5 DISCUSSION

5.1 Caspase-dependent cleavage of the pre-RC components Mcm3 and Cdc6 in apoptotic cells

It would be counterproductive and even potentially dangerous, if an apoptotic cell started replicating its DNA, thereby possibly allowing a progression of the dying cell towards mitosis. Additionally, DNA replication in this situation would consume ATP, which is needed to carry out the active apoptotic death itself (157). Depletion of ATP early in the apoptotic process was shown to prevent the activation of caspases and switch the apoptotic to a necrotic cell death (6). Preserving ATP is especially important, because the release of cytochrome c from the mitochondrial inter-membrane compartment (which was shown to occur for all mitochondria in a cell within only 5 minutes) and the resulting activation of caspases lead to a permanent loss of the proton gradient, thereby interfering with the *de novo* generation of ATP via the respiratory electron transport chain (158). Accordingly, DNA replication should be and actually is repressed in apoptotic cells. In proliferation assays, I demonstrated that DNA-replication is almost completely shut down in apoptotic BL60-2 cells, as [^{3}H]-thymidine uptake was reduced to less than 7% 24 hours after the induction of apoptosis by anti-IgM antibodies.

Diverse apoptotic processes contributing to a repression of DNA replication have been described: the apoptotic condensation of chromatin (159), the typical DNA fragmentation (133) and the specific proteolytic cleavage of proteins involved in DNA replication. These proteins include the 140 kDa subunit of DNA replication factor C that binds to the DNA replication fork and recruits PCNA, which is, in turn, needed to recruit DNA polymerases delta and epsilon (160,161). In addition, DNA polymerase epsilon (162) and the DNA helicase BLM (163), which are presumed to function in DNA replication and repair, are cleaved in apoptotic cells.

I decided to examine possible effects of apoptosis on proteins participating in the pre-RC complex. The binding of this complex to DNA is a prerequisite for the start of DNA replication. Regulation at this step would therefore be a fast way to prevent unwanted DNA replication in the apoptotic cell. I first analyzed the mRNA expression levels for the subunits of the MCM complex and Cdc6 in untreated and apoptotic BL60-2 cells and compared them to the corresponding average mRNA levels of the housekeeping genes L32 and GAPDH. I detected a decrease in mRNA amounts in apoptotic cells for all seven tested pre-RC components. The reduction was much more profound than the general

decline in mRNA level as displayed by the housekeeping genes. The amount of Cdc6 mRNA was reduced to 47% (with the housekeeping genes set to 100%) as early as 2 hours after induction of apoptosis by anti-IgM antibodies. This shows a strong negative regulation at an early stage in B cell apoptosis before the execution phase begins, suggesting a possible contribution to the inhibition of DNA replication.

Mcm3-7 and Cdc6 are also represented on the Affymetrix GeneChip microarrays that were used for the overall expression screening. Interestingly, according to the data acquired with this method the decrease in expression levels observed for Mcm3-7 and Cdc6 in apoptotic cells was marginal (factors from -1.1 to -1.6) and detectable not earlier than 12 hours after induction of apoptosis (data not shown). A similar difference was seen when expression levels of the DNA repair proteins were compared by RPA and GeneChips (Table 1). Therefore RPA seems to be the more sensitive assay, recommended for the detection of minor changes in mRNA expression.

Caspase-dependent cleavage of Mcm3 was described in response to various apoptotic stimuli (137). I therefore examined if cleavage by caspases was a mechanism, which, in addition to the observed mRNA reduction, diminished the level of functional Mcm3 protein in our system of IgM-induced B cell apoptosis. I also assessed whether other components of the pre-RC complex were cleaved. In immunoblots, the cleavage of Mcm3 and Cdc6 in apoptotic BL60-2 cells was observed, while Mcm2 and Mcm4-7 remained unchanged. The cleavage of Mcm3 occurred at 8 to 12 hours after induction of apoptosis by anti-IgM antibodies, whereas a small amount of the main cleavage fragment of Cdc6 was already visible in immunoblot after 4 hours. This represents a very early stage in IgM-mediated B cell apoptosis, which is a slow CD95-independent type of cell death (164). Cdc6 is the protein with the earliest caspase-mediated cleavage observed in IgM-stimulated apoptotic BL60-2 cells. All other proteins analyzed in this system up until this point did not display cleavage fragments earlier than 8 hours past induction of apoptosis (52,136,165). In addition, the active fragment of caspase-3 was only visible on immunoblots as late as 8 hours past induction of apoptosis (data not shown). Nevertheless, it is possible that small amounts of caspase-3 or another caspase become active earlier in the apoptotic cells and account for the fast cleavage of Cdc6.

