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- TO CATCH A KILLER -

ON THE MECHANISMS OF INTERFERON ALPHA INDUCED APOPTOSIS

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Doctoral thesis
To catch a killer - On the mechanisms of interferon alpha induced apoptosis

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"When curiosity turns to serious matters, it's called research."
- Marie von Ebner-Eschenbach

To Morfar, who would have been so proud
- and Saga, who makes me proud

ABSTRACT

A major clinical problem regarding treatment of malignant tumours is primary or secondary resistance to therapy. Anti-tumour drugs act primarily by induction of apoptosis. However, the knowledge of how various substances induce apoptosis is still incomplete, and so is the reason for the great variation in cellular sensitivity to these drugs. The aim of this thesis was to characterize the pro-apoptotic signalling mechanisms induced by IFNα and to investigate the importance of the underlying genotype on the cellular sensitivity to IFNα-treatment.

IFN can exert prominent anti-cancer activities in some malignancies. However, the mechanism(s) of IFN's anti-tumour activity is not clear, but induction of apoptosis has become a commonly accepted putative mechanism. In this thesis the molecular background to IFNα-induced apoptosis in malignant cell lines was investigated. Apoptosis induced by IFNα depends on activation of caspases, and activation of caspase-8 was found to be a triggering event in the caspase cascade. Furthermore, we show involvement of the mitochondrial pathway as demonstrated by activation of the pro-apoptotic Bcl-2 family members Bak and Bax, mitochondrial inner membrane depolarization and release of cytochrome c.

We have also shown that IFNα activates the PI3K/mTOR pathway. Signalling through PI3K/mTOR has been shown to primarily mediate survival. However, in the case of IFNα-mediated activation, this pathway is crucial for the apoptotic response. Inhibition of PI3K as well as mTOR completely abrogates IFNα-induced apoptosis. No effect from inhibition of PI3K/mTOR is observed on the IFNα-induced classical Jak-STAT signalling pathway, indicating that Jak-STAT signalling alone is not sufficient to induce the apoptotic response to this cytokine. Furthermore, the antiviral effects of IFNα-treatment are unaffected by inhibition of PI3K/mTOR, hence this signalling pathway is crucial for induction of specific effects, such as apoptosis.

The impact of activated oncogenes on the apoptotic response to IFNα was also investigated. Introduction of a constitutive active form of the STAT3 oncogene (STAT3C) was shown to inhibit IFN's pro-apoptotic activity. The result of STAT3C expression is sustained STAT3/3 dimerization and nuclear translocation. STAT3C also rescued from the IFN-induced downregulation of STAT3/3 dimers, possibly explaining its ability to interfere with IFN-induced apoptosis. Furthermore, the presence of the HPV-16 E7 oncogene was shown to sensitize cells to apoptosis induced by IFN.

Delineation of the molecular background to IFN-induced apoptosis, and the impact of oncogene activation on the cellular sensitivity to this effect, may aid in an optimized use of IFNα in the treatment of patients with cancer.

LIST OF PUBLICATIONS

This thesis is based on the following papers. In the text the papers will be referred to by their roman numerals.

I. **Thyrell L**, Erickson S, Zhivotovsky B, Pokrovskaja K, Sangfelt O, Castro J, Einhorn S and Grandér D.
Mechanisms of interferon alpha induced apoptosis in malignant cells
Oncogene, 2002 Feb 14, 21(8), 1251-1262.

II. **Thyrell L**, Hjortsberg L, Arulampalam V, Panaretakis T, Uhles S, Dagnell M, Zhivotovsky B, Leibiger I, Grandér D and Pokrovskaja K.
Interferon alpha induced apoptosis in tumour cells is mediated through the phosphoinositide 3-kinase/mammalian target of rapamycin signalling pathway
J *Biol Chem*., 2004 Jun 4, 279(23), 24152-62.

III. **Thyrell L**, Arulampalam V, Panaretakis T, Hammarsund M, Grandér D and Pokrovskaja K.
Over-expression of a constitutively activated STAT3 protects U266 myeloma cells from interferon alpha induced apoptosis
Manuscript

IV. **Thyrell L**, Sangfelt O, Zhivotovsky B, Pokrovskaja K, Wang Y, Einhorn S and Grandér D.
The HPV-16 E7 oncogene sensitizes malignant cells to interferon alpha induced apoptosis
Accepted for publication in Journal of Interferon and Cytokine Research, 2005 Jan, 25(2)

ABBREVIATIONS

$\Delta\psi_{mit}$	mitochondrial membrane potential
AIF	apoptosis inducing factor
ALL	acute lymphocytic leukaemia
ANT	adenine nucleotide translocator
Apaf	apoptosis protease activating factor
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma-2
BH	Bcl-2 homology
Bid	BH3-interacting-domain death agonist
BOP	BH3-only protein
CAD	caspase activated DNase
Caspase	cysteinyl aspartate specific protease
CDK	cyclin dependent kinase
CLL	chronic lymphocytic leukaemia
CML	chronic myelogenous leukaemia
CREB	cAMP response element binding protein
DAG	diacyl glycerol
DD	death domain
DED	death effector domain
DISC	death inducing complex
DNA	deoxyribonucleic acid
DR	death receptor
dsRNA	double stranded RNA
EGF	epidermal growth factor
ERK	extracellular regulated kinase
FADD	Fas-associated death domain
FLIP	flice (caspase-8) like inhibitor protein
GAS	gamma activation site
GF	growth factor
HPV	human papilloma virus
IAP	inhibitor of apoptosis protein
IFN	interferon
ICAD	inhibitor of CAD
ICE	interleukin-1 converting enzyme
IL-6	interleukin-6
IM	inner (mitochondrial) membrane
IRF	interferon regulated factor
ISG	interferon stimulated gene
ISGF3	interferon stimulated gene factor 3
ISRE	interferon stimulated response element

JAK	janus tyrosine kinase
JNK	c-jun NH_2-terminal protein kinase
MAPK	mitogen-activated protein kinase
MM	multiple myeloma
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
OM	outer (mitochondrial) membrane
PARP	poly ADP-ribose polymerase
PCD	programmed cell death
PDGF	platelet derived growth factor
PH	plecstrin homology
PI	propidium iodide
PIAS	protein inhibitors of activated STATs
PI3K	phosphatidyl inositol-3 kinase
PKC	protein kinase C
PKR	dsRNA-dependent protein kinase
PS	phosphatidyl serine
PT	permeability transition
PTB	phosphotyrosine binding
PtdIns	phosphatidyl inositol
PTEN	phosphatase and tensin homologue deleted on chromosome 10
PTPC	permeability transition pore complex
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SH	src-homology
Ser	serine
SOCS	suppressors of cytokine signalling
STAT	signal transducer and activator of transcription
tBid	truncated Bid
TGF	transforming growth factor
Thr	threonine
TMRE	tetramethylrhodamine ethyl ester
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand/Apo2-L
Tyr	tyrosine
VDAC	voltage dependent anion channel

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INTRODUCTION

GENERAL INTRODUCTION TO CANCER

It is estimated that in the industrial world every third person will receive a cancer diagnosis during their lifetime. In Sweden approximately 45 000 persons per year are diagnosed with cancer. Furthermore, 20 000 persons per year die from the disease. Cancer, being the second most common cause of death after cardiovascular diseases, is thus something that directly or indirectly affects us all (Cancerfonden, 2001).

Cancer is not a single disease; there are as many different cancer forms, approximately 200, as there are cell types in the human body. Except for the differences between each type of cancer disease, there is also a great variability within the same kind of tumours. Furthermore, the genetic background of individual cells in a tumour can vary as a result of selection pressure during malignant development. This variability is a main reason for the variation in response to anti-cancer treatments. The greater knowledge we achieve about the underlying causes of cancer, and the exact mechanisms of action of different anti-tumour treatments, the more likely we are to be able to treat each cancer individually and to increase the response rate to cancer treatment. In order to avoid therapy based on trial and error, pin-pointing of signalling pathways and molecular targets is necessary.

Hallmarks of cancer

It has been suggested, as reviewed by Hanahan and Weinberg, that cancer is a manifestation of six essential alterations in cell physiology, shared by most if not all human tumours. This enumeration of molecular, biochemical and cellular traits is a result of genetic alterations which are supposed to provide the tumour cells with a growth advantage. Consequently this facilitates the transformation of normal cells into tumour cells. These acquired capabilities comprise; self sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

The multistep process leading to malignant transformation is driven by genetic alterations that range from subtle point mutations to changes in, or losses or gains of, whole chromosomes. Furthermore, epigenetic changes such as aberrant promoter hypermethylation represent an important mechanism for inappropriate gene silencing in the pathogenesis of several malignancies (Galm and Esteller, 2004). The genes altered in malignant cells are primarily those responsible for driving the cell cycle machinery and regulating apoptosis. These genes are commonly known as oncogenes and tumour suppressor genes (Hanahan and Weinberg, 2000).

Oncogenes and tumour suppressor genes and their role in cancer

Oncogenes

The history of oncogenes starts approximately one hundred years ago with Peyton Rous (Rous, 1911). He found that a chicken tumour filtrate, inoculated in healthy birds, caused tumour formation. The tumour causing agent was later identified as the *SRC* oncogene carried by the Rous sarcoma virus (RSV). This latter finding was made in 1976 by Bishop and Varmus and represents the first discovery of an oncogene (Stehelin et al., 1976).

Most virus-derived oncogenes (v-onc) have normal cellular counterparts, called proto-oncogenes (c-onc), which in their normal state regulate cellular functions such as proliferation, apoptosis and differentiation (Todd and Wong, 1999). Proto-oncogenes act in a dominant manner, meaning that only one of the two alleles needs to be activated/mutated to achieve the oncogenic effect. Inappropriate activation of proto-oncogenes may occur for example by point mutations, amplifications, deletions or translocations, with the end result being expression of a protein able to stimulate abnormal growth. This uncontrolled growth causes independence of external stimuli, eventually leading to malignant transformation. Proteins encoded by proto-oncogenes include growth factors (GFs), (*e.g.* PDGF), GF-receptors (*e.g.* Her2/neu), intracellular transducers (*e.g.* PI3K, ras), transcription factors (*e.g.* myc, STAT3) and inhibitors of apoptosis (*e.g.* bcl-2) (Hanahan and Weinberg, 2000).

Tumour suppressor genes

Tumour suppressor genes (TSGs) are generally considered to be genes that suppress cell growth and proliferation (Macleod, 2000). In contrast to oncogene activation, malignant transformation is associated with inactivation or loss of these genes. The first experimental evidence for the existence of TSGs originates from an *in vitro* study published in 1969, where it was found that malignant cells could be converted into a non-malignant state when fused with normal cells (Harris et al., 1969). Additional evidence for the existence of TSGs, based on epidemiological studies of the childhood tumour retinoblastoma, was demonstrated by Alfred Knudson in 1971, who introduced the hypothesis commonly referred to as the "two hit theory" (Knudson, 1971). TSGs are referred to as recessive, meaning that both alleles have to be inactivated for the tumour suppressing activity to be lost. The first hit is phenotypically harmless whereas the second mutation causes the cell to transform. The mechanisms of inactivation are mostly genetic changes, such as deletions or point mutations, but also methylation resulting in silencing of gene expression has been shown in TSGs (Macleod, 2000).

Together with the *RB* gene, which is the TSG inactivated in retinoblastoma, the p53 gene is one of the most well characterized TSGs. p53 is regarded as the "guardian of the genome" and its protein levels increase rapidly as a response to DNA damage. This leads to cell cycle arrest and DNA repair, or induction of apoptosis when repair is not possible. Because of its important role, p53 is the most frequent target for mutations identified in human cancers. In

fact, aberrations in this gene are estimated to occur in more than 50% of all tumours (Hanahan and Weinberg, 2000).

A more recently discovered TSG, also found to be inactivated in approximately 50% of human malignancies, is the PTEN dual-specificity lipid phosphatase (Cantley and Neel, 1999). One role for PTEN is to balance GF signalling by de-phosphorylation of specific second messengers that act to promote survival and inhibit apoptosis. Thus, inactivation of this TSG results in impaired apoptotic signalling and promotion of uncontrolled cell growth. The fact that the action of PTEN also regulates signalling pathways mediating cell adhesion and migration has led to the hypothesis that loss of this TSG might contribute to tumour spreading. Hence, PTEN could be an important regulator of tumour invasiveness and metastasis (Di Cristofano and Pandolfi, 2000).

INTERFERON – PROFILING OF THE KILLER

History of interferon

Interferons were first described in 1957 by Isaacs and Lindenmann. It had long been known among virologists that individuals suffering from a viral disease rarely contracted another infection simultaneously, although the reason for this was unknown. The studies performed by Isaacs and Lindenmann, revealing the existence of interferon, included the infection of chicken chorio-allantoic membrane cells with heat-inactivated influenza virus. It was found that a soluble factor produced by the cells was able to protect from subsequent infection with live influenza virus. This phenomenon of viral interference gave rise to the name "interferon" (Isaacs and Lindenmann, 1987), which at that time was thought to be one single substance with one single effect.

Today, our knowledge about this cytokine has increased exponentially. It is a well established fact that the interferons constitute a large family of proteins and glycoproteins capable of exerting several biological effects on cells besides interfering with virus replication. Already in 1962 it was shown that IFN had anti-proliferative effects being capable of inhibiting cell growth and division (Paucker et al., 1962). These early experiments were performed using crude preparations of IFN, but have thereafter been confirmed using both highly purified as well as recombinant IFN.

The possibility to use IFN as a cancer therapy emerged as a result of the antiproliferative effects. Direct or indirect IFN-induced regression of tumour burden has been demonstrated in mice (Gresser et al., 1979; Ratner et al., 1980) and IFN, in particular IFNα, has been shown to serve as a useful therapy in several forms of malignancies (Einhorn et al., 1983; Grander et al., 1993; Lindner et al., 1997).

The early work on treatment of patients suffering from malignant diseases with IFNs was pioneered at the Karolinska Hospital by Hans Strander and co-workers in the 1970s. From these, and subsequent studies worldwide, it has firmly been shown that IFNs, alone or together with other therapeutic agents, are effective treatment in a number of malignant disorders (Einhorn and Strander, 1993; Gutterman, 1994; Borden, 1998). These include hairy cell leukaemia (HCL), chronic myelogenous leukaemia (CML), cutaneous T-cell lymphoma as well as low-grade B-cell lymphoma, mid-gut carcinoid, multiple myeloma (MM) and Kaposi´s sarcoma. There are, however, also a number of common malignant disorders, like carcinomas of the breast, lung, prostate, stomach and colon that respond poorly or not at all to IFN-treatment (Strander, 1986; Einhorn and Strander, 1993; Kirkwood, 2002).

However, despite the fact that IFNα has been used in these clinical settings for more than 20 years, the exact mechanism of its anti-tumour action, as well as the reason for lack of sensitivity to treatment of some malignancies, is still poorly understood. Increased knowledge into this field is of great importance and would lead to a more rational and efficient use of IFN as an anti-tumour agent.

The interferon family

The interferon family is constituted of two major categories; type I and type II IFNs. The human family of type I IFNs include at least five different subtypes, IFNα, IFNβ, IFNε, IFNκ and IFNω (Pfeffer et al., 1998; Pestka et al., 2004). Type I IFNs can be produced by most cells in the body as a result of various types of stimuli. Apart from viruses, both bacteria and parasites have been shown to induce production of IFN (DeMaeyer and De Maeyer-Guignard, 1988). Furthermore, dsRNA which is produced at some stage during most viral infections also induces IFN-production (Stewart and Vil\010Dek, 1979).

