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Serum Response Factor Cleavage by Caspases 3 and 7 Linked to Apoptosis in Human BJAB Cells*

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Apoptosis involves the cessation of cellular processes, the breakdown of intracellular organelles, and, finally, the nonphlogistic clearance of apoptotic cells from the body. Important for these events is a family of proteases, caspases, which are activated by a proteolytic cleavage cascade and drive apoptosis by targeting key proteins within the cell. Here, we demonstrate that serum response factor (SRF), a transcription factor essential for proliferative gene expression, is cleaved by caspases and that this cleavage occurs in proliferating murine fibroblasts and can be induced in the human B-cell line BJAB. We identify the two major sites at which SRF cleavage occurs as Asp^{245} and Asp^{254}, the caspases responsible for the cleavage and generate a mutant of SRF resistant to cleavage in BJAB cells. Investigation of the physiological and functional significance of SRF cleavage reveals that it correlates with the loss of c-*fos* expression, whereby neither SRF cleavage fragment retains transcriptional activity. Moreover, the expression of a noncleavable SRF in BJAB cells suppresses apoptosis induced by Fas cross-linking. These results suggest that for apoptosis to proceed, the transcriptional events promoting cell survival and proliferation, in which SRF is involved, must first be inactivated.

Cell growth, proliferation, and differentiation are regulated by numerous, diverse, extracellular signals that modulate gene expression. The proto-oncogene c-*fos* represents a classic example of an immediate early (IE)[1] gene, induced in response to growth factors and other mitogenic stimuli (1–3). Contained within the promoter of c-*fos* and of other IE genes is the serum response element (SRE), which is essential for strict transcriptional control. Serum response factor (SRF) is a ubiquitously expressed, 67-kDa protein that binds, as a dimer, to the central element of the SRE, the CArG box ($CC(A/T)_6GG$) (4). SRF belongs to the MADS box family of transcription factors, named after four proteins identified with a common structural domain (MCM1, Agamous, Deficiens, SRF), all of which regulate transcription via the recruitment of auxiliary factors (5, 6). The conserved core domain of SRF is sufficient for protein dimerization, DNA binding, and recruitment of ternary complex factors (TCFs). The TCFs form a subset of the *ets* family of proteins and include Elk-1, Sap1, and Net/Sap2/Erp (7). In response to activation of the mitogen-activated protein kinase signaling pathway, TCFs are rapidly phosphorylated, resulting in a dramatic increase in their transactivation potential (8–10).

In addition to its role at the SRE, SRF has been shown to regulate transcription in a TCF-independent manner. The exact function of SRF in this mode of gene expression is unclear. Within SRF, several amino-terminal phosphorylation sites have been identified (11–14). More specifically, it has been demonstrated that phosphorylation of SRF at Ser^{103} correlates with c-*fos* expression (15, 16). A carboxyl-terminal transactivation domain has also been mapped and shown to interact with components of the basal transcription machinery, in particular with Rap74, the large subunit of TFIIF (17, 18). TCF-independent transcriptional activation by SRF is regulated by the Rho family small GTPases RhoA, Rac1, and Cdc42hs (19). These small G-proteins regulate several cytoskeletal processes, and recent data have suggested that the convergence of LIM kinase and RhoA signaling, via actin treadmilling, results in the transcriptional activation of some SRF-dependent genes (20). The mechanism by which this occurs has not yet been characterized in detail, and RhoA appears to function independently of Rac1 and Cdc42hs, implying that additional signaling pathways may be involved. The CArG box is frequently found in the promoters of muscle-specific genes. In this context, it has been demonstrated that SRF is essential for myogenic differentiation. Inhibition of SRF by microinjection of anti-SRF antibodies or the expression of antisense SRF RNA repressed muscle marker gene expression and blocked the differentiation of myoblasts to myotubes (21, 22). Moreover, homozygous SRF−/− mouse embryos fail to develop mesoderm (23).

Apoptosis, or programmed cell death, is the process by which surplus or potentially harmful cells are removed from an organism in a nonphlogistic manner. Loss of the tight control governing this process can result in inflammation, cancer, stroke, and many neurodegenerative disorders. Caspases, or cysteine-dependent aspartate-directed proteases, form an integral part of the apoptosis machinery (24). In response to some extracellular stimuli, such as Fas ligand, TNFα, or genotoxic agents, caspases are activated in a hierarchical cleavage cascade. Once activated, they target and inactivate numerous intracellular

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[1] The abbreviations used are: IE, immediate early; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor; STAT, signal transducers and activators of transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid; DAPI, 4′,6′-diamidino-2-phenylindole; DM, double mutant; ZVAD, benzyloxycarbonyl-Val-Ala-Asp.

substrates, including structural components of the cytoskeleton, DNA repair enzymes, protein kinases, and transcription factors as stricken cells are disassembled and removed by phagocytosis (25).

It is also known that caspases have additional roles beyond apoptosis, in line with their initial identification as relatives of the interleukin-1β-converting enzyme (26, 27). For example, cleavage of GATA-1, mediated by caspases, has recently been demonstrated to block erythroid differentiation (28). In addition, caspases have been shown to cleave the transcription factors NF-κB, resulting in the down-regulation of NF-κB-dependent transcription, and STAT1, suggesting a role for caspases in mediating transcriptional responses (29–31).

Here we show that SRF also serves as a target for caspases in several cell types. Two adjacent cleavage sites recognized by caspases 3 and 7 allow SRF to be cleaved into two major fragments. Expression of the corresponding fragments reveals that they are unable to activate SRE-dependent transcription. Moreover, expression of a noncleavable SRF mutant suppresses apoptosis. Our results suggest that SRF is a focal point for cross-talk between signals promoting cell survival and proliferation and those promoting cell death.

