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# 1. Genomic organization and characterization of the human inhibitor of apoptosis genes *hiap1* and *hiap2*

by

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## 2. Abstract

Evolution from single celled to multicellular and complex organisms brings with it several challenges. Chief among these is the necessity to control cellular proliferation, differentiation and cell death. These controls ensure the proper development of anatomical structures and the cellular homeostasis of the organism. Multicellularity also entails the active elimination of cells which have outlived their usefulness or have become malignant by virtue of being oncogenic, microbially infected or otherwise dangerous. Although initiating programmed cell death, or apoptosis, is catastrophic to the affected cell, failure to do so may be detrimental to the viability of the organism as a whole.

Ultimately most, if not all, apoptotic death results from the activation of a series of proteases. These powerful proteases, termed caspases, orchestrate the regulated disassembly of the apoptotic cell. In order to ensure the capacity to inhibit or prevent the inappropriate induction of cell death, several negative regulatory mechanisms have evolved. Quite recently, it was found that a novel class of anti-apoptotic proteins are direct caspase inhibitors. The mammalian inhibitor of apoptosis (IAP) family is comprised of, minimally, four core members, all of which contain three protein-protein interaction domains termed BIR domains (Baculoviral IAP repeat). It is through these domains, originally described in the baculoviral counterpart of the mammalian IAPs, that their interaction with caspases is mediated.

Two of the mammalian members, HIAP1 and HIAP2, are the subject of the following investigations. First the complete genomic organization of both *hiap1* and *hiap2* was elucidated. Results from this study indicated that both genes lie in tandem (head to tail) on the genome (at 11q23) separated by approximately 7 kbp. Furthermore, it was revealed that it is likely that these genes, as well as that of *xiap*, arose by gene duplication. By comparison to the murine organization, it was also determined that these duplication events occurred prior to the divergence of the species'. It was furthermore found that both genes possess unusually long 5'UTR's. Although the presence of several possible initiation codons within of their respective 5'UTR's would preclude translation initiation by traditional scanning, no evidence was found to support the presence of a standard internal ribosome entry site (IRES) upstream of either open reading frame.

Studies into the transcriptional regulation of these genes found that although *hiap2* transcription was unresponsive to a variety of stimuli, that of *hiap1* was markedly enhanced by agents which activate NF-κB. Analysis of the 5' region of *hiap1* has revealed the presence of two conserved putative NF-κB binding sites as well as an NF-IL-6 (C/EBPβ) site and a putative NF-κB negative regulatory element. Gel shift analysis has shown that all are able to bind their respective factors although reporter gene analysis coupled with site directed mutagenesis has shown that only the NF-κB and NF-IL6 sites are functional under the conditions examined.

Partial characterization of a naturally occurring *hiap1/hiap2* double knockout cell line derived from the Burkitt's lymphoma line BJAB was also carried out. By comparing the effect of TNFα on both the derivative and parental line, it was found that the presence of these genes alleviated the negative impact of TNFα on cellular proliferation. Furthermore, transient re-introduction of *hiap1* into the derivative line or anti-sense *hiap1* into the parental line partially reversed their phenotype with respect to TNFα response.

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## 7. List of Abbreviations and Acronyms (Alphabetical)

Not included in the following list are abbreviations denoting common reagents and solutions (e.g. PBS, TAE, EDTA, SSC).

| | |
|---|---|
| 4E-BP | Eukaryotic initiation factor 4E binding protein |
| A1 | Bcl2 related protein A1 (Bcl2A1) |
| AIF | Apoptosis inducing factor |
| AICD | Activation induced cell death |
| AITR | Activation inducible TNF receptor family member |
| APAF | Aptotic protease activating factor |
| ATAR | Another TRAF associated receptor |
| ATP | Adenosine triphosphate |
| ASK1 | Apoptosis signal-regulating kinase 1 |
| BAC | Bacterial artificial chromosome |
| BAD | Bcl2 antagonist of cell death |
| BALT | Bronchial associated lymphoid tissue |
| BAK | Bcl-2 homologous antagonist/killer |
| BAX | Bcl2 associated X-protein |
| Bcl2 | B-cell lymphoma-2 |
| Bcl-$X_L$ | Bcl2 related gene (long) |
| BCMA | B-cell maturation antigen |
| Bfl1 | Bcl2 homologue from fetal liver-1 |
| BH(domain) | Bcl2 homology (domain) |
| BID | BH3 interacting domain death agonist |
| BIK | Bcl2 interacting killer |
| BIR(domain) | Baculoviral IAP repeat (domain) |
| Bir | Baculoviral IAP repeat |
| BMP | Bone morphogenic protein |
| Boo | Bcl-2 homologue of ovary |
| BRUCE | BIR repeat containing ubiquitin-conjugating enzyme |
| CAD | Caspase activated DNase |
| CARD | Caspase (activation and) recruitment domain |
| CARDIAK | CARD-containing interleukin (IL)-1β converting enzyme (ICE) associated kinase |
| CART | C-rich domain associated with ring and traf |
| CASP | Caspase |
| CASPER | Caspase eight related |
| CAT | Chloramphenicol acetyl transferase |
| CEBPβ | CCAAT/enhancer binding protein |
| CED(1-9) | *C. elegans* death (protein)(1-9) |
| cDNA | Complementary DNA |
| c-FLIP | Cellular FLICE inhibitory protein |
| CHX | Cycloheximide |
| Cip1 | CDK interacting protein |
| CMV | Cytomegalovirus |
| CNS | Central nervous system |
| CRADD | CASP2 and RIPK1 domain containing adaptor with death domain |
| CrmA | Cytokine response modifier A |
| csp | Caspase |
| CSP | Caspase |
| CTAR | C-terminal activation region |

| | |
|---|---|
| dAPAF1 | Drosophila Apaf-1 |
| DARK | Drosophila Apaf1-related killer |
| DAXX | Fas death domain associated protein |
| DCP | Drosophila caspase |
| DcR | Decoy receptor |
| DD | Death domain |
| DDC theory | Duplication-degeneration-complementation theory |
| DED | Death effector domain |
| DENN | Differentially expressed in normal and neoplastic tissues |
| DEX | Dexamethasone |
| dFADD | Drosophila FADD |
| DFF | DNA fragmentation factor |
| DIABLO | Direct IAP binding protein with low pI |
| DIAP | Drosophila IAP |
| Dif | Dorsal-related immunity factor |
| DISC | Death inducing signalling complex |
| DIVA | Death inducer binding to vBcl-2 and Apaf-1 |
| DNA | Deoxyribonucleic acid |
| DNA-PK | DNA dependant protein kinase |
| DR | Death receptor |
| DREDD | Death related ced3/nedd2-like gene |
| drICE | Drosophila ICE |
| DRONC | Drosophila Nedd2-like caspase |
| dsODN | Double stranded oligodeoxynucleotide |
| dTRAF | Drosophila TRAF |
| EBV | Eptein Barr virus |
| Edar | Ectodysplasin A receptor |
| eIF4E | Eukaryotic initiation factor 4E |
| Epr-1 | Effector cell protease receptor 1 |
| EST | Expressed sequence tag |
| EMSA | Electrophoretic mobility shift assay |
| FADD | Fas associated death domain |
| FAK | Focal adhesion kinase |
| FasL | Fas ligand |
| Fas | Fas Antigen, Apo1, CD95 |
| FISH | Fluorescence *in situ* hybridization |
| FLICE | FADD like ICE |
| F/T | Freeze/thaw |
| GALT | Gut associated lymphoid tissue |
| GCKR | Germinal centre kinase related |
| GCMCC | German Collection of Micro-organisms and Cell Cultures |
| GEF | Guanine nucleotide exchange factor |
| GITR | Glucocorticoid-induced TNFR related gene |
| GR | Glucocorticoid receptor |
| GSK3β | Glycogen synthase kinase 3β |
| GSP | Gene specific primer |
| GTP | Guanosine triphosphate |
| GV | Granulosis virus |
| HAC1 | Homologue of Apaf-1 and ced-4 |
| HIAP1 | Human inhibitor of apoptosis –1 |
| HIAP2 | Human inhibitor of apoptosis –2 |

| | |
|---|---|
| HSP | Heat shock protein |
| HUGO | Human Genome Organization |
| IAP | Inhibitor of apoptosis |
| ICAD | Inhibitor of caspase activated DNase |
| ICE | Interleukin –1 converting enzyme |
| IFN | Interferon |
| IκB | Inhibitor of NF-κB |
| IKK | IκB kinase |
| IKKAP1 | IκB kinase associated protein-1 |
| IKK-i | Inducible IKK |
| IL-1R | Interleukin-1 receptor |
| ILK | Integrin linked kinase |
| IRES | Internal ribosome entry site/sequence |
| i-TRAF | TRAF inhibitor |
| JNK | c-jun N-terminal kinase |
| LARD | Lymphocyte associated receptor of death |
| LCL | Lymphoblastoid cell line |
| LMP1 | Latent (infection) membrane protein –1 |
| LTβ | Lymphotoxin β |
| MADD | Map-kinase activating death domain protein |
| MALT | Mucosa associate lymphoid tissue |
| MAP2K | MAP kinase kinase |
| MAP3K | MAP kinase kinase |
| MAPK | Mitogen activated protein kinase |
| MAPKK | MAP kinase kinase |
| MAPKKK | MAP kinase kinase kinase |
| MEK | MAPK/ERK kinase |
| MEKK | MAPK/ERK kinase kinase |
| MKK | MAP kinase kinase |
| MIAP | Mouse inhibitor of apoptosis |
| MNPV | (Multinucleocapsid) nucleopolyhedrosis virus |
| MOI | Multiplicity of infection |
| MYA | Million years ago |
| MZBCL | Marginal zone B-cell lymphomas/leukemias |
| NAIP | Neuronal apoptosis inhibitor protein |
| NEMO | NF-κB Essential Modulator |
| NF-IL6 | Nuclear factor (for) interleukin 6 |
| NF-κB | Nuclear factor kappa B |
| NIK | NF-κB inducing kinase |
| NK (cell) | Natural killer (cell) |
| NPV | Nucleopolyhedrosis virus |
| ROS | Reactive oxygen species |
| NRE | Negative regulatory element |
| NRF | NF-κB repressing factor |
| OCIF | Osteoclast inhibitory factor |
| OD | Optical density |
| O/N | Overnight |
| OPG | Osteoprotegrin |
| p90rsk1 | 90 kDa ribosomal S6 kinase-1 |
| PBMC | Peripheral blood mononuclear cells |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| PDGF | Platelet derived growth factor |
| PDK | PI3K dependent kinase |
| PDTC | Pyrrolidine dithiocarbamate |
| pfu | Plaque forming units |
| PH domain | Pleckstrin homology domain |
| PKB | Protein kinase B |
| PKR | Double-stranded-RNA-activated protein kinase |
| PI3K | Phosphatidylinositol 3-kinase |
| PT | Permeability transition |
| PTEN | Phosphatase and tensin homologue deleted on chromosome ten |
| PUR | Puromycin |
| RACE | Rapid amplification of cDNA ends |
| RAIDD | RIP associated ICH1/CED3 homologous protein with a death domain |
| RANK | Receptor activator of NF-κB |
| RICK | RIP-like interacting CLARP kinase |
| RING | Really interesting new gene |
| RIP | Receptor interacting protein |
| RNA | Ribonucleic acid |
| RPA | Ribonuclease protection assay |
| rpm | Revolutions per minute |
| RT | Room temperature |
| RTK | Receptor tyrosine kinase |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| RZF | RING (zinc) finger |
| SAPK | Stress activated protein kinase |
| SEK1 | SAPK and ERK kinase 1 |
| SHIP | SH2 containing inositol phosphatase |
| SMA | Spinal muscular atrophy |
| Smac | Second mitochondria-derived activator of caspase |
| SMN | Survival motor neuron |
| SODD | Silencer of death domains |
| ssODN | Single stranded oligodeoxynucleotide |
| TAB1 | TAK1 binding protein 1 |
| TAC1 | Transmembrane activator and CAML interactor |
| TAK1 | TGFβ activated kinase 1 |
| TANK | TRAF associated NF-κB activator |
| TBK1 | TANK binding kinase 1 |
| TES | Transforming effector site |
| TIAP | Thymus and testes IAP |
| TNFα | Tumour necrosis factor alpha |
| TNFRI | Tumour necrosis factor receptor I |
| TNFRII | Tumour necrosis factor receptor II |
| TNFRSF(1-16) | Tumour necrosis factor receptor superfamily member 1-16 |
| TPL-2 | Tumour progression locus 2 |
| TRADD | TNFRI associated death domain protein |
| TRAF | TNF receptor associated factor |
| TRAIL | TNF related apoptosis inducing ligand |
| TRAMP | TNF receptor related apoptosis mediating protein |
| TRANCE | TNF-related activation-induced cytokine |

| | |
|---|---|
| TRID | TRAIL receptor without an intracellular domain |
| TRIP | TRAF interacting protein |
| TRUNDD | TRAIL receptor with a truncated death domain |
| TRX | Thioredoxin |
| TUNEL | Terminal deoxynucleotidyl transferase (TdT) -mediated dUTP-biotin nick end labeling. |
| UTR | Untranslated region |
| UV | Ultraviolet |
| VEGF | Vascular endothelial cell growth factor |
| WAF1 | Wild-type p53-activated fragment 1 |
| XIAP | X-linked inhibitor of apoptosis |

## 8. Introduction

In contrast to uniquely unicellular organisms, growth and proliferation of individual cells within a multicellular organism must be tightly regulated. Though this may be for the greater good of the organism, it is often at the expense of an individual or group of cells. Although there is some evidence for the co-ordinated growth (and death) of unicellular organisms, it is generally thought that these control growth and division by sensing/evaluating conditions within their environment and reacting in consequence, irrespective of others within their community.[1]

From its beginning, a multicellular organism is constantly remodelling itself according to a pre-determined programme. During human embryogenesis, the loss of the embryonic tail and interdigital webs are probably the most dramatic examples of tissue remodelling. Most of these structures are thought to be vestiges of different evolutionary periods in our development as a species.[2] Tissue remodelling involves not only the controlled proliferation of certain subsets of cells, to create novel structures, but also the controlled removal of certain cells, in order to dismantle pre-existing ones. Occasionally, an organism creates redundant structures during foetal development only to eliminate the unnecessary ones after birth. Such is the case of motor neurons. During development, a two-fold excess of motor neurons are sent forth to innervate the developing musculature. Only those neurons that have successfully made neuro-muscular junctions survive. The remaining neurons are either actively eliminated or more likely die off due to a lack of pro-survival signals.[2], [3] In the mature human immune system, on the order of a billion ($10^8$) lymphocytes are actively culled from the circulation every day.[4] Consequently a similar number is regenerated in order to maintain tissue homeostasis.

From the above, it is obvious the aberrant control of cell death may contribute significantly to several pathologies. A pathological resistance to apoptosis can lead to several malignancies such as the proliferative skin disorder of psoriasis[5], to benign tumours, and ultimately to metastatic cancers, leukemias, and lymphomas(6). Conversely excessive apoptosis has been implicated in a number of pathologies including, among many others, eczema[7], insulin dependent diabetes mellitus, retinitis pigmentosa, Parkinson's, Huntington's, and Alzheimer's diseases[8], as well as spinal muscular atrophy[9].

Given the importance of programmed cell death in multicellular organisms, there is no doubt that the process requires strict control. To accomplish this task, there have evolved very sophisticated methods of both promoting and inhibiting apoptosis. In general terms apoptosis can be initiated in two ways. The first of these may be termed receptor mediated apoptosis. In this model, apoptosis is initiated when a membrane bound receptor binds its cognate ligand and initiates a signal transduction cascade, ultimately leading to the activation of a group of apoptotic

effector enzymes (caspases). A second method by which the cell may initiate apoptosis may be termed non-receptor mediated apoptosis. During times of stress, the cell may initiate an apoptotic cascade originating from the mitochondrion and ultimately leading to caspase activation. Non – specific apoptotic triggers include, among others, UV irradiation and subsequent DNA damage, heat shock, or exposure to genotoxic compounds or metabolic inhibitors.

Central to the execution of apoptosis are the caspases. In virtually all models of apoptosis activation of the caspases represent a point of no return, beyond which the cell can no longer reverse the apoptotic process and is destined to die[10]. Activation of the caspases can be inhibited at several points. The Bcl2 family of proteins modulate caspase activity either by preventing or promoting the mitochondrial permeability transition[11]. The Bcl2 family of proteins also regulate caspase activation at the level of the apoptosome, a complex composed minimally of APAF1, cytochrome C, and CASP9[12]. Wherease the Bcl2 family functions mainly within the mitochondrial pathway[13], the activity of select caspases, has recently been shown to be directly inhibitable by a family of proteins termed Inhibitors of Apoptosis (IAPs). The core group of mammalian IAPs is comprised of NAIP, XIAP, HIAP1, and HIAP2. All four possess three N-terminal Baculoviral IAP repeat (BIR) domains, while the latter three possess a C-terminal RING (zinc) finger (RZF) motif. Although these may represent the core members, several other single BIR and singleBIR+RZF proteins have been described, all of which possess some anti-caspase activity. Indeed, it is now apparent that the presence of a single BIR domain is sufficient to include a protein into the IAP family[14].

At the genomic level the organization of *hiap1*, *hiap2*, and *xiap* is quite remarkable. *Hiap1* and *hiap2* lie in tandem on chromosome 11(q23). An analysis of the exonic structure reveals that all three emerged as a result of whole gene duplication events at some point prior to the divergence of the mouse and human lineages. Soon after their initial discovery it was observed that *hiap1*, *hiap2*, and *xiap* possess unusually long 5'UTRs and that one member, *hiap1*, appeared to be under the transcriptional control of NF-κB. In the following work, the full genomic organization of *hiap1* and *hiap2* is described. We have also undertaken to study both the transcriptional and translational control of *hiap1* and *hiap2* gene expression. And finally, preliminary experiments on the effect of *hiap1* on TNFα mediated cytotoxicity were carried out in a *hiap1/hiap2* null vs wild type background.

### 8.1. Apoptosis in various model organisms

#### 8.1.1. Prokaryotes

The earliest and perhaps the most primitive form of programmed cell death occurs in sporulating bacteria such as *bacillus subtilis*. When environmental conditions are such that survival of the colony is in question, the cell undergoes asymmetric cell division whereby a long lived and impermeable spore is produced. When environmental conditions improve, this spore then germinates and its life cycle is then able to continue. During late stages of sporulation, co-ordinated expression of four transcription factors, $\sigma^E$ to $\sigma^K$, complete the process. Of these, $\sigma^K$ is expressed solely in the "mother cell" and ensures transcription of genes required to initiate programmed cell death.[15],[16],[17]

#### 8.1.2. Unicellular eukaryotes

One of the several differences between prokaryotes and eukaryotes is the presence within the cytoplasm of mitochondria. Mitochondria, complete with their own genome, are thought to be of bacterial origin. The endosymbiont hypothesis states that at some point in our evolutionary past oxidative bacteria routinely parasitized neighbouring anaerobic (glycolytic) unicellular organisms. A parasitic relationship then ensued with the pairing of the glycolytic pathway of the host organism with the oxidative one of the parasite. Over time, this relationship became more symbiotic with the sharing of resources between the two organisms. As this relationship stabilized, a majority of the mitochondrial (and chloroplast) genome was transferred to the nucleus. With its essential genes now found in the nucleus, the mitochondrion ceased to be an independent organism and became truly an organelle.[17],[18]

In the early stages of this relationship, its parasitic nature, may have been more evident. It has been suggested that apoptosis in multicellular organisms originated when the parasitizing bacteria, sensing a nutrient deficiency, induced cell death in the host organism thereby releasing itself into the extracellular space. This hypothesis is not without modern-day evidence. Some Bcl2 type proteins may modulate mitochondrial membrane permeability, and hence regulate the release of the pro-apoptotic protein cytochrome c, by interacting with adenine nucleotides: the ATP/ADP ratio being an efficient sensor of energy balance.[19]

Like some prokaryotes, unicellular eukaryotes also sporulate when environmental conditions are poor. Though unlike prokaryotes, sporulation involves the formation of a "pseudo-multicellular organism". When conditions dictate, individual cells come together and form a mast-like structure whose stalk is comprised of the relatively rigid remains of dead cells. Atop

this stalk one finds the spore forming cells. These cells then sporulate, releasing their progeny into the wind.[20]

### 8.1.3. Plants

Until recently it was thought that programmed cell death occurred only in eukaryotes such as *C. elegans*, invertebrates, and vertebrates. Conceptually, programmed cell death in plants is purposefully similar to that described in other eukaryotes. Apoptosis has been observed in vascular plants to occur in the development of various tissues including bark, leaves and the vascular tissues xylem and phloem as well as following viral infection. As a host response to viral infection it has the purpose of limiting the spread of viral progeny.[21] It has been shown that the hypersensitive response described in plants has many of the hallmarks of classical apoptosis found in other eukaryotes, such as cell shrinkage, membrane blebbing, chromosome condensation[21] and protease activation.[22],[23] It has also been shown that the proteases activated during the hypersensitive response are inhibitable by the same range of protease inhibitors active against the caspases.[22] Recently, a subfamily of caspases has been described in plants, yeast, fungi, and protozoa. The meta-caspases share with the caspases several structural features as well as the same active site.[24] It remains to be determined what the substrate specificity is of the metacaspases and if, in fact, they play any role in the hypersensitive response in plants. Other similarities between plant and metazoan apoptotic pathways include the observations that menadione or heat shock induced cell death is preceded by the release of cytochrome c from the mitochondria.[25],[26] That the same basic mechanism for initiating apoptosis can be found in both metazoans and plants is indicative of the antiquity of the process.

### 8.1.4. *Caenorhabditis elegans*

From the standpoint of apoptosis, *C. elegans* is likely the most primitive multicellular organism studied. *C. elegans* has the advantage of being both small and prolific. The nematode has been so thoroughly studied that the origin and fate of every cell from every stage of its life cycle is known. Although this may, at first, seem implausible, the task is made tractable by the fact that the adult possesses only 959 somatic cells. Another 131 cells are born but are destined to die at predetermined times. Given both the minimal size of the organism and the predictability of apoptosis, *C. elegans* is arguably the most extensively studied multicellular organism. Of the twelve genes genetically identified as being involved in *C. elegans* apoptosis, three have proven to be critical in initiating the execution stage.[27] Of these, CED3 (*C. elegans* death–3) has shown itself to be the *C. elegans* homologue of CASP9[28], whereas CED4 and CED9 are homologous to

Apaf1[29]and Bcl-$X_L$[30] respectively. While CED3, CED4, and CED9 define the core of the apoptotic process, the others appear to be involved in either the commitment, engulfment, or degradation stages.[27] Virtually all of the *ced* genes have mammalian counterparts; and, where appropriate, these will be pointed out in the following chapters.

### 8.1.5. Invertebrates

Another useful organism in which to study apoptosis is the common fruit fly, *Drosophila melanogaster*. As a model organism, the fly is perhaps more complex than is *C. elegans*, though unlike the nematode, *D. melanogaster* holds much of its signal transduction machinery in common with higher vertebrates. For example, although no TNF or FAS receptors have been identified in *C. elegans* or *D. melanogaster*, *Drosophila* homologues of their downstream signalling molecules have been described. Among others, these include dTRAF1[31], DIAP1 and DIAP2[32], dFADD[33], and DREDD (dCASP8).[34] As for the receptors, although no members of the TNF receptor family have been described, this may simply be due to a lack of adaptive immunity in this organism; a process with which the TNF receptors are intimately involved. This is supported by the finding that Toll-like receptors, a range of receptors associated with innate immunity and development in the fly, have been described in vertebrates[35],[36],[37] in much the same role.

### 8.1.6. Vertebrates

In higher vertebrates, such as mammals, regulation of apoptosis is at its most complex. While no novel regulatory or mechanistic theme has been described in vertebrates, the number of pathways by which apoptosis can be initiated, inhibited, or modulated is vastly increased over that found in invertebrates or the nematode. As such the level of fine tuning which can be achieved is that much greater. Whereas the Toll receptors in both invertebrates and vertebrates may impact significantly on innate immunity[35],[36],[37], the emergence of the TNF receptor superfamily in vertebrates has allowed for the development and regulation of many aspects of adaptive immunity. In the sections that follow, many of the molecules involved in the signal transduction pathways originating from the TNF receptor superfamily are described.

## 8.2. Protein mediators, regulators and effectors of the apoptotic process

### 8.2.1 TNF Receptor Superfamily

The Human Gene Nomenclature Database maintained by the Human Genome Organization (HUGO, http://www.gene.ucl.ac.uk/nomenclature) lists 23 entries for the TNF receptor

superfamily. The TNF receptor-1 (TNFRI) is the prototype receptor for the family which bears it name. All members of the receptor, save EBV-LMP1, are type-1 transmembrane proteins[38],[39] (cytoplasmic C-terminus, extracellular N-terminus, single pass through the plasma membrane) and possess a variable number of cysteine/histidine-rich motifs in their extracellular domain.[39] The receptors may be subdivided into two categories; those containing intracellular "death domains" and those which do not. Receptors which contain a death domain include TNFRI, FAS/CD95, the death receptors(DR) 4,5, and 6 as well as the decoy receptors (DcR)1 and 2. In the following discussion, the HUGO nomenclature assignment will be noted in the text after the first mention of the gene in question (e.g. TNF receptor superfamily member 1: TNFRSF1).

**FAS/CD95 and the Decoy Receptor-3 (DcR3)**

Fas/CD95 (TNFRSF6) is the membrane receptor for FasL. Upon receptor trimerization, CD95 recruits FADD to the receptor complex via homotypic binding of their respective death domains. FADD is then able to recruit and activate CASP8/FLICE.[40] Competitive inhibition of CASP8 activation can occur when c-FLIP/CASPER, an inactive CASP8 homologue, binds FADD in its place.[41],[42]

CD95 is thought to initiate the apoptosis observed during "activation induced cell death" (AICD) of $CD4^-$ T-cells. Under these circumstances exposure of CD4+ T-cells to IL-2 causes a transcriptional upregulation of FasL and a concomitant decrease in the signalling inhibitor c-FLIP.[43] Furthermore, the majority of somatic cells express the FAS receptor on their cell surface, making them susceptible to killing by activated NK cells expressing the FASL.[44]

Mitigating the effect of FasL on target tissues is the decoy receptor-3 (TNFRSF6B). DcR3 is a secreted FasL binding protein[45]. Of note, the gene encoding DcR3 is amplified[46] or overexpressed[47] in a number of malignancies and may serve as a mechanism by way the tumour escapes immune surveillance.[46]

**TNF receptor 1 (TNFRI) and the "death receptors", DR3 and DR6**

The TNF receptor-1 (TNFRI, CD120a, TNFRSF1A) is arguably the best characterized of all the receptors in the family. The cytoplasmic portion of TNFRI possesses a protein-protein interaction motif termed the "Death Domain".[39] It is this domain through which the receptor is able to bind the death domain of the adaptor molecule TRADD (TNF receptor associated death domain).[48] TRADD is a multifunctional protein and, in turn, can concurrently bind the protein kinase RIP (receptor interacting protein)[49] and the adaptors TRAF1 and TRAF2 (TNF receptor

associated factor).[50] This complex has also been demonstrated to recruit NIK. HIAP1 and HIAP2.[51]

TNFRI is known to activate a variety of transcription factors including c-Jun (via SAPK/JNK)[52], as well as NF-κB.[53] Which transcription factor is activated likely depends on the variety of molecules recruited to the signalling complex, although the common denominator is always TRAF2.

In a signalling cascade which may have a more immediate response, TNFRI may bind the adaptor protein FADD (Fas associated death domain). In so doing TNFRI can recruit and activate CASP8, thereby initiating an apoptotic protease cascade.[54]

Another receptor highly homologous to TNFRI has been described. Downstream signalling from DR3 (TRAMP, WSL-1, LARD, TNFRSF12) is similar in all respects to TNFRI. DR3 binds the same range of signalling and adaptor molecules and activates the same transcription factors.[55] Curiously, the tissue distribution of the receptors and their respective ligands appear to be mutually exclusive. Whereas DR3 is expressed predominantly in tissue of immune origin[56], TNFRI is ubiquitously expressed. On the other hand, their ligands, Apo3L and TNFα, are expressed in an opposite manner[53]. Given the similarity in their downstream signal transduction cascades, it is tempting to speculate that the two receptors evolved in parallel in order to prevent autocrine signalling whilst maintaining the downstream signalling pathways in both tissue types.

DR6 represents another TNFRI-like molecule. Studies on DR6 show that it binds TRADD and is able to signal to both NF-κB and JNK. Beyond that, very little is known about this latest member of the TNFR superfamily including what is its natural ligand.[57]

**TNF receptor 2 (TNFRII)**

In contrast to TNFRI, TNFRII (CD120b, TNFRSF1B) is thought to transduce an apoptotic signal only under some circumstances. The extent to which TNFRII may contribute to apoptosis may be by a process termed ligand passing.[58] This process involves the movement of TNFα from TNFRII to TNFRI where it then initiates a signal transduction cascade. The cytoplasmic domain of TNFRII does not contain a death domain and, as such, it cannot recruit either TRADD nor FADD. Consequently, neither RIP nor CASP9 can be recruited. The cytoplasmic domain, however, possesses an intrinsic capacity to recruit TRAF1 and TRAF2.[59] It is through these adaptor proteins by which TNFRII is able to recruit both HIAP1 and HIAP2 to the receptor complex.[60]

**Death Receptors-4 and -5, Decoy Receptors-1 and -2**

The cytotoxic TNF-like cytokine TRAIL/Apo2L[61],[62] (TNF-related apoptosis inducing ligand) was found to bind previously undescribed members of the TNFR superfamily.[63],[64] Both DR4 (Death receptor-4, TRAIL-RI, TNFRSF10A) and DR5 (TRAIL-RII, TNFRSF10B) contain cytoplasmic death domains; and it is through these that they are able to recruit FADD and, consequently, CASP8 to both homotrimeric and heterotrimeric receptor complexes.[65],[66]

Regulation of TRAIL signalling in various tissues appears to be at the level of competition for TRAIL binding. Two more TRAIL receptors have been identified. TRAIL-R3 (Decoy receptor-1/DcR1, TRAIL receptor without intracellular domain/TRID, TNFSF10C) is a membrane bound protein lacking little if any cytoplasmic domain.[67],[68] TRAIL-R4[69] (TNFSF10D), otherwise known as DcR2[70] or TRUNND[67] (TRAIL receptor with a truncated death domain), like DcR1 is able to bind TRAIL but is unable to transmit a death signal due to a truncated cytoplasmic tail. Unlike DR4 and DR5, the tissue distribution of DcR1 and DcR2 is limited. DcR1 appears to be predominantly expressed in peripheral blood lymphocytes (PBLs) and skeletal muscle[71] whereas DcR2 is abundantly expressed in foetal liver and adult testes.[70] It should also be noted that all four TRAIL receptors reside at 8p21-22, suggesting that they arose from gene duplication events.[70]

Finally, *in vitro* TRAIL induced apoptosis can be inhibited by the secreted protein osteoprotegrin/osteoclast inhibitory factor (OPG, OCIF, TNFSF11B). As such OPG represents another decoy receptor for TRAIL.[72] Binding by TRAIL of OPG, though, prevents the latter from binding yet another TNF-like molecule, TRANCE[73],[74] (TNF related activation induced cytokine). TRANCE is expressed on the surface of activated T-cells, dendritic cells and osteoblasts. Surface expression of TRANCE allows it to bind its receptor, RANK (TNFSF11A) on the surface of macrophages allowing it to differentiate into an osteoclast (bone resorbing cell). Secreted OPG also competes with RANK for TRANCE binding thereby inhibiting the formation of the multinucleated osteoclasts and hence bone resorption in affected tissues. In binding OPG, TRAIL is able to inhibit the anti-osteoclastogenic activity of OPG.[75]

**EBV-LMP1**

Epstein Barr Virus (EBV) infection causes a rapid proliferation and transformation of peripheral B-cells *in vitro*. This phenomenon has been attributed, in part, to the expression of a virally encoded integral membrane protein (Latent membrane protein-1, LMP-1). LMP1 is a type-IV transmembrane protein (both N and C termini are cytoplasmic with multiple passes through the plasma membrane)[38] and is thought to constitutively mimic TNFR superfamily

signal transduction cascades[76], leading to an increase in NF-κB activity.[77] Inclusion into the TNFR superfamily is by virtue of its ability to engage a similar range of adaptor and regulatory proteins, such as TRADD and TRAF1/2. The net outcome of LMP1 expression is the activation of NF-κB (as well as JNK) and subsequent prevention of programmed cell death as a response to viral infection.

LMP1 is able to signal to NF-κB via two Transforming effector sites (TES) or C terminal activation regions (CTAR). TES1/CTAR1 has been demonstrated to mediate short term NF-kB activation and is responsible for the initial proliferation of B-cells following infection.[78] This site is similar to an activated TNFRII in that it is able to directly bind various members of the TRAF family.[79] The second domain mediates longer term NF-κB (and JNK)[80],[81] activation and outgrowth of infected B-cell.[78] In contrast to TES1/CTAR1, TES2/CTAR2 binds TRAF proteins only through the intermediacy of the TRADD protein.[82] Furthermore, the signalling proteins RIP[82] and NIK[83] have been associated with LMP1 signalling. As such TES2/CTAR2 mimics an activated TNFRI. In an observation which may be applicable to TNFRI and TNFRII, it would appear that although JNK activation is dependent on TRAF2, it is only activated via TES2/CTAR2[81]. Also in analogy to TNFRI, signalling via LMP1 can be abrogated by A20[84], TANK[85], and mutant TRAF2.[85]

**Other TNF receptor family members**

Although the preceding discussion highlights some of the more prominent members of the family, several more have been characterized. Some, such and the CD27, CD30 and CD40 receptors, have been extensively studied. These receptors are primarily involved in lymphocyte maturation and activation pathways[39]. Less well understood are the receptors OX40, 4-1BB, and LTβ. These receptors do not possess a death domain and as such are more like TNFRII in both structure and downstream signalling events.[39] Other TNFR family members which have more recently been described include TAC1[86] (Transmembrane activator and CAML interactor), ATAR[87] (Another TRAF associated receptor), BCMA[88] (B-cell maturation antigen), AITR[89] (Activation inducible TNF receptor family member), GITR[90] (Glucocorticoid-induced TNFR related gene), Edar[91] (Ectodysplasin A receptor) and finally TNFRSF19[92] (TNF receptor superfamily member 19). Like TNFRII, most, if not all, of these receptors bind various TRAF family members, which in turn serve to modulate the activity of NF-κB (Table1).

### 8.2.2. Adaptors

**TNF Receptor Associated Factors (TRAFs)**

The TRAF proteins are a family of six adaptor molecules found in association with a variety of receptors including, but not limited to, the TNF receptor superfamily (Table 1). In binding the receptor, the TRAF molecules likely function to recruit a set of signalling molecules unique to the receptor to which they are bound. TRAF homologues have been characterized in *C. elegans*[93] and in *D. melanogaster*[31]. In higher vertebrates, TRAFs appear to associate with the death-domain containing receptors CD95 and TNFRI via the adaptor protein TRADD (see below) whereas they bind the non death-domain receptors directly. The first TRAF molecules to be identified (TRAF1 and TRAF2) were found in association with the 75kDa TNF receptor (TNFRII).[59] Since then, four more family members have been identified as well as several TRAF inhibitors. TRAF family members are characterized by the TRAF domain: a 230 amino acid domain involved in receptor binding and oligomerization. The TRAF domain can be subdivided into two subdomains. The c-terminal 150 amino acids comprise the highly conserved TRAF-C subdomain whereas the remainder of the TRAF domain forms a coiled coil motif termed the TRAF-N subdomain. The TRAF-N subdomain of TRAF1 and TRAF2 differs from that found in the other TRAF members, and it is this domain to which HIAP1 and HIAP2 bind. The TRAF-N subdomain of TRAF1 and TRAF2 is also able to concurrently bind the TNFRI adaptor protein TRADD, and, in so doing, recruit HIAP1 and HIAP2 to this receptor.[50] TRAF2 also appears to recruit the IKK complex to the receptor.[94]

The TRAF proteins also possess a cluster of zinc fingers forming the CART domain and all but TRAF1 contain an N-terminal RING finger domain.[59],[95],[96],[97],[98],[99],[100],[101] Although very similar in structure, only TRAFs 2, 5, and 6 appear to promote the activation of NF-κB via the amino terminus whereas TRAFs 1 and 3 are generally seen as inhibitory(Table 1).[102],[103]

The requirement of an intact RING finger for NF-κB activation is exemplified by TRAF2a and TRIP (TRAF interacting protein). TRAF2a possesses an inactivating 7 amino acid insert within the RING domain generated by alternative splicing, whereas TRIP possesses an extended RING domain. Association of either molecule to TNFRII inhibits activation of NF-κB.[104],[105] Other molecules which modulate TRAF signal transduction by binding it include I-TRAF/TANK[106],[107], the TNFα inducible signalling inhibitor A20[108],[109], and the CARD containing protein Bcl10.[110]

**Table 1: Receptors to which TRAF proteins have been associated.** When available the receptors are listed numerically according to their HUGO assignment (TNFRSF#, Tumor Necrosis Factor Receptor Superfamily #). When known, the ability of a particular TRAF family member to promote or inhibit the activation of is indicated.

| HUGO assignment | CD assignment | Common name(s) | Associated TRAFs | NF-κB activation | Reference |
|---|---|---|---|---|---|
| TNFRSF1A | CD120a | TNFRI | TRAF1 (via TRADD) | | 50 |
| | | | TRAF2 (via TRADD) | | 50 |
| TNFRSF1B | CD120b | TNFRII | TRAF1 (indirect) | | 59, 111 |
| | | | TRAF2 | promotes | 59, 111 |
| | | | TRAF3 | | 112 |
| TNFRSF3 | unassigned | LT-βR | TRAF3 | | 113 |
| | | | TRAF5 | promotes | 95 |
| TNFRSF4 | CD134 | OX40 | TRAF2 | promotes | 114, 115 |
| | | | TRAF3 | inhibits | 114, 115, 116 |
| | | | TRAF5 | promotes | 114 |
| TNFRSF5 | CD40 | | TRAF1 | | 117 |
| | | | TRAF2 | promotes | 117, 118, 111 |
| | | | TRAF3 | | 117, 118, 96 |
| | | | TRAF5 (indirect) | promotes | 97, 118 |
| | | | TRAF6 (indirect) | promotes | 117, 118, 98 |
| TNFRSF6 | CD95 | Fas, Apo-1 | none | | |
| TNFRSF7 | CD27 | | TRAF2 | promotes | 119, 120 |
| | | | TRAF5 | promotes | 120 |
| TNFRSF8 | CD30 | | TRAF1 | promotes | 112, 121 |
| | | | TRAF2 | promotes | 112, 121, 122 |
| | | | TRAF3 | inhibits | 112, 121 |
| | | | TRAF5 | promotes | 122 |
| TNFRSF9 | CD137 | 4-1BB | TRAF1 | | 115, 123, 124 |
| | | | TRAF2 | promotes | 115, 123, 124 |
| | | | TRAF3 | | 124 |

**Table 1 (cont'd): Receptors to which TRAF proteins have been associated.**

| HUGO assignment | CD assignment | Common name(s) | Associated TRAFs | NF-kB activation | Reference |
|---|---|---|---|---|---|
| TNFRSF11A | unassigned | RANK | TRAF1 | | 125, 126 |
| | | | TRAF2 | | 125, 126, 127 |
| | | | TRAF3 | | 125, 126 |
| | | | TRAF5 | | 125, 126, 127 |
| | | | TRAF6 | promotes | 125, 126, 127 |
| TNFRSF14 | | ATAR, LIGHTR | TRAF2 | | 87 |
| | | | TRAF5 | promotes | 87 |
| TNFRSF16 | NA | p75(NTR) | TRAF2 | promotes | 128 |
| | | | TRAF4 | inhibits | 128 |
| | | | TRAF6 | promotes | 128 |
| TNFRSF17 | | BCMA | TRAF1 | | 129 |
| | | | TRAF2 | promotes | 129 |
| | | | TRAF3 | | 129 |
| NA | CD121 | IL-1R | TRAF6 | promotes | 130 |
| NA | unassigned | IL-17R | TRAF6 | promotes | 131 |
| NA | unassigned | IL-18R | TRAF6 | promotes | 132 |
| unassigned | NA | EBV-LMP1 | TRAF1 | promotes | 69, 123, 124 |
| | | | TRAF2 | promotes | 69, 123, 124 |
| | | | TRAF3 | inhibits | 69, 89, 123, 124 |

As originally described, the TRAF/TANK heterodimer may inhibit receptor mediated TRAF signaling.[106] Conversely, it may contribute to both NF-κB and SAPK/JNK signalling by forming alternative signalling complexes. TRAF2/TANK is able to bind and activate GCKR (germinal centre kinase related) leading to JNK activation.[135] Alternatively the TRAF/TANK complex may bind/activate TBK1/IKK-i. Activated TBK1 is then able to promote the phosphorylation and subsequent degradation of IκBα.[136] Activated TBK1 also phosphorylates I-TRAF/TANK thereby releasing TRAF2 from the complex to perhaps rejoin the receptor.[137]

At physiological concentrations, binding of HIAP1-TRAF2 complexes by Bcl10 promotes the activation of NF-κB.[110] In binding the HIAP1/TRAF2 complex, Blc10 also recruits the paracaspase, MALT1, to the complex.[24] What role, if any, this plays in NF-κB signalling remains to be determined.

**TNF Receptor Associated Death Domain (TRADD)**

TRADD is a multifunctional adaptor protein[48] capable of concurrently binding the TNFRI, FADD and RIP via its C-terminal death domain. It is also able to bind the TRAF-C domains of TRAF1 and TRAF2[51] via its N-terminal TRADD domain (N-TRADD).[138] Notably TRADD is unable to bind other members of the TRAF family of adaptors to any great extent.[50] TRAF1 and TRAF2 are also the other family members to which bind HIAP1 and HIAP2. Through its interactions with FADD and RIP, TRADD is able to recruit CASP1, CASP2 and CASP8 to the receptor complex (see FADD and RIP for details)

**Fas Associated Death Domain (FADD)**

FADD is associated with the death receptors CD95 and TNFRI. Its direct association with CD95 occurs via homotypic interactions of their respective death domains.[139],[140] Interactions with the TNFRI are mediated by its interaction with the death domain of the TRADD adaptor molecule[54]. Regardless of the receptor with which FADD is found, its role is to recruit CASP8 and/or CASP10 to the DISC (death inducing signalling complex) via their N-terminal death effector domain (DED). [40],[141],[142],[143]

**RAIDD/CRADD (RIP associated ICH1/CED3 homologous protein with a death domain/CASP2 and RIPK1 domain containing adaptor with death domain)**

RAIDD/CRADD was originally identified as an EST (expressed sequence tag) with significant homology to caspase-2. RAIDD was found to be a bipartite adaptor capable of binding both RIP (see below) via its C-terminal "death domain" as well as caspase-2 via its N-

terminal CARD[144],[145] (caspase recruitment domain). As such RAIDD. may recruit CASP2 to any receptor to which RIP is bound.

**MADD/DENN[146],[147](Map-kinase activating death domain protein/Differentially expressed in normal and neoplastic tissues)**

Like the previously described death domain containing protein, TRADD, MADD is able to bind the death domain of the TNFRI, but not it seems that of CD95. MADD is also able to bind the death domain of TRADD, although the significance of this in unclear, and may simply be due to the similarity of their respective death domains. MADD also possesses a leucine zipper motif which may function to recruit downstream signalling molecules.[146] MADD differs from other death domain adaptors in that it conceivably harbours some enzymatic activity. MADD shares significant homology to the rat guanine nucleotide exchange factor (GEF), Rab3.[148],[149] As such. MADD may directly influence the activity of an as yet unidentified monomeric G-protein and thereby mediate the observed induction of phospholipase A2 and the MAP kinase pathways.

**Silencer of death domain (SODD)**

SODD was identified as a DR3 interacting protein in a yeast two hybrid screen and an hsp70/hsc70 interacting protein. BAG-4. It was subsequently shown that SODD binds both DR3 and TNFRI via an N-terminal death domain to the exclusion of TRADD. Beyond its ability to compete with TRADD for binding of the TNFRI and DR3 receptors, SODD appears to have no secondary function. It has been shown that binding of TNFα by the TNFRI displaces SODD thereby allowing TRADD binding. Thus it appears that SODD functions to prevent aberrant signal transduction from occurring from the unbound receptor.[150] The significance of SODD binding to the molecular chaperones is not known.

**DAXX**

DAXX is a CD95 binding protein and like FADD is able to promote apoptosis from the FAS receptor. Unlike FADD, though, DAXX accomplishes this without the primary activation of CASP8 (FLICE) rather it relies mainly on the activation of the JNK pathway.[151] This is accomplished via the activation of ASK1 (Apoptosis signal-regulating kinase 1) and subsequent activation of downstream signalling pathways.[152] DAXX mediated apoptosis, but not that mediated by the FADD-CASP8 pathway, can be inhibited by the binding of DAXX to HSP27.[153]

Presumably this binding prevents its association to CD95 and hence prevents the DAXX dependent activation of ASK1.

### 8.2.3. Kinases

**Receptor Interacting Protein (RIP)**

RIP was originally characterized as a component of the FAS/CD95 receptor complex.[154] RIP was later shown to bind the TNFRI complex via TRADD and was, in fact, a serine/threonine kinase.[49] This protein is thought to bind CD95 and TRADD via mutual death domains.[154] RIP may also be recruited to either TNFRI or TNFRII by association with TRAF2.[51] Overexpression of RIP has been shown to promote apoptosis[49] as well as NF-κB activation.[155] It has also been shown that in RIP null mice, T-cells are more sensitive to TNFα induced cytotoxicity and fail to activate NF-κB, indicating a necessary role for this kinase in this context.[156] The activation of NF-κB by RIP may proceed via two pathways. The first of these involves the recruitment of NIK to the receptor complex. NIK then in turn activates, via phosphorylation, the IKK complex. Alternatively RIP may recruit IKK to the receptor complex via an association with TRAF2.[94] In contrast to this, signalling to NF-κB from the TNFRII only occurs in the absence of RIP. When present, RIP suppresses NF-κB activation and promotes apoptosis.[157]

Under circumstances where apoptosis is to be initiated, RIP may be cleaved by CASP8 (FLICE). The RIP cleavage product is unable to signal to NF-κB and instead promotes the interaction of TRADD to FADD which in turn is able to recruit more proCASP8 to the receptor complex.[158]

Although the initial cleavage of RIP may be due to minimal constitutive CASP8 activity at the receptor complex, other proteins in the same complex may promote the event. RIP2[159] (Rick[160], CARDIAK[161]) is homologous to RIP in that it possesses a homologous kinase domain although its C-terminal domain contains a caspase (activation and) recruitment domain (CARD) instead of the death domain found in RIP. Through this domain, RIP2 is able to recruit CASP1 to the receptor complex.[159],[161] Alternatively, RIP is able, through its interaction with RAIDD (described above), to recruit CASP2 to the receptor complex.[145]

RIP3 possesses the same kinase domain as does the other family members although its C-terminal is unique and is used co-localize with RIP. Unlike the other family members, RIP3 appears to suppress NF-κB activation following ligation of TNFRI by TNFα and, like RIP2, is able to recruit caspases to the receptor complex. [162],[163],[164]

**NF-κB Inducing Kinase (NIK)**

NIK was first cloned as a TRAF2 interacting protein by yeast two-hybrid screening. Sequence analysis of the NIK revealed that it shares significant homology to the MAP3K family of kinases.[165] Further screening for NIK interacting proteins revealed that NIK complexes with[166] and phosphorylates IKKα at S176.[167] IKKα, in turn, directs the degradation of the direct inhibitors of NF-κB, IkBα/β/γ, in conjunction with an activated IKKβ.[166],[168]

In the context of the IL-1 receptor NIK can be phosphorylated/activated by another MAP3K family member, TAK1.[169] This kinase was originally described in the TGFβ signalling complex where it could promote the activation of NF-κB independently of NIK.[170] Furthermore, NIK can be activated upon viral infection, independent of receptor complexes, by the double-stranded-RNA-activated protein kinase, PKR.[171]

Although NIK was the first IKK kinase identified, it has since been shown that MAP3K1(MEKK1)[172],[173],[174],[175], MEKK2, and MEKK3[176] can functionally substitute for NIK under some circumstances.