The genes encoding the human type I IFNs are all located on chromosome 9p21, including 14 distinct genes of IFNα, four IFNα pseudogenes, as well as one IFNβ, one IFNε, one IFNκ and one IFNω gene (Pestka, 1997; Weissmann and Weber, 1986; LaFleur et al., 2001; Hardy et al., 2004). The IFNα genes encode proteins of 165-166 amino acids, which share a sequence homology of 75-80%. Furthermore, all IFNα proteins generally display a high level of species specificity in their biological properties. The reason for this genetic multiplicity is not clear, but so far only quantitative differences between the activities of the various IFNs have been observed (Pfeffer et al., 1998).

In contrast to the type I IFNs, there is only one type II IFN; IFNγ (Diaz et al., 1993). The gene encoding IFNγ is located on the long arm of chromosome 12 (Naylor et al., 1983) and its product is a 146 amino acid glycoprotein. This IFN shares no homology with any of the type I IFNs. Nevertheless, it has been named and classified as an IFN, as it exhibits antiviral and antiproliferative properties. IFNγ is also crucial in eliciting a proper immune response and in pathogen clearance. Production of this cytokine seems to be restricted to antigen-stimulated T-cells and natural killer (NK) cells (Pfeffer et al., 1998; DeMaeyer and De Maeyer-Guignard, 1988).

Interferon alpha signalling

The interferon receptor

In order for interferons to exert their biological effects, binding to their respective cognate receptors is required. Type I and type II IFNs signal through distinct but related pathways. All of the type I IFNs bind to the same receptor, the Type I IFN receptor, while IFNγ binds to a different receptor, the Type II IFN receptor (Platanias and Fish, 1999).

The type I IFN receptor is composed of two major subunits, IFNAR1 and IFNAR2. The IFNAR2 exists in three differentially spliced variants. These alternatively spliced forms of the IFNAR2 gene include IFNAR2c, which is the subunit normally expressed together with IFNAR1, resulting in a functional receptor, and IFNAR2b which encodes a receptor subunit with a short cytoplasmic domain (Domanski et al., 1995). It has been shown that IFNAR2b, when overexpressed, can act in a dominant negative manner (Stark et al., 1998). The third

splice variant is IFNAR2a, a soluble form of the receptor subunit, which has been shown to block the activity of IFNs (Radaeva et al., 2002; Novick et al., 1994). A different composition of the type I IFN receptor has also been observed; the formation of a complex between IFNAR1 and IFNAR2b. Both of these type I IFN receptor compositions are capable of transducing signals and mediating the biological effects of interferons, but only IFNAR2c restores IFNα/β signalling in a mutant cell line lacking expression of the IFNAR2 gene (Platanias and Fish, 1999; Stark et al., 1998; Lutfalla et al., 1995).

The type II IFN receptor consists of two polypeptide subunits, IFNGR1 and IFNGR2. In unstimulated cells, IFNGR1 is associated with Jak1, and IFNGR2 is associated with Jak2. Binding of IFNγ induces oligomerization of the receptor subunits, which leads to the transphosphorylation and activation of the Jak's. Activated Jak's phosphorylate IFNGR1, thereby creating a docking site for STAT1. Following phosphorylation and activation of STAT1, this transcription factor is released from the receptor and forms a homodimer that translocates to the nucleus where transcription from gamma activated sites (GAS) can be initiated (Stark et al., 1998).

The Jak-Stat pathway

Since IFNα was the cytokine used in the studies presented in this thesis, the text will henceforth refer to this cytokine only.

A high affinity binding of IFNα requires both of the subunits, IFNAR1 and IFNAR2. The IFN receptor itself lacks intrinsic kinase activity, therefore it relies on the action of the constitutively associated Jak's to transmit the downstream signal (Figure 1). After binding of IFNα to the receptor, the cascade begins with phosphorylation of Tyk2, which is constitutively associated with the IFNAR1. This phosphorylation is followed by trans-phosphorylation between Jak1, which is associated with IFNAR2, and Tyk2 in order to further enhance the activation signal. The activated Jak1 and Tyk2 are thereafter responsible for phosphorylation of the cytoplasmic parts of the receptor, on tyrosine 466 (Y466) of the IFNAR1 (Stark et al., 1998).

In addition to Jak1, both STAT1 and STAT2 are constitutively associated with the IFNAR2. Binding of STAT1 depends on the presence of STAT2, but not *vice versa*. When the cytoplasmic part of IFNAR1 becomes phosphorylated, this becomes a docking site for STAT2, which binds the receptor through its src-homology 2 (SH2) domain. This new interaction positions STAT2 for phosphorylation on Y690, which in turn becomes the docking site for STAT1 and its subsequent phosphorylation on Y701. These tyrosine phosphorylations are suggested to be mediated by Jak1 and Tyk2 (Stark et al., 1998). In addition to tyrosine phosphorylation, STAT-proteins must also be phosphorylated on serine residues for efficient transcriptional activation. The transcriptional activation potential of STAT1 is dependent on the phosphorylation of residue Ser727 (Stark et al., 1998; Brierley and Fish, 2002) and in response to type I IFNs this phosphorylation has been shown to be mediated by protein kinase C delta (PKCδ) (Uddin et al., 2002) and by the p38 MAPkinase

(p38) (Sanceau et al., 2000). However, conflicting data on the involvement of p38 in the phosphorylation of STAT1 exists and need to be further investigated (Li et al., 2004).

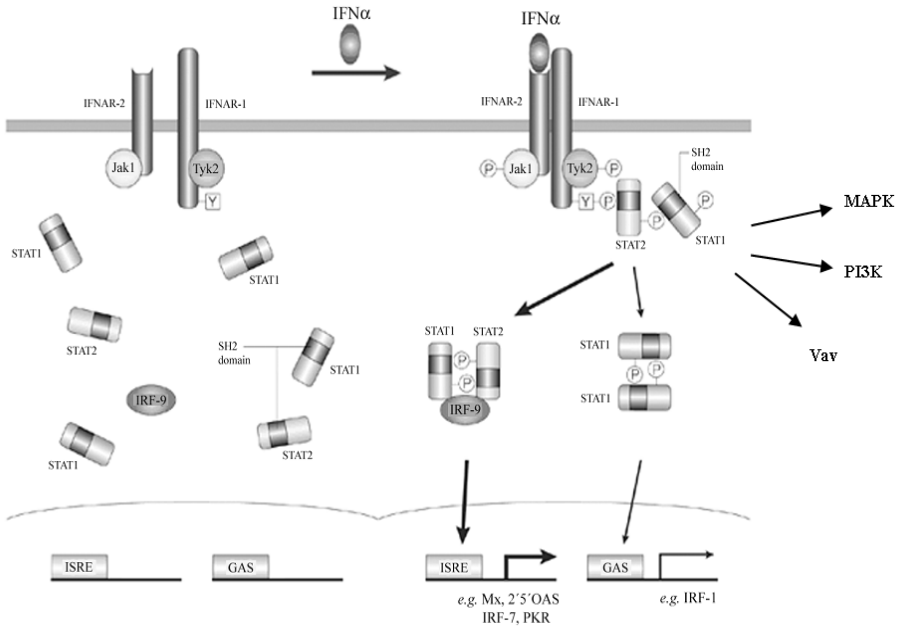

Figure 1 Signalling induced by IFNα

Binding of IFNα to the receptor results in activation of the classical Jak-STAT pathway, as well as activation of e.g. PI3K, Vav and MAPK signalling. Although not shown in the figure, STAT1 and STAT2 are constitutively associated to the IFNAR-2 before IFN-stimulation and the subsequent docking of these proteins to IFNAR-1.

The significance of functional Jak- and STAT proteins in the IFN-response has been shown in mice knockout studies. Jak1-deficient mice show perinatal lethality and a complete loss of the downstream IFN-induced signal (Rodig et al., 1998). In line with this data, Tyk2 was not phosphorylated in mutant cell lines lacking Jak1 after IFNα/β-stimulation (Imada and Leonard, 2000). Mice deficient for Tyk2 however, display only a partially reduced response to type I IFN signalling (Karaghiosoff et al., 2000). Among the STAT knockouts, all models except STAT3 are viable. STAT3 knockouts die before birth and the embryos show severe

developmental abnormalities. STAT1 and STAT2 knockouts suffer from increased susceptibility to mainly viral infections as a result of impaired IFN-signalling (Igaz et al., 2001).

The phosphorylated and activated STATs form both homodimers and heterodimers via their SH2 domains. Thereafter, they dissociate from the receptor complex and translocate to the nucleus. How this translocation is carried out is not yet clear, but when in the nucleus, binding to specific sequences in the promoters of IFN-regulated genes initiate gene transcription. Several STAT proteins are involved in Type I IFN signalling, and different compositions of STATs activate transcription of different sets of genes.

A major IFN-inducible transcription factor formed as a result of IFNα signalling is the interferon stimulated gene factor 3 (ISGF3). This complex comprises STAT1, STAT2 and IRF-9/p48, which associates with the STAT heterodimer in the nucleus. The ISGF3 complex binds with high affinity to the conserved IFN-stimulated response element (ISRE), $AGTTTN_3TTTCC$, present in the promoters of many IFN-stimulated genes (ISGs) *e.g.* MxA, PKR, 2´5´OAS, and PML, and stimulates their transcription (Brierley and Fish, 2002).

In addition to the ISGF3 complex, several other STAT complexes are formed during engagement of the type I IFN receptor. STAT3 is tyrosine phosphorylated in an IFNα-dependent manner and forms STAT3/3 homodimers or STAT3/1 heterodimers that bind to the high-affinity sis-inducible element/gamma-activated sequences (SIE/GAS), $TTCN_3GAA$, located in the promoters of different ISGs, such as IRF-1 and c-fos (Brierley and Fish, 2002; Platanias, 2003). In addition, STAT1/1 homodimers and STAT1/2 heterodimers are formed independently of IRF-9 and drive the expression of ISGs, such as IRF-1, through GAS-elements. Furthermore, STAT5 also undergoes tyrosine phosphorylation in an IFNα-dependent manner and forms STAT5/5 homodimers, which like the STAT1/1 homodimers subsequently translocate to the nucleus and bind to GAS elements to regulate transcription of certain ISGs (Erickson et al., 2002; Platanias, 2003).

Alternative signalling pathways induced by IFNα

Even though the Jak-STAT pathway is the primary IFN-dependent signalling cascade that mediates transcriptional activation of IFN-sensitive genes, research over the last few years has provided evidence for the existence of other type I IFN-activated signalling pathways.

One major signalling pathway induced by IFNα is the insulin receptor substrate (IRS) pathway, resulting in activation of phosphatidylinositol 3 kinase (PI3K). Activation of the IRS-pathway following binding of IFN to the type I receptor has been shown to occur as a result of Jak kinase dependent tyrosine phosphorylation of the IRS-1 and IRS-2 proteins (Platanias et al., 1996). The IRS-PI3K pathway appears to operate distinctively from the IFNα-activated STAT-pathway, as it has been demonstrated that IRS-proteins do not provide docking sites for the SH2 domains of STAT-proteins and IRS-activation is not required for DNA-binding of STATs (Uddin et al., 1997). There are however also studies suggesting that STAT3 acts as a mediator, coupling PI3K to the type I IFN receptor. In this system, binding of IFNα to the receptor results in tyrosine phosphorylation of STAT3 and subsequent

docking of the p85 regulatory subunit of PI3K, followed by phosphorylation and activation of this kinase (Pfeffer et al., 1997).

The vav proto-oncogene is another signalling molecule that is involved in type I IFN signalling. Vav undergoes tyrosine phosphorylation and participates in signalling induced by various cytokines by acting as GDP/GTP exchange factors for Rho/Rac molecules. This action of the vav adaptor-protein family is a direct link between receptors with intrinsic or associated tyrosine kinase activity and signalling pathways regulated by Rho/Rac proteins. The activity of vav has proven essential in the regulation of cytoskeletal, proliferative, and apoptotic pathways that determine the development of lymphoid cells (Bustelo, 2000). In the interferon system, phosphorylation of vav is regulated by Tyk-2, to which vav is constitutively associated via its SH2 domain. It has been suggested that vav is part of a type I IFN activated pathway that mediates growth inhibition since disruption of vav expression using antisense oligonucleotides reverses the antiproliferative effects of IFNα in a megakaryocytic cell line (Platanias and Fish, 1999).

Another example of a non classical Jak-STAT signal that is induced by IFN to mediate antiproliferative effects involves members of the Crk-proteins which also have SH2 and SH3 domains facilitating their interactions with other proteins. These adaptor proteins are involved in modulation of cell adhesion, cell migration and immune cell responses (Feller, 2001). Both CrkL and CrkII proteins are phosphorylated in a Tyk-2 dependent manner as a result of IFNα-signalling, and inhibition of their expression results in suppressed growth inhibition by IFN in hematopoietic cells (Uddin et al., 1996). Furthermore, CrkL has been found to act as an adaptor protein for STAT5. When phosphorylated by Tyk-2, STAT5 associates with CrkL via its SH2 domain and subsequently translocates to the nucleus for regulation of interferon stimulated gene transcription mediating growth inhibitory effects (Platanias and Fish, 1999).

The p38/MAPK pathway which participates in signalling cascades induced by a variety of cellular stimuli is also activated by IFNα. The function of p38 is required for the generation of a range of biological responses, and it has been shown that its serine kinase activity mediates phosphorylation of transcription factors and regulation of downstream gene transcription. The upstream regulatory events linking the type I IFN receptor to p38 have not been fully elucidated, but have been proposed to depend on the action of the vav protein. Another possibility is the activation of p38 by PKCδ, which has been suggested since rottlerin, a specific PKCδ inhibitor, also blocks activation of p38 (Uddin et al., 2002). Several studies indicate that activation of the p38 pathway by type I IFN signalling is essential for transcription of most, if not all, IFN-sensitive genes, however extensive research has failed to show that p38 facilitates STAT-complex formation and/or DNA binding of STAT proteins (Platanias, 2003).

In the context of alternative IFN signalling pathways, it is also important to note that type I IFN signalling is involved in crosstalk with several other signalling pathways. One such example is the cross talk between IFNγ- and IFNα/β-signalling, where pre-treatment with IFNγ strongly augments IFNα-signalling (Darnell et al., 1994). This enhancement can be at least partly explained by the increase in abundance of IRF-9 which is induced by IFNγ.

Conversely, IFNα treatment increases the cellular abundance of STAT1, making the subsequent response to IFNγ much stronger (Bluyssen et al., 1996). In a similar manner it has been observed that IL-6 also requires a constitutive sub-threshold IFNα/β-signal for efficient activation of the transcription factors STAT1 and STAT3 (Mitani et al., 2001).

Negative regulation of IFN-signalling

As for the majority of signalling cascades in eukaryotic cells, the signals generated by IFNs need to be modulated in a delicate fashion to ensure efficient shut off mechanisms. Jak proteins as well as STATs can be regulated in several ways. A number of phosphatases, such as SHP-1, have been implicated in dephosphorylation of both the receptor and the Jak´s (Platanias and Fish, 1999; Stark et al., 1998). STATs can be negatively regulated by degradation, as has been shown for instance for STAT1 which is targeted for ubiquitin/proteasomal degradation. Furthermore, alternative spliceforms of STAT-proteins can act in a dominant negative manner, thereby negatively regulating STAT actions (Imada and Leonard, 2000). Members of the suppressors of cytokine signalling (SOCS) family are upregulated in response to cytokine stimulation, generating a negative feedback loop. SOCS proteins can regulate IFN-induced signalling by several mechanisms including binding to the Jak´s, thereby inhibiting their catalytic activity. SOCS family members also act through binding to the receptor, thereby blocking STAT recruitment, and finally through the targeting of signalling proteins, *e.g.* vav, for proteasomal degradation (Krebs and Hilton, 2001; O'Shea et al., 2002). The protein inhibitors of activated STATS (PIAS) bind activated STAT dimers and block transcription (O'Shea et al., 2002). Finally, several members of the IRF family, such as IRF-2 and the interferon consensus sequence binding protein (ICSBP/IRF-8), bind to ISREs and thereby negatively regulate the expression of ISGs (Mamane et al., 1999).

