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THE ROLE OF TPPII IN APOPTOSIS CONTROL AND TREATMENT OF MALIGNANT DISEASE

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To my family

ABSTRACT

Degradation of cytosolic proteins depends largely on the proteasome, a large multi-catalytic protease complex present in the cytosol and nucleus of mammalian cells. This complex contains a catalytic core (20S) and an accessory complex (19S) that recognizes ubiquitinated substrates and translocates these to the catalytic sites in the 20S particle. The proteasome is responsible for most of cytosolic protein degradation in eukaryotic cells, of both short-lived and long-lived proteins, and some of the degradation fragments are presented bound to MHC class I molecules. In the case of an immunological challenge, mammalian cells express IFN-inducible proteasomal subunits.

Impaired proteasomal activity can be functionally compensated, at least in part, by another large cytosolic peptidase, tripeptidyl-peptidase II (TPPII). TPPII is built from a unique 138kDa sub-unit expressed in multi-cellular organisms from Drosophila to Homo Sapiens. The TPPII complex consists of repeated sub-units forming two twisted strands with a native structure of about 6 MDa. The physiological role and extent to which TPPII can contribute proteolysis is unclear, although this peptidase degrades cytosolic polypeptides and generates certain MHC class I ligands. Further, TPPII participates in regulation of apoptosis and its-over-expression in lymphoma cells increases tumor growth *in vivo*. Increased expression of TPPII is induced by proteotoxic and oncogenic stress as well as starvation.

EL-4 lymphoma cells adapted to growth in the presence of inhibitor maintain cytosolic proteolysis and cell viability by a mechanism that includes compensatory up-regulation of TPPII. However, it is unknown whether the adapted state has functional consequences at the level of MHC class I ligand generation and antigen presentation to CTLs. We have analyzed that lymphoma cells with reduced reliance on proteasomal activity no longer efficiently produced MHC class I ligands, although cytosolic proteolysis continued, and proliferation was not altered compared with control cells. This phenotype contributed to escape from tumor rejection in tumor graft experiments in syngeneic C57Bl/6 mice. Using a GFP reporter to measure proteolysis, we show in live cells that non-proteasomal serine peptidase activity participated in protein degradation, but inhibition of these enzymes failed to have a significant effect on the assembly of H-2K^{b} molecules. Continued proteolysis in proteasome-impaired cells afforded the cell the requisite housekeeping functions while preventing the full display of the usual set of MHC class I-restricted epitopes.

Many tumors overexpress members of the inhibitor of apoptosis protein (IAP) family. IAPs contribute to tumor cell apoptosis resistance by the inhibition of caspases, and are degraded by the proteasome to allow further progression of apoptosis. Impaired proteasomal activity is associated with induction of alternative cytosolic peptidases, such as TPPII and iso-peptidases. It is unclear whether this has any impact on tumor progression. In second study we have analysed apoptosis control in tumor cells with high TPP II activity, which can grow despite proteasomal inhibition. We find that EL-4 lymphoma cells that can grow in the presence of low proteasomal activity acquire apoptosis resistance due to a failure in degradation of IAPs. The rate of *in vivo* tumor

growth of such cells was strongly increased. Rapid tumor growth, as well as a delayed degradation of IAPs, could be induced by transfection of TPP II. In addition, in cells derived from large *in vivo* tumors we observed an upregulated TPPII activity and a slower degradation of IAPs. Our data suggest a novel mechanism for apoptosis resistance in tumors.

Cancer therapy frequently depends on the induction of DNA damage, e.g. treatment with γ-irradiation or DNA topoisomerase II inhibitors. Responses to double-stranded DNA brakes are controlled Phospho-Inositide-3-OH-Kinase-related-kinases (PIKKs); including the kinase mutated in Ataxia Telangiectasia (ATM). These kinases are crucial for maintenance of DNA stability and are constitutively activated in many cancer cells. To understand how cells sense stress is crucial for the possible development therapies against many pathogenic conditions, including cancer and ischemic disease. We have explored the involvement of TPPII in response to DNA damage. We found that the expression of TPPII is controlled by PIKKs. TPPII is required for stabilization of p53 and p21, and for lymphoma cell cylce arrest in response to stress. We also studied that TPPII contains a putative BRCT domain; a targeting domain for ATM-kinase phosphorylated proteins. This domain was important for p53 stabilization. We found a TPPII-dependent interaction between p53 and ATM, as well as DNA repair foci components Mre11 and 53BP1. Novel peptide-based inhibitors of TPPII caused complete *in vivo* tumor regression in mice, in response to relatively low dose of γ-irradiation. This was observed with established mouse and human tumors of diverse tissue backgrounds. Our data suggest that TPPII has an essential role in DNA damage responses, and that this peptidase can be targeted for treatment of tumors.

At last we have studied the activity of the ubiquitin-proteasome pathway in stressed cells. We observed that TPPII was essential for down regulation of proteasomal substrate degradation, and targeting of proteasomal complexes to the nucleus during stress. Our data indicate that TPPII-mediated suppression of substrate degradation may have a substantial impact on cellular processes and that this may contribute to transduction of cellular stress signals.

In conclusion, our results indicate a crucial role for TPPII in signal transduction during cellular stress, and that this peptidase complex is suitable for drug targeting.

LIST OF PUBLICATIONS

I. Benedikt Kessler*, **Xu Hong***, Jenela Petrovic, Anna Borodovsky, Nico P. Dantuma, Matthew Bogyo, Herman S. Overkleeft, Hidde Ploegh, and Rickard Glas. Pathways Accessory to Proteasomal Proteolysis Are Less Efficient in Major Histocompatibility Complex Class I Antigen Production. **Journal of Biological Chemistry, 2003, 278(12): 10013-10021.**
* contributed equally

II. **Xu Hong**, Lu Lei and Rickard Glas. Tumors Acquire Inhibitor of Apoptosis Protein (IAP)-mediated Apoptosis Resistance through Altered Specificity of Cytosolic Proteolysis.
The Journal of Experimental Medicine, 2003, 197(12): 1731-1743

III. **Xu Hong**, Lu Lei, Mikolaj Malinowski, Brita Kunert, Steven E. Applequist, Alf Grandien and Rickard Glas. Tripeptidyl-Peptidase II Controls DNA Damage Responses and Resistance to Cancer Therapy.
Manuscript

IV. **Xu Hong**, Lu Lei, Laszlo Szekely, Steven E. Applequist and Rickard Glas. Cellular Stress Induces TPPII-Dependent Suppression of the Ubiquitin-proteasome pathway.
Manuscript

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LIST OF ABBREVIATIONS

AAF-AMC	alanine-alanine-phenylalanine-amino-methyl-coumarin
APC	antigen presenting cell
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia related
BCL	B-cell lymphoma proteins
BL	Burkitt's lyphoma
CCK	cholecystokinin
CDK	cyclin-dependent kinase
CP	core particle
DUB	deubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzymes
E3	ubiquitin-ligases
ER	endoplamsic reticulum
GFP	green fluorescent protein
IAP	inhibitor of apoptosis protein
IFN	interferon
LCL	lymphoblastoid cell line
MHC	major histocompatibility complex
NLVS	4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinyl sulphone
PI	propidium iodine
PI3K	phospho-inositid-3-kinase
PIKK	phosphoinositide-3-OH-kinase-related kinase
RB	retinoblastoma protein
RP	regulatory particle
SCA	spinocerebellar ataxia
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SiRNA	small interfering RNA
Succ-LLVY-AMC	succinyl-leucine-leucine-valine-tyrosine-aminomethyl-coumarin

TAA	tumor associated antigen
TAP	transporters for antigen presentation
TNF	tumor necrosis factor
TPPII	tripeptidyl-peptidase II
TSA	tumor specific antigen
UPP	ubiquitin-protasome pathway
UPS	ubiquitin proteasome system
Z-GLA-OH	Z-Gly-Leu-Ala-OH

INTRODUCTION

1. Tumor biology – an introduction

1.1 General introduction

Cancer existed since human societies first recorded their activities. Cancer is a major problem especially today due to an aging population. Improvements in public health and medical care, has increased the chance of survival of cancer patients. However the proportion of people at the risk of cancer has increased constantly. Therefore, cancer prevention and control are major health issues. Cancer is not only a problem confined to man and higher mammals, but affects all multicellular organisms and animals. Cancer involves deregulated cell proliferation, differentiation and development, and to understand this disease will help us to understand the very basic mechanisms of life.

Johannes Mueller showed that cancers were made of cells about 140 years ago. Normal cells and tissues are under the control of growth via a fine balance between stimulatory and inhibitory stimuli. Tumor cells differ from normal cells in their lack of response to normal control mechanisms. The process of cancer development is called carcinogenesis, a multistage process. Berenblum and Shubik (1) carried out experiments in 1940s on mouse skin indicating that at least three stages for carcinogenesis exist; the first stage was initiation involving mutagenic effects of the carcinogen on skin cells; the second stage was promotion, and at last the progression. The mouse skin model indicated that carcinogenesis is a multistep process which is the case for human cancer as well (2).

1.2 The hallmarks of cancer

Cancer is a complicated disease in humans with a multi-step process resulting in various malignant characteristics. Each acquired capability for cancer is described as following:

Self-sufficiency in growth signals

All types of normal cells need mitogenic growth signals (GS) when they proliferate. These signals are transmitted into the cell by transmembrane and/or intracellular receptors that bind distinctive classes of signaling molecules: (A) diffusible growth factors (GF), such as EGF-R/erbB and HER2/neu (3,4), additionally, gross over-expression of GF receptors can elicit ligand-independent signaling (5). (B) extracellular matrix components, like their receptors (e.g. integrins) can be changed. (6-8). (C) cell-to-cell adhesion/interaction molecules (9). Tumor cells reduce their dependence on stimulation from normal tissue microenvironment by generating many of their own growth signals. There are three strategies for tumor cells to achieve autonomy: alteration of extracellular growth signals, alteration of transcellular transducers of those signals, or alteration of intracellular circuits that translate those signals into action (9).

