Association of Phosphorylated Serine/Arginine (SR) Splicing Factors With The U1-Small Ribonucleoprotein (snRNP) Autoantigen Complex Accompanies Apoptotic Cell Death

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Summary

Proteins subject to proteolysis or phosphorylation during apoptosis are commonly precipitated by autoantibodies found in the serum of patients with systemic lupus erythematosus (SLE). We screened a panel of murine monoclonal and human monospecific sera reactive with known autoantigens for their ability to selectively precipitate phosphoproteins from apoptotic Jurkat T cell lysates. Sera known to recognize the U1-small nuclear ribonucleoprotein (snRNP) complex (confirmed by their ability to precipitate U1-snRNA) selectively precipitated a phosphoprotein complex (pp54, pp42, pp34, and pp23) from apoptotic lysates. Monoclonal antibodies reactive with U1–snRNP proteins precipitated the same phosphoprotein complex from apoptotic lysates. The phosphorylation and/or recruitment of these proteins to the U1–snRNP complex is induced by multiple apoptotic stimuli (e.g., Fas ligation, gamma irradiation, or UV irradiation), and is blocked by overexpression of bcl-2. The U1–snRNP-associated phosphoprotein complex is immunoprecipitated by monoclonal antibodies reactive with serine/arginine (SR) proteins that comprise a structurally related family of splicing factors. The association of phosphorylated SR proteins with the U1–snRNP complex in cells undergoing apoptosis suggests a mechanism for regulation of alternative splicing of apoptotic effector molecules.

Components of ribonucleoproteins (RNP) such as Ro, La, heterogeneous nuclear (hnRNP), and small nuclear (snRNP) are commonly recognized by autoantibodies found in the serum of patients with autoimmune disease (1–4). These mechanisms by which these and other autoantigens escape tolerance are largely unknown. The observation that keratinocytes subjected to ultraviolet radiation express autoantigens such as Ro, La, and the U1-70 kD snRNP protein at cell surface blebs suggests that apoptotic cells may play an important role in the production of autoantibodies (5–7). This is supported by experiments demonstrating the development of autoantibodies after immunization of mice with apoptotic cells (8). Proteolytic cleavage of at least 13 known protein autoantigens by individual interleukin-1β converting enzyme (ICE) family proteases (now collectively termed cysteine protease with aspartic acid substrate specificity, or “caspases” [9]) during programmed cell death further supports this hypothesis. To date, over half of all caspase targets are autoantigens or are constituents of larger complexes that contain a protein that is cleaved, and include the U1-70 kD snRNP (10), poly A ribose polymerase (PARP; reference 11), DNA-dependent protein kinase (DNA-PK; 12), hnRNP C1 and C2 (13), lamins A, B, and C (14), the nuclear mitotic apparatus protein (NuMA; 15, 16), topoisomerases 1 and 2 (16), the nucleolar protein UBF/NOR-90 (16), and α-fodrin (17, 18).

Although proteolysis could expose novel epitopes required for the production of autoantibodies, only a fraction of the known autoantigens are cleaved during apoptosis. Recently, we reported that phosphoproteins are commonly

Abbreviations used in this paper: DNA-PK, DNA-dependent protein kinase; HI-FCS, heat-inactivated FCS; hnRNP, heterogeneous nuclear RNP; MCTD, mixed connective tissue disease; NuMA, nuclear mitotic apparatus protein; PARP, poly A ribose polymerase; PCNA, proliferating cell nuclear antigen; PVDF, polyvinylidene difluoride; RNP, ribonucleoprotein; snRNP, small nuclear RNP; SLE, systemic lupus erythematosus; Sm, Smith complex; SR, serine/arginine; SRP, signal recognition particle; TIAR, T cell intracellular antigen-related protein.
precipitated from apoptotic cell extracts by autoantibodies
derived from patients with systemic lupus erythematosus
(SLE), suggesting that protein modifications accompanying
apoptosis might generally predispose to autoantibody for-
mation (19). We previously identified seven phosphopro-
teins (termed pp200, pp54, pp46, pp42, pp34, pp23, and
pp17) in Jurkat T cells that are specifically precipitated with
autoimmune sera in response to apoptotic stimuli (19). We
also showed that a serine kinase activity is present in immu-
noprecipitates prepared from apoptotic Jurkat cell extracts
using sera from patients with SLE and SLE overlap syn-
dromes. We proposed that phosphorylation of autoantigens
may be a common sequela of apoptotic cell death, and we
postulated that these phosphoproteins, like other kinase
substrates, such as c-jun, may be involved in the effector
arm of the cell death pathway.