To evaluate whether the cleavage of Mcm3 and Cdc6 was a general apoptotic phenomenon and consistently generated the same fragments, Mcm3 and Cdc6 cleavage was analyzed in BL60-2 cells, HeLa cells and the T cell line H9 in response to different apoptotic stimuli. In all three cell lines, regardless of the apoptotic stimulus applied, I observed cleavage of Mcm3 and Cdc6. Apoptotic cleavage of Mcm3 yielded the same fragment with an apparent size of ~90 kDa in all cases. In contrast, I found a deviating

pattern for Cdc6 in apoptosis induced by anti-CD95 (Fas) antibodies in H9 cells. Cleavage, in response to this apoptotic stimulus, yielded three Cdc6 fragments (about ~50 kDa, ~45 kDa and ~35 kDa), in contrast to the other apoptotic samples, where only two fragments (~45 kDa and ~35 kDa) were generated. The ligation of death-receptors like CD95 starts a discrete apoptotic pathway, which substantially differs from the pathways induced by most other apoptotic stimuli. The deviating pattern of Cdc6 fragments might therefore be generated by a mechanism unique for death-receptor mediated apoptosis. In addition, the generation of Mcm3 and Cdc6 fragments was always paralleled by the activation of caspase-3.

Apoptotic processing of Mcm3 as well as Cdc6 did not only occur in parallel to the activation of caspase-3, but was dependent on caspase function, as two different caspase inhibitors completely blocked the cleavage of Mcm3 and Cdc6 in apoptotic cells. Based on this premise, I analyzed the effect of the three known executioner caspases on recombinant Mcm3 protein *in vitro*. I observed a cleavage by recombinant caspase-3 and caspase-7, which share the same optimal cleavage site DEVD, but not by caspase-6 (optimal sequence VEHD) (47). The specific cleavage, which was prevented by the caspase inhibitor z-DEVD-fmk, produced the same ~90 kDa-fragment that is generated in apoptotic cells. Incubation of HeLa nuclear extract with the recombinant caspases, led to identical results, further confirming the data observed with the recombinant protein.

Recombinant Cdc6 protein was cleaved by recombinant caspase-3 *in vitro* and the fragments produced corresponded to the ones generated in apoptotic cells (~45 kDa and ~35 kDa). In contrast to the data published by Pelizon et al. while these experiments were under way, that excluded a cleavage of Cdc6 contained in HeLa nuclear extract by caspase-7 (138), I examined a distinct cleavage of recombinant Cdc6 by caspase-7. Interestingly, caspase-7 selectively produced the larger protein fragment (~45 kDa), while the smaller fragment (~35 kDa) was not generated. Caspase-6 induced a very weak cleavage of Cdc6. Because of the deviating pattern observed for Cdc6 fragments in apoptotic H9 cells after crosslinking of CD95 (Fas), I examined whether caspase-8 or caspase-10, which are known to be involved in apoptosis induced by crosslinking of death-domain containing receptors, would cleave recombinant Cdc6. I did not observe a cleavage after incubation with these caspases. In addition, a combined incubation of recombinant Cdc6 with caspase-3 and caspase-8 or caspase-3 and caspase-10 generated exactly the same fragments produced during incubation with caspase-3 alone. The same results were obtained upon incubation of HeLa nuclear extract with the recombinant caspases, proving that the data did not originate from limitations associated with recombinant proteins (e.g. missing posttranslational modifications). It therefore

remains unknown which protease or combination of proteases generates the deviating pattern of Cdc6 fragments observed in apoptotic H9 cells. Although both caspase-3 and -7 were able to cleave Mcm3 and Cdc6 *in vitro*, I focused on caspase-3 for the subsequent experiments as this protein appears to be the primary effector caspase (166).

5.2 Identification of caspase-3 cleavage sites in Mcm3 and Cdc6

The two most critical determinants of specificity in the caspase-3 optimal cleavage site DEVD are the aspartic acids in position P_1 and P_4. Because there are several DxxD sites in both Mcm3 and Cdc6, I attempted to determine the specific sites used by caspases-3 in apoptotic cells. Analysis of different fragments spanning Mcm3 / Cdc6 by an *in vitro* transcription and translation assay (TNT) and the application of PCR-mediated mutagenesis enabled us to identify the decisive sites at $DAKD^{701}$ for Mcm3 and at $DEMD^{287}$ as well as $SEVD^{442}$ for Cdc6. Usage of the identified sites *in vivo* in apoptotic cells was verified by overexpression of FLAG-tagged Mcm3 and Cdc6 wildtype proteins and Mcm3 and Cdc6 proteins mutated in the identified caspase-3 sites. The mutant Mcm3 and Cdc6 proteins were not cleaved in apoptotic cells, while the wildtype proteins were processed like their intrinsic counterparts.

