Influence of active nuclear transport on caspase-3 localization and apoptotic chromatin changes

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TABLE OF CONTENTS

1 INTRODUCTION

1.1 Definition and morphology of apoptosis 1

1.2 Pathways leading to apoptosis 2

1.2.1 The extrinsic pathway of apoptosis induction by tumor necrosis factor \( \alpha \) 2

1.2.2 The intrinsic pathway of apoptosis induction 4

1.3 Caspases, the executioners of apoptosis 5

1.3.1 Effector caspase-3 6

1.3.2 Role of caspase-3 in nuclear apoptosis 8

1.4 Inhibitor of apoptosis proteins (IAPs) 8

1.5 Active nuclear transport 9

2 AIMS OF THE THESIS 12

3 MATERIALS AND METHODS 13

3.1 Materials 13

3.1.1 Chemicals and reagents 13

3.1.2 Consumable materials 14

3.1.3 Laboratory equipment and technical devices 14

3.1.4 Cell lines and microorganisms 14

3.1.5 \( \Delta \text{imp}\beta \) construct 14

3.1.6 Cell culture material 15

3.2 Methods 16

3.2.1 Purification of proteins from Saccharomyces cerevisiae 16

3.2.1.1 Transformation of \( S. \text{ cerevisiae} \) using the lithium acetate (LiAc) method 16

3.2.1.2 Protein overexpression in \( S. \text{ cerevisiae} \) 17

3.2.1.3 Lysis of yeast cells 17

3.2.1.4 Anti-FLAG affinity chromatography 18

3.2.2 Purification of proteins from E.coli 19

3.2.2.1 Overexpression of proteins in E. coli 19

3.2.2.2 Purification of His-tagged constructs 19

3.2.2.3 Gelfiltration of caspase-3 constructs 20

3.2.3 Concentration of proteins 21
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.4 Determination of protein concentration by Edelhoch</td>
<td>21</td>
</tr>
<tr>
<td>3.2.5 Linkage of TRITC-BSA with SV40 NLS via Sulfo-SMCC</td>
<td>22</td>
</tr>
<tr>
<td>3.2.6 Measurement of caspase-3 like activity</td>
<td>23</td>
</tr>
<tr>
<td>3.2.7 In vitro caspase activation assay</td>
<td>23</td>
</tr>
<tr>
<td>3.2.8 In vitro inactivation active caspase-3</td>
<td>24</td>
</tr>
<tr>
<td>3.2.9 Cell culture</td>
<td>24</td>
</tr>
<tr>
<td>3.2.10 Microinjection experiments</td>
<td>24</td>
</tr>
<tr>
<td>3.2.10.1 Microinjection of caspase-3</td>
<td>25</td>
</tr>
<tr>
<td>3.2.10.2 Co-microinjection of active caspase-3 with BIR3</td>
<td>25</td>
</tr>
<tr>
<td>3.2.10.3 Inhibition of active nuclear transport by ∆impβ (45-462)</td>
<td>25</td>
</tr>
<tr>
<td>3.2.10.4 Inhibition of active nuclear transport by wheat germ agglutinin</td>
<td>26</td>
</tr>
<tr>
<td>3.2.10.5 Inhibition of active nuclear transport by lowering of the temperature</td>
<td>26</td>
</tr>
<tr>
<td>3.2.10.6 Apoptotic stimulation with cycloheximide and TNFα</td>
<td>27</td>
</tr>
<tr>
<td>3.2.11 TUNEL assay</td>
<td>27</td>
</tr>
<tr>
<td>3.2.12 Microscopic analysis of microinjected cells</td>
<td>28</td>
</tr>
<tr>
<td>3.2.13 Scoring of chromatin condensation</td>
<td>28</td>
</tr>
<tr>
<td>4 RESULTS</td>
<td>29</td>
</tr>
<tr>
<td>4.1 Quality and functionality of purified proteins</td>
<td>29</td>
</tr>
<tr>
<td>4.1.1 Purification of caspase-3 constructs expressed in E. coli</td>
<td>29</td>
</tr>
<tr>
<td>4.1.2 Purification of ∆impβ (45-462) expressed in E. coli</td>
<td>33</td>
</tr>
<tr>
<td>4.1.3 Quality of BIR3 purification from S. cerevisiae</td>
<td>34</td>
</tr>
<tr>
<td>4.2 Intracellular distribution of caspase-3</td>
<td>36</td>
</tr>
<tr>
<td>4.2.1 Active caspase-3 induces apoptotic morphology and translocates into the nucleus</td>
<td>36</td>
</tr>
<tr>
<td>4.2.2 Nuclear translocation of caspase-3 does not depend on caspase-9 activity</td>
<td>38</td>
</tr>
<tr>
<td>4.2.3 Activity of caspase-3 is dispensable for nuclear translocation</td>
<td>39</td>
</tr>
<tr>
<td>4.2.4 The unprocessed form of caspase-3 efficiently enters the nucleus</td>
<td>40</td>
</tr>
<tr>
<td>4.3 Involvement of active transport processes in nuclear apoptosis</td>
<td>43</td>
</tr>
<tr>
<td>4.3.1 Caspase-3 entry into the nucleus depends on the hydrolysis of energy</td>
<td>43</td>
</tr>
<tr>
<td>4.3.2 Interactions with nucleoporins are essential for caspase-3 import</td>
<td>47</td>
</tr>
<tr>
<td>4.3.3 Caspase-3 does not depend on importin β for its translocation</td>
<td>49</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------</td>
</tr>
<tr>
<td>4.4</td>
<td>Inhibition of active transport processes alters nuclear apoptosis</td>
</tr>
<tr>
<td>4.4.1</td>
<td>Effect on chromatin condensation</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Induction of apoptotic DNA strand breaks</td>
</tr>
<tr>
<td>4.5</td>
<td>Inhibition of active transport sensitizes HeLa cells to</td>
</tr>
<tr>
<td></td>
<td>TNFα induced apoptosis</td>
</tr>
<tr>
<td>5</td>
<td>DISCUSSION</td>
</tr>
<tr>
<td>5.1</td>
<td>Rationale and experimental setup</td>
</tr>
<tr>
<td>5.2</td>
<td>Caspase-3 lacking the prodomain rapidly crosses</td>
</tr>
<tr>
<td></td>
<td>into the nucleoplasm</td>
</tr>
<tr>
<td>5.3</td>
<td>Nuclear entry of caspase-3 is mediated by</td>
</tr>
<tr>
<td></td>
<td>active transport processes</td>
</tr>
<tr>
<td>5.4</td>
<td>Active nuclear transport is indispensable for nuclear apoptosis</td>
</tr>
<tr>
<td>5.5</td>
<td>Active nuclear transport is essential for survival following</td>
</tr>
<tr>
<td></td>
<td>TNFα treatment</td>
</tr>
<tr>
<td>6</td>
<td>SUMMARY</td>
</tr>
<tr>
<td>7</td>
<td>ZUSAMMENFASSUNG</td>
</tr>
<tr>
<td>8</td>
<td>REFERENCES</td>
</tr>
<tr>
<td>9</td>
<td>ABBREVIATIONS</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 Definition and morphology of apoptosis

Apoptosis is an evolutionarily conserved physiological process, critically involved in the proper elimination of excessive and harmful cells in development, tissue homeostasis, host defense and disease. The orderly suicide of a cell is triggered by a wide range of stimuli such as growth factor deprivation, UV damage or apoptosis inducing ligands released by cells of the immune system. Regardless of the type of apoptosis inducer, the ensuing chain of events is highly reproducible for different cell lines.

Initially, programmed cell death was described by morphological alterations of the cell. These features include the rounding and shrinkage of the cell body due to the dismantling of the cytoskeleton, the formation of cytoplasmic protrusions (“blebbing”), condensation of chromatin and the loss of adhesion to the substratum. The remains of the cell are neatly packaged into apoptotic bodies. On the biochemical level, DNA cleavage into high molecular weight (~ 50 kb) nucleosomal (~160 bp) fragments, exposure of phosphatidyl serine on the surface of the cell and finally loss of mitochondrial function are induced, resulting in a complete breakdown of cellular function.

In a living organism, apoptotic cells are scarcely observed due to the rapid removal of apoptotic bodies by phagocytic cells. In contrast to the violent rupture of the plasma membrane in necrosis, no cellular contents are initially released from apoptotic cells. Therefore, programmed cell death is characterized by the absence of an inflammatory response.

In a healthy cell, the decision to live or die is constantly updated by the monitoring of pro- and anti-apoptotic signals. Usually, survival signals keep the cellular apoptosis machinery in check. In cases, where this balance is disturbed in favor of either direction, pathological conditions may arise. Resistance to proper apoptotic signals may cause cancer, whereas excessive triggering of the cell death program is associated with neurodegenerative and auto-immune diseases.
1.2 Pathways leading to apoptosis

Induction of apoptosis results in the initiation of a conserved intracellular proteolytic cascade that culminates in the complete collapse of cellular functions. Common effects of apoptotic stimulation are the arrest of cell cycle progression, protein synthesis as well as of DNA repair mechanisms.

Although the signals inducing programmed cell death are numerous and diverse, the modes of signal propagation can be grouped into two major pathways. The binding of death ligands to their respective receptors results in the induction of active cell killing from the outside. In contrast, the reaction to proapoptotic signals from within the cell is triggered by the release of pro-apoptotic factors from mitochondria.

1.2.1 The extrinsic pathway of apoptosis induction by tumor necrosis factor α

Pathogen infected or malignant cells pose a threat to the organism as a whole and have to be efficiently eliminated on the single cell level. The necessary countermeasures are taken by the release of apoptotic effectors by cells of the immune system such as activated macrophages and T cells \(^{13}\).

One of the best characterized extrinsic apoptosis pathways are the processes at TNF-R1. Due to the absence of any enzymatic activity of the receptor, the reaction of the cell to the binding of the ligand TNF\(\alpha\) is controlled by the composition of the assembled signalling complexes.

A defining contribution to the recruitment to the receptor is made by the presence of homotypic interaction domains in the adaptor proteins. The most important in this scenario are the death domains (DD) in the cytoplasmic region of TNF-R1 as well as in TNF-receptor associated DD (TRADD) and Fas associated DD (FADD). FADD is a bifunctional adaptor protein which carries an additional death effector domain (DED) at its N-terminus \(^{14}\).

Aggregation of signalling complexes is induced by the trimerization of the receptor, supported by the trimeric structure of the ligand itself. The ensuing binding of TRADD generates a scaffold for further accumulation of proteins \(^{15}\). Depending on the nature of the factors recruited next, the fate of the cell is decided.

In the case of FADD binding, the DED containing procaspase-8 is directed to this death inducing signalling complex (DISC) \(^{14}\), resulting in the demise of the cell as a consequence of the apoptotic cascade (figure 1.1).
Recruitment of receptor interaction protein (RIP)\textsuperscript{16,17} or TNF-R-associated factor 2 (TRAF-2)\textsuperscript{18} on the other hand, leads to a phosphorylation cascade culminating in the activation of nuclear factor $\kappa$B (NF-$\kappa$B). NF-$\kappa$Bs are a family of transcription factors, expressed in a wide variety of cells. The release of NF-$\kappa$B from its cytoplasmic anchor I$\kappa$B induces its translocation into the nucleus\textsuperscript{19}. The activation of transcription results in an upregulation in the expression of genes involved in cell survival as well as inflammation and immune response\textsuperscript{20}. In various cell types, NF-$\kappa$B is able to repress the induction of apoptosis by TNF$\alpha$\textsuperscript{21}. Therefore, the ligation of TNF$\alpha$ to TNF-R1 induces diametrically opposed effects. Extensive cross-talk between the two pathways\textsuperscript{21} and the general condition of the cell decide which pathway prevails and whether the cell survives or undergoes apoptosis.

Figure 1.1 Intracellular signal transduction following TNF$\alpha$ ligation to TNF-R1
1.2.2 The intrinsic pathway of apoptosis induction

Whereas immune cells trigger the demise of the cell from the outside, a different pathway is activated in response to the perturbation of intracellular homeostasis. At the centre of the execution of this intrinsic pathway are the mitochondria. The sensing of irreparable damage to the cell such as DNA damage following irradiation or exposure to certain chemicals, growth factor withdrawal, or loss of attachment provokes the release of cytochrome c (cyt c) from the intermembrane space $^{22,23}$.

In the presence of dATP, cytoplasmic cyt c binds apoptosis protease activating factor-1 (Apaf-1) $^{24}$. This results in the heptamerization $^{25}$ of Apaf-1 and exposure of the caspase recruitment domain (CARD) following a conformational change $^{26}$. Subsequently, the CARD containing procaspase-9 interacts with the apoptosome and is activated $^{27,28}$ (figure 1.2).

Apart from cytochrome c, several other pro-apoptotic proteins are released from the intermembrane space of the mitochondria. Apoptosis inducing factor (AIF), for example, translocates into the nucleus to induce peripheral chromatin condensation as well as high molecular weight DNA cleavage $^{29-31}$. Another protein implicated in nuclear apoptosis is EndoG, a sequence unspecific DNAs $^{32,33}$, whereas the main
function of Smac/DIABLO \textsuperscript{34,35} and Omi/HtrA2 \textsuperscript{36,37} appears to be to relieve inhibition on the effectors of apoptosis. So far, it remains controversial whether these pro-apoptotic enzymes rely on caspase activity for their release from the mitochondria \textsuperscript{32,38-40}.

The extrinsic and intrinsic activation pathways are linked at the level of mitochondria\textsuperscript{41}. Following the stimulation of the cell by a death ligand, caspase-8 cleaves Bid, a member of the Bcl-2 family. Truncated Bid then translocates to mitochondria, stimulating the release of cyt c and the pro-apoptotic factors mentioned above \textsuperscript{42,43}. Following death ligand stimulation, this pathway is favored in some cell types that do not possess sufficient amounts of caspase-8 \textsuperscript{44}.

### 1.3 Caspases, the executioners of apoptosis

At the center of the apoptotic program is a family of cysteine proteases, the so-called caspases. These evolutionarily conserved proteases are among the most selective endo-peptidases known \textsuperscript{45}. Cleavage is directed by the recognition of a tetrapeptide motif \textsuperscript{46}. Although this motif differs for every member of the family, there is an absolute requirement for an aspartate at the P1 position. The cleavage event is mediated by a nucleophilic cysteine residue in the conserved QACXG catalytic centre \textsuperscript{47,48}. Therefore, the cuts induced by caspases are restricted to a subset of cellular proteins, resulting in the orderly demise of the cell in apoptosis.

Caspases are present in healthy cells as latent precursors thereby protecting the cell from the deleterious effects of these proteases. These zymogens consist of three subunits. An N-terminally situated prodomain of varying size is followed by a large and a small catalytic subunit \textsuperscript{49}. Limited proteolysis is used to dissociate the three subdomains in a sequential two step mechanism. As demonstrated for caspase-3 \textsuperscript{50}, a first cut separates the large and the small subunits, followed by the elimination of the prodomain. The catalytic subdomains are subsequently re-ordered to allow the formation of the catalytic centre. In the mature enzyme, two identical dimers form a heterotetramer with their active sites at opposing ends \textsuperscript{51}. Since the subunits are separated by cleavage after an aspartate residue, the proteolytic maturation and auto-activation appears to be restricted to members of the caspase family. One exception is constituted by granzyme B, a serine protease involved in cell killing by cytotoxic T cells \textsuperscript{52}. 


So far, 11 human caspases have been identified that are similar in sequence and structure. These proteases are classified according to their involvement in the inflammatory response (caspases -1, -4, -5 and -11) or apoptosis. Apoptotic caspases can be further subdivided on the basis of the length of their N-terminal prodomain. Initiator caspases (-2, -8, -9 and -10) all possess long prodomains containing CARD or DED domains, allowing them to engage in homotypic interactions in the DISC or the apoptosome. The induced proximity of the proteins suffices for their transactivation. At the same time, complete proteolytic processing is not a necessary requirement for their activity. Instead, the cleavage is thought to provide stability for the emerging heterotetramer.

