ARTHRITIS & RHEUMATISM
Vol. 43, No. 6, June 2000, pp 1327–1336

SMALL NUCLEOLAR RNP SCLERODERMA AUTOANTIGENS ASSOCIATE WITH PHOSPHORYLATED SERINE/ARGININE SPLICING FACTORS DURING APOPTOSIS

KARIN OVERZET, TIMOTHY J. GENSLER, SUSAN J. KIM, MEGHAN E. GEIGER, WALTHER J. VAN VENROOIJ, K. MICHAEL POLLARD, PAUL ANDERSON, and PAUL J. UTZ

Objective. **Proteins that are phosphorylated during apoptosis are commonly precipitated by autoantibodies found in the sera of patients with systemic lupus erythematosus. We sought to determine whether scleroderma autoantigens such as small nucleolar RNPs (snoRNP) also associate with phosphoproteins in response to various cellular stressors.**

Methods. **We screened a panel of monoclonal antibodies derived from mice exposed to mercury, a well-characterized murine model of the anti-snoRNP autoimmune response, for the ability to selectively precipitate phosphoproteins from radiolabeled lysates prepared from Jurkat T cells subjected to stressful stimuli.**

Results. **Monoclonal antibodies reactive with snoRNPs precipitated a phosphoprotein complex (pp42, pp34, and pp23) from lysates prepared from apoptotic cells. Several novel phosphoproteins (pp62 and pp18) were also observed. The phosphorylation and/or recruitment of these proteins to the snoRNP complex is induced by multiple apoptotic stimuli (e.g., Fas ligation, anisomycin, or ultraviolet irradiation), an effect that is blocked by overexpression of Bcl-2. We were unable to demonstrate an association of the phosphoprotein complex with snoRNPs in cells treated with the xenobiotic agent mercury. The snoRNP-associated phosphoprotein complex is composed of serine/arginine (SR) splicing factors, including SRp40.**

Conclusion. **The association of phosphorylated SR proteins with snoRNPs in cells undergoing apoptosis suggests that the immune response to fibrillarin that characterizes a subset of patients with scleroderma may be related to cell death induced by apoptotic stimuli (e.g., Fas ligation, irradiation, or chemical toxins), or by exposure to mercury.**

The posttranslational modification of autoantigens that occurs in response to cellular stress has been implicated in the subsequent development of autoantibodies in patients with systemic lupus erythematosus (SLE), dermatomyositis, and scleroderma (1,2). Such modifications include cleavage of proteins by caspases (1,3–6), site-specific small nuclear RNA (snRNA), Y-RNA, and ribosomal RNA (rRNA) cleavage (7–9), and mercury-induced protein degradation and oxidation (10,11). RNA binding proteins, including constituents of the U1 small nuclear RNP (U1 snRNP) complex (3,12) and the Ro/La RNP (9,13), both of which are common SLE autoantigens, are subjected to posttranslational modification during cell death (1,2). Little is known regarding the fate, during apoptosis, of small nucleolar RNPs (snRNP), a target of the immune response in patients with scleroderma and in mice exposed to mercury.

Ms Overzet's work was supported by grants from the Groninger University Fund, the Marco Polo Fund, and the Dutch Kidney Foundation. Dr. Gensler's work was supported by the Arthritis Foundation and NIH Training Grant T32-AR-07530. Dr. van Venrooij's work was supported in part by the Netherlands Foundation for Chemical Research, with financial aid from the Netherlands Organization for Scientific Research and the Netherlands Technology Foundation. Dr. Pollard's work was supported by NIH grants ES-07511 and ES-08080. Dr. Anderson's work was supported by the Arthritis Foundation, NIH grants AI-33600 and CA-67929, and the Peabody Foundation. Dr. Anderson is a Scholar of the Leukemia Society of America. Dr. Utz's work was supported by a grant from the Scleroderma Foundation, Inc., the Arthritis Foundation, NIH grant K08-AI-01521, and the Arthritis National Research Foundation.

Karin Overzet, BS, Timothy J. Gensler, MD (current address: Denver Arthritis Clinic, Denver, Colorado), Susan J. Kim, BS, Meghan E. Geiger, BS, Paul Anderson, MD, PhD, Paul J. Utz, MD (current address: Stanford University School of Medicine, Stanford, California): Brigham and Women's Hospital, Boston, Massachusetts; Walther J. van Venrooij, PhD: University of Nijmegen, Nijmegen, The Netherlands; K. Michael Pollard, PhD: The Scripps Research Institute, La Jolla, California.

Address reprint requests to Paul J. Utz, MD, Stanford University School of Medicine, CCSR Building, Room 2215, Mail Code 5166, 259 Campus Drive, Stanford, CA 94305.

Submitted for publication October 12, 1999; accepted in revised form February 16, 2000.

We recently described an association of the U1 snRNP autoantigen complex with phosphorylated serine/arginine (SR) splicing factors that is induced in cells undergoing apoptosis (12). This observation suggested that hyperphosphorylated SR proteins might participate in the bypass of tolerance to spliceosomal components that characterizes patients with SLE and mixed connective tissue disease (MCTD) (14,15). We sought to extend these initial studies by analyzing selected snoRNP-specific monoclonal antibodies for their ability to coprecipitate new phosphoproteins from lysates prepared from apoptotic cells. We have confirmed that a subset of human scleroderma sera can immunoprecipitate a phosphoprotein complex from lysates prepared from apoptotic cells, as can monoclonal antibodies generated from mercury-treated mice, a murine model of the immune response to the scleroderma autoantigen fibrillarin (16,17). Our results demonstrate that snoRNPs, like the U1 snRNP lupus autoantigen complex, are dynamic structures that are influenced by cellular stressors such as irradiation, chemotherapeutic toxins, mercury exposure, and activation of death receptors such as Fas.

MATERIALS AND METHODS

Cell culture. Jurkat cells were grown in 5% CO_2 at 37°C using RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 9% heat-inactivated fetal calf serum (HIFCS; Tissue Culture Biologicals, Tulare, CA) and penicillin and streptomycin (Mediatech, Herndon, VA). Jurkat cells were grown and harvested at mid-log phase. Jurkat T cells overexpressing Bcl-2 (or empty vector), a kind gift from John Reed (La Jolla Cancer Research Foundation, La Jolla, CA), were grown in RPMI medium as described above, supplemented with G418 (Gibco BRL, Gaithersburg, MD) at a final concentration of 500 μg/ml. Protein overexpression was confirmed by Western blotting.

Metabolic labeling of Jurkat cells. Jurkat cells were incubated at a concentration of 2×10^6 cells/ml in labeling medium containing the following: 90% RPMI 1640 lacking phosphate (Gibco, Grand Island, NY), 2 m*M* glutamine (Mediatech), and 9% HIFCS that had been dialyzed to equilibrium against 10 m*M* HEPES buffer (Sigma, St. Louis, MO). ^{32}P-labeled orthophosphate (Dupont NEN, Boston, MA) was added at a concentration of 0.15 mCi/ml. Cells were incubated at 37°C for 1–2 hours to allow them to reach steady state before each treatment, unless otherwise indicated. This labeling protocol differs substantially from that used previously, in which cells were labeled overnight prior to treatment (12,18). The shorter period of preincubation with label used in the present study allows for higher specific activity labeling of the snoRNPs, thus increasing the sensitivity of the immunoprecipitation assay. 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 P40 (NP40) lysis buffer (1% NP40, 150 m*M* NaCl, 50 m*M* Tris [pH 7.8], 1 m*M* EDTA). NP40 lysis buffer was supplemented immediately before use with 1 m*M* sodium vanadate and a 100× protease inhibitor cocktail containing chymostatin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, benzamidine, and aprotinin, prepared as described elsewhere (19). All chemicals were purchased from Sigma. After addition of lysis buffer, cells were incubated on ice for 30 minutes and centrifuged in a refrigerated microfuge (5402; Eppendorf, Hamburg, Germany) at 14,000 revolutions per minute for 15 minutes, and the supernatant was used immediately for each experiment.