The caspase-3 cleavage site identified in Mcm3 is located at the C-terminus of the protein, directly following the putative nuclear localization sequence (167). Hitherto no specific functions of the protein are known to be located in the short C-terminal part that is removed by caspase-3.

The two caspase-3 sites in Cdc6 were identified in very interesting positions. The atypical caspase-3 site $SEVD^{442}$, which is cleaved very early in apoptotic BL60-2 cells, is located directly at the transition between the Cdc6 domains II and III (Figure 26) (125).

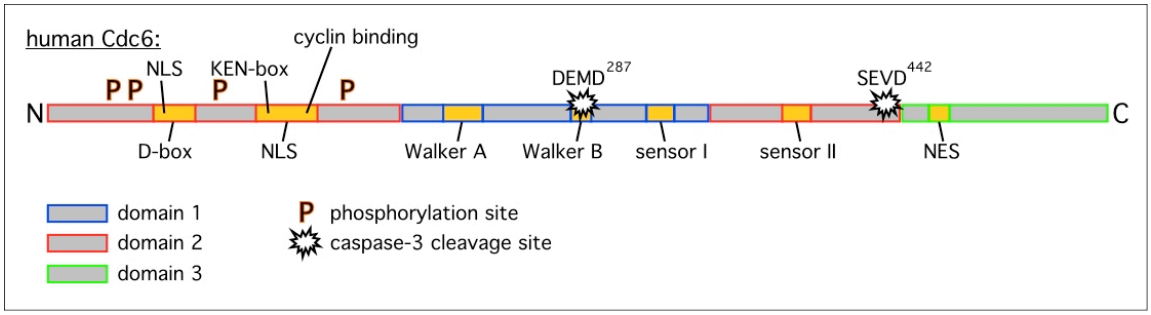

Figure 26. Cdc6 domains and caspase-3 cleavage sites. The main identified caspase-3 cleavage site SEVD442 is located at the boundary between the Cdc6 domains II and III. The second cleavage site DEMD287 resides in the Walker B site, which is involved in ATP hydrolysis by Cdc6.

As described briefly in the introduction, Cdc6 belongs to the AAA^+ superfamily of ATPases. Proteins belonging to this family generally consist of three domains with domain I and II functioning in ATP binding and hydrolysis, while domain III is a variable structure that can rotate considerably relative to the rest of the protein in an ATP dependent fashion (98). Caspase-3 cleavage of Cdc6 at $SEVD^{442}$ deprives the protein of this third domain including the putative **n**uclear **e**xport **s**equence (NES) (122) and a winged-helix structure, which is thought to mediate protein-DNA or protein-protein interactions and could therefore exhibit important regulatory functions (125).

The second caspase-3 cleavage site $DEMD^{287}$ resides in the Walker B site of the Cdc6 protein (Figure 26). This site is essential for ATP hydrolysis by Cdc6. Cdc6 mutants with inactivated Walker B site were shown to inhibit S phase progression (99). Caspase-3 mediated cleavage directly in the Walker B site separates Walker A and B from the Sensor sequences I and II, which are thought to mediate a conformational change of Cdc6 in response to ATP hydrolysis (91).

5.3 Functional analysis of apoptotic Mcm3 and Cdc6 fragments

Mcm3 and Cdc6 fragments corresponding to the ones generated from intrinsic Mcm3 and Cdc6 in apoptotic cells were prepared (Figures 14 and 18) and their properties as well as their effects on transfected cells were examined. First, the subcellular localization of the three Mcm3 variants FLAG-Mcm3wt, FLAG-Mcm3N and FLAG-Mcm3D701A was analyzed. All of them were discovered in the nuclei of transfected HeLa cells. Both FLAG-Mcm3N and FLAG-Mcm3D701A were also able to coprecipitate intrinsic Mcm4, although to a lesser extent than FLAG-Mcm3wt. The localization of the N-terminal Mcm3 fragment and the caspase-resistant Mcm3 mutant in the nucleus and their binding to Mcm4 indicates that both can fulfill at least part of the wildtype's functions e.g. participating in MCM complexes.

