Activation of bcl-2 suppressible 40 and 44 kDa p38-like kinases during apoptosis of early and late B lymphocytic cell lines

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Abstract Activation of several different kinases characterizes the induction of apoptosis. Abelson virus transformed pre-B lymphocytes undergo apoptosis within 24 h of serum deprivation, PKA activation or γ-irradiation, and the activity of two kinases of ca. 40 and 44 kDa is specifically induced during this apoptotic process. Bcl-2 expression prevents both apoptosis and the induction of these kinases. Immunologic and substrate similarities indicate that these kinases are related to the p38 family of MAP kinases. More mature cells of the B lymphocytic lineage, plasmacytomas, also exhibit induction of these kinases when apoptosis is induced by withdrawal of serum or IL-6. Treatment of the pre-B cells with ICE protease inhibitors when apoptotic stimuli are delivered prevents induction of the kinase activity, and partially inhibits apoptosis. These findings indicate that the induction of these 40 and 44 kDa p38 related kinases is a common feature of apoptosis in mouse B lymphocytic cells and may represent a step downstream of ICE proteases in the signal cascade that leads to programmed cell death.

Key words: Apoptosis; bcl-2; Interleukin 1β converting enzyme; Kinase; p38; Lymphocyte

1. Introduction

Apoptosis is a common and highly regulated mechanism of programmed cell death. Apoptosis is essential during the development of higher organisms, and failure to undergo apoptosis appears to be one of the key events that lead to the development of malignancies [1–3]. In particular, apoptosis is an important mechanism that regulates the population homeostasis in the hematopoetic cell compartment [4,5]. During the maturation of B and T lymphocytic cells, autoreactive cells are eliminated by apoptosis, and apoptosis also serves to terminate immune responses at the appropriate time [6–8]. Failure to undergo apoptosis has been linked to the development of several hematopoetic malignancies, most notably B cell neoplasms, such as follicular lymphomas, in which the anti-apoptotic gene bcl-2 was first discovered at the site of a characteristic chromosomal translocation [9,10]. The biochemical function of bcl-2 remains controversial, but it is well documented that bcl-2 can protect various cell types against a broad range of apoptotic signals [6,8].

Apoptosis can be induced by a wide variety of stimuli, including ionizing radiation, activation of several cell surface receptors or deprivation of growth factors [1,11,12]. While the mechanisms of induction of apoptosis can be quite different, the intracellular components of the apoptotic machinery are highly conserved, since various apoptotic signals seem to converge into common intracellular pathways [5,7]. Based on genetic studies in *Caenorhabditis elegans*, an ICE (interleukin 1β converting enzyme)-like protease cascade has been discovered that becomes activated during apoptotic cell death [5]. The proteases are synthesized as inactive proenzymes that become activated by specific proteolysis at aspartate residues. The activation of the cascade remains enigmatic, although the recent cloning of a Fas associated protease, termed FLICE/MACH/Mch-5, suggests that Fas may trigger apoptosis by direct activation of this protease cascade [13,14]. How these events effect the subsequent steps that result in activation of additional genes in the 'death program' is unclear.

Recent data also suggest that MAP kinases (MAPK) are involved in the regulation of apoptosis. The MAPK superfamily comprises the mitogen induced ERK kinases and the stress activated kinases, JNK/SAPK, and p38. ERK activation has been shown to counteract apoptosis in some cells [15,16]. The role of the stress activated kinases is less clear. These kinases are activated by many stimuli that also induce apoptosis, e.g. UV irradiation, tumor necrosis factor α and growth factor withdrawal. Transfection experiments showed that dominant negative JNK and p38 mutants can block apoptosis in PC12 cells triggered by NGF deprivation [15]. On the other hand, JNK activation promotes T cell survival [17]. The situation is further complicated by a methodological ambiguity stemming from the fact that in many reports JNK activity was examined using immobilized GST-c-Jun to precipitate JNK from cell lysates. This type of assay for JNK and p38 activity may be of insufficient specificity, since it is possible that kinases other than JNK and p38 bind to c-Jun. Therefore, we decided to explore this possibility using in-gel assays to detect kinases that become activated during growth factor deprivation induced apoptosis of B cells. This method

