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The novel SAR-binding domain of scaffold attachment factor A (SAF-A) is a target in apoptotic nuclear breakdown

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The scaffold attachment factor A (SAF-A) is an abundant component of the nuclear scaffold and of chromatin, and also occurs in heterogeneous nuclear ribonucleoprotein (hnRNP) complexes. Evidence from previous experiments had suggested that SAF-A most likely has at least two different functions, being involved both in nuclear architecture and RNA metabolism. We now show that the protein has a novel scaffold-associated region (SAR)-specifi bipartite DNA-binding domain which is independent from the previously identifie RNA-binding domain, the RGG box. During apoptosis, but not during necrosis, SAF-A is cleaved in a caspase-dependent way. Cleavage occurs within the bipartite DNA-binding domain, resulting in a loss of DNA-binding activity and a concomitant detachment of SAF-A from nuclear structural sites. On the other hand, cleavage does not compromise the association of SAF-A with hnRNP complexes, indicating that the function of SAF-A in RNA metabolism is not affected in apoptosis. Our results suggest that detachment of SAF-A from SARs, caused by apoptotic proteolysis of its DNA-binding domain, is linked to the formation of oligonucleosomal-sized DNA fragments and could therefore contribute to nuclear breakdown in apoptotic cells.

Keywords: apoptosis/chromatin structure/nuclear scaffold/SAF-A/SAR

Introduction

In recent years, it has become increasingly clear that the nucleus of eukaryotic cells is a highly ordered organelle. Whole chromosomes (Lichter *et al.*, 1988) and even single genetic loci (Marshall *et al.*, 1996) occupy well-define areas in the interphase nucleus, and all processes of RNA metabolism are precisely located in distinct nuclear regions (Xing *et al.*, 1993). However, even the most basic principles of internal nuclear architecture are not yet fully understood. One much debated possibility is that the internal order of the nucleus is determined by a specifi attachment of chromatin to an underlying skeleton (Gasser and Laemmli, 1987). Indeed, a proteinaceous framework, termed nuclear matrix or scaffold, can be isolated from the nuclei of nearly all cell types and organisms (reviewed in Berezney *et al.*, 1995). Much evidence has been presented on the influenc of this scaffold on the regulation of DNA replication, gene expression and RNA processing (reviewed in Nickerson *et al.*, 1995). However, its involvement in maintaining chromosomal order *in vivo* is still controversial.

Indirect evidence for a structuring role of the nuclear scaffold comes from the identificatio of specialized AT-rich DNA regions, termed SARs (scaffold-associated regions; Mirkovitch *et al.*, 1984) or MARs (matrix attachment regions; Cockerill and Garrard, 1986). These DNA regions specificall interact with scaffold preparations, suggesting that chromatin is attached to the nuclear scaffold through these sites. From the distance between individual attachment sites, it has been proposed that chromatin is organized in loops with an average size of 30 kb (reviewed, e.g. by Garrard, 1990; Laemmli *et al.*, 1992), consistent with conclusions drawn from unrelated experiments (Vogelstein *et al.*, 1980; Filipski *et al.*, 1990). In some cases, these loops coincide with units of gene expression, but attachment sites can also be located in introns of large genes (e.g. see Romig *et al.*, 1994). In fact, analysis of long stretches of genomic DNA did not reveal an obvious relationship between gene structure and the localization of SARs (Surdej *et al.*, 1990). Additional to their structural role, SARs might also have functional or regulatory roles. They frequently are observed in the vicinity of enhancers (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986), can stimulate expression of heterologous reporter genes when integrated into the genome (Stief *et al.*, 1989) and appear to be involved in regulating chromatin accessibility (Jenuwein *et al.*, 1997).

To elucidate the function of SARs *in vivo*, several laboratories have characterized proteins with specifi binding to SAR DNA. These proteins include ubiquitous, abundant proteins like topoisomerase II (Adachi *et al.*, 1989), histone H1 (Izaurralde *et al.*, 1989), lamin B1 (Luderus *et al.*, 1992), HMG I/Y (Zhao *et al.*, 1993) and nucleolin (Dickinson and Kohwi-Shigematsu, 1995), but also proteins that are expressed primarily in certain cell types, like SATB1 (Dickinson *et al.*, 1992) or p114 (Yanagisawa *et al.*, 1996). We have isolated and characterized the human nuclear proteins SAF-A (scaffold attachment factor A) and SAF-B (Romig *et al.*, 1992; Renz and Fackelmayer, 1996). SAF-A was later found to be identical to hnRNP-U (Fackelmayer *et al.*, 1994), a component of heterogeneous nuclear ribonucleoprotein particles (Kiledjian and Dreyfuss, 1992). The precise role of the protein *in vivo* has not been fully resolved yet, but evidence suggests that it most likely has at least two different functions. As a constituent of hnRNP particles, it is involved in the packaging and processing of RNA (Dreyfuss *et al.*, 1993). On the other hand, several reports have shown that SAF-A has a high binding specificit for

SAR DNA and should therefore be important for the organization of chromatin (Tsutsui *et al.*, 1993; Fackelmayer *et al.*, 1994; von Kries *et al.*, 1994). Indeed, as shown below, the double role of the protein is reflecte by its structure, as SAR-binding activity resides on a protein domain independent from the known RNA-binding motif, the RGG box described by Kiledjian and Dreyfuss (1992).

The identificatio of SAF-A as an RNA-binding protein may be surprising, but is fully compatible with recent investigations showing that 16 out of the 21 most abundant proteins of the nuclear scaffold are hnRNP proteins (Mattern *et al.*, 1996). The conclusion from these experiments is that hnRNP proteins may be required to maintain the internal nuclear architecture, an assumption further substantiated by reports describing that pre-mRNA is tightly associated with nuclear substructures (He *et al.*, 1990), possibly even as a structural component (Belgrader *et al.*, 1991). In addition, transcriptionally inactive cells like avian erythrocytes seem to be devoid of a detectable nuclear scaffold, but regain such a structure after transcriptional activation by fusion with fibroblast (LaFond *et al.*, 1983). Thus, SAF-A and other hnRNP proteins may serve as a structural and/or functional link between RNA metabolism and nuclear architecture.

Consistent with its presumed role in nuclear architecture, SAF-A is a highly abundant protein in all cells investigated. HeLa cells contain ~2×10^6 copies per nucleus, of which one half are structurally fixe by a tight and salt-stable binding to the nuclear scaffold (Fackelmayer *et al.*, 1994; Mattern *et al.*, 1996). The remaining half of the protein distributes equally between a soluble, hnRNP-associated and a DNase-extractable fraction. Indeed, we recently have presented evidence that at least 20% of the total cellular SAF-A is directly bound to DNA *in vivo* (Göhring and Fackelmayer, 1997). *In vitro*, purifie SAF-A self-assembles into filamentou polymers in the presence of DNA, thereby preferentially interacting with SAR DNA fragments (Fackelmayer *et al.*, 1994). Even more interestingly, polymers of SAF-A capture the same DNA fragments from a complex mixture of SAR and non-SAR DNA as unfractionated preparations of nuclear scaffolds (unpublished). In these scaffold preparations, SAF-A is one of the 10 most abundant proteins (Mattern *et al.*, 1996, 1997), and the only protein with known specificit for SAR DNA. Thus, SAF-A appears to be one of the major SAR-binding proteins in human cells.