Anti-tumour features

It is unclear what induces tumour regression *in vivo* following IFNα-treatment, but *in vitro* data and animal studies suggests both direct and indirect effects as possible effector mechanisms.

Indirect effects

Immunological effects

IFNs have been shown to influence a large number of functions of the immune system in both stimulatory as well as inhibitory ways. For example, stimulation of cytotoxic T- and NK cells by IFNs induce tumour cell death by upregulation of TRAIL or the Fas ligand as well as release of pro-apoptotic perforin from NK cells (Chawla-Sarkar et al., 2003). These findings suggest that IFN may induce tumour cell apoptosis indirectly by activating immune effector cells. However, data that support a role for immunological mechanisms in the anti-tumour effects of IFN have mainly been derived from animal experiments or *in vitro* studies. In humans there is yet no clear proof that immunological effects induced by IFN have an anti-tumour role. For instance, correlations between immunological functions and clinical effects are commonly not seen in patients receiving IFN-therapy (Einhorn et al., 1982).

Anti-angiogenic effects

Non-immunological indirect effects may also contribute to the anti-tumour action of IFNs. It is well known and studied that a growing tumour is dependent on the in-growth of newly formed blood vessels for supply of oxygen and nutrients. Interestingly, IFNα has been shown to inhibit the angiogenic process in animal systems (Dvorak and Gresser, 1989). Furthermore, IFNα is used in the treatment of large haemangiomas, sometimes with dramatic effects (Chan, 2004). This is a promising putative effector mechanism that might well contribute to the anti-tumour actions of IFN, at least in some solid tumours. However, the importance of this effect in clinical settings needs to be further clarified.

Direct effects

Several studies have shown a correlation between the *in vitro* susceptibility of primary malignant cells to IFN and the clinical response of the patients to IFN-therapy. This strongly supports the idea that the anti-tumour effects of IFN result mainly from direct effects on the tumour cells (Brenning et al., 1985; Grander et al., 1990; Rosenblum et al., 1986; Ferbus et al., 1990).

Effects on proliferation and differentiation

The first study to show that IFNs exert anti-proliferative effects was published in 1962 (Paucker et al., 1962). This finding has subsequently been verified in many malignant as well as non-malignant cell types of different origin (DeMaeyer and De Maeyer-Guignard, 1988). Most commonly, IFN arrests cells in the G1 phase of the cell cycle, but sometimes also causes a lengthening of the S-phase or prolongation of all cell cycle phases (Balkwill and Taylor-Papadimitriou, 1978; Roos et al., 1984). IFNα-induced cell cycle arrest seems to be mediated through a co-operation between Cip/KIP and Ink4 cyclin dependent kinase inhibitors (CKIs) as shown both in tumour cell lines (Sangfelt et al., 1997b; Sangfelt et al., 1999) and in primary leukemia cells (Szeps et al., 2003). It has furthermore been shown that IFNα downregulates telomerase activity in a number of malignant cell lines as well as in primary leukemic cells (Xu et al., 2000). This inhibition of constitutive telomerase activity, possibly resulting in proliferative senescence of the tumour cells, could be a putative anti-tumour mechanism of IFNα.

It has been suggested that during malignant transformation there is a block in terminal differentiation of the tumour cells. If so, abrogation of this inhibition might lead to a reversion of the malignant phenotype (Sachs, 1978). IFNs have been shown to induce differentiation of cells from a number of established cell lines (Rossi, 1985) and such effects have also been observed in primary malignant cells from patients with chronic B-lymphocytic leukemia (Ostlund et al., 1986) and chronic myeloid leukemia (Paquette et al., 2002). This induction of differentiation could presumably also add to the anti-tumour action in these malignancies.

Apoptotic effects

When it was found that IFNs can reduce the number of malignant cells it was initially assumed that this was due to inhibition of proliferation alone. It is, however, becoming increasingly evident that the cytotoxic effects mediated by induction of apoptosis, in many cases are responsible for the reduction in cell number following IFN treatment. Several studies indicated that IFN is a direct inducer of cell death in some tumour cell lines and also in primary cells (Manabe et al., 1993; Grander et al., 1993). In 1997 it was shown for the first time that IFNα can exert a direct apoptotic effect in malignant cells, independent of its cell cycle inhibitory activity (Sangfelt et al., 1997a). Furthermore, apoptosis induced by IFNα occurs without requirement of wild type p53 (Sangfelt et al., 1997a). It has been suggested that IFN may induce apoptosis by the death receptor pathway through a strong and sustained induction of ISGs like TRAIL and Fas/FasL (Chawla-Sarkar et al., 2003). Furthermore, in MM cell lines, long term treatment with both type I and type II IFN sensitizes cells to Fas induced apoptosis (Spets et al., 1998). It has also been shown that treatment of the MM cell line U266 with IFN results in a strong expression of Apo-2L/TRAIL, release of cytochrome c and apoptosis. This effect was partly abrogated by the presence of a dominant negative Apo-2L receptor DR5 (Chen et al., 2001). It should however also be noted that there are some studies showing that IFNα and -γ protects from apoptosis as shown in B-CLL cells both *in vitro* and *in vivo*. Protection in these cases has been suggested to be a result of Bcl-2 overexpression as well as suppression of the c-myc protein (Sangfelt et al., 1996; Milner et al., 1995; Jewell et al., 1994).

As shown in paper I of this thesis, IFNα induces apoptosis in a caspase-dependent manner. However, the upstream mechanisms leading to caspase induction remain to be clarified. The apoptosis induced by IFNα furthermore involves activation of the Bak and Bax pro-apoptotic proteins, disrupted mitochondrial membrane integrity and release of cytochrome c, and can be rescued by overexpression of Bcl-2. (Panaretakis et al., 2003; Thyrell et al., 2002). These data clearly demonstrate the importance of the mitochondrial pathway in IFNα-induced apoptosis. Recent data from our group also show the involvement of JNK and PKCδ in this process (Panaretakis *et.al.* unpublished data). Thus it seems that IFNα induced apoptosis occurs both through the death receptor, extrinsic, pathway as well as the intrinsic mitochondrial dependent pathway. As demonstrated in paper II of this thesis, in malignant cell lines of different origin, activation of the PI3K/mTor pathway is crucial for IFNα induced apoptosis (Thyrell et al., 2004). However, the relevance of these data will be discussed in further detail under the "Results and discussion" part.

Resistance to IFN-treatment

Considering the direct effects of IFN; one of the challenges when it comes to improve IFN-therapy is to understand the reasons for variability in sensitivity to treatment between different malignancies as well as between patients suffering from the same cancer type.

Like cells from continuous cell lines, primary tumour cells differ widely in their sensitivity to IFNs, some being very sensitive whereas others are more or less resistant (Grander et al.,

1993; Borden, 1998; Einhorn and Grander, 1996; Einhorn and Strander, 1977). Theoretically, reasons for resistance may be divided into non-cellular or cellular causes.

Non-cellular reasons for resistance may for example be that the bio-availability of IFN in the tumour is low. Another reason could be that the patient may develop neutralizing antibodies against IFN or that high enough doses can not be given due to side effects (Einhorn and Grander, 1996).

The lack of a cellular response to IFNs is a far more complicated issue. One cause for resistance could be defects in the IFN signalling transduction pathway. Defects in all parts of the signalling have been found in various cell systems. A cell may lack IFN-specific cell surface receptors (Aguet, 1980), or may contain defective Jak and STAT proteins (Hunter, 1993; McKendry et al., 1991), which results in cellular resistance. Another mechanism could be loss of function of important ISGs such as PKR, RNAseL or IRF-1 that have been considered as potential tumour suppressor genes (Tanaka et al., 1994). Finally resistance could be attributed to defects in genes regulating specific IFN-modulated functions, such as apoptosis or cell cycle checkpoints. The result from this is insensitivity of the cells to the direct anti-tumour effects of IFNα. One such example is evidenced by the fact that high levels of the anti-apoptotic protein Bcl-2 correlates to a poor clinical response to IFNα in patients with MM (Sangfelt et al., 1995).

DEATH SIGNALLING - APOPTOSIS

In the mid 19th century it was found that cells die in an ordered fashion during normal amphibian development. This ordered scheme of dying was later been found to occur in both invertebrates and vertebrates, and is a very well conserved process (Lockshin and Zakeri, 2001). The term programmed cell death (PCD) was suggested in 1965 (Lockshin and Williams, 1965) to describe the ordered chain of genetically controlled events leading to death of the cell. PCD was shown to occur in a predictable manner during development and also to serve as a major mechanism for removal of unwanted and potentially dangerous cells, such as virus-infected cells, self-reactive lymphocytes and tumour cells (Lockshin and Zakeri, 2001). Although some distinct morphological features of PCD had been described already in 1885, when Walther Flemming made a drawing of what he called "chromatolysis", showing a cell with clear apoptotic morphology, it was not until 1972 that the now widely used term apoptosis was coined. In a seminal article by Kerr *et.al.*, the morphological features of apoptotic cells was used to distinguish from the more chaotic, non-programmed necrotic cell death (Kerr et al., 1972).

Apoptosis *versus* necrosis

Apoptosis is associated with a number of morphological changes, including cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing and loss of adhesion (Kerr et al., 1972). Apoptotic cells furthermore display exposure of phosphatidyl serine (PS) on their surface (Martin et al., 1995). In healthy cells, PS is located to the inner leaflet of the cell membrane. During the apoptotic process, PS is externalized to provide an "eat-me" signal for engulfment by adjacent macrophages. Phagocytosis of the apoptotic cell takes place rapidly, prior to release of intracellular contents, hence without induction of an inflammatory response (Henson et al., 2001).

Biochemical features associated with apoptosis include the activation of cysteinyl aspartate specific proteases (caspases) which perform proteolytic cleavage of a number of intracellular substrates (Thornberry et al., 1992; Thornberry et al., 1997). Apoptosis in its most classical form is observed almost exclusively when caspases are activated and might thus be blocked by the use of caspase inhibitors Activation of specific endonucleases during apoptosis result in cleavage of DNA into oligonucleosomal fragments, generating the typical ladder pattern observed in gel electrophoresis, a commonly recognized hallmark of apoptosis (Wyllie et al., 1980).

Necrosis occurs after exposure to high concentrations of detergents, oxidants or high intensities of pathologic insult. The term necrosis is the conceptual counterpart to apoptosis, as it is prevented only by removal of the stimulus. No chromatin condensation is observed in necrotic cells, and the chromatin is degraded randomly yielding a smeared pattern rather than an ordered, when viewed following agarose gel electrophoresis. Cells undergoing necrosis swell, their mitochondria dilate, organelles dissolve and their plasma membranes rupture. This

leads to leakage of the cellular contents with the result being an inflammatory response. (Leist and Jaattela, 2001).

If focusing on the strict morphological criteria of apoptosis, caspases seem to be indispensable for this process to proceed. There are however many forms of "apoptosis-like PCDs" that can occur without caspase activation. The term apoptosis-like PCD is used do describe forms of PCD with chromatin condensation that is incomplete or less compact than in apoptosis (Leist and Jaattela, 2001). PCD can also occur in the complete absence of chromatin condensation, this type of cell death is termed "necrosis-like PCD" and usually involves specialized caspase-independent signalling pathways (Leist and Jaattela, 2001). Hence, caspase activation appears to be the preferred mode of execution, although in its absence or failure, there exists several other default pathways. (Lockshin and Zakeri, 2004)

Caspases – the executors of apoptosis

Since caspases bring about most of the visible changes that characterize apoptotic cell death, they are regarded as the central executors of the apoptotic process. However, although overexpression of each of the caspases can kill cells by induction of apoptosis, not all of them are normally involved in this process.

The caspase family constitutes 15 cystein proteases (Joza et al., 2002), of which 11 human enzymes are known. They all cleave their substrates after aspartic acid residues, and their substrate specificity is determined by the four residues amino-terminal to the cleavage site. Caspases reside in the cell as inactive proenzymes, zymogens, which require proteolytic cleavage to be activated (Thornberry et al., 1997). The zymogens contain three domains; the inhibitory N-terminal prodomain, a large subunit (p20) and a small subunit (p10). Activation of the proenzyme to a fully functional protease requires cleavage at two sites. The first cleavage separates the large subunit from the small, and the second cleavage removes the N-terminal prodomain (Earnshaw et al., 1999). The active caspase is a hetero-tetrameric protease, constituted of two small and two large subunits, with two active sites per molecule. Based on the length of the prodomain, caspases are divided into two major classes, the long prodomain (initiatior) caspases and the short prodomain (executor) caspases.

Caspases-2, -8 -9 and -10 are the major initiator caspases. The N-terminal long prodomains of caspases-8 and -10 contain death effector domains (DEDs) which facilitate their interactions with upstream regulators such as death receptors. The prodomains of caspases-2 and -9 contain caspase activation and recruitment domains (CARDs) that appear to be important for these caspases in promoting interactions with one another and a range of other regulatory and adapter proteins (Earnshaw et al., 1999). Activation of the long-prodomain caspases takes place via oligomerization-induced autoproteolysis. The function of the active initiator caspases is to proteolytically cleave and activate the downstream executor caspases.

Caspases-3, -6 and -7 are the major executor caspases. When activated, either by initiator caspases or other pro-apoptotic proteases such as Granzyme B, they cleave themselves,

thereby amplifying the caspase activation cascade. Furthermore, the executor caspases cleave the vast majority of proteins that undergo proteolysis in apoptotic cells, including structural components of the cytoskeleton and nucleus. For example, ICAD - the inhibitor of caspase-activated DNase (CAD) - is cleaved and thus releases CAD which translocates to the nucleus for fragmentation of DNA. Other examples of caspase substrates include the nuclear structure proteins lamin A and - B as well as PARP (poly-(ADP-Ribose) polymerase), which is involved in DNA-repair (Earnshaw et al., 1999).

The main apoptotic pathways

Although cell death can be triggered by a vast array of stimuli, the manner by which all apoptotic signals engage the cell death machinery falls under two broad categories; one beginning at the level of cell surface death receptors (the extrinsic pathway), the other is mediated by mitochondria (the intrinsic pathway) (Figure 2). Both pathways involve activation of caspases that act to cleave cellular substrates. This results in the cellular and biochemical morphological changes which together constitute the characteristics of apoptosis as discussed above.

The extrinsic –death receptor mediated- pathway

Death receptors (DRs) belong to the tumour necrosis factor (TNF) receptor gene super family. This family of receptors is defined by similar cystein-rich extracellular domains in addition to a homologous cytoplasmic sequence termed the death domain (DD). The most well characterized DRs are CD95 (also called Fas or Apo1), TNFR1, DR4 (also called TRAIL-R1) and DR5 (also called TRAIL-R2). The ligands that activate these receptors are structurally related molecules that belong to the TNF gene super family; CD95 ligand (CD95L) binds CD95, TNFα and lymphotoxin α bind to TNFR1 and TRAIL binds to DR4 and DR5 (Igney and Krammer, 2002; Debatin and Krammer, 2004).

All TNF receptor family members are homotrimeric molecules. Upon binding of their respective ligand, the receptor trimerizes as a result of the propensity for DDs to associate with one another. As a consequence, the DDs of the DRs attract the intracellular adaptor protein FADD (in the case of CD95) and TRADD (in the case of TNFR and TRAIL-R), thereby forming the death inducing signalling complex (DISC). FADD and TRADD furthermore contain a death effector domain (DED) which is essential for the recruitment of the initiator caspases-8 (initially known as FLICE) and -10 to the DISC complex (Ashkenazi and Dixit, 1998).