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Abbreviations: PKA, protein kinase A; MAP kinase, mitogen activated kinase; IL-6, interleukin-6; ICE, interleukin 1β converting enzyme

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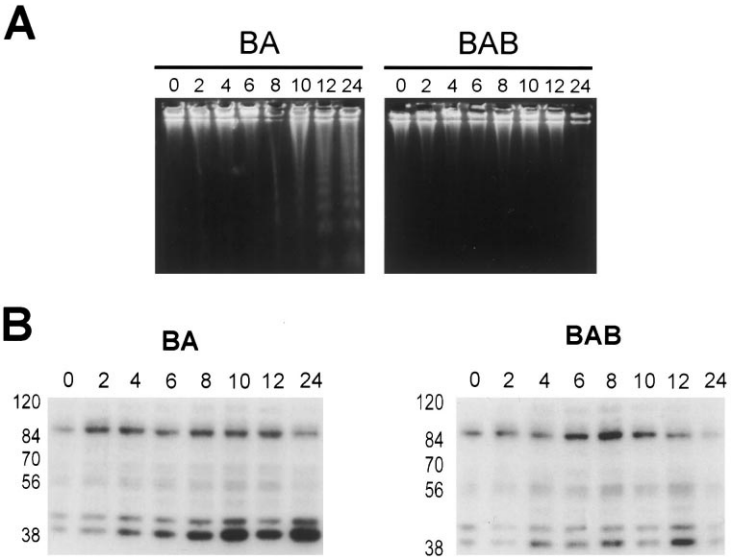

Fig. 1. A: Time course of the induction of apoptosis in BA cells and its absence in BAB cells. Cells were serum starved for the time indicated at the top of each lane and refed with medium containing serum for 18–24 h. Subsequently, DNA was isolated and loaded on a 2% agarose gel. While essentially no apoptotic laddering can be observed in the DNA isolated from BAB cells, the appearance of apoptotic ladders is easily visible in the DNA isolated from BA cells that were serum starved for at least 8 h. B: In-gel kinase assay of cell lysates from BA or BAB cells that were serum starved for the times indicated on the top. The sizes and positions of protein molecular weight markers are indicated on the left. This experiment was repeated five times with comparable results.

provides a more general survey of kinase activities, and it appears to be better suited for the purpose of screening for kinases involved in the apoptotic process.

Our results show that the kinetics of the onset of apoptosis in B cells correlates with the activation of two novel kinases with apparent molecular weights of 40 and 44 kDa. These kinases appear to be immunologically and biochemically related to the p38 kinase, since they are detectable with an anti-p38 antibody and can bind to and phosphorylate c-Jun. The activation of these kinases can be inhibited by the use of ICE-like protease inhibitors, suggesting that they are downstream of this protease cascade.

2. Materials and methods

2.1. Cell culture, virus infection, and reagents

All cells were grown at 37°C/5% CO_2 in RPMI 1640 supplemented with 10% fetal calf serum and 2 mM glutamine. The medium for TEPC 1165 was further supplemented with 1 ng/ml IL-6 (Gibco BRL). For serum starvation, cells were washed twice and subsequently cultured in the same medium without serum. Helper-free Abelson virus was generated as described previously [18]. The bcl-2 expressing plasmid, pLbclSN, was constructed by inserting the mouse bcl-2 cDNA into the LXSN retroviral expression vector. The bcl-2 expressing retrovirus was generated by transfecting pLbclSN into the Ψ2 packaging line. The cells were selected for G418 resistance with 1 mg/ml G418 (Gibco BRL) for 14 days and kept as bulk culture. To generate the BA (cells overexpressing v-abl) cell line, freshly isolated bone marrow cells from BALB/c mice were infected with helper-free Abelson virus as described [18]. To generate the BAB (cells overexpressing abl and bcl-2) cell line, BA cells were infected with LbclSN virus and subsequently selected for G418 resistance as described above.