Ultraviolet (UV) irradiation. Radiolabeled Jurkat cells were plated in 100 × 15–mm polystyrene petri dishes (Nunc, Thousand Oaks, CA) at a concentration of 2×10^6 cells/ml and irradiated in a Stratalinker 2400 (Stratagene, La Jolla, CA) at a distance of 9 cm for 18 seconds. After irradiation, cells were incubated at 37°C for the indicated times prior to harvesting.

Cellular activation. Radiolabeled Jurkat cells were treated with anti-Fas antibody 7C11 (IgM; kindly provided by Michael Robertson, Indiana University, Bloomington, IN) diluted from hybridoma supernatant (1:500 volume:volume). Anti-CD3 antibody (Coulter Immunology, Hialeah, FL) was added at a concentration of 5 μg/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. Jurkat cells were treated with 10 μg/ml anisomycin or 40 μM $HgCl_2$ (Sigma) for varying times prior to harvesting.

Immunoprecipitation and Western blot analysis. Lysates were precleared once with 25 μl of a 50% solution of protein A–Sepharose (Pharmacia, Uppsala, Sweden) in phosphate buffered saline (PBS) overnight, followed by two 20-minute preclears with protein A–Sepharose. Mouse monoclonal antibodies (5 μg purified antibody or 50 μl hybridoma supernatant) and 5 μg rabbit anti-mouse immunoglobulin, or 2 μl patient serum alone, were used in precipitation experiments. Human polyclonal antibodies were obtained from the following sources and stored at −70°C until used: anti-U3/fibrillarin (from T. Medsger and N. Fertig, University of Pittsburgh School of Medicine, Pittsburgh, PA) and anti–RNA polymerase I/III (serum KA), anti–polymerase I/III (serum IM), and anti-U3/fibrillarin (from M. Kuwana, Keio University Medical School, Tokyo, Japan).

Autoimmune sera capable of precipitating SR proteins (pp54, pp42, pp34, and pp23) were described previously (18). Mouse monoclonal antisera were obtained from the following sources and stored at −70°C until used: anti-U1A/U2B″ (9A9, described previously [20]); anti-SC35 (Sigma), which specifically recognizes the phosphorylated isoform of the SC35 splicing factor; and antifibrillarin monoclonal antibodies (72B9.D31 and 17C12.G9) and a monoclonal antibody directed against other unidentified snoRNP components (6G10.D3), all 3 of which have been described previously (11). Serum from healthy controls and scleroderma patients was a gift from P. Fraser (Brigham and Women's Hospital, Boston, MA).

Immunoprecipitations were performed in NP40 lysis buffer in a total volume of 500 μl, with rotation in a 4°C cold room for 2–24 hours. Precipitates were harvested by centrifuging for 15 seconds at 14,000 rpm in a refrigerated Eppendorf microfuge, washing 3 times with NP40 lysis buffer, resuspend-

ing in sodium dodecyl sulfate (SDS) loading buffer with 9% 2-mercaptoethanol, boiling for 5 minutes, and separating by SDS–polyacrylamide gel electrophoresis (PAGE) as described (21). Proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) for Western blotting experiments, 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 1:1,000. Bands were visualized using species-specific antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) at a dilution of 1:7,500 in 5% Blotto in PBS, and developed using enhanced chemiluminescence performed according to the manufacturer's instructions (Amersham).

DNA fragmentation. Unlabeled Jurkat cells were induced to undergo apoptosis using the above triggers in experiments parallel to those using radiolabeled cells. Cells were collected at the indicated times and centrifuged for 5 minutes at 1,000 rpm. The cell pellet was solubilized in 500 μl DNA lysis buffer (20 m*M* Tris [pH 7.4], 5 m*M* EDTA, and 0.4% Triton X-100) and incubated on ice for 15 minutes, mixing several times. After centrifuging at 4°C, 14,000 rpm for 5 minutes, 400 μl supernatant was extracted with a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol (Gibco). Next, 100 μl of 5*M* NaCl and 500 μl isopropanol were added to each tube prior to incubating overnight at −70°C. Samples were thawed and centrifuged at 14,000 rpm for 5 minutes, washed once with 70% ethanol, and dried in a Speed-Vac. Pellets were resuspended in 30 μl of Tris–EDTA buffer containing 0.1 mg/ml RNase A (Sigma) and incubated at 37°C for 30 minutes. After the addition of 10 μl loading buffer, 10 μl of each sample (corresponding to 1 million cells/lane) was separated on 0.8% agarose gels and visualized by ethidium bromide staining under UV light. A molecular size marker (1-kb ladder; Gibco) was included on each gel.

Two-dimensional phosphopeptide analysis. Two-dimensional tryptic phosphopeptide mapping was performed as described (22) using trypsin (Worthington, Freehold, NJ) at a concentration of 0.1 mg/ml in 100 m*M* ammonium bicarbonate. Plates were exposed to film at −80°C with an intensifying screen for 1–3 days.

RESULTS

Anti-snoRNP monoclonal antibodies derived from mice exposed to mercury immunoprecipitate a phosphoprotein complex from lysates prepared from anti-Fas–treated Jurkat cells. We recently identified a subset of SLE patients whose sera contain antibodies that coimmunoprecipitate, from apoptotic Jurkat T cell lysates, a complex composed of the U1 snRNP and phosphorylated SR splicing factors (12,18). SR proteins associate with the U1 snRNP following multiple different apoptotic stimuli, an event that is inhibited in cells overexpressing the apoptosis-inhibitory protein Bcl-2. Identical results were obtained using monoclonal antibodies that recognize either Sm components or the U1 snRNP–specific protein U1A, but not using antibodies specific for other RNA binding proteins such as U2B″, TIAR (23), TIA-1 (24), or the Ro/La snRNP (12). A large number of other human autoimmune sera and monoclonal antibodies failed to coimmunoprecipitate SR proteins, suggesting that this phenomenon is specific for U snRNPs. In the present study, we sought to extend these experiments by addressing whether snoRNPs, which are relatively common targets of the scleroderma autoimmune response, may also be associated with phosphoproteins during cellular stress (14,17).

In pilot experiments, we first screened a small panel (15 patients) of scleroderma autoimmune sera for their ability to precipitate phosphoproteins from cell lysates prepared from apoptotic Jurkat cells. Eleven of the sera precipitated no new phosphoproteins. However, 4 of these sera, all of which had been previously characterized by Western blot, immunoprecipitation, and immunoprecipitation RNA analysis, specifically recognized fibrillarin, a major component of the U3 snoRNP (data not shown). This pattern was reminiscent of that observed for the U1 snRNP autoimmune sera reported previously, suggesting that snoRNPs may also associate with SR proteins during apoptosis (12).