Given this prominent role among nuclear scaffold proteins, we found it interesting to investigate how SAF-A behaves during apoptosis, where a decomposition of the nuclear architecture is one of the most dramatic reactions observed. We describe here that SAF-A is cleaved relatively early in apoptosis. Cleavage destroys the DNA-binding activity of SAF-A and causes its detachment from nuclear substructures. Thus, the cleavage of SAF-A may be a critical step in apoptosis, contributing to the collapse of chromatin structure or nuclear fragmentation.

Results

SAF-A is cleaved during apoptosis

To investigate the fate of SAF-A in apoptosis, we have used a well-established model system for apoptosis, Jurkat human leukaemic T cells exposed to a monoclonal antibody against the cell surface receptor CD95 (Fas/Apo-1; reviewed in Matiba *et al.*, 1997). Polyacrylamide gel electrophoresis and immunoblotting revealed that cleavage of SAF-A begins 90 min after induction of apoptosis, and leads to an essentially complete conversion of the native 120 kDa protein to a 97 kDa cleavage product within 180 min (Figure 1C, left). An identical cleavage of SAF-A also occurs in other apoptotic model systems, e.g. Jurkat

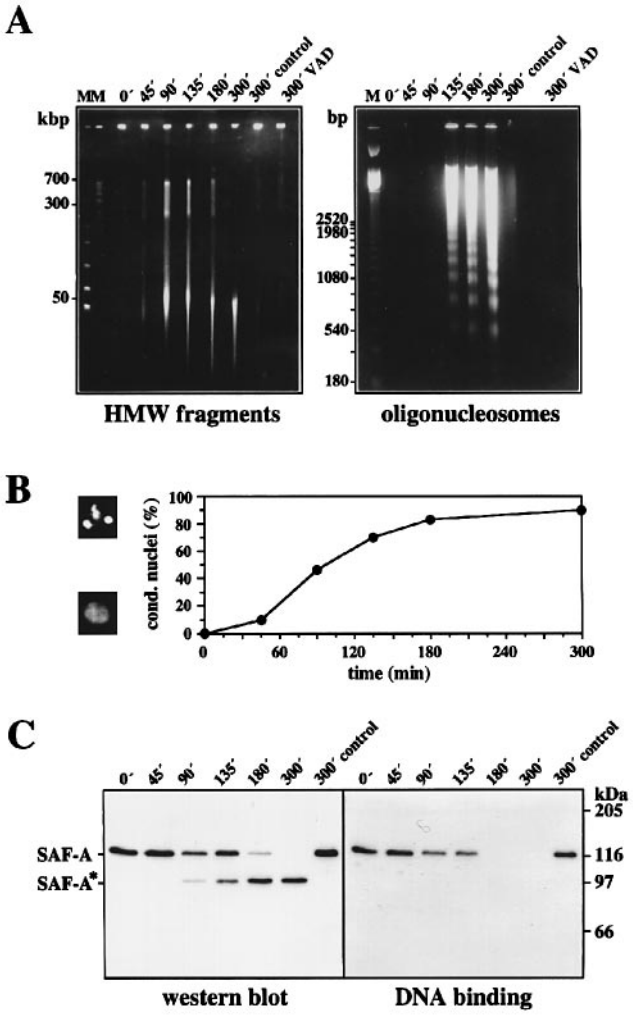

Fig. 1. Time course of apoptosis and cleavage of SAF-A. Jurkat cells were analysed for DNA cleavage, apoptotic phenotype and cleavage of SAF-A after different times of incubation with anti-CD95 antibody. (**A**) DNA cleavage, examined by fiel inversion gel electrophoresis to visualize high molecular weight chromatin fragments (left panel), or conventional agarose gel electrophoresis to visualize oligonucleosomal fragments (right panel). Per lane, DNA from 1×10^6 cells was analyzed. Control experiments: without anti-CD95 antibody (300′ control) or in the presence of the caspase inhibitor z-VAD-fmk (300′ VAD). Markers: concatemeric λ DNA (left panel, firs lane), yeast chromosomes (left panel, second lane) and a 180 bp ladder (right panel, firs lane). (**B**) Percentage of cells displaying apoptotic phenotype, scored by microscopic investigation of cells (~300 per time point) stained with Hoechst 33342. (**C**) Cleavage of SAF-A abolishes DNA binding. Total cell extracts (25 μg protein per lane) prepared at different times after induction of apoptosis were analysed for SAF-A by Western blotting (left panel), or by a Southwestern assay with labelled SAR DNA (fragment MII) in the presence of a 5000-fold excess of unlabelled *E.coli* DNA. The DNA-binding assay and Western blot were performed on the same membrane, with the DNA-binding assay first SAF-A*: stable cleavage product of SAF-A.

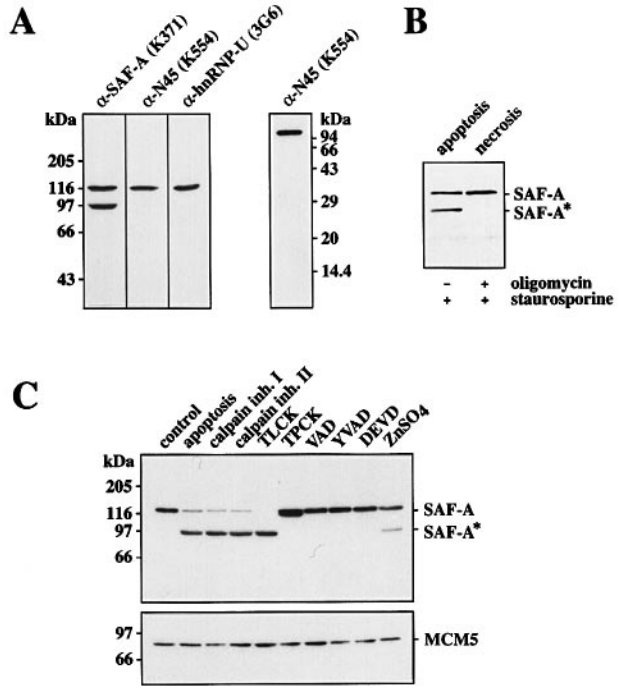

Fig. 2. Amino-terminal cleavage of SAF-A is a specifi apoptotic process. (**A**) Total cell extract prepared 150 min after induction was resolved by SDS–PAGE (7% polyacrylamide, left three panels), blotted to a PVDF membrane and analysed for SAF-A by Western blotting with polyclonal antibodies to total SAF-A [α-SAF-A (K371)], polyclonal antibodies to the amino-terminal 45 amino acids of SAF-A [α-N45 (K554)] or monoclonal antibody 3G6 to hnRNP-U/SAF-A [α-hnRNP-U (3G6)]. The same sample was analysed on a higher percentage gel (15% polyacrylamide, forth panel) with α-N45 antibody. (**B**) Jurkat cells were induced for apoptosis or necrosis by treatment with staurosporine without or with pre-treatment with oligomycin, respectively. Total cell extracts prepared after 3 h were analysed for SAF-A cleavage by Western blotting with polyclonal antibodies to total SAF-A. (**C**) Jurkat cells were induced for apoptosis in the absence or presence of different protease inhibitors. Total cell extracts, prepared after 4 h, were analysed for SAF-A cleavage by Western blotting. Non-induced cells were analysed as control (firs lane). Note the double band of full-length SAF-A in the TPCK lane, most probably a result of a covalent modification SAF-A*: stable cleavage product of SAF-A. As a loading control, the same membrane was stripped and reprobed with antibodies against replication protein MCM5, a protein not cleaved in apoptosis (unpublished).