This dependence reduction disrupts the important homeostatic mechanism for normal cells.

Insensitivity to antigrowth signals
There are multiple anti-proliferative signals including both soluble growth inhibitors and immobilized inhibitors to maintain cellular quiescence and tissue homeostasis. These signals are received by transmembrane cell surface receptors coupled to intracellular signaling circuits. Cancer cells can evade these anti-proliferative signals by disrupting the pRb pathway to liberate E2Fs and allow cell proliferation (10-18) or by over-expressing c-myc oncogene to avoid cell terminal differentiation and maintain cellular proliferation (19-21).

Apoptosis escape
The expansion of a cell population is not only determined by the rate of cell proliferation but also of cell death. Apoptosis is a major source of cell death. Tumor cells acquire resistance to apoptosis through a variety of strategies such as down regulation of pro-apoptotic regulators by mutating tumor suppressor genes such as p53 or PTEN (22-26). A mechanism for abrogating the FAS death signal has also been revealed in lung and colon carcinoma cell lines (27).

Limitless replicative potential
Hayflick demonstrated that cells in culture have a finite replicative potential (28), a process termed senescence which can be circumvented by disabling their pRb and p53 tumor suppressor proteins. Then the cells can go into a state called crisis and at last when cells acquired the ability to multiply without limit, immortalization (29).

Most types of tumor cells that are propagated in culture are immortalized, suggesting that limitless replicative potential is a phenotype that was acquired *in vivo* during tumor progression and was essential for the development of their malignant growth state (28). This phenotype is induced by telomere maintenance (30-38).

Angiogenesis
Tumor must develop an angiogenic ability by angiogenesis-initiating signals such as vascular endothelial growth factors (VEGF) and acidic and basic fibroblast growth factors (FGF1/2) to progress to a large size (39-42). However, angiogenesis is the result of the balance of positive as well as negative signals. Tumors activate the angiogenic switch by changing the balance of angiogenesis inducers and inhibitors (40, 43, 44). The mechanism of switch of angiogenesis inducer and inhibitor is incompletely understood. The VEGF gene is also controlled by complex transcriptional signals (45-47). Another angiogenesis regulator is the protease that can control the bioavailability of angiogenic activators and inhibitors by releasing bFGF (48, 49). The coordinated expression of pro- and anti-angiogenic signaling molecules, and their modulation by proteolysis, appear to reflect the complex homeostatic regulation of normal tissue angiogenesis and of vascular integrity (9).

Tumor invasion and metastasis
It has been reported that 90% of human cancer deaths is caused by tumor cell metastasis (50). Invasion and metastasis are exceedingly complex processes, and their

genetic and biochemical determinants remain incompletely understood. At the mechanistic level, they are closely allied processes, which justifies their association with one another as one general capability of cancer cells. Both utilize similar operational strategies, involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases.

Several classes of proteins involved in the tethering of cells to their surroundings such as changes in expression of CAMs and integrins (6-8, 51-55). Extracellular matrix-degrading proteases also involve tumor invasive and metastatic capability. By up-regulating protease genes, down-regulating protease inhibitor genes and converting inactive zymogen forms of proteases into active enzymes (56-60). There is a report showing that in many types of carcinomas, matrix-degrading proteases are produced not by the epithelial cancer cells but rather by conscripted stromal and inflammatory cells (58), once released by these cells, they may be wielded by the carcinoma cells (61).

Genome Instability

When tumor cells acquired the capabilities as we described above during the course of tumor progression, genome instability is acquired as well. Tumor cells lose function of genomic "caretaker" systems such as the p53 tumor suppressor protein (23, 62), which may cause incorrect chromosomal segregation during mitosis. Another interesting finding is that apoptosis may also be a vehicle of genomic instability, in that DNA within apoptotic cell bodies can be incorporated into neighboring cells following phagoctytosis (63).

In general, all types of tumors will share these common hallmark capabilities although some types of tumors will not have all these hallmarks or the capabilities acquired at different periods during their development.

1.3 Genes involved in tumorigenesis

Various types of genes are known to contribute to the development of cancer. The retroviruses containing oncogenes are related to cellular genes (proto-oncogenes). Many cellular genes can act as oncogenes when expressed inappropriately or mutated. Genes that provide negative regulatory signals in the normal cell are also implicated in the development of cancer. If such a gene requires loss or inactivation to contribute to the transformation process, then it is likely that both copies of the gene must be altered and that such tumor suppressor genes would be genetically recessive at the cellular level. This was proposed by Knudson's so-called "two-hit hypothesis" (64). In normal cells, the requirement for efficient repair mechanisms is clear. In the absence of such repair capacity, it is difficult to see how long-lived species such as man could survive daily exposure to environmental carcinogens without severe toxicity and inevitably a high cancer rate (65). Other mutator genes include genes involved in regulation of the mitotic apparatus which can also affect the rate of acquisition of other mutations. An example is aurora kinase A (AURKA, 66, 67).

Oncogenes

It has been shown that cellular genes can cause cancer when transduced by a virus, and it would not be surprising if alterations to the same genes within the host cell could have a similar effect. This indeed proved to be the case and numerous cellular genes have now been shown to act as oncogenes in human tumors. To date more than 100 mammalian genes have been reported to act as oncogenes when expressed inappropriately or altered by mutation.

Tumor suppressor genes

Tumor suppressor genes (TSGs) encode proteins whose absence, repression, expression inactivation, or mutation promotes oncogenesis. In some cases, reactivation of the function of a TSG suppresses the malignant phenotype (68, 69). This type of TSG is referred to as a gatekeeper gene. Inactivation of other TSGs (caretaker genes), leads instead to genomic instability and therefore to an increase in the mutation of other genes (may be gatekeeper genes) or influence tumor development. Caretaker genes include genes involved in DNA repair and genes that maintain the integrity of chromosomes and their numbers. Unlike gatekeeper genes, reconstitution of caretaker genes fails to override these secondary effects and does not suppress malignancy. In many cases TSGs are involved in both gatekeeping and in caretaking functions.

Knudson's "two-hit" hypothesis that both alleles of a gene have to be inactivated to contribute to transformation, holds true for some TSGs. In fact, the genes for most dominantly inherited cancers, like retinoblastoma, show this relationship. TSGs may also be inactivated by mechanisms other than mutation, such as methylation and this may occur at higher frequency than mutations. Furthermore, if mutations occur in genes that control genetic stability (such as caretaker genes), this "mutator" phenotype may contribute to the appearance of mutations and other genetic abnormalities. Finally, there is the state called haploinsufficiency, a state in which loss of a single allele significantly impairs its function (70-74).

p53

P53 was discovered in 1979, and was described as a host protein with an apparent molecular weight of 53kDa binds to T antigen in SV40 transformed cells (75). At least 50% of adult human tumors carry inactivating mutations in the p53 gene. In many cases, loss of one allele and mutation of the other is detected. Furthermore, in many of the tumors that encode wild-type p53, its levels and activity may be impaired due to alterations in other cellular factors or the expression of viral oncogenes. The amino terminus of p53 contains its transactivation domain. This region may be important for the stability of the protein. The central part of p53 contains its DNA binding region, there is a nuclear localization signal and an oligomerization domain that mediates the tetramerization of p53, which is necessary for its transcriptional activation function. The C-terminus of p53 contains a negative regulatory domain as well as lysine residues that are susceptible to ubiquitination, which involved in targeting p53 protein to degradation by the 26S proteasome. Other modifications at the C-terminus that may affect p53 stability and activity include SUMO (small ubiquitin-like modifier) conjugation and acetylation. Phosphorylation can modulate protein stability and function (23).

Wild type p53 tumor suppressor activity is mainly due to the ability to act as a transcription factor and induce the expression of a large number of proteins (76). Whether p53 can also specifically repress transcription in a direct way is still matter of controversy.

Some p53 targets are involved in inhibition of cell cycle arrest (such as p21C1P1 and GADD45) and others can cause the induction of cell death by apoptosis (Bax, etc). Other proteins induced by p53, such as histone H3, DNA polymerase-α may have important role in DNA replication (77).

The apoptotic and cell cycle arrest activities of p53 can be separated. P53 levels and activity must be tightly regulated because the primary function of it is to stop cell proliferation by arresting the cell cycle or by inducing cell death by apoptosis. The current view is that p53 levels are mainly regulated at the post-transcriptional level. P53 has a very short half-life in normal non-stressed cells due to interaction with MDM2 protein which functions as ubiquitin E3 ligase that targets p53 for degradation by the proteasome. In this way, p53 levels are kept low allowing cells to proliferate (78, 79).

In tumors that carry mutant p53, it is still susceptible to degradation by MDM2. But the ability to act as a transcriptional activator is generally abolished, and therefore MDM2 level is decreased leading to the accumulation of p53 in the cells.

In cancer cells where the p53 gene is intact, its tumor suppressor function may be blocked by the over-expression or inactivation of cellular factors or by the expression of certain oncoviral proteins including MDM2 protein over-expression.Additional defects in p53 expression may be caused by defects in the expression of $p14^{ARF}$ tumor suppressor protein, mutations in kinases such as ATM or Chk2, chromosome translocations involving the PML or infection with certain viruses such as SV40, adenovirus, and human papillomavirus (80-85).

The levels of p53 protein and its transcriptional activity increase in cells that are irradiated or treated with DNA damaging agents, including many of currently used chemotherapeutic drugs. This p53 activation is induced partly by DNA damage. By allowing DNA repair in moderately damaged cells, p53 prevents the fixation of mutations in cell populations. Hence, the loss of p53 function leads to genomic instability and the accumulation of cells with damaged DNA.

The explanation of the mechanisms by which p53 is activated when cells are subjected to stress has been an area of intense research. In response to DNA damage, the activity of certain kinases (such as ATM, Chk2, DNA-PK) is increased and p53 and /or MDM2 are modified by phosphorylation. In some instances, the phosphorylation events have been suggested to inhibit the ability of p53 and MDM2 to interact with each other or to inhibit the ability of MDM2 to carry out its function.