Next, I examined whether any of the Mcm3 variants had a pro-apoptotic effect on the transfected cells. Transfection with FLAG-Mcm3N repeatedly caused a significantly higher percentage of cells stained positive for activated caspases (here 9.4 fold induction when compared to empty plasmid) than transfection with FLAG-Mcm3wt or the caspase-resistant mutant. This finding indicates a reinforcing pro-apoptotic activity for the apoptotic caspase cleavage product of Mcm3. In addition, the percentage of cells containing activated caspases appeared to be slightly lower for the caspase-resistant mutant than for the wildtype. The effect was small and not always apparent, but could be due to the production of the apoptotic fragment which is possible from the overexpressed wildtype

but not from the mutant. In fact, generation of the Mcm3 fragment upon overexpression of FLAG-Mcm3wt was visible on the corresponding immunoblot (Figure 17B). Therefore, the following mechanism could be assumed: overexpressed FLAG-Mcm3wt and the caspase-resistant mutant FLAG-Mcm3D701A have a pro-apoptotic effect on the transfected HeLa cells (2.8 fold induction for the mutant when compared to empty plasmid). A perturbation of the endogenous pre-replicative complex due to the overexpression of one component may activate the DNA replication checkpoint and thereby cause an apoptosis inducing signal. This would then lead to limited caspase-mediated cleavage of FLAG-Mcm3wt, while the cleavage is prevented by mutation of the caspase cleavage site in FLAG-Mcm3D701A. The fragment produced from the wildtype might account for the further enhanced apoptosis rate observed (3.8 fold induction in this case).

Analysis of the four Cdc6 variants revealed a wildtype-like intracellular distribution for FLAG-Cdc6N and the caspase-resistant mutant FLAG-Cdc6D287A+D442A, which can be nuclear or cytoplasmic depending on the current stage in the cell cycle. In contrast, FLAG-Cdc6C was exclusively cytoplasmic, which is likely due to the fact that this fragment does not contain the two N-terminal **n**uclear **l**ocalization **s**equences (NLS) of Cdc6wt, but still includes the putative **n**uclear **e**xport **s**equence (NES) (122). FLAG-Cdc6N contains the two NLS but not the NES. Despite the lack of the described NES, FLAG-Cdc6N was nuclear or cytoplasmic in normal cycling cells and exclusively cytoplasmic in apoptotic cells with fragmented nuclei. These data argue against an important function for the NES predicted between amino acids 462 and 488 by Delmolino et al. (122) or at least for an additional alternative export mechanism.

Overexpression of FLAG-Cdc6wt and the caspase-resistant FLAG-Cdc6D287A+D442A had a significant pro-apoptotic effect on the transfected cells, while FLAG-Cdc6N and FLAG-Cdc6C augmented apoptosis only to a much lower extent. The pro-apoptotic effect of FLAG-Cdc6wt was also visible on immunoblot (Figure 13B), where the apoptotic Cdc6 fragment was generated from overexpressed FLAG-Cdc6wt even without additional induction of apoptosis by staurosporine. The pro-apoptotic effect of the caspase-resistant Cdc6 mutant contradicts the results of Pelizon et al., who describe an anti-apoptotic effect for their uncleavable Cdc6 after microinjection of the expression-plasmid into HeLa cells. This difference may be explained by the additional mutation generated at $LVFD^{99}$ or by the intactness of the site $DEMD^{287}$, which was neglected by Pelizon et al. (138). The intact site $DEMD^{287}$ could also explain the disappearance of the presumably caspase-resistant Cdc6 mutant in the presence of caspase-3 (Pelizon et al., Figure 1c). The fragments FLAG-Cdc6N and FLAG-Cdc6C augmented the percentage of cells with activated caspases far less than the full length proteins upon overexpression. Therefore, overexpressed

apoptotic Cdc6 fragments were not able to disturb cellular processes to the same extent as the full length wildtype and caspase-resistant proteins. This finding indirectly indicates a loss of function induced by the apoptotic cleavage of Cdc6.

