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# The 72-kDa Component of Signal Recognition Particle Is Cleaved during Apoptosis*

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**Proteins cleaved by apoptotic caspases are commonly recognized by autoantibodies found in the serum of patients with rheumatic disease. We report that the 72-kDa signal recognition particle (SRP) protein, a rare target of autoantibodies found in the serum of patients with dermatomyositis and systemic lupus erythematosus, is rapidly cleaved in Jurkat T cells treated with apoptotic (*i.e.* Fas ligation, treatment with γ or ultraviolet radiation, or co-culture with anisomycin or staurosporine) but not proliferative (CD3 cross-linking) stimuli. Cleavage of SRP 72 produces a 66-kDa amino-terminal fragment and a 6-kDa carboxyl-terminal fragment that is selectively phosphorylated on serine residues. Cleavage of SRP 72 is prevented by chemical and peptide caspase inhibitors, and by overexpression of bcl-2, an inhibitor of apoptotic cell death. Analysis of the carboxyl terminus of SRP 72 has identified a putative cleavage site (SELD/A) for group III caspases, and carboxyl-terminal serine residues that are highly conserved in phylogeny. Both serine phosphorylation and caspase cleavage of SRP 72 are observed in cells derived from human, dog, rat, and mouse. Canine SRP 72 is cleaved *in vitro* by recombinant caspase 3 but retains the ability to mediate transport of a signal peptide-containing protein into the endoplasmic reticulum lumen. The 72-kDa component of the SRP joins a growing list of autoantigens that undergo post-translational modifications during programmed cell death.**

Proteins modified by the proteases and kinases that are activated during apoptosis are often involved in both the execution phase of cell death and in the development of autoantibodies in patients with systemic lupus erythematosus and mixed connective tissue disease (reviewed in Ref. 1). For example, at least 17 proteins that are known to be cleaved by caspases during apoptosis are autoantigens, including the 70-kDa component of the U1-small nuclear ribonuclear protein complex (U1–70 kDa) (2), poly(A) ribose polymerase (3), DNA-dependent protein kinase (DNA-PK) (4), hnRNP C1 and C2 (5), lamins A, B, and C (6), the nuclear mitotic apparatus protein (NuMA) (7, 8), topoisomerases 1 and 2 (8), the nucleolar protein UBF/NOR-90 (8), and α-fodrin (9, 10) (reviewed in Ref. 1). In addition, phosphorylated serine/arginine splicing factors have recently been shown to specifically associate with the U1-small nuclear RNP autoantigen complex during apoptosis (11, 12). These observations have led to the hypothesis that proteins modified during apoptosis can be presented to the immune system in a way that bypasses tolerance to self proteins. Although the molecular mechanisms by which this occurs are not known, the data suggests that patient-derived autoantisera may be useful in the identification of proteins that contribute to the execution phase of apoptosis.

While screening a panel of human autoantisera for their ability to precipitate new phosphoproteins from apoptotic Jurkat cell lysates, we serendipitously identified several sera that precipitated phosphoproteins from extracts prepared from untreated Jurkat cells that were no longer observed when extracts were prepared from apoptotic Jurkat cells. One of these phosphorylated autoantigens has been identified as the 72-kDa component of the signal recognition particle (SRP).[1] SRP is a ribonucleoprotein complex comprising the 7 S RNA in association with six distinct polypeptides. SRP functions to recognize the signal peptide of nascent transcripts, attach the translating ribosome to the endoplasmic reticulum (ER), and facilitate translocation into the ER lumen. The 72-kDa SRP protein is essential for protein translocation. In this report we demonstrate that SRP 72 is constitutively phosphorylated on serine residues. In Jurkat cells subject to apoptotic stimuli, SRP 72 is cleaved by caspases to liberate a 6-kDa carboxyl-terminal phosphopeptide. Our results suggest that phosphorylation and caspase cleavage might regulate translocation of secretory proteins into the ER lumen during apoptosis.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—Jurkat cells were grown in 5% $CO_2$ at 37 °C using RPMI 1640 (Bio Whittaker, Walkersville, MD) supplemented with 9% heat-inactivated fetal calf serum (HI-FCS) (Tissue Culture Biologicals,

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*The human SRP nucleotide sequence reported in this paper has been deposited in the GenBank™/EBI Data Bank with accession number AF069765.*

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[1] The abbreviations used are: SRP, signal recognition particle; ER, endoplasmic reticulum; HI-FCS, heat-inactivated fetal calf serum; PAGE, polyacrylamide gel electrophoresis; SAP, shrimp alkaline phosphatase.

Tulare, CA) and penicillin and streptomycin (Mediatech, Inc., Herndon, VA). Jurkat cells were grown and harvested at mid-log phase. Rat basophilic leukemia cells, Madin-Darby canine kidney cells, mouse primary fibroblasts, guinea pig A104 cells, and monkey COS cells were grown in Dulbecco's modified Eagle's medium (Bio Whittaker) supplemented with 9% HI-FCS (Tissue Culture Biologicals) and penicillin and streptomycin (Mediatech, Inc., Herndon, VA). Adherent cells were plated the night prior to each experiment such that cells were at 50–80% confluence on the day of the experiment. Jurkat T cells overexpressing bcl-2 (or empty vector), a kind gift from John Reed (the La Jolla Cancer Research Foundation, La Jolla, CA) were grown in RPMI medium as described above, supplemented with G418 (Life Technologies, Inc., Gaithersburg, MD) at a final concentration of 500 $\mu$g/ml. Protein overexpression was confirmed by Western blotting.

*Metabolic Labeling*—Jurkat cells were incubated at a density of $2 \times 10^6$ cells/ml in labeling medium containing the following: 45% RPMI 1640, 45% RPMI 1640 lacking either phosphate (Life Technologies, Inc., Grand Island, NY), or methionine and cysteine (Life Technologies, Inc., Grand Island, NY), 2 mM glutamine (Mediatech Inc.), 5% HI-FCS, and 5% HI-FCS that had been dialyzed to equilibrium against 10 mM Hepes buffer (Sigma). $^{32}$P-Labeled orthophosphate or $^{35}$S-labeled methionine and cysteine (NEN Life Science Prodcts, Boston MA) were added at a concentration of 0.1 mCi/ml. Cells were incubated at 37 °C for 10–16 h to allow the cells to reach steady-state before each treatment, unless otherwise indicated. Prior to killing, adherent cells were labeled for 2 h at 80% confluence in 90% RPMI lacking either phosphate or methionine and cysteine, 2 mM glutamine, 9% dialyzed HI-FCS, and radionuclide at a final concentration of 0.15 mCi/ml. Cells were harvested at the end of the time course to ensure equal labeling for each treatment.

*Cell Lysis*—Jurkat cells were solubilized in Nonidet P-40 (Sigma) lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.8, 1 mM EDTA). Nonidet P-40 lysis buffer was supplemented immediately before use with 1 mM sodium vanadate (Sigma) and a 100 × protease inhibitor mixture containing chymostatin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, benzamidine, and aprotonin, prepared as described (12, 13). All chemicals were purchased from Sigma. After addition of lysis buffer, cells were incubated on ice for 30 min, centrifuged in a refrigerated Microfuge (5402; Eppendorf, Hamburg, Germany) at 14,000 rpm for 15 min, and the supernatant used immediately for each experiment. Adherent cells were trypsinized, washed with phosphate-buffered saline, and lysed as above. Identical results were obtained in other experiments performed by scraping cells from plates in the absence of trypsin.