MATERIALS AND METHODS

Plasmid Constructs—pCMV-SRF consists of the coding sequences of SRF inserted into pCMV5 (32). pCMV-Ncore and pCMV-coreC contain SRF sequences encoding amino acids 1–222 and 133–508, respectively. pBS-Ncore245 was produced by amplifying sequences encoding SRF amino acids 1–245 by PCR from pBOH$_2$-SRF (33) and inserting them as an *Eco*RI fragment into pBSKS$^+$ cut with *Eco*RI. pCMV-Ncore245 was generated by ligating the same *Eco*RI fragment into pCMV5. pCMV-mycSRFC was constructed inserting sequences encoding amino acids 252–508 into pCMV5m, a generic vector for the expression of Myc-tagged proteins.

pBO-EATA-SRF, pBO-SASA-SRF, and pBO-DM-SRF were produced by PCR-based site-directed mutagenesis with the following primers: EATA, 5′-GTGAGAGCTGTCGCTTCAAAGCCAGTGG; SASA, 5′-GGTGTCGGCGTCTGCCAGCAGTGG.

pCMV-EATA-SRF, pCMV-SASA-SRF, pCMV-DM-SRF were constructed by transferring SRF coding sequences from the respective pBO-SRF plasmids as *Eco*RI–*Xba*I fragments.

In pCMV-MSRF, SRF codons 134–169 were replaced by codons 9–44 from MCM1 (34, 35). The restriction sites for the replacement were produced by PCR-based, site directed mutagenesis with the following primers: 5′MCM, 5′-AGAAGGATCCCCTACTAATAATGGG; MCM/BSP, 5′-GCCTTTTTCATGATACCGTGC.

The plasmid pCMV-SRF-Chis has been described elsewhere (36); pcDNA3-wtSRF-Chis was constructed by transferring SRF coding sequences from pCMV-SRF-Chis as an *Eco*RI-*Xba*I fragment into pcDNA3; pcDNA3-DM-SRF-Chis was constructed by combining an *Eco*RI-*Xmn*I fragment from pBO-DM-SRF with an *Xmn*I-*Xba*I fragment from pCMV-SRF-Chis in pcDNA3.

The plasmid pFos-Luc3 contains the c-*fos* promoter from −711 to +42 fused to the firefly luciferase gene in pGL3-basic (Promega); pΔF10H-Luc is a derivative of pFOS-Luc3, from which the SRE has been deleted (37); pM20-Luc consists of a single copy of the M20 SRE (38) inserted into the deletion point in pΔF10H-Luc.

All sequences derived by PCR amplification were subsequently verified by dideoxy sequencing.

Cell Culture and Transfections—NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. NIH3T3 cells were plated out at a density of 7.7×10^4/ml and, after 24 h, transfected with 0.75 μg of the various SRF constructs using LipofectAMINE (Life Technologies, Inc.), according to the manufacturer's protocol. Eighteen hours after transfection, cells were washed and serum-starved (0.2%) for 24 h. BJAB cells were grown in RPMI supplemented as above. Cells were plated out at a density of 3.3×10^5/ml and, after 48 h, transfected by electroporation (290 V, 960 microfarads) using the gene pulser system (Bio-Rad) with 20 μg of DNA containing 4 μg of the various SRF constructs. Eighteen hours after transfection, cells were serum-starved (0%) for 24 h.

The *wt*SRF stable cell line (clone 9) was generated by electroporation of BJAB cells with pcDNA3-*wt*SRF-Chis, followed by G418 selection at 3 mg/ml for several passages. Clones were isolated from individual cells by dilution cloning in 96-well plates and identified by Western analyses with an anti-His monoclonal antibody (Qiagen).

Extract Preparation and Western Analysis—NIH3T3 and BJAB cells were treated, where appropriate, with staurosporine (250 nM) or CH11 (150 ng/ml), respectively. Cells were harvested in PBS and lysed, on ice for 20 min, in 10 mM Tris-HCl pH 7.8, 25 mM NaF, 20 mM Na_4P2O_7, 2.5 mM $MgCl_2$, 5 μM Na_3VO_4, 0.5% Triton X-100, 0.05% SDS, 2.5 mM benzamide, 200 μM phenylmethylsulfonyl fluoride, 2.2 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin. Cell debris was then pelleted by centrifugation, and the clear lysate was removed.

Samples were then analyzed by SDS-PAGE and electroblotting onto polyvinylidene difluoride membrane. Membranes were probed with N-terminal (α1122; M. E. Greenberg) or C-terminal antibodies (α1795; M. E. Greenberg) or G-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) against SRF or anti-Fos (K-25; Santa Cruz Biotechnology) and visualized by standard ECL procedures (Amersham Pharmacia Biotech) on a luminescence imaging system (Fuji). Reprobing of membranes was carried out in accordance with the manufacturer's protocol.

Luciferase Reporter Assays—BJAB cells were co-transfected with 5 μg of reporter plasmid and 5 μg of pCH110 (Amersham Pharmacia Biotech), as described above. Following serum starvation, cells were harvested in PBS and lysed in 250 mM KCl, 50 mM HEPES, pH 7.5, 0.1% Nonidet P-40, 10% glycerol. Values obtained from the luciferase assays were normalized to β-galactosidase activity in order to correct for transfection efficiencies.

Electrophoretic Mobility Shift Assays—Cell extract (15 μg) was incubated at room temperature for 10 min in 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 4% glycerol, 80 μg/ml herring sperm DNA, 15 μg/ml dI-dC with 0.5 μl of antiserum as indicated. Prior to loading onto a 5% polyacrylamide gel, reactions were incubated for a further 20 min with the SRE oligonucleotide probe (39). The gel was then visualized using a Fuji phosphor imaging system.

In Vitro Cleavage Assays—BJAB cells, untreated or incubated with 150 ng/ml CH11 for 10 h, were harvested in PBS. Cell pellets were then lysed in 25 mM HEPES, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 0.1% CHAPS as described above. Radiolabeled SRF protein was incubated with 30 μg of cell extract, in the buffer described previously, at 37 °C for 1 h. Samples were then analyzed by SDS-PAGE and visualized using a Fuji phosphor imaging system.

Determination of Percentage Apoptosis—Cells were fixed in 1% (w/v) formaldehyde in PBS until scored. Scoring was carried out by fluorescence microscopy following staining of the cells with 0.0001% (w/v) 4′,6′-diamidino-2-phenylindole (DAPI) (40).

RESULTS

The experiments described here follow on from our initial observation of SRF fragments in NIH3T3 cells transiently transfected with an expression vector for the protein. Two polyclonal antibodies, one raised against the amino-terminal portion of SRF (α1122) and the other against the carboxyl terminus (α1795), each recognized protein fragments of 30–35 kDa, approximately half the size of full-length SRF (Fig. 1, *a* and *b*, *lane 1*). To gain a first indication of the nature of these fragments, we compared their size and immunoreactivity with truncated forms of SRF expressed from suitably engineered vectors. Thus, the SRF fragment recognized by α1122 was larger than Ncore (*lane 5*) but almost identical in size to Ncore245 (*lane 4*), suggesting that it corresponds to SRF amino acids 1–245. Likewise, the SRF fragment recognized by α1795 was somewhat smaller than coreC (Fig. 1, *lane 3*) but very similar in size to SRF-C (amino acids 251–508). These observations suggest that in NIH3T3 cells SRF is cleaved in half under certain conditions and that the fragments are stable enough to accumulate in the cells.