At the DISC complex, procaspase-8 is cleaved to yield an active initiator caspase. In some cells, known as type I cells, the amount of caspase-8 is sufficient to initiate apoptosis directly, whereas in type II cells, the amount of caspase-8 is too small and mitochondria are used as amplifiers of the death signal (Scaffidi et al., 1998). The signal for involvement of mitochondria is mediated by caspase-8 dependent cleavage of the Bid protein. Bid, in its

truncated form (tBid) translocates to the mitochondrial membrane where it is involved in release of cytochrome c and subsequent caspase activation (Luo et al., 1998).

Activation of caspase-8 is also regulated by the FLIP (FLICE-like inhibitory) protein. FLIP is a caspase-8 homolog which lacks proteolytic activity. At high expression levels, such as those found in certain tumours, FLIP inhibits caspase-8 activation, presumably by saturating available recruitment sites on the DISC, preventing recruitment of caspase-8 to the complex (Chang et al., 2002).

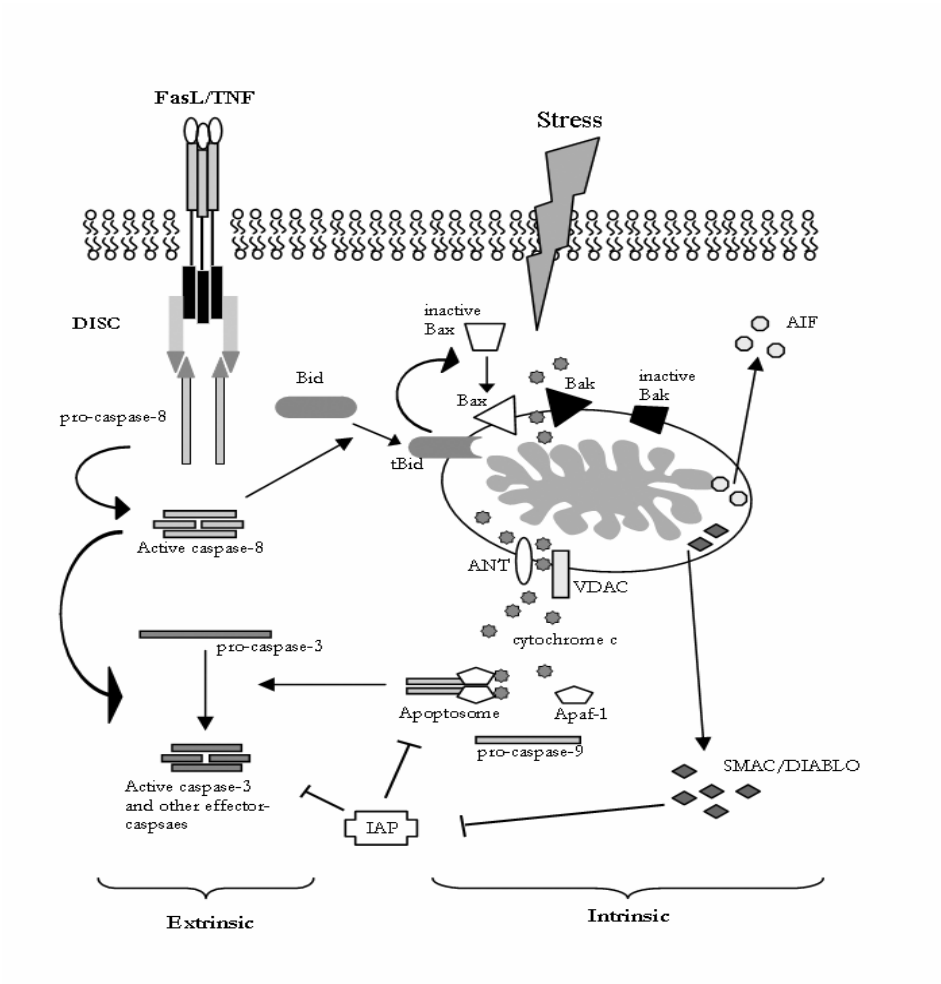

Figure 2 The main apoptotic pathways

As a result of DR signalling the extrinsic apoptotic pathway is induced, leading to activation of caspase-8 and downstream effector caspases. Cellular stress insults activate the intrinsic pathway, which converge at the mitochondria where pro-apoptotic Bcl-2 family proteins are activated, mitochondrial membrane potential is lost and cytochrome c as well as other pro-apoptotic molecules are released.

The intrinsic –mitochondria activated- pathway

The intrinsic cell death pathway is initiated as a response to multiple stress signals and depends on alterations involving the mitochondria, ultimately leading to mitochondrial dysfunction. Some of these signals are induced by cytotoxic drugs such as DNA-damaging agents as well as irradiation, hypoxia and DR-signalling.

Triggering of the mitochondrial cell death pathway results in release of pro-apoptotic proteins from the mitochondrial intermembrane space into the cytosol (the mechanism for this release is further discussed below). One of the pro-apoptotic proteins released is cytochrome c, which after entry into the cytosol stimulates formation of a complex called the apoptosome. The apoptosome, in addition to cytochrome c, contains ATP, the adaptor protein Apaf-1 and caspase-9. Binding of cytochrome c to Apaf-1 induces oligomerization of this protein which subsequently recruits pro-caspase-9 via its CARD. In turn, caspase-9 is activated autoproteolytically and triggers activation of executor caspases such as caspase-3 (Saelens et al., 2004).

The importance of cytochrome c, Apaf-1 and caspase-9 for the execution of intrinsic apoptosis in mammals has been confirmed by targeted disruption of the corresponding genes. All three knockout mice exhibit embryonic lethality or die soon after birth. Both caspase-9$^{-/-}$ (Hakem et al., 1998) and Apaf-1$^{-/-}$ (Yoshida et al., 1998) cell lines are largely resistant to intrinsic cell death signals yet sensitive to extrinsic stimuli such as Fas or TNF stimulation. These findings clearly indicate an important role for the cytochrome c/Apaf-1/caspase-9/caspase-3 pathway in cell death in response to intrinsic death signals.

The role of mitochondria in apoptosis

Opening of the PT-pore

Mitochondria are organelles with two well defined compartments; the matrix, surrounded by the inner membrane (IM), and the intermembrane space, surrounded by the outer membrane (OM). Death at the mitochondrial level is initiated by perturbation of the mitochondrial membrane and proceeds via release of cytochrome c and other death promoting proteins from the intermembraneous space (Herr and Debatin, 2001). The mechanism behind the mitochondrial permeabilization is not clearly understood and several models have been suggested to account for this effect. The key players in the different models proposed are the voltage dependent anion channel (VDAC) protein, present in the mitochondrial OM, the adenine nucleotide translocator (ANT), which is a pore-forming protein in the IM and the pro-apoptotic Bcl-2 family members Bak, Bax and Bid (Debatin et al., 2002).

VDAC is normally responsible for the transport of metabolites, such as ADP, ATP and NADH between the cytosol and the mitochondrial intermembrane space. ANT is responsible for the exchange of ATP and ADP between the mitochondrial cytosol and the intermembrane space. VDAC and ANT, together with other proteins such as cyclophilin D, form the

permeability transition pore complex (PTPC) at the contact sites between the IM and OM. Opening of the PTPC *i.e.* permeabilization of the outer and inner mitochondrial membranes results in depletion of ATP, loss of mitochondrial membrane potential ($\Delta\psi_{mit}$) and release of cytochrome c. According to this model, opening of the PTPC leads to influx of water in the mitochondrial matrix, resulting in swelling and subsequent rupture of the OM accompanied by release of intermembrane proteins (Debatin et al., 2002). However, disruption of the mitochondrial structure is a hallmark of necrosis rather than apoptosis. It has therefore been suggested that opening of the PTPC in apoptotic cells is transient, or that it only occurs in a fraction of mitochondria. This would allow for release of intermembrane proteins, such as cytochrome c, while ATP production and mitochondrial membrane integrity is preserved, thereby allowing for formation of the apoptosome and subsequent activation of caspase-9 (Martinou and Green, 2001).

Another proposed model for PTP and release of intermembrane proteins involves both VDAC and the Bcl-2 family members Bak and Bax. The pore size of VDAC in itself is not sufficient to allow passage of cytochrome c. It has therefore been suggested that binding of these pro-apoptotic proteins to VDAC induces its conformational change and increased pore size, facilitating release of pro-apoptotic proteins, and that this opening is inhibited by competitive binding to VDAC by anti-apoptotic Bcl-2 members (Debatin et al., 2002).

The third model for mitochondrial membrane permeabilization involves pro-apoptotic members of the Bcl-2 family alone, without participation of either VDAC or ANT. It has been shown that both Bak (Wei et al., 2000), Bax (Saito et al., 2000) as well as tBid (Scorrano et al., 2002) are able to homo- or heterodimerize and form pore channels in the OM. However, it is debatable whether or not these channels are sufficiently large to allow for the passage of cytochrome c into the cytosol (Debatin et al., 2002; Martinou and Green, 2001)

Proteins released from mitochondria during apoptosis

Mitochondria provide the cell with energy in the form of ATP which is produced by oxidative phosphorylation. However, besides from being guardians of survival, mitochondria also harbour noxious molecules in their intermembrane space. As the result of a wide variety of apoptosis signals, the mitochondrial outer membrane integrity is lost. Permeabilization occurs and due to both caspase dependent and independent processes there is a release from the intermembrane space not only of cytochrome c, but also of other proteins with cytotoxic activities,.

Two pro-apoptotic factors released are the Smac/Diablo and the HtrA2/Omi proteins (Du et al., 2000; Suzuki et al., 2001; Verhagen et al., 2000). Both of these proteins have been found to facilitate caspase activation through their ability to bind to, and inhibit the function of, members of the inhibitors of apoptosis (IAP) protein family. HtrA2/Omi also possesses a serine protease activity which has been found to contribute to caspase independent processes.

Other factors that have been found to be involved in caspase independent cell death include apoptosis-inducing factor (AIF) (Susin et al., 1999) and Endonuclease G (EndoG) (van Loo et

al., 2001). In fact, AIF is believed to play a central role in the caspase independent programmed cell death. This flavoprotein has an important physiological role in the regulation of oxidative processes. However, similar to the bifunctional role of cytochrome c, when AIF is released from the mitochondria in response to apoptotic stimuli, it becomes an active executioner of the cell. Following nuclear translocation, AIF is believed to induce an apoptosis-like cell death by triggering chromatin condensation. In cell free systems, incubation of isolated HeLa nuclei with recombinant AIF results in large-scale (50 kb) DNA fragmentation and chromatin condensation. Induction of nuclear apoptosis in a variety of cell types by AIF is not abrogated by pharmacological caspase inhibitors. EndoG has been found to act in a similar manner, inducing DNA fragmentation independent of caspases (Cregan et al., 2004).

Regulation of apoptosis – the Bcl-2 family

Proteins of the Bcl-2 family are central regulators of apoptosis and constitute a group thought to act primarily on the mitochondria. Members of this family possess either anti-apoptotic or pro-apoptotic functions. They are characterized by the presence of conserved sequence motifs, known as Bcl-2 homology (BH) domains. Anti-apoptotic members share all four BH domains, BH1-4. There are two groups of pro-apoptotic family members; the multidomain proteins share sequence homology in the BH1-3 regions, whereas the BH3-only protein (BOP) family members only have the BH3 domain in common (Figure 3).

An important feature of the Bcl-2 family members is their ability to form homo- as well as heterodimers. Due to the ability to form dimers, Bcl-2 family proteins are able to function either independent or together in the regulation of apoptosis. However, this feature also suggests synergistic effects as well as neutralizing competition between the pro- and anti-apoptotic members.

Pro-apoptotic proteins

The large majority of pro-apoptotic Bcl-2 proteins are localized to the cytosol. Following a death signal they translocate to intracellular membranes, mostly mitochondrial, where they either insert or interact with other proteins to stimulate apoptosis (Burlacu, 2003).

Bax and Bak

The importance of the pro-apoptotic proteins Bak and Bax has been shown in numerous studies. Both Bak deficient ($Bak^{-/-}$, $Bax^{+/+}$) and Bax deficient ($Bak^{+/+}$, $Bax^{-/-}$) cells are susceptible to pro-apoptotic stimuli induced by several agents such as etoposide, staurosporine, cisplatin and UV-irradiation, whereas $Bak^{-/-}$, $Bax^{-/-}$ double deficient cells are completely protected. These results indicate that the presence of either Bak or Bax is required for apoptosis, and that each of them efficiently promotes the apoptotic response (Wei et al., 2001).

In healthy cells, Bax is predominantly localized to the cytosol or loosely attached to membranes in an inactive form. After exposure to apoptotic stimulation, Bax undergoes conformational changes, translocates and inserts itself into the outer mitochondrial membrane, anchoring the hydrophobic COOH domain and exposing the N-terminal part to the cytosol and oligomerizes. By using specific antibodies against the exposed N-terminus it is possible to measure the levels of Bax activation (Nechushtan et al., 1999). The influence of Bax, either alone or together with other pro-apoptotic members of the family, leads to the release of cytochrome c and subsequent downstream caspase activation. The activation of Bax is thought to be mediated by tBid by an, as yet, unidentified mechanism. It has however been proposed that the BH3 domain of tBid binds to Bax and induces the conformational change which is necessary for the integration in mitochondrial membranes. Alternative Bid-independent pathways must however exist since the activation of Bax has been observed in $Bid^{-/-}$ cells (Ruffolo et al., 2000).

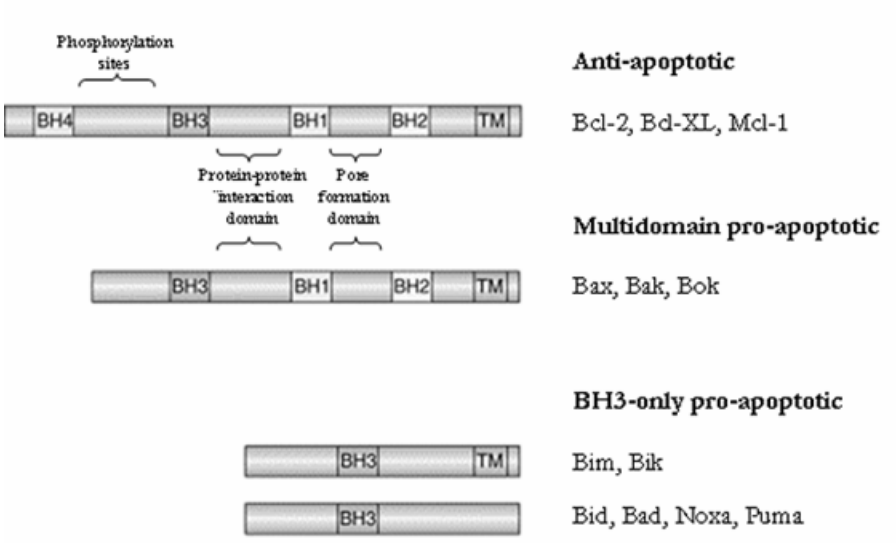

Figure 3 The Bcl-2 family of proteins

In contrast to Bax, Bak is normally associated with the mitochondrial OM in healthy cells. Upon apoptosis-induction this pro-apoptotic protein fully inserts into the membrane as a result of conformational changes. As in the case for Bax, the activation of Bak is measurable using specific antibodies against the exposed N-terminal part. Regulation of Bak has been suggested to be mediated by voltage dependent anion channel 2 (VDAC2) which specifically associates with the protein to keep it in a monomeric inactive conformation in the absence of an apoptotic stimulus. Overexpression of BH3-only proteins, such as tBid and Bad, results in the dissociation of Bak from VDAC2 *in vivo*. However, it is not clear whether this displacement is mediated directly by the BOPs (Chan and Yu, 2004).