*Ultraviolet (UV) Irradiation*—Radiolabeled Jurkat cells were plated in 100 × 15-mm polystyrene Petri dishes (Nunc, Thousand Oaks, CA) at a concentration of $2 \times 10^6$ cells/ml and irradiated in a Stratalinker 2400 (Stratagene, La Jolla, CA) at a distance of 9 cm for 12 s. After irradiation, cells were incubated at 37 °C for the indicated times prior to harvesting.

*Gamma Irradiation*—Labeled cells were placed in a 50-ml conical tube and irradiated at a dose of 3300 rad from a Cesium 137 source (Gammacell 1000; Nordion International, Kanata, Ontario, Canada). After irradiation, cells were placed in culture dishes at 37 °C and incubated for the indicated times prior to harvesting.

*Cellular Activation*—Radiolabeled Jurkat cells were treated with the following antibodies: anti-Fas antibody 7C11 (IgM; kindly provided by Michael Robertson, Indiana University, Bloomington, IN) was diluted from hybridoma supernatant (1:500, v:v); anti-CD3 antibody (Coulter Immunology, Hialeah, FL) was added at a concentration of 5 $\mu$g/ml followed by goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) at the same concentration. Cells were incubated at 37 °C for the indicated times prior to harvesting. For experiments utilizing caspase inhibitors, Jurkat cells were pretreated for 1 h at 37 °C: 10 $\mu$M YVAD-fmk (Kamiya Biomedical Co., Tukwila, WA), 10 $\mu$M ZEVD-fmk (Kamiya Biomedical Co., Tukwila, WA), 2 mM zinc sulfate (Sigma) or 1 mM iodoacetimide (Sigma). After 1 h, apoptosis was induced by the addition of 7C11, and the cells were processed as described above. Jurkat or adherent cells were treated with 10 $\mu$g/ml anisomycin or 2 $\mu$M staurosporine (Sigma) for varying times prior to harvesting.

*Immunoprecipitation and Western Blot Analysis*—Lysates were precleared once with 25 $\mu$l of a 50% solution of Protein A-Sepharose (Pharmacia, Uppsala, Sweden) in phosphate-buffered saline and 5 $\mu$g of rabbit anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h, followed by two preclears with Protein A-Sepharose overnight. Mouse monoclonal antibodies (5 $\mu$g) and 5 $\mu$g of rabbit anti-mouse IgG, or 1 $\mu$l of patient serum alone was used in precipitation experiments. Human polyclonal antibodies were obtained from the following sources and stored at −70 °C until used: T. Medsger and N. Fertig, University of Pittsburgh School of Medicine, Pittsburgh, PA, anti-SRP 54; J. Craft, Yale University School of Medicine, New Haven, CT, anti-SRP 54; anti-SRP sera M195, D175, and J141 were obtained from the University of Nijmegen Rheumatology Clinic (W. J. van Venrooij). Rabbit antisera raised against synthetic peptides derived from canine SRP 68 and SRP 72 were kindly provided by H. Lütcke and B. Dobberstein and have been described previously (14). Immunoprecipitations were performed in Nonidet P-40 lysis buffer in a total volume of 500 $\mu$l, and rotation in a 4 °C cold room for 2–24 h. Precipitates were harvested by centrifuging for 15 s at 14,000 rpm in a refrigerated Eppendorf microfuge, washing 3 times with Nonidet P-40 lysis buffer, resuspending in SDS loading buffer with 9% 2-mercaptoethanol, boiling for 5 min, and separating by SDS-PAGE as described (15). Proteins were transferred to nitrocellulose (Schleicher and Scheull, Keene, NH) for Western blotting experiments or to polyvinylidene difluoride (NEN Life Science Products) for phosphoamino acid analysis, and either exposed for autoradiography or subjected to Western blot analysis as indicated. The mouse monoclonal antibody 4D7, anti-bcl-2 (Pharmingen, San Diego, CA), was used for blotting studies at a dilution of 1:1000. Anti-SRP antibodies were used at a final concentration of 1:500. Nitrocellulose blots were blocked with 5% Blotto (Bio-Rad, Inc., Hercules, CA) in phosphate-buffered saline overnight at 4 °C. Bands were visualized using species-specific antibody conjugated to horseradish peroxidase (Amersham) at a dilution of 1:7500 in 5% Blotto in phosphate-buffered saline, and developed using ECL chemiluminescence performed according to the manufacturer's instructions (Amersham). Identical results were obtained in experiments using species-specific antibody conjugated to alkaline phosphatase and developing using colorimetric substrate. For experiments using RNase A (Sigma) or shrimp alkaline phosphatase (SAP, U. S. Biochemical Corp., Cleveland, OH) immunoprecipitates were washed 3 times with Nonidet P-40 lysis buffer and resuspended in 100 $\mu$M RNase A in Nonidet P-40 lysis buffer, or in 1 unit of SAP in 50 $\mu$l of SAP buffer provided by the manufacturer, respectively. As a control, some immunoprecipitates were also treated with SAP buffer alone at 37 °C for 30 min.

*Phosphoamino Acid Analysis*—Immunoprecipitates that had been electrophoresed and transferred to polyvinylidene difluoride were rinsed thoroughly with water, exposed for radiography, and bands corresponding to phospho-SRP 72 were excised with a razor blade. The radiolabeled bands were then subjected to acid hydrolysis and phosphoamino acids analyzed as described with the exception that two-dimensional electrophoresis was performed at 14 °C (16).

*DNA Fragmentation*—Unlabeled Jurkat or HeLa cells were induced to undergo apoptosis using the above triggers in parallel experiments to those using radiolabeled cells. Cells were collected at the indicated times and centrifuged for 5 min at 1,000 rpm. The cell pellet was solubilized in 500 $\mu$l of DNA lysis buffer (20 mM Tris, pH 7.4, 5 mM EDTA, and 0.4% Triton X-100) and incubated on ice for 15 min, mixing several times. After centrifuging at 4 °C, 14,000 rpm for 5 min, supernatants were extracted with a phenol/chloroform/isoamyl alcohol (25:24:1) mixture (Life Technologies, Inc., Grand Island, NY). Next, 100 $\mu$l of 5 M NaCl and 500 $\mu$l of isopropyl alcohol were added to each tube prior to incubating overnight at −70 °C. Samples were thawed and centrifuged at 14,000 rpm for 5 min, washed once with 70% ethanol, and dried in a Speed-Vac. Pellets were resuspended in 30 $\mu$l of Tris-EDTA buffer containing 0.1 mg/ml RNase A (Sigma) and incubated at 37 °C for 30 min. After the addition of 10 $\mu$l of loading buffer, 10 $\mu$l of each sample, corresponding to 1 million cells per lane, was separated on 0.8% agarose gels and visualized by ethidium bromide staining under UV light. A molecular size marker (1-kilobase ladder, Life Technologies, Inc.) was included on each gel.