cells treated with staurosporine (compare Figure 2B), or HeLa cells treated with cycloheximide/tumour necrosis factor-α (TNF-α) or actinomycin D (not shown). In all cases, cleavage of SAF-A occurs simultaneously with chromatin degradation (Figure 1A) and nuclear disintegration (Figure 1B), slightly before high molecular weight DNA fragments are converted to oligonucleosomes. No further degradation of the 97 kDa fragment is observed even at late time points, indicating that this fragment is the stable end product of apoptotic cleavage. A second cleavage product, expected to be a polypeptide of 20–25 kDa, could not be detected in immunoblots with a variety of SAF-A-specifi polyclonal antibodies (compare Figure 2A), suggesting that it is degraded rapidly after the initial cleavage of SAF-A.

Cleavage of SAF-A is accompanied by a loss of DNA-binding activity, as shown by incubating blotted cell extracts with radioactively labelled SAR DNA (fragment MII; Romig *et al.*, 1992, 1994) in the presence of a 5000-fold excess of unlabelled, unspecifi competitor DNA. Under these stringent conditions, only SAF-A binds to the probe, but not the many other unspecifi DNA-binding proteins present in cell extracts (Romig *et al.*, 1992). Autoradiography of the membrane reveals that full-length SAF-A binds to DNA as expected, whereas the 97 kDa fragment observed in apoptosis has lost DNA-binding activity (Figure 1C, right).

To determine whether the cleavage site is located in the amino- or carboxy-terminal region of SAF-A, we used an antibody produced against the firs 45 amino-terminal amino acids of SAF-A (N45 polypeptide, see below). Immunoblot analysis shows that this antibody recognizes full-length SAF-A, but not the 97 kDa fragment (Figure 2A). The same result is obtained with the 3G6 monoclonal antibody developed by Dreyfuss and co-workers (Dreyfuss *et al.*, 1984), which is used widely in research on hnRNP proteins. By size comparison with the N-terminal constructs characterized below, the cleavage site is most probably located between amino acids 110 and 160. This region is rich in glutamate and aspartate residues and contains two sequences with homology to caspase cleavage sites (DENGD at position 130, and DELGD at position 143). Experiments to map the cleavage site precisely are currently underway.

To demonstrate that cleavage of SAF-A is a specifi apoptotic process, we compared Jurkat cells undergoing apoptosis with cells forced to execute necrotic cell death by depletion of intracellular ATP pools (Leist *et al.*, 1997). Western blot analysis of extracts from both aliquots of cells clearly demonstrates that SAF-A cleavage occurs only during apoptosis, but not during necrosis (Figure 2B). Thus, cleavage of SAF-A is presumably dependent on the proteolytic activity of a caspase. This conclusion is supported by experiments testing a variety of specifi protease inhibitors for their ability to inhibit SAF-A cleavage *in vivo* (Figure 2C). We found that inhibitors that block SAF-A cleavage also prevent the apoptotic phenotype. Particularly, these were the peptide inhibitors DEVD-CHO (aldehyde), benzyloxycarbonyl-VAD-fmk (fluoromethylketone) YVAD-cmk (chloromethylketone; all preventing cleavage of caspase substrates) and TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone, an inhibitor of serine proteases). Notably, a partial inhibition is also observed by low amounts of zinc ions, known to prevent DNA laddering but not formation of high molecular weight DNA fragments (Cohen *et al.*, 1992). No inhibition of SAF-A cleavage and apoptotic phenotype is observed with TLCK (*N*-α-*p*-tosyl-L-lysine chloromethylketone) or calpain inhibitors I and II.

Cleaved SAF-A detaches from chromatin

We next addressed the question of how apoptotic cleavage affects the localization of SAF-A in the nucleus. Immunofluorescenc experiments with affinity-purifi polyclonal antibodies demonstrate that SAF-A is localized exclusively in the nucleus of non-apoptotic cells, displaying a granular staining as reported earlier (Fackelmayer *et al.*, 1994; Zweyer *et al.*, 1997). After induction of apoptosis, Jurkat cells undergo morphological changes in a synchronous manner, as described by Weis *et al.* (1995). In early apoptosis, when chromatin begins to collapse against the nuclear periphery, SAF-A remains nuclear, but is clearly

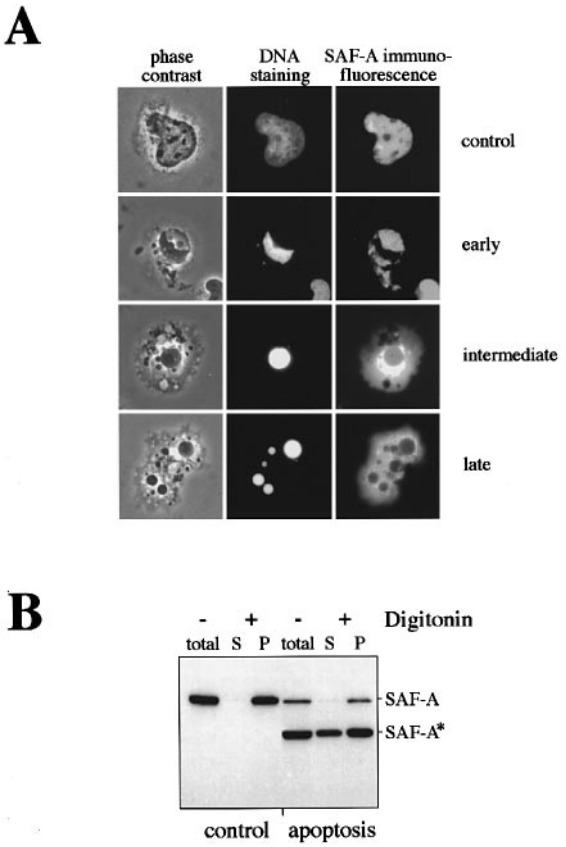

Fig. 3. Cleaved SAF-A detaches from nuclear substructures *in vivo*. (**A**) Cells induced for apoptosis and untreated control cells were analysed by immunofluorescenc with polyclonal antibody K371 (compare Figure 2) against total SAF-A. Typical images of early (45 min after induction), intermediate (90 min) and late (240 min) stages of apoptosis are shown, together with phase contrast images and DNA counterstain images with Hoechst 33258. The dimension of each frame is 20×20 μm. (**B**) Control and apoptotic cells (3 h after induction) were subjected to extraction with 10 μg/ml digitonin. Supernatant (S) and pellet (P) were analysed for their content of SAF-A or its cleavage product (SAF-A*) by Western blotting with K371 antibody. Extracts of unfractionated cells were analysed as controls (lanes 'total'). Note that a significan amount of the cleavage product, but not of uncleaved SAF-A, is solubilized.