A major problem associated with the use of human sera for such experiments is that multiple autoantibody specificities may exist within individual patient serum samples. We instead sought to confirm the results obtained with the small number of polyclonal human autoimmune sera identified in the pilot experiments described above by repeating the experiments using monoclonal antibodies specific for snoRNPs. A mouse model of the anti-snoRNP/fibrillarin autoantibody response has been established, in which mice treated systemically with the xenobiotic mercury chloride develop autoantibodies that recognize nucleolar components, particularly fibrillarin (16,17). Monoclonal antibodies derived from these animals have recently been isolated to further study the molecular mechanism by which the immune system of these animals is rendered intolerant to nucleolar antigens (11).

We tested monoclonal antibodies directed against fibrillarin (17C12, 72B9) or other unidentified snoRNP components (6G10.D3) for their ability to precipitate new radiolabeled phosphoproteins from extracts prepared from apoptotic Jurkat cells. Jurkat cells metabolically labeled with ^{32}P-orthophosphate were cultured for varying times over a 5-hour period in the absence or presence of a monoclonal antibody reactive with Fas (anti-7C11), solubilized in NP40 lysis buffer, and immunoprecipitated using each of these antibodies.

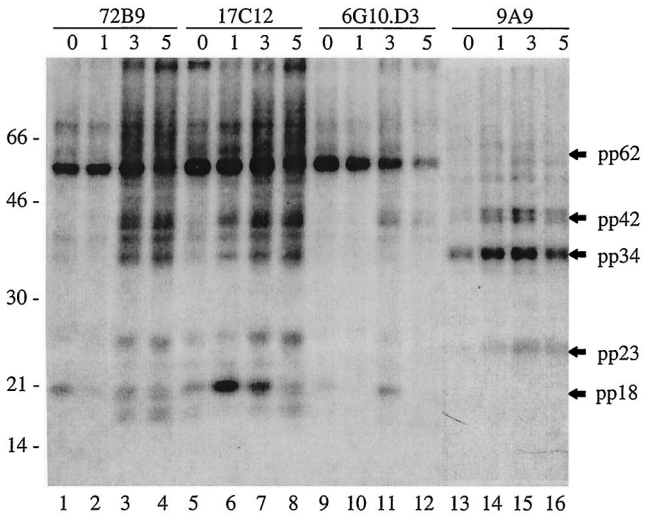

Figure 1. Immunoprecipitation by anti–small nucleolar RNP monoclonal antibodies of a phosphoprotein complex from lysates prepared from anti-Fas–treated Jurkat cells. Jurkat cells were labeled with ^{32}P-orthophosphate and lysed at varying times following the addition of anti-Fas 7C11. Proteins were then precipitated using the indicated monoclonal antibody, separated on a 12% sodium dodecyl sulfate–polyacrylamide gel, transferred to nitrocellulose, and exposed for autoradiography. Individual monoclonal antibodies are described in Materials and Methods. The time (in hours) is shown at the top of each lane. The relative migration of molecular size markers (in kd) is shown on the left. Bands corresponding to individual phosphoproteins (serine/arginine splicing factors, or pp) are shown on the right. Lanes are numbered at the bottom.

Antibodies directed against other RNA binding proteins, including U1A/U2B″ (9A9, lanes 13–16 in Figure 1 [20]), U2B″, SC35, Ro60 (25), Ro52 (26), La (26), TIAR (6E3 [23]), and TIA-1 (ML29 [24]), were used as controls (data not shown).

As shown in Figure 1, the U snRNP antibody (9A9) precipitated 4 phosphoproteins (SR splicing factors, termed pp54, pp42, pp34, and pp23) from apoptotic Jurkat cell lysates (lanes 13–16), and identical results were obtained using anti-SC35, as reported previously (12). These phosphoproteins were absent in experiments using antibodies to U2B″, La, TIAR, TIA-1, and Ro (12; data not shown). Surprisingly, all 3 snoRNP monoclonal antibodies derived from mercury-treated mice coimmunoprecipitated a similar phosphoprotein complex from lysates prepared from apoptotic Jurkat T cells (lanes 1–12). Moreover, the time course of phosphorylation was similar, but not identical, to that observed using the anti–U snRNP antibody (9A9). First, unlike the immunoprecipitation analysis using 9A9 or SC35, a prominent 62-kd band was present when immunoprecipitations were performed with the monoclonal antibodies derived from mercury-treated mice. Second, an 18-kd phosphoprotein was present in immunoprecipitations using the antinucleolar antibodies, most prominently observed with antibody 17C12 (lane 6). Finally, the intensity of the bands was identical when comparing the 3- and 5-hour time points in the 72B9 and 17C12 immunoprecipitates (lanes 3, 4, 7, and 8). The protein complex completely disappeared at a later 6-hour time point (e.g., see Figure 3A, lanes 3 and 4). In contrast, SR proteins associated with the U1 snRNP were dephosphorylated between the 3- and 5-hour time points (Figure 1, lanes 15 and 16).

Taken together, these findings suggest that antinucleolar monoclonal antibodies derived from mercury-treated mice coimmunoprecipitate a phosphoprotein complex in response to Fas ligation that resembles the SR protein complex that associates with the U1 snRNP. This complex also contains several novel phosphoproteins that uniquely associate with snoRNPs. Moreover, the dephosphorylation kinetics of the phosphoproteins are distinct from those associated with the U1 snRNP–SR complex.

A phosphoprotein complex associates with snoRNPs containing fibrillarin in response to several different apoptotic stimuli. We had previously demonstrated that, in addition to death induced by Fas ligation, phosphorylated autoantigens were also immunoprecipitated during apoptosis triggered by other stimuli, including gamma and UV irradiation, but not by T cell receptor stimulation (18). We repeated this experiment using the antifibrillarin monoclonal antibody 17C12 in immunoprecipitation experiments using lysates derived from ^{32}P-labeled Jurkat cells subjected either to apoptotic stimuli or to an activation stimulus over a 6-hour time course (Figure 2). This analysis showed that phosphorylated autoantigens were first observed in immunoprecipitates between 1 and 3 hours following Fas crosslinking (Figure 2, lanes 5–8) or UV irradiation (Figure 2, lanes 9–12). Similar results were obtained when cells were treated with the protein synthesis inhibitor anisomycin (results not shown). In contrast, the phosphoprotein complex was not precipitated with 17C12 from lysates prepared from mock-treated cells (Figure 2, lanes 1–4), or when the T cell receptor complex was ligated using a monoclonal antibody reactive with CD3, a stimulus that induces interleukin-2 production and enhances proliferation in these cells (12,18; data not shown).