In contrast, processing of the executioner caspases-3, -6 and -7 is vital for the induction of activity. Although these proteases are present in the healthy cells as dimers, steric hindrance impedes activity and auto-catalytic cleavage. Due to their short prodomains and their inability to interact with supramolecular complexes, effector caspases rely on their activation by the upstream caspases, providing an additional control point for apoptosis induction.

1.3.1 Effector caspase-3

The described pathways induced by death receptor ligation and intracellular damage both culminate in the activation of caspase-3 by caspase-8 or -9. The central position of this protease within the apoptosis apparatus is emphasized by the ability of caspase-3 to activate other caspases. The cleavage of initiator caspases such as caspase-2, -8, and -9 causes a propagation of the primary signal, resulting in positive feedback loops. Starting from a limited activation of caspase-8 or -9, the ensuing amplification of the signal constitutes an effective means to reach the point of no return in apoptosis. Additionally, downstream effectors such as caspases -6 and -7 are also efficiently activated by caspase-3, leading to the rapid dismantling of cellular structures. Only after this full activation of the apoptotic machinery, the cell will be destined to die.

On the basis of the short prodomain, caspase-3 was classified as an effector caspase. This 3 kDa prodomain was implicated in silencing the activation of the protease, although several amino acids of the p17 catalytic subunit might contribute to this regulatory mechanism.
In a first activation step, the p12 catalytic domain is removed by cleavage at IETD (aa 172 – 175) by upstream caspases. For the protease to achieve full activity, the prodomain is auto-catalytically eliminated from p20 by cleavage after ESMED (aa 25 – 28). Apart from its role as an amplifier for apoptotic signals, caspase-3 is indispensable for the proper execution of apoptosis. Since the majority of the proteins identified as substrates for the apoptotic machinery are cleaved by caspase-3, the protease was termed the central effector in programmed cell death. In the absence of caspase-3, caspase-7 is not capable of substituting for this protease, despite an analogous cleavage preference for DEVD. Caspase-3 knock-out mice die perinatally due to abnormal brain development.

The advantage of cleaving a peptide bond lies in the irreversibility of the induced change. Cleavage of a protein might result in its inactivation, activation or the alteration of its catalytic properties: (I) Caspase-activated DNAse (CAD) is released from its inhibitor ICAD to induce internucleosomal DNA cleavage in the nucleus. (II) Truncation of gelsolin results in constitutive activation leading to the disassembly of the actin cytoskeleton and ensuing plasmamembrane blebbing. (III) Anti-apoptotic Bcl-2 is cleaved to yield a pro-apoptotic fragment that promotes the apoptotic demise of the cell.

To date, more than 280 caspase substrates have been identified in all areas of cellular function such as maintenance of cellular structure, adhesion, DNA synthesis and repair, protein translation, or signal transduction. Therefore, their cleavage leads to a reliable breakdown of the cell in response to apoptotic stimulation.
1.3.2 Role of caspase-3 in nuclear apoptosis

Caspase-3 is involved in the cleavage of a multitude of substrates. Thus, the absence of its activity results in alterations of the apoptotic phenotype, especially in the nucleus. Cells lacking a functional caspase-3 gene fail to display regular apoptotic features such as plasma membrane blebbing, chromatin condensation or DNA fragmentation.\textsuperscript{128-130}

Apart from the involvement in the morphological aspects of nuclear apoptosis, caspase-3 targets numerous proteins in the nuclear compartment. Among its substrates are proteins with important roles in the maintenance of genomic stability (PARP\textsuperscript{131}, DNA-PK\textsubscript{CS}\textsuperscript{132}), DNA synthesis (DNA polymerase \(\varepsilon\)\textsuperscript{133}), replication (replication factor C large subunit\textsuperscript{134}) and RNA splicing (U1 snRNP\textsuperscript{135}).

Although there is ample evidence for caspase-3 mediated cleavage events in the nuclear compartment, little is known about the propagation of the apoptotic signal into the nucleus. In healthy cells, caspase-3 displays a mainly cytosolic distribution. Following apoptotic stimulation, a fraction of the protease enters the nuclear compartment\textsuperscript{136-138}. Whether this relocation relies on changes in the permeability of the nuclear pores as a result of nucleoporin cleavage\textsuperscript{139-141} or on active transport processes\textsuperscript{142} remains to be determined.

1.4 Inhibitor of apoptosis proteins (IAPs)

Although caspases are regulated by their proteolytic activation on a primary level, it is the binding and inhibition by inhibitors that is the back-up system to suppress unwarranted caspase activity in a cell. Originally discovered in baculoviruses\textsuperscript{75}, the evolutionarily conserved members of the inhibitor of apoptosis protein (IAP) family were identified to be potent regulators of apoptosis in response to both the intrinsic and extrinsic pathway\textsuperscript{76}. Classification as a member of the IAP family depends on the presence of one to three baculoviral IAP repeats (BIR). These domains of roughly 70 residues folded around a zinc atom were found to be essential for the modulation of apoptosis by IAPs\textsuperscript{77}. Apart from the BIRs, some IAPs possess a carboxy-terminal RING domain which exerts E3 ligase activity. RING containing proteins are therefore capable of auto-ubiquitination, as well as targeting selected proteins for degradation by the proteasome\textsuperscript{78-80}. 
So far, eight human IAPs have been identified with the most important being the structurally homologous cIAP-1, cIAP-2 and XIAP. The composition of all three is characterized by three tandem repeats of BIR and a RING domain. Among the IAP family members, the 57 kDa XIAP is the most potent inhibitor of cell death. Its protective effects have been mainly attributed to its ability to physically interact with caspases, namely caspases -3, -7 and -9. Structure-function analysis revealed the linker region between BIR1 and BIR2 to be necessary and sufficient for inhibition of active caspases-3 and -7, although by different modes. In a similar way, the BIR3 domain is able to eliminate caspase-9 activity by binding to both the zymogen as well as the processed protease.

Following apoptosis induction by Fas, XIAP is cleaved in a caspase-3 dependent manner into two distinct fragments that exhibit a significantly reduced capacity to suppress apoptosis. The anti-apoptotic potential might be further reduced by degradation of the emerging BIR1/2 domain. At the same time, XIAP function is negatively regulated by pro-apoptotic factors such as Smac and Omi/HtrA2 that are released from mitochondria in response to apoptotic stimuli.

1.5 Active nuclear transport

Eucaryotes are characterized by the sequestration of their genome into the nucleus. The spatial separation of transcription and translation poses the daunting logistical problem of managing a constant flow of proteins, RNA and even huge particles such as the ribosomes between the cytosolic and nuclear compartments. Entry into the nucleus efficiently takes place through the nuclear pore complexes (NPCs). These protein complexes of 125 MDa are composed of approximately 30 nucleoporins which are arranged in an eightfold symmetrical pattern and span the nuclear membrane. A single pore can accommodate up to 1,000 translocation events per second, demonstrating the high transport capacity of this system.

In contrast to the transport into other cellular compartments, the proteins enter the nucleus in their folded state through the nuclear pore complexes. The translocation through the single channel of the pore can occur via different pathways. Small molecules and proteins up to 40 kDa are not slowed down in their transit and pass through the pore by passive diffusion. For larger proteins, the NPC presents a tight barrier. Entry into the nuclear compartment depends on the presence of a proper import signal in the primary sequence.
Whereas the mode of translocation remains an ongoing subject for dispute, the main characteristics and mechanisms are well understood. One defining feature of active nuclear transport is the requirement for energy. This energy for translocation is provided by the small GTPase Ran that possesses low intrinsic GTPase activity. Regulation of GTP hydrolysis is conferred by RanGAP1 that is bound to the cytoplasmic fibrils of the NPC. In the nucleus, the exchange of GDP to GTP is facilitated by the RanGEF RCC1, which is immobilized in the nucleoplasm by binding to chromatin. This spatial distribution of the Ran regulating proteins leads to a steep gradient of RanGTP across the nuclear membrane that is vital for the maintenance of transport capacity.

**Figure 1.3** Nuclear-cytoplasmic transport of proteins containing a classical nuclear localization signal

- α: importin α
- β: importin β
- NLS: nuclear localization signal

One of the best characterized pathways is importin β mediated nuclear import for proteins that harbor a classical nuclear localization signal (NLS). These NLSs consist of one or two short stretches of basic amino acids. Importin α serves as a binding protein for the NLS bearing cargo and as an adaptor for the binding to importin β via the IBB domain. Crossing of the nuclear barrier is mediated by the interaction of importin β with the nucleoporins. On the nucleoplasmic side,
RanGTP serves to release the import complex from Nup153\textsuperscript{116} and displaces importin $\alpha$ from importin $\beta$\textsuperscript{117}. Subsequently, the cargo and importin $\alpha$ dissociate\textsuperscript{118}. The recycling of the import factors\textsuperscript{107,119} to the cytoplasm and RanGDP\textsuperscript{120} to the nucleus then primes the system for another round of transport (figure 1.3).

Apart from the importin mediated import, other pathways for nuclear accumulation of proteins were reported. For instance, the related transportin faithfully translocates A1 hnRNP into the nucleoplasm by binding to its M9 domain\textsuperscript{121,122}. Furthermore, the adaptor function of importin $\alpha$ is dispensable for cargo binding in some cases and importin $\beta$ can interact directly with cargo\textsuperscript{123,124}. Other proteins such as $\beta$ catenin rely on their interaction with the nucleoporins for their translocation\textsuperscript{125,126}.

A mechanism independent of cytosolic factors and GTP has been reported for the phorbol ester induced translocation of protein kinase C $\alpha$. Surprisingly, the translocation of the kinase depends on intact cytoskeleton\textsuperscript{127}. Most of these unconventional import pathways rely on poorly understood NLSs and to date unidentified import factors, signifying that there are more possibilities to enter the nucleus than the classical importin $\beta$ pathway.
2 AIMS OF THE THESIS

Caspase-3 is one of the central effectors of programmed cell death. Many features associated with apoptosis are directly connected to the activity of this protease. A subset of the numerous substrates identified for caspase-3 so far exhibit a nuclear localization. Although procaspase-3 is a mainly cytoplasmic protein, nuclear levels of the protease increase after induction of apoptosis.

Taking the absence of a consensus NLS in its primary sequence of caspase-3 into consideration, the mode of translocation into the nuclear compartment is unclear and remains the subject of ongoing discussion. Passive diffusion might ensue following the proteolytic degradation of constituents of the nuclear pore in apoptosis. However, prevention of active transport results in aberrant nuclear apoptosis, similar to that observed in caspase-3 deficient cells.

The aim of this thesis was to contribute to the understanding of the mechanisms involved in caspase-3 translocation into the nucleus. After the establishment of expression and purification conditions for recombinant caspase-3 constructs, microinjection was chosen as the experimental system for the examination of the:

(I) general intracellular distribution, steady-state levels and time course of caspase-3 accumulation in the nucleoplasm

(II) impact of proteolytic activity of caspases-3 and -9 as well as of the processing state of caspase-3 on subcellular localization

(III) involvement of active transport processes by employing different inhibitory conditions.

In addition to the localization of recombinant caspase-3, another objective was the study of general effects of active transport inhibition in apoptosis induced by either caspase-3 microinjection or exposure to the death ligand TNFα.
3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

Bachem (Weil am Rhein, Germany): benzyloxy carbonyl-Asp[OMe]-Glu-[OMe]Val-Asp[OMe]-CH$_2$F (z-DEVD-fmk); Biomol (Hamburg, Germany): Dithiothreitol (DTT), N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (DEVD-afc); BD Biosciences (Heidelberg, Germany): Bacto agar, yeast nitrogen base; Biorad (Munich, Germany): Bio-Rad protein assay reagent; ICN Biomedicals (Ohio, USA): 3-[(3-cholamidopropyl)-dimethyl ammonio]-1-propanesulfonate (CHAPS), N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES); Innogenetics (Ghent, Belgium): recombinant mouse TNF$_\alpha$; Interactiva (Ulm, Germany): SV40-NLS peptide (CYTPPKKKRKV); Merck (Darmstadt, Germany): β-mercapto ethanol, imidazole; Molecular Probes (Leiden, Netherlands): Hoechst 33342, Dextran40 Texas Red (TR); Pierce (Rockford, USA): Sulfo succinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate (Sulfo-SMCC); Polysciences (Eppelheim, Germany): Aqua/Poly Mount; Riedel de-Haen (Seelze, Germany): Dimethylsulfoxid (DMSO), paraformaldehyde (PFA); Roche (Mannheim, Germany): Pefabloc SC, In situ Cell Death Detection Kit (TMR red); Roth (Karlsruhe, Germany): Tryptone Peptone (pancreatic digest), yeast extract, Ethylenediaminetetraacetate (EDTA); Serva (Heidelberg, Germany): Coomassie brilliant blue R250, polyethylene glycol 4000 (PEG 4000); Sigma-Aldrich (Deisenhofen, Germany): amino acids, cycloheximide (CHX), cytochrome c (cyt c), dATP, FLAG affinity matrix, Isopropyl-β-D-Thiogalactopyranoside (IPTG), Tetramethylrhodamineisothiocyanate-bovine serum albumin (TRITC-BSA), wheat germ agglutinin (WGA), lactic acid, lysozyme, aprotinin, cycloheximide, Dimethyl sulfoxide (DMSO), leupeptin, pepstatin, Tris, Triton X-100, glycine; Uptima (Montlucon, France): Pierce BCA protein assay reagent.

All standard chemicals were purchased primarily from Sigma-Aldrich (Deisenhofen, Germany).
3.1.2 Consumable materials
Borosilicate glass capillaries (1.2 mm OD x 0.94 mm ID) (Clark electromedical instruments, Reading, UK); glass beads (0.25 – 0.5 mm) (Roth, Karlsruhe, Germany); cell culture flasks, plastic pipettes and 96 well plates (Greiner, Frickenhausen, Germany); 35 mm cell culture plates (Corning, Schiphol-Rijk, Netherlands); glass cover slips (VWR, Vienna, Austria); microloaders (Eppendorf, Hamburg, Germany), Amicon and Ultrafree ultrafiltration devices (Millipore, Eschborn, Germany)

3.1.3 Laboratory equipment and technical devices
Centrifuges: Eppendorf 5417R (Netheler & Hinz, Hamburg, Germany), Beckmann GS-6KR (Beckmann Coulter, Krefeld, Germany), Sorvall RC 28S (Kendro Laboratory Products, Langenselbold, Germany); Digital camera: Hamamatsu Digital Camera C 4742-95, (Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany); Fluorometer: Wallac Victor² Multilabel Counter (Wallac Instruments, Turku, Finland); FPLC and IMAC equipment: His-Trap™, G25, Superdex™ G75, Äkta™ FPLC (Amersham Biosciences, Freiburg, Germany); Imaging software: Aquacosmos 1.0 and 2.0 (Hamamatsu Photonics, Japan), Zeiss LSM Image Examiner Version 3,2,0,70 (Zeiss, Oberkochen, Germany); Microinjection equipment: Micromanipulator 5171, Transjector 5246 (Eppendorf, Hamburg, Germany), P-97 micropipette puller (Sutter instruments, Novato, CA, USA); Microscopes: Axiovert 25, Axiovert 100, LSM 510 Meta (Zeiss, Oberkochen, Germany); Photometers: SLT Spektra rainbow photometer (SLT instruments, Crailsheim, Germany), Gene Quant RNA/ DNA Calculator Pharmacia, Cambridge, GB)

3.1.4 Cell lines and microorganisms
Adherent HeLa cervix carcinoma cells were purchased from the DMSZ (Braunschweig, Germany)
E.coli M15 [pRep4] were obtained from Qiagen (Hilden, Germany)

3.1.5 Δimpβ construct
Δimpβ (45-462) in pQE60 was kindly provided by D. Görlich (Heidelberg, Germany)
3.1.6 Cell culture material

PBS, Eagle’s MEM, Trypsin-EDTA (PAA, Cölbe, Germany); Penicillin-Streptomycin (Gibco BRL Life Technologies, Eggenstein, Germany); fetal calf serum (FCS) (Boehringer Mannheim GmbH, Mannheim, Germany)
3.2 Methods

3.2.1 Purification of proteins from Saccharomyces cerevisiae

3.2.1.1 Transformation of S. cerevisiae using the lithium acetate (LiAc) method

For transformation, one clone of the uracil and leucin synthesis deficient strain NY40 was grown in 5 ml YEPD medium over night at 30°C. With this preparatory culture 25 ml of YEPD medium was inoculated and the cells incubated in a shaker at 30°C. In the logarithmic growth phase (OD₆₀₀ 0.8), a 5 ml aliquot of cells was harvested by centrifugation for 3 minutes at 3,700 rpm and 4°C. The cells were washed twice in TE buffer and then resuspended in 500 µl LiAc. To obtain competent cells, the aliquots were incubated in a shaker for 60 minutes at 30°C. For transformation, 140 µl of yeast culture was gently mixed with 10 µl of plasmid solution (5 – 10 µg). As a control, a second aliquot was transferred to 10 µl of H₂O. After 30 minutes in the incubator, 350 µl of PEG 4000 were added and the cells incubated for 60 minutes. Efficient uptake of the DNA was achieved by heat-shocking the cells for 5 minutes at 42°C. The cells were cooled to room temperature and diluted with 500 µl H₂O. After sedimentation for 2 minutes at 2,000 rpm, the pellet was washed twice with 1 ml H₂O to free the cells of LiAc and PEG. For plating, the pellet was resuspended in 100 µl of H₂O and 10 µl dispersed on selective SCD-ura plates. Colonies were visible after 3 to 4 days.

