector control (Figure 4.11). This indicated that CARDINAL might have an inhibitory effect on NFκB signalling. To determine whether this was the case, CARDINAL was co-expressed with a number of known activators of NFκB: RIP, TRADD, DR4, DR5 and Bcl-10 to determine whether CARDINAL could antagonise NFκB signals driven by these molecules. As can be seen from the data presented in Figure 4.12A CARDINAL substantially inhibited the NFκB activation in each case.

The effect of CARDINAL on NFκB signalling was dose dependent. When titrated against a fixed amount of RICK, the inhibitory effects of CARDINAL were diminished when lower levels of CARDINAL are present. However, CARDINAL still possessed a detectable amount of inhibitory activity at levels as low as 250ng (Figure 4.12B).

Importantly, CARDINAL did not have any effect on a p53 driven luciferase reporter activity (Figure 4.12C). When the NFκB activated luciferase reporter used in the above experiments was replaced by a luciferase reporter construct under the control of p53-responsive promoter elements, CARDINAL did not influence p53-induced expression of the luciferase gene. This demonstrates that the effect of CARDINAL was specific to NFκB activation.

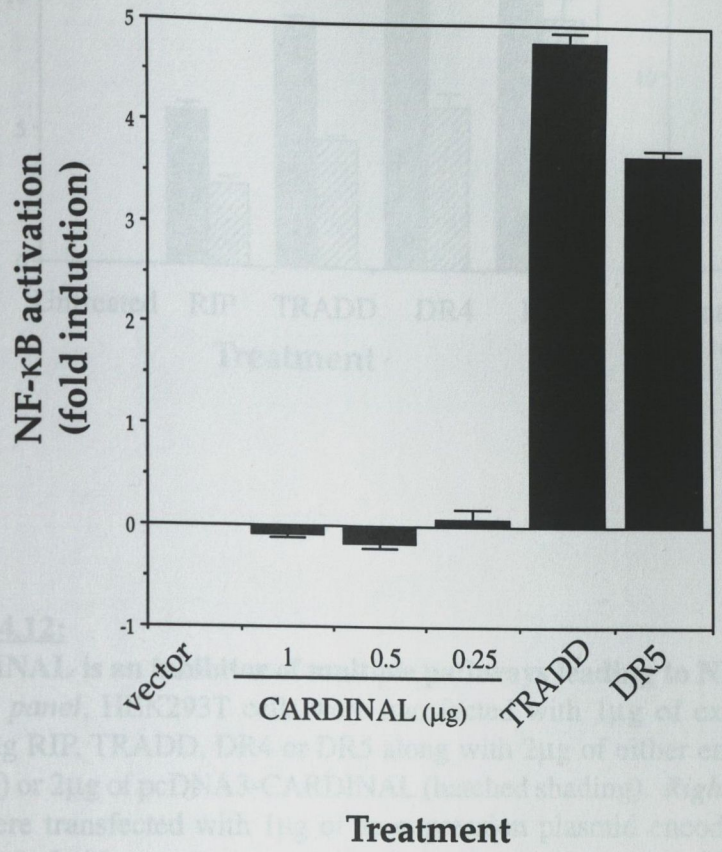


Figure 4.11:

CARDINAL overexpression is associated with sub-basal levels of NF-κB activation.

Using standard calcium phosphate precipitation, HEK293T cells were transfected with either 1μg of empty vector, or with 1μg of plasmids encoding TRADD or DR5, alongside the indicated amounts of pcDNA3-CARDINAL plasmid. All wells also received 200ng of pGL35XκB-luc and 100ng of pcMVβ reporter plasmids and were normalised to the same amount of total DNA using pcDNA3 empty vector. 24hrs after transfection, cell lysates were prepared and luciferase activities associated with activation of the NFκB-driven luciferase reporter was measured in triplicate, as described in Materials and Methods. Luciferase activity values were normalised to β-galactosidase activity values to correct for variability in transfection efficiency between wells. Results are representative of at least 6 separate experiments.

A

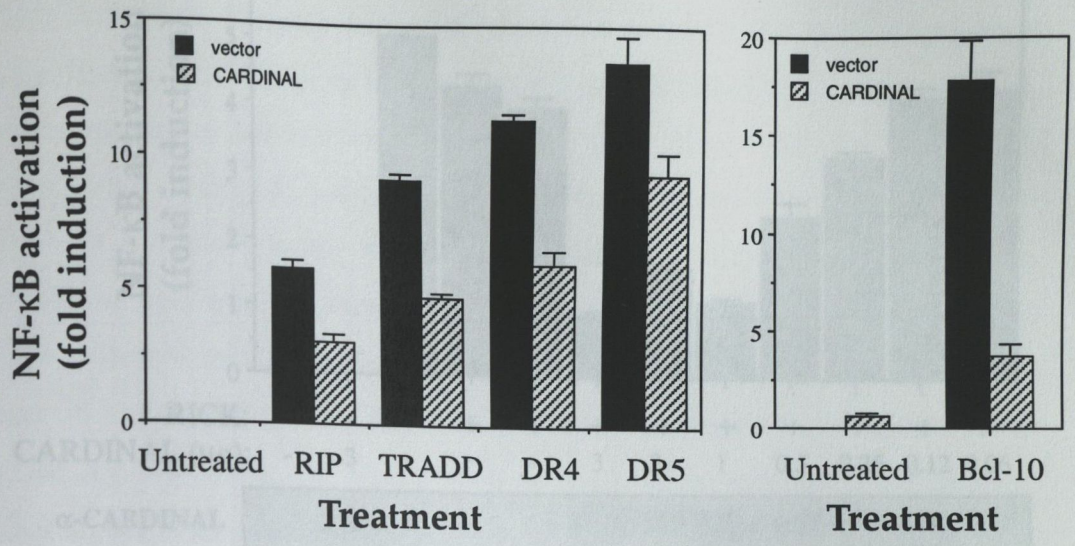


Figure 4.12:
CARDINAL is an inhibitor of multiple pathways leading to NFκB activation.
(A) *left panel*, HEK293T cells were transfected with 1μg of expression plasmids encoding RIP, TRADD, DR4 or DR5 along with 2μg of either empty vector (black shading) or 2μg of pcDNA3-CARDINAL (hatched shading). *Right panel*, HEK293T cells were transfected with 1μg of an expression plasmid encoding Bcl-10, along with 1μg of either empty vector (black shading) or 1μg of pcDNA3-CARDINAL (hatched shading). In both cases, all wells also received 100ng of pGL35XκB-luc and 50ng of pcMVβ reporter plasmids and were normalised to the same amount of total DNA using pcDNA3 empty vector. 24hr after transfection, cells were lysed and NF-κB driven luciferase reporter activities were measured, in triplicate, as described in Materials and Methods. Luciferase activity values were normalised to β-galactosidase activity values to correct for variability in transfection efficiency between wells. Results are representative of three separate experiments.

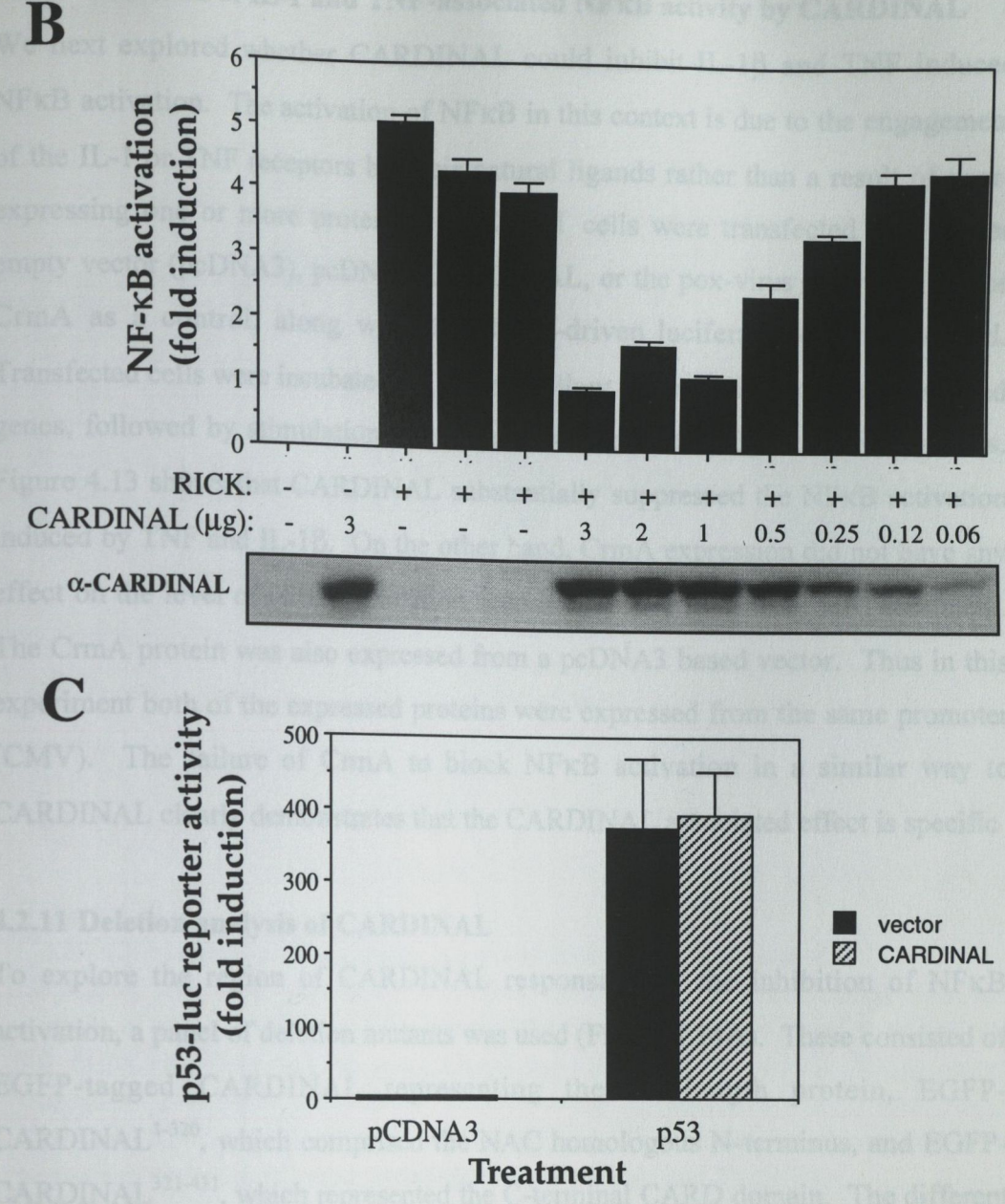


Figure 4.12:

CARDINAL is an inhibitor of multiple pathways leading to NFκB activation.

(B) HEK293T cells were transfected with 1μg of RICK expression plasmid, along with the indicated amounts of pcDNA3-CARDINAL. All wells also received the pGL35XκB-luc(100ng) and pcMVβ (50ng) reporter plasmids. Luciferase assays were performed 24hr after transfection and normalised to correct for transfection efficiency as described previously. Lysates were also assessed for CARDINAL expression by immunoblot using anti-CARDINAL rabbit polyclonal antibody (*lower panel*).

(C) CARDINAL does not inhibit transactivation of a p53 responsive luciferase reporter gene. HEK293T cells were transfected with 500ng of empty vector or 500ng of a p53 expression plasmid, along with 1μg of empty vector (black shading) or 1μg of CARDINAL expression plamid (hatched shading). All wells also received 100ng of p53-luc and 50ng of pCMVβ reporter plasmids. 24hr after transfection, cells were lysed and p53-driven luciferase reporter activities were measured (in triplicate) and normalised to β-galactosidase activity values. Results are representative of three separate experiments.

4.2.10 Inhibition of IL-1 and TNF-associated NFκB activity by CARDINAL

We next explored whether CARDINAL could inhibit IL-1β and TNF induced NFκB activation. The activation of NFκB in this context is due to the engagement of the IL-1 or TNF receptors by their natural ligands rather than a result of over-expressing one or more proteins. HEK293T cells were transfected with either empty vector (pcDNA3), pcDNA3-CARDINAL, or the pox-virus derived inhibitor CrmA as a control, along with the NFκB-driven luciferase reporter plasmid. Transfected cells were incubated for 24hrs to allow for expression of the transfected genes, followed by stimulation with either recombinant IL-1β or TNFα for 6hrs. Figure 4.13 shows that CARDINAL substantially suppressed the NFκB activation induced by TNF and IL-1β. On the other hand, CrmA expression did not have any effect on the level of NFκB activation seen in response to TNF or IL-1 treatment. The CrmA protein was also expressed from a pcDNA3 based vector. Thus in this experiment both of the expressed proteins were expressed from the same promoter (CMV). The failure of CrmA to block NFκB activation in a similar way to CARDINAL clearly demonstrates that the CARDINAL associated effect is specific

4.2.11 Deletion analysis of CARDINAL

To explore the region of CARDINAL responsible of the inhibition of NFκB activation, a panel of deletion mutants was used (Figure 4.14A). These consisted of EGFP-tagged CARDINAL representing the full-length protein, EGFP-CARDINAL$^{1-320}$, which comprised the NAC homologous N-terminus, and EGFP-CARDINAL$^{321-431}$, which represented the C-terminal CARD domain. The different truncation mutants of CARDINAL were transfected into HEK293T cells along with the NFκB reporter plasmid. As in previous experiments, neither EGFP-tagged CARDINAL nor its deletion mutants, were capable of spontaneously activating the NFκB-driven luciferase reporter plasmid (Figure 4.14B, *left panel*).

Relative to the GFP control, full length CARDINAL inhibited both TNF and IL-1β induced NFκB as before (Figure 4.14B). The N-terminus of CARDINAL inhibited NFκB activity to a greater degree while the CARD domain had no effect on its own. Thus it appears to be the N-terminus of CARDINAL that is responsible for its NFκB inhibitory function. The failure of EGFP-CARDINAL$^{321-431}$ to suppress

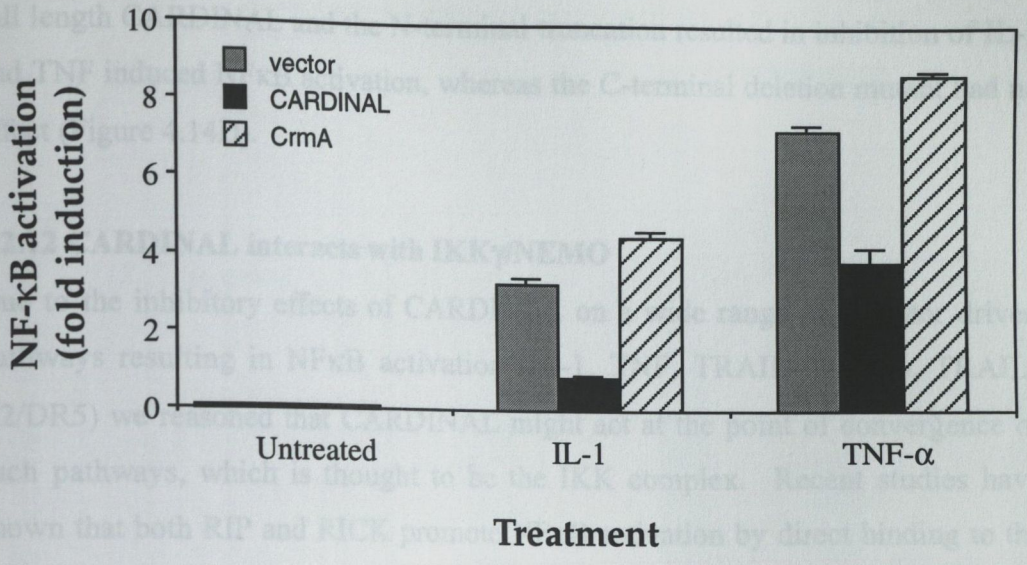


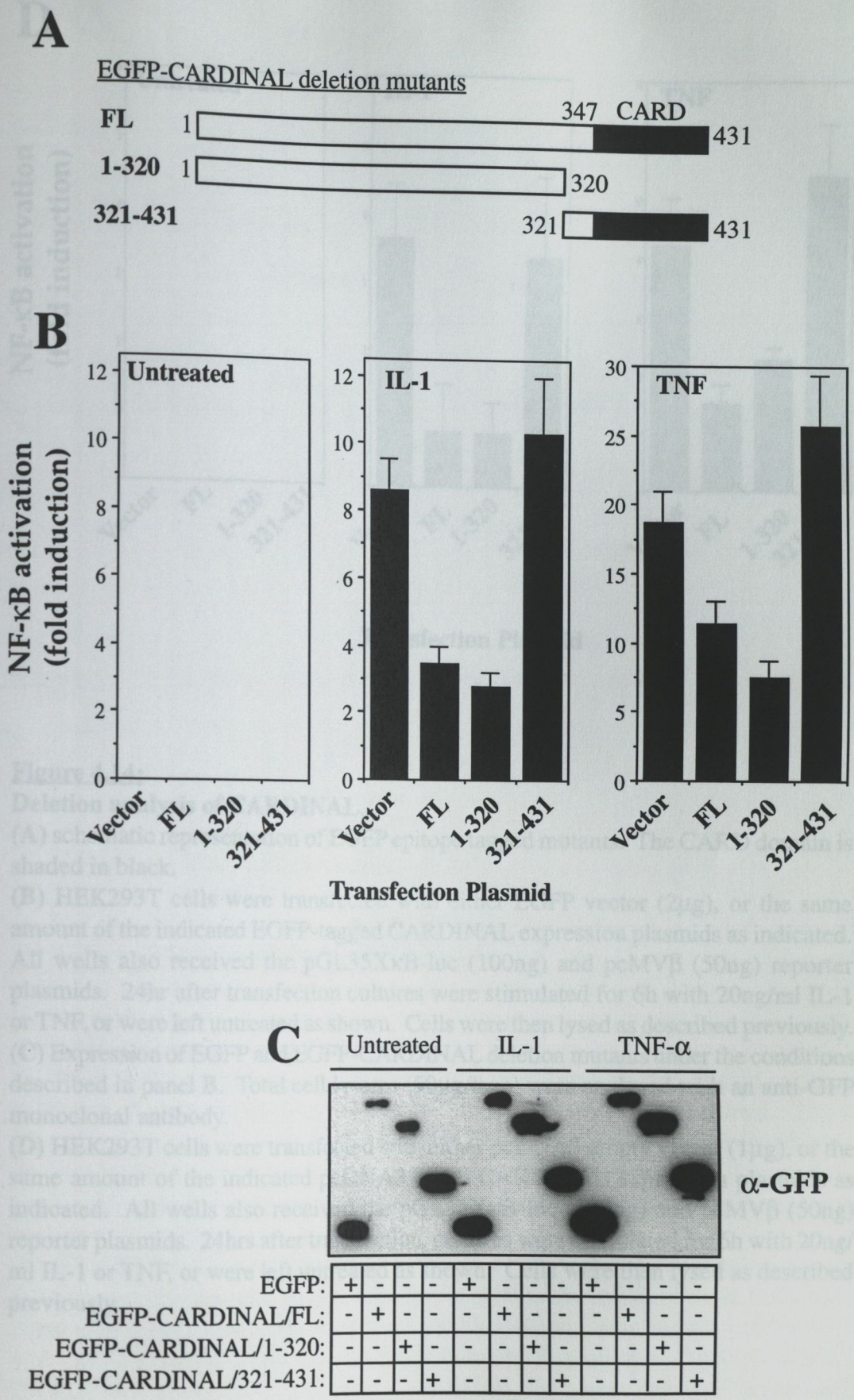
Figure 4.13:
CARDINAL inhibits IL-1 and TNF associated NFκB activation.
HEK293T cells were transfected with either pcDNA3 empty vector (2μg) or the same amount of pcDNA3-CARDINAL or pcDNA3-CrmA expression plasmids as indicated. All wells also received the pGL35XκB-luc (100ng) and pcMVβ (50ng) reporter plasmids. 24hr after transfection, cultures were stimulated for 6hrs with 20ng/ml IL-1, or TNF, or were left untreated as shown. Cells were then lysed and NFκB activation assays performed as described in Materials and Methods.