*In Vitro Caspase Cleavage and ER Transport Assay*—Canine microsomes (Promega Inc., Madison, WI) were incubated in caspase cleavage buffer with recombinant caspases (caspases 1, 2, 3, 8, and 9, or a control bacterial lysate) for 60 min at 30 °C as described (44). cDNAs encoding individual caspases were a gift of H. Li and J. Yuan, Harvard Medical School. Recombinant caspases were prepared as described and frozen at −70 °C until used (44). In a separate reaction, RNA encoding $\beta$-lactamase was *in vitro* translated in rabbit reticulocyte lysate (Promega, Inc., Madison, WI) at 30 °C for 30 min according to the manufacturer's instructions, prior to incubation with untreated or caspase-treated microsomes for an additional hour. The mixture was then separated by SDS-PAGE, transferred to nitrocellulose, and exposed for autoradiography. To ensure that the caspases were active, each was incubated with *in vitro* translated proteins that had previously been shown to be good substrates, including p35 (a gift of V. Shifrin, Scriptgen, Inc., Medford, MA), lamin A, pro-caspase 2 (a gift of H. Li and J. Yuan, Harvard Medical School), and interleukin-1$\beta$ (a gift of H. Li and J. Yuan, Harvard Medical School).

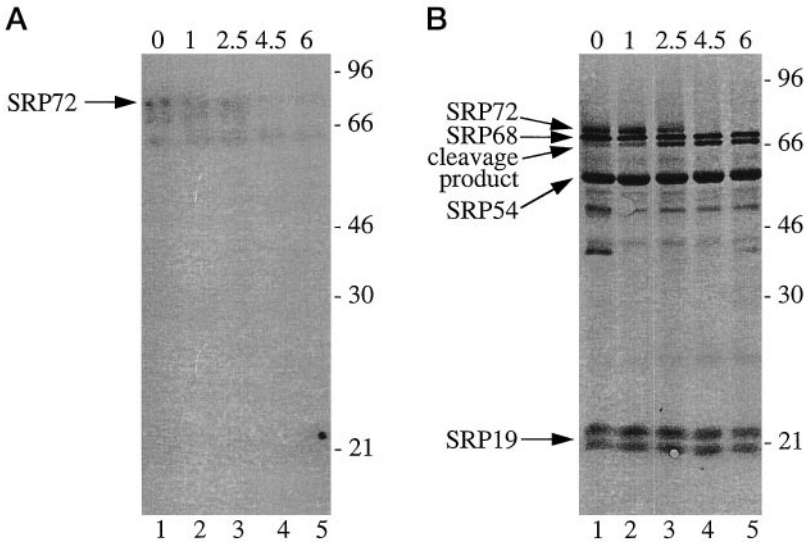


FIG. 1. **SRP 72 is a phosphoprotein that is rapidly cleaved during Fas-mediated apoptosis in Jurkat T cells.** Jurkat cells were labeled with [$^{32}$P]orthophosphate (*Panel A*) or with [$^{35}$S]methionine and cysteine (*Panel B*), treated with the anti-Fas monoclonal antibody 7C11 for the indicated times, and lysed after 6 h. Proteins were then precipitated using human anti-SRP autoantiserum JB, separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and exposed for autoradiography. The time, in hours, from initial exposure to 7C11 is indicated at the *top* of each lane. The relative migration of molecular size markers in kilodaltons is indicated on the *right side* of the panel. Components of the SRP, and the SRP 72 cleavage product, are shown on the *left side* of each panel. Lanes are numbered at the *bottom* of each panel.

## RESULTS

*SRP 72 Is a Phosphoprotein That Is Rapidly Cleaved during Fas-mediated Apoptosis in Jurkat T Cells*—We recently identified 8 phosphoproteins that are specifically precipitated from apoptotic Jurkat cell lysates using serum from patients with systemic lupus erythematosus or with monoclonal antibodies directed against the U1-small nuclear RNP complex (11, 12). While screening other human autoimmune sera for their ability to precipitate novel phosphoproteins from $^{32}$P-labeled apoptotic Jurkat cell extracts, we noted that serum derived from a patient with dermatomyositis precipitated a 72-kDa phosphoprotein from untreated Jurkat cell lysates that was no longer observed in precipitates from apoptotic Jurkat cell extracts. This serum (serum JB) had previously been shown to uniquely recognize the 54-kDa component of SRP (17). Jurkat cells metabolically labeled with [$^{32}$P]orthophosphate were cultured over a 6-h period in the absence or presence of a monoclonal antibody reactive with Fas (anti-7C11), solubilized in Nonidet P-40 lysis buffer, and immunoprecipitated using the human anti-SRP serum. Immunoprecipitates were separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and subjected to autoradiography. As shown in Fig. 1*A*, a diffuse band is observed between 70 and 80 kDa (*lanes 1–3*) that disappears from cells treated with a mAb reactive with Fas (*lanes 4* and *5*). The intensity of the band varied markedly based on labeling conditions (*e.g.* see Fig. 2). To determine whether this polyclonal antiserum was capable of precipitating all components of the SRP complex, the experiment was repeated in cells labeled with [$^{35}$S]methionine and cysteine (Fig. 1*B*). This time course reveals the disappearance of a diffuse ~72-kDa band and the concomitant appearance of a band migrating at ~66 kDa. The intensity of the bands corresponding to other SRP components (*e.g.* SRP 68, SRP 54, or SRP 19, *left arrows*) does not significantly change over the course of this experiment. This result suggests that the 72-kDa component of SRP 72 is either cleaved or dephosphorylated during Fas-mediated apoptosis.

Although a shift of 6 kDa in the electrophoretic mobility of a protein due to dephosphorylation would be unusual, the experiment shown in Fig. 2 was performed to address this possibility. Jurkat cells were metabolically labeled with [$^{35}$S]methionine and cysteine (*lanes 1–6*) or [$^{32}$P]orthophosphate (*lanes 7–13*). Cells were cultured for 6 h in the absence (−) or presence (+) of a monoclonal antibody reactive with Fas (anti-7C11) and solubilized in Nonidet P-40 lysis buffer. Following immunoprecipitation using human anti-SRP, the precipitates were incubated at 37 °C in the absence (*lanes 3, 4, 9*, and *10*), or presence (*lanes 5, 6, 11*, and *12*) of SAP or with RNase A (*lane 13*). Immunoprecipitates were washed and separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and subjected to autoradiograpy. Samples labeled with $^{32}$P were not detectable by autoradiography following treatment with SAP (*lanes 11* and *12*). SAP treatment also reduced the heterogeneity of the 72-kDa protein precipitated from $^{35}$S-labeled cells (*lane 5*). Incubation at 37 °C in the absence of SAP (*lanes 3, 4, 9,* and *10*) or in the presence of RNase (*lane 13*) did not affect the migration of SRP 72, suggesting that the 66-kDa band observed in immunoprecipitates prepared from apoptotic cell extracts is the result of proteolytic cleavage rather than dephosphorylation of SRP 72. The expected 6-kDa fragment was not detected in these experiments, nor was it detected following separation on 20% SDS-PAGE gels (data not shown). Phosphoamino acid analysis of the phosphorylated 72-kDa band (Fig. 2*B*) demonstrates exclusive phosphorylation on serine residues. Taken together, these results suggest that SRP 72 is a serine phosphoprotein that is cleaved during Fas-mediated apoptosis, and that the phosphorylation site is likely to reside within the 6-kDa cleavage product, which is no longer detectable in the immunoprecipitated SRP complex.