Well-characterized, monospecific human sera have been
used in several recent studies to identify autoantigens that
are cleaved during apoptosis (12, 16). We have used a simi-
lar approach to identify autoantigens that are selectively
phosphorylated during apoptosis. Although most of the sera
did not precipitate phosphoproteins from radiolabeled apop-
totic lysates, five sera known to recognize the U1–snRNP
complex precipitated phosphoproteins migrating with ap-
parent molecular masses of 54, 42, 34, and 23 kD by 2D-S
PAGE. A series of human autoimmune sera directed against
the U1–snRNP, but not the U2–snRNP, also coprecipi-
tated this same phosphoprotein complex. Identical results
were obtained using anti-U1A human variable domain an-
tibody fragments and monoclonal antibodies directed against
individual components of U–snRNPs. Because the relative
migration of these U1–snRNP–associated phosphoproteins
resembled the serine/arginine (SR) complex of splicing fac-
tors, we used antibodies reactive with SR proteins to pre-
cipitate phosphoproteins from apoptotic lysates. A mono-
clonal antibody specific for a phosphopeptide common to
all SR proteins (mAb104) and a monoclonal antibody spe-
cific for the phosphorylated form of the SR protein SC35
precipitated a similar phosphoprotein complex from these
lysates. The identification of SR proteins as potential sub-
strates for a serine kinase that is activated during apoptosis has
important implications for understanding cell death path-
ways, RNA splicing, and the immune response in diseases
that are characterized by the development of autoantibodies.

Materials and Methods

Cell Culture. Jurkat cells were grown in 5% CO2 at 37°C us-
ing RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supple-
mented with 9% heat-inactivated FCS (H-H-FCS; Tissue Culture
Technologies, Inc., Herndon, VA). Cells were grown and harvested at mid-
log phase. Jurkat T cells engineered to stably overexpress bcl-2
(or empty vector), a gift from John Reed (the La Jolla Cancer
Research Foundation, La Jolla, CA), were grown in RPMI me-
dium as described above supplemented with G418 (GIBCO
BR L, Gaithersburg, MD) at a final concentration of 500 μg/ml.
Protein overexpression was confirmed by Western blotting.
used: anti-Ro, anti-La, anti-Smith complex (Sm), anti-Jo-1, anti-
nucleolar, anticentromere, anti-Scl-70, anti-DNA, and anti-U1-
RNP (Arthritis Foundation/CDC Reference Sera, Atlanta, GA); anti-
Th/To, anti-U3-fibrillarin, anti-signal recognition particle (SRP), anti-
PL-7, and anti-PL-12 (T. Medsger and N. Fertig, University of Pittsburgh School of Medicine, Pittsburgh, PA); two different anti-U1/U2 monospecific sera (Sera Y and G; reference 22) and anti-SR P (J. Craft, Yale University School of Medicine, New Haven, CT); anti-N-U3mA (serum AS), and anti-U-BF (serum JO; E. Tan and C. Casiano, The Scripps Institute, La Jolla, CA); anti-RNA polymerase I/III and II (serum KA), anti-poly
merase I/III (serum IM), anti-Th/To, anti-U3-fibrillarin, anti-Ku, and anti-Scl-70 (M. Kuwana, Keio University Medical School, Tokyo, Japan); anti-ribosomal P and antihistone/U-
RNP (Immunovision Inc., Springdale, AZ); anti-U1-70-kD sn-
rNP protein (A. Rosen, the Johns Hopkins University School of Medicine, Baltimore, MD); anti-sp140, and anti-sp100 (D. Bloch, Massachusetts General Hospital, Boston, MA); seven human sera specific for the U1–snRNP complex (883, B152, B175, H34, H165, K4, and L41) and a control serum specific for both U1 and U2–snRNPs (V26) have been reported previously (23, 91). Serum from patients with SLE and mixed connective tissue disease (MCTD) with high titers of antibodies against Sm and RNP components, respectively, were provided by P.H. Schur (Brigham and Women’s Hospital, Boston, MA). Autoimmune serum capable of precipitating psp94, pp42, pp34, and pp23 (corresponding to proteins 1, 8, 11, and 12) were described previously (19). Serum from a fifth patient (patient 3) also coprecipitated proteins but was unavailable in sufficient quantity to complete the studies described below (19). The following mouse monoclonal antisera were stored at −70°C until used: anti-lamin B (E3), and anti-lamin A + B (E6; E.A. Nigg, University of Geneva, Switzerland); anti-lamin B (Calbiochem-Nova-biochem Corp., San Diego, CA); anti-polymerase II (Genzyme Laboratories, Inc., San Francisco, CA); anti-DNA-PK (D. Weaver, Dana Farber Cancer Institute, Boston, MA); two monoclonal anti-Ku antibodies (C. Zhang, Dana Farber Cancer Institute, Boston, MA); anti-Ki67 (D. Bloch, Massachusetts General Hospital, Boston, MA); anti-U1-70-kD (reference 24; S. Hoch, the Agouron Institute, La Jolla, CA); anti-Sm Y12 (J. Craft, Yale University School of Medicine, New Haven, CT); anti-U1A/U2B′′ 9A9 (25); anti-U2B′′ 4G3 (25); anti-Ro 60 2G10 (26); anti-Ro52 and anti-La SW5 (27) have been described previously; mAb104 monoclonal directed against SR proteins (reference 28; R. Reed, Harvard University School of Medicine, Boston, MA); anti-SC35 (Sigma Chemical Co.); and antifibrillarin monoclonal antibodies (728.9D.31 and 17C.12G.9) and two monoclonal antibodies directed against other U3–snRNP components (7G3.87 and 6G10.D3; K.M. Pollard, Scripps Institute, La Jolla, CA). Serum from healthy control patients was a gift from Dr. M. Fraser (Brigham and Women’s Hospital, Boston, MA). Immunoprecipitations were performed after addition of 1% BSA (Inter-
gen Company, Purchase, N.Y.) in PBS to a total volume of 500 μl and rotation in a 4°C cold room for 2–24 h. Comparison of precipitates showed no difference between incubation times for periods of up to 72 h. Precipitates were harvested by centrifuging for 15 s at 14,000 rpm in a refrigerated Eppendorf microfuge, washing three times with N-P 40 lys buffer supplemented with protease inhibitor cocktail, resuspending in SDS loading buffer with 9% 2-mercaptoethanol, boiling for 5 min, and separating by PAGE as described (29). Proteins were transferred to nitrocellu-
llose (Schleicher & Schuell, Inc., Keene, N.H.) for Western blotting or tryptic mapping experiments, or to polyvinylidene difluo-
ride (PVDF; Dupont-NEN, Boston, MA) for phosphoamino acid analysis, and either exposed for autoradiography or subjected to Western blot analysis as described (30). The anti-bcl-2 mouse monoclonal antibody 4D7 (PharMingen, San Diego, CA) was used for blotting studies at a dilution of 1:1,000. Nitrocellulose blots were blocked with 5% Blotto (Bio-Rad Laboratories, Hercules, CA) in PBS overnight at 4°C. Bands were visualized using ECL chemiluminescence performed according to the manufacturer’s instructions (Amersham Corp.).