2.2. Immunoprecipitation and kinase assays

Cell lysates (lysis buffer: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml leupeptin) and phosphatase inhibitors (20 μM β-glycerophosphate, 2 μM sodium orthovanadate, 2 μM sodium fluoride and 2 μM sodium pyrophosphate) were immunoprecipitated as described [19] using an anti-ERK serum [19] or an anti-JNK serum (Santa Cruz) coupled to protein A agarose. Precipitates were washed three times in TBST and once with kinase buffer A (20 mM Tris-HCl, pH 7.4, 20 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT). Kinase activity was determined in kinase buffer in the presence of 10 μM ATP/[γ-^{32}P]ATP (specific activity 30 Ci/mmol) using 2 μg myelin basic protein (MBP) or 2 μg GST-Jun as substrates in a total volume of 30 μl. The reactions were incubated at 30°C for 20 min, resolved on 12.5% SDS-polyacrylamide gels and the gels autoradiographed.

2.3. GST-Jun pulldown assays

Cell lysates were incubated for 2 h at 4°C with 5 μg GST-Jun fusion protein bound to glutathione-Sepharose. The precipitates were washed three times with PBS containing 1% NP-40 and 2 mM orthovanadate and once with kinase buffer B (15 mM Tris-HCl, pH 7.5, 20 mM NaCl, 7.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA). GST-Jun phosphorylation was measured as specified above in Section 2.2.

2.4. In-gel kinase assays

15 μg of cell lysate was resolved on a 10% SDS-PAGE containing 0.2 mg/ml substrate protein (myelin basic protein or GST-Jun). The gel was washed twice for 20 min with 150 ml of 50 mM HEPES pH 7.4, 5 mM 2-mercaptoethanol, and 20% isopropanol. After equilibration for 2×10 min at room temperature with 150 ml 50 mM HEPES pH 7.4 and 5 mM 2-mercaptoethanol, the proteins were denatured by incubating the gel twice for 30 min at room temperature with 150 ml of 6 M guanidine-HCl in 50 mM HEPES pH 7.4, 5 mM 2-mercaptoethanol. The proteins were renatured overnight at 4°C with three changes of 150 ml 50 mM HEPES pH 7.4, 5 mM 2-mercaptoethanol containing 0.04% Tween 20. The gel was equilibrated for 15 min at room temperature in in-gel kinase buffer (25 mM HEPES pH 7.4, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 90 μM sodium orthovanadate) and subsequently placed in fresh in-gel kinase buffer containing 250 μCi [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) for 1 h at 30°C. The gel was washed four times for 2–3 h in 500 ml 5% trichloroacetic acid/10 mM sodium pyrophosphate, dried and exposed to X-ray film.

2.5. Western blot analysis

Proteins were resolved on 10% SDS-PAGE and electroblotted. Blots were blocked with 5% non-fat dry milk for 30 min and incubated in primary and, after extensive washing with Tris buffered saline (TBS) and 1% Tween 20, in secondary antibody (Dianova) for 1 h each. The primary antibodies used were rabbit antisera against JNK

from Santa Cruz, against the C-terminus and the N-terminus of p38 from Santa Cruz, against full-length p38 (a kind gift from Brent Zanke, Smith Kline Beecham Pharmaceuticals), a mouse monoclonal antibody against v-abl from Oncogene Science and a hamster monoclonal antibody against bcl-2 from Biomol. Immunoreactive bands were visualized using the ECL system (Amersham).

2.6. Apoptosis assays

To monitor endonucleosomal DNA fragmentation, genomic DNA was isolated as described [18]. 5 μg of DNA per lane was electrophoresed at 80 V for 4 h on a 2% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). The DNA was stained with ethidium bromide. To quantify the number of apoptotic cells the APOALERT Annexin V Apoptosis Kit (Clontech) was used. Apoptotic cells were counted using a fluorescence microscope.

3. Results

To study kinases that become activated during the process of apoptosis in B cells, we used v-abl transformed mouse pre-B cells. We had determined earlier that such pre-B cell lines undergo rapid apoptosis (within 24 h) when serum is withdrawn [18]. To avoid artifacts due to the use of a cell line that had been in culture for several years, a bulk culture of freshly transformed cells (BA) was generated by infecting 10^6 BALB/c bone marrow cells with 10^7 focus forming units (ffu) of helper-free Abelson murine leukemia virus as described [18]. As bcl-2 overexpression leads to apoptosis inhibition, we generated the BAB cell line as a control: 10^7 of the BA cells were infected with LbclSN virus, a mouse bcl-2 expressing retrovirus, and selected for G418 resistance. Expression of both v-abl and bcl-2 was confirmed by Western blot (not shown).