Mercury chloride rapidly kills eukaryotic cells in a manner distinct from that of apoptosis (11). We did not observe the phosphoprotein complex in immunopre-

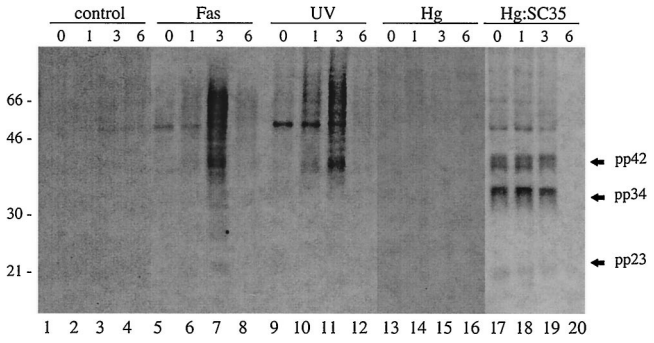

Figure 2. Association of a phosphoprotein complex with small nucleolar RNPs following several different apoptotic stimuli. Jurkat cells were labeled with ^{32}P-orthophosphate, treated with the indicated stimulus, and solubilized using Nonidet P40 lysis buffer at the times shown. Proteins were then precipitated with antifibrillarin 17C12, separated on a 12% sodium dodecyl sulfate–polyacrylamide gel, transferred to nitrocellulose, and exposed for autoradiography. The stressful stimulus is shown above each panel. The time (in hours) following each stimulus is shown above each lane. The relative migration of molecular size markers (in kd) is shown on the left. Bands corresponding to phosphoproteins (pp) are shown on the right. Lanes are numbered at the bottom. UV = ultraviolet.

cipitation experiments in which lysates prepared from Jurkat cells treated with mercury chloride were probed with 17C12 (Figure 2, lanes 13–16). This probably resulted from the inability of 17C12 to bind fibrillarin, which is rapidly modified in the presence of mercury, thus altering its immunogenicity (11). To determine whether SR proteins were phosphorylated in response to mercury treatment, the identical experiment was performed using anti-SC35 (Figure 2, lanes 17–20) and anti-U1A/U2B″ (9A9; results not shown). No phosphorylation was identified in either case, and no phosphoproteins could be observed at the 6-hour time point (Figure 2, lane 20). In fact, markedly less protein was immunoprecipitated when cells were instead labeled with ^{35}S-methionine/cysteine, a result that was confirmed by staining the nitrocellulose filters with ponceau S (results not shown). This suggests that mercury treatment of Jurkat cells may also disrupt the ability of 9A9 and anti-SC35 to directly recognize the U1 snRNP and SR proteins, respectively.

In vivo phosphorylation of snoRNP components is inhibited in UV-irradiated Jurkat cells overexpressing Bcl-2. We next asked whether the association of these phosphorylated snoRNP components could be inhibited by overexpression of the Bcl-2 protein, which has been shown to efficiently block apoptosis induced by multiple apoptotic stimuli, including gamma and UV irradiation (18,27–30). As shown in Figure 3A, Jurkat T cells stably transformed with either Bcl-2 (lanes 5–8) or empty vector (lanes 1–4) were labeled with ^{32}P-orthophosphate and subjected to UV irradiation. Cells were solubilized at the indicated times, and lysates were precipitated using the antifibrillarin monoclonal antibody 17C12. While precipitation of phosphorylated pp42, pp34, and pp23 was rapidly induced in Jurkat (neo) control cells in response to UV irradiation (Figure 3A, lanes 1–4), the intensity of the bands corresponding to the 3 phosphoproteins was unchanged in Jurkat (Bcl-2) transformants treated with this same stimulus (Figure 3A, lanes 5–8). Ectopic expression of Bcl-2 effectively inhibited apoptosis in response to UV irradiation, as judged by the induction of DNA fragmentation (Figure 3B). Taken together, these findings demonstrate that Bcl-2 effectively inhibits the association of this phosphoprotein complex with snoRNPs in response to UV irradiation.

Phosphotryptic peptide maps comparing pp42 and the SR splicing factor SRp40 are identical. We recently demonstrated that the U1 snRNP associates with SR proteins by employing a combination of immunoprecipitation and 2-dimensional phosphopeptide analysis to identify proteins in the complex (12,18). We used an identical approach to determine whether pp42, pp34, and pp23 (coprecipitated with 17C12) might also be SR proteins. Other higher molecular weight proteins could not be efficiently separated by SDS-PAGE to allow for clean excision for trypsin digests, preventing further analysis of these bands. Jurkat cells metabolically labeled with ^{32}P-orthophosphate were cultured for 3 hours in the presence of a monoclonal antibody reactive with Fas (anti-7C11), solubilized in NP40 lysis buffer, and immunoprecipitated using 17C12 or anti-SC35 (a monoclonal antibody specific for the phosphorylated form of the SR protein SC35). Labeled proteins were separated by SDS-PAGE and transferred to nitrocellulose.

Figure 4A demonstrates that 17C12 and anti-SC35 coprecipitated similar, although slightly different (an extra band was present in the 17C12 immunoprecipitate that migrated between pp34 and the pp42 doublet), phosphoprotein complexes from lysates prepared from apoptotic Jurkat cells. To confirm that the respective proteins precipitated with each antibody were identical, bands corresponding to pp42, pp34, and pp23 (from immunoprecipitates prepared independently using anti-SC35 and 17C12 [Figure 4A, lanes 1 and 2]) were localized by autoradiography, excised, and subjected to 2-dimensional phosphotryptic mapping (22). As shown for 1 of the bands (pp42), the phosphotryptic maps were identical, and were composed of 14 major