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>YEPD</td>
<td>1% yeast extract, 2% bacto-peptone, 4% glucose</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris, 1 mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>LiAc</td>
<td>0.2 M in TE buffer</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>50% in H₂O</td>
</tr>
<tr>
<td>10x amino acid mix –ura</td>
<td>2×10⁻² % adenine, histidine, tryptophan, proline, arginine, methionine</td>
</tr>
<tr>
<td></td>
<td>3×10⁻² % isoleucine, tyrosine, lysine, leucine</td>
</tr>
<tr>
<td></td>
<td>5×10⁻² % phenylalanine</td>
</tr>
<tr>
<td></td>
<td>0.1 % glutamic acid, valine, threonine, serine, aspartic acid</td>
</tr>
<tr>
<td>SCD-ura (plates)</td>
<td>0.67% yeast nitrogen base, 4% glucose, 10% v/v 10x amino acid mix –ura,</td>
</tr>
<tr>
<td></td>
<td>(1.5% agar agar)</td>
</tr>
</tbody>
</table>
3.2.1.2 Protein overexpression in S. cerevisiae

For the overexpression of recombinant proteins in yeast, the natural preference for glucose is taken advantage of. Only after depletion of this preferred energy source, galactose metabolism is induced. The required proteins are synthesized de novo. Thus, using these promoters, recombinant proteins are transcribed only in the presence of galactose.

Reagents

10x amino acid mix –ura
- 2\times 10^{-2} \% adenine, histidine, tryptophan, proline, arginine, methionine
- 3\times 10^{-2} \% isoleucine, tyrosine, lysine, leucine
- 5\times 10^{-2} \% phenylalanine
- 0.1 \% glutamic acid, valine, threonine, serine, aspartic acid

SCD-ura
- 0.67\% yeast nitrogen base, 4\% glucose,
- 10\% 10x amino acid mix –ura

SCGL-ura
- 0.67 \% yeast nitrogen base, 0.1\% glucose, 3\% glycerol,
- 2\% lactic acid, 10\% 10x amino acid mix –ura (pH 5-6)

YPGLA
- 1\% yeast extract, 2\% peptone, 0.2\% glucose,
- 3\% glycerol, 2\% lactic acid, 2\times 10^{-3} \% adenine (pH 5-6)

For efficient overexpression, two newly transformed clones were grown at 30°C overnight in 5 ml SCD-ura selective medium. This culture was diluted 1:4 in SCD-ura medium and grown for an additional 8 hours. Then, an OD$_{600}$ of 0.04 was adjusted in 200 ml of SCGL-ura and the cells grown over night. Then, an OD$_{600}$ of 1 was adjusted with SCGL-ura and the culture diluted 1:1 in YPGLA medium afterwards. After shaking for 3 hours, overexpression was induced by the addition of 8 g of galactose. The cells were harvested 5 hours later by centrifugation for 30 minutes at 4,500 rpm and 4°C. Washing of the pellet with 100 mM Tris/SO$_4$ (pH 9.2) was followed by another centrifugation step of 5 minutes at 3,000 rpm. For subsequent purification, the pellet was stored at -70°C.

3.2.1.3 Lysis of yeast cells

In order to avoid degradation of the protein, all purification steps were conducted on ice or in the cold room. The pellet was gently resuspended in 2.5 ml of resuspension buffer in the presence of protease inhibitors. The cell membranes were mechanically disrupted by vortexing seven times with 2.5 ml of washed glass beads for one minute each. After every vortexing cycle, the cells were kept on ice for one minute to cool
the suspension. The lysate was cleared by centrifugation for 30 minutes at 16,500 rpm and 4°C.

**Reagents**

- **Resuspension buffer**: 50 mM Tris/HCl, 400 mM NaCl, 0.5 mM PMSF (pH 7.9)
- **Protease inhibitor mix (1:100)**: 1 µg/ml Pepstatin A, 1 µg/ml Aprotinin, 1 µg/ml Leupeptin, 1 mM benzamidine

### 3.2.1.4 Anti-FLAG affinity chromatography

The artificial epitope (DYKDDDDK) is recognized by commercially available ANTI-FLAG® antibodies. For purification of the FLAG-tagged proteins, lysis of the cells was immediately followed by affinity chromatography using the M2 monoclonal antibody.

**Reagents**

- **TBS**: 50 mM Tris, 150 mM NaCl (pH 7.4)
- **Elution buffer**: 100 mM glycine (pH 3.5)
- **Neutralization buffer**: 1 M Tris/HCl (pH 7.5)

The affinity gel was cleaned by rinsing with 6 bed volumes (30 ml) of TBS, 1 bed volume (5 ml) of elution buffer and another 30 ml of TBS. Before loading, 5 ml of water were added to every 3 ml of yeast extract. The extract was recirculated eight times and the matrix resuspended every other time to ensure optimal binding. After a washing step with 6 bed volumes of TBS, 2 ml of elution buffer were added to the column and the matrix resuspended. After four minutes, when the matrix had sedimented, 500 µl fractions were collected in Eppendorf tubes containing 50 µl neutralization buffer. In order to achieve a quick shift in pH, the cup was inverted immediately. The column was washed with 30 ml TBS and stored in 0.1% sodium azide.

The fractions containing the highest amount of protein were pooled and concentrated to a volume of 50 µl using ultrafiltration. An exchange of buffer was achieved by addition of 1 ml PBS and followed by another round of ultrafiltration.
3.2.2 Purification of proteins from E.coli

3.2.2.1 Overexpression of proteins in E. coli

For overexpression, the pQE constructs were transformed into E.coli M15 [pRep4]. This expression strain carries the laqI repressor on a separate plasmid resulting in tight regulation of transcription of the protein of interest. Using an overnight culture, an OD\textsubscript{600} of 0.2 was adjusted in a suitable volume of selective LB. The cells were grown to a density of OD\textsubscript{600} 0.6 to 0.8 at 37°C in a shaker. With the addition of IPTG to a final concentration of 200 µM the expression of the protein of interest was induced. The cells were grown for an additional 3 hours (caspase-3 constructs) or for 4 hours (∆impβ) at 30°C. Alternatively, the expression of caspase-3 constructs was induced by 20 µM IPTG and allowed to proceed for 22 hours at 16°C. The bacteria were harvested by centrifugation at 5,000 rpm using a GS-3 rotor. The bacterial pellets were stored at -70°C.

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>1 % tryptone peptone, 1% NaCl, 0.5 % yeast extract, 0.5 % NaOH (1N)</td>
</tr>
<tr>
<td>IPTG</td>
<td>1 M stock solution</td>
</tr>
</tbody>
</table>

3.2.2.2 Purification of His-tagged constructs

Purification of polyhistidine-containing (6xHis-tagged) proteins is achieved by immobilized metal affinity chromatography (IMAC). The purification properties of the Ni-NTA (nickel-nitrilotriacetic acid) resin is based on the high affinity of the histidine residues for the immobilized nickel ions.

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>50mM NaH\textsubscript{2}PO\textsubscript{4}•H\textsubscript{2}O, 300mM NaCl, 10mM imidazol (pH 8.0)</td>
</tr>
<tr>
<td>Binding buffer</td>
<td>10 mM Na\textsubscript{2}HPO\textsubscript{4}•2H\textsubscript{2}O, 10mM NaH\textsubscript{2}PO\textsubscript{4}•H\textsubscript{2}O, 0.5 M NaCl, (pH 7.4)</td>
</tr>
<tr>
<td>Imidazole</td>
<td>2 M (pH 7.4)</td>
</tr>
</tbody>
</table>

The bacterial pellet was carefully resuspended in 20 ml of phosphate buffer. To avoid degradation of the overexpressed product, the serine protease inhibitor Pefabloc SC was added to a final concentration of 1 mM. After 30 minutes of incubation on ice in the presence of lysozyme (1 mg/ml), the bacteria were mechanically broken open by sonification. Clogging of the His-Trap\textsuperscript{™} column was avoided by pelleting cellular debris by centrifugation at 16,000 rpm at 4°C. The supernatant was loaded on a His-
Trap™ column equilibrated with 10 ml of binding buffer with 10 mM imidazole at room temperature. Used at low concentrations, the histidine analogue imidazole, reduces the binding of non-tagged host cell proteins thereby increasing the selectivity for the His-tagged proteins. After loading, the column was washed with 10 ml of the binding buffer followed by a more stringent washing step with 4 ml of binding buffer plus 60 mM of imidazole. An increase in the concentration of imidazole to 500 mM led to effective displacement resulting in the elution of the His-tagged proteins. Due to their colour, GFP-tagged proteins allowed for the collection of the protein peak in a single fraction of roughly 1 ml. Otherwise, fractions of 1 ml were collected and screened qualitatively in a Bradford assay for the highest protein concentration.

3.2.2.3 Gelfiltration of caspase-3 constructs

Gel filtration chromatography is a method capable of separating complex protein mixtures primarily based on molecular size, making it the technique of choice for the purification of biological macromolecules. For the separation of caspase-3 from unwanted by-products, a Superdex G75 (16/60) column was chosen as the stationary phase. Separation is achieved by the capacity of smaller molecules to enter the three dimensional meshwork of pores, resulting in their retardation. At the same time, large molecules are efficiently excluded, not retained and therefore travel more quickly with the mobile phase. The gelfiltration column was part of an FPLC (fast protein liquid chromatography) system.

Reagents
Phosphate buffer 66mM sodium phosphate, 0.5 mM EDTA (pH 7.6), filtrated and sonified

Gelfiltration was performed at 4°C to avoid degradation in the protein sample. The column was equilibrated with one bed volume of phosphate buffer at a flow rate of 1.0 ml/ min. The content of protein was constantly monitored by measurement of the absorption at 280 nm and fractions of 1.5 ml collected. For concentration steps, peak fractions containing the full length caspase-3 fusion protein were pooled.
3.2.3 Concentration of proteins

A fast and convenient method for the concentration of dilute protein solutions is the application of ultrafiltration. In this directional process involving semi-permeable membranes, centrifugation provides the driving force to press solvent through the membrane thereby resulting in the concentration of the retained macromolecules. Depending on the volume and desired end concentration, the sample was centrifuged in a swing bucket rotor at 2,000 rpm at 4°C for various times. The concentration of caspase-3 proteins was determined by BCA assay, adjusted to 6.9 mg/ml (100 µM) and the protein stored at -70°C. In order to remove the high salt buffer in the ∆impβ preparation, 2 ml of PBS were added to the fraction containing the highest protein concentration and the solution subjected to ultrafiltration. This procedure was repeated four times before the protein concentration was determined and aliquots stored at -70°C.

3.2.4 Determination of protein concentration by Edelhoch

The absorbance of a protein above 275 nm depends on its content of the aromatic amino acids tryptophan, tyrosine, and to a lesser extent cystines (disulfide bonds). Therefore, if the primary sequence is known, the specific extinction coefficient at 280 nm can be calculated as follows:

\[ \varepsilon_{280\text{nm}} = 5,500 \cdot n_{\text{Trp}} + 1,490 \cdot n_{\text{Tyr}} + 125 \cdot n_{\text{cystine}} \, [\text{M}^{-1} \cdot \text{cm}^{-1}] \]

For ∆impβ (45-462) the following numbers of residues were determined:

\[ n_{\text{Trp}} = 6; \, n_{\text{Tyr}} = 12; \, n_{\text{Cys}} = 12 \]

For the calculation of the coefficient, \( n_{\text{cystine}} \) was not taken into account since the number of disulfide bonds formed was not known. The maximum error by this omission amounted to 1.5 percent and was therefore negligible.

\[ \varepsilon_{280\text{nm}} (\Delta\text{imp}\beta) = 50,880 \, \text{M}^{-1} \cdot \text{cm}^{-1} \]

\( A_{280\text{nm}} \) of the protein was measured in PBS and the concentration calculated by Lambert-Beer.
3.2.5 Linkage of TRITC-BSA with SV40 NLS via Sulfo-SMCC

A widely used positive control for active nuclear import is a conjugate of the 67 kDa BSA with the strong viral SV40-NLS. This particular NLS consists of a stretch of five basic residues that are recognized by importin α. The subsequent translocation into the nucleus is mediated via the classical importin β pathway.

Red-fluorescent, commercially available TRITC-BSA was conjugated with the SV40-NLS peptide (CYTPPKKKRKV) using the heterobifunctional cross-linker Sulfo-SMCC.

**Reagents**

- TRITC-BSA 20 mg/ml in H₂O
- Sulfo-SMCC 5 mg/ml in H₂O
- SV40-NLS 7.8 mg/ml in 50 mM Tris (pH 7.0)
- Phosphate buffer 0.1 M Na phosphate (pH 7.0)
- PBS 137 mM NaCl, 13 mM Na₂HPO₄ • 2H₂O, 3 mM KH₂PO₄ (pH 7.4)

Due to the light sensitivity of the TRITC group, all reactions had to be performed in dark reaction tubes. For the primary reaction, TRITC-BSA and Sulfo-SMCC were mixed at a ratio of 4 mg to 1 mg and incubated in a shaker at 37°C for 45 minutes. Excess amounts of Sulfo-SMCC as well as free TRITC were separated from the protein by gel filtration with a G25 column equilibrated with phosphate buffer. The pink TRITC-BSA was collected in a fraction of 850 µl. For the linkage of the SV40-NLS, peptide was added to the TRITC-BSA solution in a ratio of 2 mg BSA to 1 mg SV40-NLS. PBS was added to a final volume of 3 ml and the sample rotated over night at 4°C. After concentration to 200 µl by ultrafiltration, remaining reactive groups were saturated by the addition of 4 µl β-mercapto ethanol and incubation for 45 minutes at room temperature. For the removal of this reducing agent, 2 cycles of dilution with 5 ml PBS and ultrafiltration ensued. The final concentration of TRITC-BSA-NLS (NLS-BSA) was adjusted to 3 mg/ml and aliquots stored at -20°C.
**MATERIALS AND METHODS**

### 3.2.6 Measurement of caspase-3 like activity

A fluorometric caspase assay was employed to detect the cleavage of DEVD-afc in cell lysates or of the purified protease. Upon excitation at $\lambda = 390$ nm free afc emits a yellow-green fluorescence that is detected at $\lambda = 505$ nm using a fluorometer (Wallac Victor). The rate of increase in fluorescence correlates directly with the amount of active protease.

#### Reagents

- **Dilution buffer**: 1% saccharose, 0.1% CHAPS, 50 mM HEPES
- **DTT**: 1 M
- **DEVD-afc**: 8 mM in DMSO

Purified, active caspase-3 was diluted 1:1,000 or, if necessary, 1:10,000 in cold dilution buffer. A maximum of 10 µl was transferred in duplicates to a 96 well plate. After the addition of 90 µl dilution buffer containing 10 mM DTT and 50 µM DEVD-afc, the measurement was started. Relative light units were determined 5 times at 5 minute intervals.