NFκB activation signals was not due to decreased expression levels of this protein, as this mutant was expressed at levels comparable to or even higher than the other EGFP tagged constructs (Figure 4.14C). Similar experiments were performed with the untagged versions of CARDINAL yielding comparable results, where both the full length CARDINAL and the N-terminal truncation resulted in inhibition of IL-1 and TNF induced NFκB activation, whereas the C-terminal deletion mutant had no effect (Figure 4.14D).

4.2.12 CARDINAL interacts with IKKγ/NEMO

Due to the inhibitory effects of CARDINAL on a wide range of receptor driven pathways resulting in NFκB activation (IL-1, TNF, TRAIL-R1/DR4, TRAIL-R2/DR5) we reasoned that CARDINAL might act at the point of convergence of such pathways, which is thought to be the IKK complex. Recent studies have shown that both RIP and RICK promote NFκB activation by direct binding to the regulatory subunit of the IKK complex, IKKγ/NEMO (Inohara *et al.*, 2000). As has been shown above NFκB activation through both RIP and RICK could be attenuated by CARDINAL co-expression. This suggested that CARDINAL might act at the level of IKKγ/NEMO recruitment by RICK or RIP, or at a point downstream of this.

To explore this possibility we asked whether CARDINAL could directly bind to IKKγ, providing a means whereby this CARD protein could antagonise multiple independent NFκB activation pathways. Thus HEK293T cells were co-transfected with expression plasmids encoding IKKγ in combination with EGFP or EGFP-CARDINAL. 24hrs post transfection cell lysates were made and CARDINAL was immunoprecipitated using anti-CARDINAL antibody (Figure 4.15A). Immune complexes were then probed for the presence of co-precipitated IKKγ. IKKγ was readily detectable in the CARDINAL precipitate, suggesting that CARDINAL directly interacts with this subunit of the IKK complex. Additional co-immunoprecipitation experiments with all three components of the IKK complex show that CARDINAL specifically interacted with IKKγ and not IKKα or IKKβ (Fig. 4.15B). Thus it seems that CARDINAL may act to antagonise the ability of


A
EGFP-CARDINAL deletion mutants
FL
1
347
CARD
431
1-320
1
320
321-431
321
431
B
NF-κB activation (fold induction)
Untreated
IL-1
TNF
Vector
FL
1-320
321-431
Transfection Plasmid
C
Untreated
IL-1
TNF-α
α-GFP
EGFP:
EGFP-CARDINAL/FL:
EGFP-CARDINAL/1-320:
EGFP-CARDINAL/321-431:

D

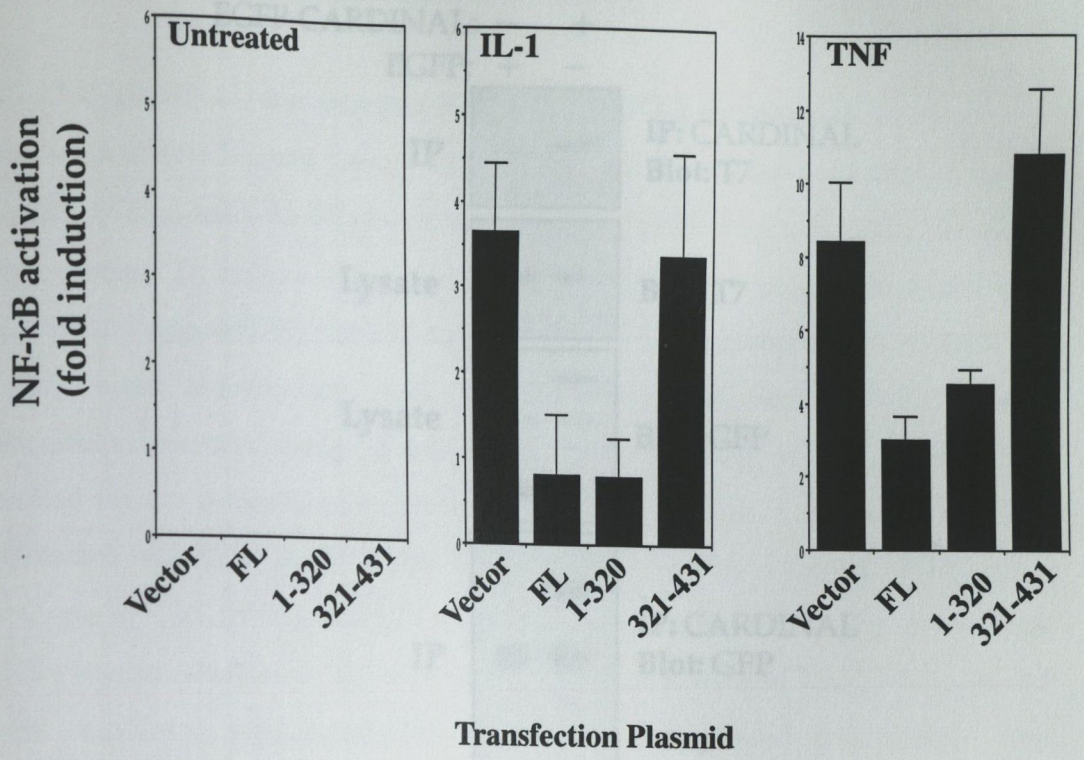


Figure 4.14:

Deletion analysis of CARDINAL.

(A) schematic representation of EGFP epitope tagged mutants. The CARD domain is shaded in black.

(B) HEK293T cells were transfected with either EGFP vector (2μg), or the same amount of the indicated EGFP-tagged CARDINAL expression plasmids as indicated. All wells also received the pGL35XκB-luc (100ng) and pcMVβ (50ng) reporter plasmids. 24hr after transfection cultures were stimulated for 6h with 20ng/ml IL-1 or TNF, or were left untreated as shown. Cells were then lysed as described previously.

(C) Expression of EGFP and EGFP-CARDINAL deletion mutants under the conditions described in panel B. Total cell lysates (50μg/lane) were analysed with an anti-GFP monoclonal antibody.

(D) HEK293T cells were transfected with either pcDNA3 empty vector (1μg), or the same amount of the indicated pcDNA3 based CARDINAL expression plasmids as indicated. All wells also received the pGL35XκB-luc (100ng) and pcMVβ (50ng) reporter plasmids. 24hrs after transfection, cultures were stimulated for 6h with 20ng/ml IL-1 or TNF, or were left untreated as shown. Cells were then lysed as described previously.

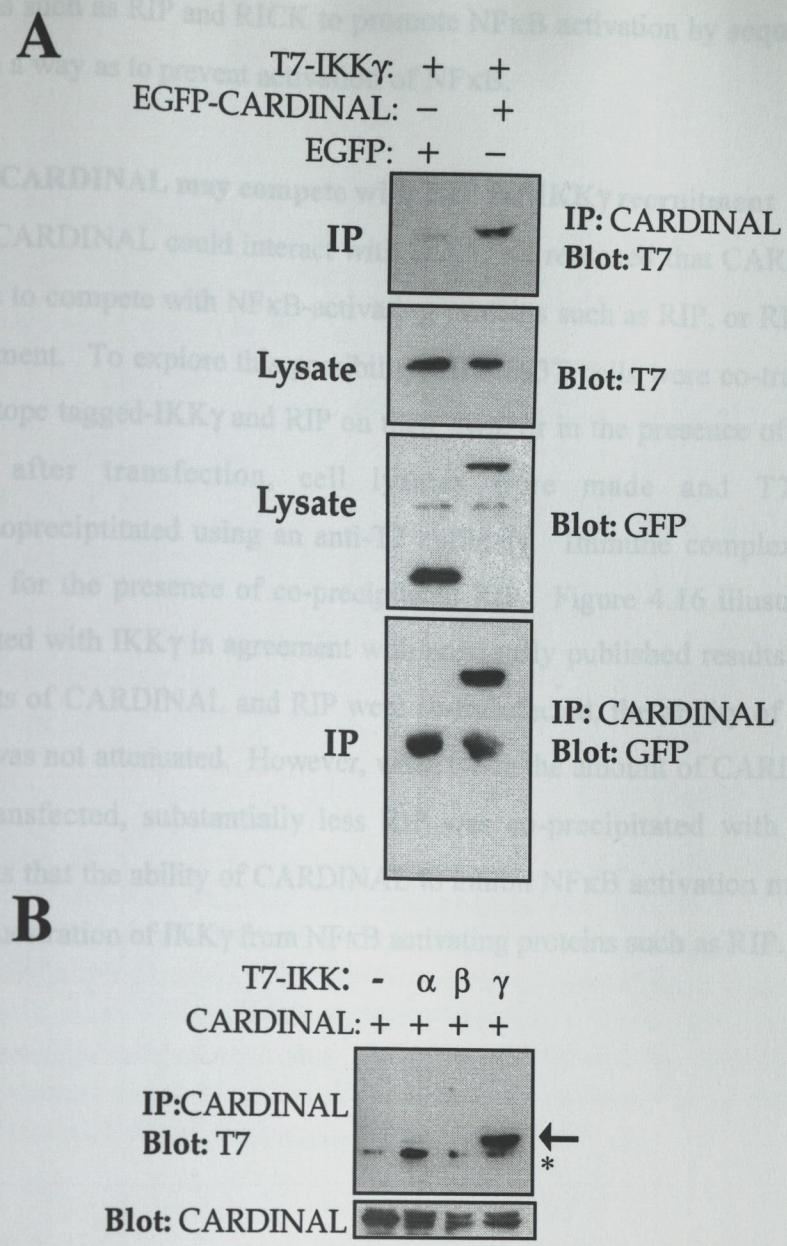


Figure 4.15:

CARDINAL interacts with IKK-γ (NEMO)

(A) HEK293T cells were transfected with 5μg of T7 epitope-tagged IKK-γ along with 5μg of plasmids encoding EGFP or EGFP-CARDINAL as indicated. 24hrs after transfection, cells were lysed and CARDINAL was immunoprecipitated with anti-CARDINAL polyclonal antibody, followed by probing with horseradish peroxidase-linked anti-T7 monoclonal antibody or anti-GFP monoclonal antibody as shown.

(B) HEK293T cells were transfected with 5μg of the indicated T7-epitope tagged IKK expression plasmids, along with 5μg of pcDNA3-CARDINAL as indicated. 24hrs after transfection, cells were lysed and CARDINAL was immunoprecipitated with anti-CARDINAL polyclonal antibody followed by probing with anti-T7 or anti-CARDINAL antibodies, as shown. Cell lysates were also assessed to confirm expression of IKKα, IKKβ and IKKγ by immunoblotting (data not shown). The asterix represents immunoglobulin heavy chain; the arrow indicates T7-tagged IKKγ.

proteins such as RIP and RICK to promote NFκB activation by sequestering IKKγ, in such a way as to prevent activation of NFκB.

4.2.13 CARDINAL may compete with RIP for IKKγ recruitment

Since CARDINAL could interact with IKKγ, we reasoned that CARDINAL might be able to compete with NFκB-activating proteins such as RIP, or RICK, for IKKγ recruitment. To explore this possibility, HEK293T cells were co-transfected with T7-epitope tagged-IKKγ and RIP on their own, or in the presence of CARDINAL. 24hrs after transfection, cell lysates were made and T7-IKKγ was immunopreciptitated using an anti-T7 antibody. Immune complexes were then probed for the presence of co-precipitated RIP. Figure 4.16 illustrates that RIP interacted with IKKγ in agreement with previously published results. When equal amounts of CARDINAL and RIP were co-transfected, the ability of RIP to recruit IKKγ was not attenuated. However, when twice the amount of CARDINAL to RIP was transfected, substantially less RIP was co-precipitated with IKKγ. This suggests that the ability of CARDINAL to inhibit NFκB activation may be through the sequestration of IKKγ from NFκB activating proteins such as RIP.

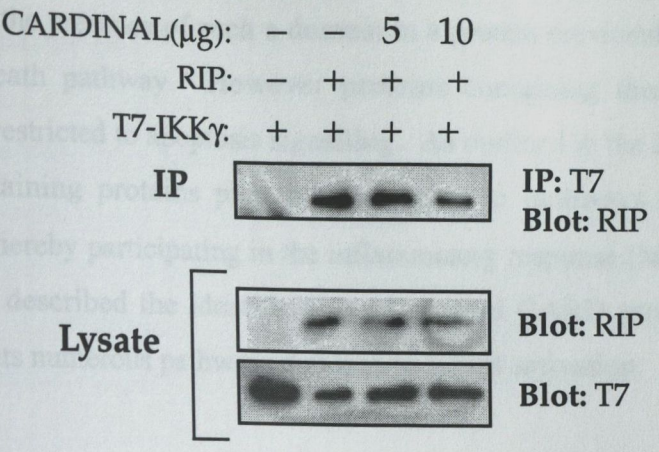


Figure 4.16:

CARDINAL may compete with RIP for IKK-γ recruitment

HEK293T cells were transfected with 5μg of T7 epitope-tagged IKK-γ along with 5μg of plasmids encoding FLAG-RIP, with empty vector 5μg or 10μg of CARDINAL as indicated. 24hrs after transfection, cells were lysed and IKK-γ was immunoprecipitated with anti-T7 antibody, followed by probing with anti-RIP monoclonal antibody or anti-T7 monoclonal antibody as shown. Results are representative of two separate experiments.

4.3 DISCUSSION

The CARD domain features prominently in apoptosis pathways (Hofmann *et al.*, 1997). Like the DED and death domain, the CARD is a protein-protein interaction domain and the presence of such a domain in a protein previously suggested a role in a cell death pathway. However proteins containing these motifs are not necessarily restricted to apoptosis signalling. As outlined in the Introduction, many CARD-containing proteins play integral roles in pathways leading to NFκB activation, thereby participating in the inflammatory response (Martin, 2001). This chapter has described the identification of a novel CARD protein CARDINAL, which inhibits numerous pathways that lead to NFκB activation.

Sequence analysis of CARDINAL revealed that the CARD domain of this protein is quite closely related to the pro-domain of caspase-1, which also contains a CARD domain (Figure 4.1C). There is also extensive homology between the N-terminus of CARDINAL and the C-terminus of the recently identified NAC protein (Figure 4.1D). Unlike many CARD proteins, CARDINAL does not promote apoptosis or NFκB activation (Figiure 4.5A; Figure 4.6). However, it has been shown here to suppress NFκB activation induced by a number of different proteins (Figure 4.12). CARDINAL binds to the regulatory subunit of the IKK signalosome, IKKγ (figure 4.15). These results suggest that CARDINAL, through recruitment of IKKγ, might act to antagonise many NFκB inducing proteins such as RIP and RICK.

4.3.1 CARDINAL and apoptosis

To characterise this protein we first investigated a role for CARDINAL in apoptosis and found that it does not display any overt cytotoxicity. In a separate study CARDINAL was cloned independently by a group who named it TUCAN (Pathan *et al.*, 2001). Their results suggest a role for CARDINAL as an inhibitor of apoptosis, more specifically as an inhibitor of the Apaf-1 apoptosome. Their model suggests that TUCAN/CARDINAL competes with Apaf-1 for caspase-9 binding. However we did not find any role for CARDINAL in suppressing apoptosis induced by a number of stimuli including Bax (Figure 4.5 C-D). This is in direct contradiction with the results reported in the TUCAN paper, where they reported a dramatic inhibition of Bax-mediated cell death by TUCAN. However, both studies

examined the expression profile of this protein, which yielded similar results, notably the high level of expression in MCF-7s. This clearly shows that both groups were working with the same protein. It can thus only be concluded that the disagreement between the two groups is perhaps due to different assays or expression systems used.

4.3.2 CARDINAL as a regulator of Caspase-1 Activation

A striking feature of the sequence of the CARDINAL protein is its high degree of homology with the pro-domain of caspase-1 (Figure 4.1C). Given the recent discovery of a number of CARD proteins that function as caspase-1 regulators it seemed a reasonable hypothesis that CARDINAL may also act to regulate caspase-1 function.

Caspase-1, the prototypical caspase, was initially reported to have a role in apoptosis when it was discovered to have a high degree of homology with the *C.elegans* cell death protein CED-3 (Miura *et al.*, 1993; Yuan *et al.*, 1993). However, it has since been recognised that the primary role of this protein is in the maturation of IL-1β (Li *et al.*, 1995). There are a number of proteins that have been reported to bind to and potentially regulate caspase-1. RICK, the CARD containing kinase has been shown to bind caspase-1 and to weakly promote caspase-1 activation (Thome *et al.*, 1998). ICEBERG and pseudoICE/COP are two small CARD proteins that are very similar to the CARD of caspase-1 (Humke *et al.*, 2000; Druile *et al.*, 2001; Lee *et al.*, 2001). Both bind to caspase-1 and have been suggested to disrupt RICK mediated oligomerisation of caspase-1, thus inhibiting its function. Finally CARD 12/Ipaf, like RICK, has been implicated as an activator of caspase-1 (Geddes *et al.*, 2001; Poyet *et al.*, 2001).

To investigate whether CARDINAL, similar to these proteins, acts as a caspase-1 regulator, we assessed whether CARDINAL has any effect on caspase-1 mediated IL-1β processing. The presence of CARDINAL seemed to increase the efficiency of IL-1β processing by caspase-1 and the CARD domain of CARDINAL potently inhibited this processing (Figure 4.7B). Moreover, when RICK was co-expressed with caspase-1, more efficient processing of IL-1β was observed, which again was

enhanced by the presence of CARDINAL, or inhibited by the presence of the CARD domain of CARDINAL (Figure 4.7C).

This observation suggested an interesting model for caspase-1 regulation by CARDINAL. We hypothesised that CARDINAL could bind caspase-1 through a CARD-CARD interaction and that the N-terminus of CARDINAL possesses an, as yet unidentified, oligomerisation domain. Thus through oligomerisation of CARDINAL, caspase-1 molecules would be brought into closer proximity, serving to activate this caspase and promote IL-1β processing. Conversely, the CARD domain inhibited the activity of caspase-1, because the truncation of the N-terminus would be likely to abrogate the oligomerisation capabilities of CARDINAL. This would cause the CARD domain to act as a decoy molecule in a similar way to ICEBERG and pseudoICE (Humke *et al.*, 2000; Druile *et al.*, 2001; Lee *et al.*, 2001).

Although this is an attractive model for caspase-1 regulation, a number of lines of evidence did not support it. Firstly, we could not find binding between caspase-1 and CARDINAL, by GST pulldown, co-immunoprecipitation or by the yeast-two-hybrid assay (Helen Conroy and Dr Helen Egan, personal communication). Secondly, the model predicts that CARDINAL induces activation of caspase-1 leading to increased IL-1β processing. However the presence or absence of CARDINAL did not affect the processing of caspase-1 in a detectable way (Figure 4.7). Finally, the original observation, pertaining to increased IL-1β processing could not be verified in the context of LPS-induced IL-1β release in THP.1 cells.

In order to verify the results obtained using HEK293T cells, stable THP.1 cell lines were generated to express EGFP, EGFP-CARDINAL or EGFP-CARDINAL $^{321-431}$. From the previous experiments it was expected that THP.1 cells stably-expressing full length CARDINAL would lead to an increase in IL-1β release, while the CARD domain would repress the release of IL-1β compared to wild type THP.1 cells. Although G418 resistant clones for all three proteins grew out, only the EGFP and EGFP-CARDINAL$^{321-431}$ cells were shown to express the protein (Figure 4.9). LPS treatment of cells expressing the CARD domain led to an increase in IL-1β release

over wild type THP.1 cells, which is in direct disagreement with the results obtained in HEK293T cells (Figure 4.10). However the EGFP-transfected lines also showed an increase in IL-1β release. Therefore compared to the GFP control, two of the clones expressing EGFP-CARDINAL$^{321-431}$ showed a decrease in IL-1β release, while the third showed an increase at lower levels of LPS. This suggests, firstly, that the presence of the EGFP protein alone was having an effect on the LPS response of these cells. Secondly, the discrepancy between the three EGFP-CARDINAL$^{321-431}$ clones suggested that none of the results from these lines could be assumed to be representative of a real effect. This is a major drawback of working with cloned cell lines. Because the cell population is grown from one or a few cells, there is a high risk that the cloned population is not representative of a normal population of cells. In this case there may be variable levels of LPS (TLR4) receptor density in each population of cells which alone could lead to variations between the LPS responsiveness of the different clones.