Phosphoamino Acid Analysis. Immunoprecipitates that had been electrophoresed and transferred to PVDF were rinsed thoroughly with water, exposed for radiography, and then appropriate bands were excised with a razor blade. The radiolabeled bands were then subjected to acid hydrolysis as described (31).

RNA Isolation and Identification. Immunoprecipitates from 32p-
labeled Jurkat cells were prepared as described above. After the third N-P 40 lys buffer wash, the immunoprecipitate was dis-
gested in a volume of 300 μl for 1 h at 37°C in a solution contain-
ing 50 μg/ml proteinase K (Sigma Chemical Co.), 10 mM Tris, pH 7.8, 10 mM EDTA, and 0.5% SDS. The RNA was iso-
lated after two extractions with a phenol/chloroform/isooamyl alcohol (25:24:1) mixture (GIBCO BRL). The RNA was precipi-
ted overnight at −70°C after the addition of 20 μl 3 M sodium acetate, 400 μl ethanol, and 10 μg transfer RNA (Sigma Chemi-
cal Co.) as a carrier. The pellet was obtained after a 15-min cen-
trifugation in an Eppendorf centrifuge maintained at 4°C. The pellet was washed once with 70% ethanol, dried in a fume hood, and subjected to PAGE on 6% sequencing gels. A small amount of whole cell lysate was also processed as above and included as an internal standard on each gel.

Two-Dimensional Phosphopeptide Analysis. Two-dimensional tryptic phosphopeptide mapping was performed as described (32) using trypsin (Worthington Biochemical Corp., Freehold, N.J.) at a concentration of 0.1 mg/ml in 50 mM ammonium bicarbonate. Plates were exposed to film at −70°C with an intensifying screen for 2 d.