BH3-only proteins

The BH3-only proteins (BOPs) are regarded as the "sensors and mediators of apoptosis". In healthy cells, BOPs are kept inactive. In response to pro-apoptotic stimuli they become, relocalized and/or post-translationally modified to gain their full apoptotic potential. Some BH3-only members are transcriptionally upregulated as in the case of Bik, Puma and Noxa which are induced by p53 as a consequence of DNA damage. The main mechanisms by which the BOPs excert their pro-apoptotic function is by binding and inactivating the anti-apoptotic proteins, *e.g.* Bcl-2 and Bcl-X_L, as well as to directly activate Bak and Bax. A variety of BOPs, such as Bim, Bad and Bid have been shown unable to induce apoptosis when expressed in Bax/Bak double deficient cells, strongly suggesting that BOPs require Bak or Bax to mediate apoptosis signals (Zong et al., 2001). Although there is no conclusive demonstration of direct binding of a BOP to Bax/Bak proteins *in vivo*, the BOPs Bid and Bim have been implicated as enhancers of Bax/Bak activation.

Bid is cleaved by caspase-8 in response to death receptor stimuli. The truncated form, tBid, is myristolated and translocates to mitochondria where it stimulates activation of Bak and Bax (Li et al., 1998). The caspase-8 mediated cleavage of Bid represents the main molecular link between DR- and mitochondria-mediated pro-apoptotic signalling. However, caspase-8 is not the only protease shown to cleave Bid. Granzyme B, a T-cell specific serine protease as well as calpain, a calcium activated cysteine protease, have both been shown to cleave Bid and thereby activate the mitochondrial pathway (Barry et al., 2000; Mandic et al., 2002).

The BH3-only protein Bad is regulated by phosphorylation. As a result of for example growth factor signalling Bad is serine phosphorylated by the Akt/PKB kinase. This phosphorylation mediates inhibition of its pro-apoptotic activity by the binding of 14-3-3 scaffold proteins (Zha et al., 1996). Upon cytokine withdrawal, Bad is de-phosphorylated and released from inhibition. The molecule is then able to interact with Bcl-2-like survival proteins and to neutralize their anti-apoptotic interactions with Bax-like death factors.

Recently a molecular link has been found between the JNK-signal transduction pathway and the Bak/Bax-dependent mitochondrial apoptotic machinery. JNK-mediated phosphorylation of Bim and Bmf was shown to be a result of UV-induced stress (Lei and Davis, 2003) and to increase apoptosis. This is suggested to depend on activation of the pro-apoptotic Bak/Bax proteins. Furthermore, JNK-dependent phosphorylation of Bim and subsequent translocation

of Bax to the mitochondria for downstream apoptotic signalling has been shown following cerebral ischemia (Okuno et al., 2004).

Anti-apoptotic proteins

The anti-apoptotic Bcl-2 family members are integral membrane proteins found anchored by their COOH-terminal hydrophobic transmembrane (TM) domain in mitochondria, endoplasmic reticulum (ER) and nuclear membranes. It has been suggested that these anti-apoptotic family members reside on intracellular membranes to control apoptosis and that they are directed to their respective organelle compartment immediately following synthesis (Schinzel et al., 2004).

There are several proposed mechanisms to explain how Bcl-2 and Bcl-X_L prevent mitochondrial perturbation. Binding of Bcl-2 or Bcl-X_L to VDAC may lead to inhibition of the PT pore opening and therefore prevention of cytochrome c release. In a similar way, Bcl-2 is able to bind ANT, thereby maintaining the ADP-ATP exchange which otherwise is prevented as a result of apoptotic stimuli. Furthermore, both Bcl-2 and Bcl-X_L can integrate in the outer mitochondrial membrane and thereby prevent lethal voltage dependent closure of VDAC (Debatin and Krammer, 2004).

Another proposed mechanism is the direct binding and inhibition of pro-apoptotic Bcl-2 family members. Interaction of Bcl-2 and Bcl-X_L with Bak and Bax inhibits the oligomerization of the pro-apoptotic proteins and/or their insertion into the mitochondrial membrane. A model in which Bcl-2 and Bcl-X_L inhibit activation of BOPs by sequestration has also been proposed (Cheng et al., 2001). Sequestration of the cell death machinery is also exemplified by the binding of Bcl-X_L to Apaf-1 and subsequent prevention of the apoptosome formation and activation of caspase-9 (Hu et al., 1998).

These anti-apoptotic proteins have to be carefully regulated since their uncontrollable inhibition of apoptosis could aid in malignant transformation. Many anti-apoptotic Bcl-2 family members are under transcriptional regulation. Several post-translational modifications also regulate their anti-apoptotic activities, *e.g.* phosphorylation of Bcl-2 leads to a conformational change which affects its activity as a negative regulator of apoptosis (Ito et al., 1997). Phosphorylation of anti-apoptotic proteins may also target them for ubiquitin-dependent degradation (Breitschopf et al., 2000). Caspase-dependent cleavage of the amino-terminus of both Bcl-2 and Bcl-X_L may occur in response to for instance Fas-ligation, etoposide and growth factor withdrawal (Burlacu, 2003). Cleavage can also be mediated by calpain, a calcium activated protease (Gil-Parrado et al., 2002). This type of regulation has been shown to convert the anti-apoptotic proteins into pro-apoptotic ones. In all cases of regulation, the net balance between the anti-apoptotic and the pro-apoptotic proteins seems to determine the cells fate and the decision whether to live or die.

Stress induced apoptosis

Cancer treatment by chemotherapy or radiotherapy causes cellular stress and ultimately cell death. A key element in the stress-induced apoptosis is p53. DNA-damage activates the protein kinases ATM and ATR, which, through Chk1 and Chk2 phosphorylate p53 to prevent binding of the p53-inhibitor mouse double minute 2 (MDM2) (Helt et al., 2004). This in turn leads to increased p53 levels and induction of p53 target gene transcription. Genes transcribed by p53 are involved in cell cycle arrest, DNA repair and apoptosis. Induction of programmed cell death is initiated if repair of damaged DNA is not achieved (Bullock and Fersht, 2001).

Two other stress pathways activated in response to chemotherapy are the mitogen activated protein kinase (MAPK) pathway and the protein kinase C (PKC) signalling cascade. Three subfamilies of MAPKs have been identified; extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38-MAPKs. It has been shown that ERKs are mainly important for cell survival and proliferation, whereas JNKs and p38-MAPKs are stress activated protein kinases (SAPKs) involved in apoptosis.

JNK and p38 signalling

JNKs are directly activated by the phosphorylation of tyrosine and threonine residues in a reaction catalysed by the dual specificity kinases MKK4 and MKK6. Regulation of apoptosis by JNKs is mediated by various mechanisms such as transcription-dependent and -independent signalling and by phosphorylation-dependent post-translational modifications

JNK-induced apoptosis by transcription-dependent signalling is mediated by phosphorylation of transcription factors such as c-Jun, leading to enhanced expression of pro-apoptotic genes. Furthermore, phosphorylation and stabilization of p53 by JNKs has been demonstrated (Fan and Chambers, 2001). Apart from transcription factors, JNK also phosphorylates key anti-apoptotic proteins *e.g.* Bcl-2 and Bcl-X_L, leading to abrogation of their functions (Herr and Debatin, 2001) or their activation, as in the case of the BOPs, Bim and Bmf (Lei and Davis, 2003).

The importance of JNKs in stress induced apoptosis was shown in *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ double knockout MEFs which were nearly completely resistant to apoptosis induced by UV irradiation and DNA damaging agents. It was observed that the resistance was localized at the mitochondrial level, with failure to induce mitochondrial depolarization, cytochrome c release and subsequent caspase activation (Tournier et al., 2000). Paradoxically, activation of JNKs does not only lead to apoptosis, it has alos been shown to be associated with cellular survival in some systems (Natoli et al., 1997; Roulston et al., 1998)

In most cases p38-MAPKs are simultaneously activated with JNKs. This activation of p38 is a result of phosphorylations of threonine and tyrosine residues made by MKK3 and MKK6. Similar to the JNK pathway, the involvement of p38-MAPK in apoptosis is also diverse. p38-MAPK signalling has also been shown to promote cellular survival as well as apoptosis and

differentiation. The choice between survival and apoptosis seems to depend on cell type and conditions (Wada and Penninger, 2004).

PKCδ signalling

Like many other signalling effectors protein kinase C is not a single entity, but a multigene family consisting of 12 different serine-threonine kinases. Most of them require diacylglycerol (DAG) and/or calcium and for their activation. The PKC family of kinases is activated by diverse stimuli and participates in cellular processes such as growth, differentiation and apoptosis. In most systems, only PKC θ, -μ and -δ act as pro-apoptotic kinases (Cross et al., 2000).

Activation of PKCδ may occur as a result of tyrosine phosphorylations (Denning et al., 1996) or proteolytic cleavage, generating a 40 kD kinase active fragment. It has been shown that cleavage can be performed by caspase-3, and the role for this caspase in activation is demonstrated in several systems. The use of selective inhibitors of caspase-3 reduced the cleavage of PKCδ and apoptosis in response to etoposide. Moreover, PKCδ inhibitors and a PKCδ dominant negative mutant inhibited both the activation of caspase-3, as well as cleavage of PKCδ, suggesting the existence of a positive feedback loop between caspase-3 and PKCδ (Blass et al., 2002; Reyland et al., 1999).

Several studies have shown a correlation between tumour growth and loss of PKCδ. Furthermore, activation of this kinase is involved in apoptosis in response to Fas, cisplatinum, etoposide and during spontaneous neuronal apoptosis. The targets of PKCδ in apoptosis are predominantly nuclear, including lamin B and DNA-dependent kinase (DNA-PK) (Cross et al., 2000). It has been shown that nuclear translocation of PKCδ is an early event in T-cell apoptosis, that can be reversed by IFNβ mediated rescue of cells from apoptosis (Scheel-Toellner et al., 1999). On the contrary, it has recently been demonstrated in our lab that apoptosis induced by IFNα in a MM cell line is dependent on the presence of active PKCδ (Panaretakis et. al., unpublished data).

The role of apoptosis in cancer development and therapy

A cell exhibiting deregulated proliferation has to escape from apoptosis in order to expand and form a tumour. Evasion of apoptosis is thus fundamental to tumour initiation, progression and maintenance, hence, deregulation of apoptosis plays an important role in tumour development. Therefore, a great number of cancer cells have gained mutations rendering them less sensitive to apoptotic signals.

An imbalance among the Bcl-2 family of proteins, in favour of the anti-apoptotic members, is a frequent phenomenon in cancer cells. Indeed, over-expression of anti-apoptotic Bcl-2 or Bcl-X_L probably occurs in more than half of all cancers. The first described example of this feature was shown in follicular B-cell lymphoma where the chromosomal translocation t(14;18) couples the Bcl-2 gene to the Ig heavy chain locus, thereby enhancing its expression

(Bakhshi et al., 1985; Tsujimoto et al., 1984). It has been shown both in *in vitro* and *in vivo* models that Bcl-2 expression confers resistance to many kinds of chemotherapeutic drugs and irradiation (Igney and Krammer, 2002). Moreover, loss of expression of Bax is also found in some colorectal cancers and in hematopoietic malignancies (Meijerink et al., 1998; Rampino et al., 1997).

Bax together with a large number of other pro-apoptotic proteins, such as Fas, Noxa, Puma and Apaf-1, is under the transcriptional regulation of p53, which is lost in more than half of all human tumours. The p53 tumour suppressor gene is under a strict regulation by several mechanisms. One of the key components of p53 regulation is the E3-ubiquitin ligase protein (MDM2), which targets p53 for proteosomal degradation. A defective pathway leading to MDM2 overexpression is found in many tumours (Oliner et al., 1992; Cordon-Cardo et al., 1994).

In the treatment of cancer, different approaches are taken when focusing on the apoptosis-evading mechanisms. Both irradiation and the chemotherapeutic drugs used today target the apoptotic machinery. However, drug resistance and, as a consequence, failure of therapy is frequently seen. A different concept for future strategies is however evolving, aiming to specifically restore the apoptotic program in cancer cells. For example, recent studies provide evidence that small molecules can restore the activity of mutated p53 and activate apoptosis in tumour cells (Bykov et al., 2002). There are however still a number of general problems that have to be solved before successful therapy that restore normal sensitivity to apoptosis in tumour cells can be fully developed. A macroscopic tumour is heterogeneous and it is likely that different cells within the tumour have acquired different mechanisms of apoptosis resistance. Moreover, multiple resistance mechanisms may evolve in single tumour cells. Furthermore, there are pharmacological and specificity related problems that have to be dealt with before these treatments will be in clinical use.

SURVIVAL SIGNALLING

There is a tight regulation of the balance between life and death in the cell. Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis. Apart from their capacity to escape apoptosis, a common phenotype of malignant cells is the overactivation of survival signalling. Hence, certain classes of signalling proteins and pathways are targeted much more frequently for oncogenic mutations than others. Some of these involve aberrations clearly targeting pathways regulating survival, such as the family of receptor tyrosine kinases (RTKs) and downstream signalling modules *e.g.* phosphatidyl inositol 3-kinase (PI3K), Ras and Jak/STAT pathways.

Receptor tyrosine kinases

In addition to their function in fundamental cellular processes such as proliferation and differentiation, the RTKs also regulate cell survival. Most of the RTKs are monomers in the cell membrane. Binding of the ligand to the extracellular domain induces receptor dimerization. This results in an increase in the kinase activity of the receptor and tyrosine autophosphorylation of the receptor subunits. The phosphorylated tyrosine residues mediate specific binding of cytoplasmic signalling proteins containing SH2 domains and phosphotyrosine binding (PTB) domains.

The proteins which are recruited in response to tyrosine phosphorylation fall into two groups; those with enzymatic activity, such as the cytoplasmic tyrosine kinases (*e.g.* Jaks, c-src and c-abl) and phospholipase c (PLC), or the group of adaptor proteins (*e.g.* the p85 subunit of PI3K and Grb2). Although several protein substrates are activated by RTKs, it appears that signalling proteins become activated by RTKs by three different general mechanisms; by conformational change (*e.g.* PI3K), by membrane translocation (*e.g.* Akt/PKB) and by tyrosine phosphorylation (*e.g.* PLCγ) (Schlessinger, 2000).

Signalling pathways activated by RTKs

In addition to activation of PLCγ and PI3K there are several other cascades induced by RTKs, such as the Ras pathway (which mainly leads to activation of Raf/MEK/ERK, PLC and PI3K (Downward, 2003)) and activated STAT signalling.

STAT signalling

STAT mediated signalling pathways were originally delineated in the context of cytokine receptors, specifically IFN and IL-6 receptors. However, STATs are also activated by GF receptors that possess intrinsic kinase activity *e.g.* EGFR, PDGFR and the Insulin receptor. STATs, mainly STAT3 and STAT5, often become persistently activated when one or more upstream tyrosine kinases become overactive due to a variety of genetic or epigenetic

alterations. In cancer cells, activation of STAT signalling by GF-receptors proceeds through a mechanism similar to that previously described for IFN-receptors. However, in addition to Jak kinases, a number of other tyrosine kinases such as the EGF receptor tyrosine kinase, the c-src kinase, the c-abl kinase, the focal adhesion kinase (FAK) and the FGF receptor kinase also have been shown to activate STAT proteins. The phosphorylated STATs dimerize and translocate to the nucleus for induction of transcription of target genes mainly mediating cell proliferation and survival, such as c-myc, cyclin D1 and cyclin D2 as well as anti-apoptotic Bcl-2 family members (Yu and Jove, 2004). There are however several lines of evidence showing that STAT activation also can promote both apoptosis and cell cycle arrest, for example as a result of IFN and EGF signalling (Sangfelt et al., 1997a; Sangfelt et al., 1997b; Chin et al., 1996). One reason for this divergence is the described antagonism between different STAT complexes on the promoters of anti-apoptotic genes, which has been reported *i.e.* for STAT1 and STAT3 (Stephanou et al., 2000).