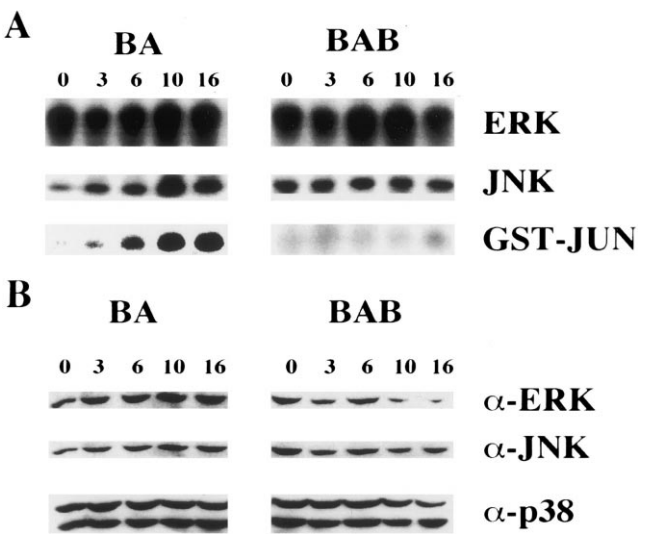

Fig. 2. Induction of Erk, JNK, and c-Jun binding kinase activity upon serum deprivation. Cell lysates of BA (abl) or BAB (abl-bcl-2) cells that had been serum starved for the time indicated at the top of the figure were precipitated using the antibodies or a GST-Jun expression protein as indicated on the right. The precipitates were washed and tested for kinase activity using either MBP as a substrate, for the immunoprecipitates, or the precipitating GST-Jun protein itself for the pulldowns. A: Kinase reactions were resolved on SDS gels and exposed to X-ray film. B: As a control for the amount of ERK, JNK, and p38 present in the cell lysates, Western blots of the crude lysates were probed with antibodies specific for the respective kinases. Antisera used were an anti-ERK1, -2 antibody [19], anti-JNK (Santa Cruz) and anti-full-length p38 (Dr. Zanke). Note that the anti-p38 antibody detects two bands (see also Fig. 3C).

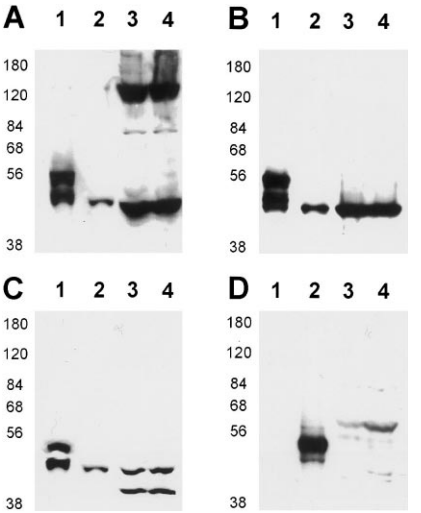

Fig. 3. p38 and JNK expression in BA and BAB cells. Duplicate Western blots were stained with antibodies against (A) the C-terminus of p38, (B) the N-terminus of p38, (C) GST-p38 (full-length p38), and (D) JNK. COS cells transfected with HA tagged p38 or HA tagged JNK-1 expression vectors were used as positive controls. Lanes 1: COS/p38, 2: COS/JNK-1, 3: BA, 4: BAB. The size and position of protein molecular weight markers is indicated on the left. This experiment was repeated three times with comparable results.

First, we determined the time point when apoptosis becomes irreversible (Fig. 1A). For this purpose BA and BAB cells were serum starved for 2–24 h, and then refed with medium containing serum. 24 h later genomic DNA was extracted and examined for endonucleosomal cleavage. While no sign of apoptotic DNA was detectable in the BAB cells, the first appearance of apoptotic DNA ladders was detected in the BA cells after serum starvation for 6–8 h. This suggests that after this period of serum starvation the apoptotic pathway has been irreversibly activated.

To examine kinases that become activated during apoptosis, cells were harvested at different time points after serum deprivation and analyzed by in-gel kinase assays using MBP substrate gels (Fig. 1B). Serum deprived BA cells, which showed first signs of apoptosis after 6–8 h, also revealed a consistent and strong activation of kinases with apparent molecular weights of ca. 40 and 44 kDa, beginning at about the same time. This size falls into the range of MAP kinases, which have been implicated in the regulation of apoptosis [15,20–22]. Therefore, we examined the activation of these kinases during B cell apoptosis.