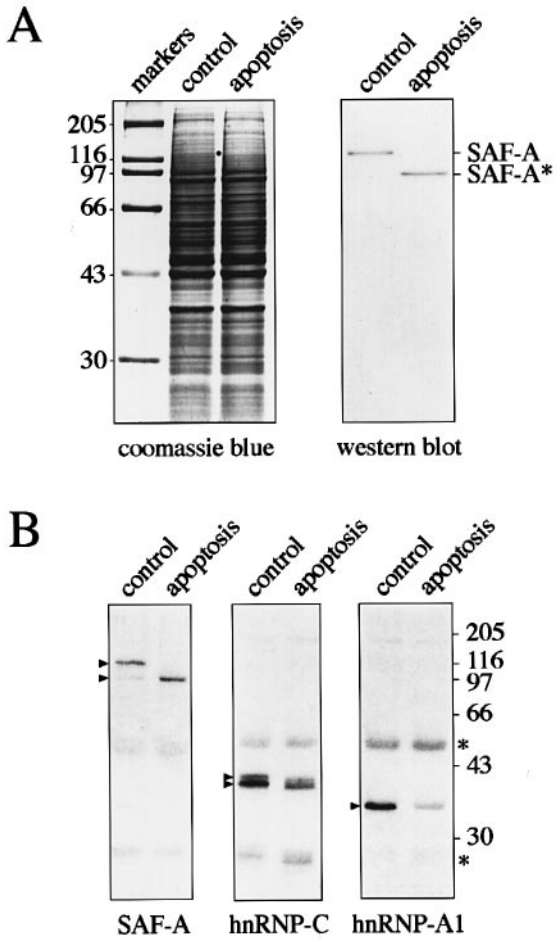

Fig. 4. Cleavage of SAF-A does not affect its association with hnRNP particles. (**A**) Total soluble extract was prepared from healthy control cells and apoptotic cells 5 h after induction. Staining with Coomassie blue reveals no apparent changes in the overall protein pattern (left panel, dot between the lanes indicates SAF-A), but immunoblotting demonstrates a complete cleavage of SAF-A in apoptotic cells (right panel). (**B**) Immunoprecipitations with 250 μg (total protein) of the extracts characterized in (A) were performed with mAB 4F4 against hnRNP-C1/C2, and the immunoprecipitated material was analysed by immunoblotting with polyclonal antibody K371 against SAF-A, or monoclonal antibodies 4F4 or 9H10 against hnRNP-C1/C2 or hnRNP-A1, respectively. Asterisks: heavy and light chain of the 4F4 antibody.

separated from DNA (Figure 3A). Thus, SAF-A is relocated rapidly at the time when cleavage occurs and contacts with DNA are resolved. At later stages of apoptosis, when the nuclear envelope breaks down, SAF-A spreads to the cytoplasm, where it remains until the latest stages of cell death (Figure 3A).

Biochemical fractionation of apoptotic and control cells by a method based on that of Adam *et al.* (1990) supports this result and demonstrates that a significan fraction of the cleavage product detaches from an insoluble nuclear structure, while full-length SAF-A is clearly bound to nuclear substructures (Figure 3B). A more detailed analysis of the subcellular localization according to several published methods (e.g. Hancock, 1974; Fey *et al.*, 1986) failed due to the increased fragility of Jurkat cells in apoptosis.

In contrast to the attachment of SAF-A to chromatin, the association of SAF-A with hnRNP particles is not compromised by apoptotic cleavage (Figure 4). This is demonstrated by immunoprecipitation of hnRNP particles from total cell extracts of healthy and apoptotic cells, using the monoclonal antibody 4F4 directed against hnRNP-C1/C2 (Choi and Dreyfuss, 1984). Western blot analysis of the immunoprecipitated material reveals that cleaved SAF-A is present in hnRNP particles from apoptotic cells in the same stoichiometry to hnRNP-C as uncleaved SAF-A in particles from healthy cells (Figure 4B). Thus, cleavage of SAF-A specificall affects its SAR DNA-binding activity, but not its binding to hnRNP particles and, presumably, to RNA (compare Figure 5B for binding of a C-terminal construct to single-stranded DNA). Additionally, our data confir the cleavage of hnRNP-C noted by others (Waterhouse *et al.*, 1996), and demonstrate that the amount of hnRNP-A1 in apoptotic hnRNP particles is greatly reduced for unknown reasons.

A novel SAR DNA-binding domain is present in the amino-terminus of SAF-A

To determine the molecular basis for the loss of DNA interaction after cleavage of SAF-A, we mapped and

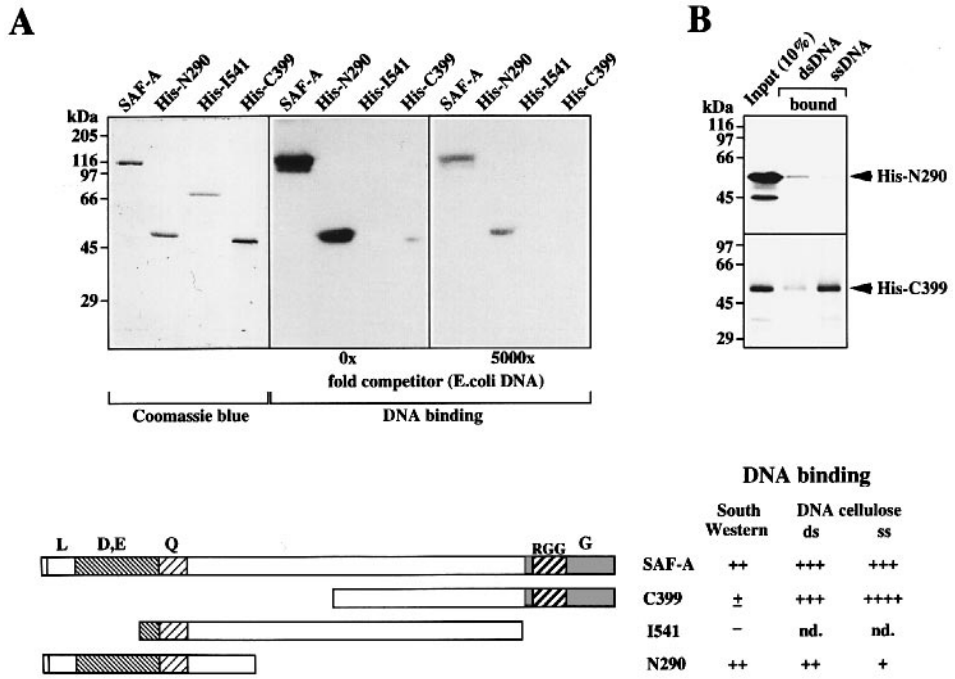

Fig. 5. The amino-terminus of SAF-A harbours an SAR DNA-binding domain. (**A**) Three overlapping partial proteins spanning SAF-A were bacterially overproduced and analysed for DNA-binding activity. First panel, proteins stained with Coomassie blue; second and third panel, Southwestern assay in the absence and presence of unlabelled competitor DNA. (**B**) Binding of ^{35}S-labelled, *in vitro* translated amino-terminal construct N290 and carboxy-terminal construct C399 to immobilized double- and single-stranded DNA (ds/ss DNA cellulose). Lower panel: schematic representation of the proteins, with shaded areas depicting regions rich in leucine (L), aspartic and glutamic acids (D and E), glutamine (Q) or glycine (G). Note the RGG box in the carboxy-terminus of the protein.