P53 activity can also be increased by a variety of stresses that do not involve DNA damage. These include hypoxia, serum starvation, heat, cold, pH changes, ribonucleotide depletion, glycerol, and inhibition of nuclear export. P53 levels can also

be increased by oncogenic signals and certain viral oncoproteins. This activation is mediated by an increase in the levels of the $p14^{ARF}$ tumor suppressor protein, an antagonist of MDM2 function. To date there is no clear mechanism for how p53 is translocated from the cytosol into the nucleus in responses to stress (86-96).

1.4 Apoptosis — an overview

The term of apoptosis was first indicated in 1972 (97) and was termed programmed cell death by Lockshin in 1974 (98). The mitochondrion plays a critical role in the induction of apoptosis, acting as an integrator of diverse biochemical signals within the cell. Cellular demolition during apoptosis is an active phenomenon and is achieved by caspases, a highly conserved family of aspartate-specific cysteine proteases. Caspases are synthesized as inactive precursors. Caspase9 is the apical caspase in the intrinsic pathway. Caspase9 activation involves the assembly of a heterotrimeric complex with cytochrome c and the CARD containing protein, APAF-1 (99, 100). The resulting heterotrimeric complex is called apoptosome. Cytochrome c is only one of a number of apoptogenic factors that are localized to the mitochondrial inter-membrane space. During apoptosis, these factors may be selectively or non-specifically released into the cytoplasm. Mitochondrial release of cytochrome c occurs transiently, and is kinetically invariant (101). Once activated, caspase9 binds to caspase3 and caspase7 (executioner caspases) via homophilic CARD interactions, resulting in cleavage of their prodomains, and activation. Caspase3 and 7, then cleave downstream caspases. DNA fragmentation factor (DFF) is a substrate for caspase3, which mediates both nuclear condensation and DNA fagmentation (102).

A number of proteins can mediate cell death in the absence of caspases. These proteins include apoptosis-inducing factor (AIF), and endonuclease G (endo G) (103). They function in parallel with caspases, to affect the morphological and biochemical phenomena that constitute apoptosis. They share the common property of being constitutively localized to the mitochondrial inter-membrane space, and undergo release upon induction of apoptosis.

Apoptosis can be initiated by extrinsic and intrinsic pathways. Two types of CD95 pathways were identified. One involves predominant signaling via the DISC, resulting in caspase 3 cleavage and apoptosis in type I cells. Type II cells rely on mitochondrial permeabilization to undergo apoptosis. Therefore apoptosis can be suppressed in Type II but not in type I cells by membrane stabilizing anti-apoptotic proteins such as Bcl-2 (Figure1).

1.5 Suppression of apoptosis

Many proteins encoded by mammalian cells antagonize the core apoptosis machinery. Over-expression of such genes may confer survival advantage with implications for cancer pathogenesis and treatment. Cells have evolved further levels of regulatory complexity through the expression of pro-apoptotic genes that antagonize endogenous apoptosis inhibitors, and this may be exploited as a therapeutic strategy to reverse apoptosis resistance.

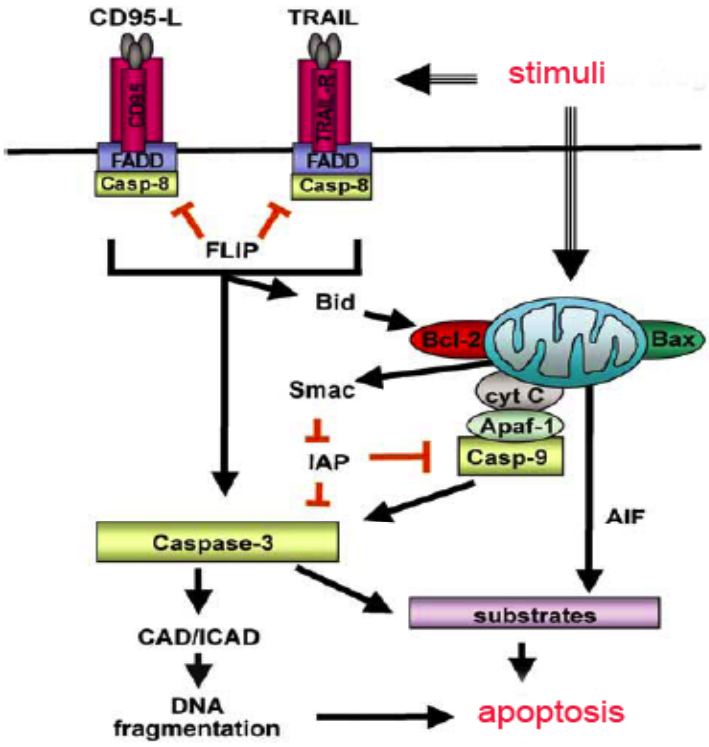

Fig 1. Apoptosis signaling pathways. Apoptosis pathways can be initiated through different entry sites, for example, at the plasma membrane by death receptor ligation (receptor pathway) or at the mitochondria (mitochondrial pathway). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors by CD95 ligand (CD95-L) or TRAIL results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8. Upon recruitment, caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases. The mitochondrial pathway is initiated by stress signals through the release of apoptogenic factors such as cytochrome *c*, apoptosis inducing factor (AIF), or Smac/DIABLO from the mitochondrial intermembrane space. The release of cytochrome *c* into the cytosol triggers caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex. The Smac/DIABLO promotes caspase activation through neutralizing the inhibitory effects to IAPs, whereas AIF causes DNA condensation. The receptor and the mitochondrial pathway can be interconnected at different levels, for example, by Bid, a BH3 domain-containing protein of the Bcl-2 family which assumes cytochrome-*c*-releasing activity upon cleavage by caspase-8. Activation of caspases is negatively regulated at the receptor level by FLIP, which block caspase-8 activation, at the mitochondria by Bcl-2 family proteins and by inhibitor of apoptosis proteins (IAPs). (Modified from Fulda, 2006, Oncogene)

Apoptosis inhibitors of the Bcl-2 family

Bcl-2 was discovered in the 1980s and is encoded on 18q21, and is expressed as a 26kDa protein comprising BH1, BH2, and BH3 domains. Bcl-2 is related to several anti-apoptotic homologues including Bcl-X_L, Bcl-W, Bcl-B, and MCL-1. Over-expression of Bcl-2 inhibits apoptosis by a wide variety of inducers including chemicals, irradiation, ultraviolet light, engagement of death receptors, and growth factor withdrawal (104). Bcl-2 plays an important role during development. Knockout of the Bcl-2 gene results in growth retardation and massive splenic involution due to excessive apoptosis. Conversely, mice over-expressing Bcl-2 exhibit increased lifespan of memory B-cells, and persistence of immunoglobulin secreting cells.

Bcl-2 interacts with Bax, Bak which are antagonistic to its activity (105,106) through a receptor/ligand-like docking in which the BH3 domain of Bax and Bak they engage with a cleft formed from BH1, BH2 and BH3 domains of Bcl-2. Thus, the BH1 and BH2 domains are required to inhibit apoptosis (107). The ratio of anti-apoptotic Bcl-2 to pro-apoptotic BAX has been likened to a rheostat that governs the cellular apoptosis threshold (105). Thus, over-expression of Bcl-2 confers an increased apoptosis threshold, whereas over-expression of BAX/BAK increases apoptosis sensitivity.

Bcl-2 protein exhibits cationic ion-channel properties and localizes to the mitochondrial outer membrane, ER, and nucleus. Bcl-2 also acts independently as an efficient inhibitor of mitochondrial permeabilization, blocking the release of apoptotic factors. Recent evidence suggests that Bcl-2 can act upstream of mitochondria to prevent caspase activation (108). The molecular basis for this action has not been elucidated.

Inhibitors of apoptosis protein family (IAP family)

The inhibitor of apoptosis protein (IAP) family comprises phylogenetically conserved homologues of an anti-apoptotic protein encoded by the baculovirus genome. These proteins, collectively termed BIR-containing proteins or BIRCs, are characterized by a baculoviral internal repeat, or BIR domain, comprising histidine- and cysteine-rich motifs. The BIR domain is essential for function, and interaction with other apoptosis-regulating proteins, including caspases and APAF-1. The structural detail of the binding interaction between the BIR-3 domain XIAP has been determined (109).

XIAP is expressed in all foetal and adult tissues, except for peripheral blood leucocytes (110). XIAP directly interacts with and inhibits caspase3 and caspase7. Moreover, it interacts with caspase9-APAF-1 apoptosome via the small p12 subunit of pro-caspase9. This interaction is required for XIAP function. The proteins c-IAP1 and c-IAP2 bind and directly inactivate caspase3 and caspase7 in a BIR domain-dependent manner (110). Additional IAP family proteins have also been identified, such as NAIP, livin, and apollon (111, 112).

During apoptosis, the proteins SMAC (second mitochondria derived activator of caspases), also called DIABLO, and OMI (serine protease 25) or HtrA2 are released from the mitochondria to depress caspases by inhibiting IAPs. SMAC/DIABLO was the first mitochondrial IAP inhibitor to be discovered, and is co-released with cytochrome c (113). OMI/HtrA2 is a mitochondrial intermembrane space protein that has close structural resemblance to the bacterial hsp HtrA, a protein involved in the endo-proteolytic degradation of denatured proteins, involved in allowing survival of the bacterial cell in high temperature environments. OMI/HtrA2 released from mitochondrial inter-membrane space in processed form, binds to XIAP (not survivin), and inhibits its caspase-inhibitor activity.

Apoptosis suppression mediated by growth factor pathways

The core apoptosis machinery is regulated by survival signals originating from growth factor receptors at the cell surface. The erbB family of growth factor receptors is a structurally related family of trans-membrane proteins with intrinsic tyrosine kinase

activity. They include EGFR, Her2, ERBB3 and ERBB4. Upon ligation, these receptors hetero-dimerize and undergo tyrosine auto-phosphorylation of the intracellular domain. This results in the recruitment of phosphoinositide-3-kinase (PI3K), which in turn is phosphorylated, mediating downstream phosphorylation and activation of AKT. Phosphorylated AKT (p-AKT) mediates anti-apoptosis by targeting down-stream pro-and anti-apoptotic factors, such as Bad, IkB, and XIAP.