Although, the effect of CARDINAL on IL-1β processing suggested an interesting potential function for this protein, there were many problems with the system used to show this effect. The processing pattern observed and used as a readout of IL-1β release was not classical. In other words the expected mature 17kD fragment of IL-1β could not be detected, only a 25kD intermediate. The ELISA results suggested that the mature subunit was not being released, or that it was being released at sub-detectable levels (Figure 4.7B). Also, processing of pIL-1β was always accompanied by an increase of the pro-form, rather than a disappearance as is seen with the cleavage of most caspase substrates. This may have been due to increased transcription of endogenous IL-1β, resulting from activation of the IL-1 receptor by the mature IL-1β that was being released from the cells. However, the ELISA results argue against this, since very low levels of mature IL-1β appeared to have been released (Figure 4.7B).

Given these internal contradictions to the initial model for CARDINAL function, and in the absence of any other supporting evidence it seemed unlikely that CARDINAL acts as a caspase-1 activating protein. Therefore we explored the role

of CARDINAL in a third area in which CARD containing proteins play a prominent role, NFκB activation.

4.3.3 CARDINAL is an inhibitor of NFκB activating pathways

The results shown here describe CARDINAL as an inhibitor of NFκB activation. When CARDINAL was co-expressed with a number of activators of NFκB including RIP, RICK and Bcl-10 it resulted in a quite dramatic suppression of NFκB activation (Figure 4.12). The three aforementioned proteins represent three distinct pathways that lead to the same outcome, that is, activation of the NFκB protein (Figure 1.3 and 1.6 in Introduction). Furthermore, CARDINAL also inhibited IL-1 and TNF-induced NFκB, which not only represents activation of the pathway through engagement of receptors naturally present on the surface of the cell rather than over-expressed proteins, but also, in the case of IL-1, is a completely separate pathway leading to NFκB activation (Figure 4.13). All of these pathways converge at the level of the IKK signalosome. Therefore, it is not surprising that CARDNAL binds to IKKγ the regulatory subunit of this complex (Figure 4.15).

At least two activators of NFκB, RICK and RIP, have been shown to bind to IKKγ, and it would be expected that other downstream activators of NFκB might act in the same way (Inohara *et al.*, 2000; Poyet *et al.*, 2000). In the case of RIP and RICK, these proteins bind to IKKγ not through their death or CARD domains (respectively) or through their kinase domains, but through an intermediate domain which has not yet been defined as having a particular motif or signal sequence. Similarly, in the case of CARDINAL, it is not the CARD domain that is responsible for the inhibitory function, but the N-terminal domain (Figure 4.14). These results suggest that CARDINAL functions to antagonise downstream NFκB activators such as RIP and RICK by competing with such proteins for recruitment of IKKγ (Figure 4.16). In doing so, CARDINAL blocks the activation of the IKK complex and downstream events leading to activation of NFκB.

4.3.4 Is there a CARD protein that binds CARDINAL?

CARD-containing proteins have typically been found to interact with other proteins through CARD-CARD interactions. No association was found between

CARDINAL and a number of caspases and known CARD-containing proteins (data not shown). This does not rule out the possibility that CARDINAL may bind an, as yet, uncharacterised CARD-containing protein, which may modulate its function. An approach to isolating such a protein would be to perform a yeast-two-hybrid screen, using CARDINAL as bait

The significance of the extensive homology between the N-terminus of CARDINAL and the C-terminus of NAC remains unclear. NAC is a member of the NBD-CARD protein family. There is disagreement as to the specific binding partners of DEFCAP/NAC (caspase2/caspase-9 versus Apaf-1) and whether this protein promotes caspase activation and apoptosis via direct or indirect means (Hlaing *et al.*, 2001; Chu *et al.*, 2001). The domain structure of NAC/DEFCAP is similar to that of Nod1/CARD4 and Nod2 containing an NBD and LRRs. This would strongly suggest that this protein is likely to act as a sensor for pathogen products, like Nod1/CARD4 and Nod2, although this remains to be determined. The latter possibility introduces a scenario where CARDINAL may act to antagonise signals routed through DEFCAP/NAC by competing for the same C-terminal binding partners, which may operate via CARD-CARD interactions.

4.3.5 Conclusions

CARD-containing proteins have been implicated in caspase activation leading to apoptosis or NFκB activation resulting in inflammation. However, this is the first report of a CARD-containing protein that serves to inhibit NFκB activation. The tissue expression pattern supports the idea that CARDINAL may be involved in some type of immune-protection function since it is quite highly expressed in a number of tissues, such as ovary and testis, that could be considered to be immune privileged sites.

Given the emerging role for CARD-family proteins as signalling intermediaries in multiple pathways that result in NFκB activation, CARDINAL may act to counteract some of the known NFκB activating molecules, such as RIP or RICK, to set a threshold for NFκB activation. Alternatively, CARDINAL may play a role in limiting the duration of NFκB activation. The exact context in which CARDINAL

participates is to be explored further. Here we have demonstrated that CARDINAL acts to inhibit a number of NFκB pathways. However, there are still a number of questions concerning CARDINAL function that remain unanswered. Is the expression of CARDINAL highly regulated or is it modified in some way to activate its function? Does CARDINAL-associated inhibition of NFκB activation pathways lead to the transcriptional repression of all NFκB regulated genes or does it only affect a particular subset of genes activated by NFκB? Further work is clearly necessary to explore how CARDINAL expression is regulated in response to pro-inflammatory stimuli and to determine the specific biological context in which CARDINAL operates. However the results shown here strongly suggest that this protein plays an important role in the negative regulation of stress responses and inflammation.

CHAPTER V

Characterisation of the role of Nod1 in the LPS response

5.1 INTRODUCTION

The previous chapter described CARDINAL as a novel CARD protein with an important role in NFκB regulation. Having assessed the ability of CARDINAL to inhibit NFκB, activated by the proinflammatory stimuli IL-1 and TNF, we wished to investigate if CARDINAL could also inhibit NFκB activated by LPS. Two CARD proteins Nod1 and Nod2 have been proposed as intracellular receptors for LPS (Inohara *et al.*, 2001; Girardin *et al.*, 2001; Martin, 2001), presenting an opportunity to analyse the role of CARDINAL in a novel LPS driven pathway resulting in NFκB activation.

5.1.1 TLR4 – The LPS receptor

The proposal that the Nod proteins act as intracellular receptors for LPS suggests a novel paradigm for LPS signalling, which up until now had been thought to act exclusively at the membrane level through the Toll-like receptor TLR4. TLR4 is a member of a family of ten receptor molecules that share a similar structure possessing a series of leucine rich repeats (LRRs) in the extracellular region, a transmembrane domain and an intracellular TIR domain, that is also found in the intracellular region of the IL-1 receptor (IL-1R) (Rock *et al.,* 1998). *In vitro* studies have implicated both TLR4 and TLR2 as the receptors for lipopolysaccharide (Chow *et al.*, 1999; Kirschning *et al.*, 1998). However studies on the *lps* mutant mouse, confirmed that a mutation in TLR4 was responsible for the LPS resistant phenotype, suggesting that TLR4 was the sole receptor for LPS (Poltorak *et al.*, 1998). Studies of the TLR2 knockout mouse suggest that TLR2 is a receptor for peptidoglycan, a component of gram-positive bacterial cell walls (Takeuchi *et al.*, 1999). In fact the consensus is that each member of the Toll-like receptor family recognises a different pathogen derived component.

5.1.2 Nod1 and Nod2 – intracellular receptors for LPS?

Given that the phenotype of the *lps* mutant mouse, due to a mutation in one gene TLR4, results in complete resistance to LPS induced shock, the physiological relevance of an intracellular LPS receptor may seem slight. However, a number of lines of evidence underscore the significance of this discovery. Firstly, not all cells are naturally responsive to LPS. For example, epithelial cells in the gut are exposed

to bacteria and flora on an ongoing basis, yet do not activate an inflammatory response (Pugin *et al.*, 1993). These cells do not express TLR4 and in this way they have adapted to their surroundings (Naik *et al.*, 2001). However if the same cells are infected with the invasive bacteria *Shigella flexneri* they have been shown to activate NFκB and the release of cytokines such as IL-8 (Philpott *et al.*, 2000). The same effect was seen when these cells were microinjected with LPS (Philpott *et al.*, 2000) suggesting that an intracellular receptor for LPS might exist.

The main candidate for such a receptor is Nod1 and its close relative Nod2. When cells that are not normally LPS responsive are transfected with Nod1 or Nod2 and then induced to uptake LPS they were shown to activate NFκB (Inohara *et al.*, 2001; Girardin *et al.*, 2001).

5.1.3 Nod2 is mutated in Crohn's disease

Nod2 is located on chromosome 16, which has been implicated as the chromosome responsible for harbouring mutations that predispose to Crohn's disease (Hugot *et al.* 1996). Crohn's disease is an inflammatory bowel disorder associated with chronic and persistent inflammation in the gut (Truelove and Pena, 1976). The chromosomal location of Nod2 and its proposed role in LPS signalling prompted a candidate gene approach to investigate if Nod2 was mutated in this disorder. This study and two independent studies which scanned the chromosome for mutations associated with Crohn's identified three mutations all located in the LRR region of Nod2 (Ogura *et al.*, 2001a; Hugot *et al.*, 2000; Hampe *et al.*, 2001). These mutations cause frame shift and missense mutations in the protein. It would be expected from the role of Nod2 that such mutations would create a constitutively active Nod2 protein, which is hyperresponsive to LPS. In fact the opposite is true, the mutant does not activate NFκB in response to LPS to the same level as the intact protein. The authors thus speculate that this inability of the cell to respond to LPS may promote an increased adaptive immune response (Ogura *et al.*, 2001).

Nod2 has also been shown to be mutated in Blau's syndrome (Miceli-Richard *et al.*, 2001). Blau's syndrome, like Crohn's disease, is an inflammatory condition but it affects different organs, being characterised by early-onset arthritis and skin rash (Blau, 1985). Three mutations have been found in the NBD of Nod2 that are

responsible for this syndrome (Miceli-Richard *et al.*, 2001). Exactly how these mutations lead to increased inflammation in skin and joint areas has not been tested.

The striking phenotypic effects of a number of mutations in this one gene suggest that Nod2 plays an important role in the regulation of the inflammatory response. This is in favour of the proposal that Nod2 and Nod1 act as intracellular receptors for bacterial pathogens.

5.1.4 NBD-LRR – a feature of plant disease resistance proteins

The structures of the Nod proteins are also supportive of their role as pathogen receptors. Both proteins are members of the NBD-CARD sub-group of CARD proteins (Figure 1.5 in Introduction). The structures of both Nod1 and Nod2 are similar to that of Apaf-1, containing a CARD domain, an NBD and a series of leucine rich repeats (LRRs) in place of the WD40 repeats found in Apaf-1. It has been shown that LPS binds to the LRRs analogous to the way in which cytochrome *c* binds to the WD40 repeats of Apaf-1 (Figure1.6 in Introduction; Inohara *et al.*, 2001). The combination of the NBD and LRR domains is found in many plant disease resistance genes or R proteins. These R proteins bind to infectious *avr* proteins and hence act as sensors for infection in plants (Dangl and Jones, 2001; Staskawicz *et al.*, 2001).

5.1.5 Aims of Chapter

As recently identified members of the CARD family of proteins, Nod1 and Nod2 have emerged as molecules of potentially huge significance, representing a novel pathway and physiological response to LPS. Therefore the aim of this chapter was to further explore the proposal that Nod1 is an intracellular LPS receptor that drives NFκB activation and also to investigate the ability of CARDINAL to act as an antagonist of this novel route to NFκB activation.

5.2 RESULTS

5.2.1 Nod1 subcellular localisation

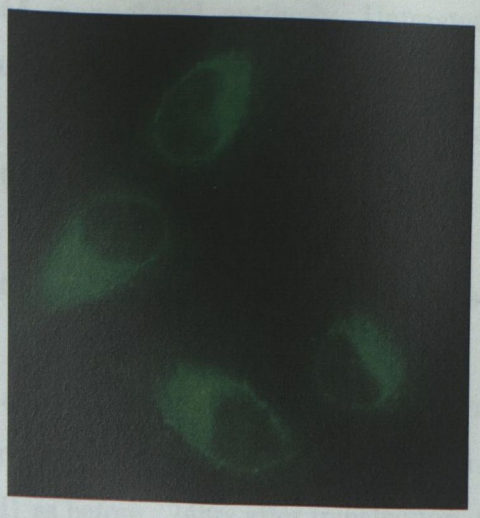
To determine the subcellular localisation of Nod1 we transfected HeLa cells with a HA-tagged vector encoding full length Nod1 and the expressed protein was detected using a monoclonal anti-HA antibody. The results revealed that the protein was found distributed throughout the cytoplasm and the nucleus, although in some cells Nod1 appeared to be excluded from the nucleus (Figure 5.1A). Nod1 also exhibited a bright perinuclear staining-pattern in the majority of cells. Another notable feature of the Nod1 distribution was the appearance of bright punctate spots in the cytoplasm. These may represent Nod1 complex or signalosome formation, but it must be noted that these punctate spots were not present in all Nod1 expressing cells.

To investigate if the subcellular distribution of Nod1 changed in response to LPS treatment, HeLa cells were again transfected with HA-tagged Nod1 and 24 hours post-transfection the cells were either left untreated or treated with LPS for 2hrs. Cells were then fixed and stained with anti-HA antibody to measure Nod1 expression. From Figure 5.1B it is apparent that there was no obvious difference between untreated cells and LPS treated cells with respect to Nod1 subcellular distribution. As in the untreated cells, bright punctate spots were observed in LPS treated cells, but again these were not present in all cells that expressed Nod1.

5.2.2 The NFκB sub-unit p65 moves to the nucleus with TNF and IL-1 treatment

We next endeavoured to determine the effect of Nod1 on the redistribution of the NFκB subunit p65 (RelA). When the IKK signalosome is activated, this results in the phosphorylation and targeted proteasomal degradation of the NFκB inhibitor IκBα. In unactivated cells NFκB is complexed to IκBα masking the nuclear localisation signals on NFκB. Degradation of IκBα allows NFκB to relocate to the nucleus and activate transcription of various target genes (Figure 1.7 in Introduction). We thus investigated if we could visualise the relocalisation of endogenous NFκB to the nucleus by studying the activity of the NFκB subunit p65 in response to the stimuli IL-1 and TNF. HeLa cells were either left untreated or

A



B

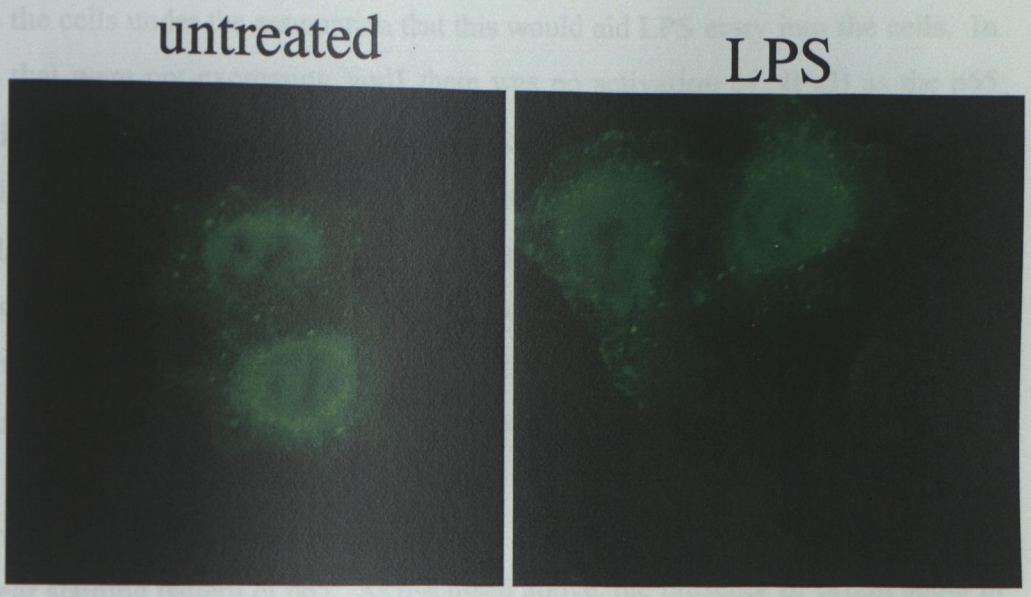


Figure 5.1:
Subcellular localisation of Nod1.
(A) HeLa cells were transiently transfected with HA epitope-tagged Nod1 (400ng). 24hrs after transfection, cells were fixed and stained with an anti-HA monoclonal antibody to evaluate Nod1 expression. Cells were photographed under UV microscopy using a FITC filter.
(B) HeLa cells were transiently transfected with HA epitope-tagged Nod1 (400ng). 24hrs after transfection cells were either left untreated or treated with LPS (10ug/ml) for 2hrs. Cells were fixed and stained for Nod1 expression with an anti-HA monoclonal antibody.

treated with IL-1 or TNF for 20mins. p65 subcellular distribution was analysed by staining with an anti-p65 monoclonal antibody. Untreated cells exhibited a distinctive cytoplasmic and non-nuclear pattern (Figure 5.2 A and C). In strong contrast, treatment with IL-1 and TNF induced translocation of p65 from the cytoplasm to the nucleus (Figure 5.2 B and D).

5.2.3 p65 relocalisation to the nucleus after LPS treatment is associated with Nod 1 expression

To confirm that Nod1 is an intracellular receptor for LPS we investigated the effect of LPS on p65 distribution in cells that overexpress Nod1. HeLa cells were transfected with HA-Nod1 as before. 24hrs after transfection, cells were either left untreated, or treated with LPS for 15mins, 30mins, 1hr or 2hrs (Figure 5.3). The DNA-lipid complexes, formed by the transfection agent Fugene, were not removed from the cells under the assumption that this would aid LPS entry into the cells. In cells that were not expressing Nod1 there was no activation of NFκB as the p65 staining was entirely cytoplasmic and non-nuclear (Figure 5.3A). In the cells where Nod1 was overexpressed there was a significant increase in the number of cells that displayed nuclear staining for p65 suggesting that Nod1 expression alone is sufficient to activate NFκB (Figure 5.3B). Treatment of these cells with LPS led to a further increase in the number of cells displaying nuclear staining of p65, which became more profound with increased duration of LPS treatment (Figure 5.3C-E).

There was an increase over background of Nod1 transfected cells that showed a nuclear staining pattern of p65. As discussed above, the presence of bright spots in the cytoplasm of Nod1 expressing cells may represent complex formation, but they were not present in all cells. It is tempting to speculate that these spots are only present in cells where NFκB is activated either by Nod1 alone or by LPS treatment of Nod1 transfected cells. However, when microscopic observations of these cells were made no notable association was observed between cells that showed this Nod1 staining pattern and nuclear p65 staining.