**Apoptosis signal-regulating kinase (ASK1)**

ASK1 is a member of the MAP3K family of signal transduction kinases and signals to both SAPK/JNK and p38 via the MAP2Ks, SEK1(MKK4) and MKK3/MKK6 respectively.[177] Activation of ASK1 can be mediated through association with G-protein coupled receptors[178], DAXX[152], as well as through association with TRAFs 2, 5, and 6.[179] Whereas ASK1 is able to promote apoptosis by signalling to SAPK/JNK and p38, it has also been implicated in the negative regulation of NF-κB activation from the IL-1 receptor.[180] Binding of the IL-1R causes an association between TRAF6, TAK1, and NIK leading to the activation of IκB kinase by the latter. ASK1 antagonizes this signalling pathway by disrupting the TRAF6-TAK1 complex. The activity of ASK1 is negatively regulated by its association with the redox sensitive protein thioredoxin (Trx).[181] TNFα signalling through TRAF2 causes a transient increase in ROS within the cell which in turn causes a dissociation of ASK1 from Trx. Release of ASK1 allows it to bind to and transduce a signal from TRAF2.[181] ASK1 mediated signal transduction can also be inhibited by the binding of the phosphorylated kinase to the 14-3-3 proteins[182] or the cell cycle regulator p21(Cip1/WAF1).[183]

**Phosphatydylinositol-3-kinase (PI3K)**[184]

Signals emanating from the various receptors found at the plasma membrane can impinge on a number of different signalling pathways. One of these involves phosphatidylinositol–3 kinase (PI3K). PI3Ks can be subdivided into three groups. Class I members are heterodimeric proteins formed by the association of a wortmannin sensitive[185] 110 kDa catalytic subunit as well as one of several smaller regulatory subunits ranging in size from 50 kDa to 101 kDa. Class I may be further subdivided into two subclasses: class Ia is comprised of three catalytic members (p110α/β/δ) whereas p110γ is the only known member of class Ib. Class II members are approximately twice the size of class I members and do not heterodimerize with a regulatory subunit. Finally class III PI3Ks, like class I enzymes, possess a regulatory subunit.

Of all the PI3Ks, only those comprising Class I appear be important with regard to anti-apoptotic signaling.[184] Members of the class Ia kinases are activated upon binding to phosphotyrosine residues on activated receptor tyrosine kinases (RTKs), such as those for PDGF[186] and VEGF.[187] Class Iβ kinases, on the other hand, bind G-protein coupled receptors, such as those for several peptide hormones, neurotransmitters, odorants, and light.[184] Upon receptor stimulation, PI3K converts phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) to phosphatidylinositol (3,4,5) triphosphate (PI(3,4,5)P3) at the plasma membrane. PIP3 then directs the localization of PIP3 dependant kinase-1 (PDK-1) and PDK-2 to the plasma membrane via their respective pleckstrin homology (PH) domains (PIP3 binding domains). Also recruited to the membrane via its PH domain is protein kinase B (PKB), otherwise known as Akt. The close association of these kinases allows for the phosphorylation of PKB by PDK-1 at Ser308 and by an as yet unidentified PDK-2 at Ser473.[184]

The action of PI3K is antagonized by the phosphatase activities of both PTEN (phosphatase and tensin homolog deleted on chromosome 10) and $p150^{SHIP}$ (SH2 containing inositol phosphatase). PTEN was originally identified as a tumour suppressor gene. PTEN is a dual specificity enzyme in that it is both a protein tyrosine phosphatase and a lipid phosphatase. PTEN is able to promote anoikis (apoptosis arising from the loss of adhesion to the extracellular matrix or neighbouring cells) in metastatic cells by dephosphorylating and, hence, deactivating FAK (focal adhesion kinase), a kinase involved in integrin signaling.[188] In the absence of PTEN, FAK and its downstream signal transduction cascade, which involves the activation of PI3K, remain active, irrespective of cellular adhesion.[189] PTEN also acts in direct opposition to PI3K by removing the D3 phosphate from PI(3,4,5)P3, thereby regenerating PI(4,5)P2. At this level, loss of PTEN results in the sustained elevation of PIP3 subsequent to signal induced activation of

PI3K. For its part, SHIP removes the D5 phosphate generating PI(3,4)P2. Although PKB is able to bind PI(3,4)P2, it does so with reduced affinity (Figure 1).[184]

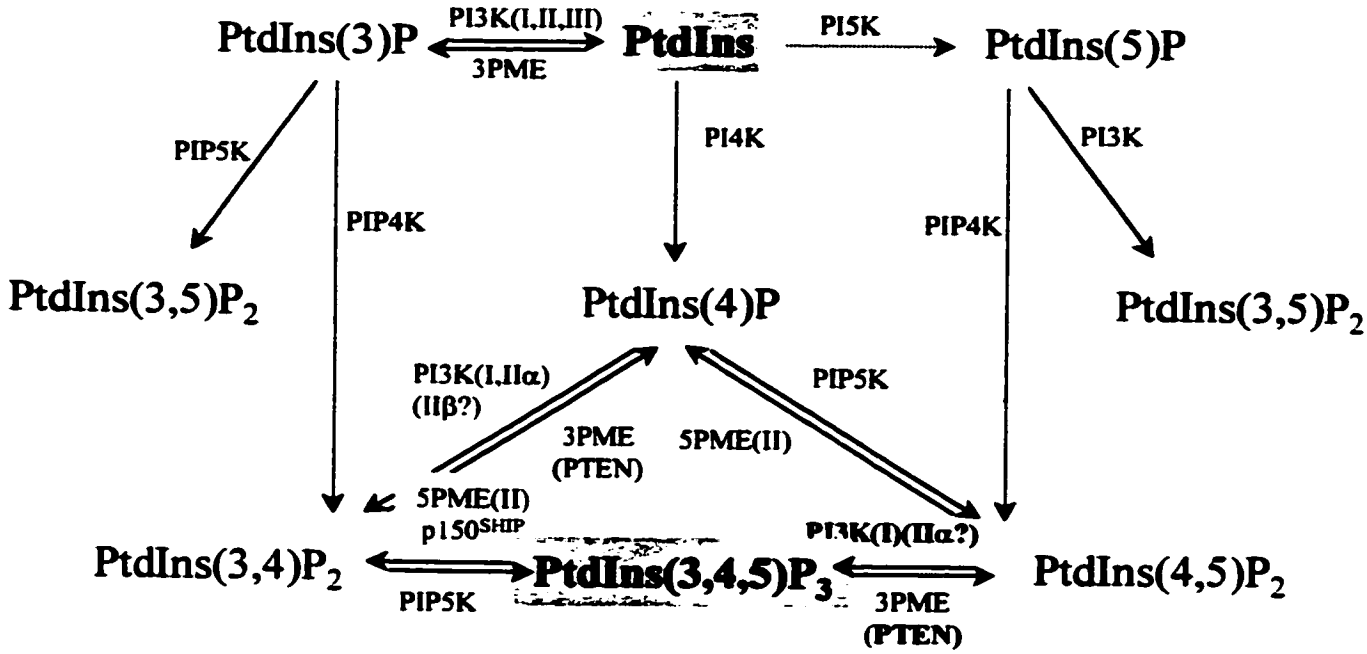
**Inhibitor of κB kinases (IKK)**

The phosphorylation and subsequent degradation of the NF-κB repressors, the IκB family (discussed in the section on NF-κB), is mediated by two closely related[190] kinases, IKKα[166] (IκB kinase α) and IKKβ.[191] The IκB kinases are found in high molecular weight (700 kDa) signalling complexes which include NIK and the scaffold protein NEMO[192]/IKKγ[193]/IKKAP1[194] (NF-κB Essential Modulator, IκB kinase γ, IκB kinase associated protein-1). Reconstituted IKKβ dimers preferentially phosphorylate IκBα, followed by IκBβ1, IκBε and IκBβ2.[195] Although the IKK complex is able to phosphorylate (IκB)/p105[196] and (IκB)/p100[197], the relative efficiency of this reaction is unknown.

Signalling through the IKK complex is thought to be initiated by one of the MAP3K family members such as NIK(Ser176)[167], MEKK1[172],[173],[174],[175], MEKK2, MEKK3[176], MLK3[198], TAK1[170], as well as an IKK related kinase NAK[199] (NF-κB activating kinase), PKCθ[200], and casein kinase II.[75] The HTLV1 (human T-cell leukemia virus-1) encoded protein Tax activates NF-κB in infected T-cells by first binding to IKKγ[201] and subsequently to IKKα/β[201],[202],[203] thereby possibly promoting/stabilizing the interaction of NIK with IKKα/β.[56],[204] The double stranded RNA-dependant protein kinase, PKR, has also been shown to influence NF-κB activity via the IKK complex[205], independently of its kinase activity[206], and thus may contribute to the cellular response to viral infection. In order to prevent continued degradation of IκB, in the absence of upstream signalling events, autophosphorylation of the carboxy-terminal serine cluster serves to decrease IKK activity.[207] In the case of TNFRI mediated activation of NF-κB, the IKK complex may be recruited directly to the receptor via its interaction with TRAF2[94], A20 or RIP.[208]

In addition to its IκB kinase activity, it has emerged that the IKK complex also serves to phosphorylate Ser536 in the transactivation domain of NF-κB/p65[209], thereby increasing its transactivation potential. Recently, this activity has been demonstrated to proceed via protein kinase B (PKB/Akt).[210] It has also been shown that PKB mediated phosphorylation of IKKα, at T23 rather than the serine targets of the MAP3Ks, is critical for TNFα mediated NF-κB activation some cell types[211], but not others.[212] Signalling from G-protein coupled receptors[213] (GPCRs) and receptor tyrosine kinases[212] (RTKs) have likewise been shown to activate NF-κB via PKB/Akt. As such, it would appear that serine phosphorylation commits the IKK complex to

**Figure 1: Phosphatidylinositol metabolism.** Pathways leading from phosphatidylinositol (PtdIns) to the generation of PtdIns(3,4,5) triphosphate (PtdIns(3,4,5)$P_3$) (shaded in grey). The major activities of phosphatidylinositol 3-kinase (PI3K) and PTEN are indicated in bold.


PtdIns(3)P
PI3K(I,II,III)
3PME
PtdIns
PI5K
PtdIns(5)P
PIP5K
PIP4K
PI4K
PIP4K
PI3K
PtdIns(3,5)P2
PtdIns(4)P
PtdIns(3,5)P2
PI3K(I,IIα)
(IIβ?)
3PME
(PTEN)
5PME(II)
PIP5K
5PME(II)
p150SHIP
PI3K(I)(IIα?)
PtdIns(3,4)P2
PtdIns(3,4,5)P3
PtdIns(4,5)P2
PIP5K
3PME
(PTEN)

IκB phosphorylation and subsequent degradation, whereas threonine phosphorylation of IKK serves to augment its NF-κB kinase activity.

Gene knockout studies of both kinases individually have demonstrated that the loss of IKKα is perinatally lethal[214]. Interestingly, NF-κB activation by pro-inflammatory cytokines in cells derived from these mice is maintained[215] albeit at somewhat reduced levels.[214] Although under normal physiological conditions IKKα mediates IKKβ activation[200], IKKβ homodimerization obviously compensates for the lack of IKKα in this model. The skin abnormalities seen in the IKKα knockout are thought to be due to the elimination of an IKKα specific, NF-κB independent signalling pathway. The IKKβ knockout, though, is embryonic lethal, displays marked inhibition of NF-κB activation and severe liver degeneration[216],[217], a phenotype reminiscent of some NF-κB knockouts.[216]

Another IKK-like protein is represented by the cytokine inducible kinase IKK-i. IKK-i binds to TANK/TRAF2 complexes and phosphorylates TANK in its TRAF2 binding domain. It is thought that this releases TRAF2 from the inhibitory effects of TANK and allows it to rejoin the TNFR signalling complex. Furthermore, IKK-i was shown to directly phosphorylate Ser36 of IκBα and promote that of Ser32.[136],[218]

**Protein kinase B/Akt (PKB)**

Protein kinase B (PKB/Akt) is a serine/threonine kinase involved in transducing anti-apoptotic signals from a variety of receptors, including the TNFRI[211], receptor tyrosine kinases such as VEGF-R[219] and PDGF-R[212],[220], G-protein coupled receptors[221], as well as integrins.[222] Three human homologues of PKB have been identified thusfar (Akt1-3) and appear to be regulated in a similar fashion.[223],[224] Activation of PKB occurs at the plasma membrane. Binding of PI3K generated PIP3 via its PH domain (pleckstrin homology) brings it in close proximity to the similarly localized kinases PDK-1 and PDK-2. Phosphorylation of PKB at both Ser308 and Ser473 are necessary events for full PKB activation. Phosphorylation at Ser308 is generally mediated by PDK-1, whereas Ser473 appears to be phosphorylated by a number of kinases.[184] It may also be that rather than distinct kinases, PDK-1 and PDK-2 may represent activities and that a variety of enzymes may rightfully be described as PDKs. Under certain circumstances, CaM-KK (Ca+/calmodulin-dependant protein kinase) can phosphorylate PKB at position 308[225] and ILK1 (integrin linked kinase) can perform the same task at Ser473.[222] It has

also been shown that PKB itself can phosphorylate the PDK-2 site[226] perhaps following signal induced oligomerization of PKB at the plasma membrane.[227]

Once activated, PKB has been demonstrated to have a number of cytoplasmic targets. Phospho-inactivation of the Forkhead family of transcription factors, represented by FKHRL1, has been postulated to reduce the expression of the FAS ligand (CD95L)[228],[229], and in so doing may reduce AICD (antigen/activation induced cell death) of T-cells. Phosphorylation of the Bcl2 homologue, BAD, displaces it from Bcl-$X_L$ and Bcl2 heterodimers thereby allowing for their anti-apoptotic homodimerization[230]. Under the direction of the insulin receptor, PKB also phospho-inactivates GSK3β[231] (glycogen synthase kinase 3) thereby promoting the translocation of the glucose transporter GLUT4 to the plasma membrane[232] as well as the activation of the βcatenin-Lef/Tcf signal transduction cascade.[233] PKB is also involved in the regulation of translation by releasing the eukaryotic initiation factor 4E (eIF4E) from its inhibitory binding proteins (4E-BPs).[234],[235]

Yet another target of PKB is the IκB kinase complex. Threonine phosphorylation of IKKα by PKB has been demonstrated to be necessary for the activation of NF-κB by a number of stimuli.[211],[212] In the end, of all the targets of PKB, it may be the activation of IKKα which may prove to have the most important impact on cell survival. Negative regulation of PKB may be at the level of proteolysis. It has been proposed by several groups that the CASP3 dependent cleavage of PKB allows apoptosis to proceed unencumbered by the anti-apoptotic products of PKB signaling.[236],[237],[238]

### 8.2.4. Bcl2 family of proteins and the permeability transition pore[11],[13],[12]

The anti-apoptotic protein Bcl2 was first identified as a potential oncogene by virtue of being translocated to the IgH locus on chromosome 14, t(14;18) (q32;q21), in a number of acute B-cell lymphomas.[239] Bcl2 now represents the archetype of an ever growing family of apoptotic regulators. The family is comprised of both pro-apoptotic and anti-apoptotic members. It is commonly thought that the ratio between individual family members may be critical in determining the fate of a given cell subsequent to a specific insult.[12] Within Bcl2 are four homology domains (BH1-4) as well as a C-terminal transmembrane domain. The latter is thought to be important in localizing various family members to the appropriate intracellular membranes, principally the mitochondria, endoplasmic reticulum, and the nuclear envelope. The BH domains mediate homo- and hetero-dimerization of the various members.

Several Bcl2 family members have been shown to form selective ion channels/pores in artificial membranes. It is thought that perhaps hetero-oligomerization of pre-existing pores with the pro-apoptotic members may disturb these in such a way as to affect the permeability of the membrane to a variety of solutes within the inter-membrane space. Alternatively, *de novo* formation of pores composed of pro-apoptotic members, such as BAX or truncated BID (tBID), may as well alter the permeability of the membrane to these solutes. The latter, tBID, being the product of proteolytic processing by activated CASP8. The various Bcl2 family members may also affect the permeability of a "conditional" pore. The permeability transition (PT) is a phenomenon associated with apoptosis whereby the transmembrane potential ($\Delta\Psi m$) of the inner membrane collapses. This collapse has been attributed to the formation of a large, multicomponent structure termed the permeability transition pore. Although the pore has only loosely been defined, its constituents include both inner and outer membrane components.[11],[240] It is thought that the PT pore may allow for the release of pro-apoptotic molecules from the inter-membrane space in either of two ways. First, the pore might directly mediate their transfer. A second more catastrophic mechanism involves the swelling of the "inner mitochondria" due to its inherent hypo-osmolarity and larger surface area (cristae). Eventually the expansion of the "inner mitochondria" causes the rupture of the outer mitochondrial membrane and release of the inter-membrane constituents. Bcl2 has been shown to inhibit the collapse the inner transmembrane potential whereas BAX appears to promote it. It may be that rather than forming pores themselves, they may contribute to the (ion) selectivity of the PT pore.[11],[13],[240]

Another role for the Bcl2 family may be to directly inhibit the activation of proCASP9 by Apaf-1. It has been shown that two Bcl2 homologues, Bcl-$X_L$ and Boo/DIVA, individually form a ternary complex with proCASP9 and Apaf-1.[241],[242],[243] In the nematode, *C. elegans*, CED9 (Bcl-$X_L$ homologue), CED3 (CASP9 homologue), and CED4(Apaf-1 homologue) form a similar complex whereby the activation of the caspase is inhibited. This being said, there exists some evidence that although Bcl-$X_L$ does in fact bind Apaf-1, it does not prevent activation of proCASP9.[244]

Studies have shown that disruption of the CED9/CED4 (Bcl-XL/Apaf-1 homologues respectively) is required for the induction of apoptosis in *C. elegans*. This disruption is mediated by EGL-1, yet another Bcl2 homologue. It would appear that CED9 preferentially binds EGL-1 thereby liberating CED4 and allowing it to bind and activate CED3 (proCASP9).[245] Similarly, both Bik and Bak (pro-apoptotic Bcl2 homologues) are able to disrupt the anti-apoptotic Boo(DIVA)/Apaf-1 complex and promote activation of proCASP9.[243] In contrast to this, work by Inohara has shown that DIVA(Boo) functions to promote apoptosis by displacing Bcl-$X_L$ from

Apaf-1.[242] In their hands, unlike BIK and BAK, DIVA binds Apaf-1 directly thereby displacing the resident anti-apoptotic homologue. Although DIVA(Boo) binds Apaf-1 it would appear that it does not interfere with Apaf-1 mediated activation of CASP9.[242]

### 8.2.5. Inhibitors of Apoptosis

#### NF-κB/IκB[246]

Although NF-κB can be induced in both pro-apoptotic and anti-apoptotic paradigms, activation of NF-κB, on the whole, will likely be found to suppress apoptosis. Originally identified as a transcription factor binding to the intronic enhancer element of the immunoglobulin kappa light chain[247], the NF-κB family of proteins has been extended to five mammalian members: RelA (p65), RelB, c-Rel (p75), NF-κB1 (p50), and NF-κB2(p52). Two *Drosophila* homologues, *dif* and *dorsal*, have also recently been cloned.[246],[248] These transcription factors form inactive homo- and hetero-dimers and, by blocking their nuclear localization signals, are retained within the cytoplasm in association with one of five ankyrin repeat containing inhibitors (IκBs).[249],[250] Along with the three classical inhibitors, IκBα[251], IκBβ1 (and IκBβ2)[252],[253],[254], as well as IκBε[253],[255], are p100/IκBδ and p105/IκBγ and the *Drosophila* homologues, *cactus* and *relish*.[256] The two IκBβ isoforms are generated by alternative splicing of their 3' end. The human-specific splice isoform IκBβ2 lacks a portion of the C-terminal PEST sequences present in all IκB isoforms, save IκBε.[254] Inclusion of p100, p105, and *relish* into the IκB family is based on sequence similarity of their C-terminus to the previously described IκBα and IκBβ molecules and their ability to retain NF-κB monomers in the cytoplasm.[257],[258],[164] Setting p100 and p105 apart from the others is the observation that co-translational processing of these inhibitors yields the NF-kB monomers p52 and p50 respectively.[259],[260],[261],[262],[263] In the case of relish, DREDD mediated endoproteolytic cleavage releases an endogenous NF-κB like domain, REL-68[264]. p100 and p105 also differ from the other IκB isoforms in that, by virtue of already harbouring an NF-κB-like moiety, they bind only single NF-κB monomers. This is also likely to be true for full length relish.[265] Consequently, binding of NF-κB by either p100 or p105 forms a pseudoheterotrimer.[246],[248] Furthermore, alternative splicing of the gene encoding p105 (*nfkb1*) has been demonstrated to yield the several related IκBγ inhibitory subunits.[266],[267],[268] Generation of IκBδ has been postulated to occur by selective proteolysis of the p100/κB pseudoheterotrimer yielding a short lived heterotrimer composed of IκBδ proper and an NF-κB dimer subunit.[269] It should be noted that the individual

inhibitors display some specificity with respect to their preferred binding partners (Table 2). although this has not been rigorously studied.

Stimulation by cytokines, mitogens, or other NF-κB inducers causes the IκB subunits to be phosphorylated[270] either by the ~700 kDa IκB kinase complex (see section on IKK) or a variety of other kinases including: inducible IKK (IKK-i, Ser36 of IκBα)[136], the 90 kDa ribosomal S6 kinase (p90rsk1, Ser32 of IκBα)[271],[272], DNA dependant protein kinase (DNA-PK, Ser36 of IκBα)[273],[274], and the MAP3K, TPL-2 (NF-κB/p105).[275] Phosphorylation of the IkBα subunit at S32 and S36[276],[277] and its subsequent polyubiquitination at K21 and K22[278],[279],[280] by an E3-complex[281],[282],[283] leads to its rapid degradation by the 26S proteasome.[284] The relevant residues for IκBβ are S19, S23[277], K9[285] and for IκBε are S18, S22, K6[255] respectively. It would appear, though, that ubiquitination of IkBβ/ε at K9/6 is not required for their signal induced degradation.[255],[285] In the case of p100 and p105, signal induced degradation liberates the p52 and p50 moiety in association with its previously bound monomer.[196],[286] Although it has not been demonstrated for either IκBβ nor IκBε, the signal induced degradation of IκBα does not appear to adversely affect the association of the bound NF-κB subunits.[287]

Sequence analysis has shown that, unlike other IκB isoforms, neither IκBβ2 nor IκBε possess C-terminal PEST sequences. PEST sequences are protein domains rich in proline (P), glutamic acid (E), serine (S), and threonine (T) and are thought to be integral to the turnover of many, if not most, short lived proteins which contain them.[38] Although the implication of this has not yet been fully addressed, IκBβ2 appears to be markedly resistant to signal induced degradation when compared to its full length counterpart IκBβ1.[254] Furthermore, the PEST sequences of IκBα and IκBβ have been demonstrated to contribute to both the binding of NF-κB dimers as well as their basal turnover.[288],[289],[290] It has also been shown that newly-synthesized IκBβ is hypophosphorylated within its PEST sequences and that IκBβ, in a hypophosphorylated state, is able to bind to active NF-κB dimers whilst maintaining both the nuclear localization signal (NLS) and transactivation potential of the unbound dimer as well as shielding it from the inhibitory effects of *de novo* IκBα.[289],[291] In a similar fashion, the IκB-like nuclear proto-oncogene Bcl3 is able to outcompete IκBα and bind either p50 or p52 homodimers and subsequently retain them within the nucleus.[292],[293],[294],[295] In so doing, Bcl-3 is able to impart a transactivation domain to these transcriptionally silent members.[293]

Once liberated from their inhibitory proteins the NLSs of NF-κB dimers are revealed and they translocate to the nucleus[250]. It has been shown that, at least for the p65 subunit, the transactivation potential of NF-κB is increased by phosphorylation of its transactivation domain

**Table 2: The various IκB isoforms and their known binding partners.**

| Isoform | NF-κB binding partners | Reference |
|---|---|---|
| IκBα | p65/x | 253 |
| | cRel/x | 253 |
| IκBβ | p65/x | 253 |
| | CRel/x | 253 |
| IκBγ | p50/p50 (in vitro) | 267 |
| | cRel/cRel (in vitro) | 267 |
| | p65 | 267 |
| | cRel | 267 |
| IκBγ1 | p50 | 268 |
| IκBγ2 | p50 | 268 |
| IκBε | p50/p65 (in vitro) | 296 |
| | p50/cRel (in vitro) | 296 |
| | p65/p65 | 288 |
| | p65 | 296 |
| | p50 | 296 |
| | p52 | 296 |
| | RelA | 255 |
| | cRel | 255, 296 |
| p100 | p50 | 257, 258 |
| | cRel | 257, 258 |
| | p65 | 258 |
| | all | 297 |
| p105 | p50 | 298 |
| BCL3 | p50/p50 | 270 |
| | p52/p52 | 270 |

by the IKK complex itself[209],[299] as well as perhaps other kinases such as casein kinase II.[300],[301] Very little is known about the sequence specificities of the various NF-κB isoforms and no modification of the canonical consensus sequence has been put forward for any of the members. It remains, though, that some genes are preferentially activated by selected NF-κB isoforms and not by others.

In the case of TNFα alone, the induction of NF-κB is transient in nature and involves only the signal-induced degradation of IκBα[302], conceivably due to the substrate specificity of the IKK complex.[195],[197] Release of NF-κB from its inhibitory subunits causes the κB mediated transcriptional upregulation of a number of κB responsive genes including IκBα[270],[303],[304],[305],[306], IκBε[288], and p100.[307] Newly synthesized IκBα is actively transported into the nucleus by an NLS independent mechanism[308],[309] where it binds to nuclear NF-κB dimers, and subsequently exits with its cargo by virtue of an N-terminal nuclear export signal[310], thus forming an autoregulatory loop. Sustained signalling, though, allows for a wider range of NF-κB isoform activation by virtue of enhanced degradation of other IκB isoforms (Table 2). Furthermore, *de novo* synthesis of IκBβ is able to prolong NF-κB dependant transcription by sequestering NF-κB dimers within the nucleus.[291],[311] Alternatively, when apoptosis is to be initiated, caspase dependant cleavage of IκBα can produce an inhibitor refractory to signal induced degradation.[312]

### p35/HRF-1

p35 (Host range factor-1) was first described in a (multinucleocapsid) nucleopolyhedrosis virus affecting *Autographa californica* (AcMNPV). p35 is able to inhibit apoptosis of the infected cell, initiated as a host defence mechanism against infection.[313] In the time following its discovery, p35 has been shown to be a suicide inhibitor of the caspases.[10] It is now presumed that p35 functions to prevent apoptosis by the inhibition of an as yet unidentified lepidopteran caspase.[10]

### CrmA

CrmA (cytokine response modifier A) is a product of the cowpox virus and functions, like p35, to prevent inflammation and apoptosis initiated by the host following infection. Again, like p35, CrmA has been shown to be a suicide inhibitor of several mammalian caspases.[10],[314]

**Inhibitor of Apoptosis (Proteins) (IAPs)**

The Inhibitors of Apoptosis comprise a family of proteins capable of preventing programmed cell death under a variety of experimental conditions. The original IAP was isolated from the granulosis virus affecting *Cydia pomonella* (CpGV, Cp-IAP) by virtue of its ability to block apoptosis induced by p35 deficient AcMNPV (*Autographa californica* MNPV).[315] Similar results were later obtained for IAPs isolated from the *Orgyia pseudotsugata* NPV.[316] Other baculoviral IAPs have now been described in *Bombyx mori* NPV, *Buzura suppressaria* NPV[317], *Lymantria dispar* MNPV[318], and *Trichoplusia ni* GV[319] , although their ability to fully complement p35 is in question.[317] AcMNPV also encodes an IAP although this inhibitor is apparently non-functional. It may be that the advent of p35 in the AcMNPV genome rendered Ac-IAP redundant and subject to mutational inactivation.[320]

All baculoviral IAPs isolated thusfar contain at least two N-terminal Cys/His motifs of approximately 80 amino acids termed "baculoviral IAP repeat" (BIR) domains and a C-terminal RING zinc finger motif. Indeed the presence of the BIR domains suffices to characterize a protein as being a member of the IAP family.

Baculoviruses likely acquired IAPs from their cellular hosts. Evidence for this comes from the isolation of an IAP from a *Spodoptera frugiperda* (Fall army worm) cell line (Sf21). Sf-IAP is organized in the same fashion as the baculoviral IAPs in that it possesses two N-terminal BIR domains as well as a RING finger domain.[321] Two IAP homologues have been identified in the common fruit fly *Drosophila melanogaster*. The first of these, DIAP1, is similar to that encoded by the baculoviruses in that it possesses two BIR domains and a RING zinc finger motif, while the second IAP, DIAP2, possesses a third BIR domain.[32]

The IAP family was extended to vertebrates with reports from several laboratories demonstrating the existence of at least four other members. *Hiap1*, *hiap2*, *xiap*, and *naip* form the nucleus of the mammalian IAP family (Table 3). Other mammalian members include the single BIR containing proteins Apollon[322](BRUCE)[323], Survivin[324](TIAP)[325], and Livin[326] (Figure 2).

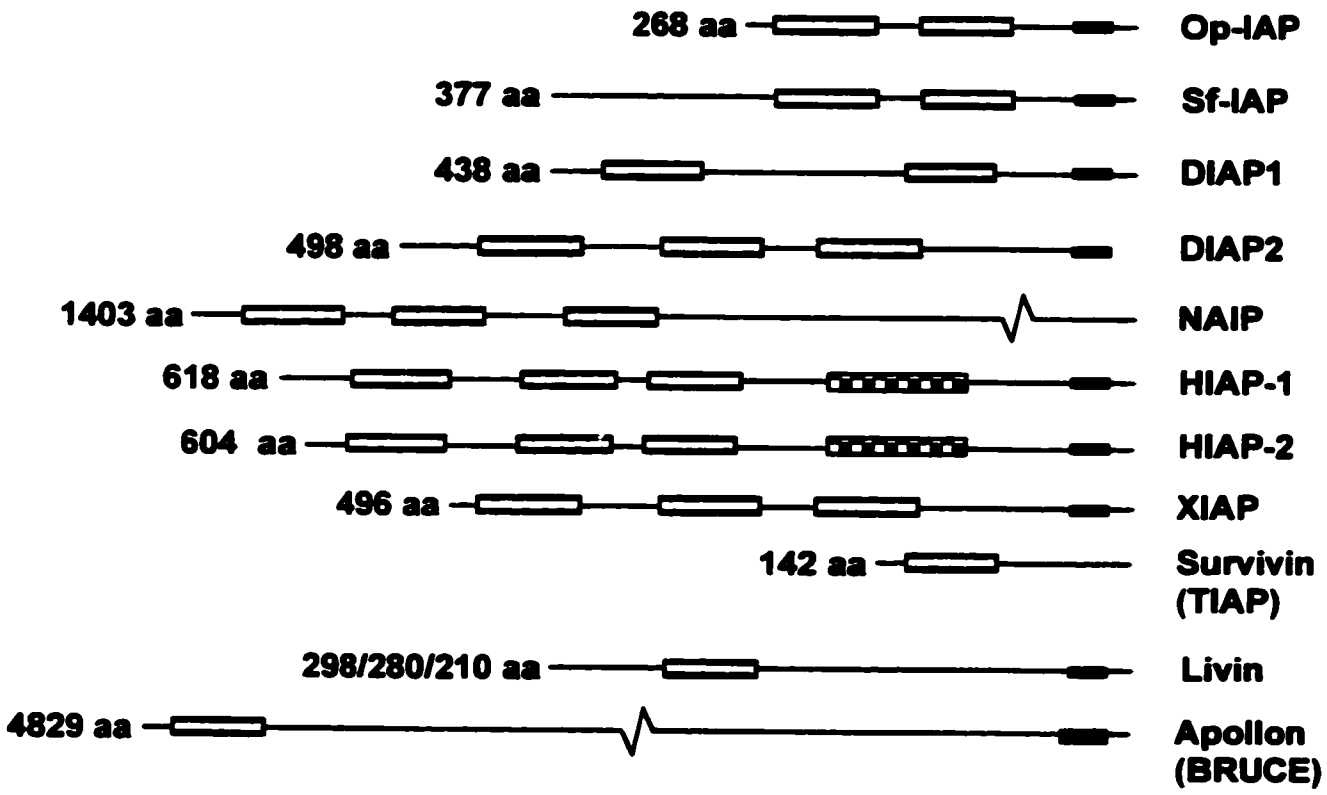
Work by AE Mackenzie and others have shown that the BIR domains in isolation function to inhibit the activation of caspases 3, 7, and 9, and in this way serve to inhibit the execution of apoptosis[327],[328] (J. Maier in preparation). Another intriguing function of the IAP family of proteins is that some, if not all, members may prove to be ubiquitin ligases. As such, they may function to promote the degradation of IAP binding partners. Curiously the IAPs also appear to be able to promote their own polyubiquitination, and in so doing, may both limit their accumulation within the cell and accelerate apoptosis once its been initiated.[329]

**Table 3: Genbank IAP nomenclature as well as those currently or previously in use.**

NB: The genbank assignment for the murine homologues of *api1* and *api2* are reversed such that murine *api2* is the equivalent to human *api1*.

| Genbank | Common names | Reference |
|---|---|---|
| *naip* | *naip* | 330 |
| *api1* | *hiap2* | 331 |
| | *c-iap1* | 60 |
| | *mihc* | 332 |
| *api2* | *hiap1* | 331 |
| | *c-iap2* | 60 |
| | *mihb* | 332 |
| *xiap* | *xiap* | 331 |
| | *miha* | 332 |
| | *ilp* | 333 |

**Figure 2: Human, invertebrate, insect and viral IAPs.** Individual protein domains are as indicated (inset). The length of each protein (in amino acids) is indicated to the left of each.




BIR domain $X_3RX_{20-23}GX_{11}CX_2CX_{16}HX_6CX_3$

RING (Zinc) Finger domain $CX_2CX_{9-39}CX_{1-3}HX_{2-3}C\text{-}X_2CX_{4-48}C\text{-}X_2C$

CARD domain $MX_7LRX_2RX_2LX_3LX_5LDXLX_4(V/I)LX_5(E/D)XIX_6$
$(QRK)X_3L(L/I)D(L/I)X_2RGX_2AX_3FX_3LXX_4LX_3L$

UBC domain

### 8.2.6. Promoters of apoptosis

#### HID, GRIM and, REAPER

These proteins appear to be negative regulators of *D. melanogaster* IAP function. Mutation analysis has revealed that IAP mutants which fail to bind HID, GRIM, or REAPER are able to suppress apoptosis initiated by these proteins,[334] the implication being that by binding DIAP1 HID, GRIM, and REAPER inhibit the anti-apoptotic function of DIAP1, thereby promoting cell death. It is noteworthy that the promoter region of *rpr* (REAPER encoding gene) contains a p53 response element[335] thus providing a link between induction of p53 and the IAPs.

Recently, two reports have described a functional equivalent of the *D. melanogaster* HID, GRIM, and REAPER proteins. DIABLO[336]/Smac[337] was found by co-immunoprecipitation to bind XIAP, HIAP1, and HIAP2, as well as OpIAP *in vivo*. Immunofluorescence studies show DIABLO to be predominantly associated with the mitochondrion, however upon UV irradiation, it distributes to the cytosol. It is postulated that DIABLO may promote apoptosis by antagonizing the anti-caspase activity of the various IAP family members.[336]

#### APAF1/CED4/DARK

The apoptotic protease-activating factor-1 (Apaf1[29]/CED4/dAPAF1[338],[339],[340]) is a cellular adaptor protein associated with the prodomain of proCASP9/CED3/Dredd[338],[341] (caspases are discussed in a later section). Under conditions where cytochrome c is released by the mitochondria, the Apaf1/CASP9 complex binds cytochrome c and is activated.[342] Activation of CASP9 then in turn leads to the activation of other caspases, most notably CASP3. Homozygous deletion of *apaf1* is embryonic lethal (dpc 16.5) and leads to malformation of the digits, eye, and head as well as overgrowth of the brain. All of these phenotypic changes can be attributed to a failure to induce programmed cell death during embryogenesis.[343],[344] Furthermore, *apaf-1* deficient cell lines show reduced activation of caspases 2, 3, and 8[344], presumably due to an inability to activate CASP9. Although Apaf-1 has been tightly linked to apoptosis initiated from the mitochondria, it would appear to have a negligible role in CD95/FAS induced apoptosis.[344]

### 8.2.7. Executioners

#### Caspases/CED-3[10]

Caspases (cysteinyl aspartate-specific proteinases) are a family of proteinases comprised of 14 highly homologous members. The prototype caspase is the protease "interleukin-1 converting enzyme" (ICE, CASP1). This protease was first described as being involved in the maturation of pro-inflammatory cytokines[345]. Although all caspases cleave their targets after aspartate residues

found within a four amino-acid minimal recognition sequence, they can be subdivided into three groups based on both substrate specificity and protease function (Table 4).

Group I, which includes caspases 1, 4, and 5, appears to be involved in maturation of pro-inflammatory cytokines containing the consensus sequence WEHD/YVAD.[346] Group II caspases are termed "effector" caspases (caspases 3, 7, and 2 as well as CED-3) due to their apparent involvement in cleavage of cellular targets with the consensus sequence DE(V)D[346] during apoptosis. It is also believed that activation of these caspases marks a point of no return, after which the cell is committed to undergo apoptosis. Group III caspases include caspases 6, 8, 9, 10, and granzyme B. These proteases cleave (VL)ExD substrates[346] and are termed activator caspases due to their role in amplification of the caspase cascade (activation of other Group III caspases) and in the activation/proteolysis of Group II caspases. Two members of this group, CASP8 and CASP10, appear to initiate the protease cascade from an activated receptor. In this model, an activated receptor, be it TNFRI, Fas, or one of the other death domain containing receptors, recruits FADD, either directly or through an intermediary. FADD, in turn, recruits CASP8 or CASP10 to the DISC (death inducing signalling complex) via mutual death effector domains (DED) where it is proteolytically activated. Instead of possessing a DED within their prodomain, CASP9, CED3, and CASP2 possess a CARD (Caspase (activation and) recruitment domain). Through this domain, these caspases are able to bind other CARD containing proteins such as Apaf-1 for CASP9/CED3 or RAIDD, as is the case for CASP2. Group III caspases also have a role in the cleavage of non-DxxD apoptotic substrates.

All caspases begin as zymogens consisting of a small prodomain at the N-terminus followed by a 20 kDa regulatory domain and a 10 kDa catalytic domain, each separated by consensus caspase cleavage sites. Processing of the zymogen, generally by Group II caspases and/or autoproteolysis, allows for the formation of an active heterotetramer comprised of two regulatory subunits and two catalytic subunits.

In addition to the mammalian and single *C. elegans* caspases, several *D. melanogaster* caspases and caspase-like proteases from *C. elegans* have also been described. These include Drosophila caspase-1 (DCP-1)[347], DREDD/DCP-2[34], drICE[348], and DRONC.[349] For its part, DREDD/DCP-2 is functionally most like CASP8 or CASP10 in that it binds the Drosophila homologue of FADD, dFADD, via an N-terminal death effector domain (DED). Furthermore, all of the Drosophila caspases, save DREDD, have been shown to be inhibitable by a *Drosophila* IAP homologue, DIAP1.[350],[351],[352],[353]

**Table 4: Caspase subfamily groupings.** *Placement of DRONC as a Group III caspases is by virtue of its ability to activate drICE, whereas DREDD may be classified as a Group III member due to its functional similarity to caspases 8 and 10. Caspase 11, identified in the mouse, is postulated to be the murine homologue of Caspase 4. Caspases 12-14 have also been identified although the substrate specificity has not been determined.

| Group | Caspases | Specificity | Function |
|---|---|---|---|
| I | 1, 4, 5 | WEHD | Maturation of proinflammatory cytokines |
| II | 2, 3, 7, CED3 DrICE, DCP-1 | DEVD | Effector caspases |
| III | 6, 8, 9, 10, Granzyme B DRONC* DREDD* | (VL)ExD | Activator caspases |

Via alternative splicing, the three caspase-like protease genes isolated *from C. elegans* code for six different proteins. *Csp1* generates three different isoforms (A, B, and C). *csp2* generates two isoforms (A and B), while *csp3* does not undergo alternative splicing. Although the N-terminal portion of the active site of both CSP1 (SACRG) and CSP2 (VCCRG) isoforms diverge from the caspase consensus, (QAC(R/Q/G)G), both are likely to be active proteases. Furthermore, CSP1B is able to cleave the CASP1 substrate Z-YVAD-AFC. CSP3, though, does not possess a recognizable active site and, therefore, is not likely to act as protease.[354]

**Paracaspases and Metacaspases**

Caspases have until very recently been thought to exist only in higher metazoans such as nematodes, insects, and vertebrates. The recent discovery of caspase-like molecules in a variety of organisms ranging from bacteria through to vertebrates has perhaps shed some light into the evolutionary origins of the caspases proper. Although all share the conserved His/Cys catalytic diad of the caspases, they can be divided into three groups.[24]

Paracaspases have been identified in humans, zebrafish, nematodes, and slime moulds. Save for that encoded by slime moulds, the paracaspases all encode an N-terminal death domain and at least one immunoglobulin repeat domain. Notably, sequence analysis of the human paracaspase has revealed it to be in fact *malt1*; a gene previously identified in oncogenic translocations involving *hiap1*.[355] Through its association with Bcl10, MALT1 has been shown to contribute to NF-κB activation by associating with pre-existing HIAP1-TRAF2 complexes.[110] The ability of MALT1 to promote the activation of NF-κB appears to reside in its protease activity, as mutation of the conserved cysteine severely diminishes this ability.[24] The HIAP1-MALT1 fusion resulting from the t(11;18)(q21;q21) translocation may obviate the need for Bcl10 in the signalling complex in order to activate NF-κB. It should be noted though, that fusions retaining the immunoglobulin repeats, and hence the ability to bind Bcl10, possess a greater capacity to activate NF-κB than do those lacking these repeats.[24]

For their part, the meta-caspases, found in plants and yeasts, as well as the bacterial cysteine proteases have yet to be shown to have any function in promoting or modulating apoptosis. The meta-caspases lack the N-terminal death-domain of the paracaspases. Instead, some carry an N-terminal proline rich region and, in addition to this, one has been shown to possess an N-terminal zinc finger domain.[24]

At this point it is not known what are the substrate specificities or indeed what are any of the substrates for these classes of caspase-like proteases.

**DNA fragmentation factor (DFF)**

One of the salient features of apoptosis is the degradation of chromosomal DNA into oligonucleosomal sized fragments. This effect, termed chromosomal laddering, due to the appearance of the DNA following agarose gel electrophoresis, is caused, in large part, by endonuclease activation. DNA fragmentation factor (DFF-40)[356] (CAD[357]/CPAN[358] Caspase activated DNase/Caspase activated nuclease) is a 40 kDa double-stranded DNA specific endonuclease. The enzymatic potential of DFF-40 is maintained[359], although inhibited[357],[360],[361], by its association with the molecular chaperone DFF-45/ICAD-L. The interaction between DFF-40 and DFF-45 occurs via an N-terminal domain shared by both proteins.[362] Activation of DFF-40 occurs via the CASP3/CASP7 mediated proteolysis of DFF-45.[363],[364] Once released from its inhibitory subunit, DFF-40 binds to histone H1 (the internucleosomal histone) where it cleaves the neighbouring DNA.[365] Studies on the sequence specificity indicate that DFF-40 is a broad spectrum endonuclease although there appears to be some preference for symmetrical cleavage sites with respect to purine and pyrimidine content.[366]

DFF-40 recognition sequence:

5' R(72), R(74), R(66), Y(61)! R(65), Y(67), Y(75), Y(75)

Alternative splicing of the DFF-45 message yields a shorter protein termed DFF-35[361] (ICAD-S). DFF-35 lacks a nuclear localization signal in its C-terminal region. As such, DFF-35 retains the ability to bind DFF-45 and may function to sequester the nuclease in the cytoplasm.[367] Furthermore, DFF-35 appears unable to act as a DFF-40 chaperone, in that the enzymatic potential of DFF-40 is lost in binding DFF-35.[368] Other DFF-45 like proteins include CIDE-A and CIDE-B (Cell death inducing DFF45-like effector).[369] These proteins share homology with the N-terminus of DFF45 and are able to interact with it. Both proteins are able to homo- and hetero-dimerize and promote apoptosis. Unlike DFF-40 though, CIDE-B is localized to the mitochondria.[370] How this DFF homologue functions in the mitochondria to promote apoptosis remains unclear.

**Apoptosis inducing factor (AIF)**

AIF, which promotes non-caspase dependant apoptosis, normally resides within the intermembrane space of the mitochondria. Under the appropriate conditions, AIF is released from this space, perhaps in conjunction with cytochrome c, and is translocated to the nucleus where it mediates chromatin condensation and large-scale DNA fragmentation (fragments of approx. 50

kbp).[371] Further degradation of the DNA into oligonucleosomal sized fragments is accomplished by DFF40.[372] In addition to its ability to promote both chromosomal condensation and degradation, forced extra-mitochondrial expression of AIF also promotes the release of cytochrome c from the mitochondria and the appearance of phosphatiydylserine on the outer leaflet of the plasma membrane.[373] AIF is a flavoprotein and, like the caspases, possesses significant homology to proteins in a number of phyla including other vertebrates, non-vertebrate animals, plants, fungi, eubacteria, and archaebacteria.[374] It has been shown that the apoptogenic potential of AIF is independent of it's oxido-reductase activity. AIF, with an intact redox domain though devoid of prosthetic groups, is able to induce an apoptotic phenotype in purified HeLa nuclei.[373] Although it would appear that AIF is able to promote cell death, it remains unclear how this is mediated or what binding partners or targets may exist for this protein.

### 8.3. Apoptotic and Anti-apoptotic signalling pathways

To this point, many of the receptors or proteins have been discussed more or less in isolation. Where appropriate, the proteins to which they bind have also been described, as well as some aspects of their downstream signalling cascades. In this section, a series of figures attempts to illustrate the major components and interactions of the various signal transduction cascades leading either from a variety of TNF receptor superfamily members or the mitochondrion. A brief description of each of the pathways, as they appear, may be found in the legend accompanying each figure. Furthermore, detailed descriptions of the individual proteins depicted in the following figures may be found in the preceding sections.

**Figure 3A: TNF receptor 1: NF-κB activation**

Under unstimulated conditions, signalling from the TNFRI is suppressed by binding of SODD to the receptor monomers. Signal induced trimerization causes the recruitment of several signalling and adaptor molecules to the receptor. At the base of these, TRADD binds the death domains of the trimerized receptor. Associated with the trimerized receptor are NIK, RIP, TRAF2 and HIAP1. Assembly of the receptor complex causes the activation of PI3K and subsequent conversion of PIP2 to PIP3 at the plasma membrane. PIP3 then serves as an anchor for the recruitment and activation of PDK1 and PDK2 which, in turn, activate PKB. Phosphorylation of IKKα and IKKβ by both activated PKB and NIK (at the receptor complex) serve to activate the IKK complex. As an alternative to this signalling mechanism, TRAF2 may bind TANK. This complex then, in turn, binds to and activates IKK-i (TBK1). IKK-I then promotes both the phosphorylation of the IKK complex and the phospho-dependent dissociation of the TRAF2/TANK complex. The liberated TRAF2 may then rejoin the signalling complex. The activated IKK complex then promotes the phosphorylation/ubiquitin dependent degradation of the IκB isoforms and phosphoactivation of NF-κB (p65).

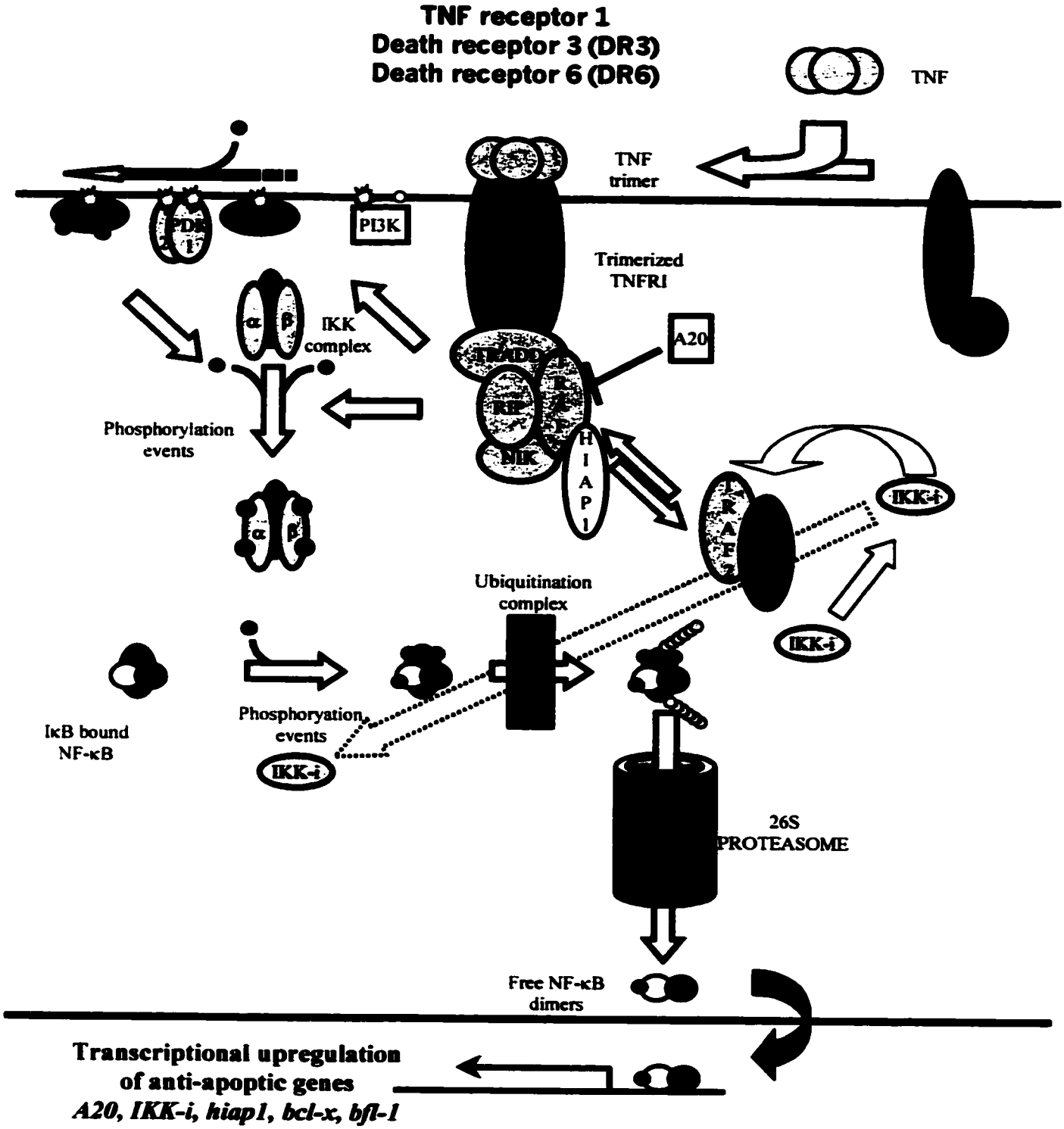

TNF receptor 1
Death receptor 3 (DR3)
Death receptor 6 (DR6)
TNF
TNF trimer
PI3K
Trimerized TNFRI
IKK complex
A20
Phosphorylation events
HIAP1
IKK-i
Ubiquitination complex
IκB bound NF-κB
Phosphoryation events
IKK-i
26S PROTEASOME
Free NF-κB dimers
Transcriptional upregulation of anti-apoptic genes
A20, IKK-i, hiap1, bcl-x, bfl-1

**Figure 3B: TNF receptor 1: JNK/SAPK and p38 activation**

Signal induced trimerization of the TNFRI receptor causes the assembly of a signalling complex comprised of TRADD, NIK, RIP, TRAF2, and HIAP1. Association of TRAF2 with TANK causes is dissociation from the receptor complex. The TRAF2/TANK complex is then able to bind and activate GCKR, which in turn serves to activate the JNK and p38 transcription factors. Alternatively, binding of ASK1 to TRAF2 promotes the TRAF2 dependent activation of JNK.