Results

A autoimmune Sera Precipitate Phosphoproteins from Lysates Prepared from Jurkat T Cells Undergoing Fas-induced Apoptosis. Sera reactive with known autoantigens were first tested for their ability to precipitate the expected protein or complex from extracts prepared from 32P-labeled Jurkat cells, as detected by SDS-PAGE followed by autoradiographic exposure. Sera that precipitated ambiguous patterns of proteins were subjected to further analysis by Western blotting of both whole cell extracts and immunoprecipitates prepared as above, to confirm that the well-char-
acterized sera precipitated the expected target antigen. Most of the sera were derived from patients with autoimmune disease. In addition, six murine monoclonal antibodies re-
active with known autoantigens (Ku, DNA-PK, lamin A, and Ki67, and PCNA) were included in this screen. Jurkat cells metabolically labeled with 32P-orthophos-
phate were cultured for 3 h in the absence or presence of a monoclonal antibody reactive with Fas (anti-7C11), solubi-
lized in N-P 40 lys buffer, and immunoprecipitated using patient serum or monoclonal antibodies as previously de-

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Autoimmune Sera Coprecipitate U-snRNP Proteins. Immunoprecipitates were prepared from ^35S-labeled lysates prepared before (−) or 3 h after (+) Fas ligation (Fig. 1 B). Although immunoprecipitates prepared from apoptotic and nonapoptotic lysates contained ^35S-labeled proteins migrating similarly to the phosphoproteins identified in Fig. 1 A, we were unable to establish the relationship between the ^32P- and ^35S-labeled proteins. Nevertheless, we did not observe the appearance of new ^35S-labeled proteins corresponding to components of the phosphoprotein complex. Moreover, all five sera precipitated U-snRNP components (e.g., the A, B, B', and C proteins, indicated by arrows) from labeled Jurkat cell extracts (Figs. 1 B and 2, also see below). Six other sera from patients with SLE and MCTD possessing high titers of antibodies against Sm or RNP components were unable to reproducibly precipitate the U1-snRNA molecule from labeled Jurkat cell extracts (data not shown). The phosphorylation of these proteins did not result from a nonspecific, general increase in kinase activity after Fas engagement, as ^32P-labeled, whole cell extracts prepared from untreated and apoptotic cells were identical when analyzed by SDS PAGE (data not shown). Moreover, this pattern was similar to that observed using four distinct sera described in our previous report, suggesting that these four proteins (termed pp54, pp42, pp34, and pp23) may be previously unrecognized components of U-snRNP complexes (19). As reported previously (19), the constitutive phosphorylation of La (Fig. 1 A, R0 and L0) was unaltered in cells undergoing apoptosis.
Jurkat cells metabolically labeled with $^{32}$P-orthophosphate and solubilized in NP-40 lysis buffer. After immunoprecipitation with the indicated serum, RNA was extracted and separated on 6% sequencing gels before drying and autoradiographic exposure. The relative migration of known RNA moieties is depicted on the right side of the figure. The serum specificity is indicated above each lane. Lanes are numbered at the bottom of the panel. Lanes 1–4, patients 1, 8, 11, 12 (19); U-serum 1, Immunovision antihistone/RNP; U-serum 2, CDC/AF reference serum 4 (anti-U1-RNP); U-serum 3, serum Ga; U-serum 4, serum Ya; U-serum 5, CDC/AF reference serum 5 (anti-Sm); U-serum 6, anti-U1-70 kD serum (gift of A. Rosen).

Figure 2. Coprecipitation of U1–snRNA using selected autoantisera. Jurkat cells were labeled with $^{32}$P-orthophosphate and solubilized in NP-40 lysis buffer. After immunoprecipitation with the indicated serum, RNA was extracted and separated on 6% sequencing gels before drying and autoradiographic exposure. The relative migration of known RNA moieties is depicted on the right side of the figure. The serum specificity is indicated above each sample. Lanes are numbered at the bottom of the panel. Lanes 1–4, patients 1, 8, 11, 12; U-serum 1, Immunovision antihistone/RNP; U-serum 2, CDC/AF reference serum 4 (anti-U1-RNP); U-serum 3, serum Ga; U-serum 4, serum Ya; U-serum 5, CDC/AF reference serum 5 (anti-Sm); U-serum 6, anti-U1-70 kD serum (gift of A. Rosen).