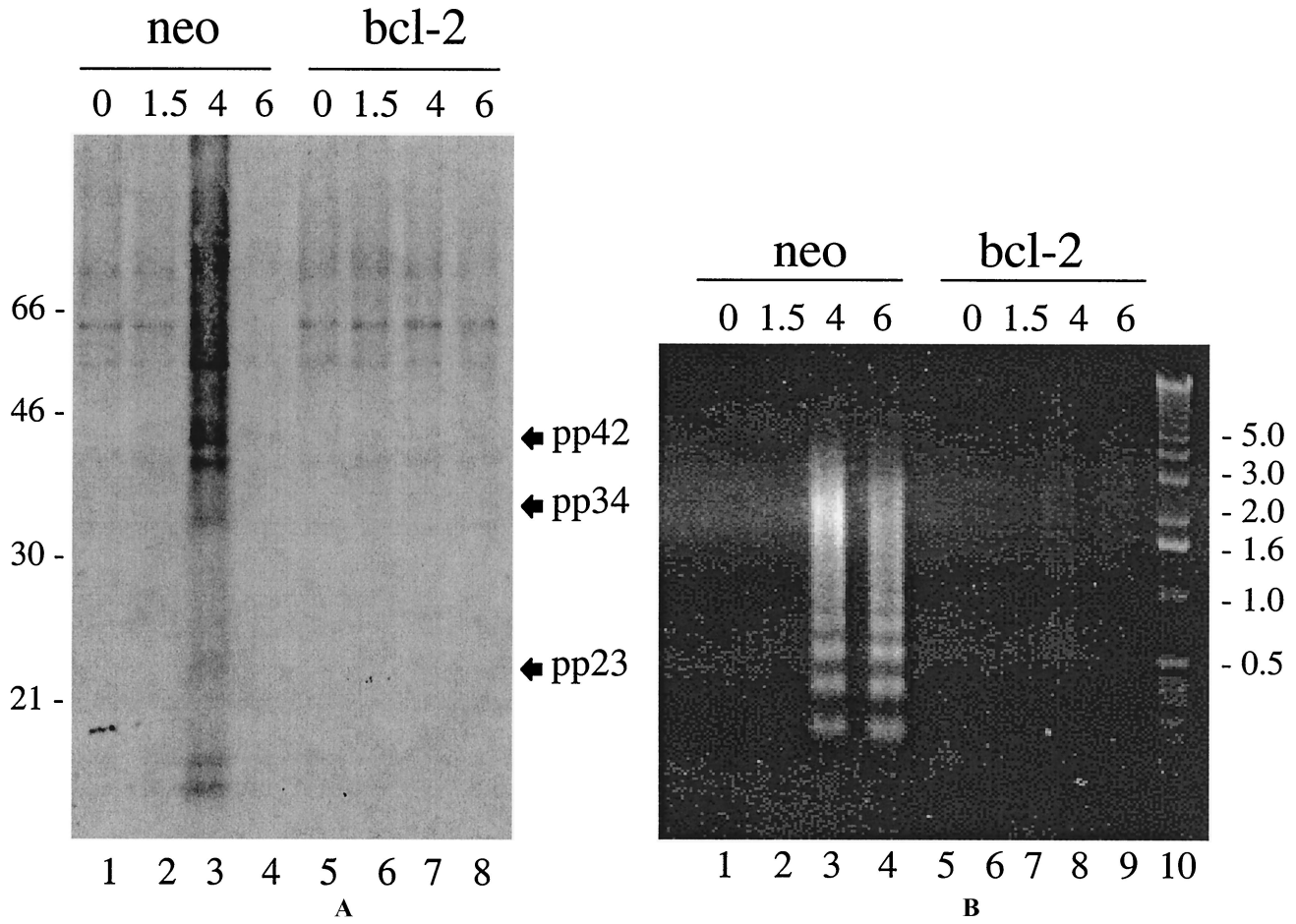

Figure 3. Inhibition of in vivo phosphorylation of small nucleolar RNP components in UV-irradiated Jurkat cells overexpressing Bcl-2. **A,** Jurkat (neo) control transformants (lanes 1–4) or Jurkat (bcl-2) transformants (lanes 5–8) were labeled with ^{32}P-orthophosphate, subjected to UV irradiation, solubilized in Nonidet P40 lysis buffer, precipitated using antifibrillarin 17C12, separated on a 12% sodium dodecyl sulfate–polyacrylamide gel, transferred to nitrocellulose, and exposed for autoradiography. The relative migration of molecular size markers (in kd) is shown on the left. Bands corresponding to pp42, pp34, and pp23 are shown on the right. The time (in hours) from initial exposure to UV irradiation is shown at the top of each lane. Lanes are numbered at the bottom. **B,** Jurkat (neo) control transformants (lanes 1–4) or Jurkat (bcl-2) transformants (lanes 6–9) were treated as in **A** and processed for DNA ladders as described in Materials and Methods. DNA was separated on an 0.8% agarose gel and visualized by UV light following ethidium bromide staining. Size standards are shown in lane 10. Lane 5 was not loaded. The relative migration of molecular size markers (in kb) is shown on the right. The time (in hours) from initial exposure to UV irradiation is shown at the top of each lane. Lanes are numbered at the bottom. See Figure 2 for definitions.

phosphopeptides (Figure 4B). Similar results were reproducibly obtained when we compared phosphopeptide maps corresponding to pp34 and pp23 (data not shown). Moreover, maps comparing labeled, biochemically purified SR proteins (31) with the maps shown in Figure 4B were identical, definitively identifying pp42 and pp23 as the SR proteins SRp40 and SRp20, respectively. Taken together, these findings demonstrate that snoRNPs containing fibrillarin, a scleroderma autoantigen, associate with SR splicing factors during apoptosis.

DISCUSSION

Since the initial demonstration by Casciola-Rosen and colleagues that SLE autoantigens are clustered in cell surface apoptotic blebs following UV irradiation (32), there has been great interest in elucidating the role played by apoptosis and other forms of cell death in the pathogenesis of systemic autoimmune diseases. In addition to relocalization of autoantigens to apoptotic blebs, there is now ample evidence that mod-