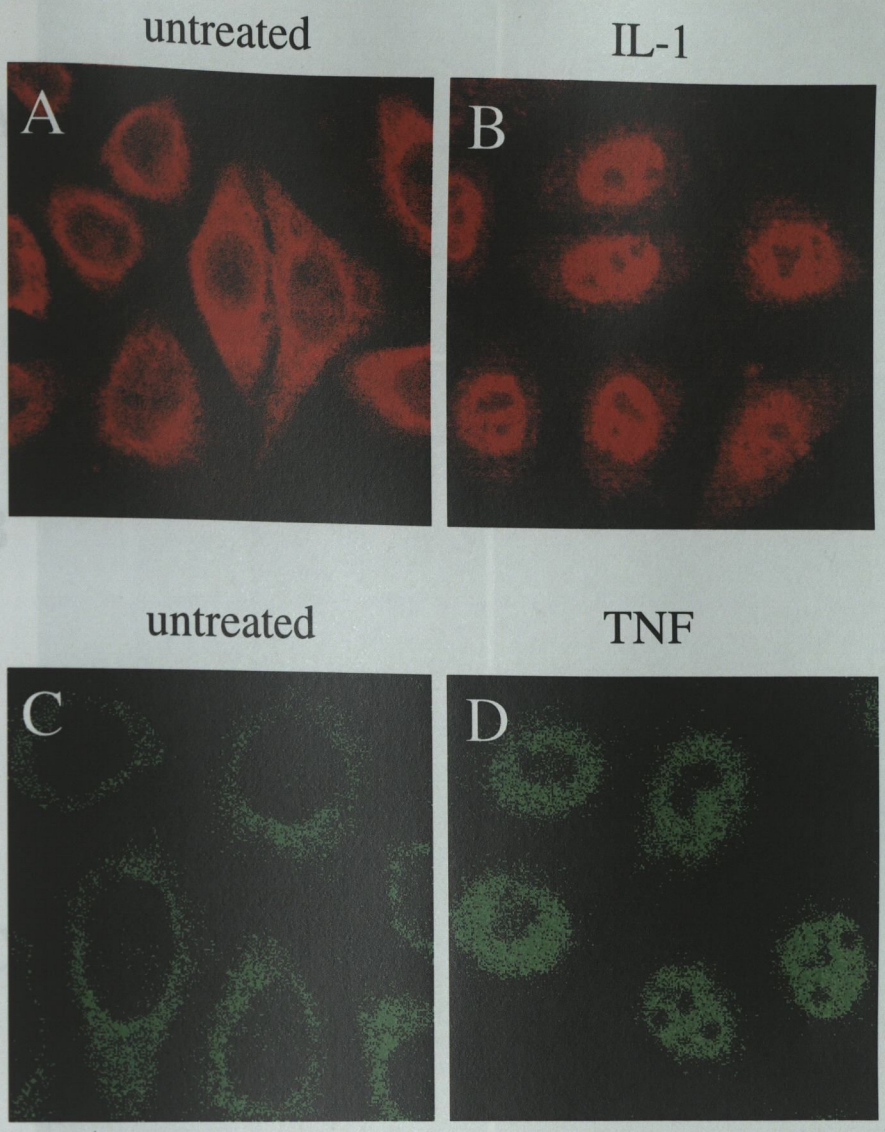
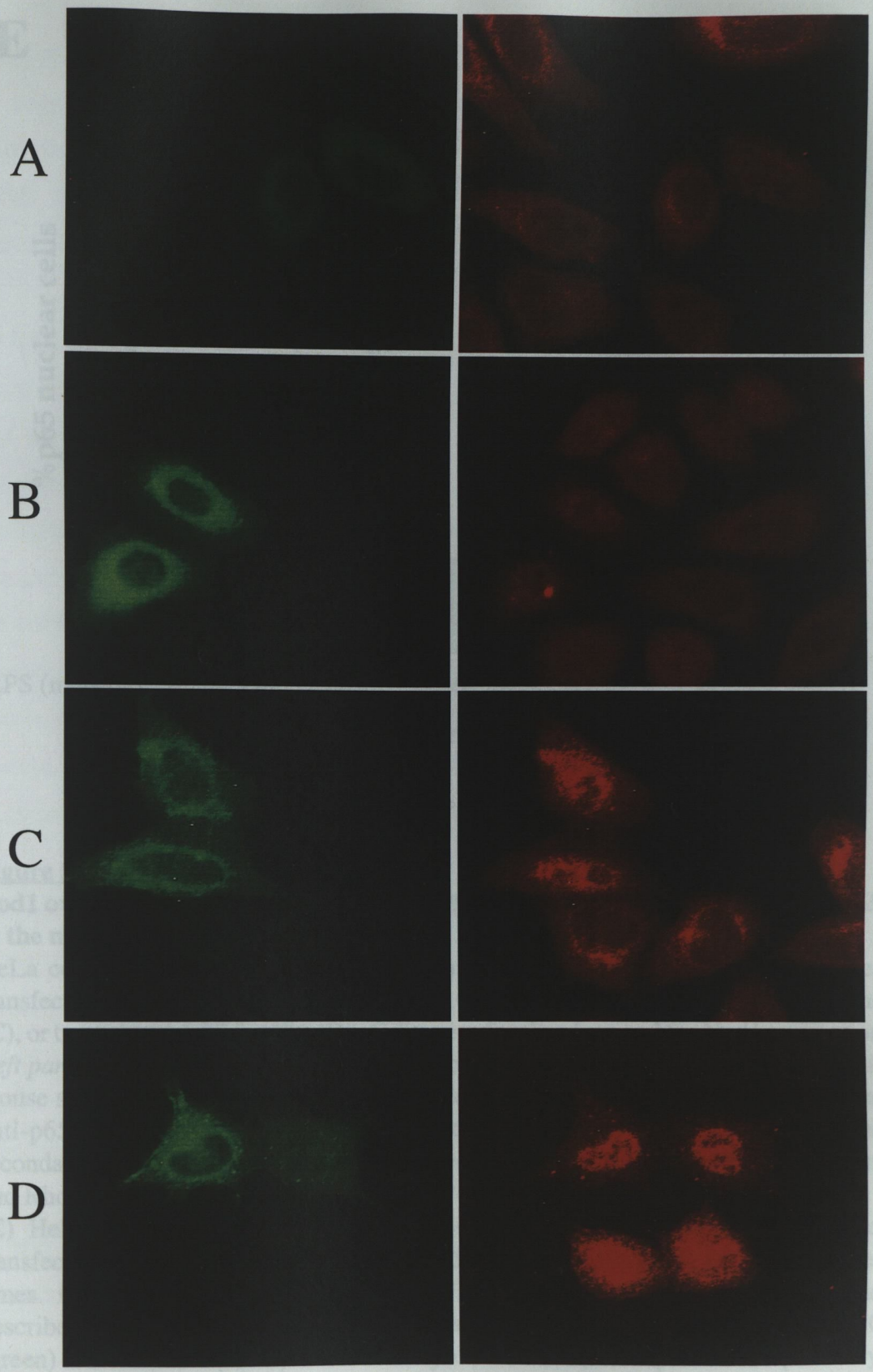


Figure 5.2:
TNF and IL-1 induced relocalisation of NF-κB p65 to the nucleus.
HeLa cells were either left untreated (**A** and **C**), were treated with IL-1 (20ng/ml) (**B**), or TNF (20ng/ml) (**D**) for 20mins. Cells were fixed and immunostained for NF-κB p65 expression using a p65 monoclonal antibody. Cells in (A-B) were stained with a Rhodamine coupled secondary antibody and were viewed using a confocal microscope under the rhodamine (red) channel. Cells in (C-D) were stained with a FITC coupled secondary antibody and were viewed using a confocal microscope under the FITC (green) channel.


Nod-1
p65
A
B
C
D

E

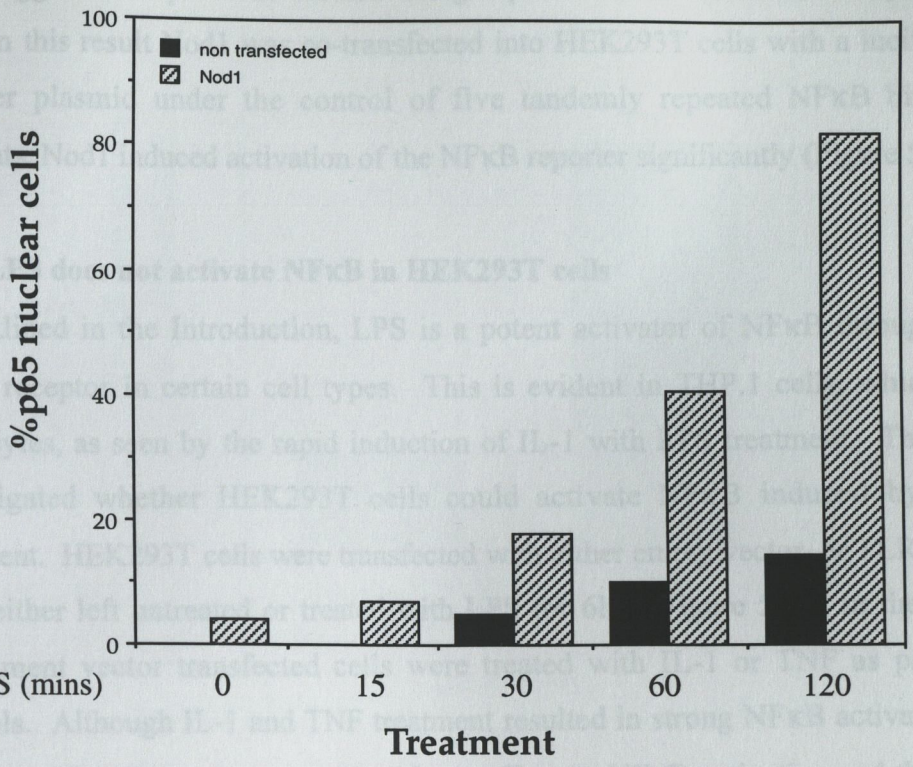


Figure 5.3

Nod1 overexpression is associated with LPS-induced relocalisation of NFκB p65 to the nucleus

HeLa cells were transfected with HA-epitope tagged Nod1 (400ng). 24hrs after transfection cells either left untreated **(A-B)**, treated with LPS (10μg/ml) for 30mins **(C)**, or treated with LPS for 2hrs **(D)**. Cells were fixed and stained for Nod1 expression (*left panels)* with a monoclonal anti-HA antibody followed by FITC conjugated anti-mouse secondary antibody, and for p65 expression (*right panels*), with a polyclonal anti-p65 antibody followed by staining with a rhodamine conjugated anit-rabbit secondary. Cells were examined using a fluorescent microscope under the FITC (green) and Rhodamine (red) filters to analyse Nod1 and p65 expression respectively.

(E) HeLa cells were transfected with HA-epitope tagged Nod1 (400ng). 24hrs after transfection cells either left untreated treated with LPS (10μg/ml) for the indicated times. Cells were fixed and stained for Nod1 expression and for p65 expression as described above. Cells were examined using a fluorescent microscope under the FITC (green) and Rhodamine (red) filters to analyse Nod1 and p65 expression respectively. The localisation of p65 was assessed in cells expressing Nod1 and in untransfected cells. The percentage of Nod-1 expressing cells (hatched boxes) and untransfected cells (black boxes) exhibiting p65 nuclear staining was determined.

5.2.4 Nod 1 induces NFκB activation

The above results demonstrate that Nod1 can activates endogenous NFκB. This has been suggested in previous studies using reporter based luciferase assays. To confirm this result Nod1 was co-transfected into HEK293T cells with a luciferase reporter plasmid under the control of five tandemly repeated NFκB binding elements. Nod1 induced activation of the NFκB reporter significantly (Figure 5.4).

5.2.5 LPS does not activate NFκB in HEK293T cells

As outlined in the Introduction, LPS is a potent activator of NFκB through the TLR4 receptor in certain cell types. This is evident in THP.1 cells, which are monocytes, as seen by the rapid induction of IL-1 with LPS treatment. Thus we investigated whether HEK293T cells could activate NFκB induced by LPS treatment. HEK293T cells were transfected with either empty vector, or TLR4, and were either left untreated or treated with LPS for 6hrs (Figure 5.5). In the same experiment vector transfected cells were treated with IL-1 or TNF as positive controls. Although IL-1 and TNF treatment resulted in strong NFκB activation as expected, LPS did not have any significant effect on NFκB activation and this was not altered significantly by the presence of co-transfected TLR4 (Figure 5.5).

5.2.6 Activation of NFκB by Nod1 is augmented by LPS treatment

To assess the effect of LPS on NFκB activation when Nod1 is overexpressed, HEK293T cells were transfected with Nod1, either alone, or in the presence of LPS (Figure 5.6). Three strains of LPS were tested, *E.coli*, *Salmonella typhimurium*, and *Shigella Flexia*. As was seen before, Nod1 activated NFκB at higher levels but this decreased proportionately as the amount of Nod1 transfected was lowered. However, in the presence of LPS, the fold induction of NFκB was greatly increased and was especially evident at the lowest concentrations of Nod1. LPS, as before, had no effect on NFκB activation in HEK293T cells when Nod1 was not present.

5.2.7 CARDINAL inhibits Nod1 induced NFκB

CARDINAL has been shown to inhibit a number of pathways leading to NFκB activation. CARDINAL can effectively inhibit RICK-induced NFκB activation, and RICK is recruited by Nod 1. Therefore we investigated whether CARDINAL

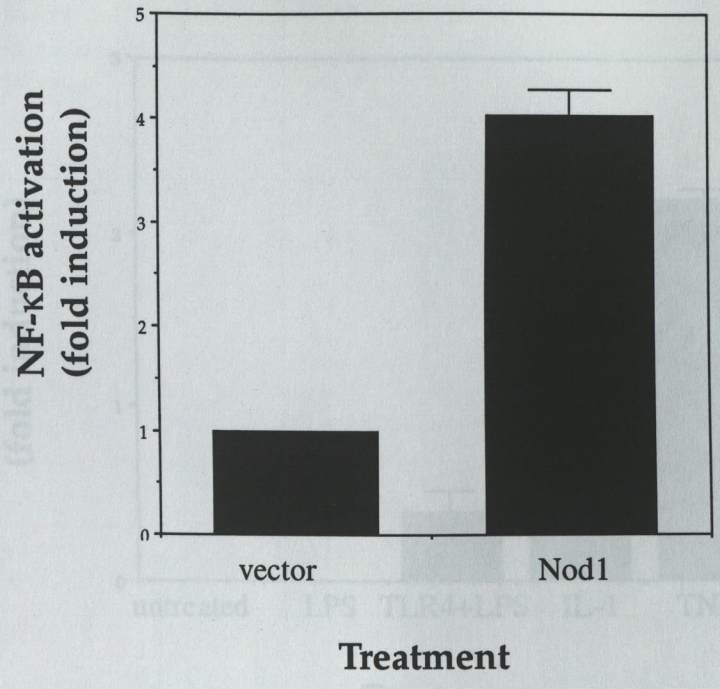


Figure 5.4

Nod1 associated activation of NF-κB.

HEK293T cells were transfected with 1μg of pcDNA3 empty vector or the same amount of pcDNA3.Nod1. Each well also received 100ng of pGL35XκB-luc and 50ng of pcMVβ reporter plasmids. 24hrs after transfection cells were lysed and NF-κB driven luciferase reporter activities were measured, in triplicate as described in Materials and Methods. Luciferase activity values were normalised to β-galactosidase activity values to correct for variability in transfection efficiency between wells. This result is representative of at least three experiments.

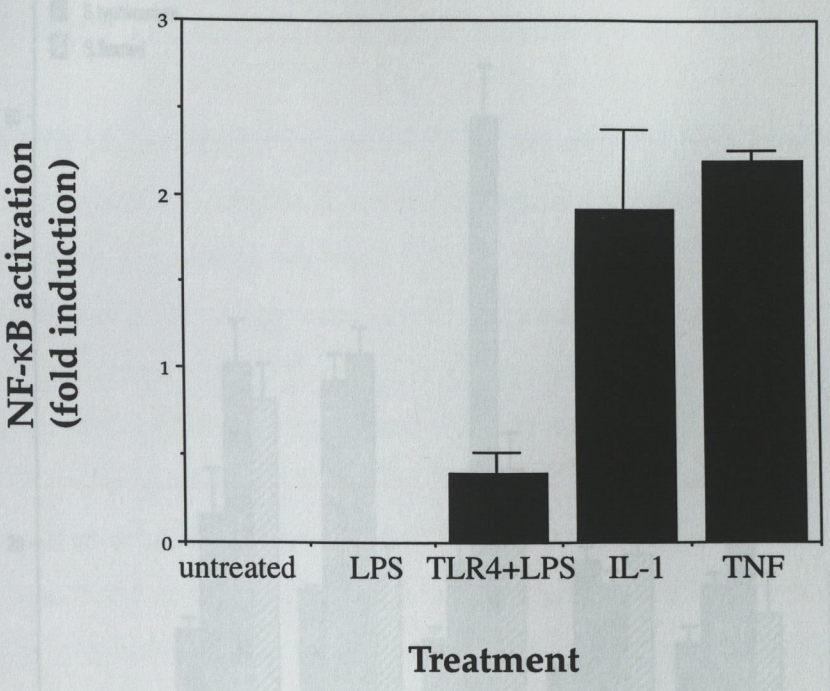


Figure 5.5

LPS treatment of HEK 293T cells does not acitvate NFκB.

HEK293T cells were transfected with 1μg of pcDNA3 empty vector or the same amount of pcDNA3.TLR4. Each well also received 100ng of pGL35XκB-luc and 50ng of pcMVβ reporter plasmids. 24hrs after transfection cells were treated with LPS (5μg/ml), TNF (20ng/ml) or IL-1 (20ng/ml) for 6hrs. Cells were lysed and NF-κB driven luciferase reporter activities were measured., in triplicate as described in the Materials and Methods. Luciferase activity values were normalised to β-galactosidase activity values to correct for variability in transfection efficiency between wells. This result is representative of at least three experiments.

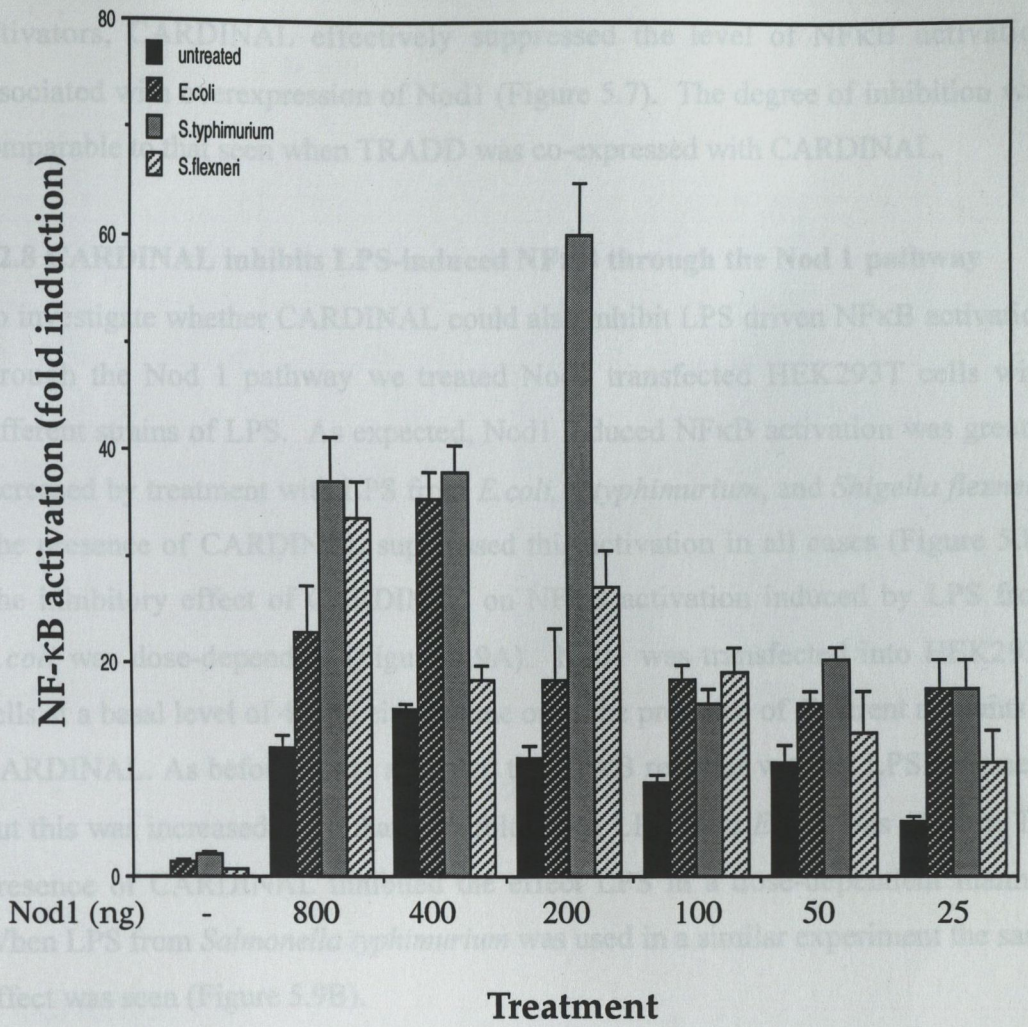


Figure 5.6

Nod1 sensitises HEK293T cells to LPS induced activation of NF-κB.