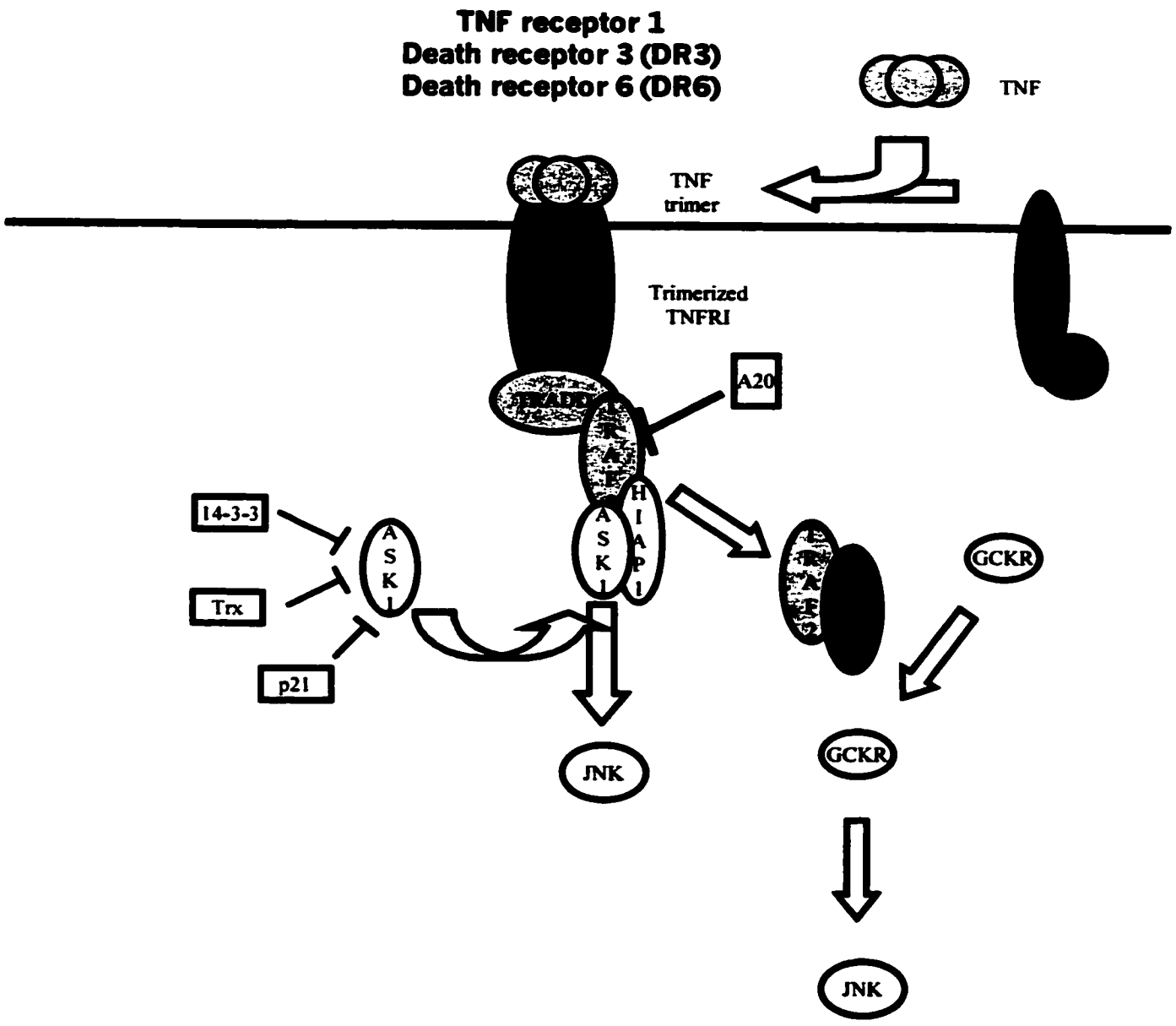

TNF receptor 1
Death receptor 3 (DR3)
Death receptor 6 (DR6)
TNF
TNF trimer
Trimerized TNFRI
A20
14-3-3
Trx
p21
ASK1
HIAP1
GCKR
JNK
GCKR
JNK

**Figure 3C: TNF receptor 1: Caspase activation**

Signal induced trimerization of the TNFRI receptor causes the assembly of a signalling complex comprised of TRADD, NIK, TRAF2, RIP, RIP2, and RAIDD. Alternatively, binding of TRADD may recruit the adaptor protein FADD to the receptor complex. Association of CASP1 to RIP2, CASP2 to RAIDD, and CASP8/10 to FADD cause the activation of these caspases. Of these, only CASP8 and 10 have been shown to be definitively apoptotic. Activation of proCASP8 then leads to the proteolytic processing (activation) of proCASP3 and BID. Activation of CASP3 leads to proteolysis of a variety of apoptotic substrates. tBID for its part destabilizes the mitochondria and may effect the formation of the permeability transition pore. Conversion of proCASP3 to CASP3 (active) is not inhibited by the IAPs, although its subsequent proteolytic activity is inhibited.

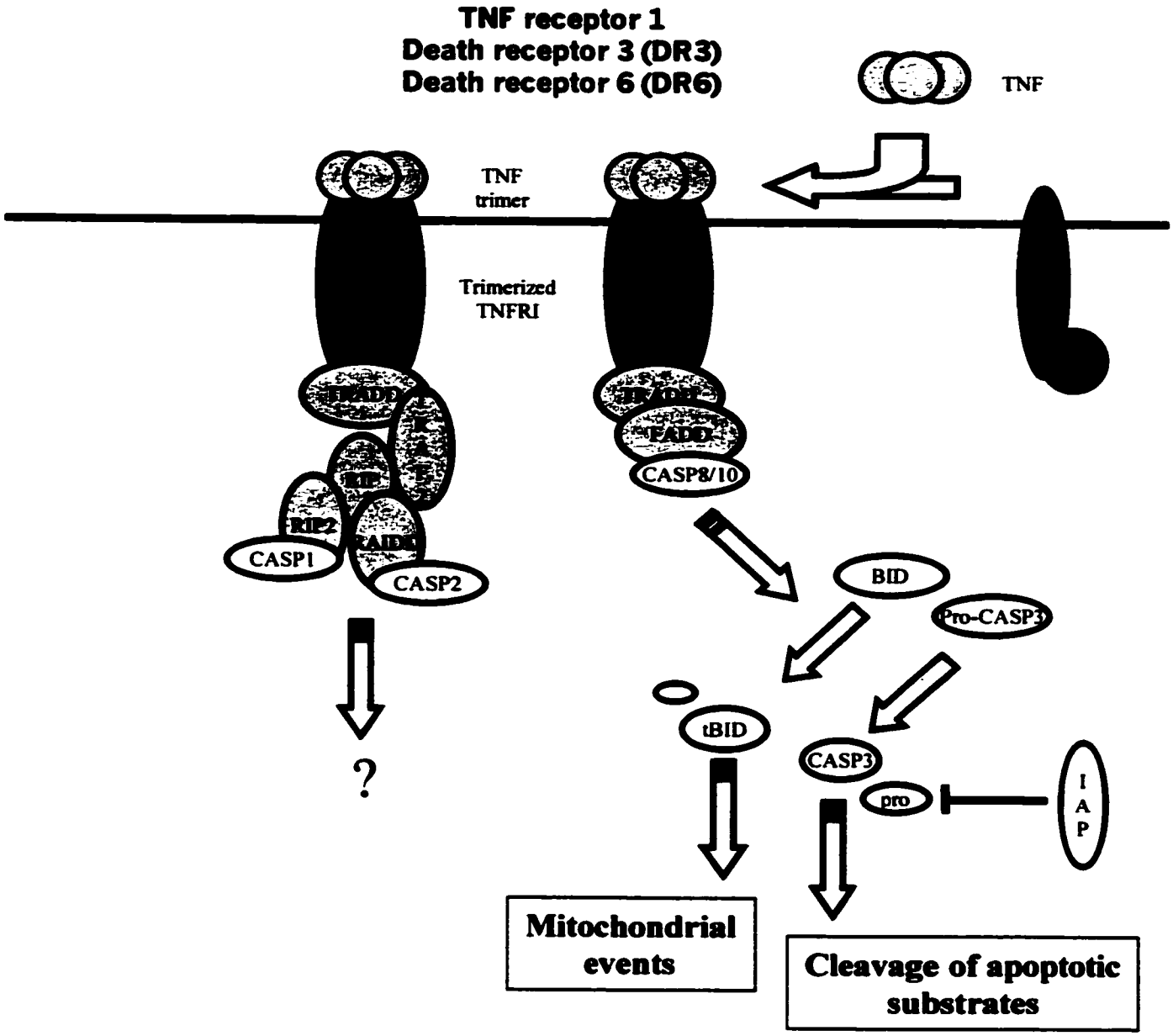

TNF receptor 1
Death receptor 3 (DR3)
Death receptor 6 (DR6)
TNF
TNF trimer
Trimerized TNFRI
FADD
RIP
RIP2
CASP1
CASP2
?
FADD
CASP8/10
BID
Pro-CASP3
tBID
CASP3
pro
IAP
Mitochondrial events
Cleavage of apoptotic substrates

**Figure 3D: TNF receptor 1: Monomeric G protein activation**

Signal induced trimerization of the TNFRI receptor may recruit the adaptor protein MADD to the receptor. MADD possesses significant homology to GEFs (guanine nucleotide exchange factor) and, as such, may regulate the activity of an as yet unidentified monomeric G-protein.

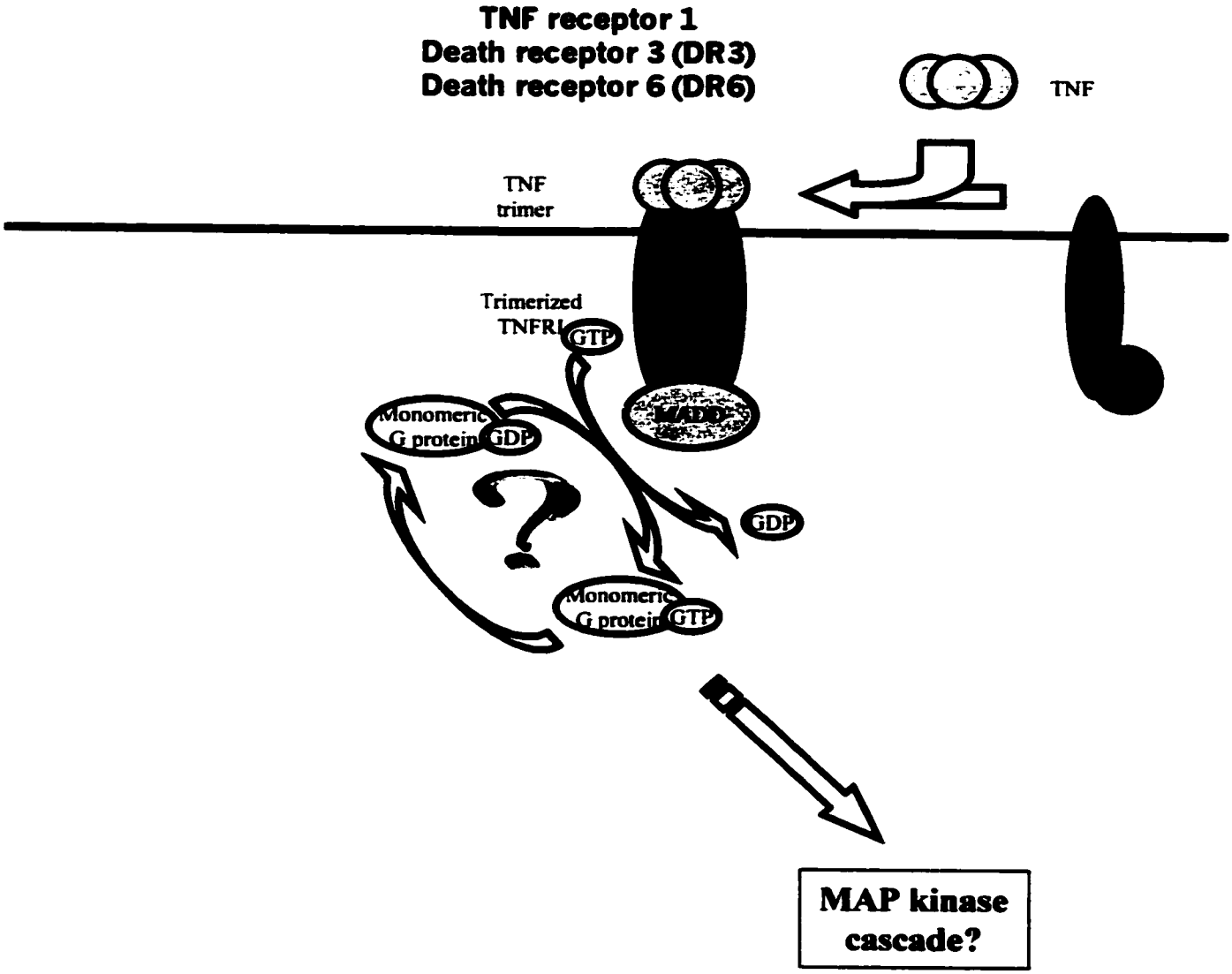

TNF receptor 1
Death receptor 3 (DR3)
Death receptor 6 (DR6)
TNF
TNF
trimer
Trimerized
TNFRL
GTP
Monomeric
G protein
GDP
?
GDP
Monomeric
G protein
GTP
MAP kinase
cascade?

**Figure 4: FAS (CD95): Downstream events**

Signal induced trimerization of CD95 may recruit the adaptor protein FADD or DAXX to the receptor. FADD serves as an adaptor protein for the recruitment of proCASP8 and(or) proCASP10. Activation of proCASP8 then leads to the proteolytic processing (activation) of proCASP3 and BID. Activation of CASP3 leads to proteolysis of a variety of apoptotic substrates. tBID for its part destabilizes the mitochondria and may effect the formation of the permeability transition pore. Conversion of proCASP3 to CASP3 (active) is not inhibited by the IAPs, although its subsequent proteolytic activity is inhibited. Recruitment of the DAXX adaptor protein to the trimerized receptor leads to the activation (and recruitment) of ASK1. ASK1, in turn, mediated the activation of the p38 and JNK pathways. Competitive binding of the CD95 ligand (CD95L) by DcR3 may serve to modulate FAS mediated apoptosis. Alternatively, binding of FADD by c-FLIP may abrogate signal transduction from the activated receptor.

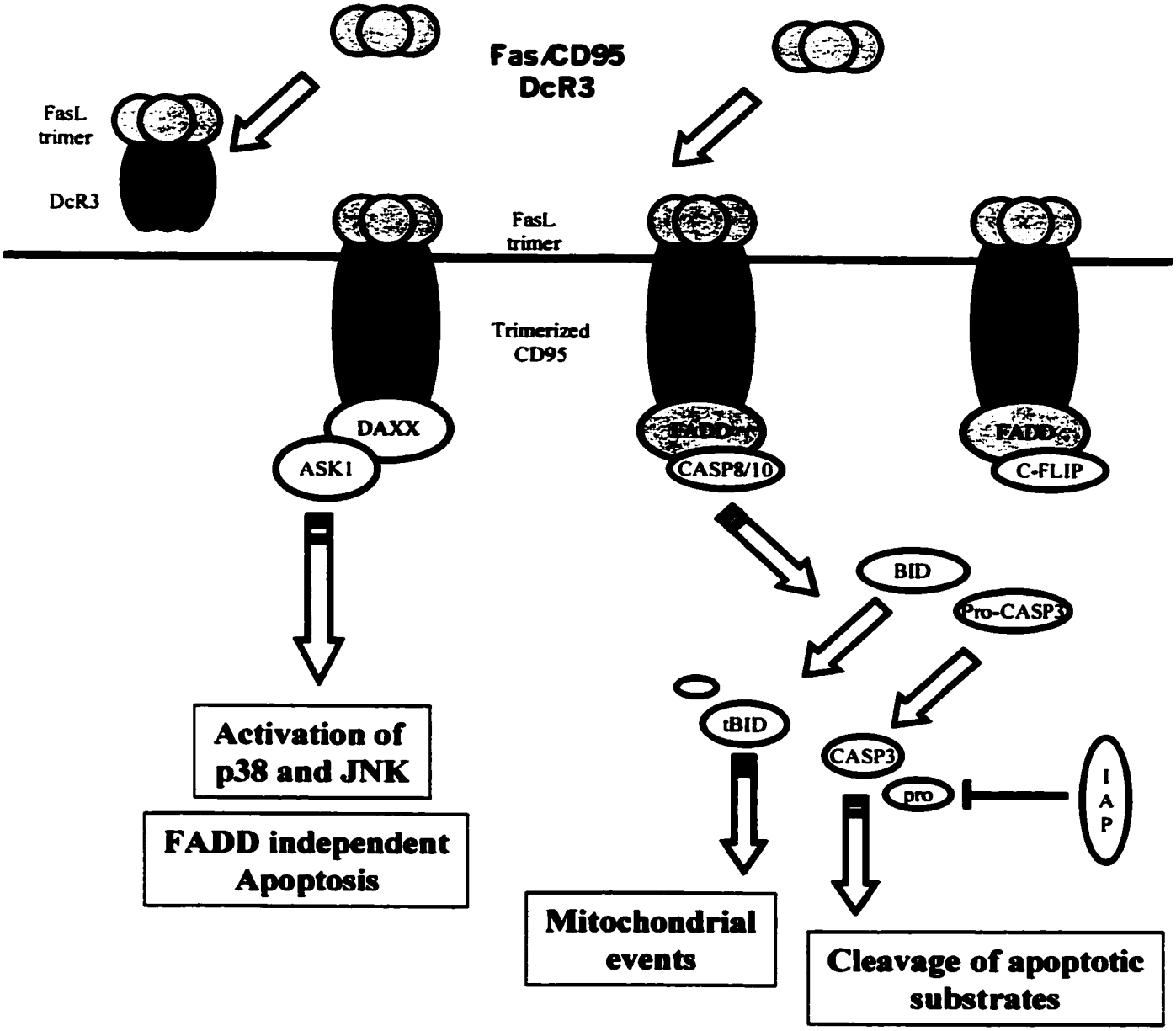

Fas/CD95
DcR3
FasL
trimer
DcR3
FasL
trimer
Trimerized
CD95
DAXX
ASK1
FADD
CASP8/10
FADD
C-FLIP
BID
Pro-CASP3
tBID
CASP3
pro
IAP
Activation of
p38 and JNK
FADD independent
Apoptosis
Mitochondrial
events
Cleavage of apoptotic
substrates

**Figure 5A: TNF receptor 2: NF-κB activation**

Signal induced trimerization or TNFRII causes the recruitment of several adaptor molecules to the receptor. Associated with the trimerized receptor are both TRAF1 and TRAF2, as well as HIAP1 and HIAP2. Signalling from TNFRII results in the activation of NF-κB through an as yet ill-defined pathway. Given that the receptor can bind many, if not all, of the transduction molecules involved in NF-κB activation from TNFRI, it would not be unreasonable to assume that many of the pathways would be similar (shaded in grey).

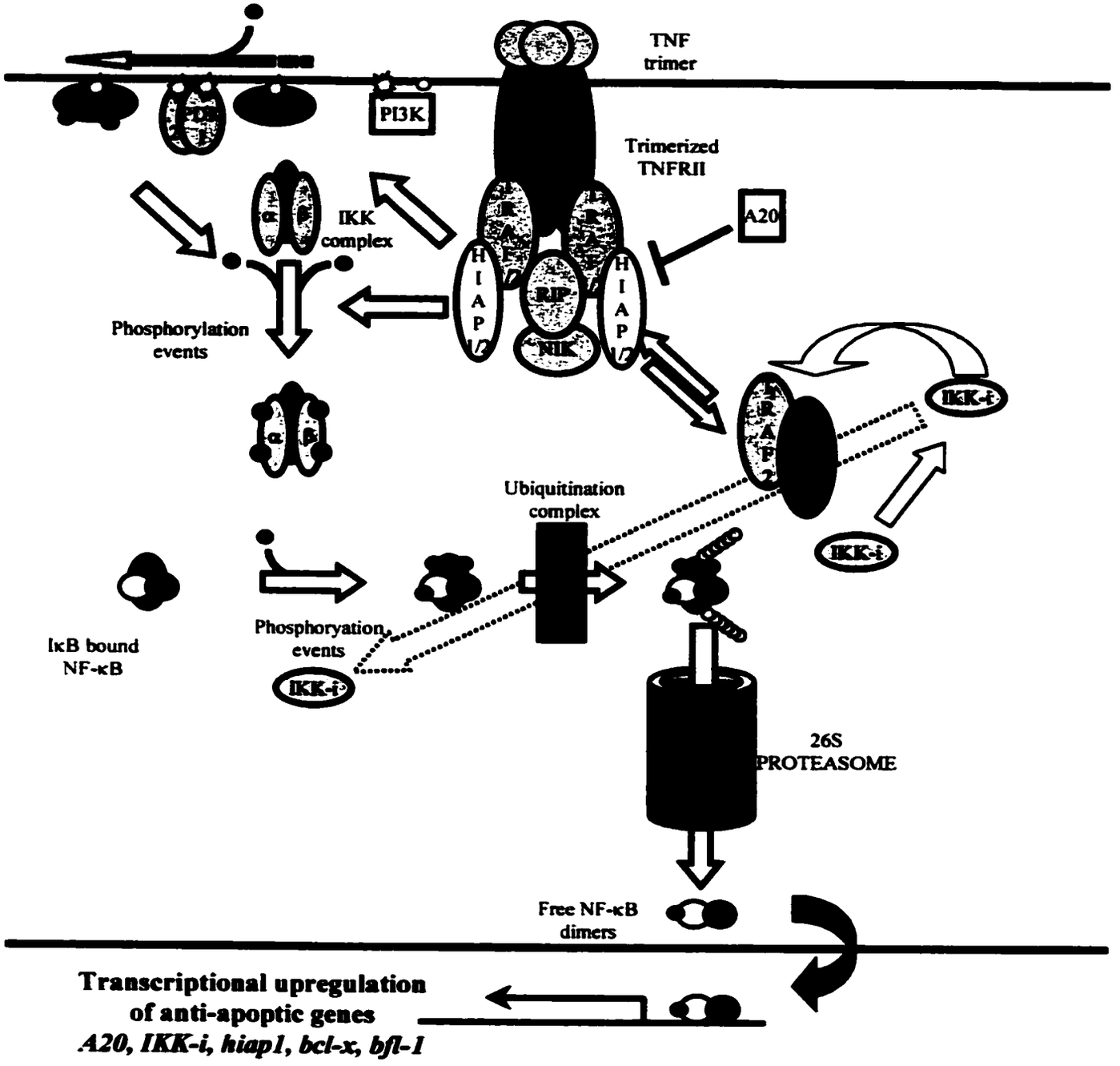

TNF receptor 2
TNF trimer
PI3K
Trimerized TNFRII
A20
IKK complex
Phosphorylation events
HIAP
RIP
NIK
TRAP2
IKK-i
Ubiquitination complex
IκB bound NF-κB
Phosphoryation events
26S PROTEASOME
Free NF-κB dimers
Transcriptional upregulation of anti-apoptic genes
A20, IKK-i, hiap1, bcl-x, bfl-1

**Figure 5B: TNF receptor 2: Caspase activation**

Association of RIP to TNFRII may lead to the recruitment of adaptor proteins generally regarded as being associated with TNFRI. RIP can associate with both RIP2 and RAIDD and, as such, may recruit CASP1 and CASP2, respectively, to the receptor complex.

**TNF receptor 2**

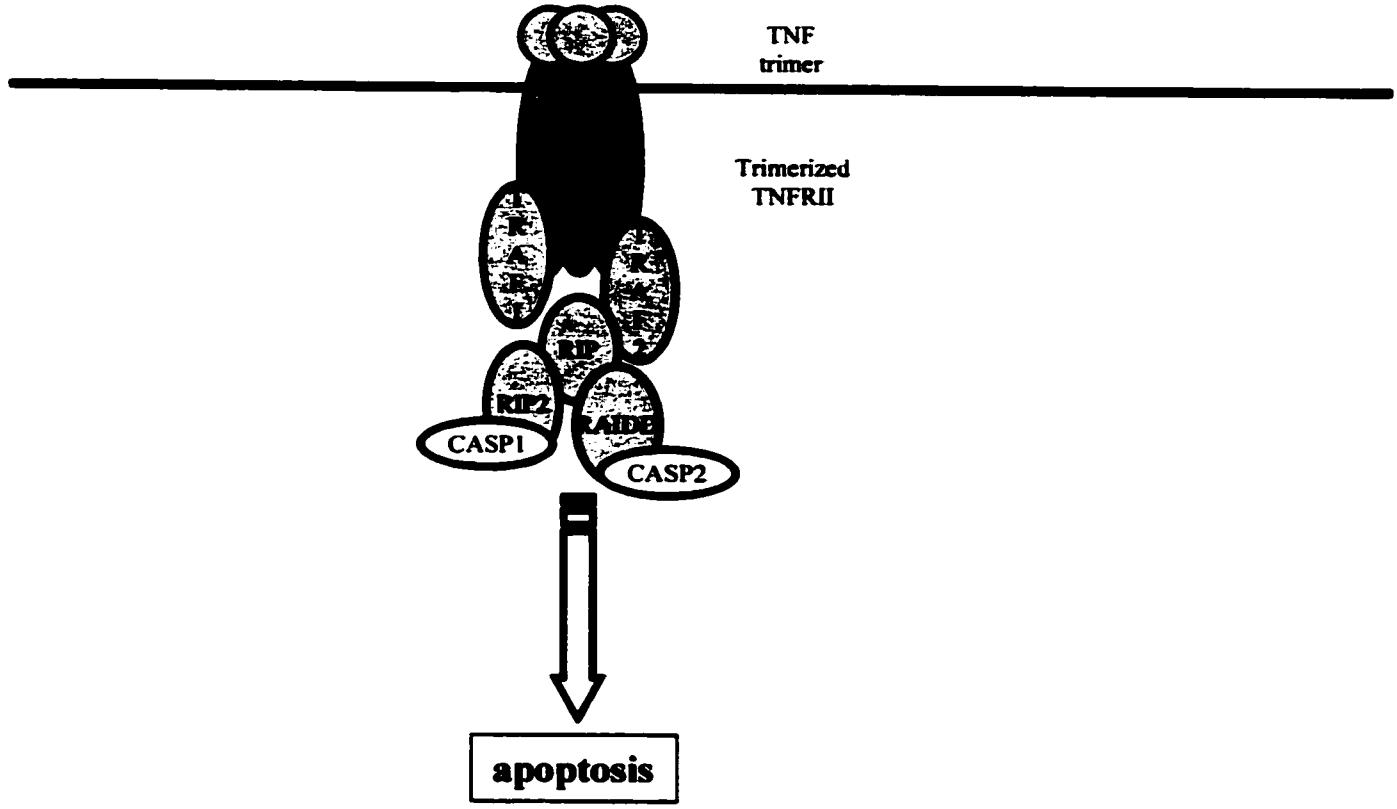


**Figure 6: TRAIL and TRANCE signalling.**

Binding of TRAIL to its receptors, DR3 and DR4, cause these to trimerize and recruit the FADD adaptor protein. This then allows for recruitment and proteolytic activation of proCASP8 or proCASP10 at the receptor complex. Activation of proCASP8 then leads to the proteolytic processing (activation) of proCASP3 and BID. Activation of CASP3 leads to proteolysis of a variety of apoptotic substrates. tBID for its part destabilizes the mitochondria and may effect the formation of the permeability transition pore. Conversion of proCASP3 to CASP3 (active) is not inhibited by the IAPs, although its subsequent proteolytic activity is inhibited. Binding of TRAIL to its receptor(s) may be antagonized by presence of the membrane bound decoy receptors, DcR1 and DcR2. As these have no intracellular domain, they serve to modulate (or prevent) TRAIL signalling.

For its part, binding of TRANCE, present on the surface of osteoblasts, to its receptor, RANK, promotes the differentiation of haematopoetic precursors into osteoclasts and hence bone resorption. TRANCE is also present on the surface of activated T-cells. Interaction of these with RANK expressing dendritic cells (DC) serves to activate the latter.

Osteoprotegrin (OPG) is a soluble decoy receptor for both TRAIL and TRANCE and, as such, inhibits TRAIL induced apoptosis and osteoclastogenesis. It is not currently known whether OPG normally functions to inhibit the activation of dendritic cells by activated T-cells.

**Death receptors 3 and 4**

**Rank (TRANCE receptor)**

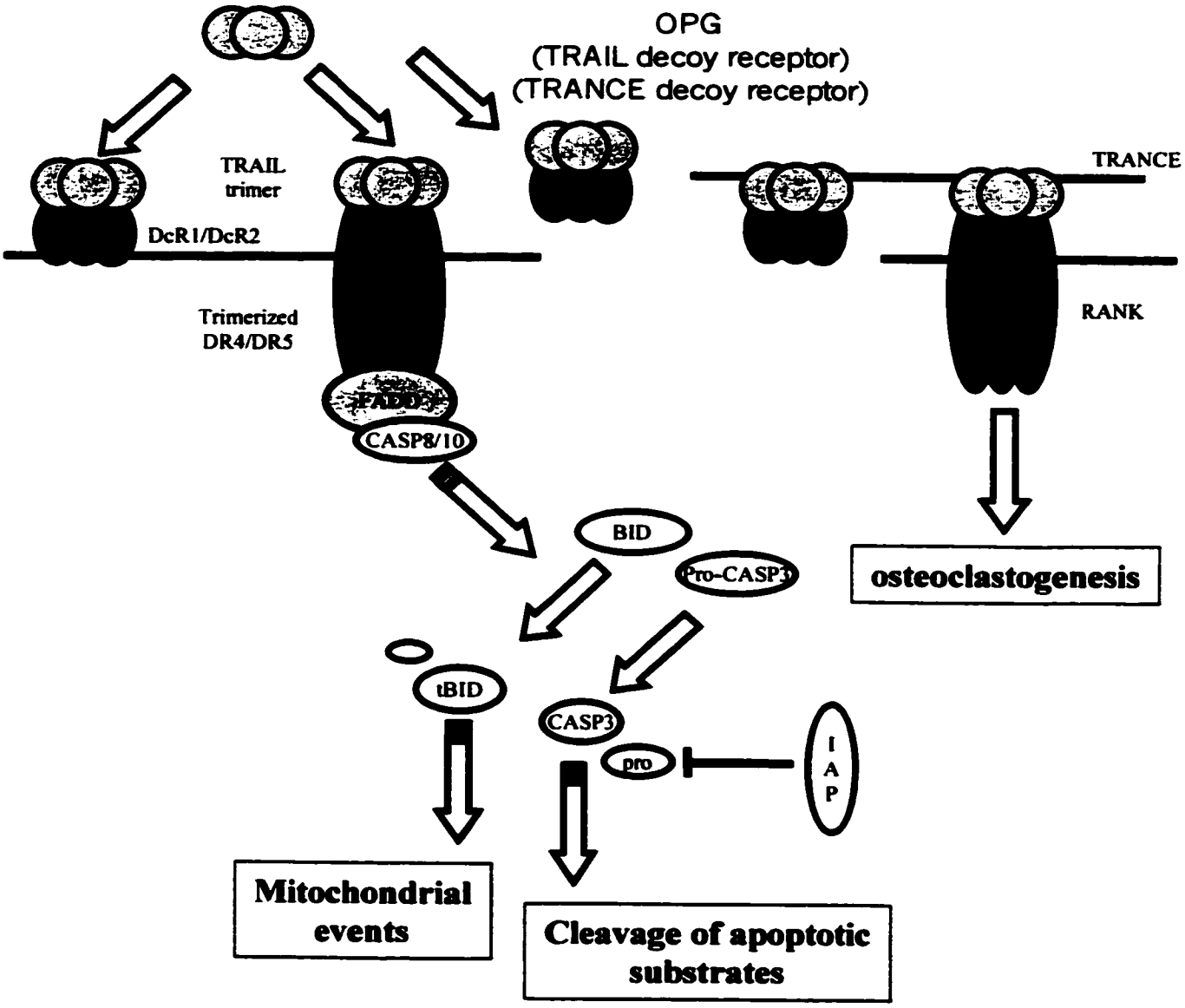


**Figure 7A: EBV-LMP1 signalling from TES1/CTAR1**

Signalling through the TES1/CTAR1 of the Epstein Barr virus Latent (infection) Membrane Protein 1 (LMP1) results in NF-κB activation. This is primarily mediated by the activity of TRAF2. Binding of TRAF2 by TANK inhibits TRAF2 mediated NF-κB activation. Signal transduction via TES1/CTAR1 is also dependent on NIK. As in signalling from TNFRI, it is presumed that NIK acts directly on the IKK complex. Data presented in the results section also implicate a role for the PI3K pathway in the activation of NF-κB from LMP1 (shaded in grey).

**Epstein Barr Virus**
**Latent Membrane Protein 1**
**(EBV-LMP1)**
**Signalling from TES1/CTAR1**

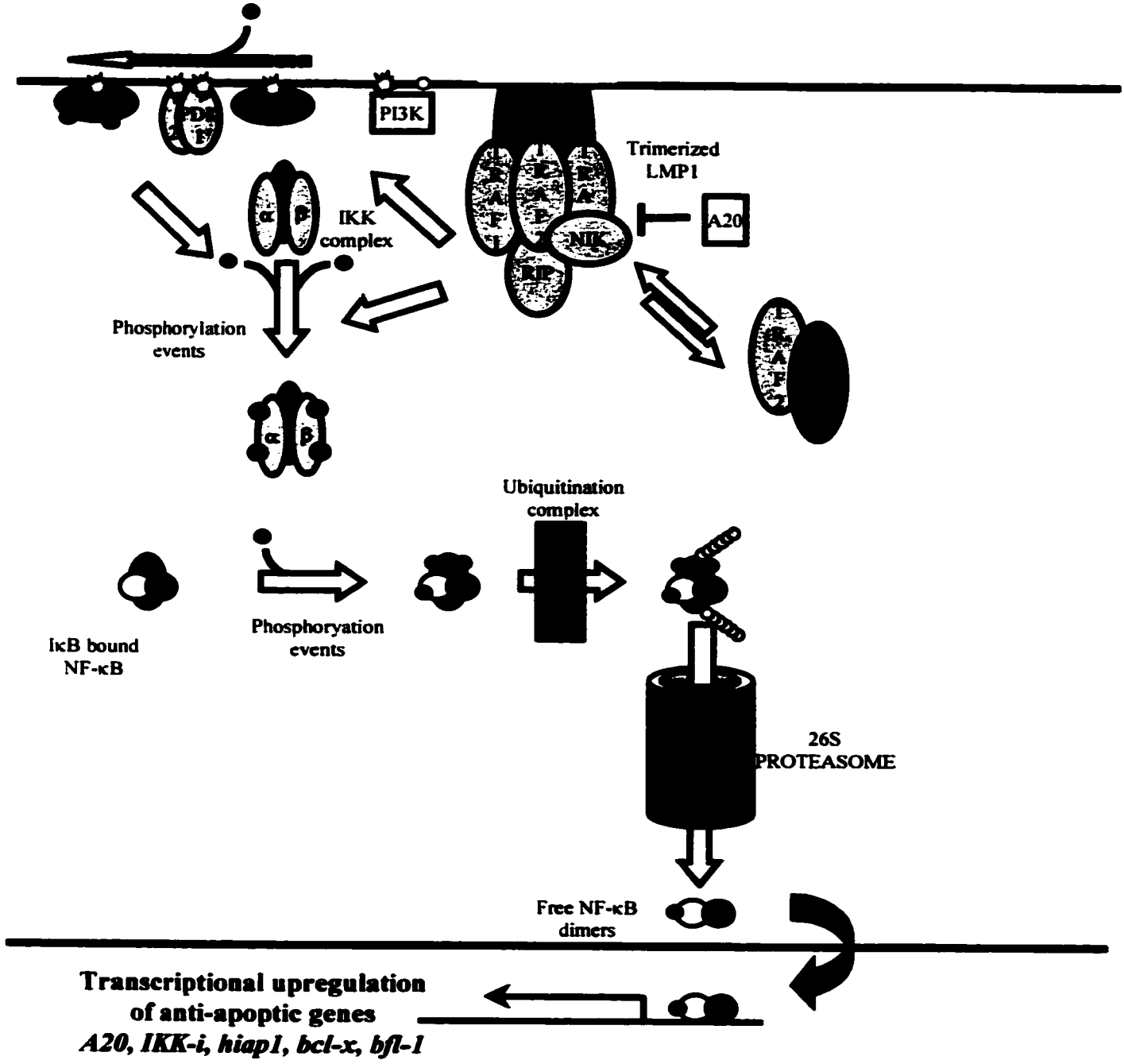


**Figure 7B: EBV-LMP1 signalling from TES2/CTAR2**

Signalling through the TES2/CTAR2 of the EBV encoded LMP1 results in both NF-κB and JNK activation. Binding of TRADD to the C-terminus of LMP1 serves to recruit NIK, RIP and TRAF2 to this region of the protein. Binding of TRAF2 by TANK inhibits TRAF2 mediated NF-κB activation but may be the basis for JNK activation via GCKR (shaded in grey). Signal transduction to NF-κB via TES2/CTAR2 is also dependent on NIK. As in signalling from TNFRI, it is presumed that NIK acts directly on the IKK complex. Data presented in the results section also implicate a role for the PI3K pathway in the activation of NF-κB from LMP1 (shaded in grey).

**Epstein Barr Virus**
**Latent Membrane Protein 1**
**(EBV-LMP1)**
**Signalling from TES2/CTAR2**

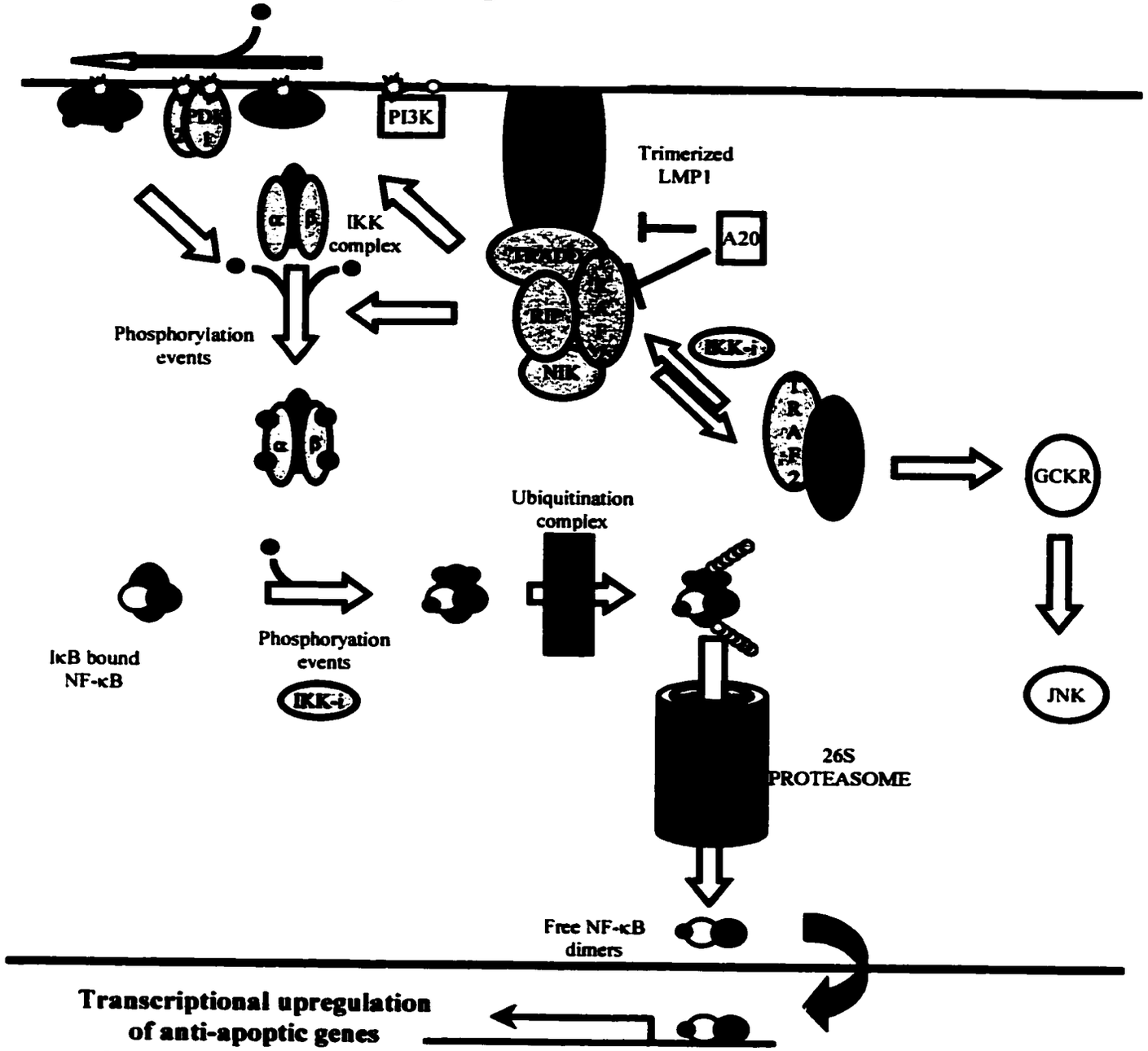
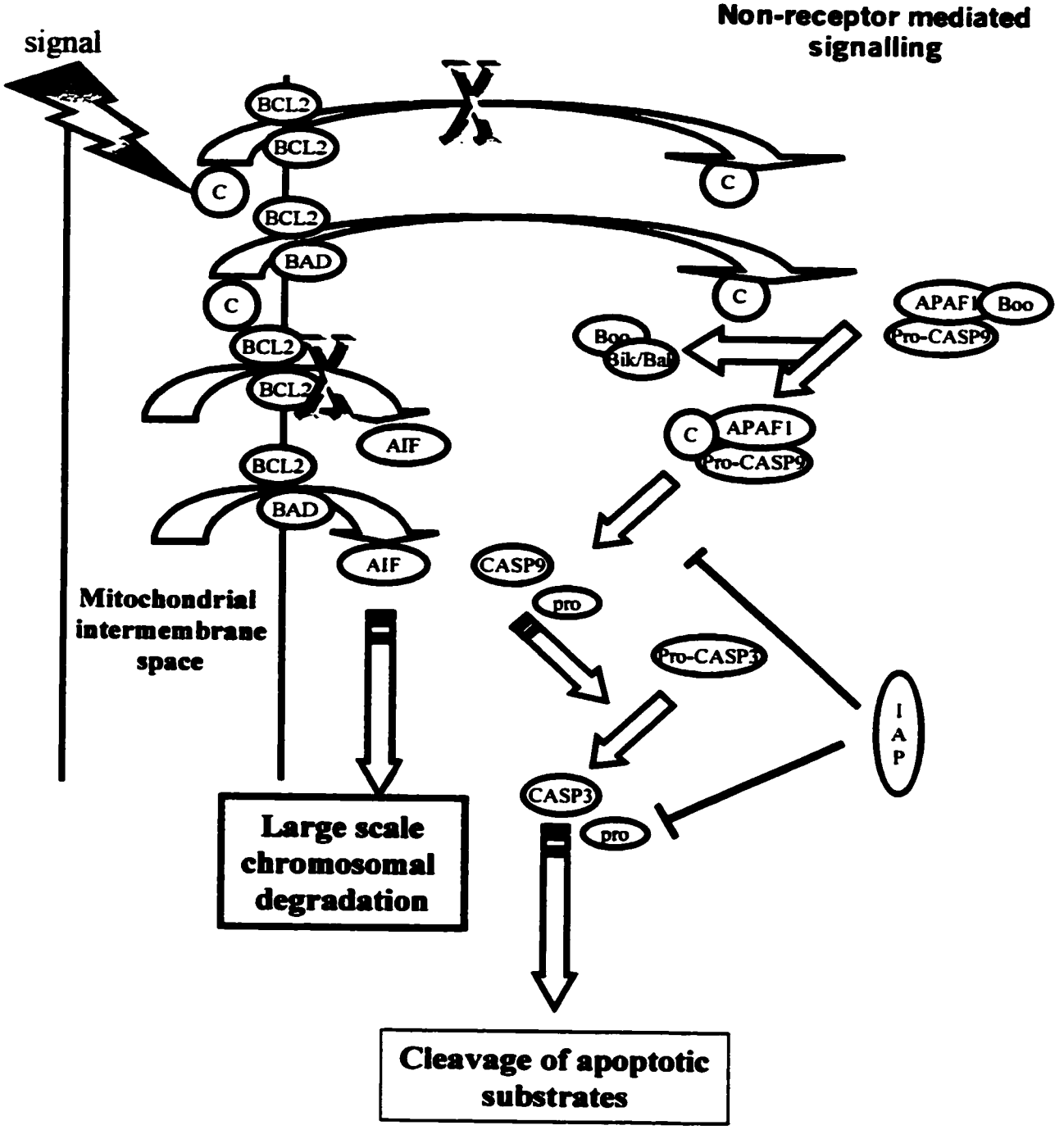


**Figure 8: Non-receptor mediated signalling**

In the resting (non-apoptotic) state cytochrome c, AIF, and proCASP3 are retained in the mitochondrial intermembrane space by the anti-apoptotic Bcl2 family members (Bcl2 in this figure). Under stimulated conditions, the anti-apoptotic members may heterodimerize (oligomerize) with pro-apoptotic members such as BAD, BID and BAX (BAD in this figure). This heteromerization is thought to affect the permeability of the pore formed by the Bcl2 homo-oligomers such that cytochrome c, AIF, and proCASP3 can exit the intermembrane space. Alternatively, the pro-apoptotic members may affect the structure and permeability of the "permeability transition pore", a loosely defined complex of both inner and outer mitochondrial membrane components (see text). Once released from the mitochondria, AIF translocates to the nucleus where it begins the process of chromosomal condensation and large scale degradation. For their part, proCASP9 and cytochrome c associate with Apaf1, where the former becomes proteolytically activated. This activation process may be inhibited by anti-apoptotic members of the Bcl2 family such as Boo and Bcl-$X_L$. Preferential association of these anti-apoptotic members with yet other pro-apoptotic members, such as Bik or Bak, may relieve this inhibition. Activated CASP9 then targets downstream caspases (CASP3 in this figure) for proteolytic activation. Proteolytic activation proCASP9 can be inhibited by HIAP1, HIAP2 and XIAP as can the proteolytic activity of CASP3.


signal
Non-receptor mediated signalling
BCL2
BCL2
C
BCL2
BAD
C
BCL2
BCL2
AIF
BCL2
BAD
AIF
C
C
C
APAF1
Boo
Pro-CASP9
Boo
Bik/Bak
C
APAF1
Pro-CASP9
CASP9
pro
Pro-CASP3
I
A
P
CASP3
pro
Mitochondrial intermembrane space
Large scale chromosomal degradation
Cleavage of apoptotic substrates

## 8.4. The Inhibitors of Apoptosis (IAPs)

### 8.4.1 The IAP genes

The mammalian Inhibitors of Apoptosis genes (IAPs) are a family of genes who's founding member, *naip*, was isolated as a potential candidate gene in the neurodegenerative disorder spinal muscular atrophy.[330] A subsequent database search, with the BIR domains (Baculovirus IAP repeat) of *naip* as a probe, revealed the presence of other members of the IAP family. These included *xiap*, *hiap1* and *hiap2*. Concomitant searches by other groups, either biochemically based or genetically based, also detected the presence of these genes. These latter members, depending on the reporting group, have been identified by a variety of names (see Table 3). Recently several other IAPs have been characterized. These include the single BIR containing proteins apollon[322](BRUCE)[323] and survivin[324](TIAP)[325] as well as another IAP, Livin, encoding a single BIR domain and C-terminal RING (zinc) finger domain.[326]

**Neuronal apoptosis inhibitory protein (*naip*)**

As stated above, *naip* was molecularly cloned as part of positional cloning effort to identify a gene whose mutation would underlie the neurodegenerative disease spinal muscular atrophy (SMA). The identification of NAIP as an apoptotic inhibitor fits well with the conceptual model of SMA[331]. During the latter stages of neuromuscular development, neurons which fail to successfully form neuromuscular junctions with their target muscle fibres die off via apoptosis.[3] In the absence of a neural specific apoptotic inhibitor, the post-natal atrophy of motor neurons would continue unchecked, leading to the SMA phenotype. Supporting a role for NAIP in the pathogenesis of SMA is the finding that NAIP-like immunoreactivity is expressed in those areas predominantly affected in SMA patients, such as the anterior horn cells of the spinal cord, the motor cortex, as well as the choroid plexus of the adult human brain.[375]

An argument against a primary role for NAIP in SMA pathogenesis is that part of a neighbouring gene, survival motor neuron (*smn*), is homozygously deleted in virtually all SMA patients whereas *naip* deletions occur in only a percentage of patients. It has also been shown that individual homozygous deletions of both *naip* and *smn* have been reported in phenotypically normal individuals, typically parents of probands[376] (proband: first/eldest identifiable individual within a family displaying a phenotype associated with a genetic disorder). Confounding the problem surrounding the discrimination between *naip* and *smn* as the "SMA gene" is the observation that the region encompassing both genes is duplicated. Furthermore, FISH analysis of chromosomes from phenotypically normal individuals has demonstrated that *naip* can be found

in as many as six copies and *smn* in as many as five. This notwithstanding, in the majority of the population, both *naip* and *smn* reside side by side in tandem at 5q13.1.[377] That two copies of both genes can be found on a single chromosome argues against the fact that deletion of one copy of *smn* and/or *naip* is enough to cause the SMA phenotype. Resolution of this comes from the observation that the centromeric copy of *naip* is a pseudogene, lacking exons 5 and 6, whereas that of *smn* (*smn(c)/smn2*) displays reduced splicing efficiency due to a C/T transition in exon 7, resulting in a reduction in mature transcript from this locus.[378],[379] It should be noted at this point that the major chromosomal abnormality affecting SMA patients is an exon 7-8 deletion in the telomeric *smn* locus. Although not now thought to be the causative defect in SMA patients, deletions in *naip* are thought to affect both disease severity and progression as they tend to be associated with the more severe forms of the disease.[9]

Studies of the *naip* gene in mice have led to the discovery that susceptibility to infection by the intracellular pathogen, *Legionella pneumophila*, is linked to the *naip* locus. Defining the exact gene involved has been hampered, though, by the fact that although *smn* is present in only one copy, *naip* can be present in as many as seven copies.[380] Of these seven, only three have the potential to be fully functional.[381] Supporting a role for *naip* in the pathogenicity of *L. pneumophila* in legionnaires disease is the finding that macrophages, the primary site of *L. pneumophila* infection, are one of the few tissues apart from the CNS which express *naip* message abundantly (Figure 14, SSY unpublished results).