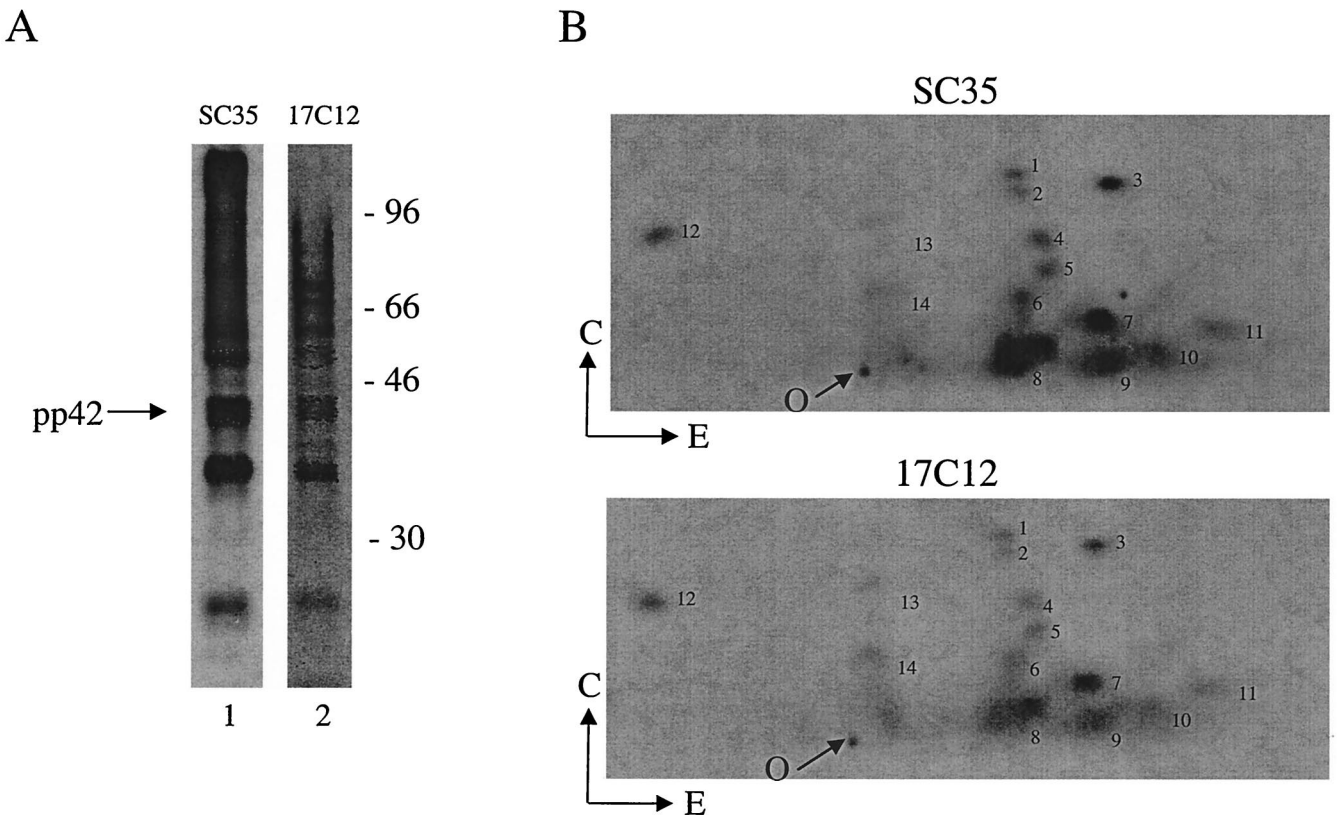

Figure 4. Equivalence of phosphopeptide maps comparing pp42 and SRp40. **A,** Jurkat cells were labeled with ^{32}P-orthophosphate and lysed 3 hours after the addition of anti-Fas 7C11. Proteins were then precipitated using the indicated monoclonal antibody, separated on a 12% sodium dodecyl sulfate–polyacrylamide gel, transferred to nitrocellulose, and exposed for autoradiography. The relative migration of pp42 is shown on the left. The relative migration of molecular size markers (in kd) is shown on the right. **B,** Two-dimensional phosphotryptic map of pp42. The 42-kd bands from the anti-SC35 immunoprecipitate (lane 1 in **A**) and the 17C12 immunoprecipitate (lane 2 in **A**) were excised and digested with trypsin. Phosphopeptides were separated electrophoretically at pH 1.9 in the first dimension, and by thin-layer chromatography in the second dimension, prior to autoradiographic exposure. Peptides are numbered from 1 to 14. Direction of separation is shown by **arrows**. C = chromatography; E = electrophoresis; O = origin (see Figure 2 for other definitions).

ifications of autoantigens during apoptosis may also be involved in the genesis of autoantibodies in several diseases, including SLE and dermatomyositis (1,2). For example, many autoantigens are cleaved in vivo and in vitro by a family of proteases (caspases) at sites containing an aspartic acid residue at the P1 position (33,34). Another protease, the cytotoxic granule protein granzyme B, has recently been shown by Andrade et al (35) and Casciola-Rosen et al (36) to uniquely cleave most autoantigens at sites that are not recognized by caspases. Other modifications of autoantigens, such as DNA cleavage (37), DNA methylation (38), RNA cleavage (7–9), protein transglutamination (39), citrullination (40), and ubiquitination (41), have also been associated with cell death. This largely observational body of evidence is now supported by in vivo immunization studies in which mice immunized with syngeneic (42) or human (43) apoptotic cells develop antibodies that recognize autoantigens, including Ku, U snRNPs, and ribosomes.

Unlike the situation with SLE, there exists only circumstantial evidence that apoptosis may contribute to the immunogenicity of autoantigens in scleroderma. Several autoantigens commonly targeted in scleroderma are cleaved by caspases during apoptosis, including DNA-dependent protein kinase, heterogeneous nuclear RNP C1 (hnRNP C1), hnRNP C2, and UBF NOR-90 (4,6,44). Topoisomerase I (Scl-70), antibodies to which are a principal diagnostic test for scleroderma, is also cleaved during apoptosis (6). Interestingly, topoisomerase I has recently been shown to possess a kinase activity, distinct from its DNA unwinding activity, that is capable of phosphorylating SR proteins such as those

that coimmunoprecipitate with snoRNPs (45). Experiments are in progress to test whether the native or cleaved forms of this scleroderma autoantigen may be involved in the kinase cascade that leads to SR protein phosphorylation during apoptosis.

In addition to apoptosis, other forms of nonapoptotic cell death have been associated with autoimmune disease, particularly scleroderma. For example, Casiano and colleagues have recently demonstrated that many autoantigens are cleaved following necrotic stimuli such as thermal injury (46). A second example is that of mercury-induced cell death, which is morphologically and biochemically distinct from apoptosis. Mice exposed to mercury chloride develop antinucleolar autoantibodies that specifically recognize an epitope on the protein fibrillarin that is indistinguishable from that recognized by human scleroderma autoimmune sera (11,16,17). The immune response is genetically restricted to the H2A region of the major histocompatibility complex (MHC) of H-2^S mice (47). Mercury has been shown to localize to the nucleoli of treated cells (10), to modify fibrillarin in a reaction that requires the presence of Cys^{105} and Cys^{274} (11), and to decrease the affinity of autoantibodies for fibrillarin when analyzed by immunoprecipitation (11). This observation suggests the reason we were unable to demonstrate coimmunoprecipitation of SR proteins with antifibrillarin antibodies following exposure of cells to mercury—the antibodies are no longer capable of recognizing and immunoprecipitating fibrillarin. However, this observation does not address the conceptual issue of why these antibodies fail to recognize antigen that has been modified by the same toxin.

Several mechanisms have been proposed to explain how mercury exposure breaks tolerance. For example, there may be direct activation of autoreactive T cells by binding of metal to the MHC and/or peptide. Alternatively, stable interaction of metal with self proteins, which then undergo selective or novel proteolysis by antigen-presenting cells, may also play a critical role (for review, see refs. 1 and 11).

SR proteins are highly conserved RNA binding proteins that contain an SR-rich motif, usually in the carboxyl terminus, that can be reversibly phosphorylated by several different kinases (see below) (48). Members of the SR protein family play important roles in both constitutive and alternative messenger RNA (mRNA) splicing, and their function is regulated by reversible phosphorylation of their SR domains. SR proteins interact with other SR domain–containing proteins such as the U1–70 kd factor (49), an important constituent of the U1 snRNP. U1–70 kd is a major autoantigen in SLE and MCTD (14). SR proteins such as SC35 localize principally to nuclear speckles, although they also shuttle between the nucleus and cytoplasm (50–54).

Our findings suggest that SR proteins may also reside in the nucleolus, where they may interact with snoRNPs containing fibrillarin. Alternatively, it is also possible that snoRNPs relocalize to another cellular compartment during apoptosis (e.g., within the nucleus or within apoptotic blebs), where they then associate with SR proteins, as has been observed for U1–70 kd and SC35 (ref. 33 and Overzet et al: unpublished observations). Fibrillarin is a constituent of many snoRNPs, including the U3 snoRNP, which mediates the rate-limiting step in the biogenesis of rRNA (55). We speculate that SR proteins may function in the nucleolus to regulate various steps in rRNA splicing, in a manner analogous to the regulation of mRNA splicing by the interaction of SR proteins and the U1 snRNP. It is not known which snoRNPs specifically associate with SR proteins during apoptosis, and experiments to address this issue are ongoing.

Human autoimmune sera have been exceedingly useful probes in identifying molecules that are cleaved during apoptosis (4,6). We have used a similar approach to uncover apoptosis signaling pathways by identifying proteins, including SR splicing factors, that are phosphorylated in response to apoptotic stimuli. The kinase(s) responsible for initiating this signaling cascade is currently unknown. In addition to topoisomerase I (45), 2 other prominent families of kinases with the ability to phosphorylate SR proteins have been identified. SR protein kinases (SRPKs) are highly conserved kinases with homologs in mice (56) and yeast (57,58). Both human kinases (SRPK1 and SRPK2) can regulate splicing of mRNA as well as relocalization of splicing factors such as ASF/SF2 and SC35 (59–62). The second kinase family, clk/sty kinases, is also highly conserved from yeast to humans and consists of 3 human and 4 mouse isoforms (63–68). Unlike SRPKs, clk/sty kinases themselves contain an SR domain and can be regulated by phosphorylation (69). It is currently not known whether one of these kinases or an as-yet-unidentified kinase is responsible for the phosphorylation of SR proteins during apoptosis. Future experiments will be aimed at unraveling this novel signal transduction pathway, identifying which snoRNPs associate with SR proteins in vivo and in vitro, and determining the role played by SR proteins in breaking tolerance to autoantigens such as fibrillarin.