*The Carboxyl Terminus of Human SRP 72 Is Cleaved during Fas-mediated Apoptosis*—To confirm the results obtained by immunoprecipitation experiments, we performed Western blotting experiments on unlabeled Jurkat cell lysates prepared from untreated or anti-Fas treated cells. Jurkat cells were treated with anti-Fas monoclonal antibodies for varying times over a 5-h period prior to detergent lysis, separation by SDS-PAGE, and transfer to nitrocellulose. The transferred proteins were then individually blotted with antibodies directed against different epitopes of the canine SRP 72 protein (14). As shown in Fig. 3*A*, an antibody raised against synthetic peptides corresponding to amino acids 561–576 of canine SRP 72 (SRP 72-3, *left panel*) recognizes full-length human SRP 72 (*lane 1*); this band decreases in intensity and a 66-kDa cleavage product (*lanes 3–6*) is observed beginning at 2 h following addition of anti-Fas antibodies. Western blot analysis using an antibody raised against amino acids 657–671 of canine SRP 72 (Fig. 3*B, SRP 72-4, middle panel*) recognizes full-length human SRP 72, which disappears during Fas-mediated apoptosis. Unlike the results using antibody 72-3 (Fig. 3*A*), Western blot analysis using antibody 72-4 (Fig. 3*B*) does not detect the concomitant appearance of a 66-kDa band. The relative migration of SRP 68, in contrast, is unaffected over the course of this experiment (Fig. 3*C, right panel*). Analysis of the amino acid sequence of

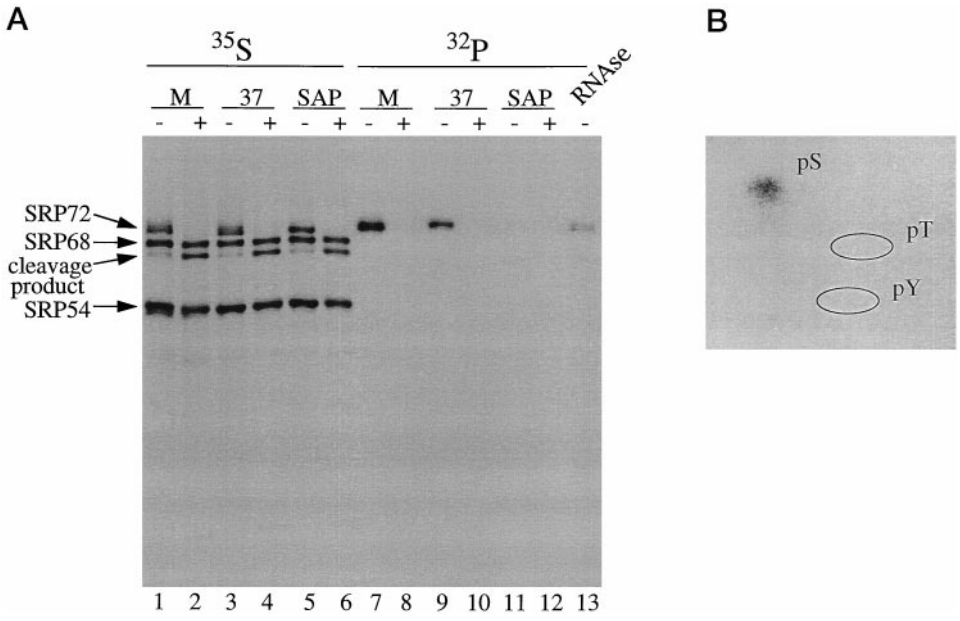


FIG. 2. **The phosphorylation site on SRP 72 is located in its carboxyl terminus.** *A,* Jurkat cells were labeled with [$^{35}$S]methionine and cysteine (*lanes 1–6*) or [$^{32}$P]orthophosphate (*lanes 7–13*). Cells were lysed either before (−) or 6 h after (+) the addition of 7C11 (anti-Fas) prior to immunoprecipitation using anti-SRP serum JB. The immunoprecipitates were washed and treated with *SAP*, SAP buffer at 37 °C (*37*), RNase A (*RNase*), or mock treatment (*M*). Immunoprecipitates were then washed again, separated on an 8% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride, and subjected to autoradiographic analysis. Components of the SRP and the SRP 72 cleavage product are shown on the *left side* of the panel. Lanes are numbered at the *bottom* of the panel. *B,* phosphoamino acid analysis of SRP 72. The band corresponding to SRP 72 (*lane 7*) was excised from the membrane and subjected to acid hydrolysis. Phosphoamino acids were separated by two-dimensional electrophoresis in pH 1.9 buffer in the horizontal dimension, followed by pH 3.5 buffer in the vertical dimension prior to autoradiographic analysis. Migration of phosphoamino acid standards are labeled with *circles* as follows: phosphoserine (*pS*), phosphothreonine (*pT*), phosphotyrosine (*pY*).

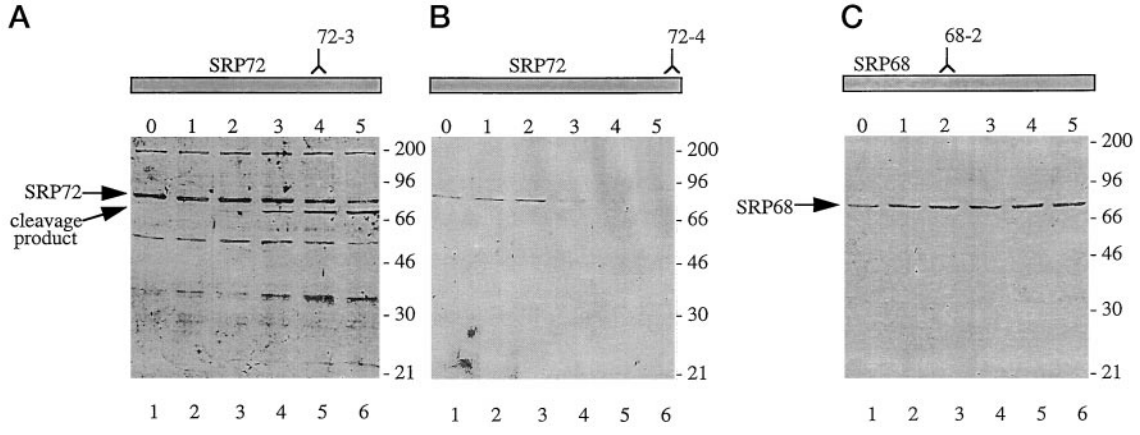


FIG. 3. **SRP 72 is cleaved at its carboxyl terminus.** Jurkat cells were incubated with 7C11 (anti-Fas) over a 5-h time period and harvested by Nonidet P-40 lysis at the times indicated at the *top* of each figure (in hours). Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using anti-SRP 72-3 (*Panel A*), anti-SRP 72-4 (*Panel B*), or anti-SRP 68-2 (*Panel C*). The relative migration of molecular size markers in kilodaltons is indicated on the *right side* of each panel. SRP 68, SRP 72, and the SRP 72 cleavage product are shown on the *left side* of the panel. Epitope mapping of each antibody as described previously (14) is shown *above* each panel (antibody symbols). The SRP 72 and SRP 68 molecules are depicted as a *bar*, with the amino terminus on the *left* and the carboxyl terminus on the *right*.

canine and human SRP 72 reveals a conserved candidate caspase cleavage site ($^{614}$SELD/A$^{618}$) at a position that is consistent with the result shown in Fig. 3 (Table I, see "Discussion"). This result demonstrates that human SRP 72 is specifically cleaved at the carboxyl terminus (between amino acids 576 and 671 in the corresponding human sequence) during Fas-mediated apoptosis, and further implies that the serine phosphorylation site is localized to a 6-kDa domain of the carboxyl terminus.