**X-linked inhibitor of apoptosis (*xiap*)**

*Xiap* is located at Xq23[382], whereas its mouse homologue, *miap3*, maps to the syntenic region XA3-A5.[383] Within the coding region, the intron/exon structure is conserved between *miap3* and *xiap*[383] (M. Lagacé unpublished results). Expression of *xiap* message appears to be relatively uniform in all tissues tested[331], although it has emerged that it may be both regulated and regulate NF-κB.[384],[385] Interestingly, the *xiap* message possesses an unusually long 5'UTR. In the mouse, *miap3* is preceded by a 5'UTR of greater than 5 kb[383], whereas the 5'UTR of *xiap* exceeds 1.6 kb (M Lagacé unpublished results). In order to correctly initiate translation, it was found that the *xiap* message possesses a powerful internal ribosome entry site (IRES).[386] It is thought that by using IRES mediated translation initiation, which is CAP independent, the cell is able maintain expression of key proteins during times of stress, such as heat shock, ischaemia, UV radiation and viral infection. Indeed, several members of the picornavirus family employ IRES mediated translation initiation as a mechanism to ensure translation of their genome following infection. The picornaviral protease 2A inhibits CAP dependent protein translation by

cleaving the CAP binding domain from the translation initiation factor 4G (eIF4G). As such, it loses the ability to promote CAP-dependent translation whilst retaining the ability to promote IRES mediated translation initiation.[387]

Unlike the remaining human IAP homologues, *haip1* and *hiap2*, *xiap* possesses several pseudogenes (M. Lagacé in preparation). One of these, a processed pseudogene, gives rise to the testes specific transcript, *testes-iap*. Although possessing the full *xiap* coding region, mutations in the 5' end of the gene only allows for an abridged open reading frame beginning at BIR3[388] (M. Lagacé in preparation).

**Human inhibitor of apoptosis 1 and 2 (*hiap1* and *hiap2*)**

With the discovery of *naip* came, in short order, that of both *hiap1* and *hiap2*. Like *xiap*, *hiap1* and *hiap2* encode three BIR (baculoviral IAP repeat) domains as well as a RING zinc finger domain.[331] In addition to these, both encode a putative caspase (activation and) recruitment domain (CARD).[389] Soon after the discovery of the core IAP family came the realization that at least one member, *hiap1* (and potentially *xiap*), is regulated by NF-κB.[390] FISH (Fluorescent *in situ* hybridization) analysis has shown that both *hiap1* and *hiap2* reside at 11q22-23.[382] This finding is of significant importance as several lymphoid malignancies have been reported to bear cytogenetic abnormalities in this region. One such malignancy is that associated with the mucosa of the gut. Recent reports have implicated a *hiap1* translocation event as the etiological event giving rise to the malignancy.[355],[391] A more detailed discussion of both the lymphoma and the *hiap1* translocation follows.

**Involvement of *hiap1* translocations in the oncogenesis of MALT lymphomas**

MALT lymphomas (mucosa associated lymphoid tissue lymphoma) are a subset of marginal zone B-cell lymphomas/leukemias (MZBCL) which are themselves a subset of non-Hodgkin's B-cell lymphomas. The marginal zone comprises the outermost segment of the white pulp within the spleen.[4] Lymphomas arising within the spleen are termed splenic MZBCLs, whereas those arising from within the lymph node are termed nodal MZBCLs (also monocytoid B-cell lymphoma), and finally those arising from similar areas within the mucosa of the gut are termed extranodal MZBCLs or more recently MALT lymphomas. MALT lymphomas are usually localized and non-invasive. Typically, they arise in the small intestine, stomach (GALT, gut associated lymphoid tissue), lung (BALT, bronchial associated lymphoid tissue), salivary gland and the thyroid.

In the digestive system, MALT lymphomas are often associated with acquired lesions of the lymphoid tissue coincident with *H. pylori* infection.[392] Interestingly, MALT lymphomas of the digestive tract can be successfully treated with antibiotics.[393] These serving to eliminate the bacterial infection and consequently the local inflammatory response. Presumably it is this response which, in some way, contributes to oncogenesis.

Of particular note is the observation that the most common cytogenetic abnormality associated with MALT lymphomas is a t(11;18)(q21;q21) translocation involving *hiap1*.[391],[394] This translocation disrupts the *hiap1* gene after exons 7 or 8. The resulting fusion with MALT1/MLT encodes a BIR only protein under the transcriptional control of the intact *hiap1* promoter.[355],[395],[396],[397] It should be noted that truncated forms of the IAPs, lacking the C-terminal RZF, have been shown to possess greater anti-apoptotic properties than their full length counterparts.[32],[398] It is here hypothesized that chronic local stimulation by pro-inflammatory cytokines caused by local *H. pylori* infection induces pathological expression of a truncated form of HIAP1 leading to enhanced resistance to apoptosis. The MALT1/MLT-HIAP1 fusion (the result of the reciprocal translocation) is likely of little pathological effect, as in at least one case the translocation was not balanced. In this patient, the 3' terminus of *hiap1* and the entire *hiap2* gene was deleted during the exchange.[355] Although this potentially leaves the N-terminal portion of MALT1/MLT as the etiological agent, it is not thought to be expressed.[355]

MALT1 has recently been shown to be involved in signal transduction leading from the TRAF2 adaptor protein. Both *in vivo* and *in vitro* analysis has shown it to bind the CARD containing protein, Bcl10.[24] That MALT1 binds Bcl10 is interesting in that, by homology, MALT1 has been shown to be a caspase-like protein and that this interaction is CARD dependent. Bcl10, in turn, has been shown to bind and promote the interaction between TRAF2 and HIAP1 and HIAP2.[110] The net effect of the Bcl10/TRAF2/HIAP is reduced JNK activation, enhanced NF-κB activation and inhibition of apoptosis. Forced Overexpression of Bcl10 though, destabilises the HIAP1/TRAF2 complex and promotes lymphocyte apoptosis.[110] Coincidentally, Bcl10 translocations are themselves common in MALT lymphomas. The t(1;14)(p22;q32) translocation disrupts the Bcl10 promoter and places the gene under the control of the immunoglobulin heavy chain locus.[399] Alternatively, t(1;2)(p22;p12) brings Bcl10 into the vicinity of the immunoglobulin kappa light chain locus.[400] Of note, Bcl10 is frequently mutated in many of the translocations reported thusfar. Truncating mutations occurring before the caspase recruitment domain fail to activate NF-κB or promote apoptosis whereas CARD containing mutants retain the ability to activate NF-κB yet do not promote apoptosis.[399],[401] Curiously, in

the absence of mutation, the translocation of wild type Bcl10 to an immunoglobulin locus would promote apoptosis due to enhanced expression.[110][401]

It is of interest to note that, under non pathological conditions and expression patterns, the CARD protein, Bcl10 binds MALT1 and presumably recruits it to the HIAP1-TRAF2 complex where it promotes activation of NF-κB.[24] The HIAP1/MALT1 fusion conceivably bypasses the requirement for Bcl10 in the signalling complex by directly recruiting MALT1. It remains to be determined what role or substrates exist for the paracaspase MALT1.

### 8.4.2. The IAP Proteins

All of the core group of IAPs, namely *naip*, *xiap*, *hiap1* and *hiap2*, code for proteins with three N-terminal BIR domains. These are thought to mediate protein-protein interactions with a variety of targets including caspases and TRAF proteins. All of the core group, with the exception of NAIP, also possess a C-terminal RING zinc finger motif which has been proposed to function as a E3-ubiquitin ligase.[329] A third domain, the caspase recruitment domain (CARD), has been ascribed to both HIAP1 and HIAP2.[389] In addition to these are the single and double BIR containing proteins from yeast species and *C. elegans*.

#### Neuronal Apoptosis Inhibitory Protein (NAIP)

NAIP is an approximately 150kDa protein possessing three N-terminal BIR domains and a long C-terminus containing a potential ATP/GTP binding site. Apart from this site, the C-terminus does not share homology with any other protein. Surprisingly, given its potential involvement in the pathology of spinal muscular atrophy[330] and apoptosis[331], very little is known about the nature NAIP, what its binding partners are, or what its molecular role is in the cell.

Recently, it has been reported that, unlike the remaining core IAP members, NAIP is unable to function as a caspase inhibitor.[327] This finding, though, is not universally accepted (J Maier in preparation).

#### XIAP and testes-IAP

XIAP was initially characterized by virtue of its similarity to NAIP. In addition to the three BIR domains encoded *naip*, *xiap* also encodes a C-terminal RING finger domain.[331] The anti-apoptotic activity of XIAP[331] may in large part be due to its ability to directly inhibit the activation of both CASP3 and CASP7.[402] Interestingly, direct inhibition seems to be mediated by the BIR domains alone. These inhibitory BIR fragments appear to be generated during the

apoptotic process by caspase or calpain mediated cleavage of XIAP[403] (H. Gibson unpublished data). Furthermore, XIAP, HIAP1 and HIAP2 have recently been shown to mediate their own degradation via an intrinsic E3-ubiquitin ligase activity.[329] Like the latter two, this activity is localized to the RING finger domain of the protein.[329] Although no XIAP ubiquitination targets have arisen, save XIAP itself, homology to the HIAP1 activity indicates a possible role in the ubiquitination of caspase family members.[404]

In addition to its connection to caspase inhibition, XIAP has also been implicated in BMP (bone morphogenic protein) signalling. By serving as a bridge between the bound BMP receptor and the TAB1/TAK1 (TAK1 binding protein 1/ TGFβ activated kinase 1) pair, XIAP may facilitate BMP mediated upregulation of NF-κB.[405] In support of this, XIAP has been shown to co-localize with both TAK and the IκB kinase complex.[385]

Inhibition of XIAP function has been demonstrated by DIABLO/Smac, a functional homologue of the *D. melanogaster* proteins HID, GRIM, and REAPER. REAPER has been shown to bind DIAP1 thereby preventing its association with either drICE (Drosophila ICE) or DRONC.[334] In a homologous fashion, DIABLO/Smac is able to antagonize IAP-CASP3 binding, and in so doing, liberate the latter from IAP mediated inhibition.[336],[337] Similarly, a novel protein denoted XAF (XIAP associated factor) appears to mediate much of the same effects.[406],[407]

**HIAP1 and HIAP2**

The signalling pathway to which HIAP1 and HIAP2 contribute was first ascertained in 1995, when it was found that, via their BIR domains, they bind two members of the TRAF protein family in association with the TNFRII.[60] Subsequent investigations have shown that, by virtue of their interaction with TRAF2, HIAP1 and HIAP2 may also contribute to TNFRI signaling.[50],[408] Conceivably, the action of both HIAP1 and HIAP2 at the plasma membrane may be steric in nature. Independent of their involvement in preventing receptor mediated apoptosis, both HIAP1 and HIAP2 have been shown to inhibit apoptotic signalling pathways originating from the mitochondria, as exemplified by their ability to prevent etoposide induced apoptosis.[409] Whereas Bcl2 is able to accomplish this by preventing the necessary release of cytochrome c from the mitochondrion, HIAP1 and HIAP2 appear to directly inhibit the activation of CASP3 and CASP7.[327] Furthermore HIAP1 and HIAP2 may indirectly inhibit the activation of CASP8 and CASP9.[409]

A unique feature of HIAP1 and HIAP2 is the presence of a caspase (activation and) recruitment domain (CARD)[389]. CARD containing proteins include Apaf-1 and its homologues

( Ced4, DARK/HAC1/dApaf-1), as well as RAIDD, RIP2, and BCL10 (a translocation partner in MALT lymphomas). CASP2, CASP9, and CED3 have also been shown to possess CARD (domains). In most cases, the CARD is thought to mediate homotypic interactions between the proteins. It may be that the CARD allows the heterodimerization of HIAP1 or HIAP2 to non-caspase targets.

Until recently, the role of the RING (zinc) finger motif at the C-terminus of XIAP, HIAP1 and HIAP2 was not known. It had been previously shown that, by eliminating this domain, the anti-apoptotic activity of several family members could be increased.[32] It has now been shown that the RING finger is critical in mediating the E3-ubiquitin ligase activity of these members. It has equally been shown that both HIAP2 and XIAP (and possibly by extension HIAP1) are able to mediate their own polyubiquitination.[329] Should this ubiquitination be a trans effect, it would be concentration dependant and may serve to limit their accumulation. Signal induced processing of the IAP in question could eliminate the E3 function thereby allowing for its greater accumulation and, hence, greater anti-apoptotic activity. For its part, the RING finger of HIAP1 in isolation has been shown to promote the mono-ubiquitination of CASP3 and CASP7 and may, therefore, possess some intrinsic E2 activity (ubiquitin conjugating enzyme).[404]

**Single BIR containing proteins**

Similar in structure to the testes-IAP described above, the IAP Livin contains a single BIR domain and a RING finger motif. Livin is expressed in a variety of tissues early in development but appears restricted to the placenta in the adult. Expression of Livin has also been observed in cancer cell lines. Like XIAP, HIAP1, HIAP2 and NAIP, the anti-apoptotic activity of Livin resides with its ability to inhibit caspase activation in a BIR dependent manner (CASP 3, 7, and 9).[326]

One of the more interesting discoveries made of late is that XIAP, HIAP1 and HIAP2 can function as E3-ubiquitin ligases. Complementing this finding is that a single BIR containing protein, Apollon[322] and its mouse counterpart, BRUCE[323] (BIR repeat containing ubiquitin conjugating enzyme), are functional E2-ubiquitin conjugating enzymes. It is therefore tempting to speculate that the IAPs may be able to interact with BRUCE to promote the degradation of target proteins.

TIAP (testes/thymus IAP) and survivin also encode single BIR containing proteins from mouse and human respectively.[324], [325] Survivin(TIAP) has been localized to the mitotic spindle where it has been proposed to prevent the initiation of a default apoptotic pathway at this critical juncture of the cell cycle. Although Survivin(TIAP) possesses no intrinsic enzymatic activity, it

has been shown to inhibit the activity of CASP3.[325] Overexpression of survivin is a poor prognostic indicator in several malignancies.[410],[411],[412],[413],[414] This point may lead one to conclude that ectopic expression of survivin may promote oncogenesis by preventing the mitotic catastrophe normally associated with aberrant progression through, or initiation of, the cell cycle. Interestingly, survivin is extensively homologous to the non-coding strand of the Epr-1 (effector cell protease receptor 1) gene, the cellular receptor for clotting factor Xa.[415] An examination of the Survivin/Epr-1 locus has suggested that the Epr-1 and Survivin genes arose by tandem duplication of an ancestral gene and that they are expressed from distinct genes and in opposing directions.[416] Conceivably, expression of survivin, apart from its inherent anti-apoptotic capacity, may contribute to the transcriptional regulation of Epr1 or, conversely expression of Epr1 may affect expression of survivin.[416] This is supported by the observation that expression of Survivin/Epr-1 is mutually exclusive.[416] The *Caenorhabditis elegans* encoded BIR-1 protein can also be grouped with TIAP/survivin in that it possesses only a single BIR domain. Embryos lacking BIR-1 failed to complete cytokinesis and, as such, their cells became multinucleated.[417] Another single BIR containing protein, deterin, is encoded by *D. melanogaster*. Little is known about the cellular localization or function of deterin other than, like survivin and the core IAPs, it is able to inhibit the activation of CASP3.[418]

A third group of BIR containing proteins are encoded by the yeast species *Schizosaccaromyces pombe* and *Saccharomyces cerevisiae*, as well as by *C. elegans*. Although these proteins, denoted bir1[419], BIR1[419] and BIR-2[417] respectively, possess two BIR domains instead of the single domain encoded by *tiap/survivin*/BIR-1, they appear to function in similar pathways. Overexpression of BIR1 in *S. cerevisiae* caused a more rapid progression through the cell cycle, whereas deletion of any of these inhibited cytokinesis and cell cycle progression.[419]

### 8.5. Summary

Apoptosis has developed in multicellular organisms to accomplish several tasks. Among these, one finds embryonic sculpting, maintenance of several tissues such as the epidermis, the various epithelial linings and cells of the immune system. As well, apoptosis is used to eliminate excess, unwanted, damaged, infected or malignant cells. In order to regulate apoptosis, a wide spectrum of signalling molecules has evolved. Central to the present work are those whose function is to modulate signal transduction events originating with the TNF receptor superfamily.

Although apoptosis can be initiated by a variety of mechanisms, there appears to be two major pathways by which the cell enters into its final stages. The first, and arguably the oldest, pathway commences at the mitochondrion with the release of cytochrome c (as well as AIF and

CASP3), which then in turn serves as a cofactor with Apaf1 in the activation of CASP9. This pathway appears to be regulated both at the mitochondrial level and at the level of Apaf1 by both the pro- and anti-apoptotic members of the Bcl2 family. The second pathway(s) originates at the plasma membrane with the TNFR superfamily. In likely the most direct path from the plasma membrane to apoptosis, ligation of CD95 (Fas receptor) by FasL serves to activate the apical caspase, CASP8, via the intermediary FADD. This activation can be regulated by the competitive binding to FADD of the CASP8 like decoy FLIP/CASPER. Furthermore, a decoy FasL receptor has been described. Alternatively, CASP8 can be activated by recruitment to trimerized TNFRI, and its functional homologues DR3 and DR6, via association with TRADD bound FADD. Two other caspases, CASP1 and CASP2, have also been proposed to localize to TNFRI via their association with RIP family members. Perhaps limiting or regulating the activity of TNF two decoy receptors have been described for TNFRI. Regardless of the mechanism by which the caspase cascade is initiated, the ultimate endpoint of both pathways is the activation of CASP3; generally thought to be the main executioner of apoptosis.

Although activation of the caspase cascade can be pre-empted by the variety of mechanisms described above, once initiated, the cascade may also be quenched by caspase binding of many, if not all, of the IAPs including the single BIR domain proteins survivin and deterin. The first signal transduction pathway associated with the IAPs was that of the TNFRII. Association of HIAP1 and HIAP2 to the TNFRII occurs via the adapter proteins TRAF1 and TRAF2. It has since been shown that HIAP2 can also be found in association with TNFRI and that both HIAP1 and HIAP2, in association with TRAF1 and TRAF2, are required for inhibition of TNFα mediated cell death. Given their role as caspase inhibitors, it is likely that HIAP1 and HIAP2 function at the plasma membrane to inhibit the activation of CASP8 and that some, or all of the core IAPs, may function to inhibit CASP9 activation subsequent to cytochrome c release.

An alternative signalling pathway from the TNFR family involves not caspase activation but rather that of various transcription factors including, but not limited to, NF-κB, and c-jun. Transcription factor activation is likely the major mechanism of action of the remaining (non death domain containing) TNFR superfamily members. All of the signal transduction potential of these receptors seems to lie with the ability of the various TRAF family members to associate with downstream signalling kinases. Co-operative signalling though the TRAF2/RIP/NIK/IKK pathway and the PI3K/PKB/IKK pathway serve to promote NF-κB activation and thence the transcription of several anti-apoptotic genes, including Bcl-$X_L$[420], Bfl-1[421], A20[108],[422], *hiap1*[423],[409],[424] and potentially that of *hiap2*[409] and *xiap*[384] as well.

### 8.6. The present work

In the present study, the complete genomic structure of *hiap1* and *hiap2* was determined. This was accomplished by genomic library screening and subsequent restriction analysis of the resulting phage clones. A contiguous array of clones encompassing the entire *hiap* locus was thus generated. The accuracy of the array was assured by comparative restriction analysis of phage clones, genomic DNA, as well as a single bacterial artificial chromosome (BAC). Furthermore, the intron/exon structure of each gene was ascertained by sequence analysis of both the coding region cDNA and the corresponding genomic DNA as well as by RACE (both 5' and 3'). When appropriate, transposon mediated genomic sequencing[425] was carried out in order to determine the genomic sequence of a given region.

Following the genomic characterization, investigations concerning the method of translation initiation were carried out. This was performed mainly by utilizing a dicistronic construct into which was inserted various fragments of the 5'UTR proximal to the translation initiation site of each of the genes. The data generated demonstrated that although it is unlikely that translation of either *hiap1* nor *hiap2* is initiated by traditional ribosome scanning or ribosomal re-initiation, neither is it initiated by internal ribosome entry as we know it.

Work by others demonstrated that the level of *hiap1* could be regulated by the activity of NF-κB. The effects of various drugs, including TNFα, cycloheximide, puromycin, and pyrrolidine dithiocarbamate, on the level of *hiap1* transcription indicated that NF-κB directly influenced the rate of *hiap1* transcription. The identification and characterization of several transcription factor binding sites, including two NF-κB sites, within the 5' region of *hiap1* further emphasized the role of this transcription factor in the regulation of *hiap1* gene expression.

During the course of this work, it was observed that both *hiap1* and *hiap2* were deleted in a derivative strain of the BJAB Burkitt's lymphoma cell line. This allowed for some initial investigations into the potential role of HIAP1 and HIAP2 in TNFα mediated signal transduction. From these experiments, it was observed that, in the absence of these genes, TNFα inhibited the growth of this cell line, an effect which could be reversed by the transient re-introduction of *hiap1*.

## 9. Results

### 9.1. Genomic organization

In order to determine the structure of the genomic region surrounding both *hiap1* and *hiap2*, a genomic southern blot analysis was carried out. Using the previously isolated *hiap1* coding region cDNA to generate probes, the size of *EcoRI* restriction fragments containing both *hiap1* and *hiap2* coding region exons were determined. From these data, it was found that the coding regions were dispersed among 5 fragments of 9, 8, and 6.5, 6, and 0.75 kbp in size (Figure 9).

Following the Southern blot analysis, a human genomic λ-phage library was screened with the *hiap1* cDNA in order to obtain phage clones spanning the genomic locus. Restriction analysis of the resulting clones with a variety of enzymes followed by comparative alignment of their respective restriction fragments generated a probable contiguous phage array. The continuity of the array was assured by Southern blot analysis of the various phage clones. By utilizing the *hiap1* coding region as a probe against the contiguous array, two regions of hybridization were observed corresponding to *hiap1* and, by cross-hybridization, *hiap2* (Figure 10). Sequence analysis of both the *hiap1* and *hiap2* cDNAs revealed several restriction sites that when mapped to the array served as a benchmark for that particular region of the gene. From this, it was observed that *hiap1* and *hiap2*, in fact, lie in tandem on the genome, thus confirming the results obtained by FISH analysis.[382]

A single bacterial artificial chromosome (BAC) clone encompassing both the *HIAP1* and *HIAP2* genes was obtained by PCR-based screening of a BAC library using 3'UTR specific primers for each of *HIAP1* and *HIAP2* (Genome Systems, BAC control #15633). Long range mapping (*EagI*, *PmeI*, *PacI*, *NotI*, *FseI*, and *AscI*) was accomplished by restriction digestion of BAC derived, as well as genomic DNA, followed by pulsed field gel electrophoresis and Southern blot analysis (Figure 10).

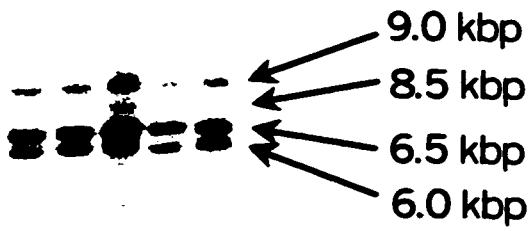
Following restriction mapping, the appropriate genomic phage clones served as templates for intron/exon boundary determinations. Sequencing primers were designed from within the previously determined coding region. Divergence of the genomic sequence from that of the known cDNA was presumed to be indicative of a coding region intron. Sequence comparison of the coding region intron/exon boundaries revealed that all are exactly conserved between all of *hiap1*, *hiap2*, and *miap1*. This also holds true for *miap2* save for the boundary between exons 6 and 7 (Table 5)[426].

In order to obtain genomic sequence information of the regions encompassing the terminal regions of *hiap1* various genomic phage fragments corresponding to these were subjected to transposon mediated sequencing.[425] These results then serve as a baseline for comparison to

data generated by 3'RACE (rapid amplification of cDNA ends), 5'RACE and primer extension. Expressed sequence tags (ESTs) and data obtained by 3'RACE indicate that up to 5 polyadenylation signals are used to generate the *hiap1* message(s). These sites generate 3'UTR lengths of 611, 1007, 2209, 2290, and 2803 nucleotides respectively. 5'RACE results indicate that the 5'UTR is divided amongst three exons. Divergence of the 5'RACE derived sequence from that of the genomic sequence was presumed to be indicative of a 5'UTR intron. Exon 2 is of 87 nucleotides in length and appears to be alternatively spliced. This was evidenced by the isolation of several cDNA clones which either contain or lack this exon. Furthermore, unlike *hiap2*, *hiap1* does not appear to commence at a specific site. Whereas primer extension analysis of *hiap1* did not generate a discreet band, both 5'RACE and cDNA sequencing results indicate that transcription initiation sites are dispersed over approximately 40 bp and that transcription likely proceeds from a TATA-less promoter. The maximum length of the 5'UTR has thus been determined to be 4568 nucleotides (4481 nt excluding exon2). Taken together, these data indicate that *hiap1* is carried on messages of 6990, 7386, 8588, 8669 and 9182 nucleotides. Alternative splicing of exon 2 would reduce these lengths by 87 bp. Northern blot analysis of *hiap1* reveals that the second polyadenylation signal, generating a message of ~7.3 kb, is generally not used. Given the resolution of the technique, Northern analysis failed to distinguish between the higher molecular weight messages. As such, no information on their relative abundance could be determined (Figures 10, 11, and 12).

In an experimental design similar to *hiap1*, the UTR structure of *hiap2* was determined by a combination of transposon mediated genomic sequencing, 3'RACE, 5'RACE (Figure 12), as well as primer extension experiments (Figures 12 and 13). When taken together, these results indicate use of a single polyadenylation site, giving rise to a 3'UTR of 470 nucleotides, whereas the 5'UTR is of 2286 nucleotides. Furthermore the 5'UTR is divided into two exons. These data indicate a total message length for *hiap2* of 4547 nt. This is in agreement with the size of the *hiap2* message as observed by Northern blot analysis (Figure 11).

**Figure 9: *hiap1* and *hiap2* genomic Southern blot.** Lanes 1-5 *EcoRI* digest of normal human genomic DNA. Probe=*hiap1* coding region.


9.0 kbp
8.5 kbp
6.5 kbp
6.0 kbp


0.75 kbp

**Figure 10: Physical and organizational map of the *hiap* locus.** Panel A: Physical map. Black rectangles correspond to *EcoR1* fragments containing coding region exons as observed by Southern blot (scale and endonuclease legend: upper inset). Panel B: Genomic organization of *hiap1* and *hiap2*. Location of exons are indicated by black rectangles. Exon length and number is indicated below each (scale: lower inset).

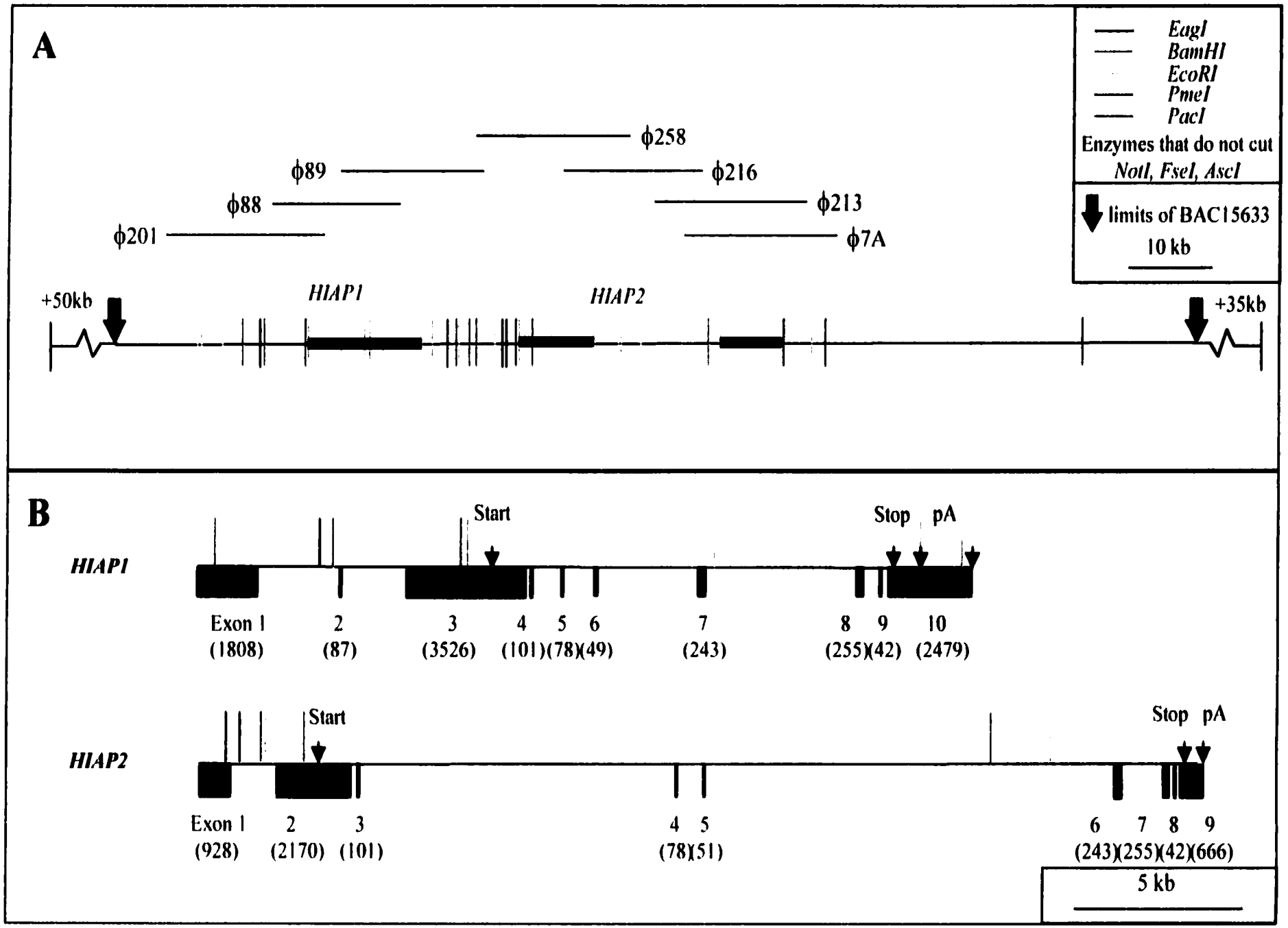
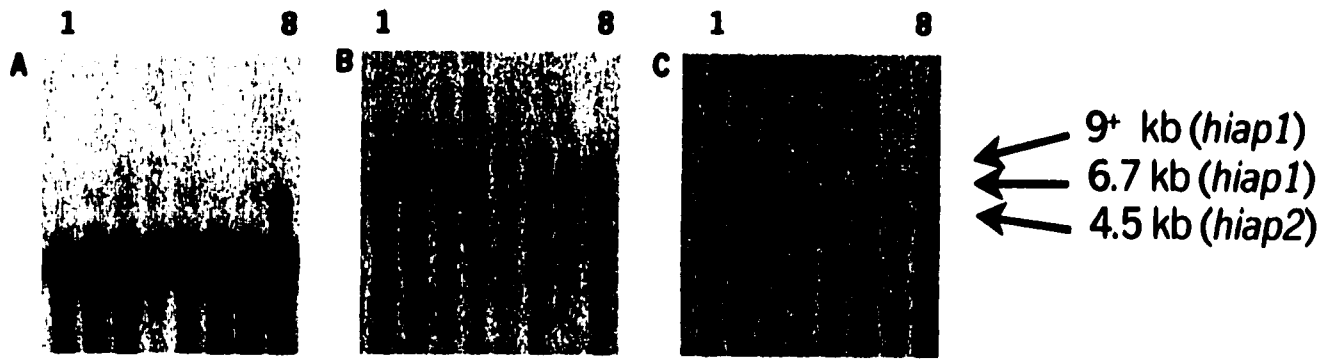

A
EagI
BamHI
EcoRI
PmeI
PacI
Enzymes that do not cut
NotI, FseI, AscI
limits of BAC15633
10 kb
ϕ258
ϕ89
ϕ216
ϕ88
ϕ213
ϕ201
ϕ7A
+50kb
HIAP1
HIAP2
+35kb
B
HIAP1
Start
Stop
pA
Exon 1 2 3 4 5 6 7 8 9 10
(1808) (87) (3526) (101) (78) (49) (243) (255) (42) (2479)
HIAP2
Start
Stop
pA
Exon 1 2 3 4 5 6 7 8 9
(928) (2170) (101) (78) (51) (243) (255) (42) (666)
5 kb

**Figure 11: *hiap1* and *hiap2* Northern blots.** Panel A: Actin control; probe=actin coding region. Panel B: *hiap1* and *hiap2*; probe=*hiap1* coding region. Panel C: *hiap2*; probe=*hiap2* 3'UTR. Data by Charles A. Lefebvre




Actin control

*hiap1* and *hiap2*

*hiap2* (specific)

1. Spleen
2. Thymus
3. Prostate
4. Testis
5. Ovary
6. Small Intestine
7. Colon
8. Peripheral blood leukocytes

**Figure 12: *hiap1* and *hiap2* RACE.** Size and position of RACE products are as indicated. The numbers beside each product identify the individual clone. PE = primer extension product. Black vertical lines indicate exon boundaries. Red vertical lines indicate translation start and stop sites. U45878, OU20D10.x1, and AS90A01.xl are dbEST accession numbers.

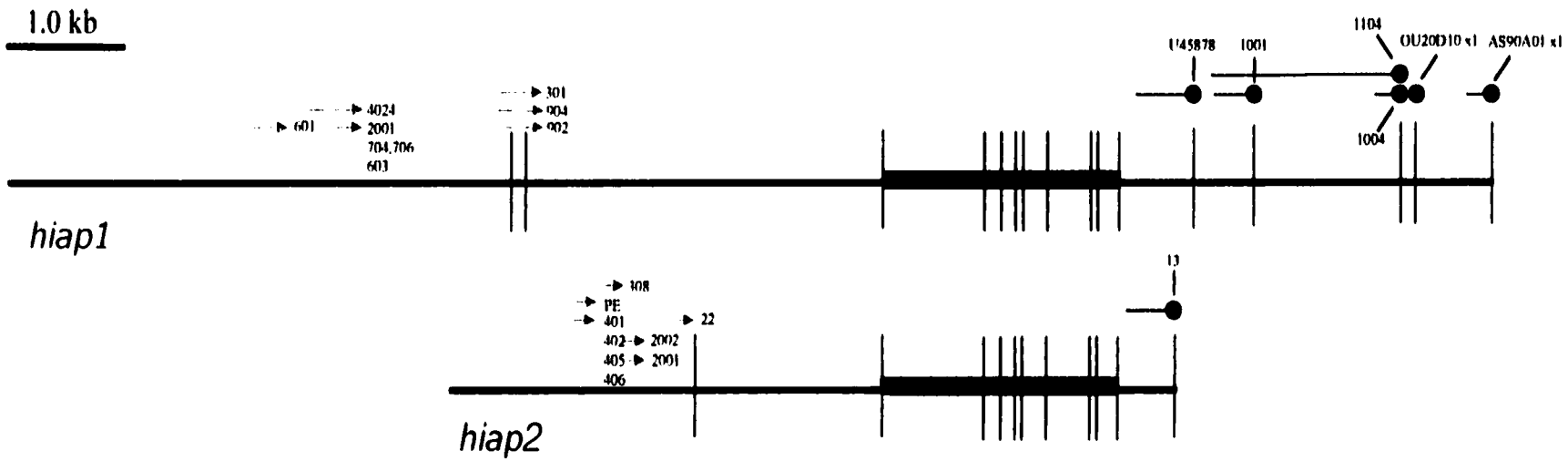

1.0 kb
1104
1145878
1001
OU20D10 x1
AS90A01 x1
4024
601
2001
704,706
603
301
004
002
1004
hiap1
13
308
PE
401
22
402
2002
405
2001
406
hiap2

**Figure 13: *hiap2* primer extension.** Extension was performed as described in the Methods section. The reverse primer was located 614 to 634 nucleotides upstream of the 5'UTR intron/exon boundary of *hiap2*. Reverse transcription from this primer resulted in a band of 321 bp (arrow, lane 5). Lanes 1–4 are the dideoxy sequencing products used to ascertain the transcription start site (T, G, C, A respectively).

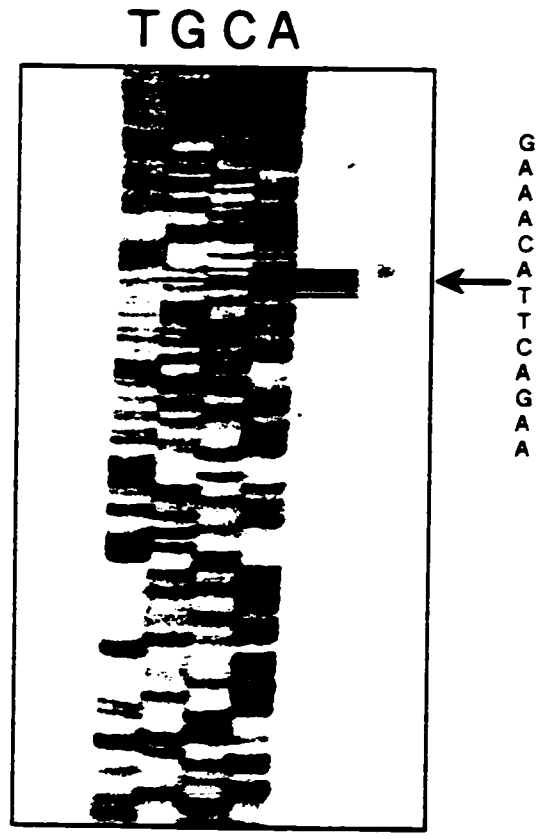

TGCA
GAAACATTCAGAA

**Table 5: Intron/Exon boundary sequences for *miap1*, *hiap1*, *hiap2*, and *miap2*.**
Exon numbering is as for *hiap1*. N/A = not applicable. *hiap1* transcription termination signals arising from polyadenylation readthrough are indented. Mouse data from Liston *et al*, 1997[426]

| | donor site | | acceptor site |
|---|---|---|---|
| *miap1* | | | 1. TGGGAGTTCC |
| *hiap1* | | | TCAGATGATC |
| *hiap2* | | | AGACGTATAT |
| *miap2* | | | AAAAGTAAAT |
| 1. | | N/A | 2. |
| | TGGGCAGCAG | gtgggcaagg..tggagtgcag | TGGTGTGATC |
| | | N/A | |
| | | N/A | |
| 2. | GTGGGCTGCG | gtgagtacgc..tcttatttta | 3. GTATCAGCCT |
| | GGAAATACAG | gtgcgtgcca..ttttgaacag | GTTTACAAAG |
| | CCGCACTGCG | gtgagtgctg..ttcttttcag | ATTTACAACC |
| | | N/A | |
| 3. | TATTATACAG | gtaagaaaca..tgaattttag | 4. GACACAGTGA |
| | TATTATGTGG | gtaagaaact..ataattttag | GTAACAGTGA |
| | TATTATGTGG | gtaagaagca..tttcctgaag | GTCGCAATGA |
| | | N/A | |
| 4. | GGTTTCCAAG | gtaacggctt..gatgctttag | 5. GTGTGAGTAC |
| | GGTTTCCAAG | gtaaatgttt..tacattttag | GTGTGAGTAC |
| | GGTTTCCAAG | gtaattgttt..tatgttttag | GTGTGAGTTC |
| | GGTTTCCAAG | gtaattggtt..cacattttag | GTGTGAGTTC |
| 5. | ACTTGAGCAG | gtaggaggcc..tttcctgtag | 6. CTATTATCTA |
| | ACTTGAACAG | gtagggcaag..ttttctgcag | CTGCTATCCA |
| | TCTTGAACAG | gtaaatacat..cttgtttcag | CTGTTGTCAA |
| | TCTTGAGCAG | gtaaactgat..tatttcatag | CTGTTGTCCA |
| 6. | GACGC------AGCAA | tatgtataat..tccatatagt | 7. TCGTGCATTT |
| | GAGTC------ATCAA | gtaagtacaa..ttccatatag | TTATCCATTT |
| | GACCC------ACCAA | gtatgtatga..tttcaaatag | TTATTCATTT |
| | GACCCTACAG | gtaagtagga..atttttttag | AGACAGTGGTGCATTT |
| 7. | GAGGAGTCAG | gtacagagta..cttcataaag | 8. ATGATCTAGC |
| | AAAGAATCAA | gtatgtagat..ttttataaag | ATGATTTATT |
| | ATGGCATCAG | gtatttgggg..tttaatgaag | ATGATTTGTC |
| | ATGGCATCAG | gtatccacgt..ttaaatgaag | GTGACTTATC |
| 8. | GATATATTTG | gtaagtgctt..cctcccccag | 9. TGCAACAGGA |
| | CATTTATTTG | gtgagtgata..tctcccttag | TGCAACAGGA |
| | AACTTATTTG | gtgagtttgt..ctttcctcag | TGGATAAGAA |
| | AACTTATTTG | gtgagtttgt..ctttcttcag | TGGAAAAGAA |
| 9. | GACATTGCAG | gtaatggcgc..ctctttacag | 10. CTCTACCAAT |
| | GATGTTTCAG | gtaatagtac..ctctttctag | ATCTACCAGT |
| | AGAGTTTCAG | gtaaaacaaa..ttgttattag | GTCTGTCACT |
| | GACGTTTCAG | gtaaaataga..ctgttctcag | GCTTGTCATT |
| 10. | (AATAAA)GCCACATAATTACCTCTTTTTTTTTAAAG | | |
| | (AATAAA)GCAACAAAAATTACTCTT | | |
| | (AATAAA)TCTGTTTCATATTGTCA | | |
| | (AATAAA)TAGCTATTATAACC | | |
| | (AATAAA)TCACCATAGATAAT | | |
| | (AATAAA)TAATTAAAAAGAAAGAAAA | | |
| | (AATAAA)GTGCTTTAAAAAG | | |
| | (AATAAA)TCTTTTTAAAAAGTGT | | |

### 9.2 Gene expression analysis

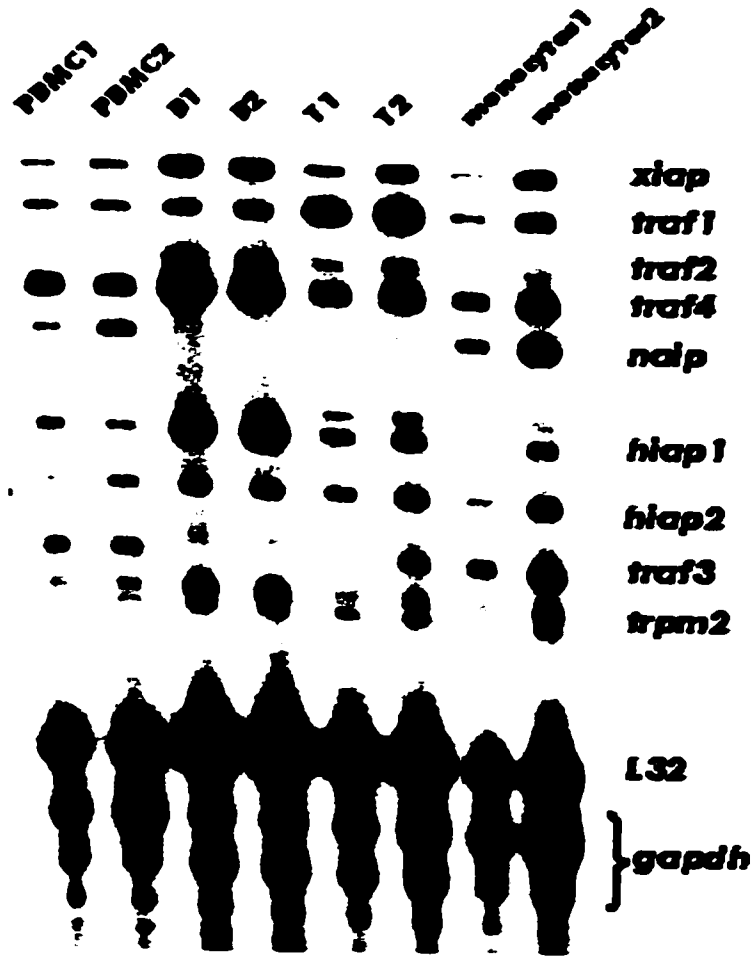
Following the isolation of the *hiap1* and *hiap2* open reading frames. the tissue distribution of the various IAP family members were determined by northern blot analyses. These data show a significant expression of both *hiap1* and *hiap2* in tissues of the immune system such as the spleen and thymus (Figure 11). In order to determine more quantitatively the tissue distribution of *hiap1* and *hiap2*, multiple tissue expression arrays (dot blots) were probed with a 3'UTR fragment of each gene. Specificity of the probe was assured by hybridization to a commercially obtained Northern blot (data not shown, Clontech MTN). Data from these experiments confirm the above observations and include kidney, liver, small intestine and lung as tissues with high expression. Conversely, expression appears to be lowest in tissues of the central nervous system. The data also indicate that, with a single exception, the expression of *hiap1* is higher than that of *hiap2*. Furthermore, the tissue distribution of each appears to be similar (Table 6).