Figure 3. U1-specific autoantisera coprecipitate the U1–snRNA molecule and pp54, pp42, pp34, and pp23 from apoptotic extracts. (A) Jurkat cells were labeled with $^{32}$P-orthophosphate and lysed in NP-40 lysis buffer. After immunoprecipitation with the indicated serum, RNA was extracted and separated on 6% sequencing gels before drying and autoradiographic exposure. Patient sera specific for the U1–snRNP complex were used in lanes 1–7. A patient serum (V26, lane 8) capable of precipitating both the U1– and U2–snRNPs is shown for comparison. The relative migration of the U1– and U2–snRNPs is depicted on the right side of the figure. (B) Jurkat cells were labeled with $^{32}$P-orthophosphate and lysed either before (−) or 3 h after (+) the addition of anti-Fas (7C11) before immunoprecipitation using sera derived from the indicated patient. Immunoprecipitates were separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and subjected to autoradiographic analysis. Sera correspond to the seven U1-specific autoantisera shown in Fig. 3A. The relative migration of molecular size markers in kilodaltons is indicated on the left side of the figure. Bands corresponding to pp54, pp42, pp34, and pp23 are shown on the right side of the panel. A high molecular mass complex is indicated with a large arrowhead. Lanes are numbered at the bottom of the figure.
precipitated either the U1–snRNP or any of the four phosphoproteins (data not shown). Several sera also precipitated phosphoproteins between 96 and 200 kD that were no longer detected after Fas stimulation (e.g., B152, H34, and K4, Fig. 3B, lanes 3, 7, and 11), again suggesting that these sera likely recognize heterogeneous epitopes of the U1–snRNP particle. It is unknown if this represents dephosphorylation, caspase cleavage, or dissociation of these phosphoproteins from the immunoprecipitate after the apoptotic stimulus. These results suggest that the U1–snRNP is a dynamic particle that is altered by caspases (U1-70 kD protein; reference 10), and potentially by kinases (pp54, pp42, pp34, and pp23; reference 19) and phosphatases (the high molecular mass protein complex shown in Fig. 3B) during apoptosis.

Monoclonal antibodies directed against U1–snRNP components precipitate pp54, pp42, pp34, and pp23 from extracts prepared from apoptotic Jurkat cells. (A) Jurkat cells were labeled with 32P-orthophosphate and lysed either before (+) or 3 h after (-) the addition of anti-Fas 7C11. Proteins were then precipitated using the indicated autoimmune serum, separated on a 12% SDS–polyacrylamide gel, transferred to nitrocellulose, and then exposed for autoradiography. The relative migration of molecular mass markers in kilodaltons is indicated on the left side of the gel. Lanes are numbered at the bottom of the panel. Lanes corresponding to pp90, pp54, pp42, pp34, and pp23 are shown on the right side of the panel. Lanes are numbered at the bottom of the panel. (B) The identical experiment in 35S-labeled Jurkat cells. The relative migration of molecular size markers in kilodaltons is indicated on the left side of the gel. Lanes are numbered at the bottom of the figure. (C) Phosphoamino acid analysis of pp54, pp42, pp34, and pp23. Jurkat cells were labeled with 32P-orthophosphate, treated with the anti-Fas monoclonal antibody 7C11, and solubilized using NP-40 lysis buffer after 3 h. Proteins were then precipitated with the anti-U1A/U2B′′′′ monoclonal antibody 9A9, separated on a 12% SDS–polyacrylamide gel, transferred to PVDF, and exposed for autoradiography. Individual phosphoproteins were localized on the membrane, excised, and then 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 before autoradiographic analysis. Individual proteins are labeled on the side of each panel. Migration of phosphoamino acid standards are labeled with circles as follows: phosphoserine (pS), phosphothreonine (pT), phosphotyrosine (pY). (D) Anti-U1A antibody fragments coprecipitate pp54, pp42, pp34, and pp23 from apoptotic Jurkat cell lysates. Labeled Jurkat cell extracts were prepared as above. Proteins were precipitated using the indicated anti-U1A antibody fragments, separated on a 12% SDS–polyacrylamide gel, transferred to nitrocellulose, and then exposed for autoradiography. The relative migration of molecular mass markers in kilodaltons is indicated on the right side of the gel. Lanes corresponding to pp54, pp42, pp34, and pp23 are shown on the left side of the panel.
clonal antibody (9A9) that recognizes an epitope common to both U1A and U2B' for their ability to precipitate pp54, pp42, pp34, and pp23 from apoptotic extracts Jurkat cells metabolically labeled with 32P-orthophosphate were cultured for 3 h in the absence or presence of a monoclonal antibody reactive with Fas (anti-7C11), solubilized in NP-40 lysis buffer, and individually immunoprecipitated using each of these antibodies; control antibodies directed against other RNA-binding proteins included monoclonal antibodies against R o60, R o52, La, and the anti-T cell intracellular antigen-related protein (TIA-1) antibody 6E3 (33). As shown in Fig. 4A, the Smith antibody Y12, and the 9A9 monoclonal antibody specific for the U1A and U2B' proteins (both of which recognize components common to both U1- and U2-snRNPs) precipitate all four phosphoproteins from apoptotic Jurkat cell lysates (Fig. 4, A and B, lanes 2 and 8). These bands are absent in the lanes corresponding to the immunoprecipitation using anti-U2B' (Fig. 4, A and B, lane 6) and anti–U1-70 kD monoclonal antibodies (A and B, lane 4, see Discussion). Interestingly, increased phosphorylation of a 90-kD protein is observed after Fas stimulation when immunoprecipitates are prepared using anti-U2B' (4G3) antibody (Fig. 4, A and B, lane 6), and on a short exposure of the lanes corresponding to the U1A/U2B' immunoprecipitate (Fig. 4, A and B, lanes 7 and 8, data not shown), suggesting that a specific phosphoprotein (henceforth called pp90) is associated with the U2-snRNP during apoptosis. Bands corresponding to pp90, pp54, pp42, pp34, and pp23 are absent using monoclonal antibodies directed against TIA-1 (6E3, Fig. 4, A and B, lane 10), Ro60 (lane 12), Ro52 (lane 14), La (lane 16), or the putative apoptosis effector TIA-1 (data not shown), another autoantigen that is known to be reversibly phosphorylated during Fas-mediated apoptosis, but at an earlier time point (19, 34, 35). The same experiment performed in cells labeled with [35S]methionine and cysteine (Fig. 4B) demonstrates no difference between immunoprecipitates prepared from apoptotic and nonapoptotic cell extracts, consistent with the results shown in Fig. 4B. Phosphoamino acid analysis of all four proteins precipitated using anti-U1A/U2B' (9A9) demonstrates exclusive phosphorylation of pp54, pp42, pp34, and pp23 on serine residues (Fig. 4C).