Variations in the level of SRF fragments indicated that SRF cleavage might be a regulated event, influenced perhaps by the growth state of the cells. We also noticed a cluster of six aspartate residues in the middle of SRF. In this context, caspases, a group of aspartate-directed proteases activated in cells undergoing apoptosis, are known to target numerous key proteins as the process unfolds. To assess a potential role for caspases in the cleavage of SRF, transfected NIH3T3 cells were treated with staurosporine for various times in the presence or absence

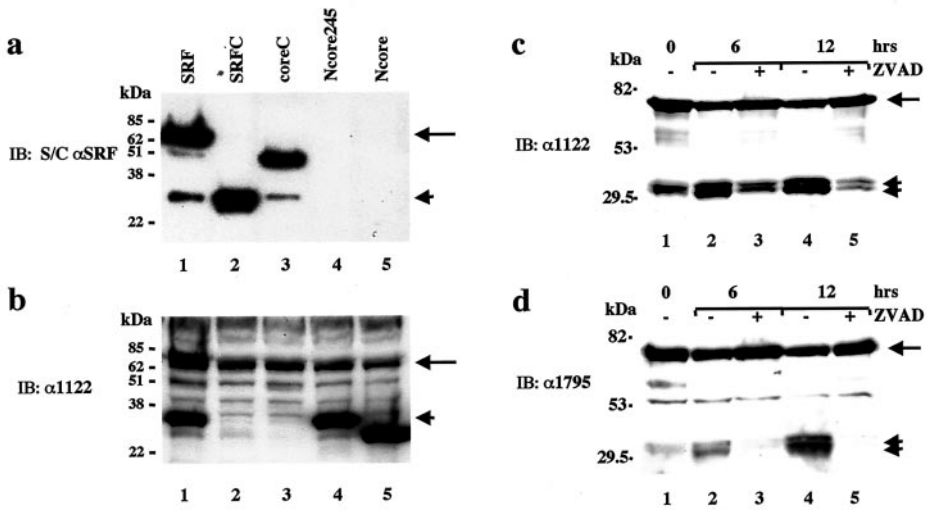

FIG. 1. **SRF cleavage products in murine fibroblasts.** *a*, NIH3T3 cells were transfected with expression vectors for full-length SRF (*lane 1*), SRFC (*lane 2*), coreC (*lane 3*), Ncore245 (*lane 4*), and Ncore (*lane 5*). Cells were harvested after 48 h, and lysates were separated on a 12.5% SDS-polyacrylamide gel and analyzed by immunoblotting (*IB*) with an antibody against the carboxyl terminus of SRF (Santa Cruz Biotechnology). *b*, as in *a*, except that an amino-terminal SRF antibody (α1122) was used. The 67-kDa band detected in all lanes is endogenous SRF. *c*, NIH3T3 cells were transfected with an expression vector for SRF and serum-starved for 24 h (*lane 1*) prior to treatment with staurosporine alone (*lanes 2* and *4*) or with ZVAD (*lanes 3* and *5*). Cells were harvested at the times indicated, and lysates were separated on a 10% SDS-polyacrylamide gel and analyzed by immunoblotting with an antibody against the amino terminus of SRF. *d*, as in *c* except that a carboxyl-terminal SRF (α1795) antibody was used.

of ZVAD, to drive cells into apoptosis and inhibit caspase activity, respectively. As seen in Fig. 1, *c* and *d*, staurosporine caused an increase in the yield of SRF fragments after 6 and 12 h (compare *lanes 2* and *4* with *lane 1*), which was strongly counteracted by ZVAD treatment (*lanes 3* and *5*), implicating caspases in this phenomenon. In this gel, it is also apparent that each antibody detects two SRF fragments, indicating that SRF is cleaved at two or more sites by caspases active in NIH3T3 cells.

In parallel analyses, NIH3T3 cells were found to enter apoptosis with delayed and heterogeneous kinetics in response to staurosporine (result not shown). However, a more efficient means of inducing apoptosis by ligation of Fas proved to be impossible, since flow cytometric analysis revealed low surface expression of the receptor on NIH3T3 cells and a corresponding failure of the antibody (Jo-2) to elicit apoptosis (not shown). For this reason, we chose to analyze caspase cleavage of SRF further in a more appropriate cell system.

The human mature B cell line BJAB expresses high levels of Fas on the cell surface and can be induced to undergo apoptosis upon receptor cross-linking by the agonistic monoclonal antibody CH11 (41). Thus, 4 h after treatment with CH11, >50% of BJAB cells have entered apoptosis, as assayed by DAPI staining and cell counting (see Fig. 2*a*), and by 10 h this number has risen to >90%. To determine if this process induces SRF cleavage, lysates were prepared from BJAB cells either untreated or incubated for various times with CH11. Cleavage of endogenous SRF was assayed by immunoblotting. Four hours after CH11 cross-linking, SRF fragments of approximately 30 kDa are detected and persist for the next 6 h (Fig. 2*b*, *lanes 4–6*, *arrowheads*). The appearance of these bands is blocked by ZVAD (*lane 7*), indicating that they are caspase cleavage products. However, very little decrease in the amount of full-length SRF is apparent. The prominent 38-kDa species seen in untreated cells are unrelated to SRF, since they are not detected by antibodies specific to the carboxyl terminus of SRF (not shown), nor are they detected in a DNA-binding assay (see below).

The integrity of endogenous SRF in CH11-treated BJAB cells was also investigated by electrophoretic mobility shift assay with a radiolabeled DNA probe corresponding to the human c-*fos* SRE (Fig. 2*c*). The intensity of the SRF dimer complex is reduced by 4 h and continues to decrease up to 10 h (*arrow*). Moreover, two faster migrating bands appear at 2 h and decay over the same period (*lower arrowheads*). All three of these complexes are supershifted by α1122 (*lanes 10–12*), which recognizes the amino terminus of SRF, while α1795 supershifts only the upper two complexes (*lanes 13–15*). The simplest interpretation of these observations is that the lower complex corresponds to a homodimer of amino-terminal SRF fragments, the DNA-binding domain (amino acids 133–222) residing in the amino-terminal half of SRF, while the intermediate complex is a heterodimer in which just one SRF molecule is truncated.