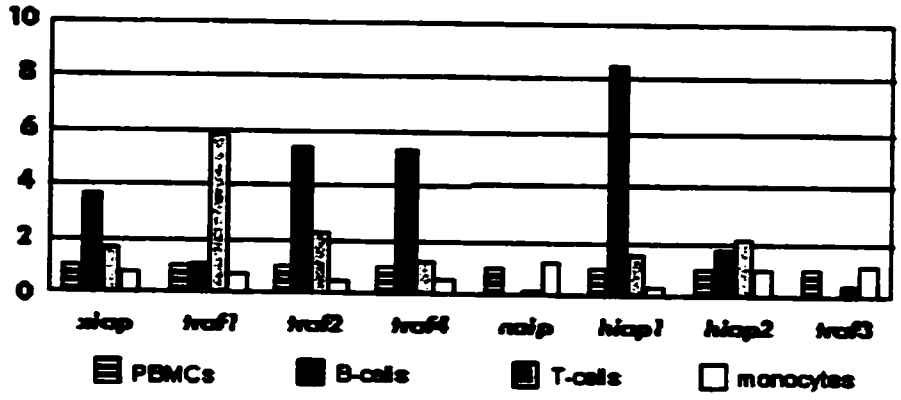
Next, the relative level of expression of the various IAP and TRAF messages in peripheral blood B-cells, T-cells and monocytes was determined. Ribonuclease protection assay of the isolated cell subsets demonstrated that expression of *hiap1* appears to be by far the highest in B-cells, with significant expression in T-cells, and only minimal expression in monocytes. Similarly *xiap* expression is highest the B-cell population, followed by T-cells and monocytes (Figure 14). In contrast, *naip* expression appears to restricted simply to the monocyte population. These data also demonstrate that expression of *hiap2* varies relatively little between the various populations although it is somewhat elevated in lymphocytes. Not surprisingly, expression of the TRAF messages is also population specific with *traf1* being predominantly expressed in T-cells whereas *traf2* and *traf4* are highest in B-cell populations and *traf3* in monocytes

**Table 6: Relative tissue distribution of *hiap1* and *hiap2*.** Values are represented as a percentage (background corrected) of the signal obtained from 100 ng of genomic DNA (100%)

| tissues | *hiap1* | *hiap2* | *hiap1/hiap2* |
|---|---|---|---|
| whole brain | 19.14 | 10.40 | 1.84 |
| amygdala | 36.19 | 13.93 | 2.60 |
| caudate nucleus | 18.61 | 17.22 | 1.08 |
| cerebellum | 15.66 | 19.23 | 0.81 |
| cerebral cortex | 18.57 | 17.24 | 1.08 |
| frontal lobe | 18.87 | 16.20 | 1.17 |
| hippocampus | 15.87 | 15.76 | 1.01 |
| medulla oblongata | 28.78 | 19.05 | 1.51 |
| occipital lobe | 12.09 | 5.60 | 2.16 |
| putamen | 19.21 | 10.66 | 1.80 |
| substantia nigra | 18.52 | 17.54 | 1.06 |
| temporal lobe | 18.31 | 21.00 | 0.87 |
| thalamus | 18.02 | 17.96 | 1.00 |
| subthalamic nucleus | 20.54 | 15.03 | 1.37 |
| spinal cord | 23.27 | 17.87 | 1.30 |
| heart | 32.69 | 11.77 | 2.78 |
| aorta | 20.42 | 8.02 | 2.55 |
| skeletal muscle | 34.63 | 12.81 | 2.70 |
| colon | 50.47 | 32.60 | 1.55 |
| bladder | 26.14 | 18.99 | 1.38 |
| uterus | 22.59 | 20.58 | 1.10 |
| prostate | 52.47 | 31.25 | 1.68 |
| stomach | 48.02 | 25.01 | 1.92 |
| testis | 23.74 | 12.16 | 1.95 |
| ovary | 54.87 | 22.04 | 2.49 |
| pancreas | 22.38 | 14.90 | 1.50 |
| pituitary gland | 71.16 | 42.05 | 1.69 |
| adrenal gland | 45.66 | 32.43 | 1.41 |
| thyroid gland | 55.05 | 28.31 | 1.94 |
| salivary gland | 34.91 | 24.78 | 1.41 |
| mammary gland | 25.99 | 14.97 | 1.74 |
| kidney | 324.86 | 100.33 | 3.24 |
| liver | 167.77 | 51.46 | 3.26 |
| small intestine | 102.51 | 42.01 | 2.44 |
| spleen | 46.29 | 25.31 | 1.83 |
| thymus | 72.30 | 42.13 | 1.72 |
| peripheral leukocyte | 21.33 | 19.73 | 1.08 |
| lymph node | 49.41 | 23.87 | 2.07 |
| bone marrow | 53.26 | 23.64 | 2.25 |
| appendix | 42.85 | 10.15 | 4.22 |
| lung | 244.24 | 77.00 | 3.17 |
| trachea | 46.11 | 22.38 | 2.06 |
| placenta | 28.27 | 23.91 | 1.18 |
| fetal brain | 25.05 | 9.86 | 2.54 |
| fetal heart | 20.29 | 8.33 | 2.44 |
| fetal kidney | 37.22 | 20.46 | 1.82 |
| fetal liver | 47.83 | 20.51 | 2.33 |
| fetal spleen | 34.48 | 19.94 | 1.73 |
| fetal thymus | 68.11 | 28.88 | 2.36 |
| fetal lung | 72.22 | 31.91 | 2.26 |

**Figure 14: Ribonuclease protection assay of whole blood fractions.** Whole blood was fractionated and RNA extracted as described in the Methods section. The RNA was subjected to RPA analysis to determine the expression pattern of the various IAPs in the different fractions. The resulting blot was subjected to autoradiography (Panel A) and quantitative phosphoimage analysis (Panel B). The results were then quantified using ImageQuaNT analysis software (Molecular Dynamics). Data have been corrected for background signal and normalized against the signal obtained from *gapdh* (data not shown). The data represent the average intensity of the protected message fragments from duplicate isolations, as indicated, relative to *gapdh*.


PBMC1
PBMC2
B1
B2
T1
T2
monocyte1
monocyte2
xiap
traf1
traf2
traf4
naip
hiap1
hiap2
traf3
trpm2
L32
gapdh


10
8
6
4
2
0
xiap
traf1
traf2
traf4
naip
hiap1
hiap2
traf3
PBMCs
B-cells
T-cells
monocytes

### 9.3 Translational regulation of *hiap1* and *hiap2*

Results from studies on the genomic organization of *hiap1* and *hiap2* revealed that both possess unusually long 5'UTRs. These were determined to be approximately 4.5 kb and 2.3 kb for *hiap1* and *hiap2* respectively. As 46 possible initiators lie upstream of the true HIAP1 initiator, traditional scanning can be excluded as mechanism of translation initiation. As for *hiap2*, 14 possible ATG start codons are present in its 5'UTR. These all possess a minimal consensus of ANNATG. The full consensus being GCCACCATGG.[427] In order to determine whether these translational blocks were overcome by internal ribosome entry (internal ribosome entry site (IRES) mediated translation initiation, several constructs were created incorporating fragments of their respective 5'UTRs. The first of these included approximately 1kb of *hiap1* 5'UTR proximal to the initiation codon. This was inserted into the dicistronic plasmid pMH (by M. Holcik) (Figure 15). The resulting construct displayed no IRES activity when the insert was positioned in the sense orientation. When placed in the anti-sense orientation, though, significant expression of the second cistron was observed (Figure 16). In order to determine whether this activity is due to cryptic promoter activity of the insert, it was placed into the reporter vector pCATbasic(Xho). Significant expression of the reporter genes was, also, observed from this construct (Figure 16), indicating that some cryptic promoter activity is present in the 5'UTR of *hiap1* albeit in the opposing direction.

Given that approximately 50 nt separates the cloning site within the pMH plasmid and the initiation codon, a second dicistronic construct was created. The resulting plasmid, pSY, eliminates this space thereby maintaining the genomic architecture surrounding the initiation codon. Furthermore, the second cistron in pSY lacks an initiating codon, thus reducing background expression in the absence of an insert.

Individual 1kb fragments terminating within the first few codons for each of *hiap1* and *hiap2* were inserted "in frame" into the pSY vector (Figure 17). Although the *hiap1* derived fragment again failed to show any IRES like activity, the construct containing the *hiap2* derived fragment showed modest expression from the second cistron (Figure 18). In order to determine if the observed expression was indeed mediated by an IRES-like element, the *hiap2* derived dicistronic plasmid was co-transfected with a protease2A expression plasmid, CMV-2A. Protease-2A specifically cleaves the CAP binding domain from the general initiation factor 4G (eIF4G). As such, protease-2A inhibits CAP dependent translation initiation. Cleaved eIF4G retains the ability to promote IRES dependent translation initiation. As shown in Figure 19, expression of the second cistron of hiap2-pSY is CAP-dependant, implying that it is not expressed via internal

ribosome entry. In contrast to this, expression from an IRES control plasmid, EMCV-pMH, was unaffected by protease-2A (Figure 19).

**Figure 15: pMH dicistronic vector and insert.** A 1 kbp genomic fragment (P4603-P4604) of the *hiap1* 5'UTR was inserted, in both orientations, into both the pMH dicistronic vector as well as the pCAT basic(Xho) vector. Both pMH and pCATbasic(Xho) were engineered by Dr. Martin Holcik. PGK-CAT was engineered by Chris Storbeck.

Vector: pMH

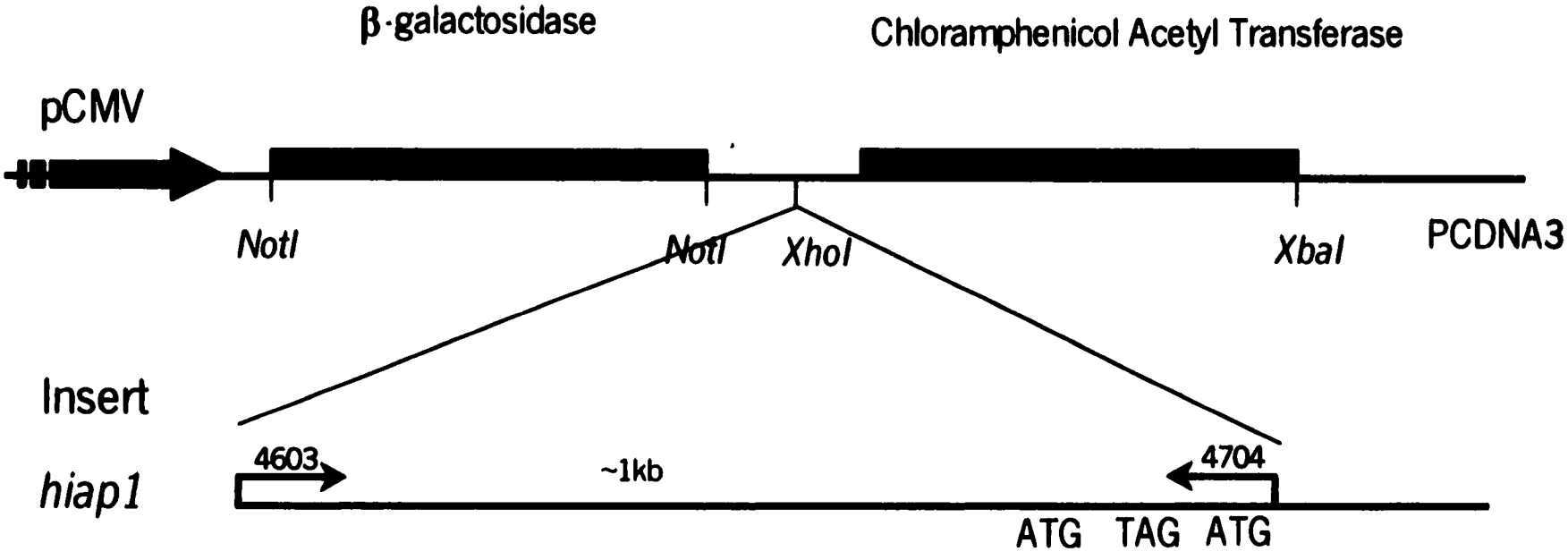
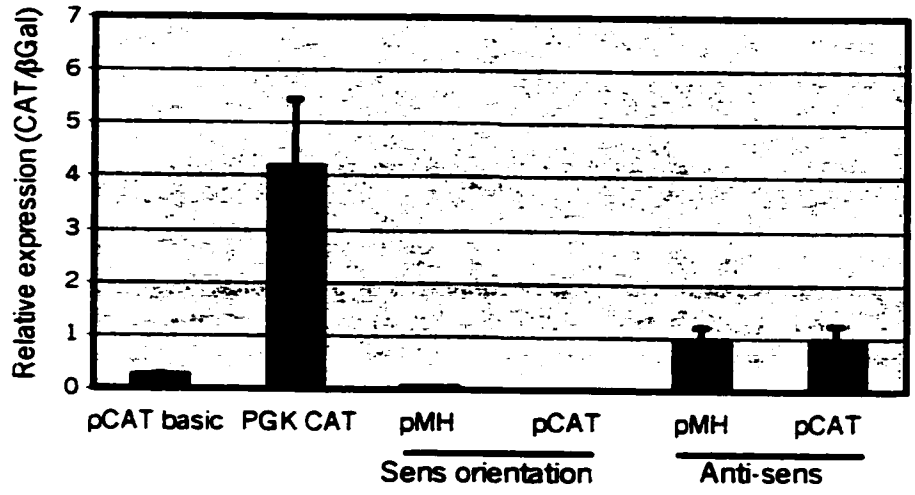

β-galactosidase
Chloramphenicol Acetyl Transferase
pCMV
NotI
NotI
XhoI
XbaI
PCDNA3
Insert
hiap1
4603
~1kb
4704
ATG TAG ATG

**Figure 16: Antisense promoter activity of *hiap1* 5'UTR.** HeLa cells were grown to 70% confluence in 6-well plates. Triplicate wells were transfected with 1 μg total DNA as indicated. Transfections were performed using Lipofectamine Plus and Opti-MEM (GibcoBRL) according to the manufacturer's protocol. Panels A and B: Results of two separate transfection experiments. Data represent relative CAT expression (vs. βGal) in arbitrary units. PCATbasic (empty vector) and PGK-CAT data result from co-transfection with pSVβGal (Promega) (0.5 μg each). Error bars represent standard deviations.

A


Relative expression (CAT/βGal)
7
6
5
4
3
2
1
0
pCAT basic
PGK CAT
pMH
pCAT
pMH
pCAT
Sens orientation
Anti-sens

B

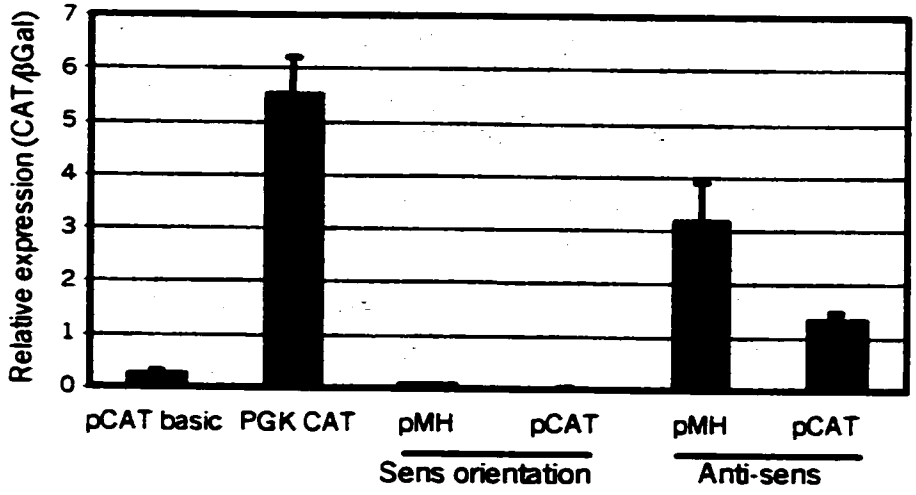

Relative expression (CAT/βGal)
7
6
5
4
3
2
1
0
pCAT basic
PGK CAT
pMH
pCAT
pMH
pCAT
Sens orientation
Anti-sens

**Figure 17: pSY dicistronic vector and inserts.** 1 kbp genomic fragments of both the *hiap1* (P4603-P4778) and *hiap2* (P4601-P4787) 5'UTR were inserted into the pSY dicistronic vector in the sens orientation only.

Vector: pSY

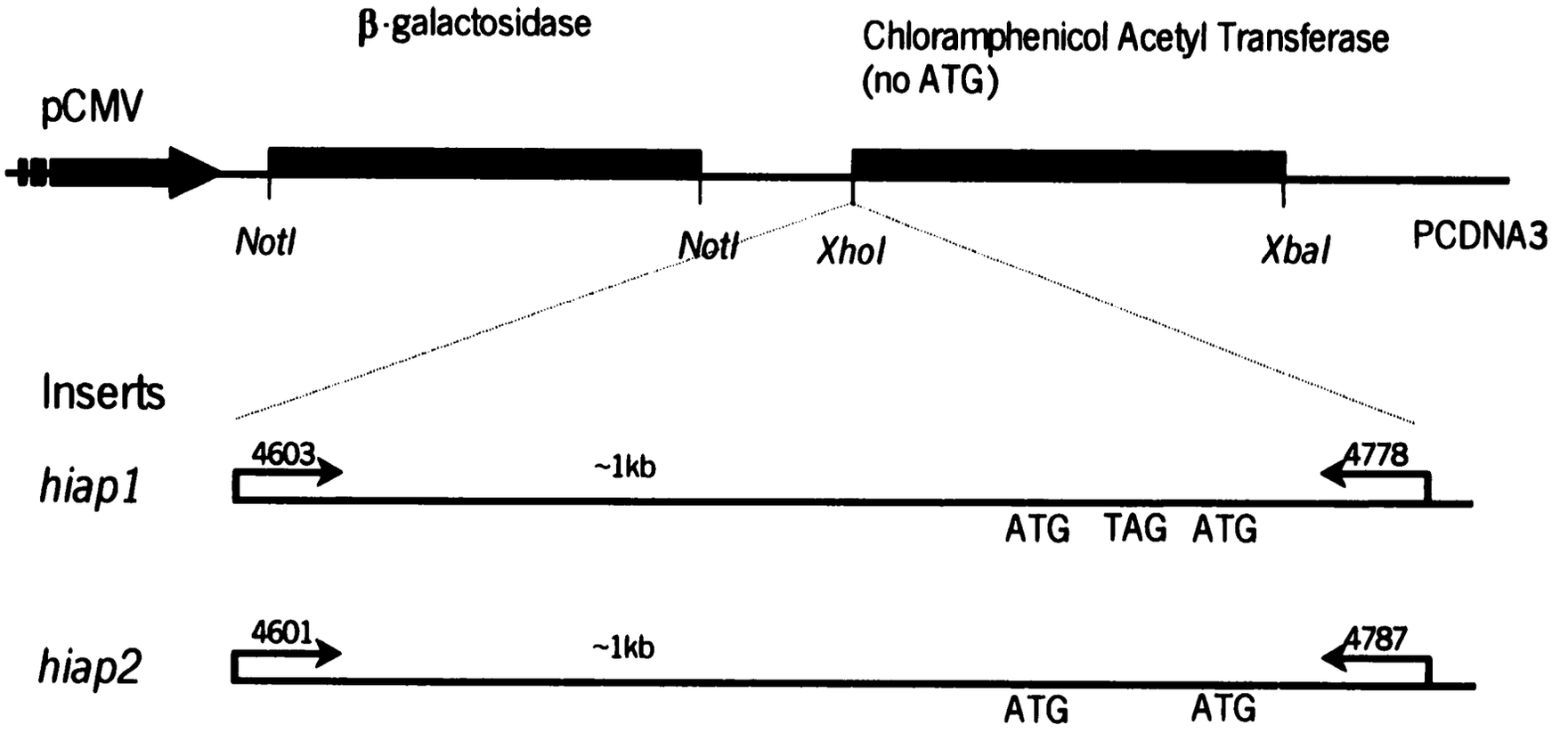

β-galactosidase
Chloramphenicol Acetyl Transferase
(no ATG)
pCMV
NotI
NotI
XhoI
XbaI
PCDNA3
Inserts
hiap1
4603
~1kb
4778
ATG TAG ATG
hiap2
4601
~1kb
4787
ATG ATG

**Figure 18:** Internal ribosome entry activity of *hiap1* and *hiap2* 5'UTR in pSY. HeLa cells were grown to 70% confluence in 6-well plates. Triplicate wells were transfected with 1 μg total DNA as indicated. Transfections were performed using Lipofectamine Plus and Opti-MEM (GibcoBRL) according to the manufacturer's protocol. Data represent relative CAT expression (vs. βGal) in arbitrary units. Error bars represent standard deviations.

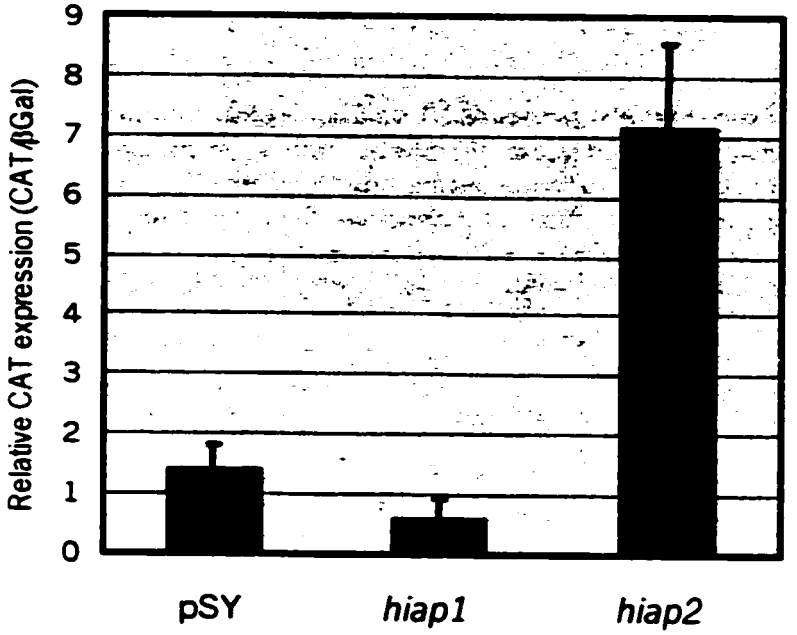
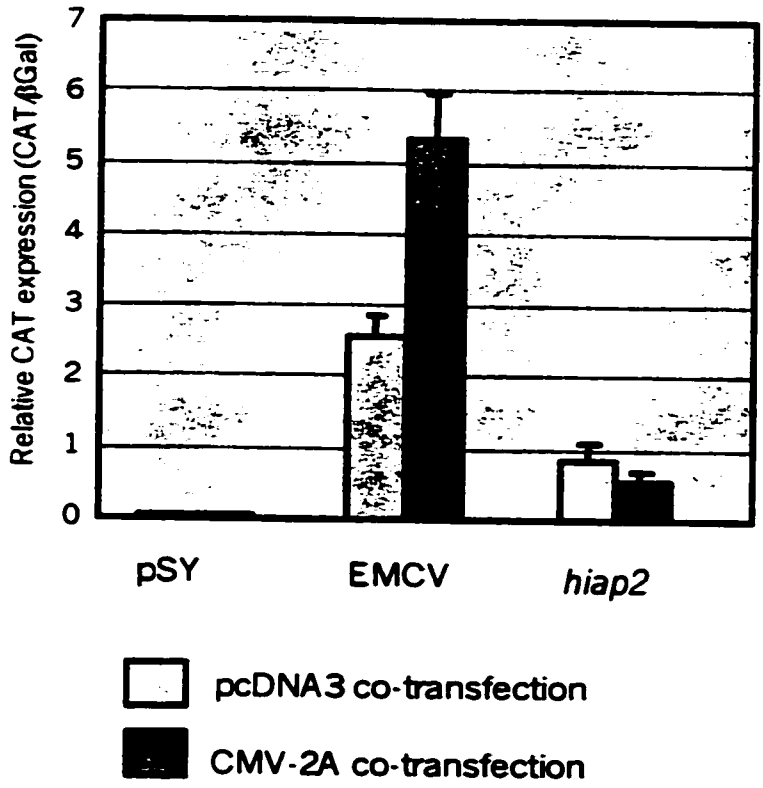

Relative CAT expression (CAT/βGal)
9
8
7
6
5
4
3
2
1
0
pSY
hiap1
hiap2

**Figure 19: Effect of picornaviral protease 2A on *hiap2*–pSY expression.** HeLa cells were grown to 70% confluence in 6-well plates. Triplicate wells were transfected with 2 μg total DNA as indicated. Transfections were performed using Lipofectamine Plus and Opti-MEM (GibcoBRL) according to the manufacturer's protocol. Data represent relative CAT expression (vs. βGal) in arbitrary units. Error bars represent standard deviations.


Relative CAT expression (CAT/βGal)
7
6
5
4
3
2
1
0
pSY
EMCV
hiap2
pcDNA3 co-transfection
CMV-2A co-transfection

### 9.4 Transcriptional control of *hiap1* and *hiap2*

In the present study, the signalling pathways leading to the transcriptional upregulation of *hiap1* were examined. Figure 20 (lanes 6 and 7) demonstrate that of *hiap1* and *hiap2* only the former is upregulated by TNFα in HeLa cells, and that the effect can be suppressed with the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC).[428] Although the transactivation of *hiap1* by TNFα has been previously shown to be dependant on the activity of NF-κB,[390],[409] it is not known whether this action is a consequence of direct NF-κB binding to promoter/enhancer elements within the *hiap* locus or whether κB-dependant synthesis of other transcriptional intermediaries is required. Dissection of these two possibilities was accomplished by incubation of HeLa cells with the translational inhibitors puromycin (PUR) and cycloheximide (CHX). Translational inhibition mimics activation of NF-κB by inhibiting the synthesis of IκB, a labile repressor of NF-κB, thus allowing for NF-κB translocation to the nucleus.[429] By abrogating *de novo* protein synthesis, these compounds also limit the activity to the transactivation of primary target genes. Figure 20 (lanes 2-5) demonstrates that translational inhibition alone can mediate an increase in the level of *hiap1* message whereas that of *hiap2* remains relatively unchanged. Pretreatment with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC)[428] reduces the effect of translational inhibition indicating that it is due to transcriptional upregulation rather than mRNA stabilization (Figure 20). In order to eliminate the possibility that translational inhibition affects *hiap1* message stability, thereby contributing to the perceived upregulation, HeLa cells were incubated with actinomycin D, following TNFα or puromycin stimulation. The degradation of the *hiap1* message was monitored by ribonuclease protection assay (RPA). Figure 21 shows that the stability of the message is not altered by either TNFα or puromycin.

Analysis of the *hiap1* 5' region as well as the corresponding region in the mouse (Genbank accession AW106060) revealed the presence of two conserved NF-κB sites within the 5' UTR (Figure 22). A similar analysis of the promoter region of *hiap2* also revealed the presence of a several putative transcription factor binding sites (Figure 23). These though apparently do not modulate *hiap2* transcription under any of the conditions tested. Electrophoretic mobility shift analysis (gel shift) with the putative *hiap1* NF-κB binding sequences (κB1 and κB3 in Figures 22 and 24), as well as that of a previously characterized non-conserved sequence within the *hiap1* 5'UTR[423] (κB2 in Figure 24), demonstrate that although all are able to specifically bind NF-κB(p50), κB3 does so with greater affinity followed by κB1 and κB2. In this assay, it is demonstrated that, at a fifteen fold excess, κB3 is able to outcompete both κB1 and κB2 for

binding to p50. whereas in reciprocal experiments, κB1 only moderately diminishes κB3 binding to p50 (Figure 24).

Further analysis of the genomic region upstream of the described NF-κB sites revealed the presence of both an NF-κB repressing factor (NRF) binding site (negative regulatory element, NRE) as well as an NF-IL6 (C/EBPβ) binding site. Gel shift analysis has demonstrated that both elements can specifically bind their respective factors (Figure 25).

In order to determine the relative contribution of the each of the elements, a 3.3 kbp genomic fragment including the entire first exon and terminating at the junction with the first intron of *hiap1*, was inserted into the pCAT3basic vector to yield H1P.4pCAT. Using H1P.4pCAT as a template, sequential site directed mutagenesis of the various transcription factor binding sites, alone and in combination, was performed. In contrast to the results obtained by gel shift analysis indicating that κB3 has a greater affinity for p50 than does κB1, mutagenesis of kB1 and kB3 individually demonstrates that κB1 appears to have a greater influence on *hiap1* transcription than does κB3 (Figure 26, Panel A). Although NRF is able to bind the NRE, mutagenesis of the NRE in isolation has revealed that it plays little or no role in either the basal transcriptional activity nor the inducibility of the *hiap1* promoter by TNFα (Figure 26, Panel B). Similarly, mutagenesis of the NRE in combination with κB1, κB3, or both has no effect on *hiap1* promoter driven CAT expression (data not shown). Although deletion of both NF-κB binding sites has a profound effect on TNFα mediated transcriptional upregulation of *hiap1*, mutagenesis of the NF-IL6 (C/EBPβ) binding site diminished somewhat the effect of TNFα (Figure 26, Panel C), the most profound effect occurring when all three sites are altered. That NF-κB indeed mediated the increase in H1P.4pCAT gene expression upon treatment with TNFα was confirmed by either co-transfection with an IκBα "super repressor" or treatment with PDTC. In both cases, TNFα mediated upregulation of the reporter gene was severely inhibited as was basal transcriptional activity (Figure 27). The ability of κB1 to act as an enhancer element was investigated by placing it 3' to a reporter gene construct, pCATenhancer. As shown in Figure 28, κB1 acts in a orientation independent manner to enhance transcription from a heterologous promoter in response to TNFα, a known NF-κB inducer.

Given the previously documented involvement of PI3K in NF-κB signalling by TNFα, we sought to determine whether P13K plays a role in the transactivation of *hiap1* by TNFα. We pre-incubated HeLa cells with the PI3K inhibitor LY294002 followed by TNFα treatment. In parallel experiments, HeLa cells were transfected with pCI-LMP1, a constitutively active member of the TNFR superfamily, in the presence of LY294002. Following TNFα treatment and pCI-LMP1

transfection, expression of *hiap1* was analyzed by RPA. Figure 29 demonstrates that the transcriptional upregulation of *hiap1* by either TNFα or LMP1 is sensitive to PI3K inhibition. Transfection studies with the H1P.4pCAT construct confirmed the sensitivity of the cloned *hiap1* promoter to LY294002 (Figure 30), indicating a necessary role for this kinase in the signalling process.

Whereas LMP1 is able to upregulate *hiap1* in an NF-κB dependant fashion, it would appear that the effect is suppressed in EBV transformed lymphoblastoid cell lines. A comparison between the levels of *hiap1* and *traf1* gene expression in LCL's and peripheral B-cells demonstrates that although *traf1* gene expression is markedly elevated by EBV infection, *hiap1* expression remains unaffected (Figure 31).

It has been reported that dexamethasone (DEX) alone or in combination with interferon-γ is able to upregulate *hiap1* in the airway epithelial cell line A549.[430] Further investigation has revealed that PDTC was able to abolish the effect of DEX on *hiap1* transcription (Figure 32). Of note is that DEX/INFγ was able to mediate an upregulation of the NF-κB responsive gene *traf1*. In order to determine whether dexamethasone responsive elements within the H1P.4pCAT construct were responsible for the observed effect, we evaluated the effect of DEX on CAT expression from both this construct as well as a mutant one lacking both the NF-κB and NF-IL6 sites (H1P.4pCAT136) in A549 cells. In contrast to the RPA results (Figure 32) and previous results by others[430], DEX reduced expression from the native construct, presumably by transrepression of NF-κB dependant expression. Removal of the NF-κB responsive elements (Figure 26) not only reduced basal transcriptional activity, as previously demonstrated, but also abrogated the repressive effect of DEX (Figure 33), thus implying that the DEX responsive element(s) lies outside the cloned promoter region. It may also be that, in the absence of NF-kB binding sites, constitutive expression is too low to observe the repressive effect of DEX on the cloned promoter region.

**Figure 20: Ribonuclease protection assay. Effect of TNFα, puromycin, cycloheximide and PDTC on *hiap1* expression.** HeLa cells were grown to 70% confluence in 6-well plates. The cells were then pre-treated for 1 hr with 500 μM PDTC followed by incubation with either 15 μg/ml puromycin, 15 μg/ml cycloheximide, or 30 ng/ml TNFα for 6 hr. Following incubation, the RNA was harvested via Trizol and analyzed via RPA according to the manufacturers' instructions. The results were visualized on a Storm 860 PhosphorImager and quantified using ImageQuaNT analysis software (Molecular Dynamics). Data have been corrected for background signal and normalized against the signal obtained from *gapdh*(data not shown). Lanes are as follows: 1. control HeLa, 2. Puromycin, 3. PDTC followed by puromycin, 4. Cycloheximide, 5. PDTC followed by cycloheximide, 6. TNFα, 7. PDTC followed by TNFα.

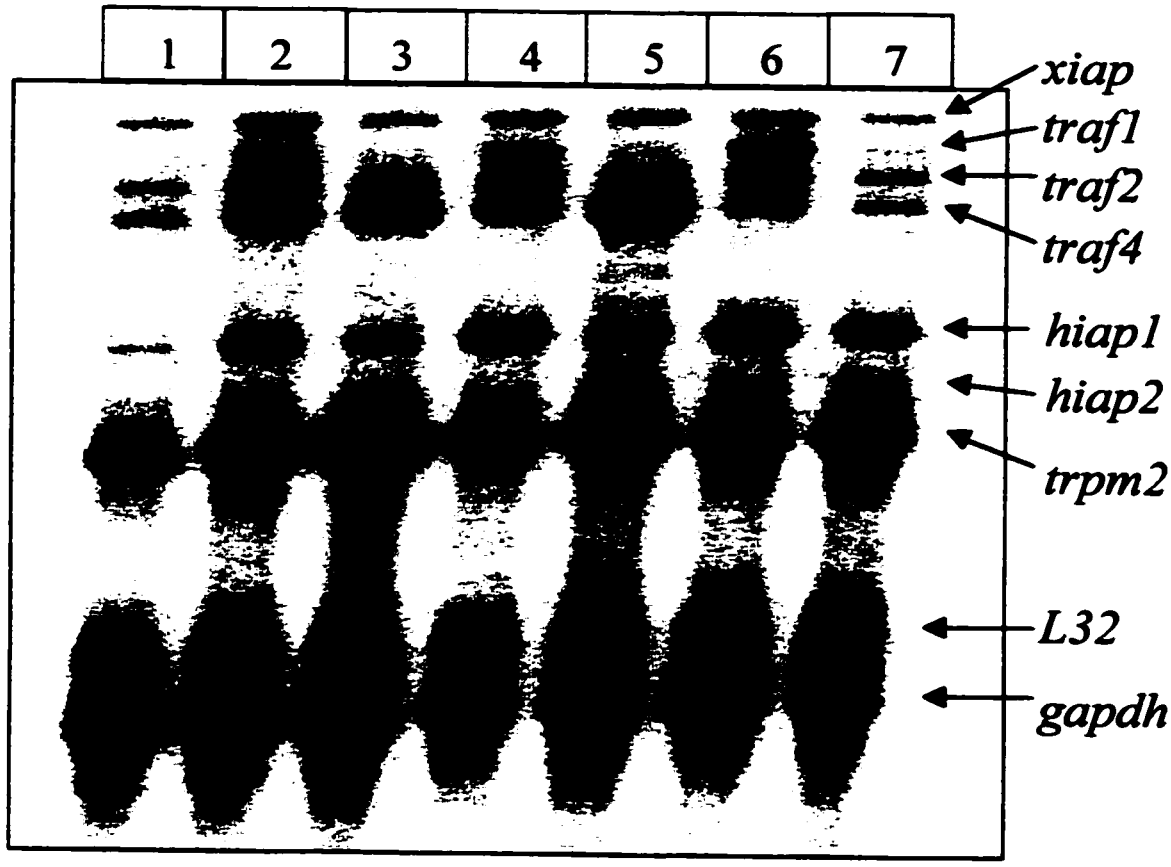

1
2
3
4
5
6
7
xiap
traf1
traf2
traf4
hiap1
hiap2
trpm2
L32
gapdh

**Figure 21: Stability of *hiap1* message following TNFα or puromycin treatment**. HeLa cells were grown to 70% confluence in 6-well plates. The cells were then incubated with TNFα (30ng/ml) or puromycin (15 μg/ml) for 6 hr followed by the addition of actinomycin D (10μg/ml) to inhibit further transcription. At the times indicated, RNA was harvested via Trizol and analyzed via RPA (hAPO5 template set) according the manufacturer's instructions. The results were visualized on a Storm 860 PhosphorImager and quantified using ImageQuaNT analysis software (Molecular Dynamics). Data have been corrected for background signal and normalized against the signal obtained from *gapdh* (data not shown).

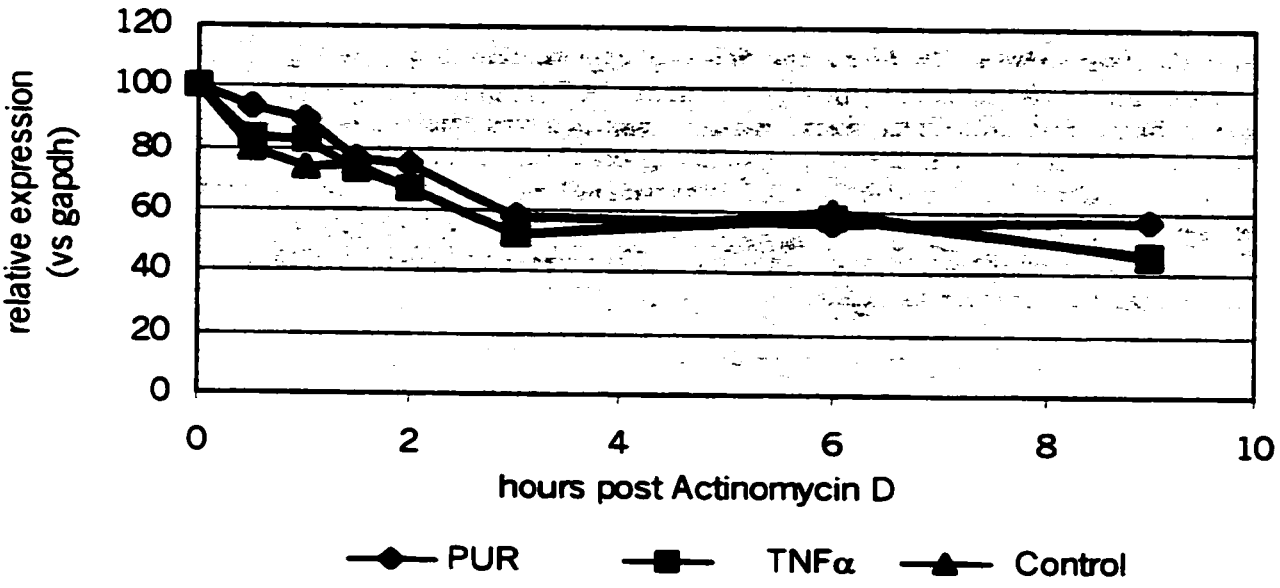

120
100
80
60
40
20
0
relative expression
(vs gapdh)
0
2
4
6
8
10
hours post Actinomycin D
PUR
TNFα
Control

**Figure 22: Alignment of the *hiap1* and *miap1* 5' region.** Shown are the NF-IL6, NRE, κB1, and κB2 elements. Regions of homology within the 5'UTR are shaded light grey. The coding region is shaded dark grey. The mouse message sequence was obtained from GenBank (accession AW106060). The human sequence was obtained from GenBank (accessions AC019309.3, AP000942.3, and AP001167.2) as well as by transposon mediated sequencing of the relevant genomic fragment (ϕ201 *EcoRI-EagI*).
The distance between the homologous regions (2.8 kb) was determined after elimination of known introns from the human sequence.

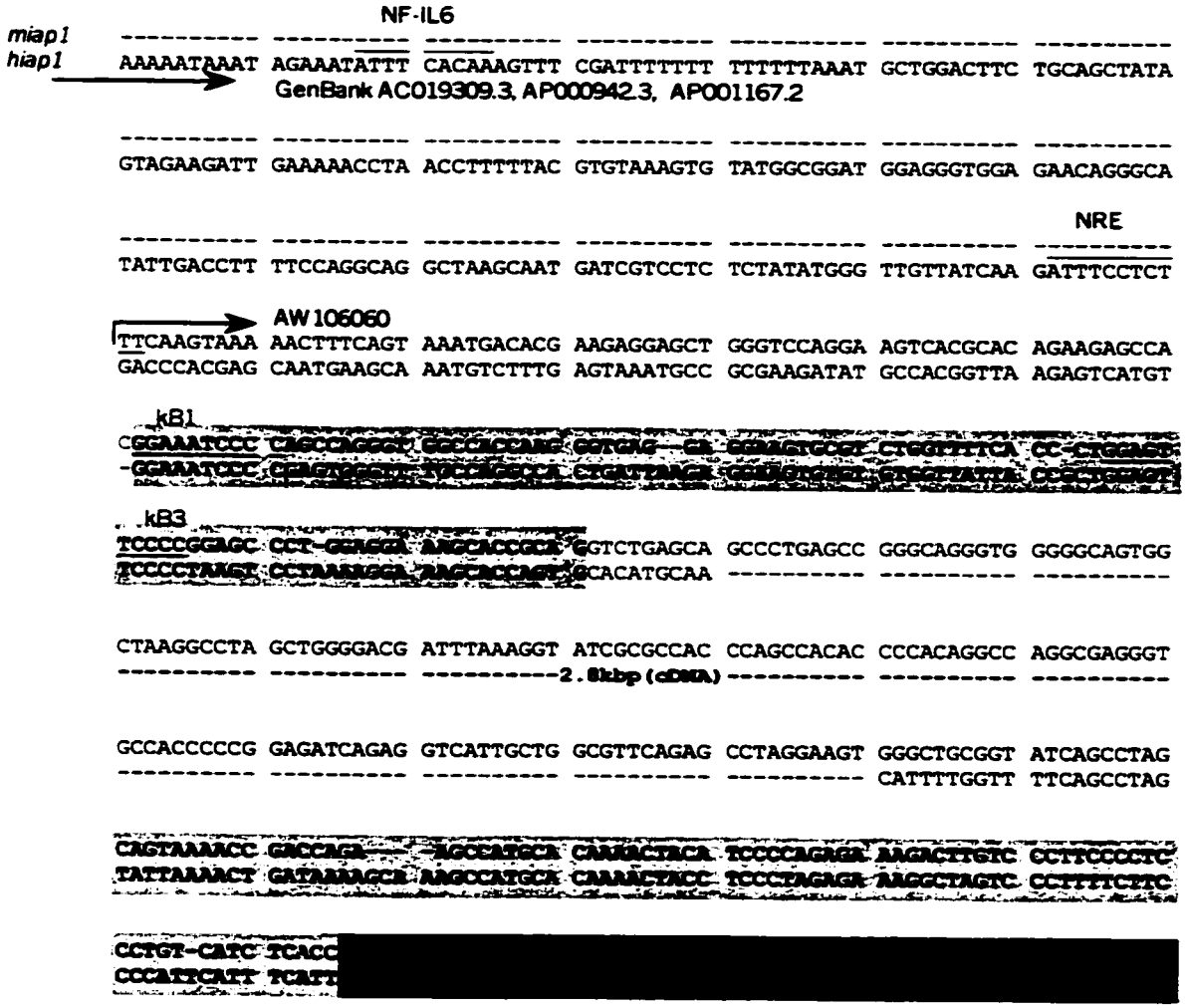

NF-IL6
miap1
hiap1
AAAAATAAAT AGAAATATTT CACAAAGTTT CGATTTTTTT TTTTTTAAAT GCTGGACTTC TGCAGCTATA
GenBank AC019309.3, AP000942.3, AP001167.2
GTAGAAGATT GAAAAACCTA ACCTTTTTAC GTGTAAAGTG TATGGCGGAT GGAGGGTGGA GAACAGGGCA
NRE
TATTGACCTT TTCCAGGCAG GCTAAGCAAT GATCGTCCTC TCTATATGGG TTGTTATCAA GATTTCCTCT
AW106060
TTCAAGTAAA AACTTTCAGT AAATGACACG AAGAGGAGCT GGGTCCAGGA AGTCACGCAC AGAAGAGCCA
GACCCACGAG CAATGAAGCA AATGTCTTTG AGTAAATGCC GCGAAGATAT GCCACGGTTA AGAGTCATGT
kB1
kB3
TCCCCGGAGC CCT-GGAGGA AAGCACCGCA GGTCTGAGCA GCCCTGAGCC GGGCAGGGTG GGGGCAGTGG
TCCCCTAAGT CCTAAAGGA AAGCACCAGT GCACATGCAA
CTAAGGCCTA GCTGGGGACG ATTTAAAGGT ATCGCGCCAC CCAGCCACAC CCCACAGGCC AGGCGAGGGT
2.8kbp (cDNA)
GCCACCCCCG GAGATCAGAG GTCATTGCTG GCGTTCAGAG CCTAGGAAGT GGGCTGCGGT ATCAGCCTAG
CATTTTGGTT TTCAGCCTAG
CCTGT-CATC TCACC
CCCATTCATT TCATT

**Figure 23: Promoter region of *hiap2*.** Indicated (bold and underlined) are binding sites for NF-IL6, SP6, AP1 and NF-κB.