*SRP 72 Is Cleaved following Multiple Apoptotic Stimuli but Not following T Cell Receptor Stimulation*—To determine whether cleavage of SRP 72 is a general consequence of apoptosis, we determined the effect of several apoptotic stimuli on its relative migration in SDS-polyacrylamide gels (Fig. 4). Jurkat cells were treated in the absence (*lane 1, M*) or presence (*lane 2, F*) of anti-Fas mAb 7C11; the antibiotic anisomysin (*lane 3, A*); staurosporine, a broad-spectrum protein kinase inhibitor (*lane 4, S*) (18); γ-irradiation (*lane 5, X*); or ultraviolet irradiation (*lane 6, U*). Cells were harvested after 5 h and processed independently for protein (Fig. 4*A*) and DNA analysis (Fig. 4*B*). All 5 stimuli induced the formation of oligosomal DNA ladders (Fig. 4*B, lanes 2–6*) as compared with mock treated cells (Fig. 4*B, lane 1*) or cells treated with anti-CD3 (data not shown) (12). Similarly, SRP 72 is specifically cleaved following treatment with each apoptotic trigger (Fig. 4*A*) but not following CD3 stimulation (data not shown). γ-Irradiation, and to a lesser extent Fas stimulation, led to incomplete cleavage at this time point (Fig. 4*A, lanes 2* and *5*). Similar results

TABLE I

*Comparison of the carboxyl terminus of SRP 72 between several organisms*

The 3′ end of the gene encoding canine SRP 72 (14) was used to screen GenBank™ sequences using the FASTA program (40) at the GENESTREAM network server IGH Montpellier, France. Alignment of open reading frames corresponding to the retrieved DNA sequences was performed using LALIGN. Canine (GenBank™ accession number X67813) (14), *S. mansoni* (GenBank™ accession number L32975) (41), *C. elegans* (GenBank™ accession number U80840) (42), and *S. cerevisia* (L35178) (43) sequences have been reported previously. A mouse sequence from an expression sequence tag (EST) was found during this screen (GenBank™ accession number W08330), as were several human EST sequences. The human EST corresponding to GenBank™ accession number W95856 was used for alignment of the human SRP sequence, and was 100% identical to the 3′ end of the full-length human gene recently isolated in our laboratory (P. J. Utz, M. Hottelet, I. Miller, and P. Anderson, unpublished observations). This sequence has been deposited in GenBank™ (accession number AF069765). Eight conserved serine residues are labeled (*). Highly conserved serine residues are underlined. Potential caspase cleavage sites are labeled with bold print. The terminal amino acid for complete sequences is numbered on the right side of the table.

| | | |
|---|---|---|
| `GTQGATAGAS**SELDA**SKTVSSPPTSPRPGSAATVSASTSNIIPPRHQKPAGAPATKKKQQQKKKKGGKGGW` | 671 | Human |
| `GTQGATAGAS**SELDA**SKTVSSPPTSPRPGSAATASASTSNIIPPRHQKPAGAPATKKKQQQKKKKGGKGGW` | 671 | Canine |
| `GTQGATAGAS**SELDA**SKAVSSPPTSPRPGSAATISSSASNIVPPRHQKPAGAPATKKKQQQKKKKGGKSGW` | | Mouse |
| `GPQGQITGE-**SEWDA**A-IRSPKVKIPEDGSAGSTPKQMSN----------TAKQQQKKGRKKGR--` | 707 | *S. mansoni* |
| `GTQGSSSAN-PNVEYV-TASPNSPRPLPGPVAEGPRQQRP----------NFQKQKKKKNASKF-` | 694 | *C. elegans* |
| `-TQGGAMNKKSEQALD----------ISKKGKPTVNKKPKNKKKGRK--` | 646 | *S. cerevesia* |

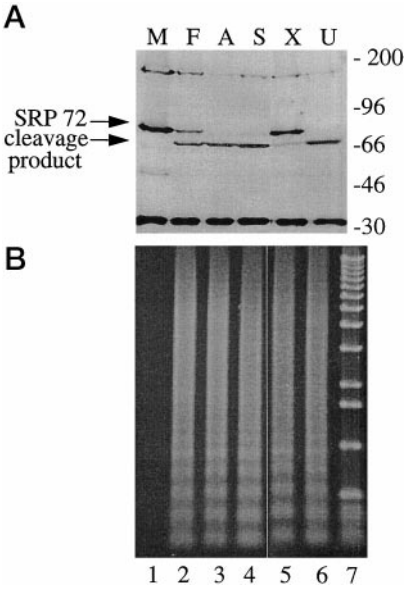


FIG. 4. **SRP 72 is cleaved following multiple different apoptotic stimuli.** Jurkat cells were subjected to various apoptotic stimuli and harvested for protein analysis by Nonidet P-40 lysis (*Panel A*) or for DNA analysis (*Panel B*) after 6 h. *A,* lysates were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using anti-SRP 72-3. The relative migration of molecular size markers in kilodaltons is indicated on the *right side* of the panel. SRP 72 and the SRP 72 cleavage product are shown on the *left side* of the panel. The stimulus is indicated *above* the panel as follows: Mock treatment (*M*), anti-Fas 7C11 (*F*), anisomycin (*A*), staurosporine (*S*), γ-irradiation (*X*), ultraviolet irradiation (*U*). *B,* DNA was separated on a 0.8% agarose gel and visualized by UV light following ethidium bromide staining. Size standards are shown in *lane 7*. Lanes are numbered at the *bottom* of the panel.