The apparent persistence of full-length SRF in BJAB cells seen in Fig. 2*b* is inconsistent with the loss of SRF-SRE complexes (Fig. 2*c*). To resolve this inconsistency, we generated a stable cell line that overexpresses a His-tagged version of SRF. When these cells were treated with CH11 and analyzed for SRF cleavage (Fig. 2*d*), we observed almost complete cleavage of SRF-Chis after 10 h (*lane 4*), in line with the loss of DNA binding. We also observed a nonspecific band migrating slightly faster than SRF-Chis in all lanes, which we believe co-migrates with endogenous SRF and masks its disappearance in Fig. 2*b*. From these observations, we infer that SRF is completely processed in apoptotic BJAB cells.

SRF cleavage was also observed *in vitro* when ^{35}S-labeled proteins were incubated in apoptotic BJAB cell lysates. As shown in Fig. 3*b*, incubation of SRF with lysates from CH11-treated cells (*lane 3*), but not from untreated cells (*lane 2*), led to the appearance of four SRF fragments of around 35 kDa, corresponding in size to those detected in NIH3T3 cells.

The data shown in Fig. 1 indicate that SRF cleavage occurs in the middle of the molecule, carboxyl-terminal to the DNA binding domain, referred to as core^{SRF} (42). The amino acid sequence of this region of SRF (amino acids 222–271) is depicted in Fig. 3*a*. Within this 10% of SRF, there are six aspartate residues, of which several could constitute sites of caspase cleavage. Within the remainder of SRF, there are only six additional aspartates. Mutations were therefore introduced into SRF to remove two possible caspase consensus sites at

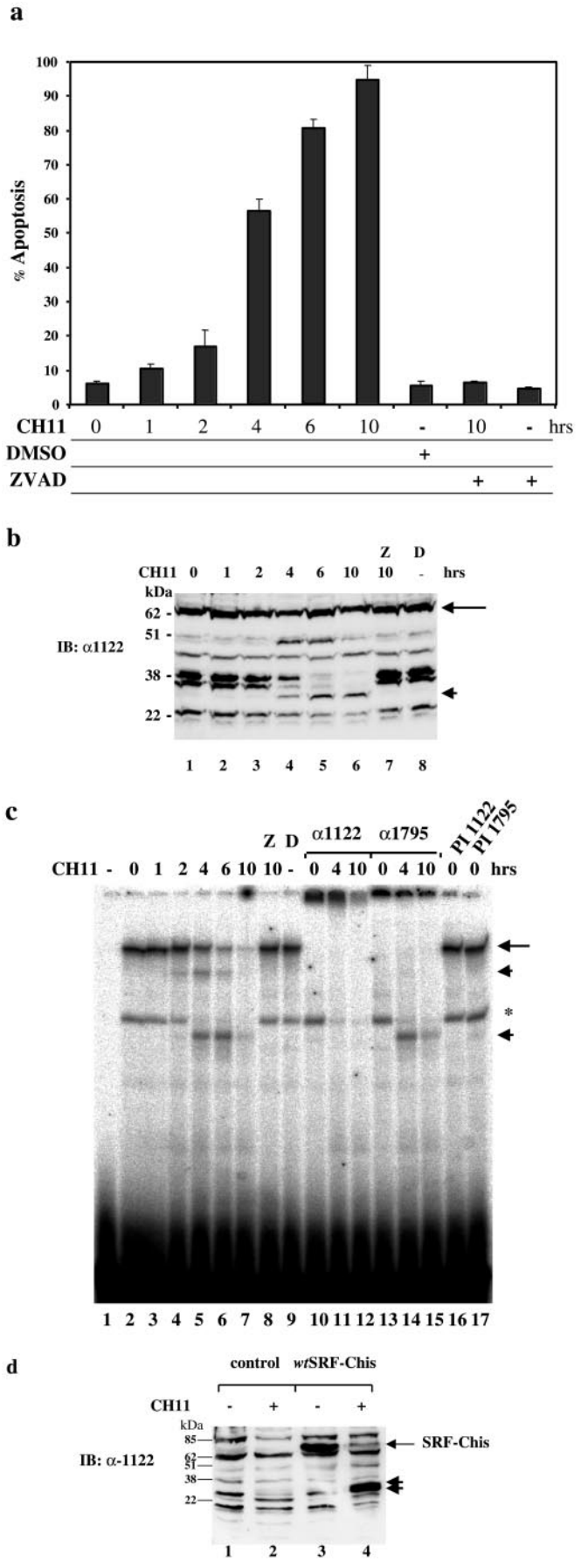

FIG. 2. **Induction of SRF cleavage by Fas ligation on B cells.** *a*, human BJAB cells, grown under normal conditions, were treated with the agonistic anti-Fas antibody CH11 alone or in the presence of ZVAD.