characterized its DNA-binding domain. For this purpose, Southwestern assays with three overlapping recombinant fragments of SAF-A were performed in the absence and presence of unlabelled competitor DNA. As seen in Figure 5A, the amino-terminal fragment N290 (aa 1–290) has a strong and specifi binding to the SAR probe, indistinguishable from that of full-length SAF-A. The internal fragment I541 (aa 145–677) does not bind to DNA, and weak binding was observed in the carboxy-terminal fragment C399 (aa 408–806), most probably due to the RGG box, an RNA-binding motif described by Kiledjian and Dreyfuss (1992). To compare DNA binding of our amino- and carboxy-terminal fragments of SAF-A, we have repeated their experimental approach. For that purpose, *in vitro* translated protein fragments were incubated with immobilized nucleic acids, and bound proteins were analysed by gel electrophoresis (Figure 5B). We found that the carboxy-terminal fragment has a higher affinit for single-stranded DNA, whereas the amino-terminal fragment prefers double-stranded DNA. However, when analysed with this assay, binding of the amino-terminal construct N290 to DNA is much weaker than binding by the carboxy-terminal construct C399. This is in apparent contrast to the results of Southwestern assays (see Discussion), and has prompted us to investigate the amino-terminal DNA-binding domain in more detail.

To this end, we have constructed and synthesized a set of polypeptides from the amino-terminus of SAF-A, expressed as secreted fusion proteins (Löwenadler *et al.*, 1987) to bypass solubility problems encountered with His-tagged proteins. The resulting recombinant proteins were then tested for DNA binding in comparison with purifie full-length SAF-A (Figure 6). To rule out a possible interference of the 15 kDa ZZ tag, we also synthesized the firs 290 amino acids as His-tagged protein as in Figure 5. We found that truncation from 290 amino acids to 247 amino acids has no effect on DNA binding, but further truncation to 158 amino acids results in a complete loss of DNA-binding activity. Interestingly, loss of activity is also observed by a truncation from the amino-terminus of the protein. Thus, two regions in the amino-terminus of SAF-A are necessary for DNA binding, and a deletion of either of these regions results in a loss of activity.

Having identifie the amino-terminal construct N247 as the shortest fragment of SAF-A that binds to DNA, we next investigated its binding specificit for SAR DNA (Figure 7) by two independent approaches. First, a Southwestern assay with N247 and purifie SAF-A was performed in the absence or presence of increasing amounts of unlabelled bacterial competitor DNA, demonstrating that *Escherichia coli* DNA only weakly competes for binding to SAR DNA (Figure 7A and B). Indeed, when an equimolar mixture of SAR and non-SAR DNA is used as labelled probe, protein N247 binds only the SAR fragment in the presence of excess bacterial competitor DNA (Figure 7C). In the absence of competitor DNA, both DNA species are equally bound, reflectin the general DNA-binding activity previously described for full-length SAF-A (Fackelmayer *et al.*, 1994).

In a second approach, recombinant N247 was bound to IgG–Sepharose beads through its ZZ tag, and the amount of bound SAR or non-SAR DNA was quantifie at increasing amounts of competitor DNA (Figure 7D). The ratio of protein to DNA was titrated to use saturating amounts of protein at low competitor DNA concentration, but limiting amounts at a 100-fold or higher excess of

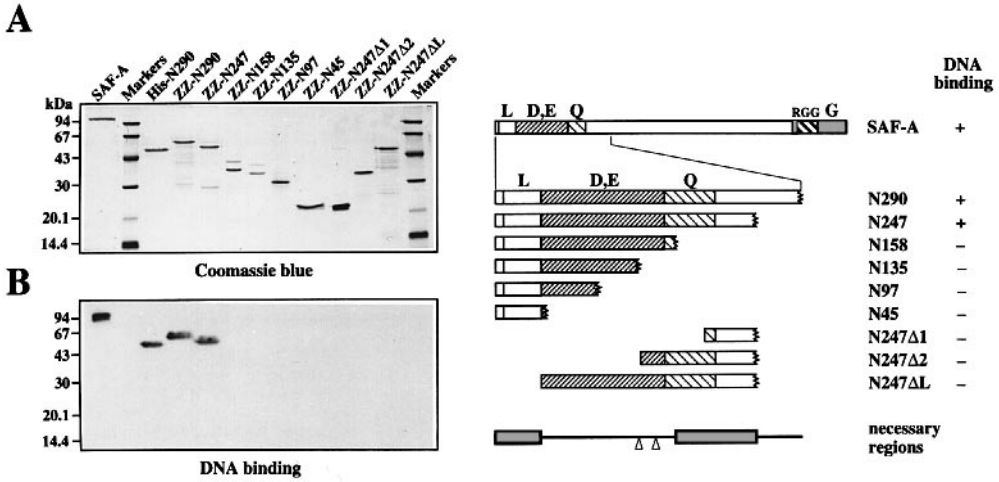

Fig. 6. Fine mapping of the SAR DNA-binding domain of SAF-A. (**A**) Amino-terminal constructs of SAF-A were bacterially overproduced as fusion proteins with either a ZZ tag or a 6×His tag, resolved on 15% polyacrylamide gels and visualized by staining with Coomassie blue. As a control, purifie human SAF-A was applied to the firs lane. (**B**) A parallel gel was blotted to a PVDF membrane, and incubated with labelled SAR DNA to analyse the proteins for DNA-binding activity. The exposure time of the autoradiograph was 5 h. The right panel is a schematic representation of the investigated partial proteins (see legend to Figure 4). Regions necessary for DNA binding are depicted below, as well as the position of two sequences similar to caspase cleavage sites (open arrowheads).