PI3K and mTOR signalling

PI3K

Phosphatidylinositol-3 kinases constitute a lipid kinase family characterized by their ability to phosphorylate the 3´-OH position of the inositol ring of phospholipids. There are multiple isoforms of PI3Ks in mammalian cells and these can be divided into three subclasses; Class I PI3Ks are heterodimers composed of a catalytic subunit, p110, and a regulatory/adaptor subunit, p85. Class II PI3Ks, with an unknown biological function *in vivo*, and class III PI3Ks which are thought to regulate vesicle transport (Cantrell, 2001). Class I PI3Ks can be further divided into subclass IA, which is activated by RTKs, and subclass IB, which is activated by G-protein coupled receptors (Blume-Jensen and Hunter, 2001).

Four isoforms of the p110 subunit have been described (α, β, γ and δ), and three mammalian genes, p85α, p85β and p55γ, encode seven different adaptor proteins generated by alternative splicing. Binding of a growth factor to its receptor and subsequent tyrosine phosphorylation of the receptor subunits attract the regulatory p85 subunit which binds either the receptor or adaptor proteins *e.g.* IRS-1 and -2, through its SH2 domain. This process recruits the p110 catalytic subunit to the plasma membrane where the generation of lipid second messengers takes place (Figure 4). The preferred substrate for class I PI3Ks is PtdIns(4,5)P_2 and the primary product of their action is PtdIns(3,4,5)P_3. This lipid is however further metabolised by lipid phosphatases to produce PtdIns(3,4)P_2. PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are produced within seconds to minutes after PI3K activation and thereafter function as signalling intermediates. The PtdIns bind to PH domains of target proteins and can allosterically modify their activity or induce relocalisation of the protein to the plasma membrane where activation occurs by additional kinases (Cantrell, 2001; Fruman et al., 1998).

Akt

One important protein being activated as a result of PI3K signalling is Akt/PKB, which upon membrane translocation becomes activated as a result of phosphorylations. For its full activation, Akt needs to be phosphorylated on two sites. PDK-1 is responsible for phosphorylation of Akt on Thr308, however, phosphorylation of Akt on Ser473 is mediated

by an as yet unidentified kinase. One candidate for Ser 473 phosphorylation is the serine/threonine kinase integrin linked kinase (ILK) (Persad et al., 2001). There are also studies proposing that phosphorylation of Ser473 is a result of Akt trans-phosphorylation (Toker and Newton, 2000). In relation to survival signalling, activated Akt mediates downstream signals mainly aiming at inhibiting apoptosis and inducing proliferation (Figure 4). For example, Akt mediated phosphorylation inactivates the pro-apoptotic proteins Bad and caspase-9 as well as the forkhead family of transcription factors (FKHR). The latter induces the expression of pro-apoptotic factors such as the Fas ligand. Furthermore, Akt phosphorylates and activates the transcription factor CREB (cyclic AMP response element-binding protein) and the IκB kinase (IKK), a positive regulator of NF-κB, and both of these transcription factors positively regulate expression of genes with anti-apoptotic activity (Fresno Vara et al., 2004).

mTOR

Another important kinase involved in survival signalling is the mammalian target of rapamycin (mTOR), which belongs to the well conserved TOR family of protein kinases. Rapamycin is an immunosuppressant drug which interacts with its intracellular receptor, the FK-506 binding protein (FKBP12). This complex binds the c-terminus of TOR-proteins from several species, thereby inhibiting TOR activity (Chen et al., 1995). The c-terminal half of mTOR contains the kinase domain, which has sequence similarity with the catalytic domain of PI3K, placing it in the family of PI3K related kinases (PIKK). However, as many of the other PIKK family members *e.g.* ATM and ATR, mTOR has not been shown to possess lipid kinase activity.

Two of the well known downstream targets of mTOR are the p70S6K and 4E-BP1 proteins. p70S6K is activated as a result of multiple phosphorylations, probably involving other kinases as well. Activation of p70S6K and the resulting phosphorylation of the 40S ribosomal protein subunit S6 drives translation of 5´TOP mRNAs. These mRNAs constitute a small family of transcripts that encode primarily ribosomal proteins and components of the translational apparatus. Thus, by controlling 5´TOP mRNA translation, mTOR upregulates the translational machinery. 4E-BP1 is a translational repressor, which when phosphorylated by mTOR releases the elongation factor eIF-4E. This subsequently leads to enhanced translation of certain mRNAs such as the one encoding for Cyclin D1, thus facilitating proliferation (Schmelzle and Hall, 2000). There are several studies showing that mTOR can act as an inhibitor of apoptosis. For example, rapamycin can sensitize cancer cells to apoptosis induced by cisplatin, an effect that is probably mediated by the inhibition of mTOR dependent phosphorylations of Bad by p70S6K (Wan and Helman, 2002). On the contrary, mTOR has also been shown to be involved in induction of apoptosis, *e.g.* by phosphorylating p53 on Ser15. This phosphorylation results in p53 dependent transcriptional activation and induction of pro-apoptotic proteins such as Bax, leading to activation of the mitochondrial cell death pathway. Rapamycin can furthermore inhibit apoptosis induced by UVA or UVB irradiation, as well as taxol-induced apoptosis of human B-cell lines (Castedo et al., 2001; Castedo et al., 2002). It thus seems like mTOR has a pleiotropic function in the regulation of apoptosis. This function appears to be dictated by the cellular context *e.g.* cell type and activation state, as well as by multiple downstream targets, including p53, Bad and Bcl-2.

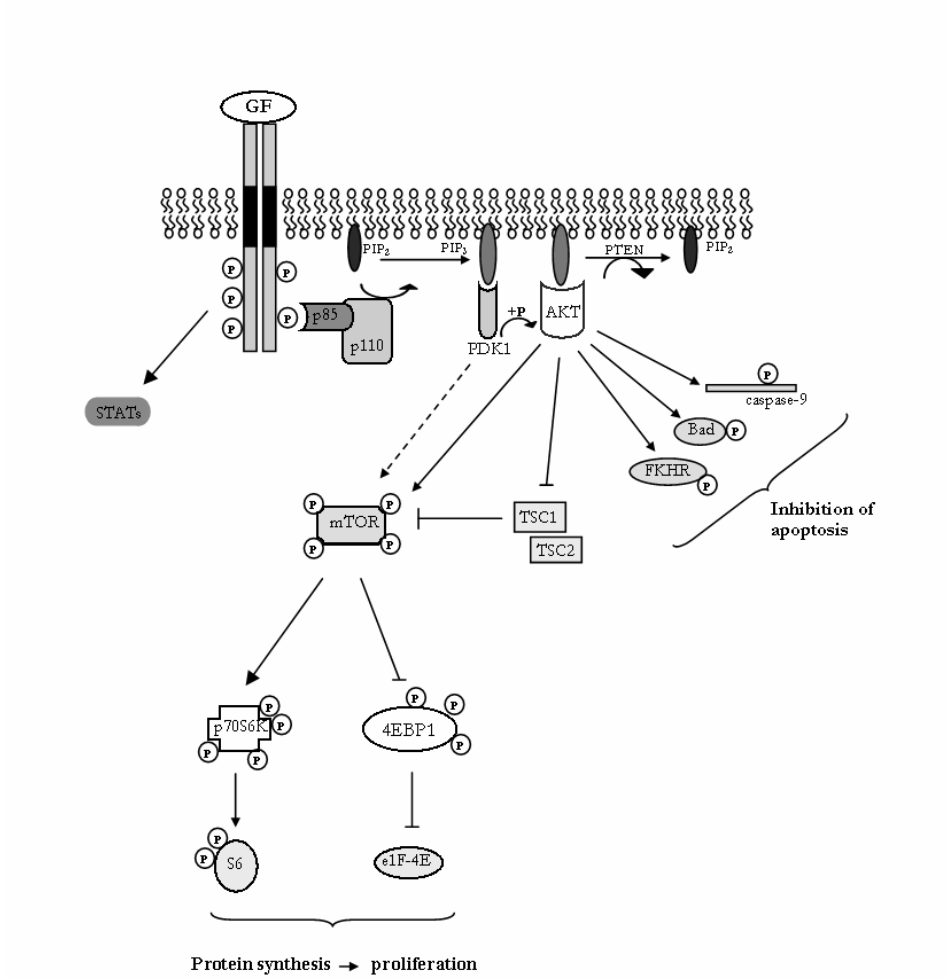

Figure 4 Survival signalling

Upon e.g. GF stimulation, PI3K is activated. This leads to generation of lipid second messengers and downstream activation of target proteins which mainly act to increase survival. Although not shown in the picture, other proteins (e.g. IRS) can act as adaptors for PI3K activation.

mTOR activity is regulated by GFs. Signalling by insulin and other GFs, result in a rapamycin sensitive phosphorylation of p70S6K and 4E-BP1. Furthermore, there is strong evidence that GF induced mTOR activity is mediated by PI3K, since experiments using the pharmacological PI3K-inhibitors LY294002 and wortmannin inhibited phosphorylation of the mTOR substrates p70S6K and 4E-BP1 (Chung et al., 1994). It has been shown that Akt stimulates growth by phosphorylating the tuberous sclerosis complex 2 (TSC2) thus inhibiting the formation of a TSC1:TSC2 complex. When functional, this complex inhibits mTOR activity and phosphorylation of p70S6K and the 4E-BP1 repressor (Inoki et al., 2002). The inhibition is a result of TSC2 acting as a GTPase-activating protein (GAP) for Rheb. Rheb, in its GTP-bound state, positively regulates mTOR by an as yet, not defined mechanism (Castro et al., 2003). It has earlier been shown that p70S6K is also phosphorylated by PDK-1, but the interaction between the TSC1:TSC2 complex and Rheb with mTOR provides the first mechanistic link between PI3K and mTOR signalling.

Raptor appears to serve as an adaptor protein that recruits mTOR substrates. It binds p70S6K and 4E-BP1 and is necessary for the in vitro phosphorylation of 4E-BP1 by mTOR as well as efficient phosphorylation of p70S6K (Hay and Sonenberg, 2004). The interaction between raptor and mTOR is also abrogated by rapamycin. mTOR activity is in part regulated by amino acid availability and it has been suggested that the nature of the mTOR-Raptor complex changes upon amino acid deprivation, inhibiting binding of mTOR to its downstream substrates.

The role of survival signalling in cancer

PI3K and mTOR

The oncogenic role of deregulated class IA PI3Ks and Akt is probably accounted for by their ability to induce multiple effects on both cell survival and cell cycle. The gene encoding the p110 catalytic subunit of PI3K has been found to be amplified in ovarian and cervix cancers. No mutations of the Akt-gene have been detected in mammals. However, the Akt-gene has been found to be amplified in a number of human malignancies *e.g.* ovarian, stomach, breast and stomach tumours (Fresno Vara et al., 2004).

Growth factor receptors are commonly overexpressed or permanently active in human cancers. One such example is the RTK ErbB2/Her2/Neu, which is amplified in a significant percentage of breast cancers as well as other human tumours (Harari and Yarden, 2000). This overexpression leads to constitutive PI3K/Akt signalling mediating cellular proliferation and survival. However, constitutive PI3K/Akt signalling may also occur independently of receptors, as in the case of cells expressing constitutively active Ras, which binds and activates the p110 catalytic subunit of PI3K.

The lipid phosphatase PTEN (Phosphatase and tensin homologue deleted on chromosome 10) is a tumour suppressor gene negatively regulating PI3K signalling. The function of PTEN is to dephosphorylate PtdIns(3,4,5)P_3, the second messenger generated by PI3K (Figure 4).

Loss of PTEN activity, which is detected in a large number of advanced stages of human tumours, leads to permanent PI3K/Akt activation (Di Cristofano and Pandolfi, 2000).

There are several lines of evidence implicating mTOR in cancer. Some of the proteins linked to mTOR signalling have been shown to have transforming potential *e.g.* PI3K and eIF-4E (Thomas and Hall, 1997). One mechanism by which mTOR can contribute to cancer development is through its effects on cell cycle in conjunction with its possible anti-apoptotic activity. There is strong evidence that mTOR is required for cell cycle progression, and inhibition of mTOR activity by rapamycin arrests cells in the G1 phase of the cell cycle. This effect of mTOR on the cell cycle is mediated, at least in part, by the increased translation of mRNAs encoding positive regulators of cell cycle progression, such as cyclin D1 and c-myc, and by decreased translation of negative regulators thereof, such as the cyclin-dependent kinase inhibitor p27 (Hay and Sonenberg, 2004).

Furthermore, a connection between STAT3, which is constitutively activated in many tumours including myelomas and squamos cell carcinomas, and mTOR has been found. In mammalian cells, mTOR controls the transcriptional activator STAT3, since serine phosphorylation of STAT3 and STAT3 dependent transcription is rapamycin sensitive. The activation of STAT3 by mTOR may be direct, as mTOR efficiently phosphorylates Ser727 in a STAT3 peptide and rapamycin treatment of cells transfected with a STAT-responsive promoter reporter decreased activation of the reporter to the same degree as a STAT3 Ser727Ala mutant (Yokogami et al., 2000).

STATs

Stat proteins, particularly STAT3 and STAT5 are frequently overactivated in a variety of primary human solid tumours and hematological malignancies. Genetic evidence for a role of STAT3 in transformation was provided by the finding that a constitutively activated mutant form of this protein, STAT3C, transforms fibroblasts in culture, allowing them to form tumours in mice (Bromberg et al., 1999). Constitutive activation of STAT3 has been detected at high frequency in diverse human cancer cell lines and primary tumours. The first links between STATs and human cancer came from the findings that constitutive STAT3 activity is required for the growth of head and neck cancer cells and of MM cells (Catlett-Falcone et al., 1999; Grandis et al., 1998). In the case of MM cells it was shown in the U266 cell line that inhibition of constitutive active STAT3 signalling induced apoptosis. In the same cell line, we have shown (Paper III) that induction of apoptosis by IFNα involves downregulation of DNA-binding STAT3 homodimers, and that expression of STAT3C protects from apoptosis induced by this cytokine. Protection from apoptosis correlated to high expression levels of the Mcl-1 protein. IL-6 autocrine or paracrine loops have been identified as a cause of constitutive STAT3 activity in certain myeloma (*e.g.* U266) and prostate cancer cell lines (Catlett-Falcone et al., 1999; Mora et al., 2002).

Furthermore, STATs can be overactivated as a result of deregulated Jak´s and non-receptor tyrosine kinases such as the Src and Abl kinases. Aberrant Jak activation has been found to involve a translocation in acute lymphocytic leaukemia (ALL). This translocation results in a

fusion protein, Tel-Jak2, which possess constitutive kinase activity (Ho et al., 1999). When introduced into hematopoietic cell lines, this fusion protein results in constitutive activation of STAT1, -3 and -5. The non-receptor tyrosine kinase Src mediates constitutive activation of STAT3 and results in transformation (Bromberg et al., 1998). Furthermore, v-src activates STAT3 in mammary epithelial cell lines. In addition, the oncogenic fusion protein BCR-Abl has been shown to activate STAT1 and STAT5 in hematopoietic cell lines (Carlesso et al., 1996).

There are several effects of constitutive STAT signalling in malignant cells. STAT3 contributes to malignancy, at least in part by preventing apoptosis by upregulating several target genes such as Bcl-X_L (Catlett-Falcone et al., 1999). Inhibition of STAT3 signalling in MM cells blocked the expression of Bcl-X_L and sensitized them to Fas-induced apoptosis. Both STAT3 and STAT5 have been shown to regulate Bcl-X_L and Mcl-1 also in several other tumour cell lines. Blocking of either STAT3 or STAT5 has been shown to downregulate Mcl-1 expression and to induce tumour cell apoptosis. Activated STAT3 also induces the expression of another anti-apoptotic protein, survivin – a member of the IAP family (Yu and Jove, 2004).