1.6 Effective cancer therapy based on apoptosis biology

Apoptosis resistance is a major factor involved in failure of systemic cytotoxic therapy. This phenomenon is multi-factorial. Local factors within a tumor such as low pH and poor perfusion may limit drug bioavailability. Mutations leading to up-regulation of drug efflux pumps such as multi-drug resistance proteins (MDR-1, MRP, LRP) (115-117) may significantly affect intracellular bioavailability. Chemotherapeutic drugs typically affect cell death via damage sensors associated with DNA, cellular metabolism, or microtubule stabilization, and mutations in specific genes attenuate apoptosis induction. Common patterns of dysregulation of the core apoptosis pathway have been identified recently. Mutations leading to inhibition of the final common pathway to apoptosis will effectively suppress cell death induced by most cytotoxic stimuli. To overcome this problem a functional dissection of the core death apparatus in specific cancers is required, to increase understanding of how such a process can be reversed. Some of the major damage in core apoptosis signaling of cancer cells and their potential as therapeutic targets are discussed following. (Figure 2 shows a possible pathway for the induction of apoptosis in cancer cells).

Therapy based on p53 induction and /or activation

There are very promising approaches to selectively kill tumor cells lacking p53 or carrying inactivating mutations. Drugs that bind to and reactivate mutant p53 may be used. Alternatively, one may use adenoviral vectors expressing wildtype p53 or viruses that selectively replicate in p53-deficient cells. Since MDM2 is an important inhibitor of p53 function, a way to increase p53 levels in a non-genotoxic way is by impairing MDM2 function. Expression of MDM2 can be decreased with antisense RNAs. To inhibit the activity of proteasome is another way to increase p53 levels although this increase is not associated with the activation of p53 transcriptional activity. Developing small molecules that inhibit ubiquitination of p53 by MDM2 or that mimic the function of the $p14^{ARF}$ tumor suppressor (such as TPPII, will be discussed later), may also be suitable approaches. Another effective way is to induce the accumulation of p53 in the nucleus, where p53 acts as transcriptional activator. The nuclear export inhibitor leptomycin (LMB) has been shown to be an extremely potent activator of p53 transcriptional activity (68, 69)

The p53 mutation rate in haematological malignant diseases and childhood cancer is significantly lower, compared to other forms of tumors. This may be one reason for the much better prognosis of children with cancer. However, it is important to consider the DNA damaging effects of many current therapies when considering the treatment of young patients. Sturm. et al (118) have shown for the first time that treatment of B-CLL patients with DNA damaging alkylating agents correlates with the appearance of

mutations in p53. The appearance of these mutations is significantly associated with poor outcome and drug resistance. That's why it is essential to search for novel non-genotoxic activators of the p53 response to improve the treatment of these cancers, in order to not risk that their p53 function is abolished by mutation. The side effects of current p53 based chemotherapeutic drugs that induce p53 responses in normal tissues, are at least partially responsible for side effects such as gastrointestinal dysfunction or hair loss. Therefore, it is necessary to take a radically different approach and aim to decrease the devastating effects of p53 induction in normal tissues to improve quality of life of patients during treatment. Using drugs like the p53 inhibitor pifithrin-α to protect normal cells from current therapies provide an example of this approach.

IAPs based therapy in cancer

Studies have shown that IAPs inhibit procaspase-9 cleavage which leads to caspase-9 activation *in vitro* (119) and can reduce apoptosis sensitivity. Another interesting study showed that XIAP inhibits caspase-3 activity by cleaving procaspase-3, a caspase downstream of both caspase-8 and caspase-9, which leads to apoptosis resistance during two types of DNA damage responses (DNA damage induced by γ-irradiation and 1-[β-D-arabinofuranosyl]cytosine (ara-C) (120). That suggests XIAP is necessary for blocking caspase-3 activity induced through the caspase-8 signaling pathway in response to genotoxic stress. Further, this indicates that IAPs functions as inhibitor of caspase-9 and downstream of procaspase-9 by a mitochondria-independent mechanism. This implicates that IAPs may be important for cancer apoptosis therapy induced through mitochondrial-dependent or/and –independent pathways.

Some tumors have reduced expression of caspase9, resulting in reduced caspase activation potential. Expression of IAPs appears to be widespread among malignancies. Anti-sense oligonucleotides that down regulate IAPs increase apoptosis sensitivity. Peptides of Smac/DIABLO an endogenous IAP inhibitor can sensitize several tumor models to chemotherapy *in vivo*, suggesting utility of this approach in the clinic. Small molecule Smac/DIABLO mimetics have showed a chemo-sensitizing efficacy, which is an exciting development (121-124).

Bcl-2 family protein based novel cancer therapy

Cancers with over-expressed Bcl-2 and Bcl-X_L have the potential to suppress mitochondrial permeabilization. Down regulation of Bcl-2 or Bcl-X_L using anti-sense oligonucleotides has been reported in patients with Non-Hodgkin's lymphoma and melanoma, validating the approach as a novel therapeutic strategy. Anti-sense Bcl-2 facilitates apoptosis induced by chemotherapeutic drugs, and represents a novel strategy for chemo-sensitization (125-127). This approach is currently being evaluated in clinical trials in a range of solid and haematological malignancies. Development of bi-specific antisense oligonucleotides targeting Bcl-2 and Bcl-X_L simultaneously have been shown to overcome the potential problem of redundant expression. Some cancers have Bax gene mutations and reduced expression of Bax is associated with poor response to chemotherapy (128). Currently small molecule antagonists that inhibit interactions between BcL-2/Bcl-X_L and BAX/BAK have been developed. The first generation of small molecule BAK- BH3 peptide mimetics (BBPMs) have been

discovered (129, 130). Second-generation small molecule peptides ("drugable") will soon enter clinical evaluation.

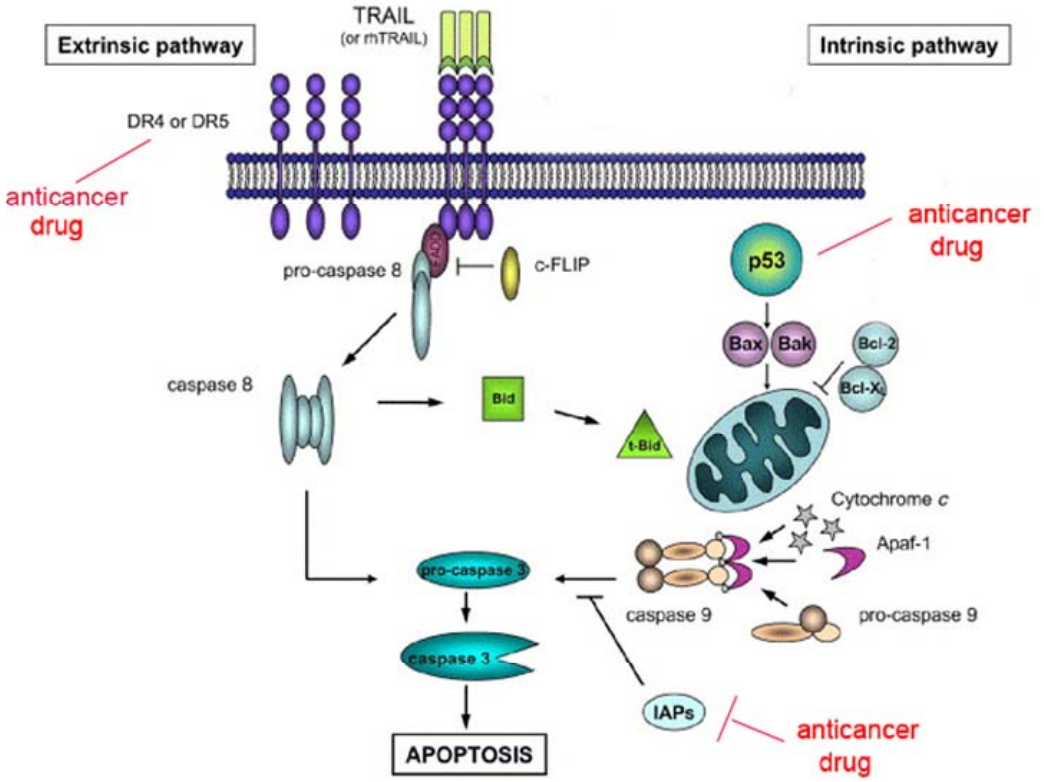

Fig 2. Apoptotic pathways that induce cancer cell death by anti-cancer drugs. The death receptor initiated apoptosis pathway is referred to as the extrinsic apoptosis pathway. TRAIL binds as a homotrimer to DR4 and DR5, which results in trimerisation of the receptors and subsequent assembly of a death-inducing signaling complex (DISC). Drugs such as agonistic antibodies of DR4 and DR5 can be used for promoting apoptosis. When the intrinsic apoptotic pathway is activated, pro-apoptotic members of the Bcl-2-gene family translocate to the mitochondria, causing subsequent release of cytochrome *c* and other mitochondrial factors into the cytosol. In the cytosol, cytochrome *c* binds the adaptor protein Apaf-1 and pro-caspase 9 in the presence of dATP. It hereby forms the apoptosome signaling complex, in which caspase 9 is activated and can activate subsequently the effector caspases 3, 6 and 7. Crosstalk exists between the extrinsic pathway and the intrinsic or mitochondria-initiated apoptosis pathway through Bid. Activated caspase 8 will cleave Bid, which then translocates to the mitochondria to induce cytochrome *c* release. Anticancer drugs such as small inhibitor molecules of Bcl-2, IAPs or agonists of p53 can be used to induce apoptosis in cancer cells (Modified from Duiker, 2006, Eur. J. Cancer)

Modulation of signal transduction for therapy of cancer

Agonist monoclonal antibodies directed to TRAIL receptors DR1 and DR2 are currently undergoing early clinical evaluation. The FADD/caspase8 inhibitor FLIP-L is expressed at high levels in melanoma, and effectively suppresses apoptosis signaling

via the DISC. FAS- ligand has hepatotoxicity in preclinical models, and this is not a valid target for anticancer drug development (131-133).