### 3.2.7 In vitro caspase activation assay

A useful method for the testing of inhibitors for caspases-9 and -3 is the reconstitution of the intrinsic apoptosis pathway in vitro. For the assembly of the apoptosome in a cell free system, exogenous dATP and cytochrome c have to be added to cytosolic preparation. As a result, caspase-9 is activated and in turn cleaves caspase-3. The activity of the latter protease is quantified by DEVD-afc cleavage. The preparation of cytosolic extract was performed as described previously.

#### Reagents

- **Cytosol (S-100)**: concentration > 9 mg/ml
- **Cytochrome c**: 100 µM stock, 10 µM final concentration in H$_2$O
- **dATP**: 10 mM, 2 mM final concentration in H$_2$O

6.5 µl of cell lysate was mixed with 3 µl dATP and 1.5 µl cytochrome c. The final reaction volume of 15 µl was adjusted by the addition of H$_2$O or inhibitor. After an incubation of 30 minutes at 30°C, duplicates of 5 µl were transferred to a 96-well plate and DEVD-afc cleavage measured.
3.2.8 In vitro inactivation active caspase-3

For inhibition of caspase activity, their substrate specificity is exploited. The substrate recognition sequence for caspase-3 is DEVD. z-DEVD-fmk is a non-cleavable analogue of the caspase-3 substrate recognition sequence and binds irreversibly to the active site, resulting in non-competitive inhibition of the protease.

Reagents
z-DEVDfmk 10 mM in DMSO (stock), diluted in PBS
DTT 1 M in H$_2$O

500 μM DEVD-fmk was added to 4 mg of recombinant active caspase-3 (8.6 molar excess of the inhibitor). As a stabilizer 13 mM DTT was included allowing for optimal accessibility of the active site for the inhibitor. The inhibition mix was incubated over night at 4°C. Measurement of residual DEVD-afc cleavage confirmed the reduction in caspase-3 activity.

3.2.9 Cell culture

HeLa cells were grown in Eagle’s MEM containing 10% heat inactivated FCS and 10% penicillin/ streptomycin at 37°C in an incubator with 5% CO$_2$ in a humidified atmosphere and passaged every other day by eightfold dilution into fresh medium. For microinjection experiments, the cells were plated on sterilized glass cover slips (16 mm diameter) at a density of 4•10$^4$ cells/ cm$^2$ for experiments on the following and 2•10$^4$ cells/ cm$^2$ for experiments on the second day.

3.2.10 Microinjection experiments

Before microinjection, the cells were transferred to 35 mm dishes containing 2 ml medium supplemented with 25 mM HEPES, pH 7.2. Unlike the bicarbonate buffering system that requires the use of a CO$_2$ incubator, the HEPES buffering system can be used without CO$_2$. Therefore, this system was chosen to protect the cells from changes in pH during the microinjection procedure. In order to prevent clogging of the capillary, the respective protein solutions were centrifuged for 10 minutes at 13,000 rpm at 4°C in a table top centrifuge and the supernatant transferred to a separate cup.
Generally, more than 100 cells were injected on each cover slip, except when the set time limit was exceeded. The injections were carried out on a heated stage under visual control. The cells were incubated at 37°C for the desired period with $t_0$ being the time of the injection of the first cell.

15 minutes before the end of the incubation, the cells were stained with Hoechst 33342 (0.6 mg/ml final concentration), a cell-permeable, minor groove–binding DNA stain that fluoresces upon binding to DNA. Then, the cells were fixed in 3% PFA in PBS for 15 minutes at room temperature and the cover slips mounted with Aqua/Poly-Mount. If the non-fixable Dextran40 Texas Red (Dextran40) was included in the experiments, the fixation process had to be foregone. As an alternative, the slides were transferred to 4°C after mounting for 30 minutes in order to stop cellular processes.

3.2.10.1 Microinjection of caspase-3

Caspase-3 (2.9 mg/ml), DEVD-fmk treated caspase-3 (2.9 mg/ml) and its active site mutant (3.4 mg/ml) were co-microinjected with Dextran40 (1 - 2 mg/ml) for better microscopic visualization. For the study of the time course of caspase-3 take-up into the nuclear compartment, the cells were incubated for a total of 15, 30 or 60 minutes respectively. The very short incubation time of 15 minutes required the addition of Hoechst 33342 directly to the HEPES buffered medium and microinjection was allowed to proceed for a maximum of 10 minutes.

3.2.10.2 Co-microinjection of active caspase-3 with BIR3

BIR3 (0.9 mg/ml) was co-microinjected with active caspase-3 (3 mg/ml) and Dextran40 (2 mg/ml). For the study of the time course of caspase-3 entry into the nucleus, the cells were treated as described in 3.2.10.1.

3.2.10.3 Inhibition of active nuclear transport by $\Delta$imp$\beta$ (45-462)

Recombinant caspase-3 (2.9 mg/ml) was co-microinjected with $\Delta$imp$\beta$ (20 mg/ml) and Dextran40 (1 mg/ml) and incubated for 60 minutes at 37°C. For the investigation of DNA strand breaks (TUNEL), Dextran40 was omitted and the incubation period extended to 3 hours.
For the assessment of the role of active nuclear transport in apoptosis, the cells were microinjected with \( \Delta \text{imp}\beta \) (20 mg/ml) and Dextran40 TR to allow for microscopic detection. The injection was followed by incubation with TNF\( \alpha \) (100 ng/ml) for 3 hours with or without pre-incubation with CHX (100 \( \mu \)M).

3.2.10.4  **Inhibition of active nuclear transport by wheat germ agglutinin**

Active transport processes can be completely interrupted using the lectin wheat germ agglutinin (WGA). In contrast to in vitro systems, the cells could not be preincubated with WGA. Instead, the lectin was co-microinjected with the protein of interest at a final concentration of 1 mg/ml. A stock solution of 6 mg/ml in PBS was prepared, aliquoted and stored at -20°C.

3.2.10.5  **Inhibition of active nuclear transport by lowering of the temperature**

Active nuclear transport is an energy-consuming process and can therefore be stopped by incubation on ice due to impeded ATP-cleavage.

For the cooling of the cells, the hollow plate holder for the 35mm dishes was connected to approximately 2 meters of tubing. Adjacent to the microinjection microscope, a styrofoam container was set up in which the majority of the tubing was submerged in saturated (23%) NaCl solution at -14°C. The saturated NaCl solution in the tubes was circulated by a roller pump. Additionally, the temperature of the microinjection stage was lowered by cool packs. With this set up, the temperature of the metal plate holder could be kept at -3°C for up to an hour.

Before microinjection, cover slips were transferred to pre-cooled medium and chilled for 5 minutes on a metal plate on ice. Since active nuclear transport is known to be a rapid process, injection of one well was allowed to commence for 5 minutes before the cells were incubated on ice. For each experiment, a minimum of 4 wells were injected with the last one serving as a 37°C control. At the end of microinjection, the temperature of the medium was found not to exceed 8°C. As an internal control, NLS-BSA (1 mg/ml) was co-microinjected with active or mutant caspase-3 (2.9 mg/ml). On the basis of the exclusion or import of this protein, the success of the experiment was judged.
3.2.10.6 Apoptotic stimulation with cycloheximide and TNFα

A commonly used means of committing HeLa cells to apoptosis is the stimulation of the extrinsic pathway via TNFα. To allow for efficient apoptosis induction, the translation inhibitor cycloheximide (CHX) has to be added. Following microinjection, the cells were transferred to medium containing 100 µM CHX. After an incubation time of 30 minutes, TNFα was added to a final concentration of 100 ng/ ml. After an appropriate incubation period (3 to 5 hours) at 37°C, the cover slips were mounted.

Reagents
CHX 10 mM in H2O
TNFα 100 µg/ ml in NaCl/ HSA
NaCl/ HSA 0.1 % HSA, 0.9 % NaCl in H2O

3.2.11 TUNEL assay

One of the biochemical hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units of roughly 160 base pairs. The generated DNA fragments can be identified by their elongation in an enzymatic reaction. In this so-called TUNEL assay, terminal deoxynucleotidyl transferase (TdT) efficiently catalyzes the polymerization of nucleotides to free 3'-OH DNA. Red fluorescing TMR (tetramethylrhodamine) labelled nucleotides, incorporated into the nascent nucleotide polymers, ensure easy visualization on the single cell level by fluorescence microscopy.

Reagents
PBS
3% PFA in PBS
Permeabilisation buffer: 0.1 % TritonX-100 in 0.1 % sodium citrate
dUTP and TdT solution

The cells were washed carefully in PBS and then fixed with PFA for 1 h at room temperature. In order to open the cell membranes for the subsequent labelling reactions, the cover slips were incubated in freshly prepared permeabilisation buffer for 5 minutes on ice. The buffer was removed by rinsing the cover slips twice with PBS. After careful removal of remaining PBS, labelling commenced with the addition of 50 µl of the reaction mix per cover slip. The cells were incubated in a dark, sealed
and humidified chamber at 37°C. After 1 hour the reaction mix was carefully collected and disposed of separately due to its toxicity. Three washing steps with PBS ensued. For subsequent microscopy the cover slips were mounted in Aqua/ Poly Mount.

3.2.12 Microscopic analysis of microinjected cells

Following the microinjection experiment, the cover slips were analyzed using a fluorescence microscope equipped with 40x (Plan-Neofluor; numerical aperture 1.3) and 63x objectives (Plan-Apochromat; numerical aperture 1.4) and filters for FITC (for GFP), TRITC (for Texas Red, TMR) and Hoechst fluorescence. For the quantitation of the distribution of fluorescence intensity, confocal microscopy was employed using a 63x objective (Plan-Apochromat; numerical aperture 1.4). The emission of the fluorescence markers was successively visualized by excitation with the following lasers: Argon/2 (488 nm) for TR and TRITC, HeNe (543 nm) for GFP, Laser diode (405 nm) for Hoechst fluorescence.

In general, all injected cells were documented using a digital imaging system. All pictures were background corrected, except for TUNEL stains.

3.2.13 Scoring of chromatin condensation

In order to determine the overall apoptosis rate in experiments that included TNF-α stimulation, more than 200 non-injected, randomly selected cells were counted. A cell was scored as apoptotic if the nucleus displayed chromatin condensation. Generally, the rate of chromatin condensation was classified according to Kihlmark et al. In stage I cells, slight chromatin condensation can be observed at the periphery of the nucleus. Stage II is defined by thick interconnected strands of chromatin, whereas in stage III, the chromatin is packed into separate lumps, resembling a grape.
4 RESULTS

4.1 Quality and functionality of purified proteins

The examination of subcellular localization in a microinjection system demands a great deal from the overexpression and purification process of the proteins of interest. Firstly, the yield of the protein needs to be sufficiently high to allow for adequate concentration. This is especially important in the case of fluorescence tagged proteins, since microscopic detection depends on the amount of injected protein. Furthermore, the purification procedure should result in proteins that are able to execute their regular cellular functions such as binding, inhibition or protease activity for instance. Therefore, purification under non-denaturing conditions is highly desired. Another important issue is the homogeneity and purity of the protein of interest. Truncated by-products might lead to a falsification of results and need to be removed using adequate techniques. To match all the aforementioned criteria, the expression of each protein had to be adjusted to produce maximum yields.

4.1.1 Purification of caspase-3 constructs expressed in E. coli

For the investigation of the subcellular localization of active caspase-3, a previously generated construct was used. Since it was shown that the prodomain inhibits the activation of the protease \cite{68}, this construct encompassed only the two catalytic subdomains p17 and p12 (aa 29-277). Additionally, caspase-3 was N-terminally fused to GFP for intracellular tracking of the fusion protein. This 28 kDa protein forms a fluorescent pigment in the absence of additional proteins, substrates, or co-factors. As a backbone, the pQE30 vector was used, providing the 60 kDa fusion protein with an N-terminal His-Tag. Similarly, an active site mutant (C163S) was overexpressed in E. coli.

Because the incubation temperature is known to greatly influence the quality and yield of protein, the overexpression of caspase-3 in E. coli was carried out at different temperatures. At 16°C and 30°C, a large fraction of protein was rendered inaccessible for purification due to formation of inclusion bodies, as was evident from the green color of the pelleted cellular debris.
RESULTS

Figure 4.1 IMAC of His-tagged caspase-3
SDS-PAGE analysis of His-Trap™ purification of active caspase-3. Approximately 1/100 vol. (10 µl) of the pooled 500 mM imidazol elution fraction was subjected to analysis on a 12 % SDS-PAGE gel and stained with Coomassie. A) Overexpression at 30°C for 3 h B) Overexpression at 16°C for 22 h
GFP p17p12: full length product; p17, p12: catalytic subunits of caspase-3; DP: degradation products

Furthermore, apart from the desired full length protein of roughly 60 kDa, a significant amount of smaller fragments was eluted from the HisTrap™ column (figure 4.1). Since the His-Tag is situated N-terminally, it stands to reason that these isolated proteins were C-terminal degradation products of caspase-3. Furthermore, this main fraction displayed an approximate size of 30 kDa. GFP is known to be comparatively stable in the presence of proteases, leading to the assumption that these by-products mainly consist of His-tagged GFP.

Judging from the intensity of the bands on the SDS-PAGE, the conclusion can be drawn that less than 50 percent of protein was isolated as the full length product at 30°C (figure 4.1 A). In comparison, incubation at 16°C led to an increase in the ratio of full-length caspase-3 protein (figure 4.1 B) as well as an overall increase in yield. Additionally, the active caspase-3 fusion protein was isolated in its processed form, as can be observed from the 47 and 12 kDa bands. In contrast, proteolytic processing did not occur at 30°C due to the shorter expression time.

Taking all the mentioned aspects into consideration, the incubation temperature of 16°C was found to be more favorable for the expression of caspase-3 GFP fusion proteins.

Due to the fact that the overexpression of caspase-3 constructs did not result in a homogenous product, a second purification step was indispensable. For cellular
RESULTS

Localization experiments, a contamination of caspase-3 with by-products such as GFP had to be avoided. With a molecular weight of 28 kDa, this protein is known to be capable of diffusing freely throughout the cell. As a result, nuclear fluorescence would be scored as false positive nuclear accumulation of caspase-3 protein.

Since the by-products mainly displayed a mass of approximately 30 kDa on the SDS-PAGE (figure 4.1), the difference in size to the full-length protein allowed for their efficient elimination in a Sephadex G75 gel filtration run. The elution profile for caspase-3 that was expressed in E. coli at 16°C is shown in figure 4.2 A.

No protein content was detected by Coomassie staining in the first peak, leading to the assumption that this fraction represents bacterial DNA-protein complexes. In a second peak (approximately 50 ml), the main fraction of the full-length caspase-3 was eluted. Judging from the green color of the eluate, the third peak that contained GFP, thereby confirming the assumption made on the basis of the SDS-Page (figure 4.1 A).

Since all the peaks were very sharp and well-defined, contamination by the other species could be minimized by pooling the respective peak fractions. The result was an efficient separation of the full length protein from contaminating by-products.

**Figure 4.2 Purification of recombinant caspases by gel filtration**

**A)** Profile of a representative gel filtration run (Sephadex G75) of His-Trap™ purified active caspase-3. Shown is the OD$_{280}$ trace.

**B)** Analysis of purity after gel filtration and ultrafiltration by SDS-PAGE. 1.5 µg of concentrated active (lane 1) and mutant caspase-3 (lane 2) caspase-3 protein was separated on a 12% gel and stained with Coomassie.

GFP p17p12: full length product; p17, p12: catalytic subunits of caspase-3
As can be observed on the SDS-PAGE in figure 4.2 B, the caspase-3 constructs could be isolated to a high level of purity employing gel filtration. Contamination by other protein species was found to be negligible. In addition, caspase-3 expressed at 30°C was completely processed during concentration by ultrafiltration, whereas caspase-3 protein overexpressed at 16°C was isolated in its active form from the bacteria. These findings served as a hint that the recombinant caspase-3 was capable of auto-activation under increased local concentrations in bacteria or in ultrafiltration (figure 4.2 B).