To facilitate comparison of our data with recent reports on p38 and JNK activation, we also assayed the kinase activity isolated by GST-Jun pulldowns from cells harvested at different times after serum deprivation. In parallel experiments, we examined the activity of the classical MAP kinases, ERK-1 and -2, as well as of JNK isolated by immunoprecipitation. We could not directly examine p38 activity by immunocomplex kinase assay, since no antibody against p38 suitable for immunoprecipitation kinase assays was available. In addition, we monitored the appearance of apoptotic DNA ladders. As shown in Fig. 2A, while no induction of ERK activity could be observed during the time course of the experiment, a kinase activity that can be precipitated with an anti-JNK antiserum was induced up to four-fold between 8 and 12 h after serum

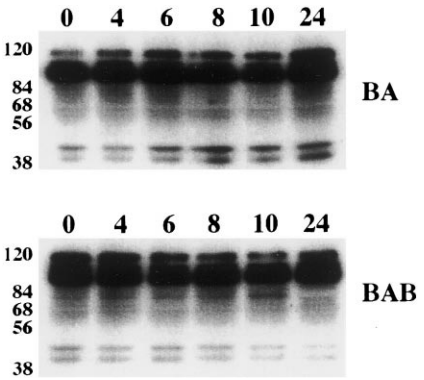

Fig. 4. In-gel kinase assay using GST-Jun as a substrate of cell lysates from BA or BAB cells that were serum starved for the times indicated at the top. The sizes and positions of protein molecular weight markers are indicated on the left.

withdrawal. Another kinase activity was pulled down with GST-Jun several hours earlier. This activity was consistently induced to a much higher level (10–20-fold), and at the time points that coincide with the minimal time of serum starvation required for the irreversible induction of apoptosis in these cells. Since the changes observed in activity were evident after a rather long period of time (6 h and more), we wanted to determine if they might be due to changes in the amount of protein present. To this end, we performed Western blot analysis using antibodies against ERK, JNK, and p38. As shown in Fig. 2B, the amount of these kinases did not change significantly during the time course, with exception of a decrease in ERK in the BAB cells. Western blot analysis of the proteins associated with GST-Jun in the pulldown assays revealed that both p38 and JNK were present in the complex (data not shown).

p38 seems to be activated, at least in part, by signals that also activate JNK [23]. Furthermore, p38's apparent molecular weight of 38 kDa is close to the molecular weight of the apoptosis associated kinases described here. Therefore, expression of p38 and JNK in BA and BAB cells was examined by Western blotting using different anti-p38 and anti-JNK antibodies (Fig. 3). HA tagged p38 (lane 1) and JNK-1 (lane 2) expressed in COS-1 cells were used as positive controls. Endogenous p38 can be detected in both COS controls (lanes 1 and 2), in BA (lane 3), and BAB (lane 4) cells as a ca. 44 kDa protein with antibodies against either the C- (Fig. 3A) or N-terminus (Fig. 3B) of p38. The bands at ca. 120 and 80 kDa in Fig. 3A are only detected by the antibody against the C-terminus of p38 and hence do not seem to represent p38 related proteins. In the HA transfected COS cells two p38 specific bands are detectable. The smaller one, migrating at about 44 kDa, represents the endogenous p38, whereas the larger band corresponds to HA-p38, whose triplicate HA tag increases the molecular weight by approximately 4 kDa.