EGFR is over-expressed in some cancers, and is a poor prognostic factor for survival. Constitutive EGFR activation effects PI3K-dependent phosphorylation and acitivaiton of AKT, with concomitant inactivation of BAD. The EGFR is a target for therapy. There are monoclonal antibodies such as cetuximab, and small molecule inhibitors of receptor tyrosine kinases such as ZD1839 and OS1774. These molecules rely for their activity on suppressing all downstream events from a receptor within the PI3K pathway. Her2 (ERBB2) is a prognostic factor in breast cancer, and a humanized monoclonal antibody to this receptor, termed herceptin, has been validated as an effective therapy in the management of breast cancer. There is evidence however, that crosstalk between the PI3K and MAPK pathways may promote constitutive activation of PI3K and AKT, and that this may not be prevented by specific inhibition of the ERBB receptors alone. This mechanism could account for the failure of response to EGFR inhibitors in some patients. Phosphorylated AKT can be measured *in vivo* in cancers, and represents a putative therapeutic target. The small molecule SRI13368 is a specific AKT inhibitor, and more novel small molecules are in development. The receptor tyrosine kinase c-kit is constitutively activated in gastrointestinal stromal tumors (GISTs), and mediates intracellular survival signals via the PI3K pathway. GISTs exhibit chemoresistance. A small molecule tyrosine kinase inhibitor (Gleevec, STI571) has demonstrated efficacy in CML (112, 134-137).

Application of novel knowledge relating to mammalian apoptosis is helping to define the important pathways that determine the apoptosis-resistant phenotype of the most common but difficult to treat cancers. Despite the complexity of regulation of apoptosis pathways, functional genomics, and target validation, using small molecules, are identifying new potentially therapeutic molecules at an advancing rate. This will inevitably increase the number of new agents entering clinical trial, and thus will increase the possibility of effective drug discovery.

1.7 The immune system and cancer

In the mid of the 20th century the theory of immune surveillance against tumors was put forward. These experiments suggested that tumors contained antigens recognized as foreign by the immune system. This suggests the possibility to boost immunity to these antigens by deliberate immunization. There are two types of tumor antigens, tumor associated antigens (TAAs) and tumor specific antigens (TSAs). TAAs can be found in both normal and cancerous cells while TSAs are encoded by mutated cellular genes or by viral oncogenes and the products of these genes are not expressed by non-transformed cells (138, 139). These antigens are processed by the antigen processing pathway, which can be divided into 4 steps: 1. protein degradation; 2. peptide transport into the ER; 3. peptide assembly with MHC class I molecules; 4. transport of the MHC class I peptide complex on the cell surface (140, 141).

In many tumors, interference with antigen presentation is observed; this occurs in more than half of all tumors (142). So however effective an anti-tumor immunization regime may be, it will be ineffective if the target epitopes are no longer presented to effector T

cells by the tumor cells. Interference with antigen processing include mutations in MHC class I heavy chain, β_2-microglobulin, defects in TAP as well as immunoproteasomal sub-unit expression (143). Moreover, tumor-bearing animals and cancer patients show an accumulation of immature myeloid cells which interfere with the function of tumor-specific T cells through generation of nitric oxide and arginine depletion (141, 144).

Cancer immunotherapy can be divided into active versus passive and specific versus non-specific (145). There is evidence suggesting that optimal strategies for induction of anti-tumor responses should target tumor antigens to APCs. This has led to immunotherapy based on the use of antigen-loaded and activated DCs (146). Some particular materials, may promote entry of antigen into the cytosol and endogenous antigen processing. The recognition that “danger signals” is essential for initiation of immune responses, has also suggested that modulation of the inate immune system is important. There are new immunization strategies being developed based on new adjuvants which can be used for human (147, 148). Overall, this is a very active field of research and there is no consensus yet for how anti-tumor immune responses are best induced.

2. The proteasome

Proteasomes purified from many different species are almost identical, and the basic components of the proteasome are conserved in all eukaryotes. Proteasomes are ubiquitous to life (149, 150).

Intracellular protein degradation is one of the most tightly regulated processes in living systems. A number of regulatory pathways are regulated by timely removal of critical proteins. These proteins include proteins involved in cell cycle, transcriptional regulation, DNA repair, development and differentiation, long-term memory, stress response, cell signaling, antigen presentation, and viral infection (140, 151, 152). Other proteins, like misfolded or malfunctional proteins (e.g. DRiPs), must also be degraded. In eukaryotes, these proteins are degraded in an ATP-dependent manner by a single 2.5 MDa multisubunit enzyme, the proteasome. (153-155).

The 26S proteasome is a multi-catalytic enzyme complex expressed in the nucleus and cytoplasm of all eukaryotic cells. It consists of a 20S core particle and one or two 19S regulatory particles. Each 19S subunit is capable of binding the poly-ubiquitin chain and cleaving it from the protein substrate. The substrate is then unfolded and fed into the proteolytic core (153). The 20S core particle is a barrel-shaped particle composed of 4 stacked rings. The two outer rings (called α rings) are composed of 7 α subunits (α1-α7) that control the access to the catalytic chamber and interact with regulatory complexes. The catalytic chamber is formed by the two inner β rings, each of which contains 7 β subunits (β1- β7) and 3 of them (β1, β2, β5) contain active sites. These active sites are termed chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolase-like (PGPH) (156). Proteins are degraded by the core particle in a progressive manner, generating peptides of 3–24 amino acids in length (157) (Fig3).

Intracellular proteins degraded by the proteasome generate antigenic peptides presented by MHC class I molecules (158). Upon immune responses, several stimuli such as IFN-γ, TNF-α induces 3 subunits of the 20S proteasome from β1, β2, β5 to LMP2, MECL-1 and LMP7 respectively (159). These iso-forms, named immunoproteasomes, have an enhanced capacity to generate peptides (160, 161). In addition, there is another regulatory complex called PA28 linked with immune response (162-164).

The proteasome plays an important role in proteolysis of many substrates, including regulatory factors such as cyclins, cyclin-dependent kinase inhibitors, transcription factors (p53), oncoproteins (Bcl-2, IAPs), tumor suppressors (Rb), and structural proteins. (165, 166) are covalently conjugated to a cascade of ubiquitin chain (E1, E2, E3) that targeted for proteasome degradation (167, 168). These enzymes specifically conjugate the lysine side chain or N-terminus of the protein (140, 169). It is reported that modification of proteins by poly-ubiquitin chain at lysine 63 plays a role in cell signaling, DNA repair but not degradation by the proteasome (170, 171). There is at least one example of a protein (ODC) which is targeted for 20S proteasome degradation in a ubiquitin-independent way (172).

Recent work in different cell systems has indicated that the proteasome occupies discrete cellular compartments. Differential localization is a way to regulate gene expression in cell. This evidence showed that proteasome can be localized in different compartments under different cell conditions. In yeast, proteasome locoalized at the unclear periphery for cells undergoing mitotic growth (173). In mammalian cells, proteasome are abundant in cytosol and nucleus (174). In neuro-degenerative diseases, the proteasome appear at the MTOC in response to aggresome formation (175).

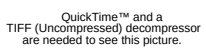

Fig 3. The 26S Proteasome (a) Two-dimensional average of the 26S proteasome from *Drosophila melanogaster* obtained from electron microscopic images of negatively stained specimens. Image analysis of a large dataset of particles revealed the flexible linkage of the 19S caps to the 20S core. The figure shows two states of the complex at the maximum amplitude of the wagging movement superimposed, one in color-scale, the other in gray-scale. The direction of the motion is indicated by the white arrows. From statistical analysis, it can be deduced that the motions of the left and right 19S caps are not correlated. The scale bar is 20 nm.(b) The 3D structure of negatively stained 19S caps was calculated using the method of random conical tilting. The figure shows a composite model of the electron microscopic structure of the 19S caps combined with the low-pass filtered 20S proteasome. (Adapted from Baumeister, 1998, Cell)

Our knowledge of proteasome regulation and activity has been assisted by the use of proteasome inhibitors. They range from small peptide-based structures (that can be modified to vary target specificity) to large macromolecular inhibitors that include proteins. These reagents have played an important role in advancing our understanding of proteasome's catalytic mechanism.

Inhibition of the proteasome may arrest or retard cancer progression by interfering with the ordered degradation of cell-cycle proteins. Indeed, the inhibition of proteasome function results in apoptosis (176-182). Preclinical studies demonstrated a differential sensitivity of cancer cells to proteasome inhibition (176, 177, 183-185) suggesting that proteasome inhibitors may function as drugs to target processes involved in diseases progression with potential clinical application. This thesis will discuss the rationale for proteasome inhibition in the treatment of cancer (Fig 4).