Yet, the question whether the extensive purification protocol resulted in loss of proteolytic activity remained to be answered.

The activity of the purified protease was tested in a DEVD-afc cleavage assay. The average specific activity of the protease was calculated at 10.4 ± 2.0 U/mg, signifying an acceptable variance between the different preparations. The activity of caspase-3 was found to be highest when overexpression proceeded at 16°C. Due to the mutation of the active site, no DEVDase activity could be detected in mutant caspase-3.

In conclusion, the purification protocol employing successive IMAC and gelfiltration steps yielded pure and highly active caspase-3 for use in microinjection experiments.
4.1.2 Purification of ∆impβ (45-462) expressed in E. coli

Active nuclear import proceeds through the nuclear pore complex (NPC). Importin β mediates translocation by direct interaction with NPC components and transports importin α with the NLS carrying cargo into the nucleus. The construct used for inhibition studies was essentially described by Kutay et al. In short, the nucleotide sequence corresponding to the amino acids 45 to 462 of importin β was cloned into the pQE60 vector, providing the recombinant protein with a C-terminal His-tag.

The expression of this 45 kDa protein in E.coli was very efficient. The elution fraction containing the highest protein content was subjected to ultrafiltration and the final protein concentration calculated to 39.9 µg/ µl by Edelhoch. In contrast to caspase-3, a second purification step was unnecessary since the preparation of ∆impβ from E. coli was found to be free of contaminating degradation products (figure 4.3 A).

A convenient way to test the inhibitory capability of the overexpressed truncation mutant was the co-microinjection with NLS-BSA. Import of SV40 NLS conjugated proteins is known to be accomplished via the importin β pathway. Therefore, microinjection along with the dominant negative inhibitor resulted in complete abrogation of NLS-BSA accumulation in the nucleoplasm (figure 4.3 B).

Figure 4.3 ∆impβ is recombinantly expressed and purified
Recombinant ∆impβ was purified from E.coli by His-Trap™ and the elution buffer exchanged for PBS by ultrafiltration. A) 2.3 µg of the final product was subjected to SDS-PAGE on a 12% gel and visualized by Coomassie stain. B) As a control for the functionality of ∆impβ, HeLa cells were injected with ∆impβ (20 mg/ ml) and BSA-NLS (1.5 mg/ ml) and incubated for a total of 30 minutes. 15 minutes before mounting, DNA was counterstained with Hoechst 33342 (0.6 mg/ ml). The injected fluorescence markers were visualized by fluorescence microscopy. Data represents three experiments. Scale bar, 10 µm.
4.1.3 Quality of BIR3 purification from S. cerevisiae

A potent inhibitor of caspase-9 could be isolated from XIAP: The BIR3 subdomain was found to bind the protease and thereby inhibits its activation with a $K_i$ of about 11 nM.

For the overexpression of BIR3, the coding sequence of the BIR3-RING domains (aa 242 – 497 of XIAP) was cloned into a yeast expression vector (pESC-URA) and transferred into an S. cerevisiae mutant strain (NY40).

The resulting fusion protein with a C-terminal FLAG epitope was purified by affinity chromatography using an immobilized Anti-Flag antibody (see 3.2.1.4). With this single step procedure, the 30 kDa BIR3 was isolated in high purity (figure 4.4).

The overexpressed protein was subjected to testing of its biological activity employing two different assays.

In a first testing system, the in vitro reconstitution of the intrinsic apoptosis pathway, leads to the formation of the apoptosome followed by caspase-9 activation and caspase-3 proteolysis. Addition of different amounts of recombinant BIR3 resulted in the complete suppression of DEVD-afc cleavage by activated caspase-3 (figure 4.5 A). The mechanism for XIAP inhibition of caspase-9 is the direct binding to both the proform and the active protease, resulting in the complete abolition of its cleavage capacity. The BIR3 subdomain is sufficient for conveying this inhibition resulting in the inability of caspase-9 to activate caspase-3.
Figure 4.5 Recombinant BIR3 is biologically active

A) HepG2 lysate was incubated for 30 minutes at 30°C with dATP (1 mM) ± cytochrome c (10 µM) in the absence or presence of BIR3 (2.2 µg or 4.4 µg). After incubation for 30 minutes at 30°C, DEVD-afc cleaving activity was measured.

B) HeLa cells were microinjected with BIR3 (1.1 mg/ ml) and Dextran40 (5 mg/ ml). After injection, the cover slips were transferred to fresh medium and CHX (100 µM) added. After incubation of 30 minutes, TNFα (100 ng/ml) was added and the cells incubated for 5 hours. The rate of apoptosis was assessed by counting the rate of injected cells with condensed nuclei visualized by Hoechst 33342 stain. As a control, at least 150 uninjected cells from the same well were assayed. The data represents two experiments (three wells each) ± SD.

In order to check the inhibitory capacity of BIR3 in a cellular environment, recombinant protein was microinjected into HeLa cells and apoptosis induced by CHX/ TNFα treatment. Incubation in the presence of inhibitor of caspase-9 led to a reduction in apoptotic cells from 46 percent in the control to 16 percent in BIR3 injected cells (figure 4.5 B). Employing this experimental system, a complete inhibition of apoptosis induction could not be expected. In contrast to the in vitro activation assay, TNFα stimulation leads to a primary activation of caspase-3 by caspase-8. Caspase-9 is activated secondarily via the tBid pathway. Its inhibition by BIR3 causes a slow-down in apoptosis induction 149 due to the disruption of an activation loop involving caspase-3 and caspase-9 28,65.

In conclusion, this experiment together with the in vitro activation assay demonstrates that the recombinant BIR3 protein was biologically active and a potent inhibitor of caspase-9. More importantly, it is capable of inhibiting activation of endogenous caspase-9 at the concentration used.
4.2 Intracellular distribution of caspase-3

4.2.1 Active caspase-3 induces apoptotic morphology and translocates into the nucleus

An advantage of direct microinjection of active caspase-3 into the cytoplasm of HeLa cells is the direct apoptosis induction without the necessity for the diverse upstream processes that are known to vary for different stimuli. Hence, any changes in cellular morphology can be directly attributed to the proteolytic activity of active protease. Microinjection of active caspase-3 resulted in the drastic shrinkage of the cell body accompanied by its rounding and subsequent detachment from the substratum. A feature that was observed only in a subset of cells was the blebbing of the plasma membrane, possibly due to rapid dismantling of the actin cytoskeleton.

In parallel to these cytoplasmic changes, increasing chromatin condensation could be detected in the nuclear compartment. Starting with slight peripheral condensation after 15 minutes (figure 4.6, top panel), the chromatin was later compressed into thick, interconnected strands (figure 4.6, middle panel) finally leading to a complete collapse of chromatin structure. After 60 minutes all the chromatin was found to be concentrated in a few separate lumps in the majority of cells (figure 4.6, lower panel). In comparison to the processes taking place in the cytoplasm, nuclear apoptosis took longer to complete. Whereas the rounded cells can be observed after 15 to 30 minutes, the majority of cells reached chromatin condensation stage III after 60 minutes of incubation.

Although the activation pathway was inversed by the injection of the effector caspase-3, the cells displayed all the morphological features associated with apoptosis (figure 4.6).

The observed alterations in cellular morphology were a direct result of caspase-3 activity, proving that this protease is the central effector in apoptosis. Microinjection of this protease alone is sufficient to emulate the regular morphological features of apoptosis.

Concerning the subcellular distribution of caspase-3, GFP fluorescence of the fusion protein was detectable in the nuclear compartment from the earliest observation point. After 15 minutes of incubation, the caspase-3 fusion protein had reached an equal distribution throughout the cell (upper panel figure 4.6). These observations are in sharp contrast to the localization of the biologically inert co-injection marker.
Dextran40. This polysaccharide with a molecular weight of 40 kDa was conspicuously absent from the nucleus at all time points. Taking the molecular weight of caspase-3 into consideration, this finding is crucial. Two caspase molecules are known to form the active heterotetramer \(^{51}\). As a result, the actual size of the recombinant caspase-3 had to be calculated at 120 kDa, three times the size of Dextran40.

The conclusion to be drawn from this observation is that caspase-3 is able to enter the nucleus without a significant increase of the size exclusion limit of the nuclear pores. Additionally, this translocation was found to be rapid and the accumulation complete after 15 minutes.

![Figure 4.6 Recombinant active caspase-3 induces apoptotic morphology and translocates into the nucleus](image)

HeLa cells were microinjected with active caspase-3 (3 mg/ml) and Dextran40 (2 mg/ml) for up to a maximum of 10 minutes and incubated at 37°C for the remaining time. DNA was counterstained with Hoechst 33342 (0.6 mg/ml) 15 minutes before mounting. Microinjected cells were visualized by fluorescence microscopy. The cells presented are representatives of the whole injected population from three experiments. Scale bar, 10 µm.
4.2.2 Nuclear translocation of caspase-3 does not depend on caspase-9 activity

As was demonstrated in the former chapter, active caspase-3 is capable of entering the nuclear compartment. One possible mechanism for this translocation was previously suggested by Faleiro et al.\textsuperscript{150}. In their proposed model, caspase-9 which is activated earlier in the intrinsic pathway increases the size exclusion limit of the nuclear pores, allowing caspase-3 to reach its nuclear targets by passive diffusion.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.7}
\caption{Co-microinjection of BIR3 does not influence apoptosis induced by active caspase-3. HeLa cells were microinjected with active caspase-3 (3.0 mg/ ml), BIR3 (0.9 mg/ ml) and Dextran40 (2 mg/ ml) for a maximum of 10 minutes. DNA was counterstained with Hoechst 33342 (0.6 mg/ ml) 15 minutes before mounting.}
\end{figure}

A) Microinjected cells were visualized by fluorescence microscopy. The cells presented are representatives of the whole population. Scale bar, 10 \(\mu\)m.

B) The number of cells displaying stage III chromatin condensation was obtained as a percentage of the whole population. Data represents the average of three experiments ±SD.
To test this model, active caspase-3 was co-microinjected with the caspase-9 inhibitor BIR3. As was shown earlier (figure 4.5), the concentration used is sufficient to potently prevent caspase-9 activation. Yet, the distribution pattern of caspase-3 is not changed in the presence of BIR3 (figure 4.7 A). The GFP fluorescence of the fusion protein was readily detectable in the nucleus after 15 minutes and did not further increase with longer incubation. Moreover, chromatin condensation was not influenced by the presence of the inhibitor of caspase-9. After one hour of incubation, 60 percent of the injected cells reached condensation stage III regardless of the presence or absence of BIR3 (figure 4.7 B).

Hence, it can be concluded, that caspase-9 has no influence on caspase-3 localization in the microinjection system. Moreover, its activity appears to be dispensable for nuclear apoptosis on the morphological level.

### 4.2.3 Activity of caspase-3 is dispensable for nuclear translocation

In the course of apoptosis, active nuclear transport is known to break down as a result of the relocalization of transport factors. Several components of the NPC such as Nup153, LAP2 and POM121 were identified as caspase-3 targets. Taking this into consideration, caspase-3 activity might result in an increase of the size exclusion limit of the NPC. Thus, its translocation into the nuclear compartment might be a result of passive diffusion.

To test this hypothesis, active caspase-3 was preincubated with 500 µM of z-DEVD-fmk. This concentration of the irreversible inhibitor is sufficient to inhibit activity of the recombinant protease completely as was examined by a DEVD-afc cleavage assay. Therefore, the protease retained its active conformation without the ability to cleave any target proteins.

As a result, microinjection of the z-DEVD-fmk treated caspase-3 did not lead to the typical apoptotic changes. Even after one hour of incubation, the HeLa cells displayed regular, flattened morphology as well as evenly distributed chromatin (figure 4.8).

In line with the observations for cells injected with active caspase-3, TR fluorescence of Dextran40 could not be detected in the nuclear compartment. Most importantly, caspase-3 was found to be capable of entering the nuclear compartment at all time points investigated. The distribution pattern was similar to that of active caspase-3 with comparable fluorescence intensities in the cytoplasm and the nucleus. At the
same time, passive diffusion is ruled out due to the complete exclusion of the Dextran40 co-injection marker from the nuclear compartment.

In summary, the conclusion drawn from this experiment is that proteolytic activity of caspase-3 is dispensable for the observed translocation into the nuclear compartment.

![c3 GFP, Dextran, Overlay, DNA](image)

**Figure 4.8 Proteolytic activity of caspase-3 is not a prerequisite for nuclear translocation**  
Active caspase-3 (4 mg/ml) was incubated over night with 500 µM DEVDfmk and 13 mM DTT. For microinjection, addition of Dextran40 (1 mg/ml) resulted in a final concentration of 3.4 mg/ml for caspase-3. The microinjected cells were incubated at 37°C for 30 minutes or 60 minutes. DNA was stained with Hoechst 33342 (0.6 mg/ml) 15 minutes before mounting. Injected HeLa cells were visualized by fluorescence microscopy. The images show representative cells from three (30 minutes) and two (60 minutes) experiments. Scale bar, 10 µm.

**4.2.4 The unprocessed form of caspase-3 efficiently enters the nucleus**

Caspases are present as zymogens in a healthy cell. Upon induction of cell death, their proteolytic activation leads to a rearrangement of the subunits. The dramatic change in structure might enhance the capacity of caspase-3 to enter the nuclear compartment.

To test this, a catalytic site mutant, in which the p17 and p12 subunits are not separated to form the active conformation, was microinjected into HeLa cells. Since this protein did not possess intrinsic cleavage activity, changes in cellular morphology associated with apoptosis could not be observed. The cells displayed flattened morphology along with equally distributed chromatin (figure 4.9). As was found for active caspase-3, the fluorescence of the proteolytically inactive mutant is detectable in the nuclear compartment at all observation points. The
translocation process is complete after 15 minutes, and no further accumulation of the caspase fusion protein could be observed after 30 or 60 minutes.

These microscopical findings were further substantiated by the confocal analysis of the spatial distribution of the fluorescence markers. TR fluorescence of Dextran40 was reduced to background levels in the nuclear compartment. At the same time, the nuclear membrane did not seem to pose an obstacle for mutant caspase-3, for the GFP fluorescence of the fusion protein was evenly distributed throughout the cell (figure 4.9 B).

Obviously, the absence of proteolytic processing and hence the arrangement of the two subunits that comprise mature caspase-3 did not influence the localization of the protease.
Figure 4.9 A catalytic site mutant of caspase-3 efficiently enters the nucleus

HeLa cells were microinjected with mutant caspase-3 GFP (3.4 mg/ml) and Dextran40 TR (1 mg/ml) for up to 10 minutes and incubated at 37°C for the remaining time. DNA was counterstained with Hoechst 33342 (0.6 mg/ml) 15 minutes before mounting. The cells presented are representatives of the whole injected population from four experiments. Scale bar, 10 µm.

A) Microinjected cells incubated for 15 and 30 minutes were visualized by fluorescence microscopy.

B) Confocal microscopy of representative cells incubated for 60 minutes. For the overlay image, the fluorescence intensity profile (arbitrary units) of mutant caspase-3 (green) and Dextran40 (red) corresponding to the arrow drawn through the cell is shown. Cytoplasmic (C) and nuclear (N) areas are indicated.

mut c3 GFP: mutant caspase-3 GFP
4.3 Involvement of active transport processes in nuclear apoptosis

4.3.1 Caspase-3 entry into the nucleus depends on the hydrolysis of energy

It has been suggested that several caspases are translocated into the nucleus by active transport\textsuperscript{152,153} and that facilitated translocation is required for apoptosis\textsuperscript{142}. Due to the size of the recombinant caspase-3, involvement of passive diffusion into the nuclear compartment was excluded as the mechanism for entry. This conclusion was further substantiated by the failure of the smaller Dextran40 to cross the nuclear membrane. Therefore, the involvement of active transport processes had to be considered as a distinct possibility for caspase-3 translocation into the nucleus.