ACKNOWLEDGMENTS

The authors thank members of Dr. Anderson's laboratory staff for insights and helpful comments; S. Wax and M. Pieczyk for critical review of the manuscript; the Brigham and Women's Hospital Clinical Immunology Laboratory; J. Craft, M. Kuwana, T. Medsger, N. Fertig, B. Dobberstein, A. Rosen, P. Fraser, and M. Robertson for providing antibodies used in this study; and J. Reed for the gift of the Bcl-2– and neo-overexpressing Jurkat cells.

REFERENCES

1. Utz PJ, Gensler T, Anderson P. Death, autoantigen modifications, and tolerance. Arthritis Res 2000;2:101–14.
2. Rosen A, Casciola-Rosen LA. Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. Cell Death Differ 1999;6:6–12.
3. Casciola-Rosen LA, Miller DK, Anhalt GJ, Rosen A. Specific cleavage of the 70 kDa protein component of the U1 small nuclear riboprotein is a characteristic biochemical feature of apoptotic cell death. J Biol Chem 1994;269:30757–60.
4. Casciola-Rosen LA, Anhalt GJ, Rosen A. DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. J Exp Med 1995;182:1625–34.
5. Casciola-Rosen LA, Nicholson D, Chong T, Rowan K, Thornberry N, Miller D, et al. Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. J Exp Med 1996;183:1957–64.
6. Casiano CA, Martin SJ, Green DR, Tan EM. Selective cleavage of nuclear autoantigens during CD95 (Fas/Apo-1)-mediated T cell apoptosis. J Exp Med 1996;184:765–70.
7. Houge G, Robaye B, Eikhom T, Golstein J, Mellgren G, Gjertsen B, et al. Fine mapping of 28S rRNA sites specifically cleaved in cells undergoing apoptosis. Mol Cell Biol 1995;15:2051–62.
8. Degen WGJ, van Aarssen Y, Piepers P, Pruijn GJM, Utz PJ, van Venrooij WJ. The fate of the U1 small nuclear RNP complex during anti-Fas induced apoptosis: specific cleavage of the U1 snRNA molecule. Cell Death Differ 2000;7:70–9.
9. Rutjes SA, van der Heijden A, Utz PJ, van Venrooij WJ, Pruijn GJM. Nucleolytic degradation of the small cytoplasmic Y RNAs during apoptosis. J Biol Chem 1999;274:24799–807.
10. Casciola-Rosen LA, Wigley F, Rosen A. Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: implications for pathogenesis. J Exp Med 1997;185:71–9.
11. Pollard KM, Lee D, Casiano CA, Bluthner M, Johnston M, Tan EM. The autoimmunity-inducing xenobiotic mercury interacts with the autoantigen fibrillarin and modifies its molecular and antigenic properties. J Immunol 1997;158:3521–8.
12. Utz PJ, Hottelet M, van Venrooij WJ, Anderson P. Association of phosphorylated SR proteins and the U1-small nuclear ribonuclear protein autoantigen complex accompanies apoptotic cell death. J Exp Med 1998;187:547–60.
13. Rutjes SA, Utz PJ, Broekhuis C, van der Heijden A, van Venrooij WJ, Pruijn GJM. The La (SS-B) autoantigen, a key protein in RNA biogenesis, is dephosphorylated and cleaved early during apoptosis. Cell Death Differ 1999;6:976–86.
14. Von Mühlen CA, Tan EM. Autoantibodies in the diagnosis of systemic rheumatic diseases. Semin Arthritis Rheum 1995;24:323–58.
15. Neugebauer KM, Merrill JT, Wener MH, Lahita RG, Roth MB. SR proteins are autoantigens in patients with systemic lupus erythematosus: importance of phosphoepitopes. Arthritis Rheum. In press.
16. Hultman P, Enestrom S, Pollard KM, Tan EM. Anti-fibrillarin antibodies in mercury treated mice. Clin Exp Immunol 1989;78:470–7.
17. Takeuchi K, Turley SJ, Tan EM, Pollard KM. Analysis of the autoantibody response to fibrillarin in human disease and murine models of autoimmunity. J Immunol 1995;154:961–71.
18. Utz PJ, Hottelet M, Schur P, Anderson P. Proteins phosphorylated during stress-induced apoptosis are common targets for autoantibody production in patients with systemic lupus erythematosus. J Exp Med 1997;185:843–54.
19. Karwan R, Bennett JL, Clayton DA. Nuclear RNase MRP processes RNA at multiple discrete sites: interaction with an upstream G box is required for subsequent downstream cleavages. Genes Dev 1991;5:1264–76.
20. Habets W, Hoet M, de Jong B, van der Kemp A, van Venrooij WJ. Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally-induced mouse monoclonal antibodies. J Immunol 1989;143:2560–6.
21. Laemmli E. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
22. Medley Q, Kedersha N, O'Brien S, Tian Q, Schlossman S, Streuli M, et al. Characterization of GMP-17, a granule membrane protein that moves to the plasma membrane of natural killer cells following target recognition. Proc Natl Acad Sci U S A 1996;93:685–9.
23. Taupin J, Tian Q, Kedersha N, Robertson M, Anderson P. The RNA-binding protein TIAR is translocated from the nucleus to the cytoplasm during Fas-mediated apoptotic cell death. Proc Natl Acad Sci U S A 1995;92:1629–33.
24. Tian Q, Streuli M, Saito H, Schlossman S, Anderson P. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. Cell 1991;67:629–39.
25. Veldhoven CIA, Pruijn GJM, Meilof J, Thijssen J, van der Kemp A, van Venrooij WJ, et al. Characterization of murine monoclonal antibodies against 60 kDa Ro/SS-A and La/SS-B autoantigens. Clin Exp Immunol 1995;101:45–54.
26. Pruijn GJM, Thijssen J, Smith P, Williams D, van Venrooij WJ. Anti-La monoclonal antibodies recognizing epitopes within the RNA-binding domain of the La protein show different capacities to immunoprecipitate RNA-associated La. Eur J Biochem 1995;232:611–9.
27. Boise L, Gottschallk A, Quintans J, Thompson C. Bcl-2 and Bcl-2-related proteins in apoptosis regulation. Curr Top Microbiol Immunol 1995;200:107–21.
28. Itoh N, Tsujimoto Y, Nagata S. Effect of bcl-2 on Fas antigen-mediated cell death. J Immunol 1993;151:621–7.
29. Reed J. Bcl-2 and the regulation of programmed cell death. J Cell Biol 1994;124:1–6.
30. Sentman C, Shutter J, Hockenberry D, Kanagawa O, Korsmeyer S. Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 1991;67:879–88.
31. Zahler A, Lane W, Stolk J, Roth M. SR proteins: a conserved family of pre-mRNA splicing factors. Genes Dev 1992;6:837–47.
32. Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface blebs on cultured keratinocytes. J Exp Med 1994;179:1317–30.
33. Nicholson D, Thornberry N. Caspases: killer proteases. Trends Biol Sci 1997;22:299–306.
34. Alnemri E, Livingston D, Nicholson D, Salveson G, Thornberry N, Wong W, et al. Human ICE/CED-3 protease nomenclature. Cell 1996;87:171.
35. Andrade F, Roy S, Nicholson D, Thornberry N, Rosen A, Casciola-Rosen LA. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. Immunity 1998;8:451–60.