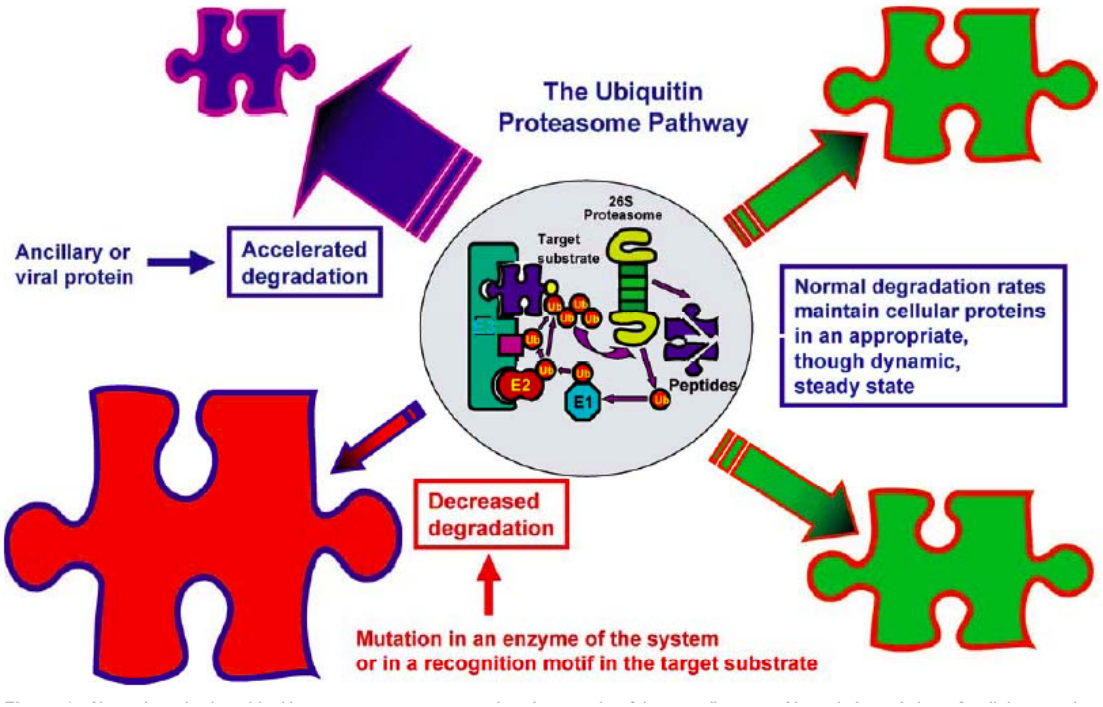

Fig 4. Aberrations in the ubiquitin-proteasome system and pathogenesis of human diseases. Normal degradation of cellular proteins maintains them in a steady state level, though, this level may change under various pathophysiological conditions (upper and lower right side). When degradation is accelerated due an increase in the level of an E3 (Skp2 in the case of p27, for example), or overexpression of an ancillary protein that generates a complex with the protein substrate and targets it for degradation (the Human Papillomavirus E6 oncoprotein that associates with p53 and targets it for degradation by the E6-AP ligase, or the cytomegalovirus-encoded ER proteins US2 and US11 that target MHC class I molecules for ERAD), the steady state level of the protein decreases (upper left side). A mutation in a ubiquitin ligase (such as occurring in Adenomatous Polyposis Coli [APC], or in E6-AP [Angelmans' Syndrome]) or in the substrate's recognition motif (such as occurs in ß-catenin or in ENaC) will result in decreased degradation and accumulation of the target substrate (Adapted from Ciechanover A, 2006, Exp. Bio. Med).

3. Tripeptidyl-peptidase II (TPPII)

TPPII was discovered in 1983 and it is an exopeptidase that removes tri-peptides from free N-termini of oligopeptides (186). Different species have different TPPII, although there is a high degree of conservation between species; from fruit flies to humans (187, 188). In fruit fly, oligomeric complexes are formed by the 150 kDa subunits which form a superstructure, composed of two segmented, twisted strands. Mammalian cells have 138 kDa subunits, but are likely to form similar three-dimensional structures. Each strand of subunits consists of 11 segments which enclose a central channel (189) (Figure 5). Electron microscopy studies have shown that TPPII is a large complex (190), and its activity needs complex formation of two oligomeric strands. The activity of TPPII is regulated by association and dissociation of the complex (191).

TPPII is a serine peptidase of the Substilisin type (192), and its physiological role is to participate in intracellular protein turnover in cooperation with the proteasome and cytosolic exo-peptidases (193). It is believed that TPPII degrades oligo-peptides that are proteasomal degradation products. Tri-peptide formation represents a degradation intermediate between degradation by the proteasome and the liberation of free amino acids. There are no native proteins yet reported that can be degraded by TPPII, and no clear data showed yet if TPPII is physically connected with the proteasome. TPPII is expressed to different levels in different cells (194) , and is localized in the cytoplasm and the plasma membrane (186, 187, 195). TPPII has been reported to at least in part substitute functions of proteasome, in mouse EL-4 lymphoma cells adapted to grow in lethal concentrations of the proteasome inhibitor NLVS (195, 196). These adapted EL-4 cells are still able to degrade ubiquitinated proteins and produce MHC class I ligands (196).

Besides its role in protein turnover, TPPII plays a role during apoptosis. In macrophages, TPPII promotes the maturation of pro-caspase-1 and thereby seems to participate upstream of this caspase (197). Further, TPPII reduce the rate of proteasomal degradation of c-IAP-1 and XIAP after treatment with etoposide (198). In addition, TPPII is upregulated in BL and BL-like cells that have impaired proteasome activity (199). Stavropoulou et al. reported that by using small interfering RNAs (siRNA) of TPPII, Burkitt's lymphoma cells significantly reduced their growth even though the reduction of TPPII expression was less than 90% (200). TPPII may have a role in antigen presentation as well. It has been shown TPPII is essential for production of the immunodominant HIV epitope HIV-Nef (201-207) in human dendritic cells. Cells treated with specific TPPII inhibitors have a strong reduction in presentation of this peptide while inhibition of the proteasome did not affect the presentation of this epitope (208).Therefore, TPPII may act in combination with or independently of the proteasome to generate antigens that evade processing by the proteasome pathway. Some of these functions of TPPII are likely to be dependent on its weak endopeptidase activity which may allow the generation of longer peptides from intact proteins or polypeptide precursors (195, 208, 209). It has been reported the TPPII inhibitor butabindide inhibits degradation of the neuropeptide cholecystokinin (210). However, butabindide was showed later to not be stable in serum. A future challenge will be to design novel inhibitors of TPPII that can be used *in vivo*.

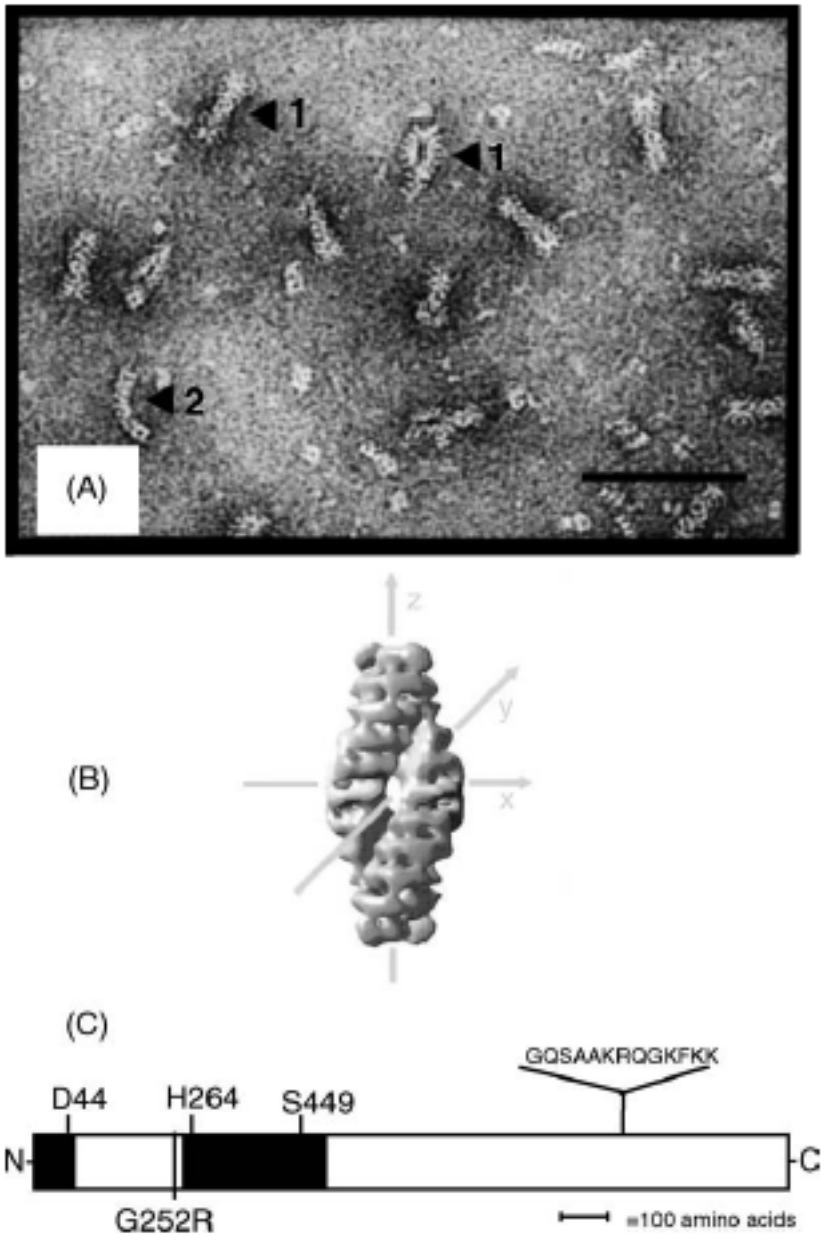

Fig 5. Structure of TPPII. (A) Electron micrograph of human TPPII negatively stained by ammonium molybdate. Two different orientations of the complex (1) and a single strand (2) are indicated (reproduced with permission from (190), © The Biochemical Society). (B) A three-dimensional reconstruction of TPPII from fruit flies based on cryo-electron microscopic images (reproduced with permission from (189), © Nature). (C) Outline of the domain structure of human TPPII. The subtilisin-like catalytic parts are black. D44, H264, and S449 indicate the catalytic amino acid residues and G252R the mutation that interferes with complex formation. The position of 13 extra amino acids, included as a result of alternative splicing, is indicated (Adapted from Tomkinson, 2005, IJBCB).

AIMS OF THE THESIS

The defined aims of my study were:

1. To determine whether the production of MHC class I ligands occurs irrespective which pathways are responsible for protein breakdown.

2. To determine whether an altered specificity of cytosolic proteolysis, with a reduced reliance of the proteasome, affects transduction of apoptotic signals.