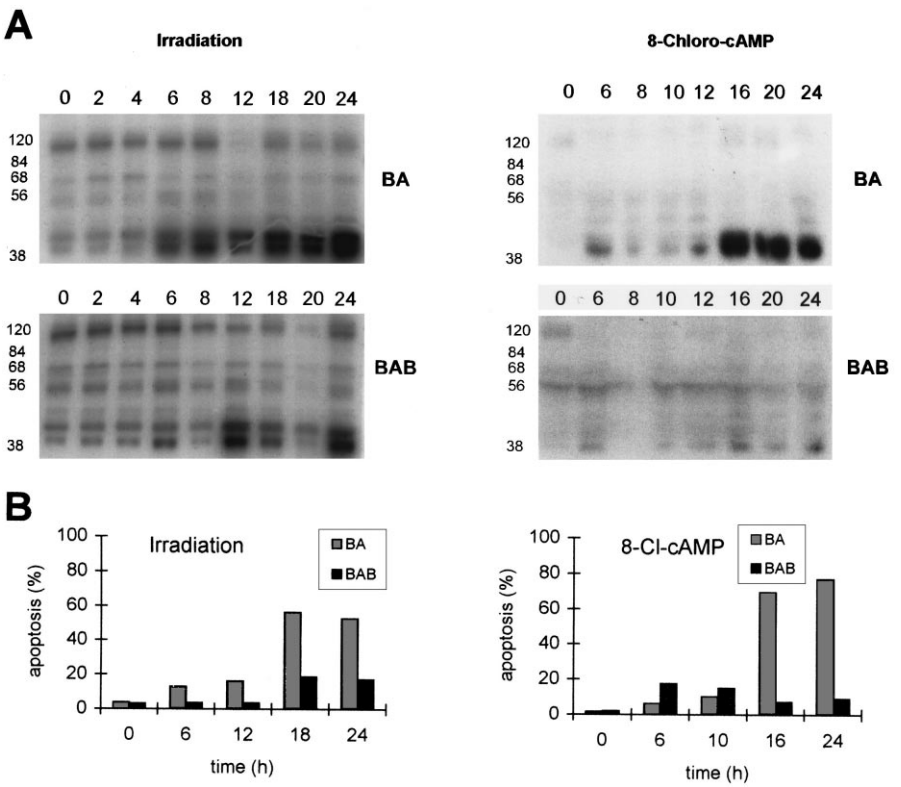

Fig. 5. Time course of the induction of apoptosis in BA cells and its absence in BAB cells. Cells were either irradiated with 20 Gy of γ-rays and harvested after the time indicated or stimulated with 100 μM 8-Cl-cAMP for the time indicated. A: In-gel kinase assays of the cell lysates. The size and position of protein molecular weight markers are indicated on the left. This experiment was repeated two times, and the kinetics of induction of p40 and p44 were consistent with the results shown here, while BAB cells showed only occasional dark bands at various time points after irradiation, depending on the experiment. B: Percentage of apoptotic cells after treatment. The cells were washed with PBS and resuspended in binding buffer. Annexin-V-FITC was added and incubated for 20 min in the dark. Stained cells were then counted in a fluorescence microscope. 300 cells were counted for each point.

A polyclonal antibody raised against full-length p38 recognizes the transfected HA tagged p38 as well as the endogenous p38 of the COS cells in Fig. 3C. In the BA and BAB cells this antiserum detects the 44 kDa band and an additional smaller band of about 40 kDa. This pattern corresponds to the pattern seen in in-gel kinase assays in Fig. 1. The α-JNK antibody specifically detected a 48 kDa protein in HA-JNK-1 transfected COS cells (lane 2, Fig. 3D). It totally failed to react with HA-p38 or endogenous p38 in the COS control cells, thus confirming the specificity of this antibody for JNK. In BA and BAB cells the anti-JNK antibody preferentially detected a 56 kDa protein. As at least 10 differentially spliced JNK transcripts have been described [24], the 56 kDa protein most likely represents an isoform of the JNK protein that is predominantly expressed in the BA and BAB cells.

In sum, this Western blot analysis suggests that the 40 and 44 kDa kinases activated during B cell apoptosis are different from JNKs, but may be related to p38 kinases. The p44 band probably corresponds to the known p38, as it is detected by all three antisera used. The p40 band does not react with the antisera made against synthetic peptides derived from the C- or N-terminus of p38. It is recognized, however, by a polyclonal serum raised against a full-length p38 protein.

If the two kinases described here were in fact members of the p38 family, they should also be able to accept GST-Jun as a substrate in in-gel assays. To this end, cell lysates of serum starved BA and BAB cells were examined in an in-gel kinase assay using GST-Jun as a substrate. In this experiment, as was observed in Fig. 1B using MBP as a substrate, the activity of the two kinases was induced several fold in the BA cells, while no significant increase could be observed in the BAB cells (Fig. 4). To prove that the incorporation of ^{32}P in the bands is due to substrate and not to autophosphorylation, we also performed an in-gel assay omitting any substrate in the gel. Under these conditions, where autophosphorylation still occurs, no kinase activity of the p40/44 kinases could be detected (data not shown).