The failure of the U1-70 kD monoclonal antibody to precipitate pp54, pp42, pp34, and pp23 from apoptotic lysates appeared to be inconsistent with the hypothesis that these phosphoproteins are specifically associated with the U1-snRNP during apoptosis. To address this apparent paradox (see Discussion), we used two previously described human variable domain antibody fragments directed against a different, unique component of the U1-snRNP (the U1A protein) and repeated the immunoprecipitation experiments (21). Both antibodies (Fig. 4D, lanes 1-4) coprecipitate a phosphoprotein complex containing pp54, pp42, pp34, and pp23, but not pp90. A control antibody fragment directed against bovine serum albumin precipitates only a faint, nonspecific 60-kD protein (Fig. 4D, lanes 5 and 6). Taken together, these results demonstrate an association between a phosphoprotein complex (containing pp54, pp42, pp34, and pp23) and the U1-snRNP during apoptosis and suggests that pp90 may be associated specifically with the U2-snRNP during apoptosis.

A ssociation of pp54, pp42, pp34, and pp23 with U1-snRNPs A companies A poptosis but N ot T Cell R eceptor Stimulation. Previously, we had demonstrated that, in addition to death induced by Fas ligation, phosphorylated autoantigens are also immunoprecipitated during apoptosis triggered by other stimuli including gamma and UV irradiation, but not by T cell receptor stimulation (19). We repeated this experiment using the anti-U1A/U2B' (9A9) monoclonal antibody in immunoprecipitation experiments using 32P-labeled Jurkat lysates prepared from cells subjected to apoptotic stimuli or an activation stimulus over a 5-h time course (Fig. 5). This analysis reveals that phosphorylated autoantigens are precipitated beginning at the 3-h time point after Fas cross-linking (Fig. 5, lanes 1-4) or UV irradiation (Fig. 5, lanes 11-14), and much less intense bands are observed 5 h after gamma irradiation (Fig. 5, lanes 5-7), consistent with our initial observations (19). In contrast, ligation of the T cell receptor complex using a monoclonal antibody reactive with CD3, a stimulus that induces IL-2 production and enhances proliferation in these cells (data not shown), induced neither precipitation of phosphoproteins (Fig. 5, lanes 8-10) nor DNA fragmentation (data not shown and reference 19) over the course of this experiment.