3. To determine whether targeting of TPPII is a possible strategy in the treatment of tumors, and if so, by what mechanism.

4. To determine if stress-induced alterations in the specificity of cytosolic proteolysis affects the efficacy of the ubiquitin-proteasome pathway.

RESULTS AND DISCUSSION

Paper I: Pathways Accessory to Proteasomal Proteolysis Are Less Efficient in Major Histocompatibility Complex Class I Antigen Production

The 26S proteasome contributes to the generation of MHC class I ligands. Upon an immunological challenge such as IFN-γ, the LMP2, LMP7 and MECL-1 (β1i, β2i and β5i subunits replace Y, X and Z (β1, β2 and β5)) and form the immunoproteasome. This can lead to increased MHC class I ligand production preferred for MHC class I binding (153,160, 211, 212). However, the majority of potential MHC class I ligands are not efficiently processed (213, 214). This may depend on proteolysis by cytosolic proteases inefficient at generating the requisite cleavage products. Thus, MHC class I processing may be regulated by differential participation of non-proteasomal peptidases in cytosolic protein degradation. It has been shown that a large cytosolic peptidase, TPPII can in part compensate impaired proteasomal activity (193-196). We here aimed to know whether and how tumor cells with inhibited proteasomal activity can survive and escape from immune system recognition.

We made stable EL-4 transfectants expressing Ub-R-GFP to monitor proteasomal degradation of a protein substrate in live cells (215). In line with data from yeast mutants and previous studies using Ub-R-GFP (215-217), efficient inhibition of proteasomal activity was sufficient for accumulation of fluorescence. Further, we observed a significant effect on R-GFP accumulation upon treating EL-4.Ub-R-GFP cells with both NLVS and AAF-CMK in combination. These data suggested that non-proteasomal oligopeptidase activity indeed contributes to cytosolic proteolysis when cells have insufficient proteasomal activity.

To examine if residual proteasomal activity influences the viability of EL-4ad cells, we used mouse EL-4 lymphoma cells with reduced proteasome activity, from continuous treatment with a covalent proteasome inhibitor—NLVS (196). We used Ada-Ahx3-Leu3-VS, an inhibitor that covalently modifies all proteasomal subunits with comparable efficiency (218). We found that virtually all retrievable proteasomal activity was inhibited, but EL-4ad cells still proliferated normally. We then investigated H-2D^b ligand production in EL-4ad cells. We found that EL-4ad cells had a lower production of H-2D^b ligands compared with control EL-4 cells, since a minor fraction of H-2D^b molecules continued transport from the endoplasmic reticulum within 120 min after onset of the chase. Despite normal proliferation, tumor cells can therefore avoid production of most H-2D^b ligands by reduced reliance on proteasomal activity.

To test whether the oligopeptidase TPPII has role in generating MHC class I ligands, we performed a pulse-chase experiment with [^{35}S]methionine metabolic labeling and precipitation of H-2K^b molecules while cells treated with AAF-CMK, a inhibitor of TPPII. We found this treatment had minor effects on the assembly and transport of H-2K^b molecules in EL-4ad cells and also in control EL-4 cells. This may suggest that TPPII support protein degradation, but has a minor role in the production of MHC class I ligands. However, it must be pointed out that AAF- CMK is designed as a general

serine peptidase inhibitor and is not specific for TPPII.

We examined whether the adapted phenotype of EL-4ad cells was selected during growth *in vivo*. We found an increased formation of EL-4ad tumors in mice, in comparison to control EL-4 cells. The tumor-forming ability of EL-4ad cells was dependent, at least in part, on escape from immune recognition because both EL-4ad and control EL-4 cells formed tumors in mice with a deficiency of perforin and RAG-1 (PKOB/RAG -/-). This implicates that tumor cells adapted to lower proteasomal activity can proliferate independently of proteasomal activities and have reduced immune recognition *in vivo*.

This study shows that tumor cells may avoid efficient production of MHC class I ligands and immune recognition by modulation of proteasomal activity. It is possible for mammalian cells to partly escape from production of MHC class I ligands by aversion to pathways of protein degradation involving proteases other than the proteasome.

It is interesting that TPPII can contribute to maintaining proteolysis when the proteasome is inhibited allows for mammalian cells to alter the spectrum of cleavage fragments in the cytosol (193-196). This notion was further supported by the up-regulation of several de-ubiquitinating enzymes in EL-4ad cells, observed otherwise in cells suffering from acute proteasomal inhibition (219). When the proteasome is blocked, tumors may fail to produce certain immunodominant ligands (220, 221). Our data suggested that EL-4ad cells use this phenotype to avoid immunological rejection during the formation of tumors *in vivo*.

Other investigators have shown that in Burkitt's lymphomas, the oncogene c-*myc* induces down regulation of a number of components of the MHC class I-processing pathway, including down-regulation of proteasomal chymotryptic activity in combination with up-regulation of TPPII, thereby linking this deficiency in antigen processing directly to oncogene c-myc expression (199, 222). These studies reveal a new strategy for tumor escape from the immune system by reducing MHC class I processing through a reduced reliance on proteasomal activity. Intracellular protein turn-over is thereby not unconditionally linked to the production of MHC class I ligands.

Paper II: Tumors Acquire Inhibitor of Apoptosis Protein (IAP)-mediated Apoptosis Resistance through Altered Specificity of Cytosolic Proteolysis

Previous results show that when cells grow in the presence of proteasome inhibitors apoptosis is induced. We previously found that tumor cells adapted to grow with proteasomal inhibitors can proliferate normally. Does this correlate with continued transduction of signals that depend on ubiquitin-dependent proteolysis? More specifically, how are apoptotic signals transduced in cells growing in the presence of inhibited proteasomal activity?

We used EL-4ad cells (196), to test whether these were able to properly control apoptosis. We observed that EL-4ad cells failed to undergo apoptosis when exposed to serum starvation for 36 h, and even continued low levels of proliferation in comparison to EL-4 control cells. We tested several apoptotic stimuli, such as serum starvation, TNF-α and etoposide. EL-4ad cells had inefficient activation of caspase3, 8, and 9 as well as an absence of DNA fragmentation.

Since many reports describe that IAPs must be degraded by the proteasome subsequently to mitochondrial cytochrome c release to allow activation of caspase 9, and further progression into apoptosis (223-229), we tested if EL-4ad could degrade XIAP, in response to etoposide treatment. We found degradation of XIAP in EL-4 cells, whereas this was very slow in EL-4ad cells. This implicated that inhibited proteasomal activity caused slow degradation of IAP molecules in EL-4ad cells.

Smac/DIABLO is an IAP inactivator (210, 230, 231). The apoptosis resistant phenotype of EL-4 ad cells was reversed by increased expression of these Smac/DIABLO molecules. These observations suggest that the inadequate degradation of IAP molecules inhibit the transduction of apoptotic signals in EL-4 ad cells. *In vivo* experiments also showed that tumors of EL-4 ad cells were rapidly growing while growth of EL-4 control tumors were significantly slower. We also killed animals and excised cells from large in vivo tumors for later analysis (denoted EL-4/tumor cells).

We next studied whether the proteasomal activity in EL-4 ad cells, as well as whether TPPII increased activity in these cells and regulated the growth of EL-4 tumors in vivo. We used EL-4 wild type cells transfected with either control vector or with pcDNA-TPPII, described previously (232). We tested TPPII activity by peptidase assays with either proteasomal or TPPII inhibitors (210, 233). We found that TPPII transfectants conferred a reduced reliance of proteasomal proteolysis, since EL-4. TPPII proliferated in the presence of NLVS as well as AdaAhx3L3VS, whereas EL-4pcDNA3 cells did not. EL-4.TPPII transfected cells displayed a failure in degradation of both XIAP and c-IAP-1 compared with EL-4pcDNA3 cells during etoposide treatment. Further, TPPII-transfected cells showed a strongly increased rate of *in vivo* tumor growth. This supports the notion that apoptosis resistance linked to TPPII was responsible for the observed effects on growth of EL-4 tumors *in vivo*.

It has been shown that poor nutritional conditions in the tumor microenvironment contribute to selection of apoptosis resistant cells (234). In order to see if poor nutritional conditions may affect the activity of TPPII in tumor cells, we found high levels of TPPII protein in EL-4 cells growing in starvation medium, which correlated with a strong increased TPPII activity. Further, EL-4 cells adapted to proliferation in starvation medium were resistant to apoptosis and expressed high levels of c-IAP-1. These data showed that apoptosis resistance mediated by IAP molecules allows a limited proliferation during serum starvation.

Our data suggest that reduced activity of proteasome proteolytic pathways and increased TPPII activity contributes to apoptosis resistance in tumor cells by inefficient degradation of IAP molecules. This may suggest a link between the microenvironment and signals that alter the specificity of intracellular proteolysis. In conclusion, a down

regulation of proteasomal activity may allow continued cell survival, and even cellular proliferation, despite inhibition of apoptosis signal transduction.

Paper III: Tripeptidyl-Peptidase II Controls DNA Damage Responses and Resistance to Cancer Therapy

To date cancer therapy often depends on the induction of tumor cell DNA damage and following tumor cell apoptosis. In paper II, we found that tumor cells can avoid apoptosis by slowing IAP molecule degradation of the proteasome. In that situation, the tumor cell up-regulated TPPII activity to support cell viability. Our question in the continued study was how TPPII affects the treatment of cancer during DNA damage responses.

First we tested if XIAP was controlled by endogenously expressed TPPII because our previous data showed that over-expression of TPPII causes increased expression of c-IAP-1 and XIAP in EL 4.pcDNA3-TPPII cells (198). We used the pSUPER system to induce stable siRNA-mediated down regulation of TPPII, and obtained EL-4.wt (empty vector) versus EL-4.TPPII^{i} cells with inhibited TPPII expression (238). We found here that expression of XIAP was substantially higher in EL-4.pSUPER cells than in EL-4.TPPII^{i} cells following treatment with etoposide, in line with previous data (198, 235).