HEK293T cells were transfected with 800ng of pcDNA3 empty vector or the indicated amounts of pcDNA3.Nod1. Each well also received 100ng of pGL35XκB-luc and 50ng of pcMVβ reporter plasmids and were normalised to the same amount of total DNA using pcDNA3 empty vector. Cells were either left untreated or treated with 10μg/ml LPS from *E.coli, Salmonella typhimurium* or *Shigella flexneri* for 24hrs. Cells were lysed and NF-κB driven luciferase reporter activities were measured, in triplicate as described in the Materials and Methods. Luciferase activity values were normalised to β-galactosidase activity values to correct for variability in transfection efficiency between wells.

could also inhibit Nod1 induced activation of NFκB. HEK cells were transfected with Nod1, or with Nod1 and CARDINAL. As has been seen for other NFκB activators, CARDINAL effectively suppressed the level of NFκB activation associated with overexpression of Nod1 (Figure 5.7). The degree of inhibition was comparable to that seen when TRADD was co-expressed with CARDINAL.

5.2.8 CARDINAL inhibits LPS-induced NFκB through the Nod 1 pathway

To investigate whether CARDINAL could also inhibit LPS driven NFκB activation through the Nod 1 pathway we treated Nod1 transfected HEK293T cells with different strains of LPS. As expected, Nod1 induced NFκB activation was greatly increased by treatment with LPS from *E.coli, S.typhimurium*, and *Shigella flexneri*. The presence of CARDINAL suppressed this activation in all cases (Figure 5.8). The inhibitory effect of CARDINAL on NFκB activation induced by LPS from *E.coli* was dose-dependent (Figure 5.9A). Nod1 was transfected into HEK293T cells at a basal level of 400ng either alone or in the presence of different amounts of CARDINAL. As before, Nod1 activated the NFκB reporter without LPS treatment, but this was increased more than two-fold when LPS from *E.coli* was present. The presence of CARDINAL inhibited the effect LPS in a dose-dependent manner. When LPS from *Salmonella typhimurium* was used in a similar experiment the same effect was seen (Figure 5.9B).

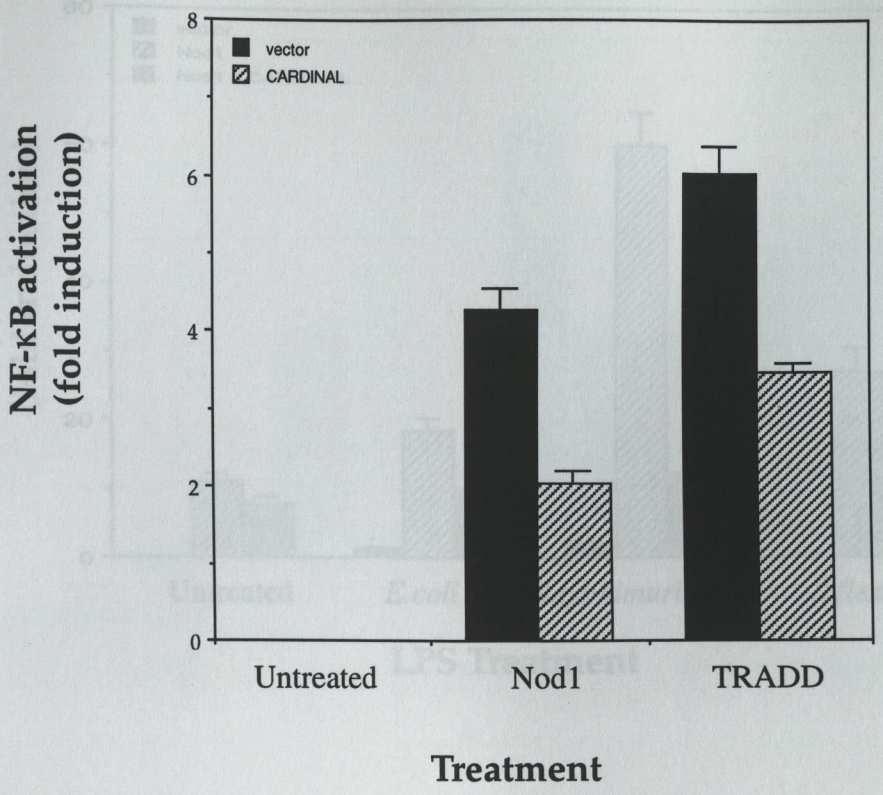


Figure 5.7:
CARDINAL inhibits constitutive Nod1 associated NF-κB activation.
HEK293T cells were transfected with 1μg of expression plasmids encoding Nod1 or TRADD along with 2μg of either empty vector (black shading) or 2μg of pcDNA3-CARDINAL (hatched shading). Each well also received 100ng of pGL35XκB-luc and 50ng of pcMVβ reporter plasmids and were normalised to the same amount of total DNA using pcDNA3 empty vector. 24hr after transfection, cells were lysed and NF-κB driven luciferase reporter activities were measured, in triplicate as described in the Materials and Methods. Luciferase activity values were normalised to β-galactosidase activity values to correct for variability in transfection efficiency between wells.

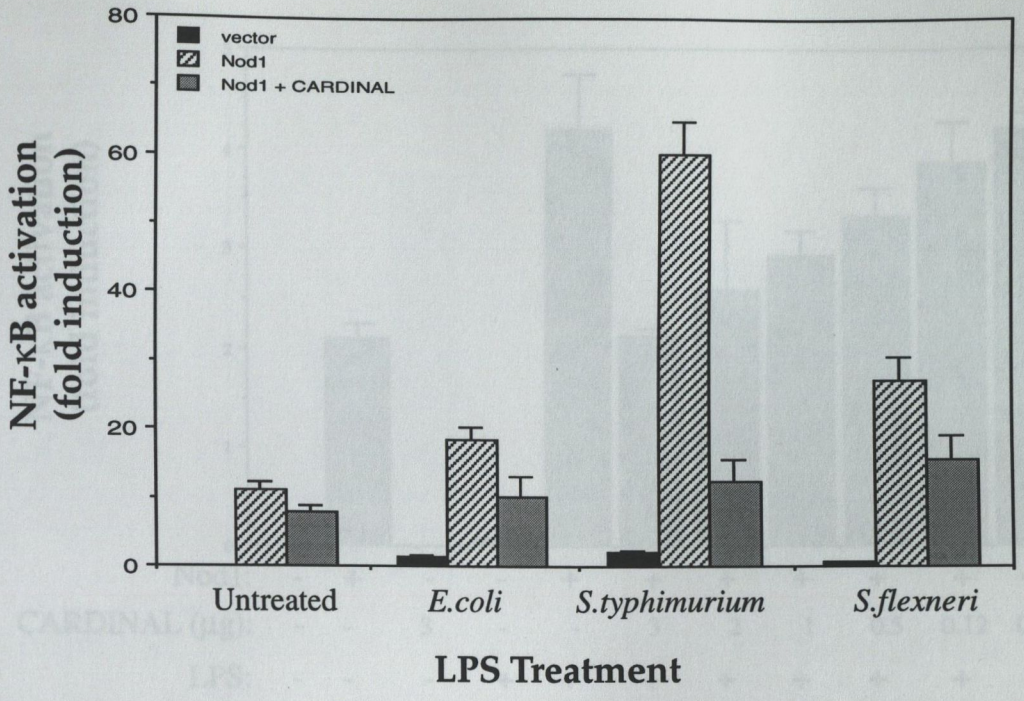


Figure 5.8:

CARDINAL inhibits LPS driven Nod1 associated NF-κB activation.

(A) HEK293T cells were transfected with 200ng of pcDNA3 empty vector or 200ng of pcDNA3- Nod1-HA along with 400ng of either empty vector (black shading) or 400ng of pcDNA3-CARDINAL (hatched shading). Each well also received 100ng of pGL35XκB-luc and 50ng of pcMVβ reporter plasmids and were normalised to the same amount of total DNA using pcDNA3 empty vector. Cells were treated with the indicated strains of LPS (10μg/ml) for 24hrs as before. 24hr after transfection, cells were lysed and NF-κB driven luciferase reporter activities were measured, in triplicate as described in the Materials and Methods. Luciferase activity values were normalised to β-galactosidase activity values to correct for variability in transfection efficiency between wells.

A

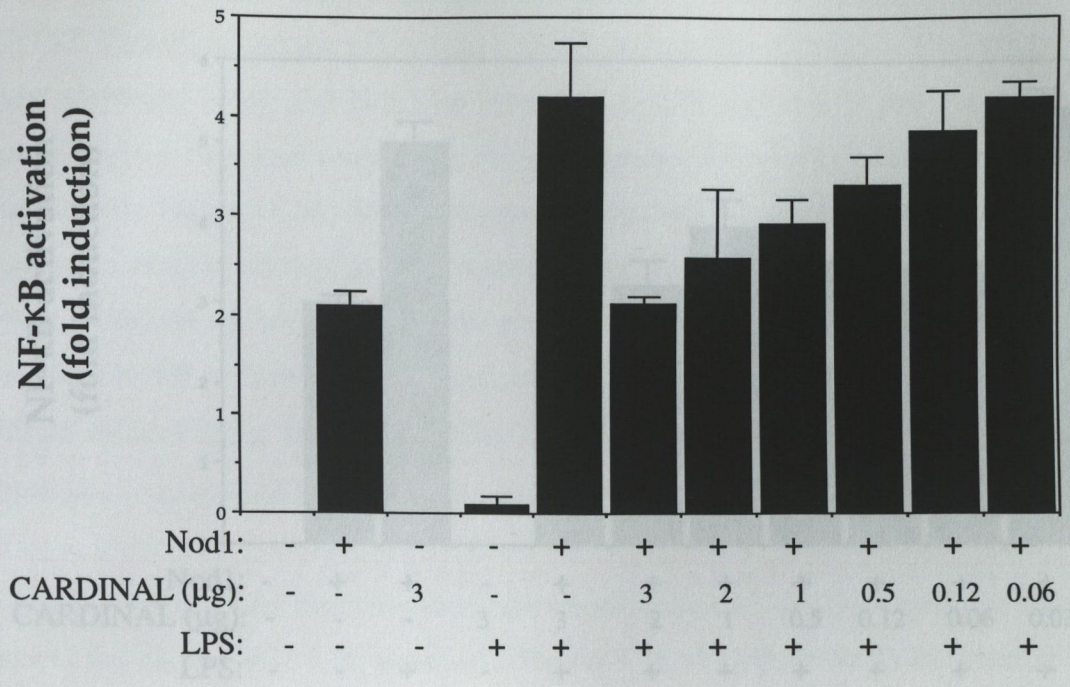


Figure 5.9:

CARDINAL inhibition of *E.coli* LPS induced Nod1 mediated activation of NFκB is dose-dependant.

(A) HEK293T cells were transfected with 400ng of HA-Nod1 expression plasmid, along with the indicated amounts of pcDNA3-CARDINAL. All wells also received the pGL35XκB-luc(100ng) and pcMVβ (50ng) reporter plasmids. Cells were treated for 24hrs with *E.coli* LPS (10μg/ml). Luciferase assays were performed 24hr after transfection and normalised to correct for variation in transfection efficiency as described previously.

B

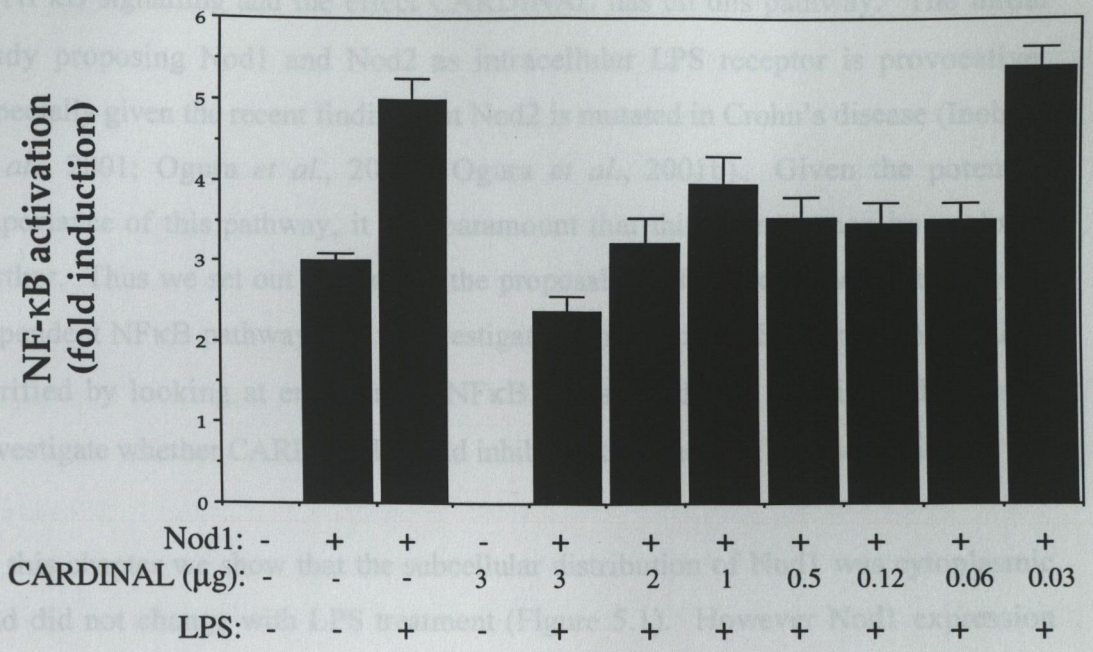


Figure 5.9:

CARDINAL inhibition of *Salmonella typhimurium* LPS induced Nod1 mediated activation of NFκB is dose-dependant.

(B) HEK293T cells were transfected with 200ng of HA-Nod1 expression plasmid, along with the indicated amounts of pcDNA3-CARDINAL. All wells also received the pGL35XκB-luc(100ng) and pcMVβ (50ng) reporter plasmids. Cells were treated for 24hrs with *Salmonella typhimurium* LPS (10μg/ml). Luciferase assays were performed 24hr after transfection and normalised to correct for variation in transfection efficiency as described previously.

5.3 DISCUSSION

5.3.1 Introduction

The aim of this chapter was to investigate the role of the NBD-CARD protein Nod1 in NFκB signalling and the effect CARDINAL has on this pathway. The initial study proposing Nod1 and Nod2 as intracellular LPS receptor is provocative, especially given the recent finding that Nod2 is mutated in Crohn's disease (Inohara *et al.*, 2001; Ogura *et al.*, 2001a; Ogura *et al.*, 2001b). Given the potential importance of this pathway, it was paramount that this phenomenon be explored further. Thus we set out to confirm the proposal that LPS could activate a Nod1-dependent NFκB pathway and to investigate if the Nod1-LPS interaction could be verified by looking at endogenous NFκB. A second aim of this study was to investigate whether CARDINAL could inhibit LPS induced-NFκB activation

In this chapter we show that the subcellular distribution of Nod1 was cytoplasmic and did not change with LPS treatment (Figure 5.1). However Nod1 expression was associated with a high degree of movement of p65 (Figure5.3) to the nucleus similar to with events that were observed when cells are treated with TNF or IL-1 (Figure 5.2). The proportion of cells with p65 nuclear staining was increased with LPS treatment (Figure 5.2E). Moreover we confirmed published results that Nod1 could activate NFκB and that this activation was enhanced by LPS from a number of stains of bacteria (Figure 5.6). Finally we demonstrated that CARDINAL could inhibit both Nod1 mediated NFκB activation and LPS-induced activation of NFκB via Nod1 (Figure 5-7-5.9).

5.3.2 LPS activation of Nod1 induced relocalisation of NF-κB p65 to the nucleus

The fact that we show here that Nod1 could induce movement of the NFκB p65 subunit to the nucleus, which was greatly enhanced by LPS treatment, gives strong corroborative evidence to the results of the NFκB reporter assays (Figure 5.3). In this study the readout was an effect on an endogenous protein, thus it is very unlikely that the effect seen is a spurious activation of the NFκB luciferase reporter construct. Whereas the reporter assays rely on accumulation of the luciferase protein over a period of time (in this case 24hrs), using the immunostaining approach the

effects of LPS on Nod1 induced NFκB could be seen as early as 30 minutes after LPS stimulation.

5.3.3 Nod1 subcellular distribution – Evidence for complex formation?

Nod1 has been proposed to respond to LPS in a similar manner to the way Apaf-1 responds to cytochrome *c* (Figure 1.6 in Introduction; Martin, 2001; Inohara *et al.*, 2001). LPS binds to the LRRs enforcing oligomerisation of a number of Nod1 molecules (Inohara *et al.*, 2001). In this way Nod1 may recruit RICK molecules in an induced proximity model (Inohara *et al.*, 2000). RICK in turn recruits IKKγ, the regulatory subunit of the IKK signalosome, leading to activation of NFκB. The subcellular distribution pattern of Nod1 did reveal, in some but not all cells, the existence of bright punctate spots in the cytoplasm that may represent complex formation (Figure 5.1). However these spots were not present in all Nod1-transfected cells and the presence of the spots did not seem to be associated with NFκB activation, indicated by translocation of p65 to the nucleus. Thus the significance, if any, of these spots is unclear.

Although Nod1 has been proposed as an intracellular LPS receptor it has not been formally demonstrated that this protein is not a membrane-associated protein. From the subcellular localisation studies shown here, it is clear that this protein is found predominantly in the cytoplasm and also in the nucleus and is not a membrane protein (Figure 5.1).

5.3.4 CARDINAL inhibits LPS induced Nod1 associated NFκB activation

We also show here that CARDINAL can effectively inhibit the NFκB activation induced by LPS through Nod1 (Figure 5.8 – 5.9). This supports the hypothesis proposed in the previous chapter that CARDINAL is an inhibitor of multiple pathways leading to NFκB activation. Nod1 operates upstream of the IKK signalosome, and the ability of CARDINAL to interrupt the Nod1 pathway is in concordance with results presented in Chapter IV that placed CARDINAL function at the point of the IKK signalosome.

5.3.5 Conclusions

In conclusion, evidence presented here strongly suggests that Nod1 functions as an intracellular LPS receptor, an activity that can be inhibited by CARDINAL. Further work is necessary to clarify what the outcome of this pathway is. In particular it would be of interest to know which subset of genes are transcriptionally upregulated by NFκB as a result of LPS induced activation of Nod1. Obvious candidates would be IL-1 and IL-8, which have been shown to be upregulated by LPS in different contexts.

CHAPTER VI

GENERAL DISCUSSSION

6.1. INTRODUCTION

In recent years the related conserved protein domains, DED and CARD have been identified in a number of proteins that participate in signalling pathways that lead to caspase activation or NFκB activation. This thesis has investigated the functional properties of two of these proteins: the novel CARD-containing protein CARDINAL and the NBD-CARD protein Nod1.

The yeast-two-hybrid screen, using caspase-10 as the bait, aimed to identify a novel binding partner for caspase-10. The role of caspase-10 in cell death pathways remains obscure and it was hypothesised that the identification of a specific binding partner for this molecule would help to resolve the function of this caspase. Unfortunately the yeast-two-hybrid screen failed to identify a strong binding partner for caspase-10 highlighting a number of drawbacks of this approach. The appearance of misleading false positives, the likely misfolding of the bait protein in yeast and the low transformation efficiency of the screen probably all contributed to the failure of this screen.

Therefore, a database interrogation approach was used to identify novel death fold-containing proteins, using the BLAST search tool. We thus identified CARDINAL, a novel CARD-containing protein. As was described in Chapter IV, CARDINAL acts as an inhibitor of NFκB activation that can inhibit multiple pathways that lead to the activation of this transcription factor. CARDINAL seems to operate at the point of convergence of multiple NFκB activation pathways, which is the IKK signalosome. The NFκB activators RIP and RICK have been shown to bind to IKKγ (Inohara *et al.*, 2000) and here we have shown that CARDINAL also binds to IKKγ.