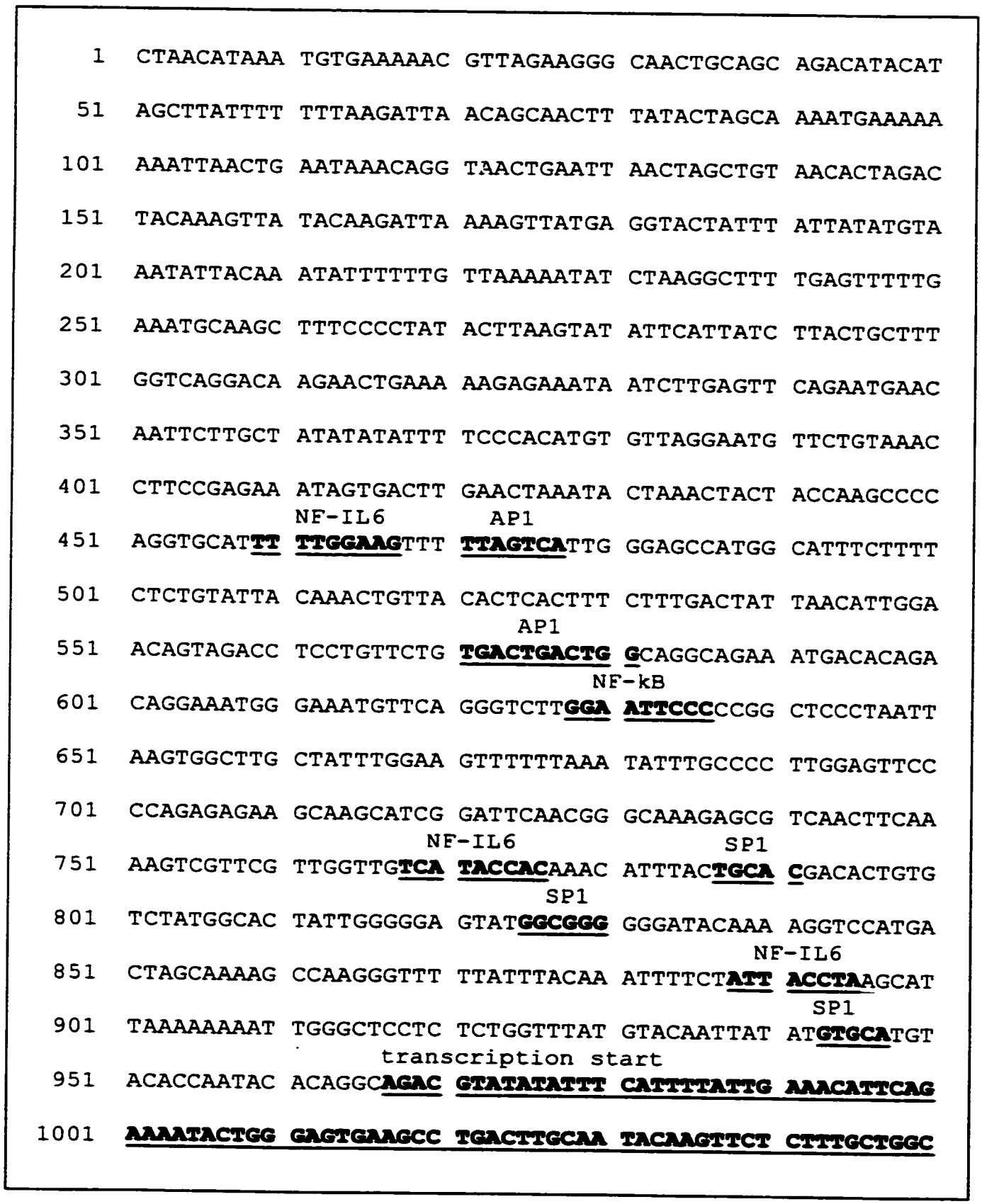
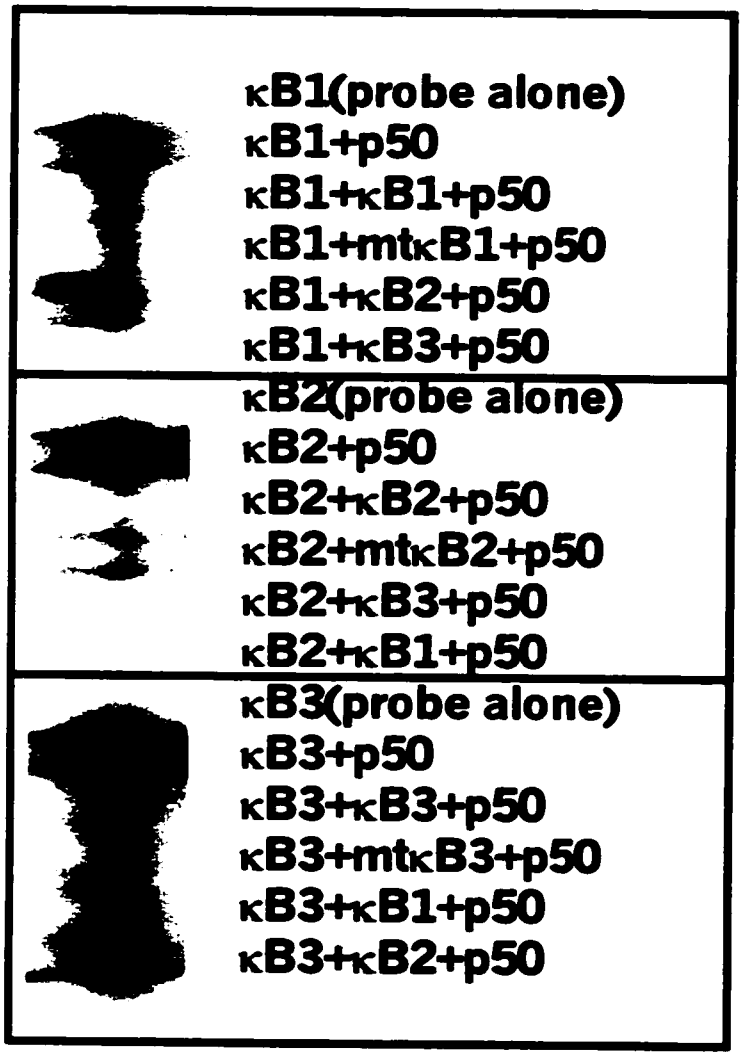

1 CTAACATAAA TGTGAAAAAC GTTAGAAGGG CAACTGCAGC AGACATACAT
51 AGCTTATTTT TTTAAGATTA ACAGCAACTT TATACTAGCA AAATGAAAAA
101 AAATTAACTG AATAAACAGG TAACTGAATT AACTAGCTGT AACACTAGAC
151 TACAAAGTTA TACAAGATTA AAAGTTATGA GGTACTATTT ATTATATGTA
201 AATATTACAA ATATTTTTTG TTAAAAATAT CTAAGGCTTT TGAGTTTTTG
251 AAATGCAAGC TTTCCCCTAT ACTTAAGTAT ATTCATTATC TTACTGCTTT
301 GGTCAGGACA AGAACTGAAA AAGAGAAATA ATCTTGAGTT CAGAATGAAC
351 AATTCTTGCT ATATATATTT TCCCACATGT GTTAGGAATG TTCTGTAAAC
401 CTTCCGAGAA ATAGTGACTT GAACTAAATA CTAAACTACT ACCAAGCCCC
NF-IL6 AP1
451 AGGTGCATTT TTGGAAGTTT TTAGTCATTG GGAGCCATGG CATTTCTTTT
501 CTCTGTATTA CAAACTGTTA CACTCACTTT CTTTGACTAT TAACATTGGA
AP1
551 ACAGTAGACC TCCTGTTCTG TGACTGACTG GCAGGCAGAA ATGACACAGA
NF-kB
601 CAGGAAATGG GAAATGTTCA GGGTCTTGGA ATTCCCCCGG CTCCCTAATT
651 AAGTGGCTTG CTATTTGGAA GTTTTTTAAA TATTTGCCCC TTGGAGTTCC
701 CCAGAGAGAA GCAAGCATCG GATTCAACGG GCAAAGAGCG TCAACTTCAA
NF-IL6 SP1
751 AAGTCGTTCG TTGGTTGTCA TACCACAAAC ATTTACTGCA CGACACTGTG
SP1
801 TCTATGGCAC TATTGGGGGA GTATGGCGGG GGGATACAAA AGGTCCATGA
NF-IL6
851 CTAGCAAAAG CCAAGGGTTT TTATTTACAA ATTTTCTATT ACCTAAGCAT
SP1
901 TAAAAAAAAT TGGGCTCCTC TCTGGTTTAT GTACAATTAT ATGTGCATGT
transcription start
951 ACACCAATAC ACAGGCAGAC GTATATATTT CATTTTATTG AAACATTCAG
1001 AAAATACTGG GAGTGAAGCC TGACTTGCAA TACAAGTTCT CTTTGCTGGC

**Figure 24: Electrophoretic mobility shift assay of κB1, κB2 and κB3.** Double stranded oligodeoxynucleotide probes corresponding to κB1, κB2, and κB3 were subjected to EMSA as described in the Methods section. The NF-κB probe sequences were as follows (NF-κB sites appear in bold with the mutated bases underlined): κB1 5'-TTT TGG GTC ATG GAA ATC CCC GAG TGG GTT; mt-κB1 5'-TTT TGG GTC ATG GAA ATA ACC GAG TGG GTT; κB2 5'-AGC AGA GCT TTC CCG CCA AGG GAG AAG CTT; mt-κB2 5'-AGC AGA GCT TTA TCG CCA AGG GAG AAG CTT; κB3 5'-TAT TAC CCG CTG GAG CTA CCC TAA GTC CTA A; mt-κB3 5'-TAT TAC CCG CTG GAA TTA CCC TAA GTC CTA A.


κB1(probe alone)
κB1+p50
κB1+κB1+p50
κB1+mtκB1+p50
κB1+κB2+p50
κB1+κB3+p50
κB2(probe alone)
κB2+p50
κB2+κB2+p50
κB2+mtκB2+p50
κB2+κB3+p50
κB2+κB1+p50
κB3(probe alone)
κB3+p50
κB3+κB3+p50
κB3+mtκB3+p50
κB3+κB1+p50
κB3+κB2+p50

κB3>>κB1>>κB2

**Figure 25: Electrophoretic mobility shift assay A. NRF/NRE B. NF-IL6.** Double stranded oligodeoxynucleotide probes corresponding to A. NRE and B. NF-IL6 were subjected to EMSA as described in the Methods section. The probe sequence were as follows (the relevant binding sites appear in bold with the mutated bases underlined): NRE 5'-GTT GTT ATC AAG **ATT TCC TCT GAC CCA CGA**; mt NRE 5'-GTT GTT ATC AAG **ATT TC<u>A</u> TCT <u>A</u>AC CCA** CGA; NF-IL6 5'-ATA **GAA ATA TTT CAC AAA** GTT TCG ATT TTT; mt-NF-IL6 5'-ATA **GAA ATA <u>G</u>TT <u>A</u>AC AAA** GTT TCG ATT TTT.

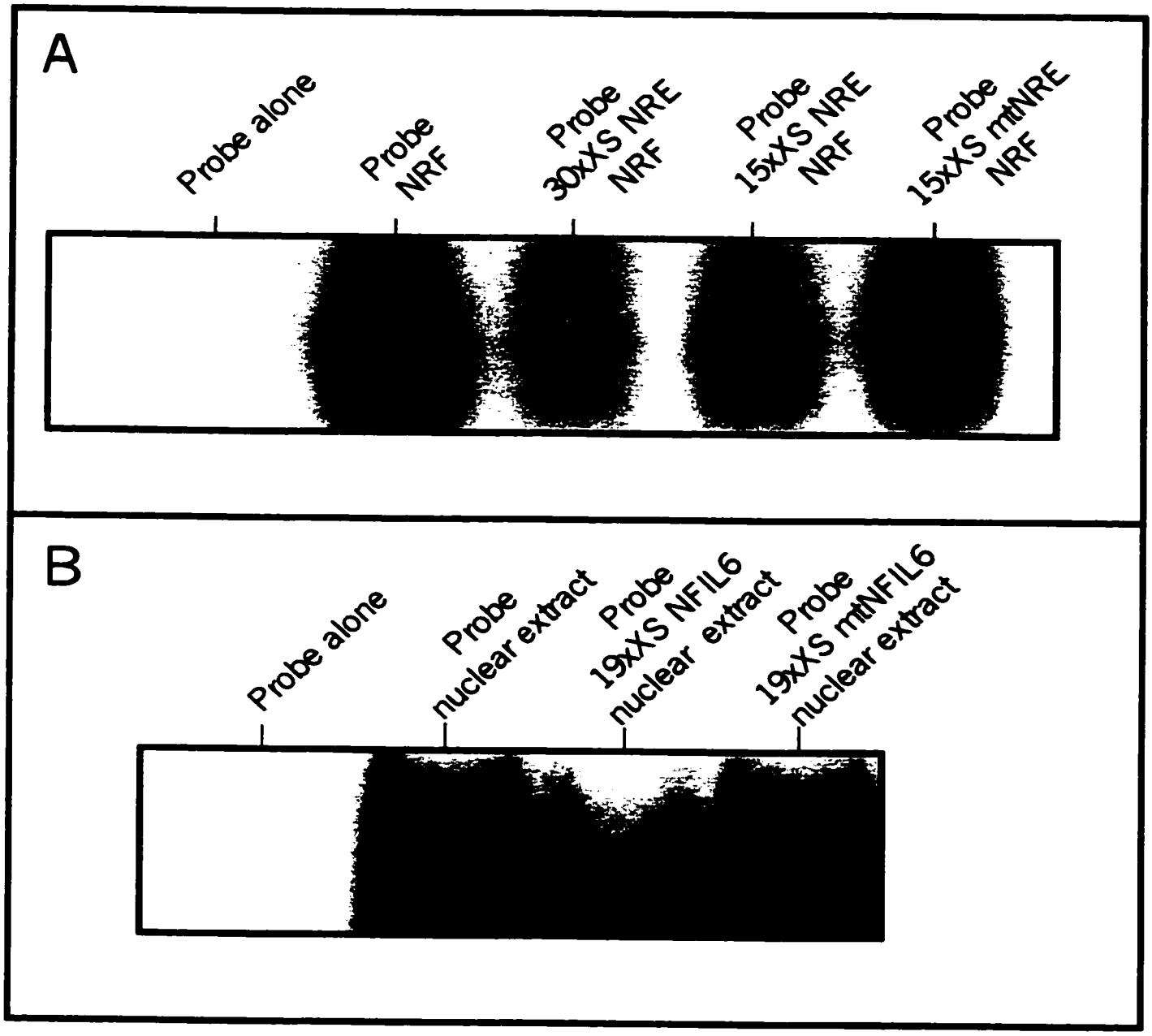

A
Probe alone
Probe NRF
Probe 30xXS NRE NRF
Probe 15xXS NRE NRF
Probe 15xXS mtNRE NRF
B
Probe alone
Probe nuclear extract
Probe 19xXS NFIL6 nuclear extract
Probe 19xXS mtNFIL6 nuclear extract

**Figure 26: Promoter analysis of the H1P.4pCAT serial mutants.** One of the H1P.4pCAT series of constructs (1 μg) was transfected in conjunction with PGKβGAL (1 μg) into HeLa cells. The cells were allowed to recover for 24 hr prior to the addition of TNFα (30 ng/ml). CAT expression was measured 48 hr post-transfection and normalized to β-galactosidase. Open boxes within the promoter diagram represent "wild type" transcription factor binding sites whereas black boxes represent mutated binding sites. White histogram bars represent basal activity, whereas black bars represent TNFα induced activity of their respective reporter construct. Values represent average of three separate transfections. A. NF-κB mutations B. NRE mutations C. NF-IL6/NF-κB mutations. Error bars represent standard deviations.

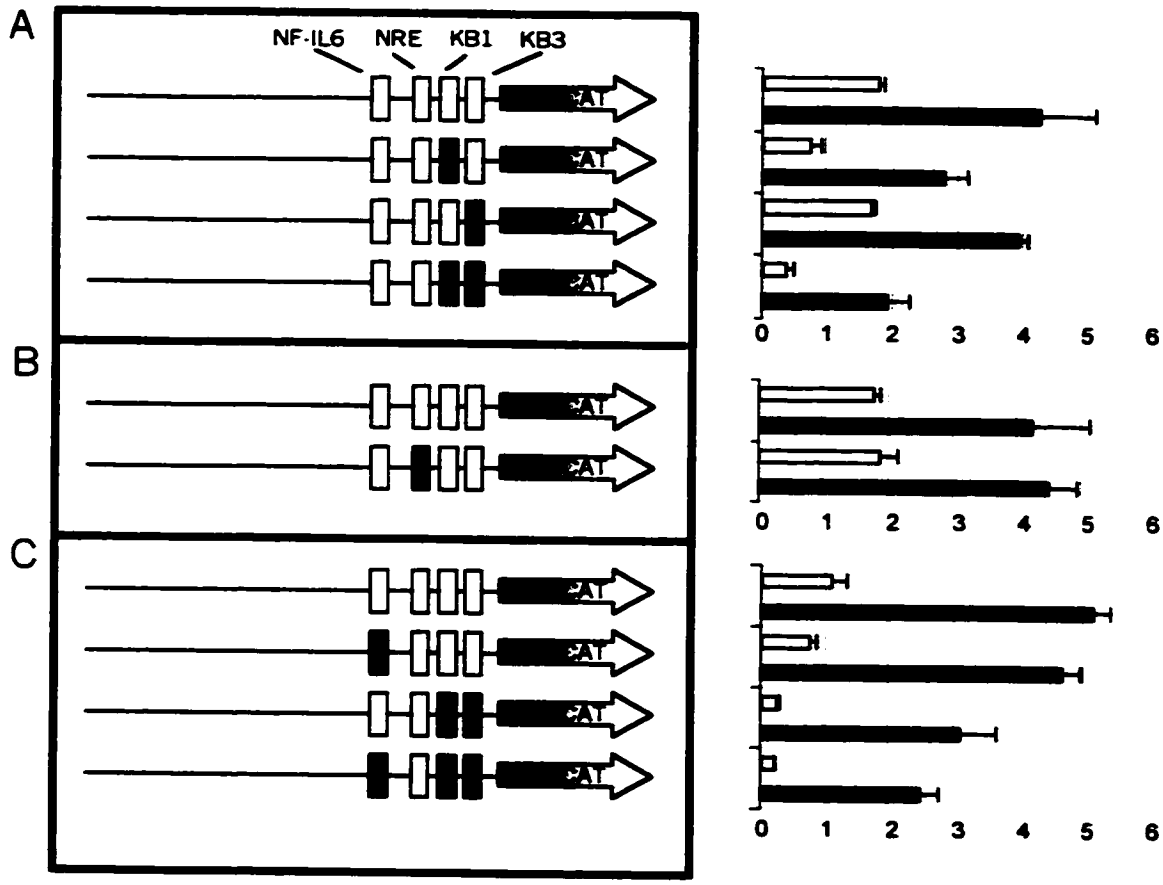

A
NF-IL6
NRE
KB1
KB3
CAT
B
C
0 1 2 3 4 5 6

**Figure 27: Effect of PDTC and IκBαSR on expression from H1P.4pCAT.** HeLa cells were grown to 70% confluence and transfected with 1μg of H1P.4pCAT as well as 1μg of pCDNA3 (control) or 1μg IκBαSR-pCDNA3. 23 hr post transfection the cells were treated with 500 μM (final) PDTC as indicated. 24 hr post transfection the cells were treated with TNFα (30ng/ml final), as indicated, and incubated for a further 6 hr. Following this incubation, the cells were harvested and β-galactosidase as well as CAT activity was determined. Error bars represent standard deviations.

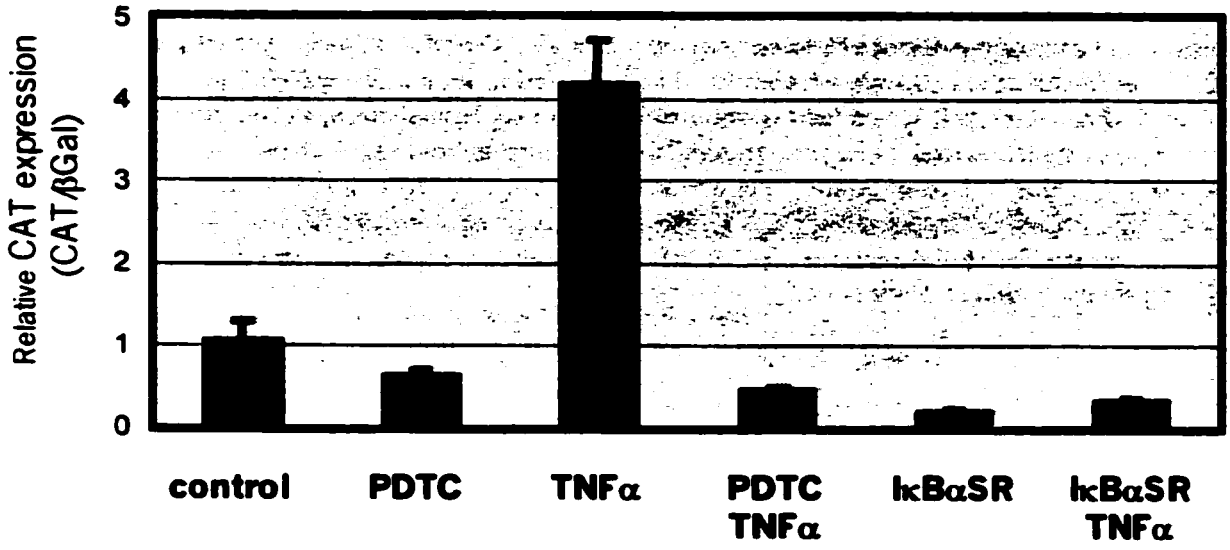
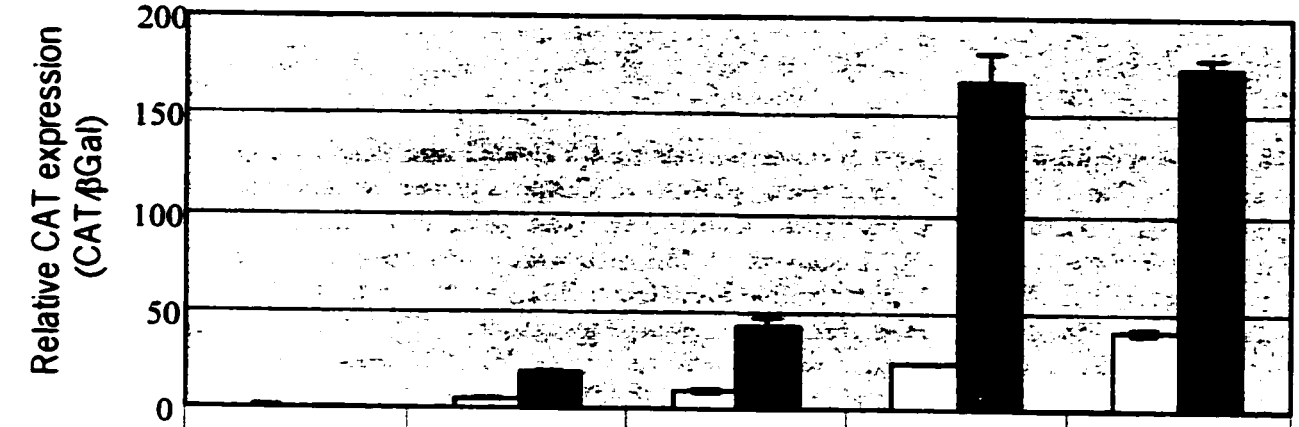

Relative CAT expression
(CAT/βGal)
5
4
3
2
1
0
control
PDTC
TNFα
PDTC
TNFα
IκBαSR
IκBαSR
TNFα

**Figure 28: Transcriptional enhancer activity of κB1.** The pCAT3promoter vector (1 μg) and pCATkB constructs (1 μg) were transfected in conjunction with pCI-lacZ (0.1 μg) into HeLa cells. TNFα (30 ng/ml) was added immediately following transfection, as indicated. CAT expression was measured 24 hr post-transfection and normalized to β-galactosidase. Values represent average of two separate transfections. control-pCAT3promoter vector; pCATκB7-single insert, forward orientation; pCAT κB8-single insert, reverse orientation; pCATκB9-two tandem inserts, forward orientation; pCATκB19-two tandem inserts, reverse orientation. Error bars represent standard deviations.




| | control | pCATkB7 | pCATkB8 | pCATkB9 | pCATkB17 |
|---|---|---|---|---|---|
| ■ no TNFα | 1.00 | 4.33 | 10.43 | 23.99 | 39.04 |
| ■ TNFα | 0.47 | 18.02 | 41.70 | 168.16 | 173.43 |

**Figure 29: Inhibition of *hiap1* transactivation by LY294002 and wortmannin**. HeLa cells were grown to 70% confluence in 6-well plates. A. The cells were then incubated with LY294002 (20 μM) for 1 hr prior to the addition of TNFα (30ng/ml). Transduction was allowed to proceed for 6 hr prior to RPA analysis. B. The cells were transfected with 1 μg pCI-LMP1 or pCI (vector control) using Lipofectamine Plus (GibcoBRL) according to the manufacturers instructions, in serum free media, and in the presence or absence of LY294002 (20 μM), as indicated. LMP1 gene expression was allowed to proceed for 12 hr prior to RPA analysis. RNA was harvested via Trizol and analyzed via RPA (hAPO5 template set) according the manufacturer's instructions. Expression of *hiap1* message was then quantified with a Storm PhosphorImager 860 and the accompanying ImageQuaNT software. Data have been corrected for background signal and normalized against the signal obtained from *gapdh* (data not shown). Error bars represent standard deviations. (C-control, LY-LY294002)

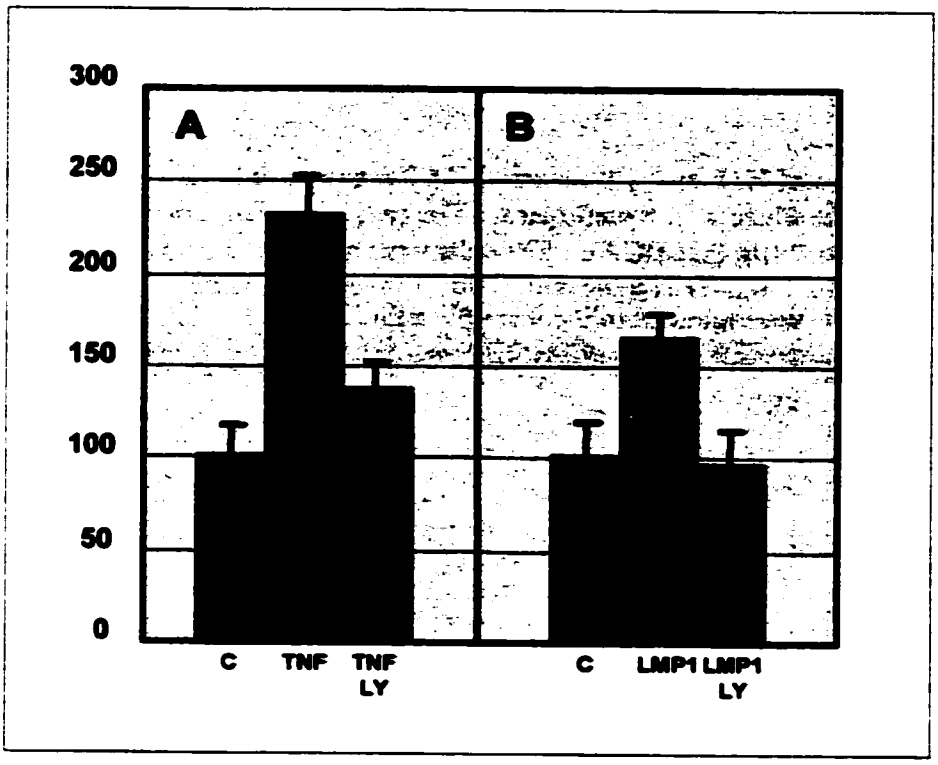

300
250
200
150
100
50
0
A
B
C
TNF
TNF LY
C
LMP1
LMP1 LY

**Figure 30: Inhibition of H1P.4pCAT directed CAT expression by LY294002.** HeLa cells were grown to 70% confluence in 6-well plates. The cells were then transfected with 1μg H1P.4pCAT along with 1μg PGKβGAL. The cells were allowed to recover for 24 hr prior to the addition of LY294002 (20 μM) as indicated. After 1 hr, TNFα (30 ng/ml) was added as indicated and the cells were allowed to incubate for a further 24 hr. CAT expression was measured 48 hr post-transfection and normalized to β-galactosidase. Error bars represent standard deviations.

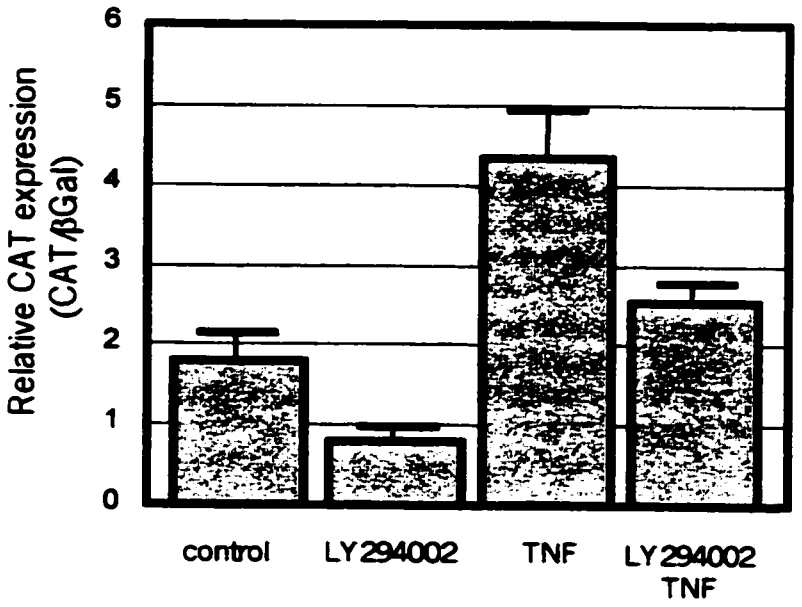
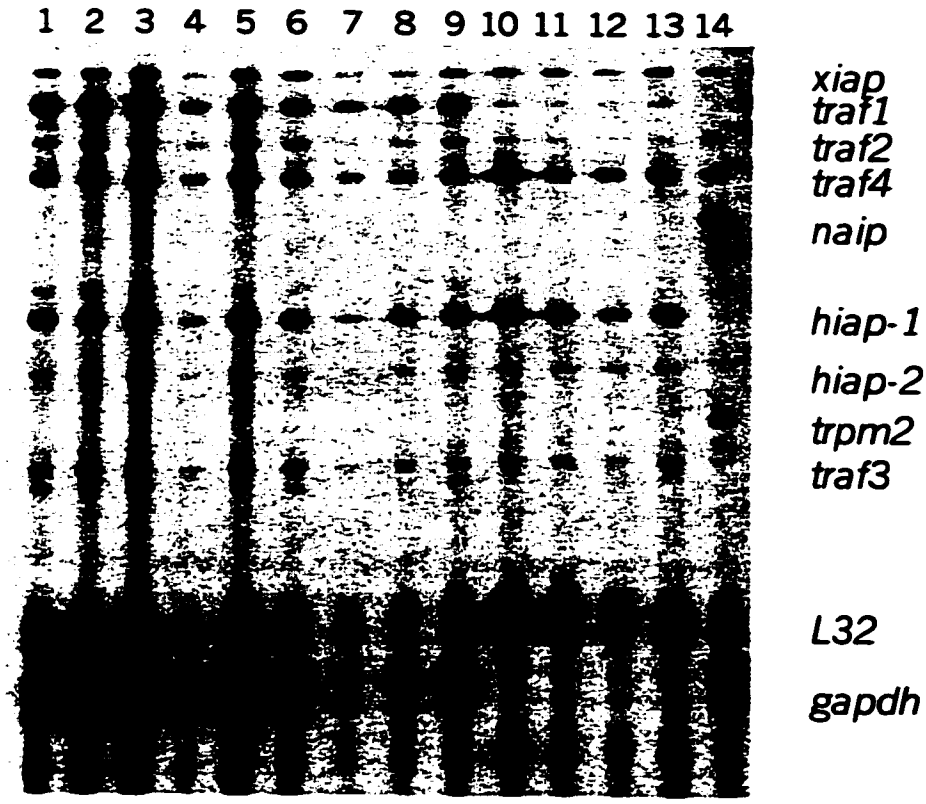

Relative CAT expression
(CAT/βGal)
6
5
4
3
2
1
0
control
LY294002
TNF
LY294002
TNF

**Figure 31: *hiap1* gene expression in PBMCs vs. LCLs.** B-cells from either lymphoblast culture or isolated from whole blood were pelleted and resuspended in 1 ml Trizol reagent (Gibco BRL). RNA was recovered as per manufacturer's protocol. RNase protection assays were carried out as per manufacturer's protocol (Pharmingen) with the analysis performed using the hAPO5 probe set and 10 μg total RNA as input. The results were visualized on a Storm 860 PhosphorImager and quantified using ImageQuaNT analysis software (Molecular Dynamics). Data have been corrected for background signal and normalized against the signal obtained from *L32* (data not shown). Error bars represent standard deviations.




Lanes 1-9 : EBV transformed PBMC's
Lanes 10-13: Peripheral blood B-cells
Lane 14 : HeLa + *naip* control

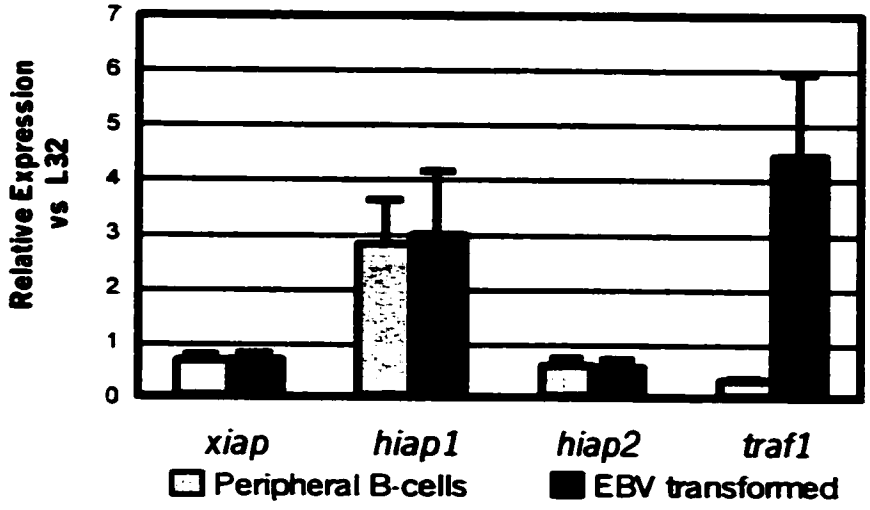


**Figure 32: Transcriptional upregulation of *hiap1* by dexamethasone.** A549 cells were grown to 70% confluence in 6-well plates. The cells were then incubated with PDTC (500 μM) as indicated for 1 hr prior to the addition of dexamethasone (12.5 μM). 6 hr post induction, RNA was harvested (Trizol) and analyzed via RPA (hAPO5 template set) according the manufacturer's instructions. The results were visualized on a Storm 860 PhosphorImager and quantified using ImageQuaNT analysis software (Molecular Dynamics). Data have been corrected for background signal and normalized against the signal obtained from *L32* (data not shown). (C-control, DEX-dexamethasone, PDTC-pyrrolidine dithiocarbamate)

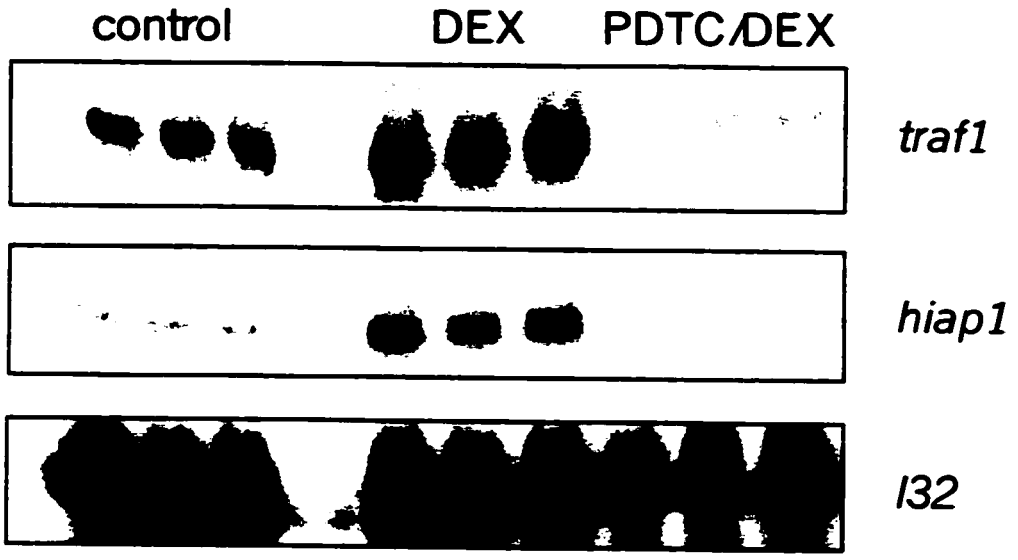
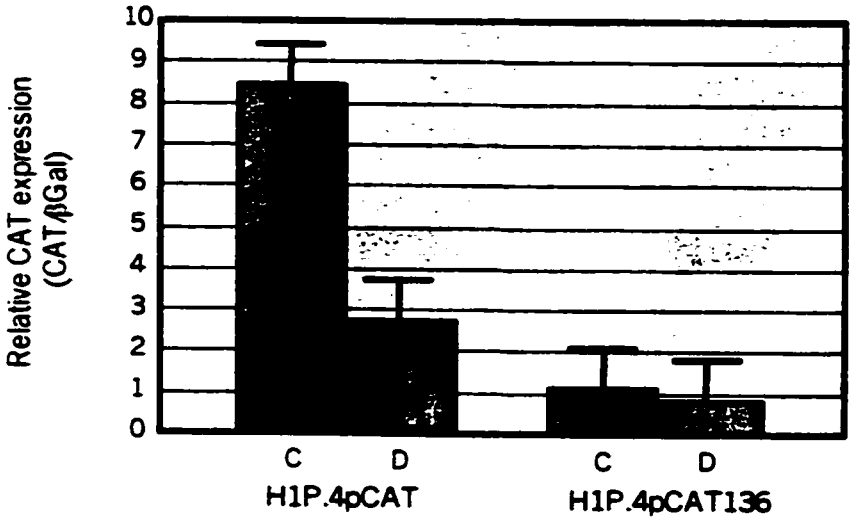

control
DEX
PDTC/DEX
traf1
hiap1
l32

**Figure 33: Inhibition of H1P.4pCAT and H1P.4pCAT136 directed CAT expression by dexamethasone.** HeLa cells were grown to 70% confluence in 6-well plates. The cells were then transfected with either H1P.4pCAT or H1P.4pCAT136 as well as PGKβGAL (1 μg each). The cells were allowed to recover for 24 hr prior to the addition of dexamethasone (12.5 μM) as indicated. CAT expression was measured 48 hr post-transfection and normalized to β-galactosidase. . Error bars represent standard deviations. (C-control, D-dexamethasone)


Relative CAT expression
(CAT/βGal)
10
9
8
7
6
5
4
3
2
1
0
C
D
H1P.4pCAT
C
D
H1P.4pCAT136

### 9.5 Analysis of the *hiap1* and *hiap2* knockout cell line BJAB(ko)

In order to establish whether or not small genomic rearrangements could account for the observed induction of *hiap1* in the Raji Burkitt's lymphoma cell line[431], we performed southern blot analysis of a panel of Burkitt's lymphoma cell lines. It was observed that the BJAB Burkitt's lymphoma cell line was deleted for both *hiap1* and *hiap2* (Figure 34). This finding was confirmed by, RPA and Western blot analysis. By contrast, a commercially available BJAB line was shown to possess both genes (Figures 35 and 36 respectively). By PCR analysis of dinucleotide repeats surrounding both *hiap1* and *hiap2*, it was determined that the area of homozygous deletion is between 2.2 cM (D11S1339-D11S923) and 4.5 cM (D11S1325-D11S917) (Figure 37, Panel A). Given the heterozygosity index of each locus, it is likely, but not definitive, that the derivative cell line in fact arose from the commercially available BJAB line (Figure 37, Panel B). Given that the derivative line appears to be hemizygous and assuming that the parental line is dizygous over the entire region analyzed (homozygous at D11S1325 and D11S917) the probability that the two cell lines arose independently is equal to the result of $(1-(H_{D1}/N_1))(1-(H_{D2}/N_2))(1-(H_{D3}/N_3))\ldots(1-(H_{Dn}/N_n))$. Where $H_D$ is the heterzygosity at any given locus, N is the ploidy at that locus in the parental line, and n is the number of loci examined. This assumes that the heterzygosity index of a given locus within a single genome is the same as that of single alleles between genomes. This analysis reveals that the probability that the two lines arose independently is 0.1209. Should the parental line, in fact, be hemizygous at D11S1325 and D11S917, the probability drops to 0.0261.

Contained within the genomic region bounded by D11S1325 and D11S1339 lie the genes encoding CASP1, CASP4, and CASP5. Although the results of the polymorphic marker analysis above allow for the possibility that these may be deleted in the derivative line, PCR amplification of a portion of their respective coding regions was possible from both the parental and derivative line. This suggests that both lines are at least hemizygous for CASP1, CASP4 and CASP5 (Figure 38).

With the identity of BJAB(ko) established, the effect of the deletion on the expression of the other *iaps* as well as the *traf* messages was determined under both constitutive and induced conditions. Although TNFα appeared to only minimally induce *hiap1* or *traf1* in both cell lines, their expression was inhibitable by LY294002. Of note, the expression of *traf1* was markedly increased in the absence of *hiap1* and *hiap2* whereas that of *traf2* appeared diminished. Expression of *xiap* appeared to be somewhat lower in the derivative cell line (Figure 39).

Given the potential involvement of HIAP1 and HIAP2 in TNFα mediated signal transduction, the effect of this cytokine on the viability of these cells was also determined. Whereas treatment of the parental line with TNFα stimulated cell proliferation, as determined by total cell counts, treatment of the derivative line inhibited it. Adenoviral mediated transient reintroduction of *hiap1* into the derivative line or anti-sense *hiap1* into the parental line effectively reversed their respective phenotypes (Figure 40).

**Figure 34: BJAB Southern.** Genomic DNA obtained from both Burkitt's Lymphoma cell lines were subjected to *EcoR1* digestion. Parallel blots were probed with either a *hiap1* or *hiap2* coding region probe, as indicated.

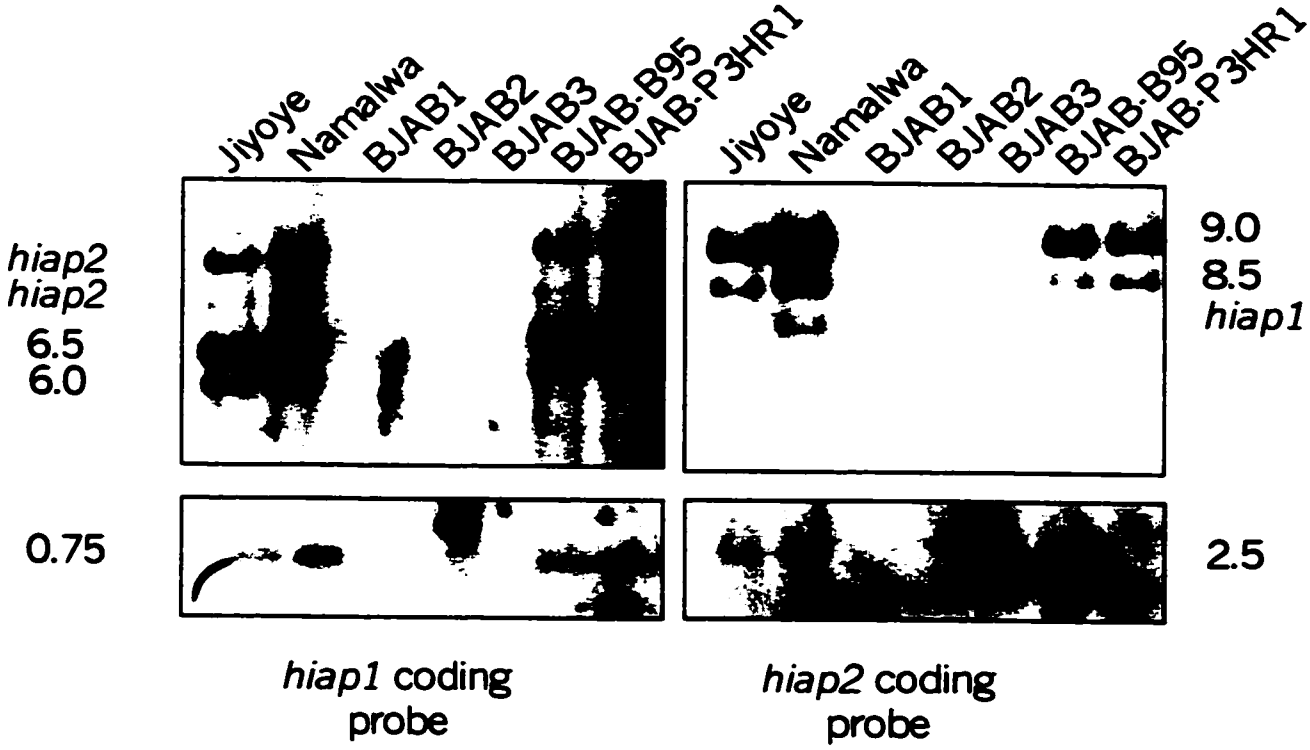
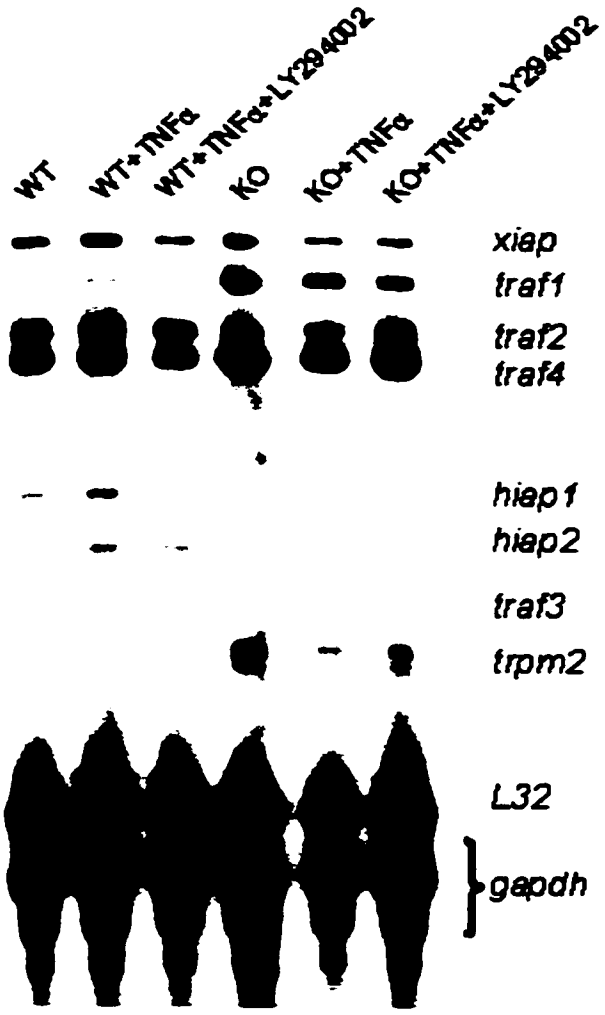

Jiyoye
Namalwa
BJAB1
BJAB2
BJAB3
BJAB·B95
BJAB·P3HR1
Jiyoye
Namalwa
BJAB1
BJAB2
BJAB3
BJAB·B95
BJAB·P3HR1
hiap2
hiap2
6.5
6.0
0.75
9.0
8.5
hiap1
2.5
hiap1 coding probe
hiap2 coding probe

**Figure 35: BJAB RPA**. 1 hr prior to treatment with TNFα (30 ng/mL), BJAB cell lines BJAB(wt) and BJAB(ko) were treated with LY294002 (20 nM). Cells were incubated for a further 6 hr prior to being harvested. Gene expression was measured by ribonuclease protection assay as described in the Methods section.


WT
WT+TNFα
WT+TNFα+LY294002
KO
KO+TNFα
KO+TNFα+LY294002
xiap
traf1
traf2
traf4
hiap1
hiap2
traf3
trpm2
L32
gapdh

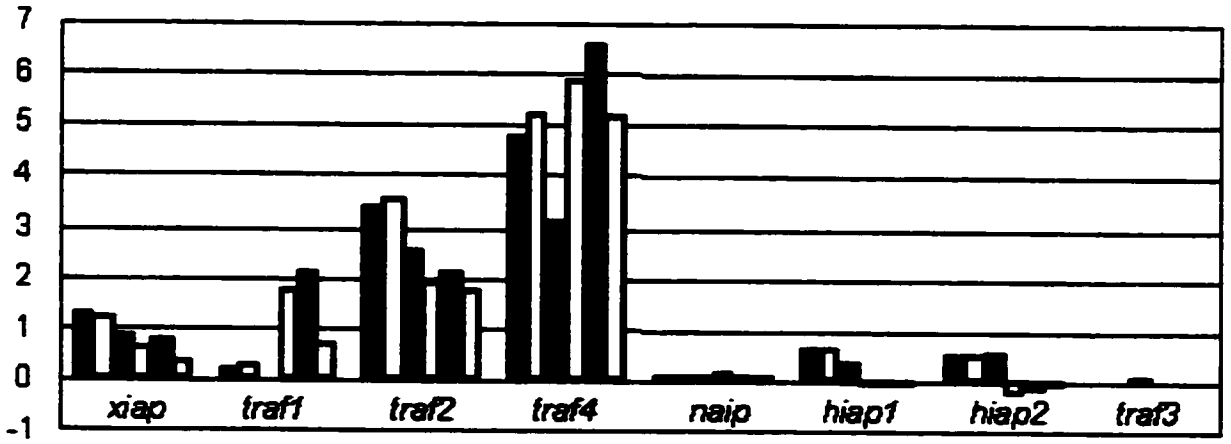
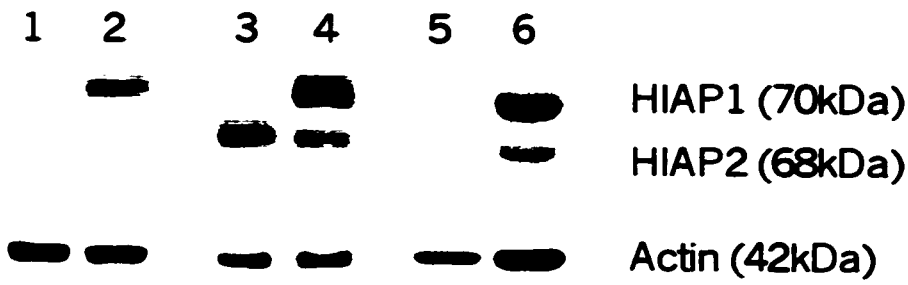

7
6
5
4
3
2
1
0
-1
xiap
traf1
traf2
traf4
naip
hiap1
hiap2
traf3

**Figure 36: HIAP1/HIAP2 Western blot**. Cell lines were grown to 70% confluence and transfected/treated as indicated. BJAB cell lines were grown to approximately "mid-log" phase. The cells were lysed and the protein concentration determined. Cell lysates were analyzed via immunoblot for expression of HIAP1 and HIAP2. Antibody=αRIAP1 (Aegera). Data by Charles A. Lefebvre.