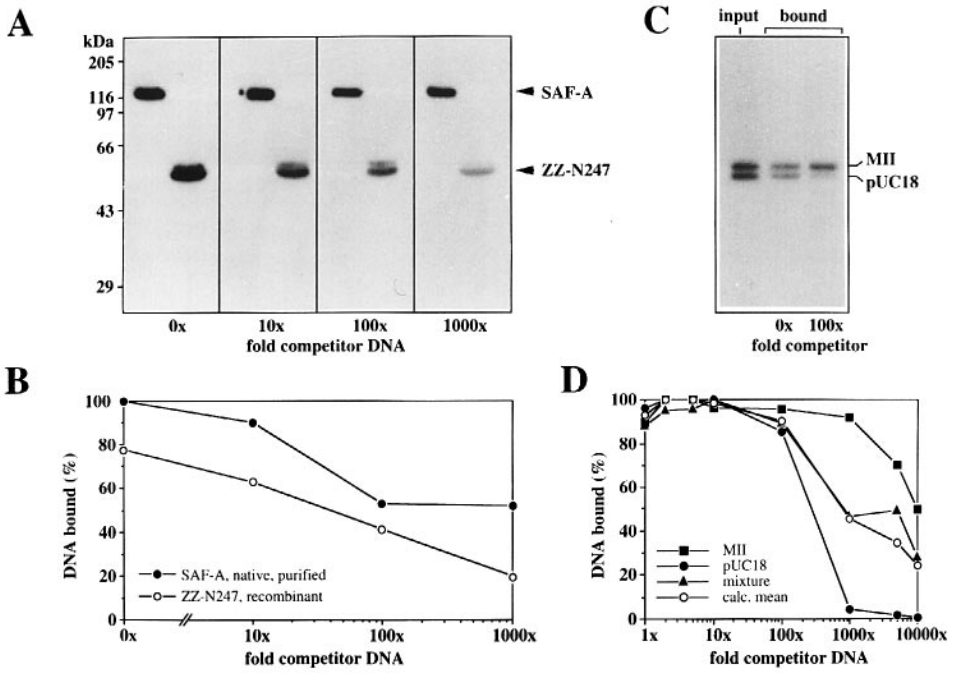

Fig. 7. Specificit of the novel binding domain. (**A**) One μg of purifie human SAF-A and recombinant N247 protein were compared for DNA binding in Southwestern blot assays in the absence or presence of increasing amounts of unlabelled *E.coli* DNA as unspecifi competitor. The exposure time of the autoradiograph was 5 h. (**B**) DNA binding in the experiment shown in (A) was quantifie by liquid scintillation counting of excised membrane pieces containing the bands of interest. Binding to native SAF-A in the absence of competitor DNA is set to 100%. (**C**) One μg of recombinant N247 protein was immobilized on a nitrocellulose membrane and incubated with an equimolar mixture of SAR and non-SAR DNA (input) in the absence or presence of a 100-fold excess of competitor DNA. Bound DNA was eluted and analysed by agarose gel electrophoresis and autoradiography of the dried gel (exposure time 6 h). (**D**) One μg of recombinant N247 protein was immobilized on IgG–Sepharose beads and incubated with labelled SAR DNA, non-SAR DNA or a mixture of both in the presence of increasing amounts of competitor DNA. The amount of bound DNA was quantifie by liquid scintillation counting.

competitor DNA. In line with the results shown in Figure 7C, saturating amounts of protein bind to non-SAR DNA as well as to SAR DNA. At a higher excess of competitor (i.e. when the protein becomes limiting), however, binding to non-SAR DNA declines sharply to undetectable levels, whereas 50% of the SAR DNA still remains bound at a 10 000-fold excess of competitor DNA. The same result was obtained with 6×His-tagged N290 immobilized on Ni-NTA–agarose (not shown), ruling out an interference by either the tag or the bead material.

We performed database comparisons and secondary structure predictions to learn more about the SAR-specifi DNA-binding domain located in the firs 247 amino acids of SAF-A. While the region between amino acids 158

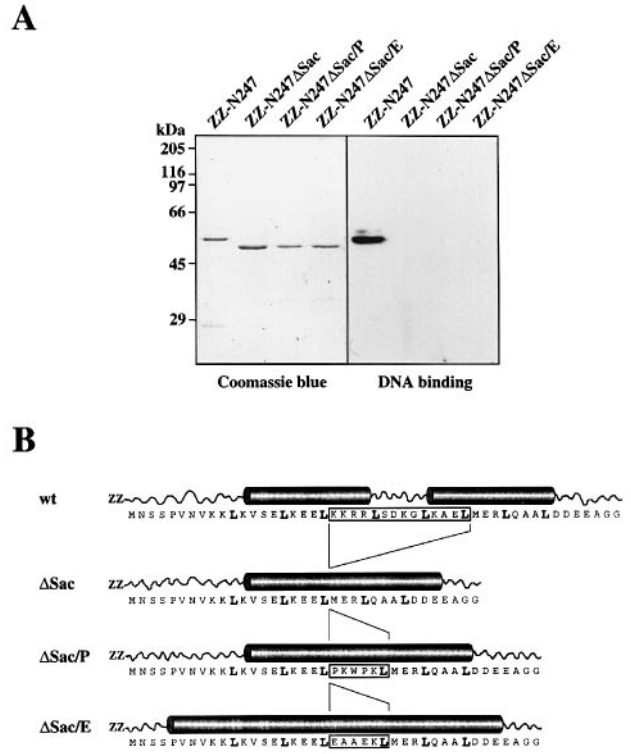

Fig. 8. The 'spaced leucine motif' is essential for DNA-binding activity. (**A**) Partial protein N247 and mutated versions were bacterially expressed and stained with Coomassie blue (left panel) or analysed for DNA binding in a Southwestern assay (right panel, autoradiography). (**B**) Schematic representation of the amino-terminal regions of the proteins analysed in (A). Note that the introduced mutations are likely to affect the secondary structure of the 'spaced leucine motif' as suggested by secondary structure predictions according to the PHD algorithm. Wavy lines, turns/unordered structure; rods, α-helical regions.

and 247 has no obvious sequence features apart from being rich in glutamine residues, the region in the firs 45 amino acids contains a conspicuous motif of spaced leucine residues, reminiscent of a leucine zipper (Figure 8). However, the leucine residues are not in register with a heptad structure necessary for protein–protein interaction of the coiled-coil type. Secondary structure predictions by the PHD algorithm (Rost, 1996) suggest that the firs 45 amino acids of SAF-A could form two α-helices, separated by a turn (Figure 8B). Based on this prediction, we have tested whether structural changes in this region would interfere with DNA binding. Three mutated partial proteins were engineered by deleting or inserting amino acids at the point where the turn is predicted. In contrast to the wild-type N247 construct, none of these mutated forms had detectable DNA-binding activity (Figure 8A). Thus, we conclude that the integrity of the spaced leucine motif is essential for specifi DNA binding.

Discussion

In the present study, we have investigated the fate of SAF-A in apoptosis. We demonstrate that SAF-A is cleaved *in vivo* at the time of chromatin collapse and nuclear fragmentation. Cleavage results in a loss of DNA-binding activity and a detachment of the cleaved protein from nuclear substructures. Analysis of the protein in the area of cleavage revealed a novel, bipartite DNA-binding domain with high specificit towards SAR DNA. The present work provides a framework for the development of a functional test to study the role of SAF-A in nuclear architecture and apoptotic nuclear breakdown.

Cleavage of SAF-A in apoptosis

When cells progress through apoptosis, a collapse of the internal nuclear structure can be seen as a transition from a textured interphase nucleus to apparently structureless, very dense chromatin spheres. Unlike the condensation of chromatin observed in mitosis due to gain of higher order structure in chromosomes, collapse of chromatin in apoptosis is most likely caused by a loss of structure (Weaver *et al.*, 1996). Therefore, proteins involved in maintaining the normal interphase structure of chromatin are probably critical targets for apoptotic proteases. One of these proteins could be SAF-A, as it is tightly bound both to the nuclear scaffold and to SAR DNA in non-apoptotic cells. Indeed, SAF-A appears to be the most abundant SAR-binding protein in nuclear scaffold preparations (Mattern *et al.*, 1996, 1997). Since the proteinaceous framework of the nuclear scaffold is probably held together by protein–protein interactions, cleavage of only a few components such as SAF-A might be sufficien for its disintegration. It is, therefore, conceivable that apoptotic cleavage of SAF-A and the accompanying loss of binding to SAR DNA is an event that facilitates nuclear breakdown. As judged from the time course of SAF-A cleavage, and its inhibition by zinc, cleavage of SAF-A occurs after formation of high molecular weight DNA fragments but before DNA laddering. Thus, it appears that SAF-A cleavage is linked to critical alterations in nuclear chromatin that occur prior to endonuclease cleavage of DNA into nucleosomal fragments (Sun *et al.*, 1994).