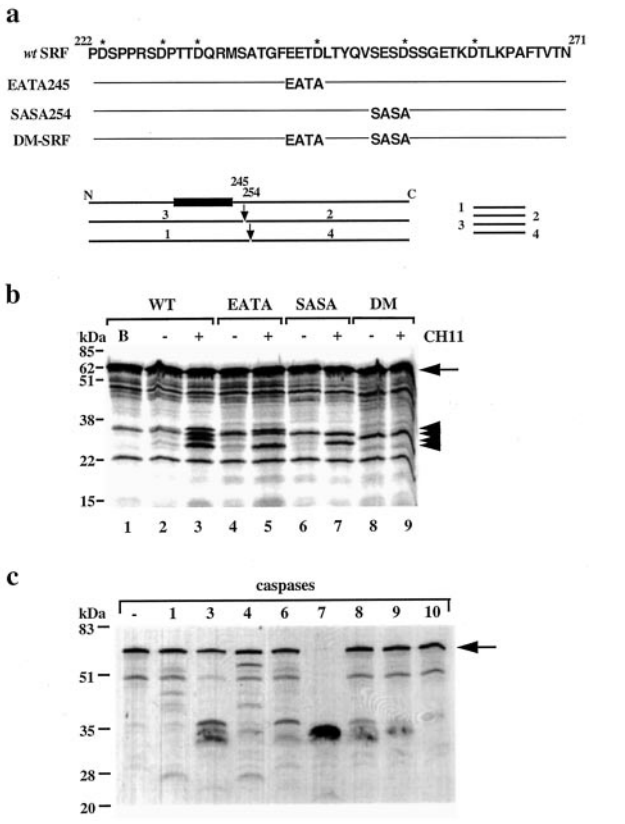

FIG. 3. **SRF cleavage by recombinant caspases.** *a*, sequence of SRF amino acids 222–271 with aspartate residues highlighted with *asterisks*. SRF mutants used in this study are also indicated. *Below* is shown a diagrammatic representation of SRF cleavage and the resultant pattern of fragments resolved by SDS-PAGE. *b*, recombinant SRF (*lanes 1–3*) or the mutants EATA (*lanes 4* and *5*), SASA (*lanes 6* and *7*), and the double mutant DM (*lanes 8* and *9*), expressed and labeled with [^{35}S]methionine by cell-free translation, were incubated with buffer B, extracts of untreated BJAB cells (−), or cells treated for 10 h with CH11 (+). Subsequently, reactions were resolved on a 12.5% SDS-polyacrylamide gel. *c*, recombinant SRF, expressed and labeled with [^{35}S]methionine by cell-free translation, was incubated alone (*lane 1*) or with recombinant caspase 1 (*lane 2*), caspase 3 (*lane 3*), caspase 4 (*lane 4*), caspase 6 (*lane 5*), caspase 7 (*lane 6*), caspase 8 (*lane 7*), caspase 9 (*lane 8*), or caspase 10 (*lane 9*). Subsequently, reactions were resolved on a 12.5% SDS-polyacrylamide gel. *WT*, wild type.

aspartate residues 245 and 254. Both individual mutants (EATA245 and SASA254) and the double mutant (DM) were then tested for cleavage by caspases present in CH11-treated BJAB cell lysates. Each single site mutant caused the loss of

Cells were harvested at the times indicated and analyzed for apoptosis by DAPI staining. Data show results from two experiments with duplicate points. *b*, as in *a* except that cells were lysed and analyzed for cleavage of endogenous SRF by immunoblotting (*IB*) with an amino-terminal SRF antibody (α1122). *c*, extracts prepared from BJAB cells, treated with CH11 for the times indicated, were incubated with a radiolabeled DNA fragment corresponding to the c-*fos* SRE alone (*lanes 2*-9) or in the presence of antibodies specific for the amino-terminal (*lanes 10–12*) or carboxyl-terminal (*lanes 13–15*) domain of SRF. The extract from untreated cells was also incubated with the corresponding preimmune sera (*lanes 16* and *17*). Complexes were resolved by electrophoresis on a native 5% polyacrylamide gel. The complex labeled with an *arrow* corresponds to the SRF homodimer; the *arrowheads* indicate complexes containing SRF cleavage fragments. The identity of the complex indicated with an *asterisk* is unclear, but it is not recognized by any of the anti-SRF antibodies. *d*, a BJAB cell clone overexpressing His-tagged SRF (*lanes 3* and *4*) and a control clone (*lanes 1* and *2*) were untreated (−) or incubated with CH11 for 10 h (+). Cells were lysed and analyzed by SDS-PAGE and immunoblotting with an amino-terminal SRF antibody (α1122). Full-length SRF-Chis and its cleavage fragments are indicated with *thin* and *thick arrows*, respectively.

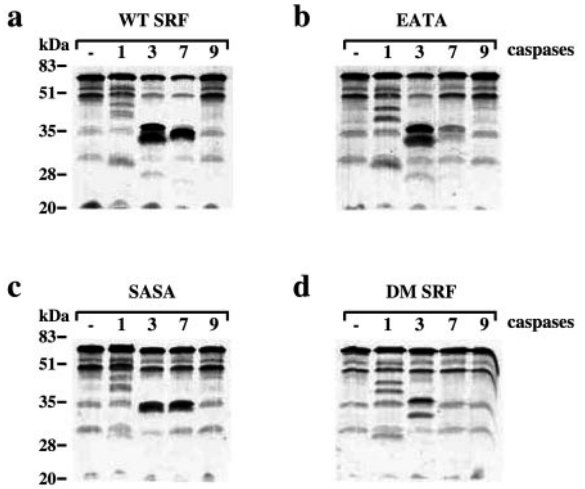

FIG. 4. **Identification of caspase cleavage sites.** *a*, recombinant SRF, expressed and labeled with [^{35}S]methionine by cell-free translation, was incubated alone (*lane 1*) or with caspase 1 (*lane 2*), caspase 3 (*lane 3*), caspase 7 (*lane 4*), or caspase 9 (*lane 5*), after which the reactions were resolved on a 12.5% SDS-polyacrylamide gel. *b*, as in *a* except with the mutant EATA-SRF. *c*, as in *a* except with the mutant SASA-SRF. *d*, as in *a* except with the mutant DM-SRF.

two SRF fragments (Fig. 3*b*, compare *lanes 7* and *5* with *lane 3*), while the double mutant abolished SRF cleavage in this assay. A similar set of results was obtained when lysates from etoposide-treated Jurkat cells were used (not shown), whereby cleavage was inhibited by the inclusion of ZVAD in the assay. Moreover, single alanine substitutions of Asp^{245} and Asp^{254} had exactly the same effect as the EATA and SASA mutations on SRF cleavage *in vitro* (result not shown). Taken together, these results demonstrate that SRF is cleaved at aspartates 245 and 254 by caspases induced in apoptotic BJAB cells and active to varying degrees in proliferating NIH3T3 cells. Furthermore, we interpret the results to indicate that SRF can be cleaved at either but not both sites.

To establish which caspase or caspases are responsible for SRF cleavage, radiolabeled SRF was incubated with each of eight active recombinant caspases and then analyzed by SDS-PAGE. This assay reveals that SRF is cleaved most effectively by caspases 3 and 7 but also, to a lesser extent, by caspases 6, 8, and 9 to yield similar fragments, suggesting that all five proteases recognize the same sites (Fig. 3*c*). In contrast, caspases 1 and 4 cleave SRF weakly to generate a distinct pattern of SRF fragments, while recombinant caspase 10 does not cleave SRF at all.