To further dissect the role of CARD proteins in NFκB signalling we investigated the role of the NBD-CARD protein, Nod1, as an intracellular LPS receptor. In this study we confirmed that the presence of Nod1 sensitises cells to LPS treatment by showing that LPS treatment of Nod1 expressing cells promotes relocalisation of the NFκB subunit p65 from the cytosol of the cell to the nucleus. We also showed that Nod1 induced activation of NFκB, measured in the luciferase reporter assay system,

is upregulated by LPS from a number of different bacterial strains. Furthermore, this mode of LPS induced activation of NFκB was suppressed by CARDINAL.

6.2 CARDINAL – A NOVEL NFκB REGULATOR

The structure of CARDINAL reveals that it contains a CARD domain and no other recognisable motif or domain. In accordance with the classification of the CARD-family of proteins outlined in the Introduction (Figure 1.5), CARDINAL is a member of the CARD-only subgroup of CARD proteins. The two other members of this group are ICEBERG and COP/pseudoICE and both of these proteins act as inhibitors of caspase-1 function (Druilhe *et al.*, 2001; Humke *et al.*, 2000; Lee *et al.*, 2001). The ability of CARDINAL to act as an inhibitor of NFκB activating pathways is consistent with its placing in this group.

This study presents data implicating a role for CARDINAL in the inhibition of multiple NFκB activating pathways. Although much of the data presented in this thesis was obtained using an NFκB reporter plasmid, the control experiments, notably using a p53 responsive reporter in place of the NFκB responsive reporter gene, suggest that this is an effect that is specific to NFκB activation. To further confirm that CARDINAL inhibits NFκB activation, electrophoretic mobility shift assays (EMSA) could be used. EMSA measures the ability of NFκB to bind a DNA probe representing the NFκB DNA binding sites. It would be predicted that the presence of CARDINAL would result in an inhibition of NFκB DNA binding activity. However, this method is more laborious, in comparison to the reporter assay system used, therefore we chose the latter method to measure NFκB activation.

The exact mechanism through which CARDINAL operates is still not entirely clear. Clearly it is important to further examine the role of CARDINAL by analysing its function at endogenous levels. The expression of ICEBERG has been shown to be upregulated in THP.1 cells in response to LPS treatment (Humke *et al.*, 2000). However unlike LPS induced expression of pIL-1β, which occurs rapidly in these cells, induction of ICEBERG expression occurs much later (Figure 4.8; Humke *et al.*, 2000). This suggests that ICEBERG functions to control IL-1 levels thereby

preventing minor proinflammatory insults escalating into conditions of persistant inflammation such as septic shock. The NFκB inhibitor, IκB, functions in a similar way. Activation of NFκB requires the degradation of IκB, but the latter is resynthesised through transcriptional activation by NFκB so it can reform a cytosolic complex with NFκB in a negative feedback loop. By monitoring the response of endogenous CARDINAL to proinflammatory stimuli such as LPS, TNF or IL-1, it would be possible to investigate whether CARDINAL operates in a similar way. The expression of CARDINAL may be upregulated in these contexts, which would suggest that this protein represents an additional level of internal control in the context of the immune response. Another way to approach this possibility would be to investigate whether CARDINAL expression is directly activated by NFκB. Attempts were made during this study to analyse the effect of proinflammatory stimuli on levels of endogenous CARDINAL expression. Unfortunately these experiments failed to yield conclusive results.

The interplay between pathways leading to NFκB activation and apoptosis has been discussed in chapter I. In particular, the observations that have been made on cells that have had their ability to activate NFκB disrupted in some way, suggest that NFκB can suppress apoptosis (Beg and Baltimore, 1996; Van Antwerp *et al.*,1996b). One could thus extrapolate from this that CARDINAL, as an NFκB inhibitor, may make cells more vulnerable to apoptotic stimuli. Obviously further work is required to investigate if CARDINAL plays a role as a mediator of the decision making process that causes a cell to commit to either the death or survival pathway. However, viewed in isolation, the fact that CARDINAL can inhibit NFκB activation is important. NFκB activation seems to confer resistance to the cytotoxic activity of TNF and certain chemotherapeutic drugs, by inducing the expression of survival genes (Van Antwerp *et al.*, 1996b; Wang *et al.*, 1996a; Wang *et al.*, 1998a). NFκB activity has also been shown to suppress the expression of MyoD, which is a regulator of skeletal muscle differentiation (Guttridge *et al.*, 2000). This can lead to muscle wasting associated with cancer, AIDS and other chronic diseases. To explore whether CARDINAL has a role in such events it would be necessary to analyse the effect CARDINAL has on specific NFκB regulated genes. Future work would aim to identify the subset of these genes that are transcriptionally repressed

by the action of CARDINAL and the biological context in which CARDINAL may influence the expression of such genes.

6.3 NOD1 and the LPS response

The physiological relevance of the role of Nod1 and Nod2 as mediators of LPS-induced NFκB activation has been demonstrated by the finding that Nod2 is mutated in certain inflammatory conditions (Hampe *et al.*, 2001; Hugot *et al.*, 2001; McGovern *et al.*, 2001; Ogura *et al.*, 2001a). Here we have confirmed that Nod1, a member of the NBD-CARD subfamily of CARD proteins, acts as a receptor for LPS. It is the combination of the NBD and LRR motifs present in Nod 1 and Nod 2 that is responsible for this function. The LRRs most likely recruit LPS and the NBD acts as an oligomerisation platform (Inohara *et al.*, 2000; Inohara *et al.*, 2001). In the TLR family of LPS receptors, each TLR has been shown to recognise a different bacterially derived product (Takeuchi *et al.*, 1999). By analogy it is likely that members of the same NBD-CARD subfamily act as pathogen sensors that recognise pathogen-associated products distinct from LPS. One candidate is the protein NAC. Although this protein has been implicated in apoptosis (Chu *et al.*, 2001; Hlaing *et al.*, 2001), the presence of a pyrin domain in its sequence may suggest a functional role in an inflammatory pathway. Thus NAC may, akin to Nod1 and Nod2, recognise an as yet unidentified bacterial-derived product resulting in the activation of NFκB. Moreover the high degree of homology between CARDINAL and the C-terminus of NAC suggests that these two proteins may operate in the same pathway. The advances made here with respect to the Nod1 pathway strongly support the proposal that intracellular receptors exist that function to recognise the presence of bacteria and their products inside the cell. Further characterisation of these proteins and the mechanisms through which they activate NFκB will greatly improve our understanding of the molecular elements that determine disease susceptibility, especially with respect to bacterial infection.

6.4 CONCLUSIONS

There are significant conclusions that this thesis supports. Specifically one could conclude that

(i) CARDINAL is a novel CARD protein that inhibits NFκB activated by multiple pathways by interacting with IKKγ, the regulatory subunit of the IKK signalosome.

(ii) Nod1 functions as an intracellular LPS receptor

(iii) CARDINAL can inhibit LPS-induced NFκB activation associated with Nod1 expression.

The characterisation of the CARD proteins described here contributes to the growing body of evidence that proteins containing the CARD motif play an indispensable role in pathways that lead to NFκB activation. These conclusions provide further support for the role of CARD proteins in the context of host mediated defence against cellular injury.

APPENDIX

Table I: Origin of plasmids used for work undertaken in the thesis

Plasmid Name	Made by/Supplied by
pcDNA3	Invitrogen
pEGFPC3	Clontech
pcDNA3.hICE	Laboratory of Dr Vishva Dixit
pAS2-1.Caspase-7pro	Dr. Mary Harte
pAS2-1.Caspase-8pro	Dr. Mary Harte
pAS2-1.Caspase-9por	Dr. Mary Harte
pAS2-1.Caspase-10(1-372)	Dr. Colin Adrain
pAS2-1.Caspase-10(1-219)	Lisa Bouchier-Hayes
pRK.FLAG-RIP	Laboratory of Dr. David Goeddel
pRK.Myc-TRADD	Laboratory of Dr. David Goeddel
pcDNA3.TRAIL-R1	Laboratory of Dr. Henning Walczak
pcDNA3.TRAIL-R2	Laboratory of Dr. Henning Walczak
pACT2.FADD	Dr. Mary Harte
pcDNA3.Bax	Laboratory of Dr. Doug Green Lab
pcDNA3.CrmA	Laboratory of Dr. Vishva Dixit
pcDNA3.T7-Bcl-10	Laboratory of Dr. Emad Alnemri
pcDNA3.T7-IKKγ	Laboratory of Dr. Emad Alnemri
pcDNA3.T7-IKKα	Laboratory of Dr. Emad Alnemri
pcDNA3.T7-IKKβ	Laboratory of Dr. Emad Alnemri
p53luc	Stratagene
pGL3.5xkBluc	Laboratory of Prof. Luke O'Neill
pcDNA3.proIL-1β	Lisa Bouchier-Hayes
pBluescript.SK+.proIL-1β	S. Galleas (Aventis)
PCR3V72Met-VSV-Stop.CARDIAK	Laboratory of Dr. Jurg Tschopp
pRCCMV.p53	Laboratory of Dr. Moshe Oren
pcDNA3.CARDINAL	Dr. Helen Egan
pEGFPC3.CARDINAL	Dr. Helen Egan
pBluescriptIISK+.KIAA0955	Kazusa DNA research institute, Japan
pcDNA3.CARDINAL(345-431)	Lisa Bouchier-Hayes
pcDNA3.CARDINAL (1-320)	Lisa Bouchier-Hayes
pEGFPC3.CARDINAL(321-431)	Dr. Helen Egan
pEGFPC3.CARDINAL(1-320)	Dr. Helen Egan
pACT2.Apaf1(1-601)	Dr. Mary Harte
pcDNA3. HA-Nod1	Laboratory of Dr. Gabriel Nunez

Table II: Primers used to generate some of the plasmids used for work undertaken in the thesis

PROTEIN	PRIMER NAME	SEQUENCE	PARENT PLASMID	RESTICTION SITE
CARDINAL (1-320)	Forward CARDINAL MetF	ACA GGA TCC GCC **ATG ATG AGA CAG AGG**	pcDNA3	BamHI (GGA TCC)
	Reverse CARDINAL Leu320R	GAC CTC GAG TTA **CAA AGT CCC ATG TCT** *		XhoI (CTC GAG)
CARDINAL (345-431)	Forward CARDINAL Ala345F	ATA GGA TCC GCC ATG **GCA GCC TTT GTG AAG**	pcDNA3	BamHI (GGA TCC)
	Reverse CARDINAL StopR	CTA GGA TCC CTC GAG **TTA CAA ATT CTG CTG** *		XhoI (CTC GAG)
proIL-1β	Forward IL-1β MetF	ATA GGA TCC GCC **ATG GCA GAA GTA CCT**	pcDNA3	BamHI (GGA TCC)
	Reverse IL-1β StopR	GGC CTC GAG **TTA GGA AGA CAC AAA TTG** *		XhoI (CTC GAG)
Caspase-10 (1-219)	Forward Caspase10 MetF	ATA GGA TCC CCG GGG **ATG AAA TCT CAA GGT**	pAS2-1	SmaI (CCCGGG)
	Reverse Caspase10 Asp219R	TGG GGA TCC CTC GAG **CTA ATC TGT TTG GGA AAC** *		BamHI (GGA TCC)

Each primer is named in the same way: protein name followed by the name and number of the first codon of the protein sequence present in the primer and the letter F or R signifies the direction of the primer.

Primers are listed in 5' to 3' direction

Kozac consensus sequence if present is underlined

Genomic sequence is in bold

Stop codons are marked with a (*)

Table III: Primers used for automated sequencing applications

Primer	Sequence
pACT2 forward screening primer*	TTC GAT GAT GAA GAT ACC CC
pACT2 reverse screening primer*	GAA CTT GCG GGG TTT TTC
T7 forward primer	TAA TAC GAC TCA CTA TAG G

* Used to screen library clones pulled in yeast two hybrid screen and also for sequencing inserts in pACT2 plasmids

REFERENCES

REFERENCES

Abbas, A.K. (1996) Die and let live: eliminating dangerous lymphocytes. *Cell* , **84**, 655-657.

Adrain, C., Slee, E.A., Harte, M.T. and Martin, S.J. (1999) Regulation of apoptotic protease activating factor-1 oligomerization and apoptosis by the WD-40 repeat region. *J Biol Chem*, **274**, 20855-60.

Adrain, C., Harte, M.T., Egan, H., Carlin, W.J., Slee, E.A. and Martin S.J. (2001) DRADD, a nuclear-localized death domain protein, interacts with caspase-9 and Rb to promote apoptosis. *Submitted.*

Ahmad, M., Srinivasula, S.M., Wang, L., Talanian, R.V., Litwack, G., Fernandes-Alnemri, T. and Alnemri, E.S. (1997) CRADD, a novel human apoptotic adaptor molecule for caspase-2, and FasL/tumor necrosis factor receptor-interacting protein RIP. *Cancer Res*, **57**, 615-9.

Alnemri, E.S., Livingston, D.J, Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. and Yuan, J. (1996) Human ICE/CED-3 protease nomenclature. *Cell*, **87**, 171.

Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.-J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R. and Martinou, J.-C. (1997) Inhibition of Bax Channel-Forming Activity by Bcl-2. *Science*, **277**, 370-372.

Baeuerle, P.A. and Baltimore, D. (1996) NF-kappa B: ten years after. *Cell*, **87**, 13-20.

Barde, Y.A. (1989) Trophic factors and neuronal survival. *Neuron*, **2**, 1525-34.

Becker, K., Schneider, P., Hofmann, K., Mattmann, C. and Tschopp, J. (1997) Interaction of Fas(Apo-1/CD95) with proteins implicated in the ubiquitination pathway. *FEBS Lett*, **412**, 102-6.

Beg, A.A. and Baltimore, D. (1996) An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science*, **274**, 782-4.

Beg, A.A., Sha, W.C., Bronson, R.T., Ghosh, S. and Baltimore, D. (1995) Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature*, **376**, 167-70.

Belvin, M.P. and Anderson, K.V. (1996) A conserved signaling pathway: the Drosophila toll-dorsal pathway. *Annu Rev Cell Dev Biol*, **12**, 393-416.

Bergeron, L., Perez, G.I., Macdonald, G., Shi, L., Sun, Y., Jurisicova, A., Varmuza, S., Latham, K.E., Flaws, J.A., Salter, J.C., Hara, H., Moskowitz, M.A., Li, E., Greenberg, A., Tilly, J.L. and Yuan, J. (1998) Defects in regulation of apoptosis in caspase-2-deficient mice. *Genes Dev*, **12**, 1304-14.

Bertin, J. and DiStefano, P.S. (2000) The PYRIN domain: a novel motif found in apoptosis and inflammation proteins. *Cell Death and Differentiation*, **7**, 1273-1274.

Bertin, J., Guo, Y., Wang, L., Srinivasula, S.M., Jacobson, M.D., Poyet, J.L., Merriam, S., Du, M.Q., Dyer, M.J., Robison, K.E., DiStefano, P.S. and Alnemri, E.S. (2000) CARD9 is a novel caspase recruitment domain-containing protein that interacts with BCL10/CLAP and activates NF-kappa B. *J Biol Chem*, **275**, 41082-6.

Bertin, J., Nir, W.J., Fischer, C.M., Tayber, O.V., Errada, P.R., Grant, J.R., Keilty, J.J., Gosselin, M.L., Robison, K.E., Wong, G.H., Glucksmann, M.A. and DiStefano, P.S. (1999) Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. *J Biol Chem*, **274**, 12955-8.

Bertin, J., Wang, L., Guo, Y., Jacobson, M.D., Poyet, J.L., Srinivasula, S.M., Merriam, S., DiStefano, P.S. and Alnemri, E.S. (2001) CARD11 and CARD14 are novel caspase recruitment domain (CARD)/membrane-associated guanylate kinase (MAGUK) family members that interact with BCL10 and activate NF-kappa B. *J Biol Chem*, **276**, 11877-82.

Blau, E.B. (1985) Familial granulomatous arthritis, iritis, and rash. *J Pediatr*, **107**, 689-93.

Bodmer, J.L., Holler, N., Reynard, S., Vinciguerra, P., Schneider, P., Juo, P., Blenis, J. and Tschopp, J. (2000) TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol*, **2**, 241-3.

Bonfoco, E., Li, E., Kolbinger, F. and Cooper, N.R. (2001) Characterization of a novel proapoptotic caspase-2- and caspase-9-binding protein. *J Biol Chem*, **276**, 29242-50.

Booth, D.R., Gillmore, J.D., Booth, S.E., Pepys, M.B. and Hawkins, P.N. (1998) Pyrin/marenostrin mutations in familial Mediterranean fever. *Qjm*, **91**, 603-6.

Bratton, S.B., Walker, G., Srinivasula, S.M., Sun, X.M., Butterworth, M., Alnemri, E.S. and Cohen, G.M. (2001) Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. *Embo J*, **20**, 998-1009.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G. and Siebenlist, U. (1995) Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science*, **267**, 1485-8.

Burns, K., Martinon, F., Esslinger, C., Pahl, H., Schneider, P., Bodmer, J.L., Di Marco, F., French, L. and Tschopp, J. (1998) MyD88, an adapter protein involved in interleukin-1 signaling. *J Biol Chem*, **273**, 12203-9.

Cain, K., Brown, D.G., Langlais, C. and Cohen, G.M. (1999) Caspase activation involves the formation of the aposome, a large (approximately 700 kDa) caspase-activating complex. *J Biol Chem*, **274**, 22686-92.

Cecconi, F., Alvarez-Bolado, G., Meyer, B.I., Roth, K.A. and Gruss, P. (1998) Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell*, **94**, 727-37.

Chai, J., Shiozaki, E., Srinivasula, S.M., Wu, Q., Dataa, P., Alnemri, E.S. and Shi, Y. (2001) Structural basis of caspase-7 inhibition by XIAP. *Cell*, **104**, 769-80.

Chau, B.N., Cheng, E.H., Kerr, D.A. and Hardwick, J.M. (2000) Aven, a novel inhibitor of caspase activation, binds Bcl-xL and Apaf-1. *Mol Cell*, **6**, 31-40.

Chen, Z., Hagler, J., Palombella, V.J., Melandri, F., Scherer, D., Ballard, D. and Maniatis, T. (1995) Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes Dev*, **9**, 1586-97.

Chien, C.T., Bartel, P.L., Sternglanz, R. and Fields, S. (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A*, **88**, 9578-82.

Chinnaiyan, A.M., Chaudhary, D., O'Rourke, K., Koonin, E.V. and Dixit, V.M. (1997a) Role of CED-4 in the activation of CED-3. *Nature*, **388**, 728-9.

Chinnaiyan, A.M., O'Rourke, K., Lane, B.R. and Dixit, V.M. (1997b) Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science*, **275**, 1122-6.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M. and Dixit, V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*, **81**, 505-12.

Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J. and Gusovsky, F. (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem*, **274**, 10689-92.

Chu, Z.L., Pio, F., Xie, Z., Welsh, K., Krajewska, M., Krajewski, S., Godzik, A. and Reed, J.C. (2001) A novel enhancer of the Apaf1 apoptosome involved in cytochrome c-dependent caspase activation and apoptosis. *J Biol Chem*, **276**, 9239-45.

Cohen, L., Henzel, W.J. and Baeuerle, P.A. (1998) IKAP is a scaffold protein of the IkappaB kinase complex. *Nature*, **395**, 292-6.

Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.*, **341**, 233-249.

Cuddeback, S.M., Yamaguchi, H., Komatsu, K., Miyashita, T., Yamada, M., Wu, C., Singh, S. and Wang, H.G. (2001) Molecular cloning and characterization of Bif-1. A novel Src homology 3 domain-containing protein that associates with Bax. *J Biol Chem*, **276**, 20559-65.

Damiano, J.S., Stehlik, C., Pio, F., Godzik, A. and Reed, J.C. (2001) CLAN, a novel human CED-4-like gene. *Genomics*, **75**, 77-83.

Dangl, J.L. and Jones, J.D. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826-33.