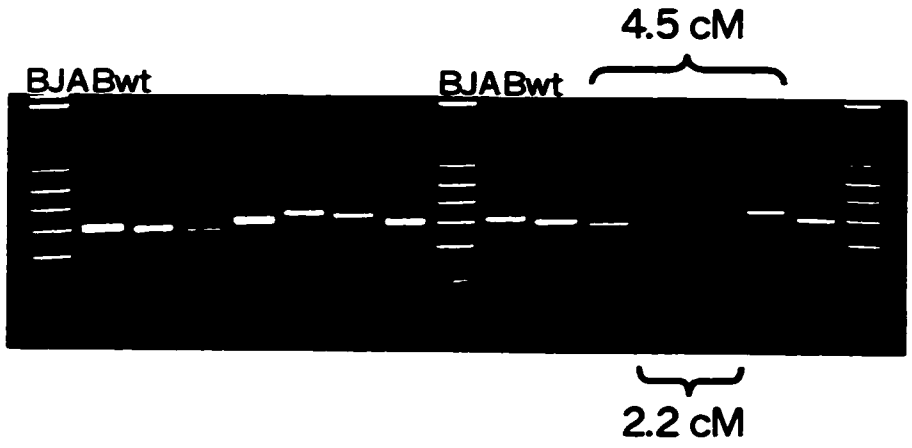

1 A549 control
2 A549 Dex (12.5μM) + INFγ(250μ/ml) for 24h

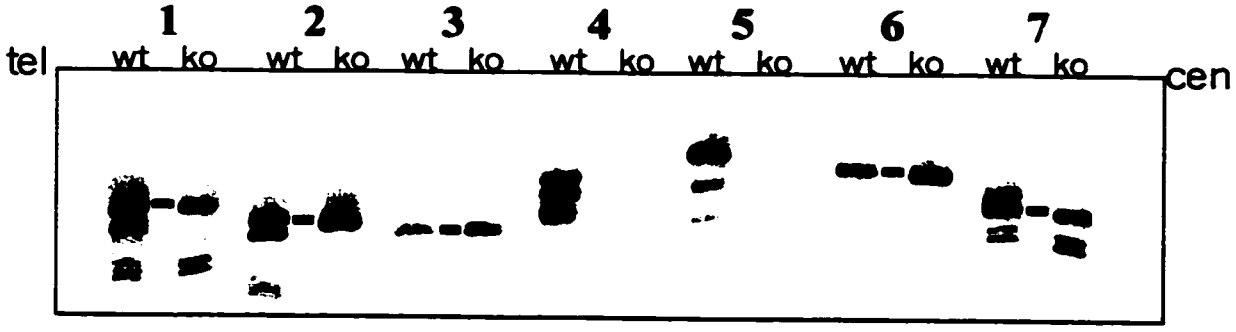
3 SF295 control
4 SF295 + CHX(10μg/ml) for 24h

5 BJAB(ko)
6 BJAB(wt)

NB:all lanes contain 20 μg total protein probed with αRiap1(1:2000)

**Figure 37: PCR analysis of BJAB(wt) and BJAB(ko) gDNA.** A. Various polymorphic chromosomal markers were amplified from BJAB(wt) and BJAB(ko) genomic DNA, as indicated. The size of the minimal and maximal homozygous deletions were estimated by the estimated distance between the negative and positive markers respectively. Source: National Center for Biotechnology Information, Human Genome Resources(http://www.ncbi.nlm.nih.gov/genome/guide/) B. Allelic differences between both cell lines was determined by polyacrylamide gel electrophoresis of radiolabelled amplification products.




1. D11S1347 (0.79)
2. D11S1343 (0.56)
3. D11S1325 (0.52)
4. D11S1339 (0.89)
5. D11S923 (0.71)
6. D11S917 (0.80)
7. D11S1311 (0.75)

**Figure 38: Genomic amplification of CASP1, CASP4, and CASP5.** The 5' end of the various caspase coding regions were amplified from BJAB(wt) and BJAB(ko) genomic DNA according to established procedures. The predicted amplification products are 100 bp, 150 bp, and 1593 bp for CASP1, CASP4, and CASP5 respectively. C1=CASP1, C4=CASP4, C5=CASP5

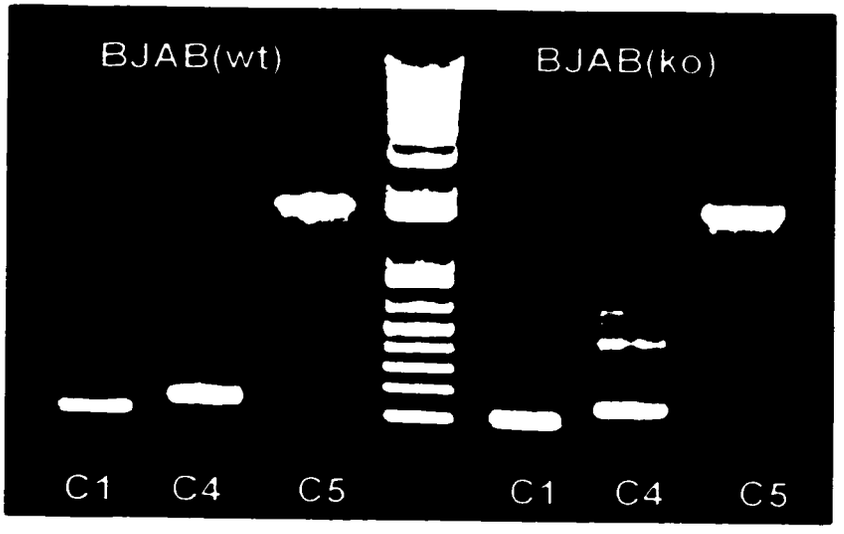
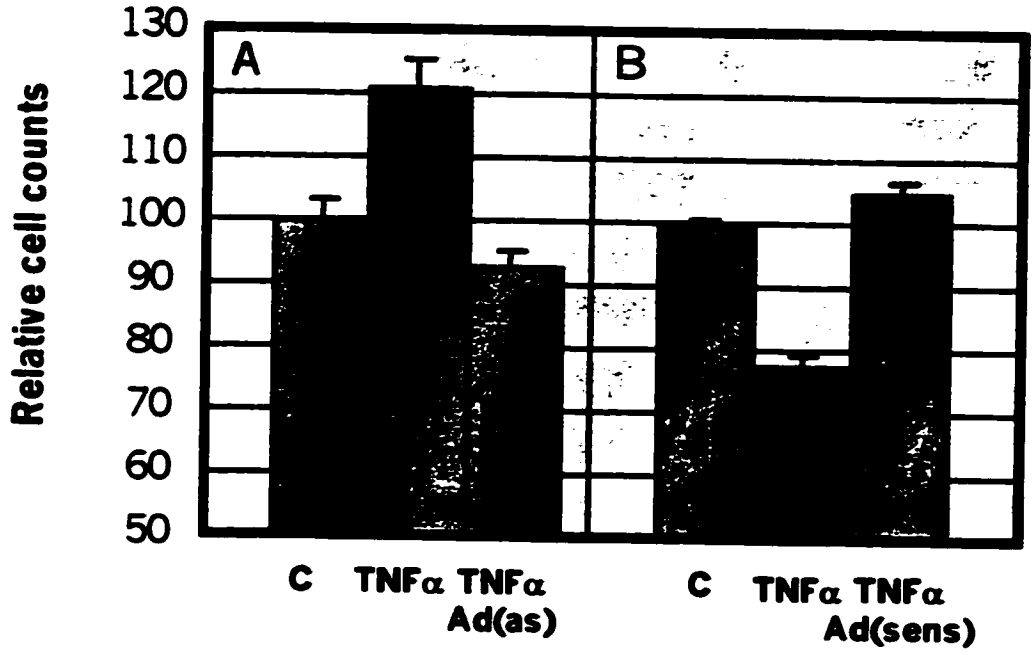

BJAB(wt)
BJAB(ko)
C1
C4
C5
C1
C4
C5

**Figure 39: BJAB death assay. Panel A: BJAB(wt) Panel B: BJAB(ko).** $2x10^5$ (1mL) cells were dispersed into each well of a 24 well plate. Cells were infected (MOI=30) with either adeno-*hiap1* (sens) or adeno-*hiap1* (antisens) as indicated (Aegera Inc). After 24 hr the cells were treated with TNFα (30 ng/mL) and incubated for a further 24 hr. Cells were then dispersed and counted on a haemocytometer. Error bars represent standard deviations.


Relative cell counts
130
120
110
100
90
80
70
60
50
A
B
C
TNFα
TNFα
Ad(as)
C
TNFα
TNFα
Ad(sens)

## 10. Discussion

### 10.1 Genomic organization

The presence of IAPs, consisting of three BIR domains and a RING (zinc) finger domain, in both the arthropod and vertebrate lineages demonstrates the relative antiquity of the gene family. The origin of the IAP family can be traced even further back, perhaps to the emergence of the metazoa, by the presence of the single BIR containing proteins in yeast.

A comparison of the coding region exons of *hiap1*, *hiap2*, and indeed *xiap* (M. Lagace in preparation) make it obvious that gene duplication events gave rise to these genes. That the mouse also possesses this same genomic organization [426],[383] reveals that the duplication event occurred prior to the divergence of the species 80-100 MYA.[432] Without further study, though, it is impossible to determine which of the three represents the "parental" gene. It is now currently thought that, in most instances, the "lifespan" of duplicated genes is but a few million years.[433] The classical model of gene duplication states that with no selective pressure being applied to the duplicated genes, they are free to acquire mutations. At some point, one of the genes will acquire a deleterious mutation rendering it essentially a pseudogene, thereby reapplying selective pressure on the remaining paralogue. An example of this can be found in the rabbit β2 globin pseudogene (ψβ2). This gene apparently arose from β2 approximately 55 MYA, survived intact for 22 million years but succumbed to inactivation at that point. With selective pressure reapplied to β2, ψβ2 has since accumulated many more mutations such that much of the duplicated gene's architecture is no longer apparent.[434] Alternatively and presumably less frequently one of the partners may acquire a novel, selectively advantageous function. Acquiring such a function would create a new gene and allow for its persistence in the genome.[434] Unfortunately, acquisition of novel functions as a sole means of retention cannot account for the number of duplications which persist within the genome.[433] A refinement of the classical model has been proposed which goes someway in bridging this gap. In the classical model, deleterious mutations in a duplicated gene propel it toward the realm of pseudogenes. If, however, the gene possesses multiple functions, point mutations may abrogate one but not all of its functions. Subsequent mutations in the duplicate gene, may abrogate yet other functions. In this model, neither gene will have acquired a novel function yet both will be retained by virtue of segregation of the parental gene's original roles among the daughter genes. Similarly, either acquisition of novel regulatory mechanisms or segregation of pre-existing ones may serve to maintain completely redundant protein coding regions. The concept of gene preservation by segregation of its various functions is termed the duplication-degeneration-complementation theory (DDC).[433] By

applying these concepts to *hiap1*, *hiap2* and *xiap* one can come to comprehend how these obviously similar genes have been retained. HIAP1 and HIAP2 are localized to the TNF receptor complexes whereas XIAP appears in the BMP signalling pathway implying an acquisition of function or perhaps more likely a segregation of function. HIAP1 and HIAP2, in contrast, are clearly homologous with respect to tissue distribution[331] (and the present work), cellular localization[60],[409], their ability to inhibit a variety of caspases[327], as well as their activity within the ubiquitination pathways.[329],[394] The most important difference between the two genes appears to be that *hiap1* is responsive to cytokine stimulation whereas *hiap2* expression appears to be more constitutive. This difference alone may provide sufficient selective advantage that both genes are maintained.

### 10.2 Gene expression and MALT lymphoma

An examination of the tissue distribution of *hiap1* and *hiap2* reveals a similar pattern of expression, though *hiap1* appears subject to wider variations than *hiap2*. That expression of *hiap1* and *hiap2* are generally depressed in the CNS argues for a reduced role for HIAP1 and HIAP2 in these tissues, perhaps their role being complemented by other IAP family members such as XIAP or NAIP. Given the similarity between both gene products, their co-localization within the TNFα signalling pathway, and their demonstrated ability to suppress caspase activation it is likely that they function in a similar fashion within equally similar signal transduction pathways. In this case, a similar tissue distribution is not unexpected. In this respect, it is interesting to note that HIAP1 and HIAP2 co-operate with TRAF1 and TRAF2 to inhibit the apoptotic signal from the TNFRI and alone are required to inhibit etoposide mediated cell death.[409] It may be that their association with the TRAF proteins brings them into proximity with CASP8 at the receptor complex, whereas homotypic association of the CARDs of HIAP1 or HIAP2 with that of Apaf1 may bring them into proximity with CASP9.

From the outset, it was observed that both *hiap1* and *hiap2* appear to be abundantly expressed within tissues of the immune system. Of particular note is that many of the non immune tissues in which *hiap1* is highly expressed also contain mucosa associated lymphoid tissues. Depending on the particular origin of the tissue, it may be referred to as GALT(gut), BALT (bronchiole), or MALT proper. In any event, these tissues form essentially extranodal centres of lymphocyte maturation or activation.[4] Recently, it has been demonstrated by several groups that the most common cytogenetic abnormality associated with lymphomas arising from these tissues involves *hiap1*. Several translocation events between *hiap1* and *malt1*, located on chromosome 18(q21), have been described[391],[396], all of which form in frame fusions. The translocations occur in two regions of *hiap1*. The first of these occurs after exon 7 of *hiap1*, while the second occurs after

exon 8 thus retaining the HIAP1 caspase recruitment domain in the resulting fusion protein. That MALT lymphomas arise in the absence of a CARD containing fusion argues against a role for that domain in the pathogenesis of this malignancy. Notably, all of the fusions described thusfar are in frame and all involve *malt1*, thus implying a role for the C-terminus of MALT1 in the oncogenic process.

Although HIAP1 is a known anti-apoptotic protein, until recently the function of the translocation partner, MALT1, was unknown. It is now known that *malt1* encodes a caspase-like protein (paracaspase)[24]. MALT1 binds Bcl10 and in so doing may be translocated to the site of HIAP1-TRAF2 interaction.[110] The HIAP1/MALT1 fusion bypasses the requirement of Bcl10 in this process by directly recruiting MALT1 to the HIAP1-TRAF2 complex. What role MALT1 plays in this complex or what substrates exist for it remain to be determined.

Interestingly Bcl10 is also translocated in many MALT lymphomas. These translocations bring *bcl10* into the vicinity of either the Ig heavy chain locus[399] or that of the Ig kappa chain locus.[400] Forced overexpression of Bcl10 promotes apoptosis and has been proposed to deplete the cell of TRAF2, and thus inhibit NF-kB activation, by sequestering it in non-productive TRAF2-Bcl2 complexes[110]. Translocations placing *bcl10* under the control of the Ig loci may be the *in vivo* equivalent of this. Circumventing the pro-apoptotic nature of overexpressed Bcl10 was the finding that it is mutated in several tumour derived samples. Mutations occurring distal to CARD of Bcl10 fail to induce apoptosis whilst maintaining the ability to activate NF-kB. Mutations affecting the CARD domain abrogate this latter activity.[399] It has been shown that both the CARD and the C-terminus of Bcl10 are required for MALT1 binding, therefore the ability of a CARD containing truncated Bcl10 to induced NF-κB is likely independent of its MALT1 binding function. This suggests the possibility that the observed ability of Bcl10 to promote the HIAP1-TRAF2 interaction, and hence, NF-κB activation[110] may be due to homotypic interaction of the HIAP1 CARD with that of Bcl10.

### 10.3 Translational regulation of *hiap1* and *hiap2*

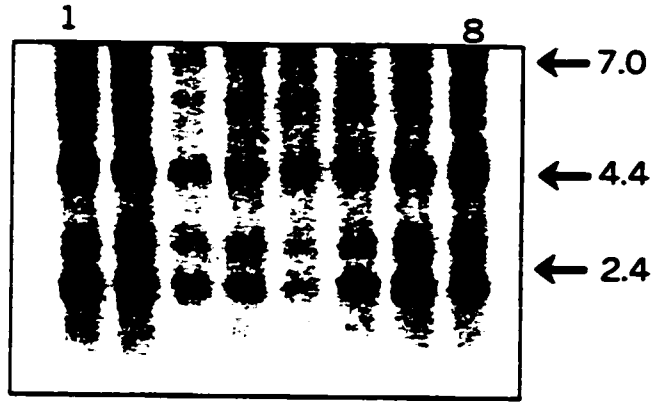
Given the observed length of the 5'UTR of both *hiap1* and *hiap2*, it is reasonable to assume that the mechanism of translation initiation would not be one of simple scanning. Apart from scanning, three other methods have been described. Similar in essence to scanning is the concept of re-initiation. Although the mechanism of re-initiation is poorly understood, some features have been described. For example, it is clear both the length and position of upstream open reading frames critically affect the efficiency of re-initiation. Furthermore, in the presence of overlapping reading frames, re-initiation can occur at sites 5' to the initial stop codon (backward

scanning). It remains unclear, though, whether the ribosome disassembles following translation of the first reading frame and whether the translation product of the first cistron plays any role in the translation efficiency of the second cistron.[435]

A third method of translation initiation is that of ribosome shunting. As in the previously described mechanisms, ribosome shunting is Cap dependant. Following recognition of the Cap structure, the initiation complex, complete with an intact ribosome, moves into proximity of the true initiation codon, and in so, doing bypasses the intervening sequence including any initiators which may be present. The actual mechanism by which this occurs remains obscure but no doubt involves recognition sequences akin to intron donor/acceptor sequences at both ends of the 5'UTR. The final mechanism of initiation is characterized by its independence of the Cap structure. As its name suggests, internal ribosome initiation allows for recognition of a sequence proximal to the start codon (internal ribosome initiation site, IRES) by eIF4E independent of Cap structure at the 5' terminus. Following internal assembly of the initiation complex, the ribosome moves to the start codon and initiates translation.[387]

An examination of their respective 5'UTRs reveals that that of *hiap1* contains 46 potential initiation sites with the minimal consensus ANNATG[427], whereas that of *hiap2* possesses 14. These would likely prove to be insurmountable obstacles to translation initiation by either traditional scanning or re-initiation. Given the observation that the *xiap* message was translated via internal initiation, the possibility that *hiap1* and *hiap2* were also was examined. As for *hiap1*, none of the 5'UTR inserts displayed any IRES like activity. This is not to say that the *hiap1* message is not translated in an IRES dependant manner. It may be that expression is cell- type specific or is inducible. That being said, HIAP1 is detectable by western blot in a number of cell types under basal conditions (Figure 36). One of the more interesting findings surrounding possible mechanisms of *hiap1* translational regulation is that several polyadenylation signals are present within the 5'UTR of *hiap1*. Northern blot analysis revealed the presence of several *hiap1* 5'UTR specific transcripts of the appropriate size, indicating that these are in fact valid transcription termination signals (Figure 40). It is interesting to speculate that perhaps signal-induced attenuation of transcription termination may play a role in cytokine mediated "upregulation" of *hiap1*. Exactly how these signals contribute to the expression of *hiap1* remains to be determined, however their location immediately upstream of the initiation codon would potentially abrogate even IRES mediated translation initiation. Whether these signals adversely affected translation initiation from the *hiap1* dicistronic construct tested is not known. Given that the *hiap1* message is indeed translated, mechanisms must exist to overcome the inhibitory effect of these signals, although they may not function in the context a dicistronic plasmid.

**Figure 40: Polyadenylation signals in the *hiap1* 5'UTR: Northern blot analysis.** Premature transcription termination was determined by hybridization of the *hiap1* 5'UTR (exon 3, P3993-P3817) to a commercially available Northern blot (Clontech, 7755-1). Bands at 2.2 and 2.7 kb likely represents transcription termination within the 5'UTR whereas the origin of the band at 4.4 kb is unknown it may represent intronic termination.




1. Cerebellum
2. Cerebral Cortex
3. Medulla
4. Spinal Cord
5. Occipital Lobe
6. Frontal Lobe
7. Temporal Lobe
8. Putamen

Interestingly, the 1kb portion of the *hiap1* 5'UTR proximal to the initiator, when placed in the antisense orientation, displays significant promoter activity. This raises the possibility that *hiap1* expression may be regulated by antisense transcription. Similar antisense transcripts have been described for FGF2[436], TGFβ2[437], SMAD5[438] as well as the EBV gene BZLF-1.[439] All of which appear or are proposed to regulate expression of their sense orientation cognate. Perhaps most relevant to the current discussion is the observation that expression of survivin and its anti-sense homologue, EPR-1, are mutually exclusive. Furthermore, transient expression of EPR-1 results in HeLa cell apoptosis thus implying a role for survivin in cell viability.[416]

In contrast to the sense orientation of the *hiap1* 5'UTR, that of *hiap2* does mediate some expression of the downstream cistron when placed in a dicistronic reporter plasmid. Like *hiap1*, this may simply be due to cryptic promoter activity of the fragment, although no evidence of smaller *hiap2* transcripts were detected by Northern blot (Figure 11).

One of the salient features of IRES driven translation is its resistance to translation inhibition by picornaviral protease 2A. This viral protease directly cleaves the translation initiation factor eIF4G.[440],[441] In so doing, 2A prevents binding of 4G to the Cap binding protein 4E, thus preventing Cap dependent translation. Through an as yet unidentified intermediary, the C-terminus of 4G is able to internally and specifically bind the 5'UTR of IRES containing messages and thereby recruit the remainder of the initiation complex.[442] Co-transfection of the *hiap2* 5'UTR containing dicistronic plasmid, pSY, with CMV driven poliovirus 2A, demonstrated that, in fact, expression from the *hiap2* 5'UTR is Cap dependant. This implies that the *hiap2* 5'UTR mediated expression of the downstream cistron in pSY is likely due to some inherent promoter activity of the fragment. How this can be reconciled with the lack of smaller transcripts, as determined by Northern blot analysis remains to be determined.

## 10.4 Transcriptional control

### 10.4.1 *hiap1*

Soon after their first characterization, it became evident that *hiap1* and perhaps *hiap2* may be transcriptionally regulated by NF-κB.[390],[409] As for *hiap1*, whether this was a direct effect on its transcriptional unit or whether NF-κB act on a different target which then in turn bound *hiap1* promoter/enhancer elements was not known. The first evidence that NF-κB might directly transactivate *hiap1* came with the observation that both puromycin and cycloheximide were able to mediate the same effect (Figure 20). Given that the turnover of IκBα is rapid, any inhibition of the protein synthesis would rapidly tilt the balance of bound versus free NF-κB. If protein synthesis is sufficiently inhibited, any effect of NF-κB on *hiap1* transcription would necessarily

be direct as, in the absence of *de novo* synthesis, no intermediate factor could be produced. Another mechanism by which inhibition of protein synthesis has been reported to augment mRNA levels is by stabilization of the message itself. Although this mechanism remains poorly understood, labile destabilizing proteins with affinity to elements within the 3'UTR of many genes appear to be sometimes involved.[443],[444],[445],[446] In order to determine whether inhibition of protein synthesis alone could alter the mRNA stability of *hiap1*, the degradation of *hiap1* (vs gapdh) was measured following TNFα and puromycin treatment. The results indicated clearly that the induction of *hiap1* by puromycin was not mediated by mRNA stabilization (Figure 21). That the effect of both cycloheximide and puromycin could be inhibited by PDTC, a known NF-κB inhibitor[428], further supports the notion that these act via direct transcriptional upregulation (Figure 20).

Computer aided scanning of the *hiap1* promoter and 5'UTR region revealed several transcription factor binding sites. Two NF-κB binding sites were observed, as well as an NF-IL6 binding site (C/EBPβ) and an (NF-κB) negative regulatory element (NRE). The latter element was shown to inhibit basal NF-κB dependant transcription from the IFNβ promoter.[429] Interestingly, although the two NF-kB sites are conserved between the mouse and human, they were identified in Genbank as being part of the mouse 5'UTR (Figure 22). Similarly, several human derived 5'RACE products extend beyond a previously reported putative transcription start site[424] . This may be due to the use of alternative upstream start sites or perhaps may simply represent aberrant or non-specific transcription initiation. As for NF-IL6, it was identified as a TNFα inducible transcription factor required, in conjunction with NF-κB, for the expression of interleukin-6 following TNFα treatment.[447],[448],[449] NF-IL6 has since been found to similarly couple with NF-κB to induce the transcription of a number of acute response genes including ICAM-1[450] and iNOS[451], as well as transcription from the HIV-1 LTR.[452],[453]

In order to determine if any or all of these sites bound their putative protein partners, they were subjected to gel shift analysis. From these results it would appear that all are able to specifically bind their respective cognates (Figures 24 and 25). Next, a CAT reporter construct was assembled encompassing all of the *hiap1* promoter region and extending to the 3' boundary of the first exon. Site directed mutagenesis analysis of the various binding sites alone and in combination revealed that indeed the putative NF-κB sites were functional as was the NF-IL6 site. The NRE, though, appeared to have no effect either on the basal transcription level nor that induced by TNFα (Figure 26). A similar observation was made with respect to the IFNβ NRE, although in that situation the NRE does inhibit basal transcription.[454] It may be that, in HeLa

cells, the basal, non-induced, level of NF-κB driven transcription is able to overcome the minimal repression offered by NRF (NF-κB regulatory factor). This is supported by the observation that PDTC is able to reduce even basal *hiap1* transcription (Figure 20 and 27). Given that the most dramatic reduction in CAT expression was achieved by eliminating κB1, this site was examined as potential enhancer element. Enhancer elements, of which NF-κB binding sites are one example, are both position and orientation independent. As such, κB1 should be able to enhance inducible expression from a heterologous promoter in such a manner. Indeed, when placed 3' to a CMV driven CAT gene κB1 was able to enhance transcription following TNFα treatment in an orientation independent manner. That expression was enhanced in the absence of TNFα argues for a basal amount of NF-κB dependant transcription (Figure 28). This last observation may also lend support to the proposal that NRF is not able to repress basal *hiap1* transcription due to constitutive low level NF-κB activation in these cells.

Until recently, the pathway by which the TNFR family members activate NF-κB has remained unknown. It has been shown that TRAF2 bound to the TNFRI complex is able to bind and activate NIK. NIK then in turn phosphorylates the IKK complex resulting in IκB phosphorylation and subsequent κB activation. Recent reports, though, have offered a complementary pathway leading to the activation of IKK. Upon trimerization of the receptor complex, PI3K becomes activated, possibly through the intermediacy of cSRC[455],[456] or a like kinase, leading to the formation of PIP3 (phosphatidylinositol 3,4,5-triphosphate) at the plasma membrane.[211] PIP3 then recruits PDK1 (phosphatidylinositol dependant kinase-1) and PDK2 to the plasma membrane where they phosphorylate PKB/akt. Activated PKB/akt then proceeds to phosphorylate the IKK complex.[184] Interestingly, the phosphorylation of IKK by PKB/akt does not occur at the same site as does that mediated by NIK. It has, likewise, recently been shown that, under the influence of PKB[210], IKK itself may phosphorylate the p65 subunit of NF-κB[209], thereby potentiating its transactivation capacity. It may be that both pathways are required for the full activation of NF-κB. Signal transduction cascades perpetuated through NIK may promote the phosphorylation and subsequent degradation of IκB isoforms, whereas PKB/akt may allow for either a change in or the expansion of the substrate specificity of the IKK complex such that p65 itself is phosphorylated.

Following the report of TNFα signalling via PI3K and PKB[211], we sought to determine whether this may be a general pathway through which all TNFR superfamily signal to the IKK complex and hence to *hiap1*. Transfection of EBV-LMP1 into HeLa cells dramatically upregulated expression of *hiap1*, as did treatment with TNFα. This, though, was not unexpected

as LMP1 is known to activate NF-κB as an anti-apoptotic mechanism. In order to determine whether TNFR mediated transcriptional upregulation of *hiap1* occurred in a PI3K dependent manner, we determined its sensitivity the PI3K inhibitors wortmannin (a mould metabolite)[457],[458] and LY294002 (a quercetin analogue).[459] The results indicate that *hiap1* upregulation by either TNFα or LMP1 was severely inhibited by these compounds, indicating a requirement for this pathway in this system (Figures 29 and 30).

To date, most of the anti-apoptotic function attributed to LMP1 revolves around the induction of Bcl2[460] and its homologues Bfl/A1[461] and Mcl-1.[462] In retrospect, the activity of LMP1 on Bfl/A1 might, in part, be due to its ability to activate NF-κB. As such, LMP1 might equally well be expected to upregulate Bcl-$X_L$ as does TAX.[420] EBV also encodes its own Bcl2 homologue, BHRF1, although its expression appears to be restricted to the lytic phase.[463],[464] Given that LMP1 represents a constitutively active member of the TNFR superfamily, *hiap1* upregulation was not unexpected, although it does add yet another mechanism by which EBV is able to evade the host's apoptotic response to viral infection.

To this point, all of the discussion surrounding the transcriptional regulation of *hiap1* has centered around the involvement of NF-κB. Recently, it was reported that dexamethasone, alone or in combination with IFNγ, can mediate *hiap1* transcriptional upregulation[430] (and Figure 32). This effect has been limited to the airway epithelial cell line A549 but might well be extended to the airway epithelia in general. Dexamethasone is a synthetic glucocorticoid whose main function is as an anti-inflammatory and immunosuppressive drug. Most of its anti-inflammatory and immunosuppressive function likely rests on the ability of the dexamethasone to antagonize the activity of NF-κB, either by upregulation IκBα[465] or by transrepression of NF-κB by the bound glucocorticoid receptor.[466],[467] Of note, dexamethasone appears to be able to upregulate expression of IκBα in the absence of any discernible glucocorticoid responsive element in its promoter region, rather it possesses between three and six NF-κB binding sites.[304],[306],[468] These results, though, remain controversial, with the effect apparently cell-type specific. It has been proposed that the glucocorticoid receptor may bind a tissue specific transcriptional co-factor which, in turn, is able to bridge the gap between the activated GR and the IκBα promoter. Work by Heck (1997) has demonstrated that NF-κB inhibition and IκBα upregulation are distinct functions and are separable by glucocorticoid analogues, with some analogues able to upregulate IκBα but not directly inhibit the activity of NF-κB.[469]

In a similar vein, dexamethasone also appears to upregulate Bcl-$X_L$ expression in a variety of cancer cell lines.[470],[471],[472] It has also recently been observed that dexamethasone may help

maintain the nuclear levels of NF-κB during serum deprivation of rat hepatoma cell lines.[473] Although a *bona fide* GRE may in time be found in the promoter region of Bcl-$X_L$, it has been shown to be inducible by NF-κB.[420] Given the above and that dexamethasone is commonly administered during chemotherapy, it has been suggested that its effect on Bcl-$X_L$ expression may interfere with the induction of apoptosis by these drugs.[472] The same arguments may as well hold true for dexamethasone in relation to elevation of *hiap1* expression in the airway epithelia.

The simplest explanation for the observed effect of dexamethasone on the expression of NF-κB responsive genes is that some facet of glucocorticoid signalling positively interacts with elements of the NF-κB signalling pathway to mediate their transcriptional upregulation. If shown to be functional, similar interactions may be at the base of the observed effect of dexamethasone on *hiap1* transcription. In the absence of such a mechanism, and with no discernable GRE to upregulate IκBα during GR mediated NF-κB inhibition, there would be no mechanism in place to replenish IκBα, following TNFα stimulation. In the case of *hiap1*, this mechanism fits well with the observed inhibition of its transcriptional upregulation by PDTC (Figure 32). In contrast to the expression of the endogenous gene however, transfection studies indicate that dexamethasone inhibits transcription from the cloned *hiap1* promoter. This is precisely the effect one would expect from any other NF-κB regulated gene. Furthermore, deletion of both NF-κB binding sites as well as the NF-IL6 site, renders the negative effect of dexamethasone on reporter gene expression insignificant. How these two results can be reconciled remains unclear at this point, although the most likely explanation is that the dexamethasone responsive element lies outside the cloned promoter region, perhaps lying in one of the downstream introns.

### 10.4.2. *hiap2*

In opposition to *hiap1*, whose expression can be modulated by a number of factors, that of *hiap2* is relatively resistant to modulation. Some reports have posited that *hiap2* is regulated by NF-κB. An analysis of the promoter region of *hiap2* reveals a single NF-κB site as well as several potential Sp1 and NF-IL6 sites, indicating a possible role of TNFα in the induction of *hiap2* (Figure 23). In contrast to previous reports, though, no significant effect on endogenous *hiap2* transcription could be observed following treatment with TNFα (Figure 20). Furthermore, expression of *hiap2* is stable following such treatments as cycloheximide, puromycin, PDTC, wortmannin, LY294002, dexamethasone, and IFNγ. That expression of *hiap2* is impervious to modulation may imply that it represents a constitutive member of the IAP family of genes. As such, HIAP2 may perform more of a "housekeeping" role by preventing random and aberrant

initiation of apoptosis, whereas under times of stress other IAPs may be brought to the fore such as XIAP (IRES mediated translation) or HIAP1 (NF-κB mediated transcriptional upregulation).

### 10.5 Characterization of the Burkitt's lymphoma cell lines BJAB(wt) and BJAB(ko)

In order to determine whether any small scale insertions, deletions, or inversions may have contributed to the observed *hiap1* upregulation in the EBV positive Burkitt's lymphoma cell line, Raji[431], a panel of Burkitt's lymphoma cell lines were subjected to Southern blot analysis. Although no genomic abnormalities were associated with Raji, it was observed that the BJAB cell line was deleted for both *hiap1* and *hiap2*. Confounding this finding was the fact that EBV positive derivative BJAB lines appeared to harbour the "wild type" genomic restriction pattern (Figure 34).

These results seemed to indicate that, subsequent to the derivation of the EBV positive substrains, the BJAB line with which we were working had lost both *hiap1* and *hiap2*. PCR analysis of a commercial strain of BJAB revealed that it harboured no such deletion. That the derivative line, in fact, was of BJAB origin was confirmed by PCR analysis of surrounding chromosomal markers.[474] This analysis also revealed the size of the homozygous deletion to be between 2.2 cM (D11S1339-D11S923) and 4.5 cM (D11S1325-D11S917) (Figure 37). This area encompasses several EST's of unknown function as well as MMP7, CASP1, CASP4 and CASP5. Although not known to be directly involved in the propagation of the apoptotic cascade, the absence of these caspases would undoubtedly be a confounding variable in any experimental paradigm utilizing these cells. PCR analysis has confirmed that both cell lines are at least hemizygous for each of the caspases in question (Figure 38).

With the identity of BJAB(ko) established, the effect of the deletion on the expression of the other IAPs as well as the TRAF messages was determined under both constitutive and induced conditions. That TNFα only marginally increased the levels of *hiap1* and *traf1* in these cells lines is not remarkable in that both are regulated by NF-κB, a constitutively active transcription factor in B-cells.[247] Although expression of *traf1* was minimally affected by TNFα it's expression was markedly higher in the derivative line. Barring any other significant differences between the two cell lines, it would appear that HIAP1 and/or HIAP2 may have a moderating effect on some aspect of *traf1* expression and a positive effect on that of *traf2*. In the absence of genuine "double knock out" cell lines or animals, these lines represent the only background in which to study signal transduction events downstream of HIAP1 and HIAP2. Transient or stable reintroduction of the deleted genes into BJAB(ko) followed by analysis of reporter gene expression will go partway towards understanding what, if any, impact these genes may have on signal transduction.

Given the reported role of HIAP1 and HIAP2 in cytoprotection following TNFα treatment, we sought to determine the effect of their loss on the viability of this cell line. The results indicate that, in agreement with previous findings, TNFα failed to induce any noticeable cytotoxicity in the parental line. In fact, TNFα caused an approximately 20% increase in total cell numbers (over control) 48 hrs post-treatment. In contrast to this, TNFα inhibited the growth of the derivative cell line by approximately 20%. Adenovirus mediated reintroduction of *hiap1* into the derivative cell line completely abrogated this line's sensitivity to TNFα. Conversely, *hiap1* anti-sense treatment rendered the parental line sensitive to TNFα (Figure 40) presumably by downregulation of *hiap1* expression. Curiously these effects were brought about by modulating the expression of *hiap1* only. It would be interesting to know if similar effects would be observed with *hiap2* or whether the results would be additive by modulating both *hiap1* and *hiap2* expression.

**11. Summary and comments**

In the present work we sought to elucidate the genomic structure of two members of the inhibitor of apoptosis gene family, *hiap1* and *hiap2*. Furthermore, we investigated various aspects of their mechanisms of transcription and translation. From this work it was found that both *hiap1* and *hiap2* share significant organizational homology, not only to themselves, but to *xiap* as well. Although neither the timing nor the sequence of events is known, it is clear that gene duplication gave rise to all three members. That all are present in the mouse genome strongly suggests that the duplications took place before the divergence of the species some 80 million years ago.

Investigations into the mechanism of *hiap1* and *hiap2* translation revealed that neither *hiap1* nor *hiap2* are translated via traditional scanning or re-initiation. Neither is *hiap1* translated via internal entry and although *hiap2* does seem to possess some qualities of internal initiation, it fails to meet the stated requirement of being protease 2A insensitive. Interestingly, *hiap1* translation may be regulated by at least two somewhat novel mechanisms. The first involves transcription termination prior to the coding region, failure to generate transcripts containing the coding region would clearly obviate translation. The second mechanism may involve anti-sense transcription originating near the translation start. The resulting anti-sense product may bind to and inhibit translation of the *hiap1* message or may serve to destabilize it. Both of these phenomena require further investigation to determine the extent to which each may contribute to the regulation of *hiap1* expression.

At the transcriptional level, *hiap1* again seems to be more complex than *hiap2*. In contrast to previous reports, we consistently failed to demonstrate any transcriptional upregulation of *hiap2*

under any paradigms, including NF-κB activation. Conversely, we have shown that *hiap1* is regulated by a number of factors including dexamethasone and directly, as well as indirectly, via NF-IL6, by NF-κB.

Analysis of the derivative BJAB cell line, BJAB(ko), shed some light on the involvement of HIAP1 in countering TNFα mediated cytotoxicity. Differential susceptibility of the parent and derivative line has demonstrated that HIAP1 alone can alleviate at least some of the cytotoxic effects of TNFα. The identification of this cell line provides us with the unique opportunity to study both apoptosis in the absence of HIAP1 and HIAP2 as well as signalling events downstream of these. In this vein, with involvement of the IAPs in the regulation of caspase activation firmly established, the function of HIAP1 and HIAP2 (and XIAP) at the plasma membrane remains somewhat elusive. Although these may also serve to modulate caspase activation at this level, the possibility exists that they may themselves transduce a signal from the receptor complex. What this signal may be and how it contributes to TNFα mediated signal transduction would be of considerable interest and importance. Furthermore, the intriguing observation that HIAP1, HIAP2, and XIAP possess intrinsic ubiquitin ligase/conjugating activity raises the possibility that they may function to target various pro-apoptotic molecules for degradation. These may include the caspases themselves, or perhaps pro-apoptotic signalling molecules within the TNFR1 receptor complex.

With the eventual conclusion of the human and mouse genome sequencing projects, work of this type described herein will be made much easier. Comparison of genomic sequence data with novel cDNAs will reduce the time required to identify individual intron/exon boundaries from several weeks or months down to a few days. Identification of genuine transcription starts sites, although aided by the presence of baseline genomic sequence, will still be hampered by technical difficulties associated with the isolation of full length cDNAs. Computer-aided mouse-to-human homology searches will likely greatly aid in the identification of key regulatory regions or sites. As the annotated genome begins to take shape, the challenge facing the molecular biologist in the future will not be in identifying novel regulatory regions or genes but rather how these regions and genes work with each other to effect a particular outcome. With this understanding comes, for the first time, the possibility of modulating single gene expression for the betterment of the patient.

It should be noted that singular gene expression is the end result of the regulated interplay of a vast number of signalling molecules. I believe that successful manipulation of gene expression, as a routine intervention, will prove to be a near insurmountable task – one which will no doubt occupy molecular biologists for the foreseeable future.

## 12. Methods
## 12.1. Genomic organization
### 12.1.1 λ-phage library screening

The strain of *E. coli* used for propagation and screening of the library differed depending on the λ phage library in use. Furthermore the λ phage strain used is different for each library. Genomic library screening was carried out using a male placenta library (Stratagene, cat# 946205) whereas cDNA library screening was carried out with a spinal cord library (Clontech, cat#HL2001b). Both the phage strain and the *E. coli* used for screening and amplification of the library were unique to the individual library.

#### 12.1.1.1. Preparation of infection competent *E. coli*

1. Grow overnight culture (30°C) of *E. coli* from single pick in 20 mL of LB culture media supplemented with the following:

| | |
|---|---|
| 1 M MgSO4 | 200 µl |
| 20% Maltose | 200 µl |

2. Harvest cells by low speed centrifugation.
3. Resuspend in 10 ml of cold 10mM $MgSO_4$
4. Measure OD at 600 nm
5. Dilute to OD600 = 0.6 with cold 10mM $MgSO_4$
6. Store bacteria at 4°C for up to 48 hrs

#### 12.1.1.2. Titration of λ phage library

1. Prepare "top agar"

NZYM media
0.7% agar
Autoclave, then cool to 48°C in waterbath until needed.

2. Warm five 150 mm NZYM agar plates to 37°C (2hrs).
3. Prepare serial dilutions of stock virus solution. Initially dilute 1ul of the stock into 1000 µl SM buffer. Follow this by subsequent dilutions of 100X, 10X, and 10X. This yields dilutions of $10^{-3}$, $10^{-5}$, $10^{-6}$, and $10^{-7}$.
4. Combine 500 µl of freshly prepared *E. coli* with 100 µl diluted phage. Also prepare a blank by combining 500 µl *E. coli* with 100 µl SM buffer.
5. Incubate at 37°C for 15 minutes NO SHAKING
6. Add 5 ml "top agar" and mix quickly
7. Pour evenly over pre-warmed NZYM agar plates.

8. Allow to cool/solidify for 20 minutes.
9. Incubate O/N at 37°C
10. Determine phage titre.

e.g. titre $= \frac{234 \text{ pfu}}{0.1 \text{ ml}} \times \frac{1}{10^{-5}}$

$= 2.34 \times 10^{8}$ pfu/µl

**12.1.1.3. Primary screen**

**Infection**

1. Prepare "top agar" as above
2. Warm 31 NZYM agar plates as above
3. Dilute stock phage to ~$3 \times 10^{4}$ pfu/100µl in a total volume of 3.5 ml SM buffer.

e.g. vol viral sock = (vol SM x $C_{final}$)/ stock titre

= (3500 µl x 300 pfu/µl)/($2.34 \times 10^{8}$pfu/µl)

= $4.48 \times 10^{-3}$µl

Therefore use 4.5 µl of the a $10^{-3}$ stock phage dilution

4. Combine 30 x 500 µl *E. coli* with 30 x 100 µl diluted phage stock.
5. Combine 500 µl *E. coli* with 100 µl SM buffer.
6. Let stand at 37°C for 15 minutes - NO SHAKING
7. To each tube in turn add 5 ml "top agar", mix quickly, and pour evenly over pre-warmed NZYM agar plate.
8. Allow to cool/solidify for 20 minutes.
9. Incubate O/N at 37°C

**Plaque lifts**

1. Remove plates from incubator and, assuming complete lysis of bacterial lawn, cool to 4°C. (SM only plate should have bacterial lawn)
2. Prepare 2000 ml each of denaturation, renaturation, and neutralization solutions.
3. To each plate individually
   - Overlay number plate with correspondingly numbered circular membrane (Hybond N® / NEN) and mark the agar according to orientation holes on membrane.
   - Let stand 30 seconds.
   - Carefully remove membrane and soak in denaturation solution for 2 min.
   - Transfer membrane to first of two neutralization solutions and soak for 2 min.
   - Transfer membrane to second of two neutralization solutions and soak for 2 min.
   - Transfer membrane to rinse solution [2X SSC] and soak for 2 min.
   - Place membrane on absorbent paper (e.g. Whatmann®) and allow to air dry for 1 hour.

4. Store plates at 4°C until further use.
5. Crosslink immobilized phage DNA to membrane by irradiating membranes under ultraviolet radiation (1200 J/cm$^2$).
6. Membranes can be stored indefinitely under dry conditions.

**Probe Hybridization, washing and mounting**

1. Place membranes in hybridization bag (e.g. Seal-a-meal)
2. Fill bag with 50-75 ml of southern blot hybridization solution.
3. Incubate at 65°C for 2-3 hours in shaking waterbath.
4. Warm a further 30-40 ml of hybridization solution.
5. Prepare four times (4X) the amount of probe described in Southern blot procedure.
6. Replace hybridization solution with 30-40 ml fresh hybridization solution.
7. Add denatured probe to membranes (see section 11.1.2.3).
8. Seal bag and incubate O/N at 65°C in shaking waterbath.
9. Remove membranes and quickly immerse all in rinse solution [2X SSC. 0.1% SDS, RT].
10. Agitate for 2 minutes at RT
11. Replace rinse solution with 1° wash solution [2X SSC, 0.1% SDS, 65°C] and agitate for 10-15 minutes.
12. Repeat step 11
13. Replace rinse solution with 2° wash solution [1X SSC, 0.1% SDS, 65°C] and agitate for 10-15 minutes.
14. Check level of background hybridization on membranes with Geiger-Mueller survey meter (0.1X setting). Background levels should be somewhat higher than for a standard Southern blot as this helps to orient the exposures with respect to orientation holes in membrane.
15. If background hybridization is sufficiently low then mount 4-5 membranes (DNA side up) on used 35x43 cm autoradiography film (as backing) and cover with cellulose wrap (Saran Wrap).
16. If background hybridization is high, replace 2° wash solution with fresh 3° wash solution [0.2X SSC, 0.1%SDS, RT] and slowly heat to ~45 °C. Continuously check level of background hybridization. Leave membranes in heated solution for a maximum of 10 min.
17. Once background hybridization is sufficiently low mount membranes as in step 15.
18. Expose mounted membranes to autoradiography film (35x43cm) O/N at -80°C
19. Develop O/N exposures.
20. Number individual membrane exposures on autoradiography film.
21. Locate and mark orientation holes on autoradiography film.

**1° phage plugs**

1. Using a light box to illuminate a single autoradiography film from below, place an appropriately numbered infected plate onto its corresponding outline (on the autorad).
2. Orient plate with respect to orientation holes.
3. Locate an area of hybridization (spot).
4. Plunge a 1 ml pipette tip with the tip cut off into the centre of the area and remove it ensuring a complete seal between the agar plug and the pipette tip.
5. Eject the agar plug into a numbered 1.5 ml microcentrifuge tube.
6. Repeat steps 1 to 5 for remaining spots.
7. To each sample (1° plug) add 500 µl of SM buffer and a single drop of chloroform (50 µl).
8. Vortex. and let stand for 2-3 hours.

**12.1.1.4. Secondary screen**

1. Dilute phage in 1° plugs to $10^{-5}$ in SM Buffer.
2. Use 100 µl of diluted phage to infect 500 µl of freshly prepared *E. coli*.
3. Remainder of infection protocol is as described for 1° infection.
4. The number of plaques obtained should be on the order of 1-4000/plate.
5. Should too many or too few plaques be obtained, repeat 2° infection with 10 fold more or less virus as appropriate.
6. Pick 2° plugs using 200 µl pipette tip with tip cut off.
7. Discard any 1° plugs which did produce a signal on the 2° infection.

**12.1.1.5 Tertiary screen**

1. Dilute phage in 2° plugs to $10^{-7}$ in SM Buffer.
2. Use 100 µl of diluted phage to infect 500 µl of freshly prepared E.coli.
3. Remainder of infection protocol is as described for 1° infection.
4. The number of plaques obtained should be less than 100/plate
5. Should too many or too few plaques be obtained, repeat 2° infection with 10 fold more or less virus as appropriate.
6. Pick 2° plugs using 200 µl pipette tip with tip cut off. It is essential that the plaque be unencumbered by surrounding plaque to ensure the purity of the resulting 3° plug.
7. Discard any 2° plugs which did produce a signal on the 3° infection.

### 12.1.1.6. Generating high titre stock

1. Dilute 3° plug to $10^{-5}$ in SM buffer.
2. Use 100 µl infect 500 µl freshly prepared *E. coli*
3. Remainder of infection protocol is as described for 1° infection.
4. Following O/N incubation check to see if bacteria are completely lysed.
5. If plate is not completely lysed perform infection again with ten fold more virus.
6. Overlay each plate with 10-15 ml SM buffer.
7. Swirl for 6 hrs at RT
8. Add 100 µl chloroform to overlayed SM Buffer (to release phage particle trapped inside bacteria)
9. Collect phage slurry in 15 ml conical tube.
10. Centrifuge phage slurry at 7000 rpm in swinging bucket rotor.
11. Collect the cleared high titre stock and store at 4oC
12. To 750 µl of the high titre stock add 250 µl glycerol, mix by hand, and store at –80°C for future use.

### 12.1.1.7. Phage DNA preparation

1. Infect 500 µl freshly prepared E.coli with 15-30 µl high titre stock or glycerol stock.
2. Let stand at 37°C for 15 min.
3. Incubate O/N at 37°C in 20 ml NZYM in shaking incubator (250 rpm).
4. Check O/N cultures. Cultures should be minimally cloudy with considerable amount of agglomerated cell debris.
5. To each culture add 300 µl chloroform, vortex.
6. Centrifuge at 3000 rpm in swinging bucket rotor for 10 min.
7. Transfer supernatant to new 50 ml conical tube
8. To each tube add RNaseA and DNaseI to a final concentration of 5 µg/ml.
9. Incubate at 37°C for 30 min.
10. To each tube add an equal volume of freshly prepared 20%PEG/2N NaCl.
11. Let stand in ice water for a minimum of 1 hour.
12. Centrifuge at 3000 rpm and 4°C in swinging bucket rotor for 20 min.
13. Resuspend pellet in 500 µl SM buffer.
14. Transfer to phage slurry to microcentrifuge tube.
15. Centrifuge at 8000 rpm in benchtop microcentrifuge for 2 min.
16. Transfer supernatant to fresh microcentrifuge tube.
17. To each tube add 10 µl of 0.5M EDTA (pH 8.0) and 10 µl of 5% SDS (10mM and 0.1% final respectively).
18. Incubate at 65°C for 15 min.
19. Extract with 1 volume phenol, followed by 1 volume phenol/chloroform, and finally by 1 vol chloroform.
20. To the extracted aqueous phase, add 1 volume of isopropanol and mix by hand (DNA should visibly precipitate).
21. Centrifuge at 14 000 rpm in benchtop microcentrifuge.
22. Wash pellet with 500 µl of 70% ethanol.