In addition to STAT3, constitutive activation of both STAT1 and STAT5 has been shown in certain human cancer cell lines and tumour tissues. STAT5 signalling promotes oncogenesis in certain diseases, such as CML and AML (Huang et al., 2002; Levis et al., 2002). Similarly to STAT3, recombinant forms of STAT5 that contain activating mutations induce some properties of transformed cells, including cytokine independent transformation, consistent with an oncogenic role for persistent STAT5 signalling.

It seems however unlikely that STAT1 promotes tumour cell growth. In many circumstances, activated STAT1 acts in a pro-apoptotic and anti-proliferative manner, and STAT1-null mice are more prone to tumour development than controls (Shankaran et al., 2001). Expression of caspases (Kumar et al., 1997), as well as of Fas and FasL (Xu et al., 1998) has also been shown to depend on the presence of a functional STAT1 protein. Moreover, it has been shown that the constitutively active form of STAT3 (STAT3C) antagonizes the pro-apoptotic effects of STAT1 induced by IFNγ in a cervical carcinoma cell line (Shen et al., 2001). It has furthermore been demonstrated that the relative proportions of STAT1 *versus* STAT3 may affect the transcriptional activity of anti-apoptotic genes. For example, STAT1 reduces the basal activity of the promoters of the anti-apoptotic genes Bcl-2 and Bcl-x both in response to IFNγ and as a result of STAT1 over-expression. STAT3 on the contrary, was shown to antagonize this effect by enhancement of Bcl-2 and Bcl- x gene expression (Stephanou et al., 2000). The biological basis for the opposing effects of STAT1 and STAT3 is unclear. Even though they bind the same optimal DNA sequence, the outcome of binding is for unknown reasons dissimilar. One possible explanation for this divergence might be the association of STAT-complexes with different sets of cofactors, resulting in opposing transcriptional outcomes. The antagonism between STAT1 and STAT3 has also been shown at the level of the receptor. As a result of IFNγ signalling, STAT3 is phosphorylated transiently in wt MEFs. However, the phosphorylation of STAT3 is much stronger and more prolonged in STAT1$^{-/-}$ cells. STAT1 and STAT3 were furthermore shown to compete for the same binding site at the

receptor. Although STAT1 is less abundant in wt cells, it has a higher affinity for binding to the receptor as compared to STAT3 and is preferentially activated in wt cells (Qing and Stark, 2004).

AIMS OF THE THESIS

The major aims of this thesis were to determine the ways used by IFNα to induce malignant cell death and the effects of genetic environment on this process. More specifically the aims were to:

- Investigate the signalling pathways used by IFNα for induction of apoptosis, with regard to both classical apoptotic pathways as well as the upstream events in this process induced upon binding of IFNα to its receptor (papers I-II).

- Investigate the effects of oncogenic signalling and the introduction or presence of activated oncogenes on apoptosis induced by IFNα (papers III-IV).

RESULTS AND DISCUSSION

Paper I Mechanisms of IFNα induced apoptosis in malignant cells

This study was initiated as a result of the first published evidence of induction of apoptosis by IFNα. It was shown that IFNα induces apoptosis in malignant cells, and that this is event is separate from the well studied effects on the cell cycle machinery induced by IFN (Sangfelt et al., 1997a). In the present study we show that IFNα-induced apoptosis involves the activation of a hierarchical caspase cascade, loss of mitochondrial membrane integrity and release of cytochrome c. By using both a general pan-caspase inhibitor as well as specific inhibitors of caspases-1, -2, -3, -8 and -9, we could show that activation of caspases is a prerequisite for apoptosis to occur and the ordering of events that took place could also be determined.

The investigations were made using three different cell lines; U266 MM cells, the SV40 transformed Rhek-1 cell line and the Burkitt lymphoma cell line Daudi. The two former cell lines respond to IFNα-treatment by induction of apoptosis. Moreover, U266 cells but not Rhek-1 cells are also cell cycle arrested in the G1-phase. Daudi cells are virtually insensitive to apoptosis induced by IFNα and respond to treatment solely by arresting in the G1/G0 phase of the cell cycle.

Apoptosis in the sensitive cell lines was accompanied by activation of a set of caspases. Caspases-1, -2, -3, -8 and -9 are activated 16-24 hours after exposure to IFN. Inhibition of all of these caspases using the pan-caspase inhibitor ZVAD-fmk reduces apoptosis by 80% after 24h of culture, clearly showing the importance of caspase activation in this process, as well as demonstrating that, at least part of the reason for the insensitivity to apoptosis in Daudi cells, is a result of the observed lack of IFN-induced caspase activation in this cell line. However, when we blocked each caspase individually, only inhibition of caspase-8 using IETD-fmk had a potent effect on the apoptotic response similar that of the pan-caspase inhibitor. Inhibition of caspase-8 also resulted in downregulated activity of caspases-2, -3 and -9. Taken together these results speak in favour of caspase-8 being the most upstream caspase in the cascade initiated by IFNα. This is reminiscent of apoptosis induced by FasL, where caspase-8 activation is an important event. Cells can respond to apoptotic stimuli either as type I cells, where activation by initiator caspases is strong and results in further activation of downstream executor caspases, or as type II cells in which the activation of initiator caspases is rather weak and the mitochondrial pathway is used to enhance the signal for downstream caspase activation (Scaffidi et al., 1998). The fact that the activation of caspase-8 is weak in comparison to caspases-2 and -3 and that mitochondrial events are seen, further strengthens the hypothesis that apoptosis induced by IFNα takes place in a type II fashion.

The IFN receptor does not contain any death domains, but it has been suggested that treatment with IFNα enhances the expression of the Fas receptor and thereby sensitizes cells to Fas-induced apoptosis (Spets et al., 1998). Furthermore, it has been shown that the Apo2L/TRAIL receptor is induced by IFNα and that it may be important for IFN-induced

apoptosis by facilitating cleavage of Bid and Bcl-2, thus causing mitochondrial cytochrome c release (Chen et al., 2001; Toomey et al., 2001). In our study, treatment with an antagonistic ab against the FasR had no effect on IFN- induced apoptosis, but the involvement of TRAIL needs to be further investigated. If TRAIL-signalling is a crucial event, this might be mediated by PML which in a recent study has been shown to be necessary for TRAIL-induction and downstream caspase activation in IFNα-treated MM cells (Crowder et al., 2004). In contrast to the effects on TRAIL, downregulation of PML using siRNA has no effect on FasR expression induced by IFNα. Another possible link between IFN and TRAIL is IRF-1, which has been shown to be strongly upregulated by IFNγ and to sensitize cells for TRAIL-induced apoptosis without increasing the receptor expression. However, the mechanism for this sensitization is not known (Park et al., 2004). IFNγ also upregulates the expression of caspase-8 in an IRF-1 dependent manner (Ruiz-Ruiz et al., 2004). If this would be the case for IFNα, which also induces IRF-1, it could explain the slight upregulation of caspase-8 protein that we observed in our study.

In U266 and Rhek-1 cells we observed release of cytochrome c and a reduction of $\Delta\psi_{mit}$ as a result of IFNα-treatment. Both of these features were absent in the Daudi cell line. An interesting notion is that in the population of cells which do not die by apoptosis, there is a hyper polarization of the mitochondrial inner membrane which is observed in the apoptosis resistant Daudi cells as well. The reason for this hyper polarization is not clear. However, a voltage dependent closure of VDAC has been shown in cells undergoing apoptosis in response to growth factor deprivation. This leads to hyper polarization of the inner membrane and subsequent damage to the outer membrane because of defective exchange of ATP and other metabolites between the mitochondria and cytosol (Vander Heiden et al., 1999). Closure of VDAC can be prevented by the anti-apoptotic Bcl-2 family members who form pores in the outer membrane and thereby facilitates charge compensation. These anti-apoptotic proteins have furthermore been proposed to prevent Bax-mediated VDAC opening (as discussed in the Introduction). If we assume that this model for opening of the PTP and subsequent release of cytochrome c release is valid for IFNα, VDAC closure is then a possible reason for the increase in $\Delta\psi_{mit}$. The competing action of Bax would thereafter theoretically open up the VDAC pore, leading to the subsequent decrease in $\Delta\psi_{mit}$ and cytochrome c release. Daudi cells, however, have been shown to harbour a mutant Bax gene, which might be a reason for this cell lines insensitivity to IFNα induced apoptosis. The increased $\Delta\psi_{mit}$ observed could however still be explained by closure of VDAC. Indeed, activation of Bax in IFNα-treated U266 cells, as well as absence of Bax activation in the Daudi cell line has in a more recent study been shown in our lab. (Panaretakis et al., 2003)

By using specific inhibitors of the caspases activated in IFNα-induced apoptosis we conclude that caspase-3 is the most downstream caspase activated since inhibition of this caspase had no profound effect on any of the other caspases investigated. This result fits well with caspase-3 being an executor caspase. Caspase-9 activation was reduced as a result of both caspase-8 and -2 inhibition, placing it downstream of these caspases in the cascade. However, since the caspase-2 inhibitor is also a partial, but much less effective, inhibitor of caspase-9, we can not exclude the possibility that caspase-9 activation occurs irrespective of caspase-2. Increasing evidence, although, points in the direction of caspase-2 acting upstream of the

mitochondria (Lassus et al., 2002; Robertson et al., 2002). Furthermore, activation of caspase-2 is in large abrogated by the inhibition of caspase-8, and is thus clearly downstream this initiator caspase in the cascade.

It has long been debated whether or not caspase-1 is involved in apoptosis. A number of studies show that this is not the case and that caspase-1 is rather involved in inflammation as a mediator of IL-1β and IL-18 processing (Garcia-Calvo et al., 1999). However, increasing evidence points in the direction of caspase-1 being a mediator of apoptosis. It has been shown that Ipaf, an activator of caspase-1, is induced in doxorubicin-mediated apoptosis. Induction of Ipaf takes place mainly in a p53-dependent manner. However, a slight p53-independent induction of this activator is observed in p53-mutant cells (Sadasivam et al., 2004). Furthermore, induction of apoptosis by tamoxifen has recently been shown to depend on IRF-1 induced caspase-1 and -3 activation. In this study, IFNα was shown to activate caspase-1. However, inhibition of this caspase had no effect on the apoptotic response. Most likely, caspase-1 is not involved in IFNα-induced apoptosis.

In conclusion, IFNα-induced apoptosis is mediated by the sequential activation of initiator caspases and executor caspases as well as involvement of the mitochondrial pathway and release of cytochrome c. Apoptosis induced by IFNα in part resembles that of TGFβ, which is also caspase dependent, as shown by inhibition using a pan-caspase inhibitor. Furthermore, TGFβ-induced apoptosis is caspase-8 dependent but independent on Fas-ligation and other DR ligands. However, inhibition of caspase-3 in a BL41 cell line does not affect TGFβ-induced apoptosis (Inman and Allday, 2000). In Hep3B cells on the contrary, caspase-3 is crucial for induction of apoptosis by TGFβ (Schuster and Krieglstein, 2002). Moreover, involvement of mitochondria in TGFβ-induced apoptosis has been shown by for example activation of Bax and release of cytochrome c, as also demonstrated in the case of IFNα-induced apoptosis.

Paper II IFNα induced apoptosis is mediated through the PI3K/mTOR signalling pathway

This study was initiated aiming to define the more upstream signalling events leading to induction of apoptosis by IFNα. Considering the multitude of pathways following IFN binding to its receptor, several signalling modules may be involved, such as the Jak/STAT, p38/MAPK as well as the PI3K/mTOR. In the present investigation we have studied the involvement of PI3K/mTOR signalling and its relation to STAT activation in the context of IFNα-induced apoptosis. It has been shown that the PI3K lipid serine kinase is activated by type I IFNs. This activation is mediated by IRS-1 and/or IRS-2 as adaptor proteins (Platanias et al., 1996; Uddin et al., 1995), but has also been shown to involve STAT3 (Pfeffer et al., 1997). However, the involvement of STAT3 as an adaptor is not an as well documented fact as the dependence on IRS-1 and IRS-2 proteins, which seem to mediate the effect in hematopoietic cell lines. IFNγ also mediates activation of PI3K, however, without prior activation of IRS-proteins (Platanias et al., 1996).

We found in this study that IFNα induces the lipid kinase activity of PI3K as measured by the formation of the lipid second messenger PtdIns(3,4,5)P_3. Furthermore, the mTOR kinase is activated, resulting in a rapid and transient phosphorylation of the downstream p70s6K and 4E-BP1 proteins, starting after 10 min of IFN exposure and returning to basal levels within 4 hours. Chemical inhibition of PI3K as well as of mTOR activity nearly completely abrogated the induction of apoptosis induced by IFNα. The inhibitory effects observed were much stronger than those using the pan caspase inhibitor in paper I. All features that are associated with apoptosis induced by IFNα are absent in cells treated with inhibitors of PI3K and mTOR, including Bak activation, loss of mitochondrial membrane potential, release of cytochrome c, activation of caspases and Annexin/PI positivity. These experiments were performed in two different cell lines, U266 MM cells, which have mutant p53, and Rhek-1 keratinocytes, which express the SV40 large T antigen that inactivates endogenous p53. To verify the results obtained with the chemical inhibitors, Rhek-1 cells were transfected with a dominant negative kinase dead (KD) mutant form of mTOR. Thereafter, the effects on IFNα-induced apoptosis were investigated. The results show that cells expressing this construct are completely rescued from apoptosis, confirming the involvement of this pathway in IFN-induced apoptosis. As a control experiment, cells were also transfected with wt mTOR. This construct, however, had no negative effect on IFNα-induced apoptosis, which could be efficiently blocked by rapamycin. All of these results demonstrating a profound effect on IFNα-induced apoptosis by inhibition of PI3K/mTOR led us to think that signalling by these kinases is an upstream event in IFN- induced apoptosis.

Traditionally, IFN-mediated changes in transcription through activation of various STAT complexes have been thought to mediate most cellular effects of IFN. The role of STAT signalling in IFN-induced apoptosis has however not been clarified. In order to determine whether or not STAT activation and signalling induced by IFNα was affected by abrogation of the PI3K/mTOR activity we investigated the effects of PI3K/mTOR inhibition on STAT

phosphorylations, STAT nuclear translocation, binding of active STAT complexes to their specific DNA target sequences as well as induction of specific IFNα target genes. We found that inhibition of PI3K or mTOR and IFNα-induced apoptosis occur in the presence of STAT phosphorylations as well as Jak/STAT mediated gene expression. Furthermore, the ability of IFNα to protect against viral propagation, generally mediated through the Jak/STAT pathway was also not impaired in the presence of either the PI3K- or the mTOR inhibitor. This suggests that the canonical Jak/STAT signalling pathway is not sufficient for the induction of apoptosis and that the PI3K/mTOR pathway induced by IFNα acts in parallel to promote apoptosis. The contribution of the Jak/STAT signalling pathway, if any, in the apoptosis induction by IFNα remains to be further investigated.

One role for IFNγ-induced PI3K activation involves phosphorylation of STAT1 on ser727. This phosphorylation functions to maximize the transcriptional potential of STAT1. The downstream effector of PI3K mediating this action seems to depend on cell type. In osteosarcoma as well as leukemic cells PI3K activates PKCδ which is responsible for the serine phosphorylation of STAT1 (Deb et al., 2003). In mesangial cells however the action is mediated by PKCε and also involves further downstream action of Erk1/2 to phosphorylate STAT1 (Choudhury, 2004). No such effect for PI3K induced by IFNα has been reported. The phosphorylation of STAT1 on ser727 was suggested to be mediated by PKCδ induced by IFNα in a system similar to ours (Uddin et al., 2002). We did not, however, observe any changes in STAT1 or STAT3 serine phosphorylations from inhibition of PI3K or mTOR. Furthermore, inhibition of PKCδ did not have any effect on STAT1 ser727 phosphorylation, but it did however efficiently block IFN-induced apoptosis, and its cleavage to the active form is a downstream effect of PI3K and JNK (Panaretakis *et.al.*, unpublished data).