On the other hand, SAF-A is not the only SAR-binding protein in human nuclei, and it is certainly not warranted to ascribe all morphological changes observed in nuclei to SAF-A cleavage. It is perfectly possible that cleavage or degradation of structurally important proteins of the nuclear scaffold may be a general phenomenon in apoptosis. Interestingly, two other SAR-binding proteins have also been shown to be targets of caspases during apoptosis, namely the lamins (Lazebnik *et al.*, 1995) and NuMA (Hsu and Yeh, 1996; Weaver *et al.*, 1996). A possible influenc of the cleavage of these proteins on their DNA-binding properties has not yet been investigated, although, like SAF-A, cleaved NuMA is clearly separated from collapsed chromatin in the nucleoplasm of apoptotic thymocytes.

In this context, one further protein deserves attention, namely DNA topoisomerase II. Apart from its enzymatic activity involved in many aspects of nucleic acid metabolism, topoisomerase II is a structural component of chromatin at all stages of the cell cycle. In interphase it is a component of the nuclear scaffold, while in mitosis it is found in the backbone of metaphase chromosomes, associated with a helically coiled scaffold with mirror symmetry between sister chromatids (Boy de la Tour and Laemmli, 1988). An ordered line-up of AT-rich sequences, presumably SARs, appears to follow the path dictated by this scaffold (Saitoh and Laemmli, 1994), and the binding specificit of topoisomerase II to SAR DNA has clearly

been demonstrated (Adachi *et al.*, 1989; Käs and Laemmli, 1992). Considering its structural role, it is interesting to note that topoisomerase II is also attacked during apoptosis. However, unlike the lamins, NuMA or SAF-A, topoisomerase II is apparently degraded by the ubiquitin proteolysis system rather than being cleaved by a caspase (Nakajima *et al.*, 1996). Certainly, a complete degradation of topoisomerase II will also contribute to the destruction of nuclear scaffold structure in apoptotic cells.

What could be the structural consequence of a disintegration of the nuclear scaffold and concomitant loss of chromatin attachment? A possible answer might come from experiments aimed at definin the role of SARs in chromosome structure (Strick and Laemmli, 1995). In these investigations, an artificia SAR 'superbinder' protein, MATH20, was used to block the interactions between SARs and their natural binding proteins in mitotic *Xenopus laevis* egg extracts. When MATH20 was added to preformed chromatids, structures resembling the chromatin spheres in apoptotic cells were observed, suggesting that the disruption of natural SAR–protein interactions leads to a reorganization of chromatin. In this case, where mitotic processes were studied, reorganization is an active, energy-dependent process possibly brought about by misguided chromosome condensation. Thus, the authors conclude that displacement of SAR-binding proteins to non-SAR locations could disrupt the SAR-mediated polymerization process of chromosome scaffold assembly, resulting in illegitimate cross-tie formation. It is not clear presently whether the similarity between the structures observed in the study of Strick and Laemmli (1995) and the structure of apoptotic nuclei is merely incidential or reflect a common mechanism. However, it is intriguing to speculate that the collapse of chromatin in apoptosis may also be initiated by a disruption of interactions between SARs and their binding proteins. Collapse of chromatin would then be a non-specifi chromatin precipitation rather than an active condensation process (that appears unlikely because of the simultaneous degradation of DNA).

The novel SAR-binding domain in SAF-A

We previously had concluded from filte binding experiments with purifie SAF-A that two independent nucleic acid-binding domains are present on the protein (Fackelmayer *et al.*, 1994). Clearly, one of these domains is the RGG box described by Kiledjian and Dreyfuss (1992) as an RNA-binding motif. Guided by the observation that proteolytic removal of the amino-terminus of SAF-A abolishes its DNA binding, we have now mapped the second domain in the firs 247 amino acids of the protein. By comparison with the activity of purified full-length SAF-A, we can safely conclude that this domain is the one that carries the SAR-specifi DNA-binding activity originally described by our laboratory (Romig *et al.*, 1992). This novel domain is separated from the RGG box by >440 amino acids, and both domains act independently of each other, as shown by our experiments with artificia constructs from either the amino- or the carboxy-terminus of SAF-A (Figure 6). Consequently, binding of SAF-A to hnRNP particles (and presumably to RNA) is not affected by apoptotic cleavage, as shown in Figure 4.

As demonstrated above, the amino-terminal domain is composed of two regions, a 'spaced leucine motif' (aa 1–45) and a less well define glutamine-rich region (aa 158–247), both of which are indispensible for DNA-binding activity. The sequence between both regions is very rich in acidic residues that are most likely not involved in DNA binding because of charge repulsion. For a firs functional characterization of the 'spaced leucine motif', we have introduced insertion and deletion mutations into the region between amino acids 20 and 34. Analysis of the modifie proteins for their DNA binding revealed that none of our constructs retains the DNA binding of the wild-type motif, suggesting that the integrity of the motif is essential for the activity. As the spaced leucine motif is reasonably similar to a leucine zipper, we have also tested whether it is a dimerization motif. Although we found low amounts of dimers of constructs carrying this motif, we remained unconvinced by this result because it was not influence by the described mutations. Interestingly, the 'spaced leucine motif' in the firs 45 amino acids of SAF-A is highly conserved between man and *X.laevis*, but database comparisons reveal no homology to any other known protein (unpublished). Thus, this region most likely serves an essential function unique to SAF-A.

Interestingly, when the isolated amino-terminus of SAF-A is investigated for DNA binding, a strong binding is only observed with immobilized protein, either on a membrane (as in Southwestern blots, compare Figure 5) or on Sepharose beads (as in the experiment of Figure 6D). If, in turn, the same protein is tested for binding in solution (Figure 5B), only weak binding to DNA is detected. This result was confirme further in filte binding and band shift experiments (not shown). A possible explanation for this effect could be that protein–protein interactions are necessary for a strong and specifi binding. It is conceivable that a partial protein that has lost the ability to form protein–protein interactions is then unable to bind to DNA in solution, but would regain activity when the attachment to a substratum mimicks these interactions by bringing the individual protein molecules into close proximity. This is interesting in the light of the peculiarities in binding of SARs to their cognate binding proteins. As Bode *et al.* (1996) recently have pointed out, many aspects of this binding are readily explained by the 'mass binding phenomenon' of Zuckerkandl and Villet (1988). Briefl , their conclusion is that low affinit binding of individual protein molecules to DNA with binding constants not far above those for non-specifi association can be multiplied to generate high specificit binding through formation of specifi protein–protein interactions.

In the context of our studies, this means that SAR binding by full-length SAF-A might be exerted by protein multimers, as characterized previously in biochemical assays (Fackelmayer and Richter, 1994; Fackelmayer *et al.*, 1994), or, *in vivo*, by arrays of SAF-A on hnRNP particles covering primary transcripts along their length. Although this is certainly a speculative hypothesis, and much future work will be necessary to test it, we fin it holds promise in resolving the presently unclear relationship between RNA metabolism and nuclear architecture.