While this work was in progress, Pelizon et al. published a caspase-dependent cleavage of Cdc6 in apoptotic cells and the generation of an additional apoptotic Cdc6 fragment of ~52 kDa. They identified the third caspase-3 cleavage site leading to the production of this fragment at $LVFD^{99}$, directly behind the second nuclear localization signal and the cyclin binding site (138). Accordingly I tried to visualize this Cdc6 fragment which was not recognized by the anti-Cdc6 antibody I used, by cleaving a radioactively labeled full length Cdc6wt protein produced in the TNT assay system with recombinant caspase-3 and through overexpression of both N- and C-terminally tagged Cdc6wt and Cdc6D287A+D442A in HeLa cells that were subsequently induced to undergo apoptosis by addition of staurosporine. The analysis of the TNT product by autoradiography and of whole cell protein extracts from the apoptotic cells with antibodies directed against the N- or C-terminal tag respectively, did not reveal the ~52 kDa fragment described by Pelizon et al.. I therefore come to the conclusion that $LVFD^{99}$ is neither a site used by recombinant caspase-3 nor in cells after induction of apoptosis.

Taken together, I was able to show cleavage of the pre-RC components Mcm3 and Cdc6 in three different cell lines in response to various apoptotic stimuli. I observed a deviating pattern of Cdc6 fragments, compared to the one described by Pelizon et al., and $LVFD^{99}$ was excluded as a site used by caspases in apoptotic cells (138). Cleavage of recombinant Mcm3 and Cdc6 by caspase-3 and -7 was examined *in vitro*. Two caspase-3 cleavage sites in Cdc6 and the single caspase-3 cleavage site in Mcm3 were identified. In overexpression experiments, the intracellular localization of the caspase-3 fragments and the caspase-resistant variants of Mcm3 and Cdc6 as well as some functional characteristics were analyzed. Furthermore, I was able to show a reinforcing pro-apoptotic function for the large apoptotic Mcm3 fragment. The cleavage of Mcm3 therefore exhibits an additional function in the apoptotic cell, which exceeds the inhibition of DNA replication.

5.4 Differential gene expression in apoptotic BL60-2 cells

As apoptosis - in contrast to necrosis - is an active process leading to an organized death of the cell, the expression of regulatory proteins that are needed in this context has to be enhanced. In contrast, the synthesis of proteins, which are not essential for the execution of apoptosis or even inhibit it, has to be downregulated (168). However, not much is known about the overall transcriptional changes in B cells following IgM-crosslinking.

Therefore, with the help of the currently available techniques for screening the differential expression of a large number of genes at once (Affymetrix GeneChips and cDNA membranes), a screen for mRNAs specifically up- or downregulated in apoptotic versus normal cycling BL60-2 cells was performed. In addition, the results found for several genes were verified in RNase protection assays.

In total I detected differential mRNA levels for 3.9% of the genes tested by GeneChip arrays and 18.1% of the genes on Atlas cDNA membranes. These numbers indicate that apoptosis did not cause an unspecific overall shutdown of transcription. On the contrary, I found more genes with increased than genes with decreased mRNA levels. This result supports the common view of apoptosis as an active organized form of cell death with the need to enhance the expression of specific genes and to shutdown others.

5.5 Repression of DNA repair genes in apoptotic BL60-2 cells

DNA fragmentation is a major hallmark of apoptosis. It leads to the irreversible degradation of chromosomal DNA, thereby preventing the uptake of biologically active DNA by the cells engulfing the apoptotic corpses (133). It would therefore be counterproductive and potentially dangerous, if an apoptotic cell started DNA repair on the fragments produced. Additionally, DNA repair in this situation would consume ATP, which is needed to carry out the active apoptotic death itself (see above) (157). Consistently, I examined the apoptotic downregulation of mRNA levels for six components contributing to cellular DNA repair mechanisms.

The mRNA amounts of three DNA ligases were reduced in apoptotic BL60-2 cells. DNA ligase I is the DNA ligase best capable of joining blunt DNA ends, whereas its activity in ligating DNA breaks with single-strand overhangs is relatively low (141). The break points produced during apoptotic DNA fragmentation are described as either blunt or with short overhangs, making them possible substrates for DNA ligase I (169). DNA ligases I has hitherto not been described in connection with apoptosis. DNA ligase III is, in addition to the mRNA reduction observed in this study, cleaved by the Ca^{2+}-dependent protease calpain in human fibrosarcoma cells after treatment with DNA damaging agents or gamma-irradiation, indicating that the removal or inactivation of this ligase might be of importance for apoptotic cell death (170). DNA ligase IV, probably the main ligase involved in non-homologous end joining (NHEJ), forms a complex with XRCC4 (132). The active form is a heterotetramer of both proteins, probably allowing a coordinated ligation of both DNA strands (131). The DNA ligase IV-XRCC4 complex needs Ku for effective DNA

binding (171). Inactivation of the LIG4 gene leads to extreme radiosensitivity of cells, inhibition of V(D)J-recombination in lymphocytes and augmented apoptosis (141).