Besides from being a mediator of apoptosis, IFNα has also been shown to promote cell survival, *e.g.* in primary B-lymphocytes, via activation of the PI3K/Akt signalling pathway (Ruuth et al., 2001). It is therefore an intriguing fact that a survival pathway, such as the PI3K pathway, is induced by IFNα to act as a mediator of cell death. We investigated the involvement of the Akt-protein, a downstream target of PI3K signalling, which in a number of studies has been shown to mediate anti-apoptotic effects by phosphorylation of target proteins. In U266 cells, we have not been able to detect any phosphorylations of Akt, which is needed for its full activation. This is in line with previously reported data which shows that treatment of U266 cells with insulin, but not IFNα, results in Akt-phosphorylation (Uddin et al., 2000). These finding strengthen the hypothesis that alternative actions besides induction of survival signalling is mediated by PI3K signalling.

One possible role for PI3K/mTOR signalling induced by IFNα might involve regulation of survival signals mediated by for example insulin and other GFs. This would result in a shift of the delicate balance within the cell to promote cell death. It has been shown that PI3K also participates in a negative feedback loop resulting in ubiquitin proteasomal mediated degradation of IRS-1 in insulin stimulated cells, but this process does not involve the action of mTOR (Zhande et al., 2002). It has also been described in insulin target tissues that chronically activated mTOR increased the Ser/Thr phosphorylation state of IRS-1. This increased Ser/Thr phosphorylation of IRS-1 via mTOR subsequently leads to increased

degradation of IRS-1 and a consequential downregulation of insulin mediated growth signalling (Haruta et al., 2000; Pederson et al., 2001). Similarly, mTOR has been shown to be involved in degradation of IRS-2, which in pancreatic β-cells has a pro-survival function. Hence, mTOR action mediates apoptosis in these cells (Briaud et al., 2004). Furthermore, IFNα-induced Jak-1 mediated tyrosine phosphorylation of IRS-1, which is required for binding of the p85 subunit of PI3K via its SH2 domain, has been shown to be negatively regulated by the serine phosphorylation of IRS-1. This serine phosphorylation of IRS-1 can be induced by several agents such as okadaic acid and chronic insulin stimulation, and one mediator has been shown to be the mTOR protein (Cengel and Freund, 1999; Hartman et al., 2001). Whether IFNα causes the activation of such a negative feedback loop remains to be investigated.

There is an apparent time lag between the activation of PI3K/mTOR, resulting in rapid phosphorylation of the downstream targets p70s6K and 4E-BP1, and the molecular hallmarks of apoptosis induced by IFNα. It has been shown by our group that IFNα-treatment results in a gradual upregulation of the anti-apoptotic proteins Mcl-1 and Bcl-X_L starting from 4 hours after IFNα induction and decreasing back to control levels after 16 hours, when the first signs of apoptosis are detected (Panaretakis et al., 2003). The Mcl-1 and Bcl-X_L proteins contain STAT-responsive elements in their promoters and upregulation of these proteins by Jak/STAT signalling might explain the fact that induction of apoptosis is fairly slow. It is possible that the other branch of signalling induced by IFNα, activation of PI3K/mTOR, may for example result in altered activity of a protease that is capable of mediating the apoptotic effects or may alternatively alter the phosphorylation of some key apoptosis regulator. Although it is possible that the induction of Jak/STAT signalling might lead to the expression of pro-apoptotic proteins, such as caspases, as has been shown in the case of IFNγ (Chin et al., 1997; Ruiz-Ruiz et al., 2004; Sironi and Ouchi, 2004). This is not likely to be the sole cause of IFNα-induced apoptosis which can be completely blocked but still have an intact Jak/STAT signalling. Future studies will thus focus on the importance of STAT activation as well as key substrates for mTOR in this process.

Paper III IFNα induced apoptosis in U266 myeloma cells involves downregulation of STAT3 homodimers

Multiple myeloma cell lines commonly depend on paracrine or autocrine production of IL-6 for their survival. This survival signalling is largely mediated by phosphorylation of STAT3 and binding of STAT3 homodimers to DNA leading to induction of anti-apoptotic genes. STAT3 is regarded as an oncogene and a large number of tumour derived cell lines, as well as primary samples from human cancers, are reported to contain constitutively activated STATs, very frequently STAT3 (Garcia and Jove, 1998). It has been shown that a STAT3C molecule, which is a constitutively active dimer generated by a substitution that introduces two cysteins, mediates cellular transformation in immortalized fibroblasts (Bromberg et al., 1999).

It has also been shown in the U266 MM cell line that autocrine IL-6 signalling results in STAT3 activation and induction of the anti-apoptotic proteins Bcl-X_L and Bcl-2 (Catlett-Falcone et al., 1999). We have previously observed that U266 cells possess a constitutive binding of active STAT3/3-homodimers to DNA. After treatment with IFNα however, there is a shift in complex formation and STAT1/1 homodimers as well as STAT1/3 heterodimers are formed, while the intensity of STAT3/3 homodimer complexes is reduced (Thyrell et al., 2004). IL-6 signalling involves Jak/STAT as well as -MAPK signalling. Since these pathways are also induced by IFNα, and crosstalk between IFN and IL-6 signalling has been demonstrated (Berger and Hawley, 1997; Mitani et al., 2001), it is conceivable that IFNα exerts its pro-apoptotic functions by shifting the delicate balance within the IL-6 regulated signalling network. It has been shown that IL-6 plays a crucial role in the survival of IL-6 dependent cells, and that blocking of STAT3 by over-expression of a dominant negative STAT3β leads to apoptosis (Catlett-Falcone et al., 1999). Hence, our observation raised the question of whether the IFNα induced downregulation of DNA-binding STAT3/3 homodimers was correlated to induction of apoptosis, especially in light of the proposed antagonism between STAT1 and STAT3 in which STAT1 acts to promote apoptosis (Stephanou et al., 2000; Shen et al., 2001).

To test this hypothesis, we transfected U266 cells with a plasmid encoding the above described constitutively activate form of STAT3, STAT3C (Bromberg et al., 1999). Both stable clones and transient transfectants were generated. The transient transfected cells expressing STAT3C were completely protected from apoptosis induced by IFNα, whereas the clones were partially protected, the degree of protection correlating with the expression levels of STAT3C in the clones. We also investigated the effect on STAT3 homodimer DNA binding in STAT3C clones and in control transfected cells. The results show that IFNα induces the reduction in STAT3-homodimers in the control transfected clones while the STAT3C transfected cells showed high levels of active STAT3 homodimers throughout the time investigated. These results speak in favour of our initial hypothesis being correct, that IFNα abrogates the IL-6 induced STAT3 activation and that this leads to induction of apoptosis.

When comparing stably transfected cells expressing the STAT3C construct with cells from the same clone expressing only endogenous STAT3 we were able to monitor differences in the response to IFNα. Although STAT3C has been considered to be constitutively DNA-binding, we found that both endogenous STAT3 and STAT3C was tyrosine phosphorylated and translocated to the nucleus in response to IFNα-treatment. After four hours, endogeneous STAT3 was dephosphorylated and disappeared from the nucleus, whereas STAT3C maintained both tyrosine phosphorylation and its nuclear localization. The sustained phosphorylation of STAT3C after IFNα-treatment was verified with western blots and furthermore correlated with an increased expression of the STAT3 induced target gene Bcl-2, and hyper-upregulation of Mcl-1 as observed in the STAT3C transfected clones. This result is in concordance with previous studies showing that blocking of STAT3 leads to decreased Bcl-X_L and Mcl-1 expression in certain cell types (Yu and Jove, 2004) and that IFNα downregulates Bcl-X_L expression in U266 cells (Chen et al., 2001; Panaretakis et al., 2003). There are, however, other anti-apoptotic proteins regulated by STAT3, such as the IAP family member survivin (Shen et al., 2001), that by downregulation could account for the induction of apoptosis induced by IFNα. This, however, remains to be investigated.

In addition we were able to investigate the mechanism for the constitutive activity of the STAT3C protein in this system. As mentioned, IFNα-treatment of STAT3C was followed by a prominent tyrosine phosphorylation and nuclear translocation of the protein. In comparison to endogeneous STAT3, the STAT3C was anchored in the nucleus for a prolonged period of time. Based on the fact that STAT3C has a sustained tyrosine phosphorylation as well as DNA binding, we suggest that tyrosine phosphorylated STAT3C has increased DNA binding capacity *per se,* alternatively dephosphorylation of the complex is attenuated. In comparison to other STAT molecules, STAT3 lacks a nuclear localization signal and it is not exactly clear how translocation of STAT3 to the nucleus is brought about. However, a recent study shows that IFNα-signalling induces a complex formation between STAT3/3 dimers and the ProTα protein. ProTα has a potent nuclear localization signal and interacts with STAT3 in a tyrosine phosphorylation-dependent manner (Yang et al., 2004). Hence, in this study, as a result of tyrosine phosphorylation and nuclear translocation, IFNα-signalling potentiated the ability of STAT3C for DNA binding and as a consequence, its anti-apoptotic effects.

No direct link between the PI3K/mTOR pathway and STAT3 regulation has been found in our studies. One possible connection between these molecules could be through the activation of PI3K using STAT3 as an adaptor. We could show that IFNα activates PI3K using IRS-1, which does not rule out the possibility that activation of PI3K in parallel could be mediated by STAT3, as has been demonstrated by another group (Pfeffer et al., 1997). Introduction of STAT3C could thus theoretically alter the activation of PI3K in response to IFN, although this remains to be investigated.

Paper IV **The HPV-16 E7 oncogene sensitizes malignant cells to IFNα induced apoptosis**

The anti-cellular effects of IFNα sometimes involve alterations of key proteins, such as oncogenes, whose expression is commonly altered in malignant cells. Therefore the possibility exists that the malignant genotype may influence the cells sensitivity to IFN. Since resistance is a common problem in the treatment of malignancies with IFNα, as well as other antitumoural agents, it is important to define markers of sensitivity. As oncogenic alterations might be decisive for response to treatment, we decided to investigate the effect of the introduction of an oncogene on IFN-induced apoptosis.

As an experimental system, we used the J3D mouse T-cell lymphoma cell line, stably transfected with the HPV-16 oncogene E7, and its mock-transfected counterpart. E7 is a potent immortalizing and transforming protein (Kanda et al., 1988; Phelps et al., 1988), with one of its main effects being targeting of pRb for degradation which leads to deregulated cell cycle progression (Boyer et al., 1996). Another effect of E7 is to interact with IRF-9 and thereby inhibit nuclear translocation of the ISGF3 complex (Barnard and McMillan, 1999).

We found that the presence of E7 sensitized the cells to IFNα-induced apoptosis, whereas the control transfected cells were completely resistant to this IFN-induced effect. In contrast, no differences were observed in the effects of IFNα on cell cycle distribution; hence, sensitization was restricted to the induction of an apoptotic response by IFNα. Furthermore, we could show that sensitization to apoptosis was specific for IFN, since both E7-transfected and control transfected cells were equally sensitive to apoptosis induced by doxorubicin. E7-mediated sensitization to IFNα-induced apoptosis was correlated to activation of caspases-2, -3, -8 and -9 as well as activation of the pro-apoptotic Bak protein. The resistance to apoptosis in the control transfected cells correlated with a nearly complete absence of both Bak activation and caspase activation. Since caspase activity and Bak activation was observed as a result of doxorubicin-treatment both in E7 and mock transfected cells, we conclude that the presence of the oncogene E7 in an unidentified manner lowers the threshold specifically for induction of apoptosis by IFNα and the switch in balance from life to death.

The presence of an activated oncogene has been reported to both cause sensitization as well as to decrease the response to anti-tumour treatment. Oncogenic sensitization by over-expression of c-myc in fibroblasts was one of the first examples of this feature. c-myc sensitizes cells to a wide range of stimuli by an as yet not completely understood mechanism that has been proposed to involve the mitochondrial pathway (Juin et al., 1999). Cisplatin has been shown to induce c-myc dependent apoptosis by a mechanism leading to activation of Ask1, p38, and Bax as well as release of cytochrome c (Desbiens et al., 2003). Activation of p38 in a similar manner, involving activation of Ask1 and downregulation of Akt activity, has also been shown as a result of sensitization by the adenoviral oncoprotein E1A (Liao and Hung, 2003). Whether HPV-16 E7 acts in a similar fashion remains to be studied.

Understanding how oncogenes sensitize cells to some apoptotic stimuli and why cancer cells survive despite this sensitization may provide a strategy with which to kill tumour cells more

selectively. One possibility is that normal cells are intrinsically protected from apoptosis by inhibitors that are inactivated in response to oncogene expression. One example is primary fibroblasts that are resistant to many anti-cancer drugs, whereas fibroblasts that express the adenoviral oncogene E1A readily die by apoptosis. E1A dependent apoptosis after etoposide treatment involves Bax translocation, cytochrome c release and activation of caspase-9. It has been found that primary fibroblasts express an unidentified inhibitor of cytochrome c release and that E1A represses this inhibitor (Duelli and Lazebnik, 2000). Recently, transcriptional upregulation of caspases-3, -7, -8 and -9 through E1A-mediated disruption of pRB function and subsequent release of free E2F was reported to contribute to both p53-dependent and p53-independent drug sensitization by E1A in normal fibroblasts (Nahle et al., 2002). No such effect has been reported for HPV-16 E7, although it seems likely that release of E2F as a result of E7-mediated degradation of pRb would have a similar effect. This could explain our data on caspase activation in the E7 transfected cells and the lack of caspase activity in the control transfected cell line. However, upregulation of caspases ought to be a general mechanism for sensitization and is thus not applicable in the case of E7-induced sensitization to IFN since it was shown that doxorubicin equally efficiently killed both E7 and mock transfected cells.

A more IFN-specific mechanism would involve inhibition of IRF-9 and the subsequent loss of ISGF3 formation and IFNα-mediated gene induction. This could lead to the induction of an IFNγ like response, mediated by SIE specific gene transcription and possibly a higher sensitivity to apoptosis in this cell line. PKR contains an ISRE site and has been suggested to mediate IFNα-induced apoptosis via the involvement of caspase-8 (Gil and Esteban, 2000). However, since it has been reported that E7 abrogates the mRNA induction of PKR by IFNα (Barnard and McMillan, 1999) PKR is probably not responsible for induction of apoptosis in this system. On the other hand, there are several pro-apoptotic SIE-responsive genes induced by IFNα, such as the PML gene, which has been shown to induce apoptosis via induction of TRAIL (Crowder et al., 2004). The importance of a skewed balance towards induction of genes containing an SIE response element in their promoters should be further investigated.

The HPV-16 E6 protein blocks p53 by targeting it for proteasomal degradation and it has been shown that cells co-expressing E7 and E6 show lower rates of apoptosis either induced by TNFα or spontaneously (Stoppler et al., 1998). The introduction of E7 only, does not have a direct effect on p53. However, the result of pRb loss is increased E2F-mediated transcription which in turn leads to expression of p14/ARF that stabilizes p53 by inhibiting MDM-2 mediated degradation (Koromilas et al., 2001). It has been proposed that the introduction of E7 sensitizes keratinocytes for apoptosis due to this p53 stabilization (Stoppler et al., 1998). However this is not the case in our system, since the J3D cells lack wild type p53.

For those of you who only made it through
the "List of publications" and "Acknowledgements"
I can reveal.....YES, the killer is in our grasp,
the interrogation progresses
and it wont be long before he finally cracks.

However, if you want the whole story, with all its twists and turns,
its blind alleys of dashed hope and confounded expectations,
its questions answered and mysteries revealed...
…I am sorry but you will have to read it all.
Although I may forgive you for not doing so!

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