23. Remove all traces of ethanol overlaying DNA pellet.
24. Dissolve DNA pellet in 50 µl water.
25. Measure DNA concentration by spectrophotometry.
    [C (µg/ml) = $OD_{260}$ x fold dilution x 35 µg $ml^{-1}$ $OD^{-1}$]
26. Dilute DNA to 1µg/µl
27. Store DNA at –20°C.

### 12.1.2. Southern blot analysis

#### 12.1.2.1. Genomic DNA

Genomic DNA for Southern blot analysis was typically prepared utilizing an automated extractor. Usually 5-10 µg of genomic DNA was used per lane. Endonuclease digestion as well as sample electrophoresis and transfer to nitrocellulose membranes (PALL B) was carried out according standard protocols.

#### 12.1.2.2 λ phage DNA

Typically 0.5 µg of DNA obtained from a λ phage library screen was digested with one or more restriction endonucleases and subjected to agarose gel electrophoresis according to standard procedures. The resulting gels were treated they would for genomic Southern analysis and subjected to two way semi-dry transfer to nitrocellulose (PALL B) as follows.

1. The gel is soaked for 1/2 hour in each of the following solutions, in turn.
   - 0.2N HCl
   - 0.5N NaOH, 1.5N NaCl
   - 0.5 M Tris(pH 7.5), 1.5N NaCl
1. Decant the final solution from the tray.
2. Overlay the gel with a nylon membrane (BiodyneB, Pall) of equal size.
3. Wet the membrane with 20X SSC and, with a transfer pipette, remove any air bubbles from between the membrane and the gel.
4. Overlay the membrane with 4 sheets of filter paper.
5. Wet the filter paper with 20X SSC and, with a transfer pipette, remove any air bubbles from between the filter paper and the underlying membrane.
6. Overlay the filter paper with dry absorbent sheets.
7. Overturn the covered gel and gently remove the tray from over of it.
8. Place the transfer stack directly on the benchtop (gel side up).
9. Repeat steps 9 through 13 on the second side of the gel.
10. Place a glass plate (or book) over the transfer stack and apply a 1kb weight on it.
11. Allow the DNA transfer to proceed overnight.
12. Following the transfer disassemble the transfer stack and utilizing a sharp pencil mark the membrane with respect to the position of the loading wells.
13. Air dry the membranes for 1 hour.
14. Crosslink the immobilized DNA to the membranes by baking at 80°C for 30 min.
15. Store membranes dry at room temperature until needed.

### 12.1.2.3. Probe synthesis

In general, probes were synthesized by random primer labelling (Rediprime II. Amersham Pharmacia) with gel purified DNA fragments (0.25-1.5 kbp) as templates. The probes were then purified on Sephadex G-50 NICK columns (Amersham Pharmacia).

### 12.1.2.4. Hybridization, washing, and mounting

The hybridization and washing steps for Southern blot analyses are essentially the same as described for probing plaque lifts (section 11.1.1.3) except for the following.

1. The volume of hybridization solution is kept to a minimum, typically less than 15 ml.
2. When washing a "phage Southern", use [0.1X SSC, 0.1%SDS] at 65°C for 15 min for the final wash.
3. When washing a "genomic Southern" the final wash should be in [0.2X SSC, 0.1%SDS]. The solution should be warmed from room temperature to 65°C while checking for background hybridization.

### 12.1.3. Northern blot analysis

Commercial multiple tissue Northern blots were obtained (Clontech) and probed with the coding region of *hiap1* as well as the 3'UTR of *hiap2*.

### 12.1.3.1 Probe synthesis

Probe synthesis for northern blot analyses is the same as described for Southern blots (section 12.1.2.3)

### 12.1.3.2 Hybridization, washing, and mounting

The hybridization steps are essentially the same as described for Southern blots and plaque lifts save the following.

1. The hybridization solution is different (see appendix)
2. Hybridization occurs at 42°C overnight rather than 65°C.
3. The final wash should be in [0.1X SSC, 0.1% SDS]. The solution should be warmed from room temperature to 42°C while checking for background hybridization.

### 12.1.4. Transposon mediated sequencing

The complete 5' sequence (genomic) of both *hiap1* and *hiap2* were obtained by transposon facilitated genomic sequencing[425] of the region immediately upstream of the initiation codon. Briefly, the DNA fragment to be sequenced was subcloned into Bluescript SK(-) (Stratagene). Subsequent to selection and amplification (in DH5α), the resulting plasmid was used to transform *E.coli* (DPWC). While in this host, the engineered plasmid is randomly fused to the resident F' plasmid (Tn $1000^{r}$, $res^{-}$) (Gold Biotechnology). Ampicillin resistant clones (resistance derived from Bluescript) were grown on selective media then mated in a conjugation reaction with *E.coli* (BW26, $Kan^{r}$, $res^{-}$) in liquid culture. Once transferred to BW26 the fused

plasmid is resolved leaving within the cloning vector a randomly inserted transposon. The multicopy plasmids obtained from the resulting Kan/Amp resistant clones served as sequencing templates. Forward and reverse sequencing of individual clones from universal primers within transposon, followed by multiple alignment of these sequences (Genetics Computer Group, 1991) generated the full contiguous sequence.

### 12.1.5. 5'RACE (Rapid Amplification of cDNA ends)(GibcoBRL)

5' RACE was carried out according to the manufacturer's protocol. Essentially total RNA was obtained from Trizol (GibcoBRL) solubilized HeLa cells. Random primed cDNAs were then generated by reverse transcription. The RNA/DNA heteroduplexes were then treated with RNaseH to liberate single stranded cDNA molecules. These were then column purified and a poly-dC "tail" was added to the 5' end with terminal deoxynucleotide transferase (TdT). The resulting "tailed" cDNA served as an amplification template. PCR amplification of the 5'UTR was then carried using a primer located within the 5'end of the target message (gene specific primer –1, GSP1) and an amplification primer (supplied) annealing to the dC "tail". The PCR products were then subjected to a 2° (nested) amplification utilizing a primer (GSP2) located upstream of the first amplification primer (GSP1). Analysis the 5' RACE products generated in both the 1° and 2° amplifications was carried out by agarose gel electrophoresis. RACE products were then cloned into PCRII or PCR2.1 (Topo T/A, Invitrogen) according to the manufacturer's protocol and end-sequenced from within the cloning vector using universal primers, typically M13 (forward) and M13 (reverse).

### 12.1.6. Primer extension (Primer Extension – Promega, Cycle Sequencing – Amersham Pharmacia)

Primer extension was performed according to the supplied instructions (Promega). Essentially the reaction consists of a reverse transcription reaction from an RNA template using a $^{32}P$ labelled primer proximal to the estimated start site. The size of the labelled cDNA generated was could then be compared to a similarly labelled molecular weight marker. In this case the labelled RT product was compared to a sequencing ladder generated from the same primer and an appropriate genomic DNA fragment (Amersham Pharmacia). As such the start site could be directly estimated by comparison to the ladder.

### 12.1.7. 3'RACE (Rapid Amplification of cDNA ends)(GibcoBRL)

3' RACE was carried out according to the manufacturer's protocol. Essentially total RNA was obtained from Trizol (GibcoBRL) solubilized HeLa cells. PolyT primed cDNAs were then generated by reverse transcription. PCR amplification of the 3'UTR was then carried using primers located within the 3'end of the target message and an amplification primer (supplied) annealing to the 3' end of the RT primer (oligo dT primer). Analysis of the 3' RACE products was carried out by agarose gel electrophoresis. RACE products were then cloned into PCRII or PCR2.1 (Topo T/A, Invitrogen) according to the manufacturer's protocol and end-sequenced from within the cloning vector using universal primers, typically M13 (forward) and M13 (reverse).

## 12.2. Gene expression analysis

### 12.2.1. Dot and Northern Blot Analysis

Tissue distribution of both *hiap1* and *hiap2* was determined by both Northern and mRNA dot blot analysis (Clontech). As such only the hybridization and subsequent washing steps were performed.

#### 12.2.1.1. Probe synthesis

Probe synthesis for northern blot analyses is the same as described for Southern blots (section 12.1.2.3)

#### 12.2.1.2 Hybridization, washing, and mounting

The hybridization steps are essentially the same as described for Southern blots and plaque lifts save the following.

1. The hybridization solution is Northern specific (see appendix).
2. Hybridization occurs at 42°C overnight rather than 65°C.
3. The final wash should be in [0.1X SSC, 0.1% SDS]. The solution should be warmed from room temperature to 42°C while checking for background hybridization.

#### 12.2.1.3. Quantification of dot blot data

The resulting blots are then visualized with a Storm 860 PhosphorImager (Molecular Dynamics) and the accompanying ImageQuaNT analysis software, v.4.1 (Molecular Dynamics). Data from individual spots are then corrected for background signal and normalized against the signal obtained from 100 ng of genomic DNA to compensate for differences in hybridization efficiency between the two probes.

### 12.2.2. Lymphocyte gene expression (Whole blood fractionation/RPA)

Typically RNA from three cell populations were isolated from whole blood: B-cells, T-cells, and lymphocyte depleted PBMCs (peripheral blood mononuclear cells). The order in which the cells were isolated was varied. All procedures were carried out with the understanding of the potential biohazard associated with blood products and appropriate safety precautions were followed.

**Isolation of washed PBMCs**

1. Dilute the whole blood as received (~200-300 ml ) with an equal volume of cold 1X PBS. 1%FBS.
2. Layer 25 ml of the diluted whole blood over 20 ml of Ficoll Paque (Pharmacia)
3. Centrifuge samples in a swinging bucket rotor at room temperature for 20 min at 1000 rpm.
4. Remover the buffy coat from each tube and collect in a tissue culture flask (~10 ml/tube)

5. Dilute collected buffy coat with an equal volume of cold 1X PBS. 1%FBS
6. Centrifuge samples in a swinging bucket rotor at room temperature for 5 min at 1000 rpm.
7. Resuspend cell pellet in a half volume of 1X PBS, 1%FBS.
8. Repeat steps 6 and 7 until a final volume of 20 ml is achieved.
9. Place washed PMBCs into ice water for later use.

**Dynal magnetic bead preparation**

For B-cell isolation α-CD19 magnetic beads were used whereas α-CD3 magnetic beads were used for T-cell isolation (Dynal).

1. Place 1ml of the bead slurry into a 1.5 ml microcentrifuge tube, and place in magnetized holder.
2. Invert holder (with tube) several times to ensure complete adherence of the beads to the side wall of the tube.
3. Remove the supernatant and replace with 1 ml of cold 1X PBS, 1%FBS.
4. Remove the tube from the holder and resuspend the beads by hand.
5. Replace the tube in the holder
6. Repeat steps 2 through 5 five times.

**Isolation of B-cell RNA.**

1. Combine 1 ml of washed α-CD19 magnetic beads with 10 ml of washed PBMCs in a tissue culture flask.
2. Incubate at 4°C for 1 hour with constant shaking.
3. Place PBMC/bead slurry on a plate magnet and continue shaking for 10 min.
4. Keeping the flask in contact with the plate magnet, carefully decant the supernatant into a fresh tissue culture flask (B-cell depleted PBMCs).
5. Remove the flask from the magnet add replenish the supernatant with 10 ml of fresh cold 1X PBS. 1%FBS.
6. Incubate bead complexes for a further 10 min.
   -(At this point isolation of T-cells cells can proceed using B-cell depleted PBMCs.)
7. Repeat steps 3 through 6 three more times
8. After the final wash. transfer bead complexes to a 15 ml conical tube and pellet by brief centrifugation.
9. Discard supernatant
10. Lyse the bound cells in 1 ml of Trizol reagent (GibcoBRL).
11. Store the lysed cells at –80°C for future RNA extraction.

**Isolation of T-cell RNA.**

1. Combine 1 ml of washed beads with 10 ml of B-cell depleted PBMCs in a tissue culture flask.
2. Remainder of procedure is as above.

**Isolation of T/B depleted PBMC RNA**

1. Transfer 2 x 1 ml of the T/B cell depleted PBMCs to a pair microcentrifuge tubes.
2. Pellet the cells at 8000 rpm in a benchtop microcentrifuge for 5 min.
3. Lyse the cells in 1 ml of Trizol reagent (GibcoBRL).
4. Store the lysed cells at –80°C for future RNA extraction.

**Ribonuclease Protection Assay**

1. RNA was extracted from lysed cells according the manufacturer's protocol (GibcoBRL).
2. RPA was carried out as described elsewhere (12.4.6.2).

## 12.3. Translational Control

### 12.3.1. Dicistronic vector construction

Various PCR generated portions of the 5'UTR of either *hiap1* or *hiap2* were inserted into the dicistronic vectors pMH or pSY. The inserts were prepared in such a way as to add *XhoI* sites at their termini for subsequent insertion into the reporter vectors. The vector pMH was constructed by M. Holcik and is essentially the bacterial β-galactosidase gene from pLitmus (Clontech) and the CAT gene from pCATbasic (Promega) inserted into pCDNA3 (Invitrogen). Between both genes lies a unique *XhoI* site for insertion of PCR generated 5'UTR fragments. The vector pSY is similar to pMH in that it is βGal/CAT dicistronic construct on a pCDNA3 backbone. These differ in that whereas pMH possesses 51nt between the cloning site and the CAT initiation codon, the second cistron of pSY lacks an initiator. In its place is a *XhoI* cloning site. As such all inserts into pSY must encode an "in frame" initiator at their 3' terminus.

### 12.3.2. Transfections

All transfections were performed according to standard procedures. Briefly, subconfluent cells (typically HeLa) were passaged one day prior to transfection into the appropriate size dish (typically 6 well format) such that they would be roughly 70% confluent the next day. Transfections were carried out with Lipofectamine or Lipofectamine Plus (GibcoBRL) and Opti-MEM serum free medium (GibcoBRL) according to the manufacturer's instructions. In general, 1-2 μg of DNA was transfected per well. When appropriate and to maintain constant transfection conditions and efficiency, an empty vector (typically pCDNA3) was co-transfected with the reporter vector.

### 12.3.3. Choramphenicol acetyl transferase activity assay

The activity of the various reporter constructs was assayed utilizing by assessing the relative CAT activity levels as follows. Typically the cells used for assay were grown in a 6-well dish.

1. At the time of assay, wash the cells with 1X PBS, three times
2. After the third wash, add 120 µl F/T buffer to each well.
3. Remove the monolayer from the well bottom using a cell scraper
4. Perform three cycle of freeze/thaw by placing each sample tube in liquid nitrogen for 1 min, followed by 1 min at 37°C, followed by 1 min of vortexing.
5. After the third cycle, pellet the cell debris by centrifugation
6. Assemble the following reaction mixture

| | |
|---|---|
| cell lysate | 50 µl |
| 0.25 M Tris (pH 7.5) | 65 µl |
| $^{14}$C-chloramphenicol | 5 µl |
| n-butyryl CoA | 5 µl |
| | 125 µl |

7. Incubate the samples for 2-12 hours
8. Terminate the reaction with the addition of 300 mixed xylenes, vortex
9. Transfer 200 µl of the upper (organic) layer to a fresh 1.5 ml microcentrifuge tube
10. Add 200 µl Tris buffer (0.25 M, pH 7.5), vortex
11. Transfer 150 µl of the upper (organic) layer to a well of a liquid scintillation plate
12. Add 600 µl organic compatible liquid scintillation fluid
13. Measure amount of n-butyryl $^{14}$C-chloramphenicol on a liquid scintillation counter.

As long as the reaction remains in the linear range this assay is allows for the quantification of CAT expression in a given well.

### 12.3.4 β-galactosidase assay

1. At the time of assay, wash the cells with 1X PBS, three times
2. After the third wash, add 120 µl F/T buffer to each well.
3. Remove the monolayer from the well bottom using a cell scraper
4. Perform three cycle of freeze/thaw by placing each sample tube in liquid nitrogen for 1 min, followed by 1 min at 37°C, followed by 1 min of vortexing.
5. After the third cycle, pellet the cell debris by centrifugation
6. Assemble the following reaction mixture

| | |
|---|---|
| cell lysate | 50 µl (50 µl F/T Buffer for blank) |
| Z buffer | 350 µl |
| ONPG | 100 µl |
| | 500 µl |

7. Incubate for several minutes (to 2hrs) until a faint yellow colour appears
8. Stop the reaction by the addition of 200 µl $Na_2CO3$ solution (0.104 g/ml)
9. Read absorbance at 420 nm.

As long as the reaction remains in the linear range this assay allows for the normalization of the reporter assay with respect to transfection efficiency.

## 12.4. Transcriptional Regulation

### 12.4.1. Vector construction

#### 12.4.1.1. NF-κB binding site insertion into pCAT3 promoter vector

In order to determine the enhancer capacity of kB1, it was inserted singly and in tandem, in both orientations downstream of the CAT reporter gene in the pCAT3promoter vector (Promega). Briefly, overlapping and complementary single stranded oligodeoxynucleotides (ssODNs) were commissioned with flanking *BamHI* restriction endonuclease sites. The κB1 site was inserted as follows. Following insertion, individual clones were sequenced from within the reporter vector to determine both the number of insertions and their orientation.

**Annealing the ssODNs**

1. Dilute each ssODN to 0.5 µg/µl in water
2. Combine 50 µl of each complementary ssODN
3. Heat the ssODNs at 95°C for 2 min in a waterbath
4. Allow the ssODN to return to RT (anneal) slowly over several hours in the waterbath

**Phosphorylation of the dsODN (T4 polynucleotide kinase, GibcoBRL)**

1. Assemble the following in a 0.5 µl microcentrifuge tube

| | |
|---|---|
| ds ODN | 25 pmol |
| water | (to 125 µl final) |
| 5X forward buffer | 25 µl |
| dATP (1mM final) | 1.25 µl |
| T4 PNK | 1 µl |
| | 125 µl |

2. Incubate at 37°C for 20 min
3. Stop the reaction by phenol/chloroform extraction
4. To the aqueous phase add the following

| | |
|---|---|
| glycogen | 1µl |
| ammonium acetate(4 M) | 12.5 µl |

5. Mix sample by hand
6. Add 375 µl cold EtOH (-20°C)
7. Mix sample by hand
8. Incubate at –70°C for 60-90 min
9. Centrifuge at 14000 rpm in a benchtop microcentrifuge for 20 min
10. Aspirate the supernatant
11. Air dry the pellet (phosphorylated dsODN) for 10-20 min
12. Resuspend the pellet in 25 µl water

**Preparation of the vector backbone**

1. Linearize the vector by restriction endonuclease digestion
2. Remove 5' phosphates by subjecting to phosphatase (CIAP ,GibcoBRL)
3. Gel purify the linearized, dephosphorylated vector
4. Resuspend in 30 µl water

**Insertion of dsODN (T4 DNA ligase, GibcoBRL)**

1. Assemble the following ligation mixtures

| | Vector (µl) | linker (µl) | water (µl) | V:I ratio |
|---|---|---|---|---|
| a. | 4 | - | 3 | |
| b. | 4 | 0.25 | 2.75 | ~1:3 |
| c. | 4 | 1 | 2 | ~1:10 |
| d. | 4 | 2.5 | 0.50 | ~1:30 |
| e. | 2 | 4.75 | 0.25 | ~1:100 |
| f. | 2 | - | 5 | |

2. To each tube add 2 µl ligase buffer, mix well
3. To each tube add 1 µl T4 DNA ligase, mix well
4. Incubate at RT for 1 hour

**Transformation of *E.coli***

Transform chemically competent *E.coli* (DH5α) using 3 µl of the above ligation mixture according to standard transformation protocols.

**12.4.1.2. Basic clonings**

**pCI-LMP1**

pCI-LMP1 was obtained by PCR amplification of the LMP1 open reading frame from a Raji (ATCC CCL86) derived cDNA library (Marathon cDNA, Clontech) using Pfu DNA polymerase (Stratagene). The resulting amplicon was inserted into the PCRII vector (Invitrogen, Carlsbad CA) and subsequently subcloned into the pCI vector backbone (Promega, Madison WI) according to standard procedures.

**H1P.4pCAT**

A PCR generated genomic fragment (3271 nt) encompassing the promoter region and the entire first exon of *hiap1* was inserted into the *BglII* site of the pCAT3basic vector (Promega) to generate the reporter H1P.4pCAT. The 5' end of the fragment corresponded to the *BglII* site in the promoter region of *hiap1* (3287 nt 5' of the exon1 E/I boundary)whereas the 3' terminus ended near the intron/exon junction of exon 1 (-16 nt). A *BamHI* site was added to the 3' terminus of the fragment. The fragment was amplified using Turbo *Pfu* DNA polymerase (Stratagene) according to the manufacturer's instructions. The fragment was then purified using a commercial PCR purification kit (Qiagen) and an adenine nucleotide was added to each end by incubation

with Taq DNA polymerase and dATP (GibcoBRL). The fragment was then inserted into PCRII (Topo T/A, Invitrogen) according to the manufacturer's instructions. The fragment was then removed from PCRII by *BglII* and partial *BamHI* digestion and inserted into pCAT3basic. Partial *BamHI* digestion was necessitated by the presence of an internal *BamHI* site in the promoter fragment. Orientation of the insertion was determined by both *BamHI* and *BglII* digestion of the resulting clone.

**Adenylation of blunt ended DNA fragments.**

1. Assemble the following in a 0.5 ml microcentrifuge tube

| | |
|---|---|
| DNA | 30 µl |
| Water | (to 50 µl) |
| 10X PCR buffer | 5 µl |
| MgCl2 | 1.5 µl |
| dATP (100 mM) | 0.5 µl |
| Taq | 1 µl |
| | 50 µl |

2. Incubate at 72°C for 20 min. (Do not heat to 95°C prior to incubation.)
3. Insert adenylated fragment directly into PCRII (as per Topo T/A instructions (GibcoBRL)).

**NRF-pCDNA3**

NRF-pCDNA3 was obtained by PCR amplification of the NRF open reading frame from a Raji derived cDNA library (Marathon cDNA, Clontech) using Platinum Pfx DNA polymerase (GibcoBRL, Rockville MD). The resulting amplicon was inserted into the PCRII vector (Invitrogen, Carlsbad CA) and subsequently subcloned into the pCDNA3 vector backbone (Invitrogen, Carlsbad CA).

### 12.4.1.3. Site Directed Mutagenesis

The *hiap1* reporter construct generated above (12.4.1.2) was subjected to site directed mutagenesis in order to remove potential binding sites for NF-kB, NRF, and IL-6 (see Figure 26). Essentially the mutagenesis was performed using the Quick Change site directed mutagenesis kit (Stratagene). The resulting amplification product was then used for direct transformation of *E.coli* (Top10F, Invitrogen, Carlsbad CA). Sequence analysis of the resulting products confirmed the sequence integrity of the construct. Before commencing completely overlapping oligodeoxynucleotide (sense and antisense) primers (30mer) were designed incorporating the desired mutation(s) midway through their sequence.

**PCR primers:**

| | |
|---|---|
| mt κB1 | 5' TTT TGG GTC ATG GAA ATA$_C$ A$_C$CC GAG TGG GTT |
| mt κB3 | 5' TAT TAC CCG CTG GAA$_G$ T$_C$TA CCC TAA GTC CTA A |
| mt NRE | 5' GTT GTT ATC AAG ATT TCA$_C$ TCT A$_G$AC CCA CGA |
| mt NF-IL6 | 5' ATA GAA ATA G$_T$TT A$_C$AC AAA GTT TCG ATT TTT |

(note: The relevant transcription factor binding site is underlined and the nucleotide present in the native sequence is represented as a subscript following the substituted base.)

### 12.4.2. Cell culture conditions

HeLa cells (ATCC CCL-2.2) were maintained in DMEM/10% FBS (Wisent) in antibiotic containing media (penicillin/streptomycin) (GibcoBRL). Pyrrolidine dithiocarbamate, wortmannin, cycloheximide and puromycin were obtained from Sigma. TNFα was obtained from Clontech. LY294002 was obtained from Calbiochem.

### 12.4.3. Transfections

All transfections were performed according to standard procedures. Briefly, subconfluent cells (typically HeLa) were passaged one day prior to transfection into the appropriate size dish (typically 6 well format) such that they would be roughly 70% confluent the next day. Transfections were carried out with Lipofectamine or Lipofectamine Plus (GibcoBRL) and Opti-MEM serum free medium (GibcoBRL) according to the manufacturer's instructions. In general, 1-2 μg of DNA was transfected per well. When appropriate and to maintain constant transfection conditions and efficiency, an empty vector (typically pCDNA3) was co-transfected with the reporter vector.

### 12.4.4. Chloramphenicol acetyl transferase ELISA assay

Activity of the various reporter constructs was assayed utilizing the CAT ELISA kit according to the manufacturer's protocol. Typically the cells used for assay were grown in a 6-well dish. Cells were lysed in 300 ml of Lysis buffer (supplied). Depending on the expected activity all of the samples in a given experiment were sometimes diluted in Sample buffer (supplied) such that the colour development was not too rapid and the values obtained fell within the standard curve. As long as the reaction remains in the linear range this assay is allows for the normalization of the reporter assay with respect to transfection efficiency.

### 12.4.5. β-galactosidase assay

This protocol is an adaptation of the one used previously (Section). The only difference being that the cell lysate is prepared by lysing the cells directly on the plate using the CAT ELISA lysis buffer (Boehringer Mannheim). As long as the reaction remains in the linear range this assay is allows for the normalization of the reporter assay with respect to transfection efficiency.

1. At time of assay, wash the cells with 1X PBS, three times
2. After the third wash, lyse the cells by the addition of 300 μl lysis buffer (supplied as a 5X concentrate).
3. Incubate the samples in the dish for 30 min
4. Pellet the cell debris by centrifugation
5. Assemble the following reaction mixture

| | | |
|---|---|---|
| cell lysate | 50 μl | (50 μl Sample Buffer for blank) |
| Z buffer | 350 μl | |
| ONPG | 100 μl | |
| | 500 μl | |

6. Incubate for several minutes (to 2hrs) until a faint yellow colour appears
7. Stop the reaction by the addition of 200 µl $Na_2CO3$ solution (0.104 g/ml)
8. Read absorbance at 420 nm.

As long as the reaction remains in the linear range this assay is allows for the normalization of the reporter assay with respect to transfection efficiency.

### 12.4.6 Ribonuclease Protection Assay (GibcoBRL/Pharmingen)

#### 12.4.6.1 Isolation of total RNA (Trizol Reagent, GibcoBRL)

Total RNA was isolated from Trizol solubilized HeLa cells according to the protocol supplied by the manufacturer. Typically, for HeLa cells grown in a 6-well dish, the RNA sample was split into two aliquots prior to isopropanol precipitation, whereas the total amount of RNA obtained from A549 cells grown in a 6-well dish was precipitated in a single tube. The amount of total RNA used for RPA should be the same for all samples. Following the final 70% EtOH wash, the RNA pellets were vacuum dried for 1 hour at room temp.

#### 12.4.6.2. RPA assay

The RPA assay described below was adapted from the Pharmingen protocol by Christine McRoberts.

**RNA preparation**

1. Resuspend dried total RNA in 10 µl hybridization buffer
2. Vortex 3-4 min to resuspend

**Probe preparation**

1. Assemble the following reaction mixture (in order given)

| | | |
|---|---|---|
| RNasin | 1 µl | on ice |
| GACU pool | 1 µl | RT |
| DTT | 2 µl | RT |
| 5x transcription buffer | 4 µl | RT |
| RPA template (hAPO-5) | 1 µl | on ice |
| α $^{32}$P UTP (no dyes) | 5 µl | RT |
| water | 5 µl | RT |
| T7 polymerase | 1 µl | on ice |
| | 20 µl | |

2. Incubate for 1 hour at 37°C
3. Terminate the reaction with the addition of 2 µl DNase
4. Incubate for 1 hour at 37°C

5. To the transcription reaction add

| | |
|---|---|
| 20 mM EDTA | 26 µl |
| Tris saturated phenol | 25 µl |
| chloroform:isoamyl alcohol(50:1) | 25 µl |
| yeast tRNA | 2 µl |

6. Vortex vigorously, centrifuge 5 min RT
7. Transfer upper phase to new microcentrifuge tube (~120 µl)
8. Add 50 µl chloroform:isoamyl alcohol (50:1)
9. Vortex vigorously, centrifuge 5 min RT
10. Transfer upper phase to new microcentrifuge tube (no chloroform)
11. To the aqueous phase add

| | |
|---|---|
| 4M ammonium acetate | 50 µl |
| ethanol (-20°C) | 250 µl |

12. Mix by inversion
13. Incubate for 1 hour at –70°C
14. Centrifuge probe for 30 min at RT
15. Discard supernatant
16. Wash probe with 200 µl 90% ethanol (-20°C) (pellet barely visible)
17. Centrifuge probe for 5 min at RT
18. Discard supernatant
19. Completely air dry probe (10-30 min)
20. Add 50 µl hybridization buffer, vortex for 2-3 min
21. Count 1 µl by liquid scintillation
22. Dilute probe to $3x10^5$cpm/µl

**Hybridization of probe to RNA**

1. To each RNA sample add 3 µl probe, vortex, spin briefly
2. Overlay samples with 2 drop light mineral oil
3. Place in 90°C waterbath
4. Immediately set waterbath temperature to 56°C and allow to cool naturally overnight (no refrigeration)

**Sample preparation**

1. Place samples in 37°C waterbath
2. Incubate for 15 min
3. To each sample add 100 µl RNase solution

| | 15 samples | 20 samples |
|---|---|---|
| RNase buffer | 1875 µl | 2500 µl |
| RNase A + T1 mix | 4.5 µl | 6 µl |

- keep on ice RNase mix on ice

4. Vortex, spin
5. Incubate samples for 30 min at 30°C
6. Transfer samples (beneath oil) to fresh microcentrifuge tubes containing 18 µl proteinase K solution

| | 15 samples | 20 samples |
|---|---|---|
| Pro-K buffer | 292.5 µl | 390 µl |
| Pro-K | 22.5 µl | 30 µl |
| Yeast tRNA | 22.5 µl | 30 µl |

7. Incubate samples for 15 min at 37°C
8. To each sample add

| | |
|---|---|
| Tris buffered phenol | 65 µl |
| chloroform:isoamyl alcohol(50:1) | 65 µl |

9. Vortex vigorously, centrifuge 5 min RT
10. Transfer aqueous phase (upper) to fresh microcentrifuge tube
11. To each sample add

| | |
|---|---|
| 4M ammonium acetate | 120 µl |
| 100 % ethanol (-20°C) | 650 µl |

12. Mix by inversion
13. Incubate 60 min at -70°C
14. Centrifuge for 30 min at RT
15. Discard supernatant
16. Wash sample with 200 µl 90% ethanol (-20°C) (pellet barely visible)
17. Centrifuge for 5 min at RT
18. Discard supernatant
19. Completely air dry sample (10-30 min)
20. Resuspend sample in 10 µl loading buffer (vortex for 2-3 min)

**Sample electrophoresis**

1. Prepare 5% TBE(1X)/polyacrylamide gel in sequencing apparatus (Sequagel, National Diagnostics/Diamed)
2. Prerun gel for 45 min at 50W (1200-1500 V). 1xTBE
3. Heat sample for 3 min at 90oC (keep on ice until loaded)
4. Rinse wells with running buffer
5. Load 6 µl of each sample into adjoining lanes.
6. Run gel for 2-3 hours (50% gel length).
7. After electrophoresis, disassemble apparatus and peel gel onto Whatmann paper.
8. Dry gel under heat (80°C) and vacuum for 1 hour
9. Mount gel and expose autoradiography film O/N at -70°C and/or expose Phosphor screen O/N at RT

**Quantification of bands**

The resulting blots are visualized with a Storm 860 PhosphorImager (Molecular Dynamics) and the protected bands quantified with the accompanying ImageQuaNT analysis software, v.4.1 (Molecular Dynamics). Normalization of each band to either *L32* or *gapdh* yields relative expression levels.

### 12.4.7. Electrophoretic Mobility Shift Assay (EMSA, Gel Shift)

Gel shift assays were performed as follows.

#### 12.4.7.1. Preparation of double stranded oligodeoxynucleotide (ODN) targets

1. Resuspend single stranded (ss)ODN to 0.5 μg/μl in deionized water.
2. Combine complementary ssODNs in equimolar amount (typically 50-100 μl each) in screw cap tube.
3. Heat ssODNs to 95°C in waterbath for five minutes.
4. Allow to cool in waterbath to 35°C over several hours.

#### 12.4.7.2. Labelling of double stranded (ds)ODNs with $^{32}$Phosphorus (kinase reaction)

1. Assemble the following reaction

| | |
|---|---|
| dsODN (1/10 dilution of annealed ODN, ~25 pmol) | 10 μl |
| deionized water | 80 μl |
| forward reaction buffer (5X) | 25 μl |
| $\gamma^{32}$P-dATP | 5 μl |
| T4 polynucleotide kinase (50 U, GibcoBRL) | 5 μl |
| | 125 μl |

2. Incubate at 37°C for 10 minutes
3. Terminate the reaction by phenol/chloroform extraction
4. To the resulting aqueous phase add

| | |
|---|---|
| glycogen (Boehringer Mannheim) | 1 μl |
| ammonium Acetate (4M, pH 7.5) | 12.5 μl |
| anhydrous ethanol (-20°C) | 375 μl |

5. Mix by hand
6. Incubate at –70°C for 3hrs
7. Centrifuge for 25 minutes at maximum speed in a microfuge
8. Remove supernatant
9. Wash pellet in 70% ethanol
10. Air dry pellet
11. Resuspend pellet in 25 μl deionized water (nominally 1pmol $^{32}$P-dsODN/μl)

#### 12.4.7.3. Binding Buffers

**NF-κB**

| | |
|---|---|
| 20 mM | Hepes (pH 7.9) |
| 100 mM | KCl |
| 0.4 mM | ethylenediaminetetraacetic acid (EDTA) |
| 5 mM | dithiothreitol (DTT) |
| 20% | glycerol |
| 0.1% | NP40 |

**NRF**

20 mM HEPES (pH 8.0)
10 mM $MgCl_2$
100 mM KCl
20% Ficoll.

**12.4.7.4. *In-vitro* translation of NRF (Promega)**

1. Assemble the following reaction mixtures

| | |
|---|---|
| TNT reticulocyte lysate (supplied) | 40 µl |
| PCR enhancer (supplied) | 2 µl |
| circular plasmid (sense, antisense, or water) | 1 µl |
| Methionine or | 2 µl |
| water | 9 µl |
| | 50 µl |

2. Incubate samples for 30 min at 30°C

**12.4.7.5. Binding reactions**

**NF-κB/ p50 (Promega)**

1. Dilute p50 1:1:2 (p50:water:Binding buffer)
2. Assemble the reaction as follows

| | |
|---|---|
| labelled target | 0.5 µl |
| cold competitor (water as a blank, as indicated) | 7.5 µl |
| Binding Buffer | 10 µl |
| diluted p50 (water as a blank, as indicated) | 2.0 µl |

3. incubate for 30 minutes at room temperature

**NRF/NRE**

1. Assemble reaction set one as follows

| | |
|---|---|
| labelled target | 0.5 µl |
| cold competitor (water as a blank, as indicated) | 7.5 µl |
| Binding Buffer | 10 µl |
| *In vitro* translated "antisense" NRF | 2.0 µl |

2. Assemble reaction set two as follows

| | |
|---|---|
| unlabelled target | 0.5 µl |
| unlabelled competitor | 7.5 µl |
| Binding Buffer | 10 µl |
| *In vitro* translated NRF | 2.0 µl |

3. Incubate for 30 minutes at room temperature

**12.4.7.6. Electrophoresis**

Resolve samples on a 6% non-denaturing polyacrylamide gel (1X TBE). Condition gel by running for 1 hour at 5 V/cm. Following electrophoresis, disassemble apparatus and dry gel under heat and vacuum on a sheet of absorbent paper (Whatmann). Mount the dried gel and expose for 30 minutes to several hours.

## 12.5. Measurement of TNFα mediated cytotoxicity

### 12.5.1. Cell lines and culture materials

The BJAB(wt) cell line was obtained from the German Collection of Micro-organisms and Cell Cultures (GCMCC, Braunschweig). The BJAB(ko) cell line is a laboratory strain of BJAB. The cells were maintained in RPMI1640/15% FBS (Wisent) with antibiotics (Penicillin/Streptomycin) (GibcoBRL). TNFα was obtained from Clontech. The *hiap1* sense and antisense adenovirus preparations are based on the pAdex1CAwt vector (Quantum) and were obtained from Aegera Therapeutics.

### 12.5.2. Isolation of genomic DNA from BJAB cell lines

DNA was extracted from cells using an automated DNA extractor (Applied Biosystems) according to the methods and procedures outlined by the manufacturer.

### 12.5.3. PCR conditions for delineation of BJAB(ko) deletion

The presence or absence of various chromosomal markers was assessed by PCR amplification of the marker in question according to common PCR procedures. The primer sequences used for the amplification of the various markers were as follows. The number in parentheses indicate the heterozygosity index of each locus.[474]

| | | | | |
|---|---|---|---|---|
| tel | D11S1347 | (0.79) | -F | 5' AGC TAT GAT TGC ACC ACT TC |
| | | | -R | 5' GGT CAA TAA GCA AAC AGA AC |
| | D11S1343 | (0.56) | -F | 5' ACT CCC ACA GTC TCT GTC CC |
| | | | -R | 5' AGC TAT GTA ATG AGC ATG GA |
| | D11S1325 | (0.52) | -F | 5' GTG TAA CCT TCA TGA ACA TC |
| | | | -R | 5' TAT CCA GGC TGC CTT ATG TC |
| | D11S1339 | (0.85) | -F | 5' AGA TGC ATA TCC CAT GGC CT |
| | | | -R | 5' CAC CCC AGA AAT GGC CCA GT |
| | D11S923 | (0.71) | -F | 5' AGC TTC ATT TTT AAC AAG TG |
| | | | -R | 5' AGC TCA TAA ATG GCC TTT GG |
| | D11S917 | (0.80) | -F | 5' TGT TGG CCA CGC TGC TCT CC |
| | | | -R | 5' AGC TTA CAC ATT TGA GGA AA |
| cen | D11S1311 | (0.75) | -F | 5' GCA GAT TAC TAA ACC ATT CT |
| | | | -R | 5' ATC TAA GGT CTT GCT GTC CC |

### 12.5.4. PCR conditions for polymorphic marker analysis of BJAB(wt) and BJAB(ko)

In order to determine whether BJAB(ko) is in fact a derivative of BJAB(wt), the size of various BJAB(wt) and BJAB(ko) alleles generated by PCR amplification of the chromosomal markers described in 3.5.3. were compared by denaturing polyacrylamide gel electrophoresis. In order to visualize the amplification products a single amplification primer from each marker was labelled with $^{32}P$. Phosphorylation of the primer (5 pmol) was accomplished using T4 polynucleotide kinase (GibcoBRL) according to the manufacturer's protocol. The resulting $^{32}P$-labelled primer was used for the PCR amplification of the chromosomal marker in question.

**Amplification mixture**

| | |
|---|---|
| gDNA | 2 µl |
| water | 34 µl |
| 10X PCR buffer | 5 µl |
| MgCl2 | 2 µl |
| Forward primer | 2 µl |
| Reverse primer | 2 µl |
| $^{32}$P labelled primer (from kinase reaction) | 2 µl |
| dNTPs (100mM) | 0.5 µl |
| Taq | 0.5 µl |
| | 50 µl |

The chromosomal markers were amplified by standard PCR methods and resolved on a TBE/urea polyacrylamide sequencing gel (6%). Following electrophoresis the gel was transferred to blotting paper and dried under heat (80°C) and vacuum. The amplified bands were then visualized by autoradiography.

### 12.5.5. PCR conditions for CASP1, CASP4, CASP5 genomic amplification

The presence or absence of the caspases was assessed by PCR amplification of the caspase in question from genomic DNA according to standard PCR procedures. The primer sequences used for the amplification were as follows. Indicated below each primer pair is the expected size of the amplification product.

CASP1 (forward) 5' ATGGCCGAAAAGGTCCTGAAG 3'
CASP1 (reverse) 5' CCCTTGTCTGTAATAATTCATC 3'
Amplification product = 100 bp

CASP4 (forward) 5' ATGGCAGAAGGCAACCACAGA 3'
CASP4 (reverse) 5' AGTTTTAGCATCGTAATATTTC 3'
Amplification product = 150 bp

CASP5 (forward) 5' ATGTTCAAAGGTATCCTTCAG 3'
CASP5 (reverse) 5' AACACCATGAAGAACATCTTTG 3'
Amplification product = 1593bp (includes a 1382 bp intron)

### 12.5.6. Adenoviral infections.

BJAB(wt) and BJAB(ko) cell lines were plated at a density of $2x10^5$/mL and infected with recombinant adeno-(s)*hiap1*, adeno-(a/s)*hiap1*, or media (mock) at an MOI of 30. The cells were then incubated for 24hrs to allow for expression of the *hiap1* insert. Following this the cells were treated with TNFα (30 ng/mL) as indicated in Figure 38 and allowed to incubate a further 24hrs. The cells were then dispersed and counted on a haemocytometer.

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## 14. Appendices

### 14.1. Common Reagents and Solutions

**10X MOPS**

0.4 M MOPS (3-[N-morpholino] propanesulfonic acid)
0.1 M NaAcetate
0.01 M EDTA
pH 7.0

**RNA sample loading dye**

1. mM EDTA pH 8.0
2. 0.25% Bromophenol blue
3. 0.25% Xylene cyanol
4. 50% glycerol

**Northern hybridization solution**

1. 5X SSPE
2. 10X Denhardt's solution
3. 100 µg/ml freshly sheared salmon sperm DNA
4. 50% formamide
5. 2% SDS

**20X SSPE**

1. 3 M NaCl
2. 0.2 M $NaH_2PO4 \bullet 7H_20$
3. 0.02 M $Na_2EDTA$
   pH 7.4

**50X Denhardt's**

1. 5g Ficoll (Type 400, Pharmacia)
2. 5g polyvinylpyrrolidone (Sigma)
3. 5g BSA
4. water to 500 ml
   - store at –20°C

**20X SSC**

1. 3 M NaCl
2. 0.3 M $Na_3citrate \bullet 2H_2O$
   pH 7.0

**50X TAE**

1. 242 g Tris base
2. 57.1 ml glacial acetic acid
3. 37.2 g $Na_2EDTA.2H_2O$ (0.1 M)
4. water to 1 L
   pH ~ 8.5

**10X TBE**

1. 108 g Tris base (890 mM)
2. 55 g boric acid (890 mM)
3. 40 ml x 0.5 M EDTA pH 8.0 (20 mM)

**TE**

1. 10 mM Tris-Cl pH 7.4, 7.5, 8.0
2. 1 mM EDTA pH 8.0

**10X PBS**

1. 80 g NaCl (1.37 M)
2. 2 g KCl (0.027 M)
3. 11.5 g $Na_2HPO_4 \bullet 7H_2O$ (0.043 M)
4. 2 g $KH_2PO_4$ (0.014 M)
5. water to 1 L

pH ~ 7.3

**GTE (miniprep solution #1)**

1. 50 mM Glucose
2. 1 mM EDTA (from 0.5 M stock)
3. 25 mM Tris-Cl pH 8.0

**SDS/NaOH (miniprep solution #2)**

1. 0.2 M NaOH
2. 1% SDS

**3M KOAc (miniprep solution #3)**

1. 60 ml 5M KOAc
2. 11.5 ml glacial acetic acid
3. 28.5 ml $dH_2O$

**Z buffer**

0.1 M phosphate buffer, pH 7.4
10 mM KCl
1 mM MgSO4
50 mM β-mercapto-ethanol

**ONPG solution**

0.1 M phosphate buffer, pH 7.4
4 mg/ml o-nitrophenyl β-D-galactopyranoside (ONPG)

**Southern blot hybridization solution**

| | |
|---|---|
| 10 % SDS | 700 ml |
| 50% PEG | 200 ml |
| 20X SSPE | 75 ml |
| water | 25 ml |

## 14.2. *Curriculum Vitae*

### Education

09/82-06/87 École Secondaire Hanmer
*Hanmer, ON*
Honours Programme

09/87-04/92 University of Waterloo
*Waterloo, ON*
BSc (Honours Biochemistry)

01/93-08/95 Laurentian University
*Sudbury, ON*
MSc (Chemistry and Biochemistry)

09/95-present University of Ottawa
*Ottawa, ON*
PhD (Biochemistry, Microbiology and Immunology)

### Awards Received

06/87 Ontario Scholar
Ontario Ministry of Education

09/95 Entrance Scholarship
University of Ottawa

### Publications

**Abstracts**

Lactic Acid Transport in the Small Cell Lung Cancer Cell Lines H69 and H69AR
(**SS Young** and H Falter) - presentation
Ontario Exercise Physiology Conference
*Barrie, ON*
1995

Genomic Characterization of the X-linked Inhibitor of Apoptosis (*xiap*) Gene
(MC Lagacé, **S Young**, C Lefebvre, P Liston, and RG Korneluk)
Human Genome Meeting (HUGO)
*Toronto, ON*
1997

Genomic Organization of the Human Inhibitor of Apoptosis Proteins
(**SS Young**, MC Lagacé, C Lefebvre, P Liston, and RG Korneluk)
Human Genome Meeting (HUGO)
*Toronto, ON*
1997

New Human *BRCA2* Isoform is Truncated and Contains a Novel Coding Exon
(MD Speevak, **SS Young**, HL Aubry, C Gilpin, and RG Korneluk)
48th Annual Meeting
American Society of Human Genetics
*Denver, CO*
1998

Genomic Characterization of the X-linked Inhibitor of Apoptosis (*xiap*) Gene and Identification of a Novel Homologous Transcript Restricted to the Testes
(MC Lagacé, **S Young**, C Lefebvre, P Liston and RG Korneluk)
Signalling in Normal and Cancer Cells
*Banff, AB*
1998

Apoptosis Related Gene Expression Following Transformation of B-cells with EBV
(**SS Young**, J Tanner, C McRoberts, and RG Korneluk)
Signalling in Normal and Cancer Cells
*Banff, AB*
1998

Akt/NF-κB pathway is responsible for direct transcriptional activation of *hiap1*
(**SS Young** and RG Korneluk)
Human Genome Meeting (HUGO)
*Vancouver, BC*
2000

**Articles**

Life and Death Decisions: The Role of IAP's in Modulating Programmed Cell Death
(P Liston, **SS Young**, AE Mackenzie and RG Korneluk)
*Apoptosis* **2**:423-441

Genomic Organization and Physical Map of the Human Inhibitors Apoptosis: *HIAP1* and *HIAP2*
(**SS Young**, P Liston, J-Y Xuan, C McRoberts, C Lefebvre, and RG Korneluk)
*Mammalian Genome* **10**:44-48

Tumor Necrosis Factor (TNF)- α Induction of the X-linked Inhibitor of Apoptosis Protein results in an Increase in NF-κB/c-REL and Tumor Suppressor Protein, p53
(JE Tanner, **S Young**, R Simms, RG Korneluk, and AE Mackenzie)
*Mol Cell Biol* – submitted

Characterization of the X-linked Inhibitor of Apoptosis (XIAP) and Identification of a Novel Testis Specific Homologue
(MC Lagacé, JY Xuan, C McRoberts, E Separovich, **SS Young**, and RG Korneluk)
*Genomics* - submitted

Integrin Linked Kinase Regulates NGF and IGF-1 Stimulation of AKT and Neurite Growth
(J Mills, M Digicaylioglu, AT Legg, CE Young, **SS Young**, AM Barr, TP O'Connor, and S Dedhar)
*J Neurosci* - submitted

A Conserved κB site regulates transcription of *hiap1* via the akt/PI3K pathway
(**SS Young** and RG Korneluk)
*J Biol Chem* – in preparation

The Epstein-Barr Virus encoded LMP1 signals through the PI3K pathway to transactivate *hiap1*
(**SS Young** and RG Korneluk)
*Virology* – in preparation

Identification of Alternatively Spliced Human *BRCA2* isoforms containing a Novel Coding Exon
(MD Speevak, **SS Young**, HL Aubry, C Gilpin, and RG Korneluk)
*Breast Cancer Research* – in preparation

**Patents (US)**

Antisense IAP nucleic acids and uses thereof
(E. LaCasse, S. Baird, M. Holcik, **S. Young** and R. Korneluk)
- filed

## 14.3. Statement of contribution of collaborators

As with any multimember research group, ideas, criticisms, and suggestions oftentimes arise through discussion with other members of the group. This is also true in the present work. With that caveat, the present work is original research performed by myself in collaboration with other members of the research group in the laboratory of Dr Robert Korneluk. Work specifically performed or data generated by persons other than myself are indicated in the text of this work where appropriate.