One main characteristic of active nuclear transport is the requirement of energy in the form of GTP. Hydrolysis of ATP and ADP by co-injection of apyrase was tested as a means to inhibit energy-dependent nuclear transport. However, the energy depletion was found to be too slow to deprive the efficient transport machinery of its driving force. As a result, the rapid accumulation of the control protein NLS-BSA in the nucleoplasm was not impeded (data not shown). Since apyrase application before microinjection of the protein of interest was not feasible, the microinjection system was determined to be not suitable for this kind of inhibition studies.

As a different approach, incubation at low temperatures was used to prevent the cleavage of GTP and ATP, thereby efficiently halting import processes.

In preparatory experiments, the degree of nuclear exclusion of a BSA-TRITC coupled with an SV40-NLS (NLS-BSA) in relation to the temperature was tested. A reduction in temperature to below 8°C resulted in complete exclusion of NLS-BSA from the nuclear compartment (figure 4.10 A), signifying an efficient inhibition of active transport processes. At higher temperatures, only a partial exclusion of the NLS-carrying substrate was achieved. Taking this into consideration, the temperature of the cell culture medium was kept below 8°C at all times during the injection procedure, using saturated NaCl solution at -14°C as a coolant.

In cells co-microinjected with caspase-3 and NLS-BSA at low temperatures, the recombinant protease was no longer capable of entering the nuclear compartment and GFP fluorescence was restricted to the cytoplasm of cells.
Figure 4.10 Incubation at low temperature inhibits nuclear accumulation of caspase-3
HeLa cells grown on coverslips were cooled on ice for 5 minutes before microinjection with active or mutant caspase-3 (2.9 mg/ml) and NLS-BSA (1 mg/ml). Microinjection was allowed to commence for 5 minutes on a cooled stage, followed by incubation on ice. After 55 minutes, the cells were fixed with 3% PFA and stained with Hoechst 33342 (0.6 mg/ml). The depicted cells are representatives of the injected population from three independent experiments.
A) Confocal microscopy was employed to analyze the localization of the microinjected proteins. Scale bar, 10 µm.
B) Fluorescence intensity profile (arbitrary units) of cellular distribution of the caspase-3 constructs (green) and NLS-BSA (red) corresponding to the arrow drawn through the cell is shown. Cytoplasmic (C) and nuclear (N) areas are indicated.
c3: caspase-3
Comparison of the spatial distribution of the fluorescence by confocal analysis confirmed the virtually complete co-localization of the two injected proteins (upper panel figure, 4.10 B). Hence, translocation of active caspase-3 into the nucleus appears to be an energy-dependent process.

To be able to draw a conclusion regarding whether the processing state of caspase-3 plays a role in its nuclear translocation, mutant caspase-3 was microinjected into HeLa cells at low temperature.

Upon incubation at 4°C, entry to the nucleus was effectively blocked for the injected proteins (lower panel, figure 4.10 B). These findings are consistent with the data obtained for active caspase-3, indicating that translocation into the nucleus is an energy-consuming process. At the same time, this finding implied that the pathway utilized is the same for the different caspase-3 forms and that the processing state of caspase-3 is not important for entry into the nuclear compartment.

An interesting consequence of injection of active caspase-3 at low temperature was the conspicuous absence of morphological changes associated with apoptosis. The cells displayed a healthy, flattened morphology and the chromatin was distributed evenly throughout the nuclear compartment (upper panel, figure 4.10 A). These observations imply that apart from ATP and GTP cleavage, incubation at low temperature results in complete abrogation of proteolytic activity of caspase-3. Under these circumstances, microinjection of active caspase-3 pre-treated with DEVD-fmk was deemed to be redundant and was not conducted as a consequence.

In order to test whether the treatment at low temperature affected the ability of the cells to display efficient nuclear import, cells were transferred to 37°C after microinjection at low temperatures (figure 4.11). This treatment did not influence the capacity of the cell to mediate nuclear translocation of the co-injection marker NLS-BSA. At the same time, mutant caspase-3 was found to be equilibrated between cytosol and the nucleoplasm, as previously demonstrated (figure 4.9).

Incubation of caspase-3 injected cells shifted from low temperatures to 37°C led to the identical changes ascertained in previous experiments (figure 4.6). After one hour of incubation, the cells displayed round morphology with several cytoplasmic protrusions. In the nucleus, the chromatin was heavily condensed. Apart from these changes, the cells displayed ready take-up of active caspase-3 into the nuclear compartment, signifying that pre-incubation at low temperature does not affect the ability of caspase-3 to induce apoptotic changes. Additionally, the translocation of the
co-injected NLS-BSA into the nucleus was found to be complete with no or only negligible levels of residual cytoplasmic fluorescence. Thus, it can be concluded that the import of the control substrate via the importin β pathway is completed even in the presence of high caspase-3 activity. In summary, the presented data indicate that efficient nuclear traffic takes place even under conditions of apoptotic proteolysis, induced by the presence of fully active caspase-3.

Figure 4.11 The capacity for nuclear import is not disrupted by incubation at low temperature
HeLa cells grown on coverslips were cooled on ice for 5 minutes before microinjection with active or mutant caspase-3 (2.9 mg/ ml) and BSA-NLS (1 mg/ ml). Microinjection was allowed to commence for 5 minutes on a cooled stage, followed by incubation at 37°C. After 55 minutes, the cells were fixed with 3% PFA and stained with Hoechst 33342 (0.6 mg/ ml). The cellular distribution of the fluorescence markers was analyzed by fluorescence microscopy. The depicted cells are representatives of the injected population from three independent experiments. Scale bar, 10 µm.
4.3.2 Interactions with nucleoporins are essential for caspase-3 import

Apart from incubation at low temperatures, another commonly used inhibitor of active transport is wheat germ agglutinin (WGA). This lectin binds to N-acetylglucosamine groups of nucleoporins, thereby unspecifically blocking transport processes \(^{146}\). At the same time, passive diffusion of molecules below the size exclusion limit of the pore is not impeded \(^{154}\). Co-microinjection of GFP along with WGA did not alter the localization of the 28 kDa protein (figure 4.12).

![Figure 4.12 WGA does not impede passive diffusion](image)

HeLa cells were microinjected with equal concentrations of GFP in the absence or presence of WGA (1 mg/ml). After one hour of incubation, the distribution of GFP in live cells was documented by fluorescence microscopy. Scale bar, 10 µm

To test whether nuclear uptake of caspase-3 could be interrupted, the protease was co-microinjected into HeLa cells with NLS-BSA and WGA. After an incubation time of one hour, the positive control NLS-BSA was found to be excluded from the nucleoplasm. Moreover, fluorescence of the GFP fusion protein was detected predominantly in the cytosolic compartment (figure 4.13 A). In comparison to microinjection of caspase-3 alone, this constitutes a significant reduction in the ratio of nuclear caspase-3 (figure 4.13 B). The observations were identical irrespective of whether the active or the mutant form of caspase-3 was injected. Nuclear uptake of caspase-3 therefore seems to be independent of proteolytic cleavage between the p17 and p12 subunits.

Consequently, the conclusion could be drawn that caspase-3 enters the nucleus through the nuclear pores. Moreover, translocation of caspase-3 appears to rely on interaction with nucleoporins, since passive diffusion was shown not to be influenced by WGA.

It has to be noted that the nuclear exclusion of the caspase-3 constructs mediated by WGA was less efficient than the incubation at 4°C. In all the experiments conducted, the nuclear levels of the GFP proteins and the positive control NLS-BSA were found to be significantly reduced compared to control cells. Yet, for both proteins, this
reduction did not reach background levels as observed for incubation at low temperatures. This might indicate that binding of WGA to the nucleoporins is not fast enough to completely shut down nuclear transport in the microinjection context. Full inhibition might not be achieved immediately upon microinjection but at a slightly later time point. By then, import of a fraction of the substrates might already have occurred, providing an explanation for the residual levels of caspase-3 and NLS-BSA in the nucleoplasm.

**Figure 4.13 Nuclear uptake of mutant caspase-3 is inhibited by co-microinjection of WGA**

HeLa cells were microinjected with active or mutant caspase-3 (2.9 mg/ml), BSA-NLS (1 mg/ml) and WGA (1 mg/ml). After a total incubation time of 60 minutes, the cells were fixed in 3% PFA and DNA stained with Hoechst 33342 (0.6 mg/ml). The data presented is representative of three independent experiments.

A) The localization of the injected proteins was visualized by confocal microscopy. The bar equals 10 µm (active caspase-3) and 20 µm (mutant caspase-3).

B) Left panel: magnification of indicated cell from A) (arrowhead). In the right panel, quantification of fluorescence intensity of caspase-3 GFP (arbitrary units) along the depicted arrow is shown.
4.3.3 Caspase-3 does not depend on importin β for its translocation

Using WGA and incubation at low temperatures, facilitated translocation events could be implicated in the entry of caspase-3 into the nucleus. In order to check for a possible translocation mechanism, a more targeted approach was utilized by the inhibition of the classical importin β pathway. Due to N- and C-terminal truncations, the ∆impβ (45-462) (∆impβ) mutant is unable to bind to importin α. Additionally, the release from the NPC is prevented by the lack of the RanGTP interaction site, thereby effectively blocking this transport pathway \(^{148}\).

![Figure 4.14 Co-microinjection of ∆impβ does not influence localization of active caspase-3](image)

HeLa cells were microinjected with active caspase-3 (2.9 mg/ ml), ∆impβ (20 mg/ ml) and Dextran40 (1 mg/ ml) for 10 minutes and incubated at 37°C for the remaining time. 15 minutes before mounting, DNA was counterstained with Hoechst 33342 (0.6 mg/ ml). The injected fluorescence markers were visualized by fluorescence microscopy. Data represents three experiments. Scale bar, 10 µm.
The presence of this inhibitor of active transport did not influence the induction of cytoplasmic apoptosis by microinjection of active caspase-3. Cellular morphology was changed as observed for cells microinjected with caspase-3 alone. Yet, in contrast to incubation at 4°C or injection of WGA, Δimpβ was not capable of denying the recombinant protease access to the nuclear compartment. Fluorescence of the GFP fusion protein was readily detectable in the nucleoplasm of injected cells (figure 4.14).

The conclusion to be drawn from this experiment is that although active transport may be involved in caspase-3 translocation into the nucleus, the mechanism used is independent of the importin β pathway.
4.4 Inhibition of active transport processes alters nuclear apoptosis

As demonstrated in the previous chapters, inhibition of active import has profound consequences for the intracellular localization of caspase-3. Since apoptosis depends on a multitude of factors, the blockage of translocation into the nucleus might influence nuclear apoptosis on a global level. Two parameters that are readily observable on the single cell level are the degree of chromatin condensation as well as the induction of apoptotic DNA cleavage.

4.4.1 Effect on chromatin condensation

A consequence of the injection of active caspase-3 into HeLa cells was the complete breakdown of chromatin structure within the short period of 60 minutes. Since caspase-3 was found to translocate into the nuclear compartment, the hypothesis was examined whether blockage of nuclear transport could alter nuclear morphology. HeLa cells were microinjected in the absence or presence of inhibitors of active import pathways. Although the overall morphology of the injected cells was not altered, co-microinjection of WGA or Δimpβ influenced the degree of chromatin condensation in the nuclear compartment (figure 4.15 A).

After one hour of incubation, less than 10 percent of cells with blocked transport had entered the final stage of nuclear apoptosis. More than 70 percent were identified as stage I, which is characterized by slight peripheral condensation of the chromatin (figure 6.15 B). These observations were found to be independent of the inhibitor used.

In the absence of the inhibitors and after the same incubation period, only a small subset of 5 percent displayed nuclei in the initial phase of nuclear apoptosis. The majority of cells injected with caspase-3 alone displayed features of advanced chromatin condensation. Thirty-five percent of the cells had entered stage II and 60 percent displayed stage III chromatin condensation (figure 4.15 B).

Taking into account that nuclear translocation of caspase-3 was not influenced by the presence of Δimpβ (figure 4.14 B), but nuclear apoptosis was significantly reduced by this dominant negative truncation mutant, it stands to reason that the presence of caspase-3 in the nucleus is not a prerequisite for chromatin condensation. The activities responsible for inducing stage III chromatin condensation, therefore, seem to originate from the cytoplasm and rely on the importin β pathway for entry into the
nucleus. At the same time, blockage of active transport fails to result in the complete inhibition of nuclear breakdown on the morphological level. This observation indicates that the induction of nuclear apoptosis can be divided into the activity of NLS-dependent factors and others that are capable of entering the nucleus by passive diffusion.

Figure 4.15 Reduction of chromatin condensation by inhibitors of active nuclear transport
HeLa cells were microinjected with active caspase (3.4 mg/ml) alone, plus WGA (1 mg/ml) or Δimpβ (20 mg/ml).
A) Nuclear morphology of injected cells assessed by fluorescence microscopy. Scale bar, 10 µm.
B) After a total incubation time of 1 hour, the degree of chromatin condensation according to Kihlmark et al. was scored by Hoechst 33342 (0.6 mg/ml) fluorescence. Data are means of three experiments ± SD.
4.4.2 Induction of apoptotic DNA strand breaks

Inhibition of active nuclear transport was shown to lead to a reduction in the degree of chromatin condensation. A second biochemical hallmark of nuclear apoptosis is the introduction of internucleosomal DNA strand breaks. To examine whether blockage of the importin $\beta$ pathway resulted in differences of the cleavage pattern, the TUNEL assay was employed.

For the examination of the induction of DNA strand breaks in apoptotic cells by TUNEL, the incubation time had to be increased from one hour to three hours. The reason for this adjustment was that in comparison to chromatin condensation, the introduction of single or double strand breaks recognized by the TUNEL reaction was significantly slower. While the majority of cells had entered stage III chromatin condensation after one hour of incubation with microinjected active caspase-3, sufficient TUNEL labelling was visible only after an incubation period of three hours in this experimental setting (figure 4.16 A).

Interestingly, even after this prolonged incubation period, the majority of $\Delta$imp$\beta$ co-injected cells (76 percent) displayed stage II chromatin condensation (figure 4.16 C) compared with 12 percent of the control cells. Eighty-eight percent of the cells microinjected with active caspase-3 alone of the cells were found to have reached the highest degree of condensation. Therefore, it can be assumed that the complete breakdown of chromatin organization might not only be delayed in the presence of the dominant negative $\Delta$imp$\beta$, but is essentially stopped as long as inhibition of importin $\beta$ dependent import is maintained.

In HeLa cells microinjected with the importin $\beta$ (45-462) truncation mutant and NLS-BSA, the localization of the control protein was still cytosolic after 4 hours of incubation (figure 4.15 B). This served as an indicator that the inhibitory capacity of $\Delta$imp$\beta$ was not critically diminished by degradation or loss of function during this time (figure 4.16 A). Our observations thereby confirmed that importin $\beta$ is not cleaved after induction of apoptosis\textsuperscript{140}.

The examination of the TUNEL staining pattern revealed a correlation between the degree of chromatin condensation and the induction of DNA strand breaks. In cells that were microinjected with caspase-3 alone, stage III morphology was accompanied by strong TUNEL staining (figure 4.16 C). In contrast, the inhibition of importin $\beta$ mediated transport not only caused a reduction in chromatin condensation
to stage II compared to the control cells, but this reduction was also accompanied by a failure of the nuclei to be stained by the TUNEL reaction. Therefore, under conditions of nuclear transport inhibition, internucleosomal DNA cleavage is impaired, presumably causing the observed reduction in the degree of chromatin condensation.