Bcl-2 Overexpression Blocks Gamma Irradiation-induced A poptosis and Precipitation of pp54, pp42, pp34, and pp23. Next, we asked whether the precipitation of these phos-

**Figure 5.** Phosphoprotein components of the U1-snRNP complex are precipitated after multiple apoptotic stimuli but not an activation stimulus. Jurkat cells were labeled with 32P-orthophosphate, treated with the indicated stimuli, and then solubilized using NP-40 lysis buffer at the indicated times. Proteins were then precipitated with anti-U1A/U2B' (9A9), separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and then exposed for autoradiography. The apoptotic stimulus is indicated above each panel. The time in hours after each stimulus is indicated above each lane. The relative migration of molecular mass markers in kilodaltons is indicated on the left side of the panel. Bands corresponding to pp54, pp42, pp34, and pp23 are shown on the right side of the panel. Lanes are numbered at the bottom of each panel.
phosphorylated U1-snRNP components could be blocked by overexpression of the bcl-2 protein, which has been shown to efficiently block apoptosis induced by multiple apoptotic stimuli, including gamma irradiation and UV irradiation (19, 36–39). As shown in Fig. 6, Jurkat cells stably transformed with either bcl-2 (lanes 1–4) or empty vector (lanes 5–8) were labeled with 32P-orthophosphate and subjected to gamma irradiation, solubilized in NP-40 lysis buffer, precipitated using anti-U1 A/U2B (9A9) monoclonal antibody. Whereas precipitated U1 A/U2B (9A9) was phosphorylated, U1A/U2B (9A9) control transformants (lanes 5–8) were unphosphorylated. Bands corresponding to pp54, pp42, pp34, and pp23 are shown on the right side of the figure. The time, in hours, from initial exposure to gamma irradiation is indicated at the top of each lane. Lane numbers appear at the bottom of the figure.

Figure 6. In vivo phosphorylation of U1-snRNP components is inhibited in gamma-irradiated Jurkat cells overexpressing bcl-2. Jurkat (bcl-2) transformants (lanes 1–4) or Jurkat (neo) control transformants (lanes 5–8) were labeled with 32P-orthophosphate, subjected to gamma irradiation, solubilized in NP-40 lysis buffer, precipitated using anti-U1 A/U2B (9A9) antibodies, separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and then exposed for autoradiography. The relative migration of molecular size markers in kilodatons is indicated on the left side of the figure. Bands corresponding to pp54, pp42, pp34, and pp23 are shown on the right side of the figure. The time, in hours, from initial exposure to gamma irradiation is indicated at the top of each lane. Lane numbers appear at the bottom of the figure.

Discussion

Autoimmune sera have been used extensively as probes to identify, characterize, and clone proteins and RNA molecules with important cellular functions. Proteins such as La and the U1-70 kD component of the U1-snRNP complex were cloned using serum from SLE patients to screen a phage expression library (41, 42), and antibodies directed against Sm proteins and the Th/To antigen have been used to identify their function in vitro by inhibiting specific steps in mRNA and nucleolar RNA processing, respectively (43, 44). More recently, serum from such patients has proven useful for the identification of proteins that are cleaved by caspases during apoptosis. We have used a similar strategy to identify proteins that may be phosphorylated during stress-induced apoptosis (19). Before our recent report (18), only a few such proteins had been identified, and several of these proteins have been implicated as critical effectors of cell death. The TIA-1 autoantigen (Utz, P.J., and P. Anderson, unpublished data) is phosphorylated by Fas-
activated serine/threonine kinase (FAST kinase) during apoptosis (35), and it is postulated that TIA-1 and a related protein, TIAR, differentially regulate RNA metabolism in response to apoptotic stimuli (Anderson, P., and N. Kedersha, unpublished observations; reference 34). Another kinase (termed JNK) phosphorylates and activates the c-jun transcription factor in response to multiple apoptotic stimuli (45–48). Overexpression of an NH$_2$-terminal deletion mutant of the c-jun protein acts as a dominant negative suppressor of apoptosis. Recent reports, however, suggest that kinases such as JNK are activated after both lethal and nonlethal stimuli, and thus may be dispensable for execution of the apoptotic program under some circumstances (49, 50).