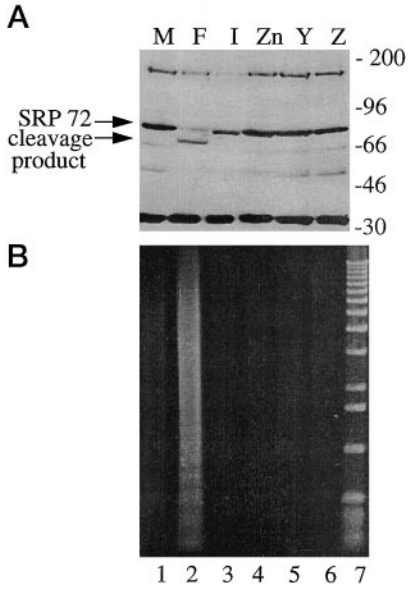


FIG. 5. **SRP 72 cleavage is inhibited by specific caspase inhibitors.** Jurkat cells were pretreated for 1 h with the indicated chemical or peptide, cultured with anti-Fas 7C11, and harvested 5 h later for protein analysis (*Panel A*) or for DNA analysis (*Panel B*). *A,* lysates were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using anti-SRP 72-3. The relative migration of molecular size markers in kilodaltons is indicated on the *right side* of the panel. SRP 72 and the SRP 72 cleavage product are shown on the *left side* of the panel. The stimulus is indicated *above* the panel as follows: mock treatment (*M*), anti-Fas 7C11 with no pretreatment (*F*), pretreatment with iodoacetamide (*I*), pretreatment with zinc sulfate (*Zn*), pretreatment with fmk-YVAD (*Y*), pretreatment with fmk-ZEVD (*Z*). *B,* DNA was separated on a 0.8% agarose gel and visualized by UV light following ethidium bromide staining. Size standards are shown in *lane 7*. Lanes are numbered at the *bottom* of the panel.

were obtained using the human autoimmune serum that precipitates the SRP complex (data not shown).

*Proteolytic Cleavage of SRP 72 Is Prevented by Caspase Inhibitors*—Protease inhibitors and specific caspase inhibitors were used to block the Fas-mediated cleavage of human SRP 72. As shown in Fig. 5, Jurkat cells were co-cultured with zinc sulfate (an inhibitor of caspases, particularly Mch2 α (caspase 6) (19)), iodoacetamide (a protease inhibitor that irreversibly blocks sulfhydryl groups (20)), YVAD (a soluble peptide inhibitor of ICE family proteases (21)), and DEVD (a soluble peptide inhibitor of CPP32 family proteases) (21), for 1 h prior to the addition of anti-Fas 7C11. Five hours later, cells were harvested and processed for DNA ladders (Fig. 5*B*) and protein (Fig. 5*A*). All 4 inhibitors effectively prevent the formation of DNA ladders (Fig. 5*B*), as well as the characteristic morphologic changes associated with cell death (data not shown). This correlates with inhibition of caspase-mediated cleavage of human SRP 72 (Fig. 5*A*), consistent with a role for apoptotic caspases in this event.

*Ectopic Expression of bcl-2 Inhibits UV-induced Apoptosis and Cleavage of SRP 72*—We next asked whether the cleavage of SRP 72 could be blocked by overexpression of the bcl-2 protein, which prevents apoptosis induced by multiple stimuli, including γ irradiation and UV irradiation (12, 22–25). As shown in Fig. 6, Jurkat T cells stably transfected with either empty vector (*left panels*) or bcl-2 (*right panels*) were subjected to UV irradiation. Cells were solubilized at the indicated times, lysates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-SRP 72-3. Whereas complete SRP 72 cleavage is rapidly induced in Jurkat (neo) control cells in response to UV irradiation (Fig. 6, *left panel*), the 66-kDa

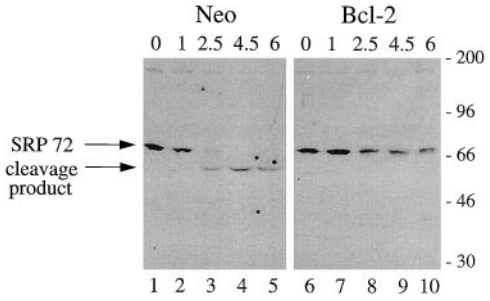


FIG. 6. **Cleavage of SRP 72 is inhibited in UV-irradiated Jurkat cells over-expressing bcl-2.** Jurkat (bcl-2) transformants (*right panels*) or Jurkat (neo) control transformants (*left panels*) were subjected to UV irradiation, cultured for the times indicated *above* each lane (in hours), solubilized in Nonidet P-40 lysis buffer, separated by SDS-PAGE, and transferred to nitrocellulose. The membrane was then blotted with anti-SRP 72-3. The relative migration of molecular size markers in kilodaltons is indicated on the *right side* of the panel. SRP 72 and the SRP 72 cleavage product are shown on the *left side* of the panel. Lanes are numbered at the *bottom* of each panel.

cleavage product is absent from Jurkat (bcl-2) transformants treated with this same stimulus (Fig. 6, *right panel*). Overexpression of bcl-2 effectively inhibited apoptosis in response to UV irradiation, as judged by the induction of DNA fragmentation (data not shown and Ref 12). Taken together, these results demonstrate that SRP 72 cleavage correlates with the induction of apoptosis, and is downstream of the inhibitory effects of bcl-2.

*SRP 72 Is Phosphorylated in Vivo, and Is Cleaved during Apoptosis, in Cells Derived from Multiple Different Mammals*—The amino acid sequence of SRP 72 is highly conserved in organisms ranging from human to yeast. To determine whether the protein is phosphorylated *in vivo* in cells derived from other organisms, we labeled cells with [$^{32}$P]orthophosphate, prepared lysates, and precipitated the SRP complex using a human autoantiserum specific for SRP 54 (serum J141). This serum was used because of the unavailability of serum JB (Figs. 1 and 2), which was derived from a patient who is now deceased. Serum J141 was characterized by immunoprecipitation of SRP from $^{35}$S-labeled extracts prepared from all 6 cell lines,[2] and by Northern blot analysis of RNAs precipitated by this antibody.[3] As shown in Fig. 7*A*, a band corresponding to phosphorylated SRP 72 is observed when immunoprecipitates are prepared from human (*lane 1*), dog (*lane 2*), rat (*lane 3*), and mouse (*lane 4*) cells. The corresponding band is absent when serum from a control patient is used to precipitate proteins from labeled Jurkat cells (*lane 5*). Similar results were obtained using 2 other SRP-specific human autoantisera in cells derived from human, dog, rat mouse, monkey, and guinea pig (data not shown). Phosphoamino acid analysis of the 72-kDa band from all 6 species demonstrates exclusive phosphorylation on serine residues, consistent with the result shown in Fig. 2*B* (data not shown). We also compared the migration of SRP 72 in cells from human, dog, rat, and mouse before and after treatment with anisomycin, a potent inducer of apoptosis (Fig. 7*B*). The induction of apoptosis was confirmed by DNA ladder analysis and by the appearance of typical morphologic changes in treated cells (data not shown). Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-SRP 72-3. SRP 72 cleavage was observed to some degree in all cells tested, confirming that SRP 72 is a highly conserved target of caspase(s) during apoptosis.