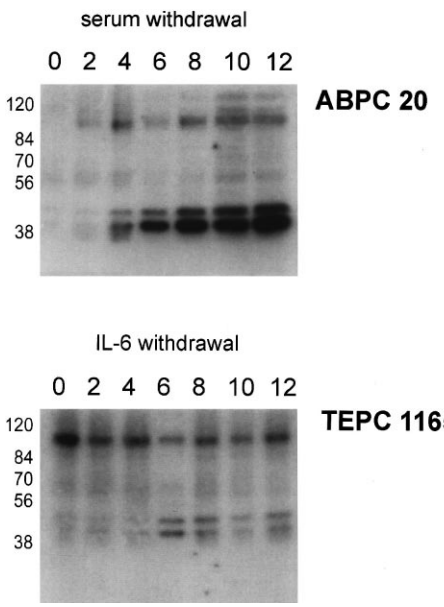

Fig. 6. Time course of the induction of apoptosis in two plasmacytoma cell lines. Apoptosis was induced in ABPC 20 cells by serum starvation, while IL-6 was withdrawn from the IL-6 dependent cell line TEPC 1165. The extent of apoptosis was determined and the cell lysates collected at the time points indicated at the top of the figures. In-gel assays were performed with the cell lysates. The sizes and positions of protein molecular weight markers are indicated on the left. This experiment was repeated three times, always showing similar results.

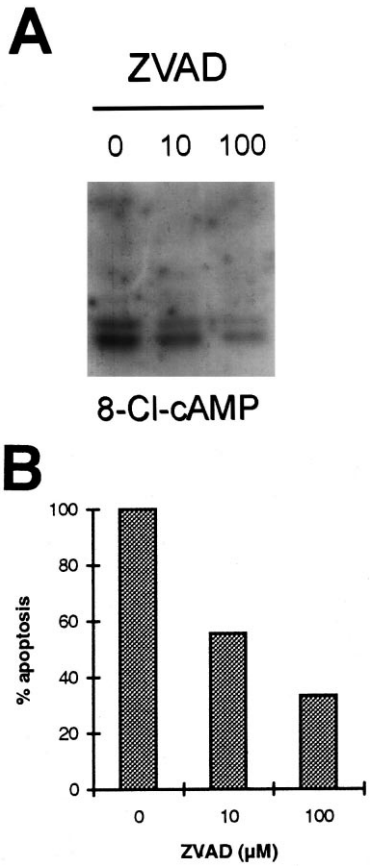

Fig. 7. Effect of inhibition of ICE protease on induction of apoptosis and kinase activity by 8-Cl-cAMP (100 μM) and their inhibition of Z-VAD. A: In-gel kinase assays of lysates of the BA cells with MBP as a substrate, after the treatments listed above. The concentrations of the inhibitors are indicated on the top. B: Percentage of apoptotic cells after 20 h of treatment. The cells were washed with PBS, treated with annexin-V-FITC, incubated for 20 min in the dark and counted in a fluorescence microscope. 300 cells were counted for each point, and the experiment was repeated twice.

To rule out the possibility that the activation of the p40/44 kinases is a peculiar consequence of serum withdrawal rather than specifically linked to apoptosis, we investigated whether these kinases could also be activated by other apoptotic stimuli. To this end, BA and BAB cells were either irradiated with γ-rays (20 Gy) or treated with 8-Cl-cAMP, which induces apoptosis via PKA mediated inhibition of Raf-1 [25] (Fig. 5A,B). Irradiation readily induced apoptosis in BA cells, which began at 6 h and peaked between 18 and 24 h (Fig. 5B). At these late time points there was also a modest increase of apoptosis in BAB cells. 8-Cl-cAMP elicited a modest rate of apoptosis in both BA and BAB cells between 6 and 10 h (Fig. 5B). The apoptosis in BAB cells, however, declined between 16 and 24 h, whereas in BA cells it progressed to very high levels. The apoptotic response was largely reflected in the activation pattern of the p40 and p44 kinases (Fig. 5A). The greater extent of apoptosis in BA cells correlated with a more pronounced kinase activation. Moreover, in these cells irradi-

ation induced early apoptosis and early onset of kinase activation. 8-Cl-cAMP triggered massive apoptosis as well as kinase activation only at the late time points. Some variation in the kinase activity could be observed during the time course (e.g. an apparent lower activity at 12 h than at 8 or 18 h after irradiation of the BA cells). These small decreases and increases, however, change from experiment to experiment and hence are not significant.