SRF cleavage by caspases was analyzed further by comparing the susceptibility of SRF mutants to a subset of recombinant caspases (Fig. 4). Caspase 1 cleaved each of the three mutants similarly to *wt*SRF, indicating that it does not recognize the sites at Asp^{245} and Asp^{254} but does recognize other sites in the protein. Cleavage of SRF by caspases 3 and 7 is influenced by both mutations. The mutations at Asp^{245} (EATA) result in the loss of two caspase 3 cleavage fragments and appear to enhance the yield of the others. The same mutant markedly reduces SRF cleavage by caspase 7. In contrast, mutations at Asp^{254} (SASA) lead to the reciprocal pattern of fragments obtained with caspase 3 but have little effect on SRF cleavage by caspase 7. The double mutant is resistant to cleavage *in vitro* by caspase 7 and is cleaved only weakly by caspase 3. Moreover, the fragment sizes suggest that caspase 3 may cleave DM-SRF at a site that is not recognized in *wt*SRF. In summary, caspase 7 cleaves at Asp^{245}, while caspase 3 cleaves predominantly at Asp^{254} but also, suboptimally, at Asp^{245} and an additional site (possibly Asp^{261}).

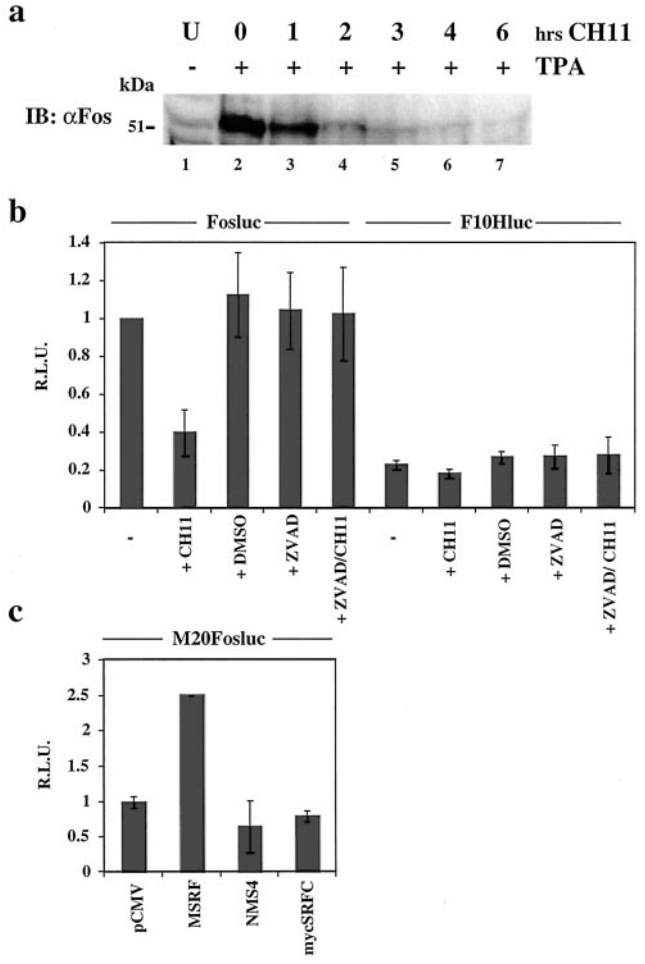

FIG. 5. **Inhibition of c-*fos* expression in apoptotic cells.** *a*, human BJAB cells, cultured for 24 h in low serum (*lane 1*), were treated directly with phorbol ester (*TPA*) (*lane 2*) or after pretreatment with CH11 for the times shown (*lanes 3–7*). Cells were lysed, and Fos expression was monitored by immunoblotting (*IB*) with an anti-Fos antibody. *b*, human BJAB cells were transfected with the firefly luciferase reporter Fos-Luc3 or the mutant ΔF10H-Luc3, which lacks the SRE, and after culture for 24 h in low serum they were treated with CH11, ZVAD, or both, as indicated (*DMSO*, dimethyl sulfoxide vehicle). After 6 h, lysates were prepared and assayed as described. Luciferase activity is expressed as the ratio of relative light units (*R.L.U.*) and the β-galactosidase activity expressed from a co-transfected plasmid, whereby the value for untreated cells is set as unity. *c*, BJAB cells were transfected with the M20Fos-Luc reporter, which contains the M20 sequence in place of the SRE, and either control vector (pCMV5), MSRF, NMS4, or SRFC. After a 24-h culture in low serum, lysates were prepared and assayed for luciferase activity. Luciferase activity is expressed as the ratio of relative light units and the β-galactosidase activity expressed from a co-transfected plasmid, whereby the value for cells transfected with pCMV5 is set as unity.

We surmised that the cleavage of SRF by caspases was likely to alter the expression of IE genes such as c-*fos*. Accordingly, we examined the inducible level of Fos protein in BJAB cells at increasing times after CH11 treatment. Cells were pretreated with CH11 for various lengths of time and then with phorbol ester to induce c-*fos* expression, which was assayed at the protein level by immunoblotting. Phorbol ester alone induced robust Fos expression (Fig. 5*a*, *lane 2*), but prior treatment with CH11 for 1 h or longer reduced and finally abolished inducible Fos expression (*lanes 3–7*). Thus, Fas cross-linking rapidly leads to the inhibition of Fos protein expression. To ascertain if the loss of Fos protein could be due to proteolysis, radiolabeled Fos protein was incubated in apoptotic BJAB cell lysates. However, no degradation of Fos was observed under

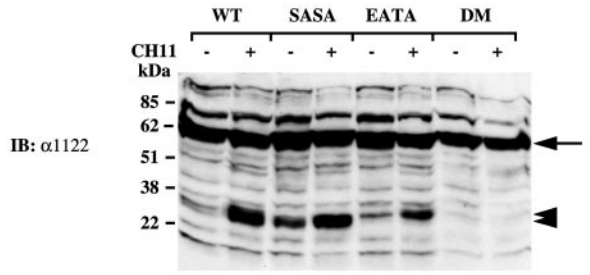

FIG. 6. **DM-SRF resists cleavage in BJAB cells.** Cells were transfected with expression vectors for SRF (*lanes 1* and *2*), EATA-SRF (*lanes 3* and *4*), SASA-SRF (*lanes 5* and *6*), or DM-SRF (*lanes 7* and *8*). After 24-h culture in low serum, cells were treated with CH11 (+) or left untreated (−), and after a further 6 h lysates were prepared and assayed by immunoblotting (*IB*) with an amino-terminal anti-SRF antibody. *WT*, wild type.