*Canine SRP 72 Is Cleaved in Vitro by Recombinant Caspase 3 but Retains the Ability to Mediate Transport of a Signal Peptide-containing Protein into the ER Lumen*—To determine whether caspase cleavage of SRP 72 disrupts the function of the particle, we first tested whether recombinant caspases could cleave SRP 72 *in vitro*. SRP 72 was not cleaved *in vitro* when the source of SRP was either Jurkat cell lysates or immunoprecipitates derived from Jurkat cell lysates (data not shown). However, incubation of purified canine microsomes (which are rich in SRP) with recombinant caspases resulted in near complete cleavage of SRP 72 by caspase 3, yielding the signature 66-kDa fragment (Fig. 8*A, lane 5*). None of the other caspases generated the 66-kDa fragment, although caspase 1 appeared to partially cleave the protein at a unique site, generating a 35-kDa product (Fig. 8*A, lane 3*). As expected, SRP 68 was not cleaved in this analysis (Fig. 8*B*). To determine whether cleavage of SRP 72 had an effect on ER transport, the experiment shown in Fig. 8*C* was performed. Untreated, caspase-treated (*lanes 1–3*), or bacterial lysate-treated (*lane 4*) canine microsomes were coincubated with *in vitro* translated $\beta$ lactamase, and the reaction mixture was analyzed by SDS-PAGE. Incubation of microsomes with caspase 1 (but not caspases 2 and 3) completely inhibited the ER transport of $\beta$ lactamase, as evidenced by the absence of processing of the 32-kDa precursor to the 30-kDa product by signal peptidase in the ER lumen. Similar treatment of rabbit reticulocyte lysate with recombinant caspases had no reproducible effect on ER transport in this assay (*lane 5* and data not shown). Taken together, these results demonstrate that SRP 72 cleavage does not grossly disrupt ER transport *in vitro*, and further suggests that caspase 1 cleaves an unidentified microsomal component that is required for ER transport.

## DISCUSSION

SRP, a highly conserved cytoplasmic complex composed of a 7 S structural RNA molecule and 6 polypeptides, mediates the targeting of secretory proteins to the endoplasmic reticulum (26, 27). The intact particle has at least three separable activities: (i) binding to newly synthesized proteins bearing signal sequence as they emerge from the ribosome; (ii) elongation arrest during translation; and (iii) binding to the SRP receptor, leading to release from elongation arrest and translocation of the targeted protein into the lumen of the endoplasmic reticulum. Biochemical mutagenesis experiments have implicated individual domains of the SRP complex in each of these three functions (28, 29). Thus, the 54-kDa polypeptide is required for binding to the signal sequence, the 14- and 9-kDa polypeptides are involved in elongation arrest, and the 68- and 72-kDa proteins have been implicated in binding to the SRP receptor and promoting the directional translocation of newly translated proteins bearing a signal sequence into the lumen of the ER (28). The role played by the 7 S RNA molecule is currently unknown.

Deletion analysis of canine SRP 68 and SRP 72 has demonstrated that these proteins associate with each other through their carboxyl termini, forming a stable complex with the 7 S RNA (14). A 57-kDa fragment that includes the amino terminus of canine SRP 72 (*i.e.* a smaller fragment than that generated by caspase cleavage of SRP 72) generated by elastase digestion is still capable of interacting *in vitro* with SRP 68, while a 42-kDa fragment is not (14). Interestingly, an elastase-generated carboxyl fragment of ~4 kDa was observed in this analysis, suggesting that a portion of the carboxyl terminus of SRP 72 is exposed when associated with other components of the SRP particle (14). Our results are consistent with those of Lütcke *et al.* (14) since caspase-cleaved SRP 72 remains associated with the SRP complex in immunoprecipitates (Fig. 1*B*), and migrates at ~11 S by sucrose gradient centrifugation when comparing complexes prepared from untreated and apoptotic

[2] P. J. Utz, M. Hottelet, and P. Anderson, unpublished data.
[3] W. J. van Venrooij, unpublished data.

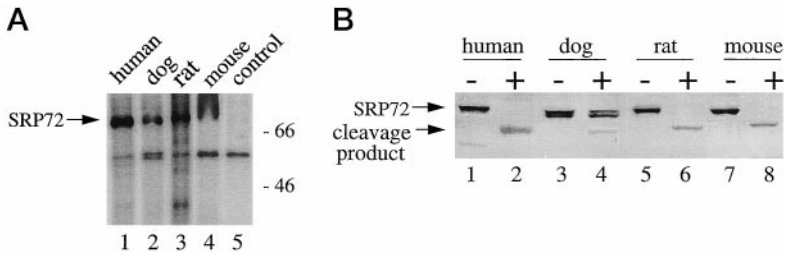


FIG. 7. **SRP 72 is phosphorylated *in vivo*, and is cleaved during apoptosis, in cells derived from multiple different organisms.** *A,* cells derived from the indicated animal were labeled with [$^{32}$P]orthophosphate and lysed after 6 h. Proteins were then precipitated using human anti-SRP autoantiserum J141 (*lanes 1–4*) or a control patient serum (*lane 5*), separated on a 12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride, and exposed for autoradiography. The relative migration of molecular size markers in kilodaltons is indicated on the *right side* of the panel. SRP 72 localization is shown on the *left side* of the panel. The animal of origin for each lane is depicted at the *top* of the panel. Lanes are numbered at the *bottom* of the panel. *B,* cells depicted in *panel A* were cultured in the absence (−) or presence (+) of anisomycin for 6 h and harvested for protein analysis. Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using anti-SRP 72-3. SRP 72 and the SRP 72 cleavage product are shown on the *left side* of the panel.