In addition, three subunits of the DNA-dependent protein kinase (DNA-PK), the catalytic subunit DNA-PKcs and the regulatory subunits Ku70 and Ku80, were negatively regulated in apoptotic BL60-2 cells. Ku70 and Ku80 proteins form a heterodimer and bind non sequence-specific with high affinity to double-stranded DNA ends (172). Ku subsequently recruits DNA-PKcs and the DNA ligase IV-XRCC4 complex to the break (132). DNA-PK phosphorylates several different proteins including Ku, XRCC4, histones, topoisomerases and RNA polymerase II *in vitro* (126,143). Autophosphorylation causes dissociation of the catalytic subunit DNA-PKcs from the regulatory subunit Ku and inactivation of the kinase *in vitro* (173). In addition to the downregulation on mRNA level, caspase-dependent cleavage of DNA-PKcs was shown, suggesting that the removal of this protein is important for the apoptotic process.

In a recent publication, overexpression of Ku70 was reported to have an anti-apoptotic effect. Ku70 was shown to bind the pro-apoptotic Bcl-2 family member Bax and to suppress its translocation from the cytoplasm to the mitochondrial membrane. Ku70 thereby prevented Bax-induced cytochrome c release from the mitochondria and attenuated or even blocked apoptosis (174). Interestingly, the overexpression of Bax was shown to sensitize the Burkitt's lymphoma cell line BL-41 for IgM-induced apoptosis (25). In addition, a reduction of the Ku70 protein level in apoptotic HEK293T cells 12 hours after UV irradiation was described (174). The reduction was independent of caspases and could therefore be mediated by a change in the mRNA expression level as observed in apoptotic BL60-2 cells. Furthermore, unusual high expression levels for Ku70 were described for cancer cells (175,176), while a significant decrease of Ku70 and Ku80 protein was reported in apoptotic but not in non-apoptotic brain cells after ischemia (177).

In summary, I observed the transcriptional downregulation of six major components of the DNA repair system in apoptotic BL60-2 cells. Although the observed mRNA decrease seemed only moderate when analyzed by GeneChip microarrays, the profound changes visible for all six genes in RPAs proved their significance. As discussed above, RPAs seem to be more sensitive in detecting minor changes in mRNA expression levels than GeneChips in general (see pre-RC components). Together with the calpain-mediated cleavage of DNA ligase III and the caspase-mediated cleavage of DNA-PKcs the reduction in mRNA levels should impair DNA repair in apoptotic cells. In addition, the diminished Ku70 mRNA level probably has a direct pro-apoptotic effect, as a resulting negative regulation of Ku70 protein would allow the mitochondrial translocation of Bax.

5.6 Differential expression of transcription regulators in apoptotic BL60-2 cells

Because apoptosis, with the exception of death-receptor-mediated apoptosis in type I cells, is dependent on the *de novo* transcription of genes whose corresponding proteins are needed for the apoptotic processes and the repression of anti-apoptotic genes, transcriptional regulators play an important role in transforming a normal cycling into an apoptotic cell. This was reflected by modified mRNA levels of several transcription regulators after IgM-crosslinking on BL60-2 cells.

The mRNA level of ID2 was enhanced in apoptotic BL60-2 cells. Overall, ID2 is described as a protein with a positive impact on proliferation, e.g. downregulation of ID2 is required to stop proliferation and allow further differentiation of T cells after successful T cell receptor (TCR) □ chain rearrangement (178). If this is true in BL60-2 cells, the upregulation of ID2 in apoptotic cells would be counterproductive, as proliferation is shut down in apoptotic cells (Figure 7). On the other hand, my findings are supported by a recent report about reduced neuronal apoptosis in ID2 knockout mice and enhanced cell death upon ID2 overexpression (179).

NF-ATc was transiently upregulated in early apoptotic BL60-2 cells. Elevated intracellular calcium levels following antigen receptor crosslinking were described to activate cytoplasmic subunits of the NF-AT complex resulting in dephosphorylation and rapid translocation to the nucleus. There, the protein associates with an inducible nuclear component belonging to the AP-1 family (145). NF-ATc seems to play a role in lymphocyte differentiation as mice lacking functional NF-ATc fail to show IL-4 production and IL-4 dependent isotype switching to IgG1 and IgE (180). NF-ATc was not mentioned in context with apoptosis so far.