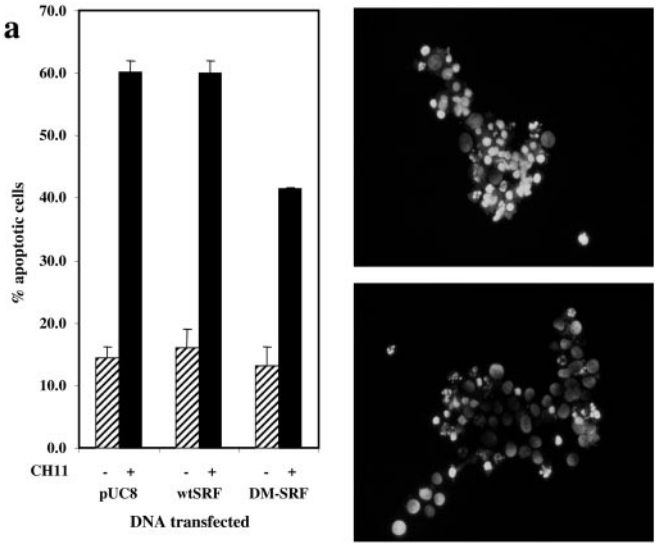

FIG. 7. **DM-SRF expression suppresses apoptosis in BJAB cells.** *a*, cells transfected with pUC8 or the expression vectors pCMV-*wt*SRF or pCMV-DM-SRF were untreated (*hatched bars*) or incubated with CH11 for 20 h (*solid bars*). Fixed cells were stained with DAPI and scored for apoptosis under a fluorescence microscope. Data are from two individual experiments, whereby each experiment was scored ($n = 200$) twice independently. *b* and *c*, representative images of CH11-treated cells transfected with pCMV-*wt*SRF and pCMV-DM-SRF, respectively.

conditions in which SRF cleavage was apparent (result not shown). Therefore, the absence of Fos in CH11-treated cells is probably due to loss of expression. To assess the extent to which this effect was due to transcriptional inhibition, the effect of CH11 on the expression of a c-*fos* reporter was measured. As shown in Fig. 5*b*, CH11 inhibited c-*fos* reporter expression by 60%, and this inhibition could be blocked by ZVAD. In contrast, a c-*fos* reporter from which the SRE had been deleted (F10H) was refractory to CH11 treatment. It should be noted that expression of the control gene against which transfection efficiency was normalized was also insensitive to CH11 treatment. These results suggest that IE promoters containing SREs are down-regulated early in apoptosis, while other promoters remain unaffected.

It remained conceivable that the down-regulation of c-*fos* expression could be due to something other than SRF cleavage. Since cleavage by caspases yields two SRF fragments, we chose to measure their direct influence on c-*fos* reporter expression. To this end, a mutant of SRF with altered DNA binding specificity was adopted. This mutant (MSRF), which was originally developed by Treisman and colleagues (38, 43), contains part of the DNA-binding domain of MCM1 and binds to a sequence (M20) poorly recognized by SRF. A reporter containing the M20 element therefore has a low basal level of activity when transfected alone into BJAB cells (Fig. 5*c*), which is elevated when MSRF is expressed in the cells. In contrast, expression of a carboxyl-terminal truncation of MSRF corresponding to the amino-terminal caspase fragment of SRF (NMS4) or the carboxyl-terminal fragment of SRF (SRFC) inhibits basal reporter expression slightly. Taken together, these results indicate that SRE-dependent c-*fos* expression is down-regulated early in apoptosis, that down-regulation is blocked by ZVAD, and that the fragments of SRF generated by caspase cleavage fail to maintain expression levels supported by full-length SRF.

Given that the mutation of two predicted caspase sites produced a mutant of SRF resistant to caspase cleavage *in vitro*, we wished to see if the mutant was also resistant to cleavage in BJAB cells undergoing apoptosis. Thus, BJAB cells expressing various forms of SRF were treated with CH11, and SRF cleavage was assessed by immunoblotting. We observed that mutation of the site at Asp^{254} had little effect on SRF cleavage *in vivo*, whereas cleavage was severely impaired by mutation of the site at Asp^{245} (Fig. 6). As expected, no cleavage fragments were generated from the double mutant, in line with the *in vitro* cleavage data.

The availability of a caspase-resistant form of SRF allowed us to assess directly the importance of SRF cleavage for the progression of apoptosis. To this end, BJAB cells transfected with a vector for either *wt*SRF or DM-SRF were cultured for 24 h in full medium and then treated with CH11. Western blotting confirmed that *wt*SRF and DM-SRF are expressed at identical levels (not shown). After 20 h, cells were fixed and stained, and apoptotic cells were counted. As shown in Fig. 7*a*, CH11 induced apoptosis in 60% of the cells transfected with either control DNA or an expression vector for *wt*SRF. By comparison, in cells transfected with the corresponding expression vector for DM-SRF, the level of apoptosis induced by CH11 was reduced to 40%. The reduction in the number of apoptotic cells due to the expression of a cleavage-resistant SRF is remarkable, because we routinely achieve a transfection efficiency of no more than 50%, as gauged by transfection of a vector for GFP. Moreover, the effect of DM-SRF expression was already apparent 10 h after CH11 treatment (not shown). From these results, we infer that SRF cleavage by caspases is an important early step in Fas-mediated apoptosis of BJAB cells.

DISCUSSION

In this paper, we have examined the cleavage of SRF, a transcription factor involved in proliferative gene expression, by caspases, the intracellular executioners of apoptosis. We find that in BJAB cells, SRF is cleaved early after Fas-induced apoptosis, at which point c-*fos* expression is down-regulated. *In vitro* SRF is cleaved most efficiently by caspase 7 but also by caspases 3 and 8, yielding two major cleavage fragments. SRF cleavage fragments fail to support SRF-dependent reporter expression, while expression of a cleavage-resistant form of SRF suppresses apoptosis.