Davis, R.J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell*, **103**, 239-52.

De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D.U., Jin, R., Jones, J., Cong, R. and Franzoso, G. (2001) Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. *Nature*, **414**, 308-13.

Degli-Esposti, M.A., Dougall, W.C., Smolak, P.J., Waugh, J.Y., Smith, C.A. and Goodwin, R.G. (1997a) The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity*, **7**, 813-20.

Degli-Esposti, M.A., Smolak, P.J., Walczak, H., Waugh, J., Huang, C.P., DuBose, R.F., Goodwin, R.G. and Smith, C.A. (1997b) Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med*, **186**, 1165-70.

Delhase, M., Hayakawa, M., Chen, Y. and Karin, M. (1999) Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. *Science*, **284**, 309-13.

Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S. and Reed, J.C. (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *Embo J*, **17**, 2215-23.

DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E. and Karin, M. (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature*, **388**, 548-54.

Dinarello, C.A. (1996) Biologic basis for interleukin-1 in disease. *Blood*, **87**, 2095-147.

Dinarello, C.A., Gelfand, J.A. and Wolff, S.M. (1993) Anticytokine strategies in the treatment of the systemic inflammatory response syndrome. *JAMA*, **269**, 1829-35.

Druilhe, A., Srinivasula, S.M., Razmara, M., Ahmad, M. and Alnemri, E.S. (2001) Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. *Cell Death Differ*, **8**, 649-57.

Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell*, **102**, 33-42.

Duan, H. and Dixit, V.M. (1997) RAIDD is a new 'death' adaptor molecule. *Nature*, **385**, 86-9.

Duan, H., Chinnaiyan, A.M., Hudson, P.L., Wing, J.P., He, W.W. and Dixit, V.M. (1996a) ICE-LAP3, a novel mammalian homologue of the Caenorhabditis elegans cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis. *J Biol Chem*, **271**, 1621-5.

Duan, H., Orth, K., Chinnaiyan, A.M., Poirier, G.G., Froelich, C.J., He, W.W. and Dixit, V.M. (1996b) ICE-LAP6, a novel member of the ICE/Ced-3 gene

Garcia-Calvo, M., Peterson, E.P., Leiting, B., Ruel, R., Nicholson, D.W. and Thornberry, N.A. (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J Biol Chem*, **273**, 32608-13.

Geddes, B.J., Wang, L., Huang, W.J., Lavellee, M., Manji, G.A., Brown, M., Jurman, M., Cao, J., Morgenstern, J., Merriam, S., Glucksmann, M.A., DiStefano, P.S. and Bertin, J. (2001) Human CARD12 is a novel CED4/Apaf-1 family member that induces apoptosis. *Biochem Biophys Res Commun*, **284**, 77-82.

Geleziunas, R., Xu, W., Takeda, K., Ichijo, H. and Greene, W.C. (2001) HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature*, **410**, 834-8.

Ghosh, S., Gifford, A.M., Riviere, L.R., Tempst, P., Nolan, G.P. and Baltimore, D. (1990) Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal. *Cell*, **62**, 1019-29.

Gietz, R.D., Schiestl, R.H., Willems, A.R. and Woods, R.A. (1995) Studies on the Transformation of Intact Yeast Cells by the LiAc/SS-DNA/PEG Procedure. *Yeast*, **11**, 335-360.

Girardin, S.E., Tournebize, R., Mavris, M., Page, A.L., Li, X., Stark, G.R., Bertin, J., DiStefano, P.S., Yaniv, M., Sansonetti, P.J. and Philpott, D.J. (2001) CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. *EMBO Rep*, **2**, 736-42.

Gogvadze, V., Robertson, J.D., Zhivotovsky, B. and Orrenius, S. (2001) Cytochrome c release occurs via Ca2+-dependent and Ca2+-independent mechanisms that are regulated by Bax. *J Biol Chem*, **276**, 19066-71.

Goldberg, Y.P., Nicholson, D.W., Rasper, D.M., Kalchman, M.A., Koide, H.B., Graham, R.K., Bromm, M., Kazemi-Esfarjani, P., Thornberry, N.A., Vaillancourt, J.P. and Hayden, M.R. (1996) Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nat Genet*, **13**, 442-9.

Goltsev, Y.V., Kovalenko, A.V., Arnold, E., Varfolomeev, E.E., Brodianskii, V.M. and Wallach, D. (1997) CASH, a novel caspase homologue with death effector domains. *J Biol Chem*, **272**, 19641-4.

Guttridge, D.C., Mayo, M.W., Madrid, L.V., Wang, C.Y. and Baldwin, A.S., Jr. (2000) NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. *Science*, **289**, 2363-6.

Hakem, R., Hakem, A., Duncan, G.S., Henderson, J.T., Woo, M., Soengas, M.S., Elia, A., de la Pompa, J.L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S.A., Lowe, S.W., Penninger, J.M. and Mak, T.W. (1998) Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell*, **94**, 339-52.

Hampe, J., Cuthbert, A., Croucher, P.J., Mirza, M.M., Mascheretti, S., Fisher, S., Frenzel, H., King, K., Hasselmeyer, A., MacPherson, A.J., Bridger, S., van Deventer, S., Forbes, A., Nikolaus, S., Lennard-Jones, J.E., Foelsch, U.R., Krawczak, M., Lewis, C., Schreiber, S. and Mathew, C.G. (2001) Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet*, **357**, 1925-8.

Haslett, C. (1992) Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. *Clin Sci (Colch)*, **83**, 639-48.

Hegde, R., Srinivasula, S.M., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A.S., Fernandes-Alnemri, T. and Alnemri, E.S. (2001) Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts IAP-caspase interaction. *J Biol Chem*, **17**, 17.

Hengartner, M.O. and Horvitz, H.R. (1994) C.elegans Cell Survival Gene ced-9 Encodes a Functional Homolog of the Mammalian Proto-Oncogene bcl-2. *Cell*, **76**, 665-676.

Hiscott, J., Marois, J., Garoufalis, J., D'Addario, M., Roulston, A., Kwan, I., Pepin, N., Lacoste, J., Nguyen, H., Bensi, G. and et al. (1993) Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. *Mol Cell Biol*, **13**, 6231-40.

Hlaing, T., Guo, R.F., Dilley, K.A., Loussia, J.M., Morrish, T.A., Shi, M.M., Vincenz, C. and Ward, P.A. (2001) Molecular cloning and characterization of DEFCAP-L and -S, two isoforms of a novel member of the mammalian Ced-4 family of apoptosis proteins. *J Biol Chem*, **276**, 9230-8.

Hofmann, K., Bucher, P. and Tschopp, J. (1997) The CARD domain: a new apoptotic signalling motif. *Trends Biochem Sci*, **22**, 155-6.

Hsu, H., Huang, J., Shu, H.B., Baichwal, V. and Goeddel, D.V. (1996a) TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity*, **4**, 387-96.

Hsu, H., Shu, H.B., Pan, M.G. and Goeddel, D.V. (1996b) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*, **84**, 299-308.

Hsu, H., Xiong, J. and Goeddel, D.V. (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell*, **81**, 495-504.

Hsu, S.Y., Kaipia, A., McGee, E., Lomeli, M. and Hsueh, A.J. (1997a) Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proc Natl Acad Sci U S A*, **94**, 12401-6.

Hsu, Y.-T., Wolter, K.G. and Youle, R.J. (1997b) Cytosol to-membrane redistribution of Bax and Bcl-xL during apoptosis. *Proc. Nat. Acad. Sci. USA*, **94**, 3668-3672.

Hu, S., Snipas, S.J., Vincenz, C., Salvesen, G. and Dixit, V.M. (1998) Caspase-14 is a novel developmentally regulated protease. *J Biol Chem*, **273**, 29648-53.

Hu, S., Vincenz, C., Ni, J., Gentz, R. and Dixit, V.M. (1997) I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. *J Biol Chem*, **272**, 17255-7.

Hu, W.H., Johnson, H. and Shu, H.B. (2000) Activation of NF-kappaB by FADD, Casper, and caspase-8. *J Biol Chem*, **275**, 10838-44.

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R. and Karin, M. (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science*, **284**, 316-20.

Hu, Y., Baud, V., Oga, T., Kim, K.I., Yoshida, K. and Karin, M. (2001) IKKalpha controls formation of the epidermis independently of NF-kappaB. *Nature*, **410**, 710-4.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.F., Sahbatou, M. and Thomas, G. (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*, **411**, 599-603.

Hugot, J.P., Laurent-Puig, P., Gower-Rousseau, C., Olson, J.M., Lee, J.C., Beaugerie, L., Naom, I., Dupas, J.L., Van Gossum, A., Orholm, M., Bonaiti-Pellie, C., Weissenbach, J., Mathew, C.G., Lennard-Jones, J.E., Cortot, A., Colombel, J.F. and Thomas, G. (1996) Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature*, **379**, 821-3.

Humke, E.W., Ni, J. and Dixit, V.M. (1998) ERICE, a novel FLICE-activatable caspase. *J Biol Chem*, **273**, 15702-7.

Humke, E.W., Shriver, S.K., Starovasnik, M.A., Fairbrother, W.J. and Dixit, V.M. (2000) ICEBERG: a novel inhibitor of interleukin-1beta generation. *Cell*, **103**, 99-111.

Imai, Y., Kimura, T., Murakami, A., Yajima, N., Sakamaki, K. and Yonehara, S. (1999) The CED-4-homologous protein FLASH is involved in Fas-mediated activation of caspase-8 during apoptosis. *Nature*, **398**, 777-85.

Inohara, N., del Peso, L., Koseki, T., Chen, S. and Nunez, G. (1998) RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis. *J Biol Chem*, **273**, 12296-300.

Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J. and Nunez, G. (1999) Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem*, **274**, 14560-7.

Inohara, N., Koseki, T., Lin, J., del Peso, L., Lucas, P.C., Chen, F.F., Ogura, Y. and Nunez, G. (2000) An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. *J Biol Chem*, **275**, 27823-31.

Inohara, N., Ogura, Y., Chen, F.F., Muto, A. and Nunez, G. (2001) Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J Biol Chem*, **276**, 2551-4.

Inohara, N., Koseki, T., Hu, Y., Chen, S. and Nunez, G. (1997) CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proc Natl Acad Sci U S A*, **94**, 10717-22.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J-L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L.E. and Tschopp, J. (1997) Inhibition of death receptor signals by cellular FLIP. *Nature*, **388**, 190-195.

Ito, A., Uehara, T. and Nomura, Y. (2000) Isolation of Ich-1S (caspase-2S)-binding protein that partially inhibits caspase activity. *FEBS Lett*, **470**, 360-4.

Itoh, N. and Nagata, S. (1993) A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J Biol Chem*, **268**, 10932-7.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. and Nagata, S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, **66**, 233-43.

Jaattela, M., Wissing, D., Kokholm, K., Kallunki, T. and Egeblad, M. (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *EMBO J*, **17**, 6124-34.

Jacobson, M.D., Weil, M. and Raff, M.C. (1997) Programmed cell death in animal development. *Cell*, **88**, 347-54.

Janicke, R.U., Walker, P.A., Lin, X.Y. and Porter, A.G. (1996) Specific cleavage of the retinoblastoma protein by an ICE-like protease in apoptosis. *EMBO J.*, **15**, 6969-6978.

Jo, M., Kim, T.H., Seol, D.W., Esplen, J.E., Dorko, K., Billiar, T.R. and Strom, S.C. (2000) Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med*, **6**, 564-7.

Kamada, S., Kusano, H., Fujita, H., Ohtsu, M., Koya, R.C., Kuzumaki, N. and Tsujimoto, Y. (1998) A cloning method for caspase substrates that uses the yeast two-hybrid system: cloning of the antiapoptotic gene gelsolin. *Proc Natl Acad Sci U S A*, **95**, 8532-7.

Keegan, L., Gill, G. and Ptashne, M. (1986) Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science*, **231**, 699-704.

Kelliher, M.A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B.Z. and Leder, P. (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity*, **8**, 297-303.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, **26**, 239-57.

Khoshnan, A., Kempiak, S.J., Bennett, B.L., Bae, D., Xu, W., Manning, A.M., June, C.H. and Nel, A.E. (1999) Primary human CD4+ T cells contain

heterogeneous I kappa B kinase complexes: role in activation of the IL-2 promoter. *J Immunol*, **163**, 5444-52.

Khoshnan, A., Tindell, C., Laux, I., Bae, D., Bennett, B. and Nel, A.E. (2000) The NF-kappa B cascade is important in Bcl-xL expression and for the anti-apoptotic effects of the CD28 receptor in primary human CD4+ lymphocytes. *J Immunol*, **165**, 1743-54.

Kirschning, C.J., Wesche, H., Ayres, T.M. and Rothe, M. (1998) Human Toll-like Receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med*, **188**, 2091-2097.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H. and Peter, M.E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J*, **14**, 5579-88.

Kischkel, F.C., Lawrence, D.A., Tinel, A., LeBlanc, H., Virmani, A., Schow, P., Gazdar, A., Blenis, J., Arnott, D. and Ashkenazi, A. (2001) Death Receptor Recruitment of Endogenous Caspase-10 and Apoptosis Initiation in the Absence of Caspase-8. *J Biol Chem*, **276**, 46639-46646.

Kitson, J., Raven, T., Jiang, Y.P., Goeddel, D.V., Giles, K.M., Pun, K.T., Grinham, C.J., Brown, R. and Farrow, S.N. (1996) A death-domain-containing receptor that mediates apoptosis. *Nature*, **384**, 372-5.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*, **275**, 1132-6.

Knudson, C.M., Tung, K.S.K., Tourtellote, W.G., Brown, G.A.J. and Korsmeyer, S.J. (1995) Bax-Deficient Mice with Lymphoid Hyperplasia and Male Germ Cell Death. *Science*, **270**, 96-99.

Koseki, T., Inohara, N., Chen, S., Carrio, R., Merino, J., Hottiger, M.O., Nabel, G.J. and Nunez, G. (1999) CIPER, a novel NF kappaB-activating protein containing a caspase recruitment domain with homology to Herpesvirus-2 protein E10. *J Biol Chem*, **274**, 9955-61.

Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T.J., Kirschner, M.W., Koths, K., Kwiatkowski, D.J. and Wiliams, L.T. (1997) Caspase-3 generated fragment of gelsolin: effector of morphological change in apoptosis. *Science*, **278**, 294-298.

Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R.A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature*, **384**, 368-72.

Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P. and Flavell, R.A. (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell*, **94**, 325-37.

Kuida, K., Lippke, J.A., Ku, G., Harding, M.W., Livingston, D.J., Su, M.S. and Flavell, R.A. (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science*, **267**, 2000-3.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.C. (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*, **371**, 346-7.

Lee, S.H., Stehlik, C. and Reed, J.C. (2001) Cop, a caspase recruitment domain-containing protein and inhibitor of caspase-1 activation processing. *J Biol Chem*, **276**, 34495-500.

Lennon, S.V., Martin, S.J. and Cotter, T.G. (1991) Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Prolif*, **24**, 203-14.

Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323-31.

Li, F., Ambrosini, G., Chu, E.Y., Plescia, J., Tognin, S., Marchisio, P.C. and Altieri, D.C. (1998a) Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature*, **396**, 580-4.

Li, H., Zhu, H., Xu, C.J. and Yuan, J. (1998b) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*, **94**, 491-501.

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J. and et al. (1995) Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell*, **80**, 401-11.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, **91**, 479-89.

Li, Z.W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R. and Karin, M. (1999) The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med*, **189**, 1839-45.

Lin, X.Y., Choi, M.S.K. and Porter, A.G. (2000) Expression Analysis of the Human Caspase-1 Subfamily Reveals Specific Regulation of the CASP5 Gene by Lipopolysaccharide and Interferon-gamma. *J. Biol Chem*, **275**, 39920-39926.

Lin, Y., Devin, A., Rodriguez, Y. and Liu, Z.G. (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev*, **13**, 2514-26.

Lucas, P.C., Yonezumi, M., Inohara, N., McAllister-Lucas, L.M., Abazeed, M.E., Chen, F.F., Yamaoka, S., Seto, M. and Nunez, G. (2001) Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway. *J Biol Chem*, **276**, 19012-9.

MacFarlane, M., Ahmad, M., Srinivasula, S.M., Fernandes-Alnemri, T., Cohen, G.M. and Alnemri, E.S. (1997) Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J Biol Chem*, **272**, 25417-20.

Martin, S.J. (2001) Dealing the CARDs between life and death. *Trends Cell Biol*, **11**, 188-9.

Martin, S.J. and Green, D.R. (1995) Apoptosis and Cancer: the failure of controls on cell death and cell survival. *Crit Rev Oncol Haematol*, **18**, 137-53.

Martin, S.J., O'Brien, G.A., Nishioka, W.K., McGahon, A.J., Saido, T., Green, D.R. (1995) Proteolysis of Fodrin nonerythroid spectrin during apoptosis. *J. Biol. Chem.*, **270**, 6425-6428.

Martins, L.M., Iaccarino, I., Tenev, T., Gschmeissner, S., Totty, N.F., Lemoine, N.R., Savopoulos, J., Gray, C.W., Creasy, C.L., Dingwall, C. and Downward, J. (2001) The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a Reaper-like motif. *J Biol Chem*, **15**, 15.

Marzo, I. *et.al.* (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science*, **281**, 2027-2031.

Masumoto, J., Taniguchi, S., Ayukawa, K., Sarvotham, H., Kishino, T., Niikawa, N., Hidaka, E., Katsuyama, T., Higuchi, T. and Sagara, J. (1999) ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *J Biol Chem*, **274**, 33835-8.

Masumoto, J., Taniguchi, S. and Sagara, J. (2001) Pyrin N-terminal homology domain- and caspase recruitment domain-dependent oligomerization of ASC. *Biochem Biophys Res Commun*, **280**, 652-5.

McCarthy, J.V., Ni, J. and Dixit, V.M. (1998) RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. *J Biol Chem*, **273**, 16968-75.

McConnell, K.R., Dynan, W.S and Hardin, J.A. (1997) The DNA-Dependent Protein Kinase Catalytic Subunit (p460) Is Cleaved During Fas-Mediated Apoptosis in Jurkat Cells. *J Immunol*, **158**, 2083-2089.

Medema, J.P., Scaffidi, C., Kischkel, F.C., Shevchenko, A., Mann, M., Krammer, P.H. and Peter, M.E. (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *Embo J*, **16**, 2794-804.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S. and Janeway, C.A., Jr. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell*, **2**, 253-8.

Miceli-Richard, C., Lesage, S., Rybojad, M., Prieur, A.M., Manouvrier-Hanu, S., Hafner, R., Chamaillard, M., Zouali, H., Thomas, G. and Hugot, J.P. (2001) CARD15 mutations in Blau syndrome. *Nat Genet*, **29**, 19-20.

Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K. and Tschopp, J. (2001) NF-kappaB signals induce the expression of c-FLIP. *Mol Cell Biol*, **21**, 5299-305.

Miura, M., Zhu, H., Rotello, R., Hartwieg, E.A. and Yuan, J. (1993) Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. *Cell*, **75**, 653-60.

Munday, N.A., Vaillancourt, J.P., Ali, A., Casano, F.J., Miller, D.K., Molineaux, S.M., Yamin, T.T., Yu, V.L. and Nicholson, D.W. (1995) Molecular cloning and pro-apoptotic activity of ICErelII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. *J Biol Chem*, **270**, 15870-6.

Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E. and Dixit, V.M. (1996) FLICE, a novel FADD-homologous

ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell*, **85**, 817-27.

Nagafuchi, S., Yanagisawa, H., Ohsaki, E., Shirayama, T., Tadokoro, K., Inoue, T. and Yamada, M. (1994) Structure and expression of the gene responsible for the triplet repeat disorder, dentatorubral and pallidoluysian atrophy (DRPLA). *Nat Genet*, **8**, 177-82.

Nagata, S. (1997) Apoptosis by death factor. *Cell*, **88**, 355-65.

Naik, S., Kelly, E.J., Meijer, L., Pettersson, S. and Sanderson, I.R. (2001) Absence of Toll-like receptor 4 explains endotoxin hyporesponsiveness in human intestinal epithelium. *J Pediatr Gastroenterol Nutr*, **32**, 449-53.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B.A. and Yuan, J. (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature*, **403**, 98-103.