The mRNA levels of the two NF-AT target genes EGR-1 and EGR-2 were also enhanced in apoptotic BL60-2 cells. Several tumor cell lines as well as primary tumor tissues express little or no EGR-1 when compared to their non-malignant counterparts and ectopic EGR-1 expression in tumor cells can severely reduce their proliferation rates and tumorigenicity. In line with these findings, EGR-1 antisense RNA enhances cell growth. EGR-1 expression was reported to prevent apoptosis after UV and ionizing irradiation in several cell types, but was also shown to augment apoptosis via p53 upregulation following stress stimuli (181,182). Mature B cells rapidly upregulate EGR-1 after stimulation with anti-IgM, which induces proliferation and differentiation in these cells. In contrast, no upregulation appears in response to BCR crosslinking in the immature B cell line WEHI-231, which undergoes apoptosis in response to this stimulus (183). EGR-2

levels were found to be low in ovarian tumor tissues compared with their normal counterpart. Ectopic EGR-2 expression was able to suppress proliferation in several tumor cell lines while EGR-2 antisense RNA increased cell growth (184). Therefore, the positive regulation of EGR-1 and EGR-2 in apoptotic BL60-2 cells is in line with the examined shutdown of proliferation after stimulation with anti-IgM antibodies. In addition, EGR-1 mediated activation of its target gene NAB2, whose positive regulation was shown on GeneChip arrays, provides a negative feedback loop, since the NAB2 protein can act as a repressor for EGR-1, EGR-2 and EGR-3 (185,186).

TAFII30, which displayed an extremely elevated mRNA amount in apoptotic BL60-2 cells, is essential for transcription of specific genes by RNA polymerase II and is present in approximately 50% of TATA binding complexes (187). Some of these genes may be involved in the apoptotic processes. An involvement of TAFII30 in apoptosis was not reported until now, except for the fact that cells lacking TAFII30 are blocked in G1 and undergo apoptosis (188).

Topoisomerase I mRNA was downregulated in apoptotic BL60-2 cells. One function of topoisomerase I is to heighten the threshold between basal transcription levels and transcription levels after binding of a positive regulatory protein. This effect is specific for genes regulated by TATA containing promoters and is mediated by an interaction of topoisomerase I with the **T**ATA-**b**inding **p**rotein (TBP) (149). Topoisomerase I is cleaved by caspase-3 at an unusual cleavage site in apoptotic cells. However, the cleavage occurs relatively late in the apoptotic process and the major cleavage fragment is still able to bind to and cleave DNA (189). The downregulation on mRNA level found in anti-IgM stimulated BL60-2 cells could therefore be an additive mechanism to downregulate topoisomerase I protein in apoptotic cells.

ETR101 was upregulated in apoptotic BL60-2 cells. ETR101 was shown to accumulate after the crosslinking of IgM or IgG molecules on immature, naïve and memory B cells (151). Its upregulation therefore seems to be part of the general cellular response to B cell receptor stimulation independent of the later outcome – apoptosis or proliferation – of this event.

Taken together, I examined altered expression levels for several transcription regulators in apoptotic BL60-2 cells, some of which were not known to be involved in apoptosis. It is probable that the corresponding proteins represent some of the tools used by an apoptotic cell to adjust its proteome according to the new requirements - to carry out an organized death of the cell.

5.7 Differential expression of genes belonging to various functional groups in apoptotic BL60-2 cells

Phosphatases, in cooperation with kinases, participate in many cellular signal transduction pathways where they modify the activity of specific target proteins. It is well known that phosphatases are involved in the signaling cascade initiated by IgM-crosslinking (79). I observed enhanced mRNA levels for the two phosphatases CD45 and DUSP5 in apoptotic BL60-2 cells. CD45 sets a relative threshold for the sensitivity to the anti-IgM induced stimulus. It dephosphorylates Src family kinases in B cells, thereby rendering them competent to participate in the kinase cascade. The increase in intracellular calcium ions, which is part of the BCR signaling cascade, is abolished in CD45 negative cells (152,190). DUSP5 expression is induced very early (0,5 h) in fibroblasts following serum stimulation or heat shock. Recombinant protein was shown to dephosphorylate ERK-1/MAP kinase, serine-phosphorylated myosin and other substrates *in vitro* (153). A recent report showed that the dual-specificity phosphatase JKAP is involved in **t**umor **n**ecrosis **f**actor (TNF) and **t**ransforming **g**rowth **f**actor (TGF) mediated activation of the c-jun kinase (191). The function of DUSP5 itself is poorly defined and a possible role in the process of apoptosis needs to be assessed.