36. Casciola-Rosen LA, Andrade F, Ulanet D, Wong W, Rosen A. Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. J Exp Med 1999;190:815–26.
37. Rumore P, Steinman C. Endogenous circulating DNA in systemic lupus erythematosus: occurrence as multimeric complexes bound to histone. J Clin Invest 1990;86:69–74.
38. Huck S, Deveaud E, Namane A, Zouali M. Abnormal DNA methylation and deoxycytosine-deoxyguanine content in nucleosomes from lymphocytes undergoing apoptosis. FASEB J 1999;13: 1415–22.
39. Autuori F, Farrace M, Oliverio S, Piredda L, Piacentini M. "Tissue" transglutaminase and apoptosis. Adv Biochem Eng 1998; 62:130–6.
40. Asaga H, Yamada M, Senshu T. Selective deimination of vimentin in calcium ionophore-induced apoptosis of mouse peritoneal macrophages. Biochem Biophys Res Commun 1998;243:641–6.
41. Marushige Y, Marushige K. Disappearance of ubiquitinated histone H2A during chromatin condensation in TGFβ1-induced apoptosis. Anticancer Res 1995;15:267–72.
42. Mevorach D, Zhou J, Song X, Elkon K. Systemic exposure to irradiated apoptotic cells induces autoantibody production. J Exp Med 1998;188:387–92.
43. Gensler T, Hottelet M, Zhang G, Schlossman S, Anderson P, Utz PJ. Monoclonal antibodies derived from BALB/c mice immunized with apoptotic Jurkat T cells recognize known autoantigens. Submitted for publication.
44. Staněk D, Vencovský J, Kafková J, Raška I. Heterogeneous nuclear RNP C1 and C2 core proteins are targets for an autoantibody found in the serum of a patient with systemic sclerosis and psoriatic arthritis. Arthritis Rheum 1997;40:2172–7.
45. Rossi F, Labourier E, Forne T, Divita G, Derancourt J, Riou J, et al. Specific phosphorylation of SR proteins by mammalian DNA topoisomerase I. Nature 1996;381:80–2.
46. Casiano CA, Ochs R, Tan EM. Distinct cleavage products of nuclear proteins in apoptosis and necrosis revealed by autoantibody probes. Cell Death Differ 1998;5:183–90.
47. Hultman P, Bell L, Enestom S, Pollard KM. Murine susceptibility to mercury. I. Autoantibody profiles and systemic immune deposits in inbred, congenic, and intra-H-2 recombinant strains. Clin Immunol Immunopathol 1992;65:98–109.
48. Fu XD. The superfamily of arginine/serine-rich splicing factors. RNA 1995;1:663–80.
49. Wu J, Maniatis T. Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell 1993;75:1061–70.
50. Cáceres J, Misteli T, Screaton G, Spector D, Krainer A. Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. J Cell Biol 1997;138:225–38.
51. Cáceres J, Screaton G, Krainer A. A specific subset of SR proteins shuttles continuously between the nucleus and the cytoplasm. Genes Dev 1998;12:55–66.
52. Hedley M, Amrein H, Maniatis T. An amino acid sequence motif sufficient for subnuclear localization of an arginine/serine-rich splicing factor. Proc Natl Acad Sci U S A 1995;92:11524–8.
53. Misteli T, Cáceres J, Clement J, Krainer A, Wilkinson M, Spector D. Serine phosphorylation of SR proteins is required for their recruitment to sites of transcription in vivo. J Cell Biol 1998;143: 297–307.
54. Romac J-J, Keene J. Overexpression of the arginine-rich carboxy-terminal region of U1 snRNP 70K inhibits both splicing and nucleocytoplasmic transport of mRNA. Genes Dev 1995;9:1400–10.
55. Kass S, Tyk K, Steitz J, Sollner-Webb B. The U3-snoRNP functions in the first step of preribosomal RNA splicing. Cell 1990;60:897–908.
56. Kuroyanagi N, Onogi H, Wakabayashi T, Hagiwara M. Novel SR-protein-specific kinase, SRPK2, disassembles nuclear speckles. Biochem Biophys Res Comm 1998;242:357–64.
57. Siebel C, Feng L, Guthrie C, Fu XD. Conservation in budding yeast of a kinase specific for SR splicing factors. Proc Natl Acad Sci U S A 1999;96:5440–5.
58. Yeakley J, Tronchere H, Olesen J, Dyck J, Wang H, Fu XD. Phosphorylation regulates in vivo interaction and molecular targeting of serine/arginine-rich pre-mRNA splicing factors. J Cell Biol 1999;145:447–55.
59. Gui J, Tronchere H, Chandler S, Fu XD. Purification and characterization of a serine kinase specific for the serine- and arginine-rich pre-mRNA splicing factors. Proc Natl Acad Sci U S A 1994;91:10824–8.
60. Gui J-F, Fu XD. A serine kinase regulates intracellular localization of splicing factors in the cell cycle. Nature 1994;369:678–82.
61. Koizumi J, Okamoto Y, Onogi H, Mayeda A, Krainer A, Hagiwara M. The subcellular localization of SF2/ASF is regulated by direct interaction with SR protein kinases (SRPKs). J Biol Chem 1999; 274:11125–31.
62. Wang H, Lin W, Dyck J, Yeakley J, Songyang Z, Cantley L, et al. SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. J Cell Biol 1998;140:737–50.
63. Colwill K, Pawson T, Andrews B, Prasad J, Manley J, Bell J, et al. The clk/sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. EMBO J 1996;15:265–75.
64. Colwill K, Feng L, Yeakley J, Gish G, Cáceres J, Pawson T, et al. SRPK1 and Clk/Sty protein kinases show distinct substrate specificities for serine/arginine-rich splicing factors. J Biol Chem 1996; 271:24569–75.
65. Duncan P, Stojdl D, Marius R, Bell J. In vivo regulation of alternative pre-mRNA splicing by the clk1 protein kinase. Mol Cell Biol 1997;17:5996–6001.
66. Nayler O, Stamm S, Ullrich A. Characterization and comparison of four serine and arginine rich (SR) protein kinases. Biochem J 1997;326:693–700.
67. Hanes J, von der Kammer H, Klaudiny J, Scheit K. Characterization by cDNA cloning of two new human protein kinases. J Mol Biol 1994;244:665–72.
68. Melcher M, Thorner J. Identification and characterization of the CLK1 gene product, a novel CaM kinase-like protein kinase from the yeast Saccaromyces cerevisiae. J Biol Chem 1996;271: 29958–68.
69. Nayler O, Schnorrer F, Stamm S, Ullrich A. The cellular localization of the murine serine/arginine-rich protein kinase CLK2 is regulated by serine 141 autophosphorylation. J Biol Chem 1998; 273:34341–8.