By a combination of deletion analysis and point mutagenesis, caspase cleavage sites in SRF were mapped to two closely spaced aspartates in the middle of the molecule. Cleavage therefore occurs just carboxyl-terminal to the DNA-binding core of SRF. Intriguingly, half of the aspartate residues in SRF (6 of 12) are clustered in this region of the protein. Apart from the core domain of SRF, which has been examined in some detail (6), little is known about either the structure or function of other SRF domains. Within the amino-terminal domain, the only feature to have been studied is a cluster of phosphorylation sites that seems to influence the rate of formation and stability of DNA complexes (11–14). Phosphorylation of one of these residues (Ser^{103}) was shown to correlate with transcrip-

tional activation of c-*fos*, but its mutation had no detectable effect on expression levels (15). A region toward the carboxyl terminus of SRF has been shown to possess transactivation potential, and this correlates with an ability to interact with the larger subunit of TFIIF, the mammalian homologue of yeast RAP74 (17, 18). Thus, caspase cleavage severs the transactivation domain of SRF from its DNA-binding domain.

We found that whereas full-length SRF enhanced SRE-dependent transcription, SRF fragments corresponding to those generated by caspase cleavage were transcriptionally inactive or slightly inhibitory to reporter expression. To rule out any influence of endogenous full-length SRF, these experiments were performed with a reporter system consisting of a modified SRF with altered DNA binding specificity and a corresponding reporter. This system has been used extensively to unravel SRE-mediated transcription responses to intracellular signaling events (38, 43). Two different mechanisms can be envisaged to account for these observations. On the one hand, the amino-terminal cleavage fragment of SRF (NMS4) is able to dimerize and bind DNA as well as full-length SRF (see *e.g.* Fig. 2*c*), but since it lacks the carboxyl-terminal transactivation domain it is unlikely to recruit a functional transcription complex. A truncated form of SRF lacking part of the transactivation domain, which is generated by alternate splicing at exon 5, has also been shown to lack transactivation potential and act as a trans-dominant inhibitor of SRF function (44). On the other hand, the carboxyl-terminal cleavage fragment of SRF (SRFC), which cannot be recruited to the promoter, may inhibit transcription by squelching basal factors, for example TFIIF (18).

The two sites in SRF recognized and cleaved by caspases conform to a weak consensus by which the P4 position preceding the conserved P1 aspartate is a small polar or negatively charged residue (45). Although these sites conform structurally to those for caspase 3 (24), it was not possible to predict unequivocally which caspase(s) would be responsible for cleavage. A comparison of active, recombinant caspases revealed that caspase 7 cleaves SRF most effectively, but cleavage at one or the other of the sites by caspases 3, 8, and 9 was also observed. In addition, a distinct, albeit weak, pattern of SRF fragments was generated by caspases 4 and 1, suggesting that other aspartate residues in SRF may serve as cleavage sites. Since the relative activity of each recombinant caspase was similar, these assays serve to predict with some accuracy the identity of the caspases responsible for SRF cleavage *in vivo*.

Caspases are activated by proteolytic cleavage in a hierarchical fashion (24). Thus, caspases activated early during apoptosis by scaffold-mediated proteolysis at death receptors or in association with the Apaf-1-cytochrome *c* complex (46, 47) activate, in turn, other caspases that cleave predominantly cellular target proteins. Consequently, the activation of initiator caspases (*e.g.* caspases 8 and 9) is thought to determine the onset of the apoptosis program, while effector caspases, including caspases 3 and 7, serve as executioners in what has become an irreversible process. However, this simple dichotomy may not strictly apply. For example, several proteins are targets for both initiator and effector caspases (24). Moreover, caspase 3 can activate procaspase 9 (48).

The presence of four SRF fragments (*e.g.* Fig. 3*b*) suggests that once SRF is cleaved by caspase 7 or caspase 3 at one site, its susceptibility to cleavage at the adjacent site is markedly reduced. In contrast, weak caspase 3 cleavage of DM-SRF *in vitro* at a suboptimal site suggests that this region in full-length SRF is acutely sensitive to cleavage. Notably, both cleavage sites are evolutionarily conserved from zebrafish to humans, although the zebrafish homologue of SRF is truncated at the carboxyl terminus and lacks residues beyond amino acid 244. In *Drosophila* SRF (blistered), mutations are present at each site, but three additional aspartate residues are available in this region of the protein.

Our results suggest that SRF cleavage may be a determining step in the apoptosis of BJAB cells. First, fragments of SRF are detectable just 2 h after CH11 treatment of BJAB cells, a time point at which morphological changes are absent. Second, the overexpression of SRF lacking caspase cleavage sites caused a 40% reduction in the induction of apoptotic cells 20 h after Fas cross-linking. The interpretation of this result is complicated by two factors. First, whereas 95% of untransfected cells are clearly apoptotic 10 h after CH11 treatment (Fig. 2*a*), only 60% of cells that survive electroporation undergo apoptosis in response to CH11. The reason for this is currently obscure. Second, only 50% of the cells take up and express the transfected DNA, which implies that the effect of DM-SRF expression on apoptosis is an underestimate. To circumvent these problems, we attempted to generate stable BJAB cell lines overexpressing *wt*SRF and DM-SRF. While the former was straightforward (Fig. 2*d*), we were unable to generate a cell line expressing DM-SRF, despite three attempts with two expression/selection systems. Nonetheless, our results show that interference with the cleavage of a single caspase target protein, in this case SRF, appears to derail the apoptosis machinery. These observations are also consistent with a previous finding that treatment of Jurkat cells with phorbol ester, a potent inducer of IE genes, prevents SRF cleavage and protects the cells against Fas-induced apoptosis (49).

Caspases promote cell death in several ways. For example, the proapoptotic proteins Bid and Bax are activated by caspase cleavage (50, 51). Several protein kinases capable of activating the stress-activated protein kinase/Jun N-terminal kinase pathway are also cleaved by caspases to produce constitutively active, proapoptotic kinases (52–54). Caspases also disrupt cell survival by inactivating survival factors, such as Bcl-2 and Bcl-X_L (55, 56), but also by targeting components of survival pathways (57–59). Survival pathways up-regulate several nuclear events, including IE gene expression, in which SRF is involved. In this context, SRF has recently been linked to expression of the Bcl-2-related survival factor MCL1 (60). Paradoxically, stress signals have also been shown to stimulate expression of c-*fos*, a paradigm for SRF-regulated IE genes (61–64). Thus, caspase cleavage of SRF may ensure the extinction of a subset of survival genes whose expression would otherwise be maintained by cell stress signals.

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Serum Response Factor Cleavage by Caspases 3 and 7 Linked to Apoptosis in Human BJAB Cells

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