Figure 4.16 Inhibition of active nuclear transport reduces apoptotic DNA cleavage
A) HeLa cells were microinjected with active caspase-3 (3.4 mg/ ml) ± Δimpβ (20 mg/ ml) and incubated at 37°C for a total of 3 hours. After fixation and permeabilization, apoptotic DNA strand breaks were labelled by TUNEL. Cells were counterstained for DNA with Hoechst 33342 (0.6 mg/ ml). Fluorescence microscopy of cells representative for the whole population. TUNEL exposures were not background adjusted. Scale bar 10 µm.
B) Control cells injected with NLS-BSA (1.5 mg/ ml) and Δimpβ (20 mg/ ml) were incubated for 4 hours at 37°C and localization of TRITC labelled BSA examined by fluorescence microscopy. Scale bar, 10 µm.
C) The degree of chromatin condensation according to Kihlmark et al. 141 and TUNEL labelling was scored by fluorescence microscopy. Data represent the means of three experiments ± SD.
4.5 Inhibition of active transport sensitizes HeLa cells to TNF\(\alpha\) induced apoptosis

Ligation of TNF\(\alpha\) to its receptor leads to the activation of pro- and anti-apoptotic pathways\(^{21}\). Therefore, translational or transcriptional inhibitors are included in many experimental systems to prevent de novo synthesis of anti-apoptotic factors. In order to elucidate the role of nuclear transport in TNF\(\alpha\) induced apoptosis, the impact of blocking the importin \(\beta\) pathway on TNF mediated apoptosis was examined.

Cells were microinjected with \(\Delta\text{imp}\beta\) along with Dextran40 to allow for their detection using fluorescence microscopy. Incubation with the recombinant inhibitor for 3 hours resulted in induction of apoptosis in 28 percent of the cells as assessed by changes in nuclear morphology (figure 4.17). In uninjected cells, in comparison, the apoptosis level was below 5 percent. The importin \(\beta\) truncation mutant therefore elicits toxicity in the cells by inhibiting nuclear transport processes.

![Figure 4.17](image)

**Figure 4.17 Microinjection of \(\Delta\text{imp}\beta\) sensitizes HeLa cells to TNF\(\alpha\) induced apoptosis**

HeLa cells were microinjected with \(\Delta\text{imp}\beta\) (20 mg/ ml) and Dextran40 (1 mg/ ml). The coverslips were transferred to fresh medium and treated as follows:

- **Control**: Incubation at 37°C for 3.5 hours;
- **+ TNF\(\alpha\)**: Addition of TNF\(\alpha\) (100 ng/ml) after 30 minutes of incubation;
- **CHX/ TNF\(\alpha\)**: 30 minutes preincubation with CHX (100 µM) before addition of TNF\(\alpha\) (100 ng/ ml).

15 minutes before mounting, DNA was counterstained with Hoechst 33342 (0.6 mg/ ml). The rate of apoptosis was assessed by fluorescence microscopy of changes in nuclear morphology. Effects of the respective treatments on uninjected cells (\(-\Delta\text{imp}\beta\)) was scored by examining nuclear morphology of >150 cells per well on the same coverslip. Data is the mean of three experiments ± SD.
In the presence of the translational inhibitor CHX and TNFα, the level of apoptotic cells rose to 32 percent in control cells. At the same time, the ratio of apoptotic cells rose to 73 percent when active nuclear transport was inhibited by ∆impβ, signifying a sensitization of the injected cells to the CHX/ TNFα treatment. Strikingly, a comparably high percentage (70 percent) of ∆impβ injected cells displayed apoptotic morphology if treated with TNFα in the absence of the translation inhibitor. In contrast, the apoptosis level in the corresponding control cells was identical to untreated cells, signifying the predominance of the anti-apoptotic pathway in reaction to TNFα stimulation alone.

![Figure 4.18](image)

**Figure 4.18 ∆impβ injected HeLa cells display reduced chromatin condensation following TNFα treatment**

HeLa cells were microinjected with ∆impβ (20 mg/ml) and Dextran40 (1 mg/ml). The coverslips were transferred to fresh medium and treated as follows:

- **Control:** Incubation at 37°C for 3.5 hours;
- **TNFα:** Addition of TNFα (100 ng/ml) after 30 minutes of incubation;
- **CHX/ TNFα:** 30 minutes preincubation with CHX (100 µM) before addition of TNFα (100 ng/ml).

15 minutes before mounting, DNA was counterstained with Hoechst 33342 (0.6 mg/ml). Morphological changes in ∆impβ injected cells were assessed by fluorescence microscopy. Bar equals 10 µm.

As described before, the ∆impβ treated cells displayed only reduced chromatin condensation after 3 hours of treatment (figure 4.18), substantiating the results obtained by microinjection of active caspase-3.

Taken together, microinjection of the importin β truncation mutant was shown to be an efficient substitute for the translational inhibitor CHX. Therefore, importin β dependent transport plays an essential role in conferring cellular resistance in response to TNFα induced apoptosis.
5 DISCUSSION

5.1 Rationale and experimental setup

Like most of the members of the caspase family, the intracellular localization of caspase-3 was determined to be mainly cytoplasmic. Depending on the author, a nuclear increase in the pro- and processed forms was observed after induction of apoptosis $^{136-138}$. This increase in nuclear DEVDase activity was to be expected, since many substrates of caspase-3 are localized in the nuclear compartment. Yet, the means of propagation of the apoptotic signal into the nucleus remains controversial. Whereas the requirement for active transport processes in apoptosis was demonstrated by Yasuhara et al. $^{155}$, Faleiro et al. $^{150}$ postulated passive diffusion of caspase-3 through a functionally impaired nuclear pore complex. In order to clarify which mechanism was responsible for translocation into the nuclear compartment, the redistribution of exogenously added caspase-3 in living cells was studied.

At first, a suitable experimental system for the introduction of active caspase-3 into living cells had to be determined. A routinely used method for the analysis of protein function is the transient transfection of the corresponding DNA construct into mammalian cells. Due to a lack of control over the temporal start, expression of the protein of interest is not synchronized on the single cell level. Yet, for processes that are executed rapidly, a defined starting point for the experiment is highly desirable. The use of inducible expression systems tends to ameliorate but not eliminate this problem entirely. The mentioned disadvantage can be overcome by direct microinjection of the protein of interest into the cytoplasm of living cells. The narrow injection window of several minutes provides a defined starting point of the experiment.

Another routinely used method for the study of transport process is the reconstitution of active import in semi-intact cells. Yet, compared to microinjection, import does not take place in the natural environment of a living cell, since the cellular membrane is permeabilized and cellular factors may be removed by several washing steps.

In contrast to experiments using semi-intact cells, the steady state levels of protein distribution can be determined using living cells $^{156}$. A significant disadvantage of the microinjection method, however, is the limitation in the number of injected cells. The
observation of cellular events has to be restricted to the single cell level, limiting the
number of available detection assays.

For the analysis of caspase-3 localization, a recombinant protein was overexpressed
in E. coli and isolated in high purity employing a two-step purification protocol.
The fusion to GFP provided a widely used means to directly follow the localization of
proteins on the single cell level by fluorescence microscopy (see 157 and references
therein). At the same time, the increase in overall size of the fusion protein to roughly
60 kDa reduced the capacity for passive diffusion. Active caspase-3 forms a dimer
51,158, thereby increasing the size of the protein to approximately 120 kDa, which lies
above the size exclusion limit of the nuclear pore complex, which is approximately
40 kDa97,98. The fusion of caspase-3 to GFP did not lead to an introduction of a
cryptic NLS, as 4x GFP, which is of comparable size, is excluded from the nuclear
compartment159. Therefore, all changes in subcellular localization could be attributed
to the protease part of the fusion protein. Furthermore, fusion to GFP did not inhibit
caspase-3 activity, since the isolated protein displayed high specific DEVD-afc
cleavage.

5.2 Caspase-3 lacking the prodomain rapidly crosses into the
nucleoplasm

Induction of apoptosis via the intrinsic or extrinsic pathways usually involves a
plethora of cellular factors that must be assembled to induce the different activation
cascades. The reasoning behind the microinjection of active caspase-3 into living
cells was the circumvention of these processes by direct induction of apoptosis. All
the induced changes were primarily provoked by the proteolytic activity of caspase-3
alone. As could be observed from microinjected HeLa cells, the recombinant
protease was able to induce apoptotic features such as the shrinkage and rounding
of the cell body, the formation of cytoplasmic protrusions and finally detachment from
the substratum. Nuclear changes included progressive chromatin condensation
accompanied by shrinkage of the nucleus.

Thus, microinjection of active caspase-3 was sufficient to induce the changes
associated with regular apoptosis on the morphological level, demonstrating that this
protease is the central effector of programmed cell death and situated at the
convergence point of the different activation pathways. Hence, this bottom up
approach to apoptosis induction demonstrated that all major morphological changes in the cell can be induced by the action of caspase-3 alone.

Apart from the morphological changes in the injected cells, the localization of the protease was of interest. As was determined by fluorescence microscopy, caspase-3 entered the nucleus rapidly, since GFP fluorescence was detected in the nucleoplasm after 15 minutes. At later time points, the caspase-3 fusion protein did not accumulate further in this compartment, implying that translocation is a fast process.

Strikingly, the co-injected 40 kDa Dextran did not equilibrate across the nuclear membrane like caspase-3, even after an incubation time of 60 minutes. This finding is in disagreement with Ferrando-May et al. Using the same cell line, the biologically inert Dextran40 was found to be able to diffuse into the nucleus. Nevertheless, in the experimental system employed, the caspase-3 fusion protein entered the nuclear compartment whereas the significantly smaller Dextran40 failed to, hinting that the size exclusion limit of the nuclear pores was not significantly altered.

One possible mechanism for allowing caspase-3 access to its nuclear targets was proposed by Faleiro et al. An increase in the exclusion limit of the nuclear pores caused by the proteolytic activity of caspase-9 should allow for passive diffusion of caspase-3 into the nuclear compartment.

The BIR3 subdomain of XIAP is a potent inhibitor of caspase-9 by direct obstruction of its active site. To test the inhibitory capacity of BIR3-RING (BIR3) domains overexpressed in yeast, two different assays were employed. In an in vitro reconstitution of the intrinsic activation pathway the presence of recombinant BIR3 did not lead to activation of caspase-3 via caspase-9.

TNFα induced apoptosis in HeLa cells leads at least in part to a direct activation of caspase-3 via caspase-8, bypassing caspase-9. The observed reduction in the apoptosis rate could be attributed to the disruption of an existing amplification loop involving caspase-3 and -9. Furthermore, the injected amount of BIR3 was sufficient to uphold inhibition of caspase-9 in the presence of mitochondrially released Smac/Diablo and Omi/HtrA2. Both apoptotic effectors are able to interact with and displace BIR3 from caspase-9, demonstrating the biological activity of the protein and the potent inhibition of caspase-9 at the concentration used.
To test the hypothesis that caspase-9 activity leads to an increase of the conductivity of the nuclear pores, active caspase-3 was co-microinjected with BIR3. Cells injected with both proteins did not display altered morphology as compared to cells injected with caspase-3 alone. Accumulation of the recombinant protease in the nucleus was rapid and complete before the first observation point at 15 minutes. As described for caspase-3 alone, Dextran40 was not observed to equilibrate across the nuclear membrane. Additionally, the degree of chromatin condensation was not decreased. Hence, an involvement of caspase-9 in the translocation of caspase-3 into the nucleus could be excluded for the microinjection system.

Although the involvement of caspase-9 in nuclear translocation of caspase-3 was ruled out, the possibility of passive diffusion into the nucleus could not yet be dismissed. In the course of apoptosis, several components of the NPC are cleaved by caspase-3$^{139-141}$, possibly resulting in a change of the size exclusion limit. Consequently, the proteolytical activity of caspase-3 was completely abrogated by pre-incubation with the irreversible synthetic inhibitor z-DEVD-fmk. Upon microinjection of this pre-treated protein, the characteristic cellular alteration associated with apoptosis could not be observed with the cells displaying healthy morphology. Strikingly, the translocation of the GFP-fusion protein was not hindered. The proteolytically silenced protease was found to be capable of entering the nucleus to the same degree as observed for active caspase-3.

As a conclusion, the cleavage of nucleoporins and the concomitant alteration of the NPC is not a requirement for allowing caspase-3 translocation into the nuclear compartment. This finding, along with the size of the fusion protein of approximately 120 kDa and the observed exclusion of Dextran40, disqualifies passive diffusion as the means for entry of caspase-3 into the nuclear compartment. As mentioned before, a similar sized 4xGFP protein is retained in the cytoplasm$^{159}$. Thus, the capacity for entering the nuclear compartment must be a quality of the caspase-3 protein.

Since activity of caspase-3 did not influence its subcellular localization, a crucial determinant might be the processing state of the protease. Although the z-DEVD-fmk treated protease lost its capacity to cleave its targets, it retained the conformation of active caspase-3. Therefore, the reasoning was that the significant conformational rearrangements upon proteolytic processing could lead to the exposure of a translocation signal. Yet, microinjection of a catalytic site mutant that is not
processed between the two catalytic subunits during purification, failed to exhibit any changes in subcellular localization compared to processed caspase-3. The extent and rate of translocation into the nucleus was not altered for the active site mutant. As observed before, equilibration of the recombinant fusion protein between cytosol and nucleus was complete within 15 minutes. Again, passive diffusion of this construct is an unlikely mechanism. The catalytic site mutant lacking the prodomain is capable of forming a dimer like active caspase-3 \textsuperscript{59}, effectively increasing the size of the mutant fusion protein to approximately 120 kDa. As a conclusion, caspase-3 lacking its prodomain translocates into the nucleus independent of its processing state and the pathways used are similar or even identical.

All the caspase-3 constructs tested for nuclear translocation were devoid of the short prodomain. For caspase-7, an accumulation in the nucleus after apoptotic stimulation was demonstrated \textsuperscript{161}. This translocation was triggered by the removal of the prodomain that exerts a negative influence on the recognition of a basic tetrapeptide NLS in the large subunit of caspase-7. Caspase-7 is the closest relative of caspase-3 with a sequence identity of 55 percent. Similar to caspase-3 \textsuperscript{68}, its activation is inhibited by its prodomain \textsuperscript{161,162}. Yet, no comparable NLS sequence could be identified in caspase-3. Using a GFP fusion protein, the exclusion of overexpressed full length caspase-3 in unstimulated Mcf-7 cells could be demonstrated \textsuperscript{150}. Upon cisplatin induction of apoptosis, this GFP fusion construct enters the nucleus in comparable levels as observed for the caspase-3 proteins described before. Conflicting observations were reported by Shikama et al. \textsuperscript{163}. Following the expression of an N-terminal GFP fusion to procaspase-3, fluorescence was detected in both the cytoplasm and the nucleus of HeLa cells. Although the authors insisted on an absence of apoptosis induction 48 hours post-transfection, they failed to provide the proper controls by Western blot. The induction of maturation of caspase-3 at high concentrations could result in the loss of the prodomain along with the fluorescence tag. Ensuing unhindered diffusion of GFP into the nuclear compartment may explain the detection of fluorescence in the nucleus.
5.3 Nuclear entry of caspase-3 is mediated by active transport processes

Passive diffusion of the caspase-3 constructs was ruled out because of their molecular size and the nuclear exclusion of the smaller Dextran40. Consequently, the distinct possibility of facilitated transport across the nuclear membrane was taken into consideration and further investigated in this work.

Several members of the caspase family such as caspase-1, caspase-2 and caspase-7 were found to translocate into the nuclear compartment. For caspases-1 and -2 this could be explained by the presence of an NLS in their long prodomain. As mentioned above, the prodomain of caspase-7 masks an NLS-like peptide that allows for nuclear translocation after apoptotic stimulation.

In order to test for the involvement of active transport processes, microinjection was performed at low temperatures. The uptake of NLS-bearing substrates is impaired by chilling, probably due to impaired hydrolysis of nucleoside triphosphates such as ATP. Since release of transport complexes from the nuclear pore complex is an energy dependent process, transport complexes are frozen in translocation and accumulate at the nuclear membrane.

To examine whether incubation at 4°C affected the cells capacity to efficiently perform nuclear import, control cells were incubated at 37°C after microinjection at low temperature. NLS-BSA accumulated in cells co-microinjected with mutant caspase-3.

This is in agreement with the finding that HeLa cells can be kept on ice for four hours without impairing their ability to perform NLS-mediated transport afterwards.