The mRNA amount of TTP (tristetraprolin) was augmented in apoptotic BL60-2 cells. TTP belongs to a group of proteins, which regulate the stability of specific target mRNAs. TTP deficient macrophages show an elevated TNF-α expression, which is the result of increased TNF-α mRNA stability. TTP was shown to bind to the **A**U-rich **re**gion (ARE) in the 3'-**u**n**t**ranslated **r**egion (UTR) of TNF-α mRNA (154). Upon re-expression of TTP in TTP-negative cells, reduced levels of reporter transcripts containing AREs from TNF-α, IL-3 or GM-CSF (**g**ranulocyte-**m**acrophage **c**olony-**s**timulating **f**actor) were seen, implicating a role of TTP in destabilizing these mRNAs (192). TTP is upregulated in apoptotic cells in response to various stimuli and continuous expression of TTP at physiological levels causes apoptosis (193). In agreement with my findings, treatment of T cells with crosslinking anti-TCR (T cell receptor) antibodies results in expression of TTP mRNA and protein, which is absent in resting cells (194).

The mRNA level of the TTP related early response gene Berg36 was strongly enhanced early in apoptotic BL60-2 cells. Berg36 antisense oligonucleotides were shown to partly inhibit apoptosis in Ramos cells, hinting at a pro-apoptotic function for Berg36 (195). Furthermore, overexpression of the mouse homologue Tis11b caused apoptosis in 3T3 cells (193). Due to the fact that the accumulation of Berg36 mRNA starts very early in apoptotic BL60-2 cells and reaches extremely high levels, Berg36 could be an important

target for differential regulation of mRNA expression in apoptotic BL60-2 cells. The definite function of the protein is presently under investigation, but a role in the regulation of mRNA stabilities similar to that of TTP is suspected.

The mRNA level of RGS1, an inhibitor of G protein function, was upregulated in apoptotic BL60-2 cells. RGS1 is activated in response to mitogens in tonsil B cells and several B cell lines. *In vivo* RGS1 levels are constantly high in a subpopulation of germinal center B cells (155,196).

The mRNA amount of the calcium binding protein NEFA was enhanced in apoptotic BL60-2 cells. Treatment with caffeine causes cells to release Ca^{2+} from the endoplasmatic reticulum. Cells show higher cytosolic calcium levels in response to caffeine when NEFA is overexpressed, suggesting that NEFA could be involved in intracellular calcium homeostasis (156). This finding is in agreement with the enhanced mRNA level of NEFA seen in apoptotic BL60-2 cells, as a low calcium level in the ER (after release of Ca^{2+} ions to the cytoplasm) was shown to trigger the incorporation of the pro-apoptotic protein Bax into the mitochondrial membrane. This again leads to cytochrome c release and activation of caspases thereby promoting apoptosis (197).

The mRNA of CD70 accumulated in apoptotic BL60-2 cells. CD70 can only be found on a limited number of B cells *in vivo*. The expression of CD70 was reported to be upregulated *in vitro* after BCR crosslinking in both naïve and memory B cells (198).

Taken together, the second part of my work represents a detailed analysis of transcriptional changes during IgM-induced B cell apoptosis. The results acquired by three different independent methods correlated well in almost all cases, rendering the data even more significant and showing that all three assays, Affymetrix GeneChips, Atlas cDNA membranes and RPAs, are valuable tools to screen the differential expression of genes. The data provide a basis for further investigation of the genes described which might be done by inducible expression systems or the siRNA (**s**mall **i**nterfering **RNA**) technique, to reveal their functional roles in the apoptotic process.

I was able to show differential mRNA levels for genes involved in DNA repair, for transcription regulators, phosphatases and genes belonging to other functional groups in apoptotic compared to unstimulated BL60-2 cells. In total, changes in mRNA levels were observed for only a small percentage of the genes analyzed, indicating their specific regulation in apoptosis. Most of the transcriptional changes appeared after 4 hours of stimulation with the anti-IgM $F(ab)_2$ fragment which is an early stage of B cell apoptosis clearly before effector caspases are activated. Therefore, the regulation of these genes probably plays an active role at the onset of cell death rather than reflecting late destructive events in the apoptotic cell.

The results observed in this study could also contribute to our understanding of lymphocyte differentiation in general, as several of the genes differentially regulated in IgM-induced cell death may also participate in other forms of B cell apoptosis like apoptosis by neglect (e.g. developing B cells lacking correctly folded B cell receptors). In addition, the identified genes with a differential regulation in apoptosis might be interesting candidates with regard to an involvement in malignant B cell diseases.