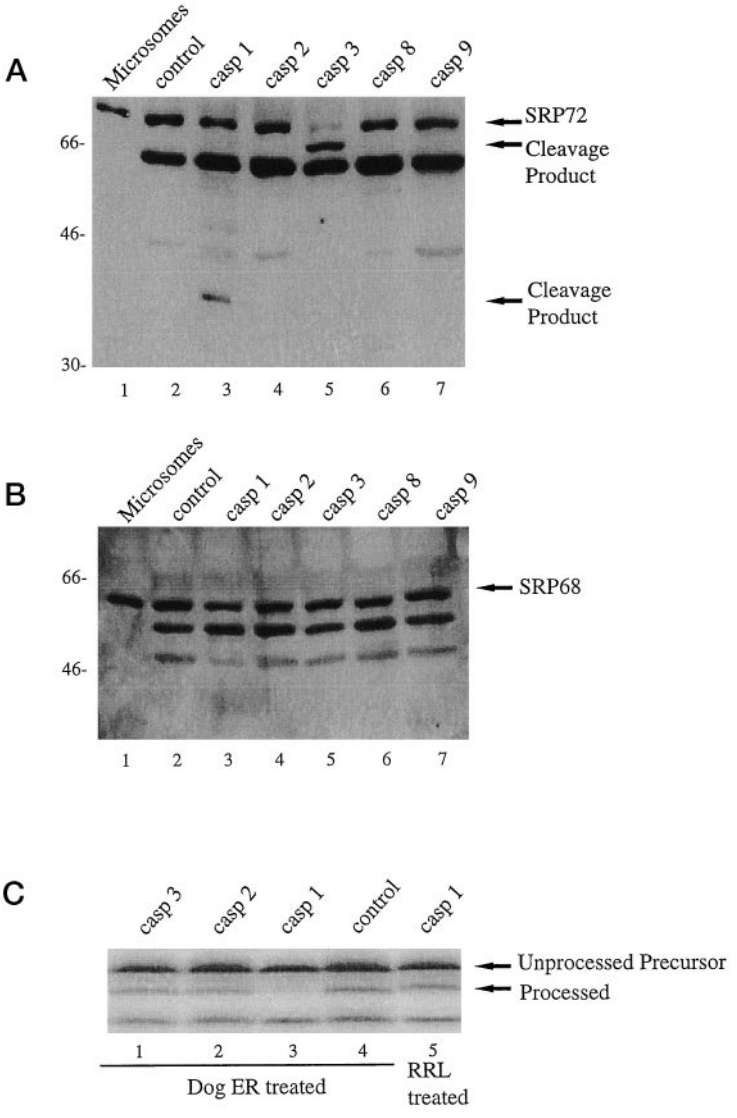


FIG. 8. **Canine SRP 72 is cleaved *in vitro* by recombinant caspase 3 but retains the ability to mediate transport of a signal peptide-containing protein into the ER lumen.** *A* and *B,* canine microsomes were incubated with recombinant caspases (*lanes 3–7*), control bacterial lysate (*lane 2*), or mock treatment (*lane 1*). The mixture was then separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using anti-SRP 72-3 (*A*) or anti-SRP 68-2 (*B*). The relative migration of molecular size markers in kilodaltons is indicated on the *left side* of each panel. SRP 68, SRP 72, and the SRP 72 cleavage product are shown on the *right side* of each panel. *C,* canine microsomes were treated with caspases as in *A* and *B*. Following treatment, microsomes were co-incubated with *in vitro* translated β lactamase for 1 h. The mixture was then separated by SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiographic analysis. As a control, rabbit reticulocyte lysate (which contains a small amount of SRP) was treated with caspase 1 (*lane 5*) prior to addition of untreated canine microsomes. β-Lactamase precursor and the 30-kDa signal peptidase cleavage product are shown on the *right side* of the panel.

Jurkat cells (data not shown). SRP has been observed to migrate in a larger complex of ~40 S (14), and it remains possible that cleavage of SRP 72 may disrupt the formation of the larger complex.

Based on biological and chemical mutagenesis experiments (14, 28), we predict that cleavage of SRP 72 during apoptosis may inhibit binding of the complex to the SRP receptor on the endoplasmic reticulum membrane. This would result in (i) globally preventing the localization of secretory proteins to the endoplasmic reticulum; and (ii) irreversible elongation arrest of newly translated proteins bearing a signal sequence. While this is unlikely to play an important role in the execution phase of most types of cell death, cleavage of SRP 72 may play a critical role when apoptosis is induced by enveloped viruses, since many viral proteins pass through the ER. This would be thwarted if SRP 72 was no longer capable of targeting nascent peptides to its receptor on the outer lumen of the ER. While the *in vitro* results shown in Fig. 8 argue against this model, it

remains possible that removal of the carboxyl terminus of SRP 72 during apoptosis may have a more subtle effect on ER transport *in vivo*, particularly since the cleaved carboxyl-terminal peptide is likely to harbor the serine phosphorylation site(s).

Targeting of secretory proteins to the ER occurs by a strongly conserved, and presumably highly regulated, mechanism. However, little is known about how this process is governed in eukaryotic cells. Protein translation is regulated in response to exogenous stress at an early step (initiation) by several kinases, including: mitogen-activated protein kinases, which repress translation by phosphorylating the mRNA cap-binding protein eIF4E (30); the interferon-inducible, double stranded RNA-regulated protein kinase, which inhibits translation by phosphorylating the eIF2 initiation factor (31, 32); the target of rapamycin which phosphorylates PHAS-1, a regulator of eIF4E; and the rapamycin-sensitive p70 S6 kinase, which modulates translation through phosphorylation of the S6 component of ribosomes (33). Our observation that SRP 72 is phosphorylated on serine residue(s) in all cell types tested suggests that a serine kinase and/or phosphatase may regulate gene expression at a later step than the translation initiation checkpoint that is regulated by the above kinases.

Our results are most consistent with caspase-mediated cleavage of the carboxyl terminus of SRP 72, generating a 6-kDa peptide containing the serine phosphorylation site(s). The fate of the 6-kDa fragment is unknown. We cannot, however, exclude the possibility that SRP 72 is a target for both a phosphatase and a caspase during apoptosis. It is also possible that a comigrating serine kinase (*e.g.* p70 S6 kinase) is coprecipitated with the SRP complex, and that the carboxyl terminus of SRP 72 is required for the interaction between the kinase and SRP. We have not consistently observed a serine kinase activity in these immunoprecipitates in *in vitro* kinase assays, however, arguing against the later possibility (data not shown).

Comparison of the carboxyl terminus of SRP 72 from different organisms (Table I) shows striking conservation of eight serine residues (labeled with an *asterisk*, *) that are distal to the proposed caspase cleavage site (*bold letters*). Three of these serine residues are conserved from *homo sapiens* to *Schistosoma mansoni* (underlined). Although SRP 72 from *Caenorhabditis elegans* and *Saccharomyces cerevisiae* lacks these conserved serine residues, both proteins have carboxyl-terminal serines (*i.e.* amino acids 660, 663, and 693, *C. elegans*; aa 620 and 627, *S. cerevisiae*) that are potential phosphorylation sites. We have not yet determined whether SRP 72 derived from yeast, *C. elegans*, and *S. mansoni* are similarly phosphorylated *in vivo*.

The results shown in Fig. 8 demonstrate cleavage of SRP 72 *in vitro* by caspase 3; however, the caspase(s) responsible for cleaving SRP 72 *in vivo* remain unidentified. Based on published data, it is most likely that SRP 72 is cleaved *in vivo* by a group III caspase such as caspase-6 (Mch 2), caspase-8 (MACH, FLICE, Mch 5), or caspase-9 (ICE-LAP6, Mch 6), reviewed in Ref. 21. Group III caspases cleave non-D*XX*D motifs characteristically found in other caspases or in components of the nuclear or cytosolic skeleton (*e.g.* actin, Gas2, and nuclear lamins (21)). The SELD/A motif of SRP 72 most resembles the SELD/A cleavage site of SREBP-2, a transcription factor involved in regulation of cholesterol metabolism that is also associated with the endoplasmic reticulum (34). In support of the hypothesis that SRP 72 is also a substrate for group III caspases, we have observed cleavage of SRP 72 (generated in an *in vitro* translation reaction) by several group III caspases *in vitro,* including caspases 8 and 9.[4] It will also be of great interest to determine if SRP 72 from *C. elegans* and *S. cerevisiae* (both of which lack a conserved caspase cleavage site), and SRP 72 from S. mansoni (which possesses such a site at $^{665}$SEWD/A$^{669}$) can be cleaved *in vivo* or *in vitro*.

Autoantibodies directed against the 54-kDa polypeptide of SRP were first described independently in 3 patients with dermatomyositis (17, 35). It is currently unknown whether phosphorylation or caspase-mediated cleavage of SRP 72 contributes to the production of autoantibodies reactive with components of the SRP complex. Several studies have identified autoantigens as constituents of membrane-bound blebs on the surface of apoptotic cells, where it has been proposed that they are ideally situated for presentation to the immune system (1, 36–38). Consistent with this hypothesis, we have observed SRP components as prominent constituents of apoptotic blebs by immunofluorescence.[4] Like SRP 72, several proteins that are modified by caspases, or become associated with highly phosphorylated proteins during apoptosis are targets for autoantibody production in patients with autoimmune disease. The presence of autoantibodies reactive with components of the SRP is associated with a particularly aggressive form of dermatomyositis (39). Whether this is a reflection of increased levels of apoptosis in affected tissues remains to be determined.

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