To test whether our findings could be extended to more mature B cell lines, we utilized plasmacytoma cell lines, where apoptosis was induced ca. 4–6 h after withdrawal of either serum, from the cell line ABPC 20, or IL-6, from the IL-6 dependent cell line TEPC1165. Again, robust activation of the 40 and 44 kDa kinases coincided temporally with the induction of apoptosis (Fig. 6). Hence, the activation of the 40/44 kDa kinases during the induction of apoptosis is seen in early and late stage B lineage cells.

To investigate where these kinases could be placed in the apoptosis pathway relative to the ICE-like proteases, BA cells were treated with the caspase inhibitor Z-VAD. BA cells were stimulated with the membrane permeable PKA activator 8-Cl-cAMP and simultaneously incubated with varying concentrations of Z-VAD. After 20 h the cells were harvested, and apoptotic cells were detected by annexin staining (Fig. 7B). Z-VAD could substantially protect BA cells from 8-Cl-cAMP induced apoptosis, and, as shown in Fig. 7A, could inhibit the activation of the 40/44 kDa kinases in a dose dependent manner. These results place the activation of the 40/44 kDa kinases downstream of the ICE protease cascade.

4. Discussion

Our results reveal two kinases of 40 and 44 kDa that are activated during the execution phase of apoptosis in pre-B and plasmacytoma cells lines, early and late stage cells of the B cell lineage. Lu et al. [26] and Cahill et al. [27] have recently reported the activation of kinases of similar molecular weights during apoptosis, but it is unclear whether these kinases correspond to the kinases described here. Other groups reported that activation of JNK and/or p38 accompanies the induction of apoptosis [15,20–22], and our results confirmed these findings. However, our data indicate that JNK was certainly not the main c-Jun phosphorylating kinase present (Fig. 3). Instead, two kinases of p40/44 molecular weight predominate in our system. Since the p44 kinase is recognized by three different p38 antisera, it most likely represents p38. The p40 kinase only reacts with an antiserum raised against the full-length p38 protein, but not with antipeptide sera specific for the N- or C-terminus, respectively. This is not unexpected, inasmuch as the members of the p38 family known at present share hardly any homology in their extreme C- and N-terminal regions, while the core kinase domain appears to be highly conserved. Hence, only antisera directed against the entire protein should be able to detect additional p38 family members. Thus, the p40 kinase may represent a novel member of the p38 family. This notion is additionally supported by the substrate specificity of this kinase. Like p38, it accepts both MBP and GST-Jun as a substrate in the in-gel assays. (Figs. 1 and 4). Furthermore, the activity of both kinases could be induced by treatment of the cells with anisomycin, a known activator of p38 (data not shown).

Our kinetic analysis showed that the activation of the p40/44 kinases occurs at the time point where apoptosis becomes irreversible (Fig. 1). By use of different apoptotic stimuli we further showed that the kinetics of apoptosis induction correlated with the kinetics of kinase activation (Fig. 5). Because endogenous expression of bcl-2 does not completely inhibit apoptosis of these cells, a small but significant amount of kinase activation can be observed. These results demonstrate that the activation of the p40/44 kinases is tightly linked to the apoptotic process. Furthermore, activation of the p40/44 kinases can be inhibited by the caspase inhibitor Z-VAD. These data suggest that the p40/44 kinases fall downstream of the caspase protease in the cascade of reactions that lead to apoptosis.

These observations point to a causal relationship between the induction of apoptosis and the activation of the p40/44 kinases. It remains open, however, whether the p40/44 kinases are involved in the induction of apoptosis or whether their activation is a consequence of the execution of the apoptotic program. The resolution of this question has to await further studies, such as the purification and direct identification of the p40/44 kinases, which is currently under way in our laboratory.

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