After an incubation time of one hour at 37°C, the cells microinjected with active caspase-3 displayed morphological alterations described before as well as complete breakdown of chromatin structure. Hence, the injection at low temperature did not reduce the capacity of caspase-3 to induce an apoptotic phenotype. Strikingly, complete accumulation of the NLS bearing BSA was also observed in the presence of active caspase-3 protein.

The completion of the import might be explained by the high velocity of the importin dependent import. 10 minutes after microinjection with an NLS coupled β-galactosidase (120 kDa) into rat hepatoma cells, the level of accumulated fusion protein was four times higher in the nucleus than in the cytosol. Hence, even in the presence of high DEVDase activity, active nuclear transport does not break down
immediately. Actually, its maintenance was found to be essential for the progression of apoptosis. Several proteins such as PKCδ, c-Abl, MST1, and Cdc6 were identified to translocate into the nuclear compartment following their cleavage by caspases 170-173, emphasizing the requirement of active nuclear transport in apoptosis.

A qualitative time point for the loss of import capacity was set at the transition from stage I to stage II, concomitant with the cleavage of the nucleoporin POM121. Inferring the same time frame for the microinjection experiments, active transport could occur even after 15 minutes of incubation. At that time point, accumulation of caspase-3 GFP fluorescence in the nuclear compartment had already peaked.

It has to be mentioned that in GFP-NLS expressing Rat2 cells, redistribution of the fluorescent substrate from the nucleus to the cytoplasm became detectable in the cytoplasm during progression from chromatin condensation stage I to stage II. The corresponding observation was not made in the majority of NLS-BSA injected HeLa cells. Although this finding could be attributed to the larger size of BSA compared to GFP or the difference in cell type, it is in line with the incapacity of Dextran40 to equilibrate across the nuclear membrane even after 60 minutes of incubation with active caspase-3 in the experiments with HeLa cells.

As for the microinjection of active caspase-3 at low temperature, the typical apoptotic features were absent, implying that the protease does not exert DEVDase activity under these conditions. At the same time, NLS-BSA was completely excluded from the nuclear compartment, demonstrating that the cooling conditions were sufficient to halt active transport processes. More importantly, the GFP fluorescence of the caspase-3 fusion proteins co-localized completely with the control protein. The observation of complete exclusion of caspase-3 protein from the nucleoplasm strongly indicated an involvement of active transport processes in the translocation of caspase-3 into the nuclear compartment. This cytoplasmic retention was observed independent of the processing state of the protease, again hinting that similar import pathways are employed by the active and mutant caspase-3 constructs.

Incubation at low temperatures therefore served as another control to exclude passive diffusion as a means of caspase-3 entry into the nucleus. Additionally, the complete exclusion of the different caspase-3 constructs demonstrated that the observed nuclear uptake was not an artifact produced by the microinjection of high amounts of recombinant protein.
Another commonly used method to test for the involvement of active nuclear import is the use of WGA. This lectin binds to N-acetylglucosamine residues on the surface of nucleoporins, thereby inhibiting their interaction with import complexes and efficient translocation. Co-microinjection of this inhibitor changed the cellular distribution of active and mutant caspase-3 constructs as compared to cells microinjected with the protease alone. In both cases, the fluorescence level of the GFP in the nucleoplasm was significantly reduced, although not to background levels, as observed in the previous experiment. Hence, the interaction of the caspase-3 constructs with nucleoporins was mandatory for efficient translocation into the nucleus. Taken together, caspase-3 traverses into the nucleus through the pore complexes, and not holes in the nuclear membrane as a result of proteolytic activity in the case of active caspase-3.

One of the most commonly used transport pathways is the import mediated by members of the importin $\beta$ family. Since two requirements for active transport, namely the energy-dependence and interaction with nucleoporins, were demonstrated to be fulfilled, the involvement of the importin $\beta$ pathway was examined. A potent inhibitor of this pathway is a truncation mutant of importin $\beta$. In addition to the elimination of the importin $\alpha$ interaction site, $\Delta$imp$\beta$ (45-462) can no longer be released from its nucleoplasmic docking site by RanGTP. With this mutant, import mediated by importin $\beta$ and the related transportin is completely blocked.

In contrast to the exclusion of NLS-BSA from the nuclei of $\Delta$imp$\beta$ injected cells, caspase-3 localization was not affected by this dominant negative mutant. Accumulation of the fusion protein in the nuclear compartment was evident at all time points investigated. The capacity to enter the nucleus was not the result of degradation of $\Delta$imp$\beta$, since importin $\beta$ is not cleaved in apoptotic cells. Consequently, involvement of both importin and transportin both disqualified as mediators of the import of caspase-3. The presence of the dominant negative inhibitor was not capable of influencing the spatial distribution of caspase-3 in microinjected HeLa cells.

It has been estimated that more than 160 transport factor binding sites are present per NPC. Karyopherins exhibit a strong preference for a subset of nucleoporins, implying that binding of the importin $\beta$ mutant would result in the blockade of
pathways with overlapping docking sites, but not the global inhibition of active transport.

Although active transport mechanisms were implicated in the nuclear entry of caspase-3, the analysis of the primary sequence of this protein did not reveal any known consensus NLS. Post-translational regulation of transport signals by glycosylation or phosphorylation, for example, can be ruled out, since the proteins were bacterially expressed. Additionally, intracellular modification upon injection would have to be extremely rapid considering the rate of translocation into the nucleoplasm observed for the caspase-3 constructs.

Although importin and transportin mediated import constitute major transport pathways, various proteins enter the nucleus by other mechanisms. As an example, the mitogen activated protease ERK2 translocates into the nucleus by directly interacting with nucleoporins, similar to the translocation of importin α and β alone. This mechanism is not feasible for caspase-3 however, since in absence of cytosolic factors, the fusion protein is retained in the cytoplasm (W. Albig, unpublished data).

In conclusion, the nature of the import pathway for caspase-3 as well as of the mediators of facilitated transport remains obscure so far.

### 5.4 Active nuclear transport is indispensable for nuclear apoptosis

Although co-microinjection of Δimpβ did not result in an alteration of caspase-3 localization, inhibition of active transport did reveal a difference in the morphology of nuclear apoptosis. Whereas more than 60 percent of active caspase-3 injected cells displayed complete breakdown of chromatin structure after 60 minutes of incubation, less than 10 percent co-injected with the dominant-negative truncation mutant had entered stage III chromatin condensation. A similar observation was made in the presence of WGA, confirming the findings of Yasuhara et al. Even after prolonged incubation in the presence of Δimpβ, chromatin condensation did not reach the level of control cells, allowing for the speculation that chromatin condensation is not only slowed, but effectively arrested under conditions of transport inhibition. Therefore, it has to be concluded that irrespective of the inhibitor of active transport, the apoptotic signal has to be relayed to the nucleus via active transport mechanisms.
In parallel to a reduction in chromatin condensation, inhibition of active import processes led to a decrease in TUNEL staining as a measure for the degradation of the DNA. Whereas stage II cells were mostly TUNEL negative, stage III nuclei were readily labelled by the TUNEL reaction, raising the possibility that the ultimate chromatin condensation can proceed only after DNA cleavage. These data are in agreement with the findings that BRL cells treated with a pan-caspase inhibitor failed to display stage III chromatin condensation and TUNEL staining after stimulation with staurosporine. Chromatin condensation was found to precede TUNEL labelling, starting at stage II.

The use of a pan-caspase inhibitor signified the involvement of caspases in the breakdown of chromatin structure. In Mcf-7 cells, nuclear DNA fragmentation is disturbed due to a functional knock-out of caspase-3. In the presence of z-DEVD-fmk, staurosporine treated Jurkat cells displayed only reduced chromatin condensation and DNA laddering, implying that the execution of these apoptotic events relies on caspase-3 activity. In conclusion, caspase-3 activity is critically involved in the execution of nuclear apoptosis either directly or indirectly by activating other effectors.

As demonstrated in this work, the presence of caspase-3 activity in the nucleus appears to be dispensable for the morphological aspects of nuclear apoptosis. The inhibition of the importin β pathway did not result in a reduction of caspase-3 translocation into the nuclear compartment. Yet, comparing the effect of WGA and ∆impβ as inhibitors of active transport processes, their impact on nuclear morphology, namely the reduction of chromatin condensation, was identical. Consequently, the origin of the apoptotic signal for the degradation of the DNA has to be placed in the cytosol. In conclusion, the activation of an apoptotic DNAse is caspase-3 dependent and takes place in the cytoplasm.

The most probable candidate for this apoptotic DNA cleavage is CAD. The localization of this apoptotic DNAse has long been the subject of dispute. The complex of ICAD/ CAD was purified from the cytoplasm of HeLa cells, but detected by another group in the nucleus of the identical cell line by immunoblotting and fluorescence microscopy. The reduction in chromatin condensation and TUNEL staining reported here serves as indirect evidence that prior to apoptotic stimulation the heterodimer is confined to
the cytoplasm. Degradation of ICAD by caspase-3 releases CAD that is subsequently transported into the nucleus in an importin β dependent manner. In the presence of the dominant-negative inhibitor of the importin β pathway, nuclear translocation of CAD, but not that of caspase-3, is disturbed, resulting in reduced chromatin condensation. The residual peripheral condensation can possibly be ascribed to AIF, an apoptotic factor released from the mitochondria. Immunodepletion of AIF and addition of ICAD to apoptotic cytosol completely abolished morphological changes in HeLa nuclei, underscoring the importance of AIF in nuclear apoptosis. For translocation of the 57 kDa AIF into the nucleoplasm, neither energy nor cytosolic factors are required. Therefore, the initial stages of nuclear apoptosis are not impeded by inhibitors of nuclear import such as WGA or Δimpβ and are efficiently executed.

5.5 Active nuclear transport is essential for survival following TNFα treatment

Ligation of TNFα to TNF-R1 elicits both pro- and anti-apoptotic responses, with the latter pathway relying on de novo synthesis of proteins such as c-IAP1, c-IAP2 and XIAP. Following an appropriate stimulus, cytoplasmic anchoring of the transcription factor NF-κB is abolished by unmasking of an NLS. NF-κB is then transported into the nucleus in an importin β dependent manner. Since transcriptional activation takes place in the nuclear compartment, the effect of blockage of the importin β dependent import pathways on TNFα mediated apoptosis was determined.

Microinjection of Δimpβ sensitized HeLa cells to apoptosis in response to CHX/ TNFα treatment, as judged by an increased apoptosis rate when compared to uninjected cells. In addition to the blockage of import pathways, Δimpβ binding to nucleoporins is known to shut down NES-dependent and mRNA export. As a result, cellular homeostasis is presumably negatively affected.

Demonstrating the importance of nuclear translocation for NF-κB for cell survival, the rate of apoptosis was not diminished in the absence of the translational inhibitor for Δimpβ injected cells. Therefore, TNFα and Δimpβ displayed a synergistic effect in promoting cell death in microinjected HeLa cells. This is in agreement with the finding
that an NLS-containing peptide competing for importin α binding of NF-κB specifically inhibits NF-κB mediated inflammatory responses \(^{186}\).

In the presence of this inhibitory peptide, TNFα and IL-6 production in response to LPS treatment was abolished in RAW macrophages. The action of the peptides was selective for NF-κB translocation since the response via the NF-κB independent Jak/STAT transcription factor pathway was not affected.

In summary, the inhibition of active transport acts as an efficient substitute for translational inhibition. The absence of NF-κB induced transcription of anti-apoptotic factors results in a sensitization of HeLa cells to TNFα induced apoptosis.

Apart from the previous finding, the proper execution of nuclear apoptosis relies on active transport processes, as demonstrated in this work. Although caspase-3 serves as the trigger for the breakdown of chromatin structure, the induction does not rely on its presence in the nucleus. Nevertheless, caspase-3 lacking the prodomain faithfully translocates into the nucleoplasm, presumably to promote cellular demise by the cleavage of several substrates.
6 SUMMARY

The subcellular localization of caspase-3 in a healthy cell was determined to be mainly cytosolic. In response to apoptotic stimuli, the apoptotic protease enters the nucleus. The mechanism employed remains the subject of controversy. In the presented study, the subcellular localization and the mechanism of nuclear entry of caspase-3 was studied employing a microinjection model.

For this purpose, recombinant caspase-3 was overexpressed in E. coli and isolated in high purity. Fusion of GFP did not affect the the proteolytic properties of the enzyme as demonstrated by strong DEVD-afc cleavage by the recombinant protease.

Injection of recombinant caspase-3 elicited the morphological features associated with apoptosis, accompanied by rapid equilibration of the apoptotic effector across the nuclear envelope. Both the inhibition of caspase-9 and caspase-3 activity failed to result in retention of the protease in the cytoplasm. Additionally, the rearrangement of the catalytic subunits following proteolytic activation of caspase-3 did not influence its cellular distribution. At the same time, the significantly smaller 40 kDa dextran was excluded from the nuclear compartment. Therefore, passive diffusion can be ruled out as the mode for caspase-3 entry into the nucleus.

The observed accumulation of caspase-3 in the nuclear compartment relied on active transport processes, as demonstrated by the requirement for interaction with nucleoporins and energy dependence. The hypothetical import factors utilized by caspase-3 do not belong to the family of importins or transportins, as inhibition of these pathways failed to block caspase-3 accumulation in the nucleoplasm.

Under conditions of import inhibition nuclear apoptosis is altered, as demonstrated by impaired chromatin condensation and DNA cleavage. The presence of caspase-3 was not required in the nucleus for the induction of these morphological features, indicating that the effectors are imported via an importin β dependent pathway from the cytosol following initiation of apoptosis.

Inhibition of the importin β pathway sensitized HeLa cells to apoptosis, probably by preventing NF-κB dependent activation of transcription. Therefore, active transport processes are indispensable for mediating cellular resistance in response to TNFα ligation, as well as the proper execution of nuclear apoptosis.


Die Inhibition des Importin β vermittelten Transports führte zu einer Sensitivierung von HeLa Zellen auf TNFα. Damit wird deutlich, dass aktive Transportvorgänge sowohl für die Resistenz gegen TNFα vermittelte Apoptose als auch für die Durchführung der Kernapoptose unentbehrlich sind.
8 REFERENCES


9 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>afc</td>
<td>7-amino-4-trifluoromethyl coumarin</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis protease activating factor-1</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculoviral IAP repeat</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>cyt c</td>
<td>cytochrome c</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DED</td>
<td>Death effector domain</td>
</tr>
<tr>
<td>DEVD</td>
<td>Aspartate-glutamate-valine-aspartate</td>
</tr>
<tr>
<td>DISC</td>
<td>death inducing signalling complex</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas associated death domain</td>
</tr>
<tr>
<td>fmk</td>
<td>Fluoromethylketone</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized ion affinity chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LiAc</td>
<td>Lithium acetate</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
</tbody>
</table>
NHS    $N$-hydroxysuccinimide
NLS    Nuclear localization signal
NPC    Nuclear pore complex
NTA    Nickel-nitrilotriacetic acid
PAGE   Polyacrylamide gel electrophoresis
PARP   poly (ADP-ribose) polymerase
PBS    Phosphate buffered saline
PEG    Polyethylene glycol
PFA    Paraformaldehyde
PMSF   Phenylmethyl Sulfonyl Fluoride
RIP     Receptor interaction protein
S. cerevisiae Saccharomyces cerevisiae
SDS-   Sodium dodecyl sulphate
Smac   Second mitochondria-derived activator of caspase
Sulfo-SMCC Sulfosuccinimidyl 4-[N-maleimidomethyl)cyclohexane-1-carboxylate
SV40   Simian Virus 40
TBS    Tris buffered saline
TdT    Terminal deoxynucleotidyl transferase
TE     TrisEDTA
TMR    Tetramethylrhodamine
TNF    Tumor necrosis factor
TNF-R  Tumor necrosis factor receptor
TR     Texas Red
TRADD  TNF-receptor associated death domain
TRAF-2 TNF-R-associated factor 2
TRITC  Tetramethylrhodamineisothiocyanate
WGA    Wheat germ agglutinin
XIAP   X-linked inhibitor of apoptosis protein
z-DEVD-fmk benzyloxycarbonyl-Asp[OMe]-Glu-[OMe]Val-Asp[OMe]-CH$_2$F