Neamati, N., Fernendez, A., Wright, S., Kiefer, J. and McConkey, D.J. (1995) Degradation of laminB1 precedes oligonucleosomal DNA fragmentation in apoptotic thymocytes and isolated thymocyte nuclei. *Journal of Immunology*, **154**, 3788-3795.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A. and et al. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, **376**, 37-43.

Nolan, G.P., Ghosh, S., Liou, H.C., Tempst, P. and Baltimore, D. (1991) DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide. *Cell*, **64**, 961-9.

O'Connor, D.S., Grossman, D., Plescia, J., Li, F., Zhang, H., Villa, A., Tognin, S., Marchisio, P.C. and Altieri, D.C. (2000) Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc Natl Acad Sci U S A*, **97**, 13103-7.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T. and Nagata, S. (1993) Lethal effect of the anti-Fas antibody in mice. *Nature*, **364**, 806-9.

Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S., Hanauer, S.B., Nunez, G. and Cho, J.H.

(2001a) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*, **411**, 603-6.

Ogura, Y., Inohara, N., Benito, A., Chen, F.F., Yamaoka, S. and Nunez, G. (2001b) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem*, **276**, 4812-8.

Oltvai, Z.N., Milliman, C.L. and Korsmeyer, S.J. (1993) Bcl-2 Heterodimerizes In vivo with a Conserved Homolog, Bax, That Accelerates Programed Cell Death. *Cell*, **74**, 609-619.

Orth, K., Chinnaiyan, A.M., Garg, M., Froelich, C.J. and Dixit, V.M. (1996) The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J Biol Chem*, **271**, 16443-6.

Ott, M., Lovett, J.L., Mueller, L. and Verdin, E. (1998) Superinduction of IL-8 in T cells by HIV-1 Tat protein is mediated through NF-kappaB factors. *J Immunol*, **160**, 2872-80.

Pan, G., Ni, J., Wei, Y.F., Yu, G., Gentz, R. and Dixit, V.M. (1997a) An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science*, **277**, 815-8.

Pan, G., O'Rourke, K., Chinnaiyan, A.M., Gentz, R., Ebner, R., Ni, J. and Dixit, V.M. (1997b) The receptor for the cytotoxic ligand TRAIL. *Science*, **276**, 111-3.

Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S.M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E.S., Kufe, D. and Kharbanda, S. (2000) Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *EMBO J*, **19**, 4310-22.

Pathan, N., Marusawa, H., Krajewska, M., Matsuzawa, S., Kim, H., Okada, K., Torii, S., Kitada, S., Krajewski, S., Welsh, K., Pio, F., Godzik, A. and Reed, J.C. (2001) TUCAN, an Antiapoptotic caspase-associated recruitment domain family protein overexpressed in cancer. *J. Biol. Chem.*, **276**, 32220-32229.

Philpott, D.J., Yamaoka, S., Israel, A. and Sansonetti, P.J. (2000) Invasive Shigella flexneri activates NF-kappa B through a lipopolysaccharide-dependent innate intracellular response and leads to IL-8 expression in epithelial cells. *J Immunol*, **165**, 903-14.

Pitti, R.M., Marsters, S.A., Ruppert, S., Donahue, C.J., Moore, A. and Ashkenazi, A. (1996) Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem*, **271**, 12687-90.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, **282**, 2085-8.

Poyet, J.L., Srinivasula, S.M., Lin, J.H., Fernandes-Alnemri, T., Yamaoka, S., Tsichlis, P.N. and Alnemri, E.S. (2000) Activation of the Ikappa B kinases by RIP via IKKgamma /NEMO-mediated oligomerization. *J Biol Chem*, **275**, 37966-77.

Poyet, J.L., Srinivasula, S.M., Tnani, M., Razmara, M., Fernandes-Alnemri, T. and Alnemri, E.S. (2001) Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. *J Biol Chem*, **276**, 28309-13.

Pugin, J., Schurer-Maly, C.C., Leturcq, D., Moriarty, A., Ulevitch, R.J. and Tobias, P.S. (1993) Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc Natl Acad Sci U S A*, **90**, 2744-8.

Pulst, S.M., Nechiporuk, A., Nechiporuk, T., Gispert, S., Chen, X.N., Lopes-Cendes, I., Pearlman, S., Starkman, S., Orozco-Diaz, G., Lunkes, A., DeJong, P., Rouleau, G.A., Auburger, G., Korenberg, J.R., Figueroa, C. and Sahba, S. (1996) Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. *Nat Genet*, **14**, 269-76.

Ray, C.A., Black, R.A., Kronheim, S.R., Greenstreet, T.A., Sleath, P.R., Salvesen, G.S. and Pickup, D.J. (1992) Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. *Cell*, **69**, 597-604.

Richards, N., Schaner, P., Diaz, A., Stuckey, J., Shelden, E., Wadhwa, A. and Gumucio, D.L. (2001) Interaction between Pyrin and the Apoptotic Speck Protein (ASC) Modulates ASC-induced Apoptosis. *J Biol Chem*, **276**, 39320-9.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A. and Bazan, J.F. (1998) A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A*, **95**, 588-93.

Rothwarf, D.M., Zandi, E., Natoli, G. and Karin, M. (1998) IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature*, **395**, 297-300.

Ruben, S.M., Dillon, P.J., Schreck, R., Henkel, T., Chen, C.H., Maher, M., Baeuerle, P.A. and Rosen, C.A. (1991) Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-kappa B. *Science*, **251**, 1490-3.

Rudolph, D., Yeh, W.C., Wakeham, A., Rudolph, B., Nallainathan, D., Potter, J., Elia, A.J. and Mak, T.W. (2000) Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. *Genes Dev*, **14**, 854-62.

Ruland, J., Duncan, G.S., Elia, A., del Barco Barrantes, I., Nguyen, L., Plyte, S., Millar, D.G., Bouchard, D., Wakeham, A., Ohashi, P.S. and Mak, T.W. (2001) Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. *Cell*, **104**, 33-42.

Saleh, A., Srinivasula, S.M., Balkir, L., Robbins, P.D. and Alnemri, E.S. (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat Cell Biol*, **2**, 476-83.

Savill, J.S., Wyllie, A.H., Henson, J.E., Walport, M.J., Henson, P.M. and Haslett, C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest*, **83**, 865-75.

Savukoski, M., Klockars, T., Holmberg, V., Santavuori, P., Lander, E.S. and Peltonen, L. (1998) CLN5, a novel gene encoding a putative transmembrane protein mutated in Finnish variant late infantile neuronal ceroid lipofuscinosis. *Nat Genet*, **19**, 286-8.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H. and Peter, M.E. (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*, **17**, 1675-87.

Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S.J., Krammer, P.H. and Peter, M.E. (1999) Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. *J Biol Chem*, **274**, 22532-8.

Shu, H.B., Halpin, D.R. and Goeddel, D.V. (1997) Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity*, **6**, 751-63.

Slee, E.A., Harte, M.T., Kluck, R.M., Wolf, B.B., Casiano, C.A., Newmeyer, D.D., Wang, H.G., Reed, J.C., Nicholson, D.W., Alnemri, E.S., Green, D.R. and Martin, S.J. (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol*, **144**, 281-92.

Song, H.Y., Dunbar, J.D., Zhang, Y.X., Guo, D. and Donner, D.B. (1995) Identification of a protein with homology to hsp90 that binds the type 1 tumor necrosis factor receptor. *J Biol Chem*, **270**, 3574-81.

Spector, M.S., Desnoyers, S., Hoeppner, D.J. and Hengartner, M.O. (1997) Interaction between the C. elegans cell-death regulators CED-9 and CED-4. *Nature*, **385**, 653-6.

Sprick, M.R., Weigand, M.A., Rieser, E., Rauch, C.T., Juo, P., Blenis, J., Krammer, P.H. and Walczak, H. (2000) FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity*, **12**, 599-609.

Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G. and Alnemri, E.S. (1996) Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. *Proc Natl Acad Sci U S A*, **93**, 14486-91.

Srinivasula, S.M., Ahmad, M., Lin, J.H., Poyet, J.L., Fernandes-Alnemri, T., Tsichlis, P.N. and Alnemri, E.S. (1999) CLAP, a novel caspase recruitment domain-containing protein in the tumor necrosis factor receptor pathway, regulates NF-kappaB activation and apoptosis. *J Biol Chem*, **274**, 17946-54.

Srinivasula, S.M., Hegde, R., Saleh, A., Datta, P., Shiozaki, E., Chai, J., Lee, R.A., Robbins, P.D., Fernandes-Alnemri, T., Shi, Y. and Alnemri, E.S. (2001) A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature*, **410**, 112-6.

Stanger, B.Z., Leder, P., Lee, T.H., Kim, E. and Seed, B. (1995) RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell*, **81**, 513-23.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L. and Galan, J.E. (2001) Common and contrasting themes of plant and animal diseases. *Science*, **292**, 2285-9.

Staub, E., Dahl, E. and Rosenthal, A. (2001) The DAPIN family: a novel domain links apoptotic and interferon response proteins. *Trends Biochem Sci*, **26**, 83-5.

Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J.A., Binder, B.R. and Lipp, J. (1998) Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. *J Exp Med*, **188**, 211-6.

Suda, T., Takahashi, T., Golstein, P. and Nagata, S. (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell*, **75**, 1169-78.

Sun, X., Lee, J., Navas, T., Baldwin, D.T., Stewart, T.A. and Dixit, V.M. (1999) RIP3, a novel apoptosis-inducing kinase. *J Biol Chem*, **274**, 16871-5.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K. and Takahashi, R. (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell*, **8**, 613-21.

Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T. and Nagata, S. (1994) Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell*, **76**, 969-76.

Takeda, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N. and Akira, S. (1999) Limb and skin abnormalities in mice lacking IKKalpha. *Science*, **284**, 313-6.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity*, **11**, 443-51.

Tan, K.O., Tan, K.M., Chan, S.L., Yee, K.S., Bevort, M., Ang, K.C. and Yu, V.C. (2001) MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains. *J Biol Chem*, **276**, 2802-7.

Tang, G., Minemoto, Y., Dibling, B., Purcell, N.H., Li, Z., Karin, M. and Lin, A. (2001) Inhibition of JNK activation through NF-kappaB target genes. *Nature*, **414**, 313-7.

Tartaglia, L.A., Ayres, T.M., Wong, G.H. and Goeddel, D.V. (1993) A novel domain within the 55 kd TNF receptor signals cell death. *Cell*, **74**, 845-53.

Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S. and Dixit, V.M. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell*, **81**, 801-9.

Thome, M., Hofmann, K., Burns, K., Martinon, F., Bodmer, J.L., Mattmann, C. and Tschopp, J. (1998) Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr Biol*, **8**, 885-8.

Thome, M., Martinon, F., Hofmann, K., Rubio, V., Steiner, V., Schneider, P., Mattmann, C. and Tschopp, J. (1999) Equine herpesvirus-2 E10 gene product, but not its cellular homologue, activates NF-kappaB transcription factor and c-Jun N-terminal kinase. *J Biol Chem*, **274**, 9962-8.

Thompson, C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456-62.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J. and et al. (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature*, **356**, 768-74.

Trauth, B.C., Klas, C., Peters, A.M., Matzku, S., Moller, P., Falk, W., Debatin, K.M. and Krammer, P.H. (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science*, **245**, 301-5.

Truelove, S.C. and Pena, A.S. (1976) Course and prognosis of Crohn's disease. *Gut*, **17**, 192-201.

Urban, M.B., Schreck, R. and Baeuerle, P.A. (1991) NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit. *Embo J*, **10**, 1817-25.

Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R. and Verma, I.M. (1996) Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science*, **274**, 787-9.

Vairapandi, M., Balliet, A.G., Fornace, A.J., Jr., Hoffman, B. and Liebermann, D.A. (1996) The differentiation primary response gene MyD118, related to GADD45, encodes for a nuclear protein which interacts with PCNA and p21WAF1/CIP1. *Oncogene*, **12**, 2579-94.

Varfolomeev, E.E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J.S., Mett, I.L., Rebrikov, D., Brodianski, V.M., Kemper, O.C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K.B., Goncharov, T., Holtmann,

H., Lonai, P. and Wallach, D. (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity*, **9**, 267-76.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J. and Vaux, D.L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell*, **102**, 43-53.

Verhagen, A.M., Silke, J., Ekert, P.G., Pakusch, M., Kaufmann, H., Connolly, L.M., Day, C.L., Tikoo, A., Burke, R., Wrobel, C., Moritz, R.L., Simpson, R.J. and Vaux, D.L. (2001) HtrA2 promotes cell death through its serine protease activity and its ability to antagonise inhibitor of apoptosis proteins. *J Biol Chem*, **16**, 16.

Vincenz, C. and Dixit, V.M. (1997) Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. *J Biol Chem*, **272**, 6578-83.

Viriyakosol, S., Tobias, P.S., Kitchens, R.L. and Kirkland, T.N. (2001) MD-2 binds to bacterial lipopolysaccharide. *J Biol Chem*, **276**, 38044-51.

Wang, C.Y., Mayo, M.W. and Baldwin, A.S., Jr. (1996a) TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science*, **274**, 784-7.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V. and Baldwin, A.S., Jr. (1998a) NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science*, **281**, 1680-3.

Wang, J., Chun, H.J., Wong, W., Spencer, D.M. and Lenardo, M.J. (2001a) Caspase-10 is an initiator caspase in death receptor signaling. *Proc Natl Acad Sci U S A*, **98**, 13884-8.

Wang, J., Zheng, L., Lobito, A., Chan, F.K., Dale, J., Sneller, M., Yao, X., Puck, J.M., Straus, S.E. and Lenardo, M.J. (1999) Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell*, **98**, 47-58.

Wang, L., Guo, Y., Huang, W.J., Ke, X., Poyet, J.L., Manji, G.A., Merriam, S., Glucksmann, M.A., DiStefano, P.S., Alnemri, E.S. and Bertin, J. (2001b) Card10 is a novel caspase recruitment domain/membrane-associated

guanylate kinase family member that interacts with BCL10 and activates NF-kappa B. *J Biol Chem*, **276**, 21405-9.

Wang, L., Miura, M., Bergeron, L., Zhu, H. and Yuan, J. (1994) Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell*, **78**, 739-50.

Wang, S., Miura, M., Jung, Y., Zhu, H., Gagliardini, V., Shi, L., Greenberg, A.H. and Yuan, J. (1996b) Identification and characterization of Ich-3, a member of the interleukin-1beta converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. *J Biol Chem*, **271**, 20580-7.

Wang, S., Miura, M., Jung, Y.K., Zhu, H., Li, E. and Yuan, J. (1998b) Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell*, **92**, 501-9.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A. and Nagata, S. (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature*, **356**, 314-7.

Watson, R., W., G. (1999) Apoptosis: its role in the systemic inflammatory response syndrome and the involvement of cytokines. In Redl, H. and Schlag., G. (ed.) *Cytokines in severe sepsis and septic shock.* Birkhauser Verlag, Basel; Boston; Berlin, pp. 213-226.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. and Korsmeyer, S.J. (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*, **292**, 727-30.

Wellington, C.L., Ellerby, L.M., Hackam, A.S., Margolis, R.L., Trifiro, M.A., Singaraja, R., McCutcheon, K., Salvesen, G.S., Propp, S.S., Bromm, M., Rowland, K.J., Zhang, T., Rasper, D., Roy, S., Thornberry, N., Pinsky, L., Kakizuka, A., Ross, C.A., Nicholson, D.W., Bredesen, D.E. and Hayden, M.R. (1998) Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J Biol Chem*, **273**, 9158-67.

Wesche, H., Henzel, W.J., Shillinglaw, W., Li, S. and Cao, Z. (1997) MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity*, **7**, 837-47.

Wiley, S.R., Schooley, K., Smolak, P.J., Din, W.S., Huang, C.P., Nicholl, J.K., Sutherland, G.R., Smith, T.D., Rauch, C., Smith, C.A. and et al. (1995) Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*, **3**, 673-82.

Williams, M.S. and Henkart, P.A. (1994) Apoptotic cell death induced by intracellular proteolysis. *J. Immunol*, **153**, 4247-4255.

Willis, T.G., Jadayel, D.M., Du, M.Q., Peng, H., Perry, A.R., Abdul-Rauf, M., Price, H., Karran, L., Majekodunmi, O., Wlodarska, I., Pan, L., Crook, T., Hamoudi, R., Isaacson, P.G. and Dyer, M.J. (1999) Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. *Cell*, **96**, 35-45.

Winfield, S.L., Tayebi, N., Martin, B.M., Ginns, E.I. and Sidransky, E. (1997) Identification of three additional genes contiguous to the glucocerebrosidase locus on chromosome 1q21: implications for Gaucher disease. *Genome Res*, **7**, 1020-6.

Wolf, D., Witte, V., Laffert, B., Blume, K., Stromer, E., Trapp, S., d'Aloja, P., Schurmann, A. and Baur, A.S. (2001) HIV-1 Nef associated PAK and PI3-Kinases stimulate Akt-independent Bad-phosphorylation to induce anti-apoptotic signals. *Nat Med*, **7**, 1217-24.

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. and Youle, R.J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol*, **139**, 1281-92.

Wu, G.S., Burns, T.F., McDonald, E.R., 3rd, Jiang, W., Meng, R., Krantz, I.D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., Hamilton, S.R., Spinner, N.B., Markowitz, S., Wu, G. and el-Deiry, W.S. (1997b) KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet*, **17**, 141-3.

Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S.T., Weil, R., Agou, F., Kirk, H.E., Kay, R.J. and Israel, A. (1998) Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell*, **93**, 1231-40.

Yan, M., Lee, J., Schilbach, S., Goddard, A. and Dixit, V. (1999) mE10, a novel caspase recruitment domain-containing proapoptotic molecule. *J Biol Chem*, **274**, 10287-92.

Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B. and Korsmeyer, S.J. (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell*, **80**, 285-91.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P. and Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science*, **275**, 1129-32.

Yin, X.M., Oltvai, Z.N. and Korsmeyer, S.J. (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature*, **369**, 321-3.

Yoshida, H., Kong, Y.Y., Yoshida, R., Elia, A.J., Hakem, A., Hakem, R., Penninger, J.M. and Mak, T.W. (1998) Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell*, **94**, 739-50.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and Horvitz, H.R. (1993) The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell*, **75**, 641-52.

Yuan, J. and Yankner, B.A. (2000) Apoptosis in the nervous system. *Nature*, **407**, 802-9.

Zauli, G., Gibellini, D., Secchiero, P., Dutartre, H., Olive, D., Capitani, S. and Collette, Y. (1999) Human immunodeficiency virus type 1 Nef protein sensitizes CD4(+) T lymphoid cells to apoptosis via functional upregulation of the CD95/CD95 ligand pathway. *Blood*, **93**, 1000-10.

Zhang, S.Q., Kovalenko, A., Cantarella, G. and Wallach, D. (2000) Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation. *Immunity*, **12**, 301-11.

Zheng, L., Schickling, O., Peter, M.E. and Lenardo, M.J. (2001) The death effector domain-associated factor plays distinct regulatory roles in the nucleus and cytoplasm. *J Biol Chem*, **276**, 31945-52.

Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V.M., and Salvesen, G.S. (1997) Target protease specificity of the Viral Serpin CrmA. *J. Biol. Chem*, **272**, 7797-7800.

Zou, H., Li, Y., Liu, X. and Wang, X. (1999) An APAF-1.Cytochrome c Multimeric Complex is a Functional Apoptosome That Activates Procaspase-9. *J. Biol. Chem*, **274**.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A. and Wang, X. (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell*, **90**, 405-13.

On the role of Caspase-Recruitment Domain proteins in Apoptosis and NFκB activation

By

Lisa Bouchier-Hayes

A thesis submitted to Trinity College Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy

Submitted December 2001

Research conducted in the Division of Molecular Cell Biology, Department of Genetics, Trinity College Dublin, Dublin 2

Thesis Supervisor: Prof. Seamus J. Martin