Materials and methods

Cell culture and apoptosis induction

Jurkat cells were grown in RPMI1640 medium containing 10% fetal calf serum at 37°C in a humidifie atmosphere, and passaged every

3 days by 5-fold dilution into fresh medium. For induction of apoptosis, Jurkat cells were pelleted for 5 min at 190 *g* and washed twice in serum-free medium before addition of anti-CD95 antibody (CH-11, Immunotech, fina concentration 100 ng/ml). Parallel induction for apoptosis or necrosis was done as described previously by Leist *et al.* (1997). For protease inhibitor assays, cells were pre-incubated with the inhibitor [100 μM for all inhibitors except z-VAD-fmk (10 μM) and DEVD-CHO (200 μM)] for 30 min before antibody addition, and cells were analysed 3 h after induction.

Digitonin fractionation

Jurkat cells were induced for apoptosis for 3 h, washed twice in cold phosphate-buffered saline (PBS), pelleted for 5 min at 190 *g* and gently resuspended in PBS containing 10 μg/ml digitonin. After 5 min on ice, cells were centrifuged again to separate soluble proteins (supernatant) and structurally bound proteins (pellet). Proteins from all fractions were purifie according to Wessel and Flügge (1984), before analysis by Western blotting.

Immunofluorescence

Jurkat cells were dispensed into 6-well dishes containing coverslips. Apoptosis was induced as described above, and cells were incubated at 37°C for different times before processing for immunofluorescence Images of early, intermediate and late apoptotic cells were taken after 45, 90 and 240 min, respectively, and represent the predominant population of cells at these time points. Cells were washed twice in PBS, fixe for 10 min at 22°C with 3.5% formaldehyde in PBS and permeabilized with 0.3% Triton X-100 in PBS for 3 min. Non-specifi binding sites were blocked by incubation with 3% bovine serum albumin (BSA) in PBS for 1 h at 22°C, before affinity-purifi polyclonal antibodies (100 ng/ml) were added for 1 h. Cells were washed fiv times in PBS, and secondary antibody (anti-rabbit–Texas red conjugate, Molecular Probes, 1:300 dilution in PBS) was added for 30 min. After fiv washes in PBS, cells were embedded in PBS containing 50% glycerol and 1 ng/ml Hoechst 33258.

Immunoprecipitation of hnRNP particles

For the experiment in Figure 4, cells were harvested by centrifugation, resuspended in lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100) and disrupted by sonication. The turbid lysate was layered over a 30% sucrose cushion in lysis buffer and centrifuged at 4000 *g* for 15 min. Immunoprecipitations were performed with aliquots of the supernatant (250 μg total protein per assay), 1 μl of ascites flui 4F4 (anti hnRNP-C) and 25 μl of protein A–Sepharose, in a total volume of 500 μl for 30 min at 4°C. Immunoprecipitations were washed six times with 1 ml of lysis buffer, before precipitated proteins were eluted by boiling in SDS–PAGE sample buffer.

Cloning, expression and purification of recombinant protein fragments

Fragments of the cDNA encoding SAF-A were cloned into the expression vectors pRSET (Invitrogen) or pEZZ18 (Pharmacia). Constructs for the proteins I541 and C399 were made by using the natural restriction fragments *Sac*I–*Msc*I and *Bgl*II–*Hin*dIII, respectively. All other constructs were cloned after PCR amplificatio of cDNA fragments by using primer-incorporated restriction sites (*Eco*RI–*Bam*HI for all clones except N290, for which *Eco*RI–*Hin*dIII was used) and proofreading *Pfu* polymerase (Stratagene). All clones were verifie by sequencing. For protein expression of constructs in pEZZ18, recombinant *E.coli* were grown in LB containing 0.2 mM $ZnSO_4$ for 30 h. Proteins were purifie from the culture medium by chromatography over IgG–Sepharose and Mono Q. Briefl , 500 ml of medium was passed over 3 ml of IgG–Sepharose (Pharmacia), followed by 200 ml of washing buffer (50 mM Tris, 150 mM NaCl, 10 mM $Na_2S_2O_5$, 5 mM EDTA, 0.05% Tween-20, pH 7.5) and 20 ml of water. Proteins were eluted with 7.5 ml of 500 mM ammonium acetate buffer (pH 3.4), immediately desalted into 10 mM Tris–HCl (pH 8.0) by gel filtratio on PD10 columns and applied to a HR5/5 Mono Q column. Proteins were eluted with a linear gradient from 0 to 1 M NaCl in 10 mM Tris (pH 8.0). Proteins, eluting between 100 and 500 mM NaCl, were diluted with an equal volume of glycerol and stored at –20°C.

For protein expression of constructs in pRSET, recombinant *E.coli* were induced at an OD_{600} = 0.8 with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 3 h, before proteins were purifie by metal chelate chromatography over Ni-NTA–agarose (Qiagen) as recommended by the manufacturer.

DNA-binding assays

Southwestern assays were performed as described previously (Romig *et al.*, 1992). For assays with *in vitro* translated proteins (synthesized using the TNT-coupled *in vitro* transcription–translation system (Promega) in the presence of [^{35}S]methionine), aliquots containing 50 000 c.p.m. of labelled protein were mixed with immobilized DNA (dsDNA or ssDNA cellulose, Sigma) in 400 μl of binding buffer (10 mM Tris, 80 mM NaCl, 2 mM EDTA, pH 8.0). After gently mixing for 30 min at 22°C, the cellulose material was pelleted in a microcentrifuge, and washed six times with 1 ml of binding buffer, before bound proteins were eluted in SDS–PAGE sample buffer. For binding assays with immobilized protein, 1 μg of recombinant N247 was bound to 50 μl of IgG–Sepharose in binding buffer (see above), washed six times and incubated with labelled DNA in the presence of unlabelled *E.coli* competitor DNA (assay volume 200 μl). After 30 min with gentle agitation, Sepharose beads were pelleted and washed as above, before the amount of bound DNA was quantifie by liquid scintillation counting.

Other methods

Field inversion and conventional agarose gel electrophoresis were performed as described by Leist *et al.* (1997). Briefl , cells at different stages of apoptosis were embedded in agarose plugs, and DNA was purifie by in-gel proteinase K digestion. During digestion, short (oligo-nucleosomal) DNA fragments diffuse out of the agarose plugs, are recovered from the supernatant of the digest and analysed by conventional agarose gel electrophoresis. Long DNA fragments and uncut genomic DNA remain in the agarose plugs that are then used for pulse-fiel electrophoresis. SDS–PAGE was performed as described by Laemmli (1970). Protein gels were stained with Coomassie brilliant blue (Sambrook *et al.*, 1989) or with Roti-White (Carl Roth, Karlsruhe). Western transfer was performed according to Towbin *et al.* (1979) with affinity-purifi antibodies (Harlow and Lane, 1988), peroxidase-coupled secondary antibody (Sigma) and enhanced chemiluminiscent detection (ECL, Amersham). Protein concentrations were determined using the Bio-Rad protein assay.

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