We further tested whether TPPII upregulation during stress was affected by PI3K-like kinases (PIKKs), a family of kinases that controls cellular responses to many kinds of stress. By western blotting analysis of EL-4 lymphoma we found that TPPII expression was increased by γ-irradiation (196, 198, 199), while cells treated with 1uM wortmannin (a PIKK inhibitor) had reduced TPPII expression. Increased TPPII expression during irradiation thereby requires signaling by PIKKs.

It is reported that PIKKs are required to halt DNA synthesis in response to DNA damage (236, 237). We found that high levels of γ-irradiation-resistant DNA synthesis in EL-4.TPPII^{i} cells up to 36 hours after exposure while control EL-4 cells had inhibited DNA synthesis. This suggested that TPPII was important for halting DNA synthesis of EL-4 cells in response to γ-irradiation.

P53 is a transcription factor that coordinates the response to many types of stress. We found that p53 was still synthesized but degraded by the proteasome in EL-4.TPPII^{i} cells using NLVS treatment following γ-irradiation. Further, we also found that p21, a transcriptional target of p53, was weakly expressed in EL-4.TPPII^{i} cells. This phenotype was also displayed in YAC-1, and ALC lymphoma cells. We noted that some of our control tumor cells had substantial levels of p53 prior to γ-irradiation, a phenomenon in line with the frequently up-regulated DNA damage response in transformed cells (239). These data suggested that TPPII expression was required for p53 efficient stabilization.

These data promoted us to test if TPPII was specifically controlled by ATM kinase. We made a stable transfectant cell line denoted EL-4.pSUPER-ATM^{i} which expressed siRNA directed against the 3'region of ATM transcripts. We found that EL-4.ATM^{i}

cells had much reduced expression of TPPII upon γ-irradiation. Previous data show that the substrate of ATM kinase is targeted by BRCA C-terminal repeat (BRCT)-domains (240-242), and we found that one region of TPPII around the GG-doublet at position 725 which match most but not all, requirements of a BRCT motif (240). So we performed site-directed mutagenesis in our pcDNA3-TPPII vector, and expressed this mutated TPPII plasmid in EL-4 cells. This resulted in substantially reduced stabilization of p53. We thereby showed that TPPII is functionally linked to the pathway of ATM kinase signaling.

There are several available catalytic inhibitors of TPPII, e.g. butabindide and AAF-CMK, but they are not suitable for use *in vivo*. We found that a tri-peptide Subtilisin inhibitor Z-Gly-Leu-Ala-OH (Z-GLA-OH) efficiently inhibited TPPII (243). To test if Z-GLA-OH had effects on TPPII inhibition *in vivo* during tumor γ-irradiation, we exposed C57B1/6 mice with established EL-4 tumors to γ-irradiation with doses of 4 Gy and we observed strong tumor regression. We also tested the treatment response to freshly induced tumors; tumors produced by using a retroviral expression system with two separate vectors encoding c-myc and Bcl-X_L. Our data support that the radio-sensitizing effect observed from Z-GLA-OH can be found in cells freshly transformed and was not dependent on specific tumor defects.

Our data strongly support that increased TPPII expression in response to DNA damage requires PIKKs signaling. TPPII controls the interaction between ATM and p53 following γ-irradiation. Our data suggest that TPPII upregulation in cells with reduced proteasomal activity or with high c-myc expression may be due to activation of PIKKs (195, 196, 199). The observed roles for TPPII in the control of IAP stability and centrosome homeostasis (198, 200, 244) may be indirect effects from PIKK signaling (245, 246). This may be important information for the development of novel therapeutic drugs.

Paper IV: Cellular Stress Induces TPPII-Dependent Suppression of the Ubiquitin-proteasome pathway

It has been shown that several forms of stress inhibit the activity of the ubiquitin-proteasome pathway. Our recent data (paper III) suggest that TPPII is important for the transduction of signals from PIKKs. Since PIKKs are important for responses to both starvation and γ-irradiation, we wanted to know whether TPPII was important for inhibition of the ubiquitin-proteasome pathway during stress.

We used EL-4 or Hela cells stably transfected with Green Fluorscent protein (GFP)-reporter substrates (215) to test the rate of proteasomal degradation during cellular stress. We found that exposure of EL-4.Ub-R-GFP and Hela.Ub-GV76-GFP cells to starvation as well as γ-irradiation led to an accumulation of GFP-fluorescence, reaching levels observed during treatment with low concentrations of NLVS. This is consistent with previous reports and suggests reduced activity of the Ubiquitin-proteasome pathway during cellular stress.

We then made EL-4.Ub-GV76-GFP cells co-transfected with pSUPER-TPPII^{i} (paper III, this thesis). We found that the induction of GFP-fluorescence was in part dependent on the expression of TPPII by exposure of cells to γ-irradiation. Further, EL-4.TPPII^{i} cells had a significantly increased ability to proliferate in the presence of proteasomal inhibitor compared to EL-4.pSUPER cells. This suggested that TPPII inhibited the degradation of proteasomal substrates.

To further address whether TPPII reduces proteasomal substrate degradation we studied degradation of a substrate that often resists degradation by the proteasome. We used Ub-R-GFP-Q112 (247) which inhibits proteasomal protein degradation and cause neurodegenerative disease (248-250). We found that EL-4 cells failed to efficiently degrade Ub-R-GFP-Q112, and treatment with NLVS increased accumulation of this substrate. We also used two different catalytic inhibitors of TPPII, Butabindide and Z-GLA-OH (155, 251), to test if they affected stability of the R-GFP-Q112 substrate in EL-4 cells. Out data indicate that the activity of TPPII suppresses their degradation. These data further suggested that TPPII mediated a stress-induced inhibition of the ubiquitin-proteasome pathway.

To study the mechanism behind the down regulation of proteasomal substrate degradation, we did immunocytochemical stainings for proteasomal complexes. We observed a homogenous 19S proteasome staining in the cytosol and nucleus in untreated EL-4.wt and EL-4.TPPII^{i} cells. Further, a strong staining of 19S was present in the nucleus in starved EL-4.wt cells, while in starved EL-4.TPPII^{i} cells, fluorescence failed to go into nucleus. Release from starvation allowed proliferation of most EL-4.wt and EL-4.TPPII^{i} cells, suggesting that these cells were not apoptotic. It is unclear that whether the TPPII controlled proteasomal substrate degradation through regulating sub-cellular localization of 19S regulatory proteasome complexes, but this is certainly a possibility

Further, also by western blotting we found that proteasomes were not biochemically detectable in cytosols from EL-4.wt cells while proteasomes of EL-4.TPPII^{i} cells were cytosolic after starvation. This shift was also found in starved Hela cells and ALL cells. Using TPPII specific inhibitor butabindide we inhibited proteasome nuclear complexes localization during starvation, also in these cells. Further,1μM wortmannin inhibited proteasome translocation into the nucleus, suggesting an involvement of PIKKs in this event. In brief, TPPII is essential for down regulation of proteasomal substrate degradation in response to stress through PIKKs signaling pathway, an event that correlates with translocating proteasomes into the nucleus. Proteasomal complexes were redistributed into the cytosol if the activity of PIKK-family kinases was blocked.

Some reports suggest that modification of sub-units of the 19S proteasome could control the cellular level of proteasome activity. It is suggested that Rad23, to carry ubiquitinated proteins to the proteasome, interacts with S2 (Rpn1) through its ubiquitin-like (Ubl) domain (252, 253). Further more, recent data suggest an ATP-driven dissociation of 19S and 20S complexes, opening another possibility for regulation (254). The process of ubiquitin-dependent protein degradation is thereby controlled by several post-translational mechanisms. Further, it will be interesting to determine whether the recently reported proteasomal ATPase-associated factor 1 (PAAF1) that

associates with sub-units of the 19S complex affects cellular growth (255). Regulating levels of proteasomal activity may turn out as an important mechanism for controlling ubiquitin-dependent processes, in addition to substrate ubiquitin conjugase interactions.

Recently TPPII was found to be the main peptidase to degrade cytosolic polypeptides longer than 15 amino acids, contributes to protein turnover substrates (256). Further, it has been shown that TPPII can allow survival of lymphoma cells with inhibited proteasomal activity due to a significant contribution to cellular protein turnover (195, 196, 199). This study suggests that TPPII is to restrain substrate degradation by the ubiquitin-proteasome pathway in cells with normal proteasomal activity. Subtilisins have many industrial applications, there are commercially available variants of subtilisin (251). This may facilitate studies and design of inhibitors of TPPII (257). Future studies may investigate the therapy resistance in clinical tumors or neuro-degenerative disease, if TPPII inhibitors can be applied for the therapy.

CONCLUDING REMARKS

In this thesis we have studied the role of TPPII and the proteasome in apoptosis signal transduction in tumor cells; and the role of TPPII in signal transduction in responses to cellular stress. The main conclusions are:

1. The production of MHC class I binding peptides is not an unavoidable consequence of intracellular protein turn over. EL-4 ad cells, that grow with inhibited proteasomal activity, were able to avoid a substantial proportion of MHC class I antigen production, especially H-2D^{b} ligands.

2. Apoptosis inhibition can occur via an altered specificity of cytosolic proteolysis. In our case, reduced reliance of proteasomal activity caused stabilization of IAPs and a much inhibited rate of transduction of apoptotic signals. Our data suggest a reversible mechanism in regulation of apoptosis resistance that drives tumor progression *in vivo*.

3. TPPII may be targeted for inhibition of tumor therapy resistance. We observe a strong radio-sensitizing effect from *in vivo* treatment with a TPPII inhibitor. We found low direct toxicity of this TPPII inhibitor, with no or minor effects in the absence of γ-irradiation. Mechanistically TPPII controls responses by PI3K-like kinases.

4. We found that TPPII is induced by cellular stress and required for suppression of the ubiquitin-protesome pathway. Inhibition of TPPII allows degradation of an otherwise degradation-resistant poly-Glutamine substrate.

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