The U-snRNPs are a group of related nuclear particles containing a unique, uridine-rich, structural RNA (termed the U-snRNA) and a core of six or more polypeptides (51). The most abundant of these, the U1-, U2-, U5-, and U4/U6-snRNP complexes are known autoantigens (22, 51–54) and play critical roles in the splicing of pre-mRNA molecules. During splicing, U-snRNPs assemble into a macromolecular structure termed a “spliceosome” whose function is to efficiently and precisely process introns from pre-mRNA before export of the mature mRNA from the nucleus. The fidelity of this complex process is facilitated by other splicing factors that transiently associate with the U-snRNP complexes, particularly the U1- and U2-snRNPs (55, 56). Splicing factors belonging to the SR family are highly conserved proteins containing one or more RNA recognition motifs (RRMs) at their NH$_2$ termini and a SR highly conserved proteins containing one or more RNA (55, 56). Splicing factors belonging to the SR family are U–snRNP complexes, particularly the U1– and U2–snRNPs by other splicing factors that transiently associate with the nucleus. The fidelity of this complex process is facilitated pre-mRNA before export of the mature mRNA from the function is to efficiently and precisely process introns from a macro-molecular structure termed a “spliceosome” whose role in RNA splicing is facilitated by other splicing factors that transiently associate with the U-snRNP complexes, particularly the U1- and U2-snRNPs (55, 56). Splicing factors belonging to the SR family are highly conserved proteins containing one or more RNA recognition motifs (RRMs) at their NH$_2$ termini and a SR repeat of varying length in their COOH termini (57). Structural analysis of the SR protein ASF/SF2 demonstrates that the SR domains are required for protein phosphorylation and constitutive RNA splicing but are dispensable for alternative splicing. Targeted disruption of the RRM domains blocks RNA binding and constitutive splicing activity (58, 59). At least eight proteins containing SR domains have been identified in humans, including the U1-70 kD protein, SR p75, SR p54, SR p40, ASF/SF2, SC35, U2AF35, and SR p20. Six of these eight proteins (SR p54, SR p40, ASF/SF2, SC35, U2AF35, and SR p20) are similar in size to the proteins described above (pp54, pp42, pp34, and pp23). It has been postulated that SR proteins enhance splicing by binding to the U1–snRNP during the formation of a commitment complex, thus stabilizing the spliceosome assembly (60–62). Individual SR proteins can substitute for the U1–snRNP in vitro splicing (63), and SR proteins have been implicated in regulation of both constitutive and alternative splicing of several mRNAs (57, 64–69).

Reversible protein phosphorylation is thought to regulate both constitutive and alternative mRNA splicing. Experiments using phosphatase inhibitors, nonhydrolyzable ATP analogues, or purified phosphatases in in vitro splicing reactions demonstrate a requirement for reversible protein phosphorylation for mRNA splicing (70–72), and several kinases capable of phosphorylating SR proteins have been identified. The U1-70 kD snRNP protein is an in vivo and in vitro substrate for an unidentified serine kinase that copurifies with the U1–snRNP complex (73). A second kinase, SR protein kinase-1 (SRPK-1), capable of phosphorylating multiple different SR proteins has also been identified (40, 74–76). Interestingly, this kinase is active during mitosis, phosphorylates substrates exclusively on serine residues, copurifies with snRNP complexes, and disrupts both nuclear speckles and in vitro pre-mRNA splicing (40). All five of the known in vitro substrates for SR PK-1 are identical in size to the proteins described in this report and include SR p55 (pp54), SR p40 (pp40), SC35 (pp34), ASF/SF2 (pp34), and SR p20 (pp23; references 19, 40). A related kinase, Clk/Sty, has also been shown to phosphorylate SR proteins in vitro (76, 77). Despite these
intriguing reports, to date there have been no studies directly linking a serine kinase to the phosphorylation of splicing factors during stress-induced apoptosis. Experiments designed to identify whether SR PK-1, Clik/Sty, the U 1-70 kD kinase or a novel serine kinase is responsible for the apoptosis-specific phosphorylation of SR proteins, and the role that this modification plays in apoptosis and alternative mRN A splicing, are in progress.

The evidence suggesting that pp54, pp42, pp34, and pp23 are components of the U1–snRNP is compelling. First, all 4 autoimmune sera from our initial report (19) and 20 sera described herein simultaneously precipitate all 4 phosphoproteins (Figs. 1 and 3 B) together with the U1 RNA (Figs. 2 and 3 A), from lysates prepared from Fas-treated Jurkat cells. Second, two different monoclonal antibodies (Y12 and 9A9) that recognize core (Sm) components of the U1–snRNP complex also precipitate these same four phosphoproteins from extracts prepared from apoptotic Jurkat cells, whereas monoclonal antibodies directed against six other RNA-binding proteins do not (Fig. 4). Third, two human variable domain antibody fragments directed against overlapping epitopes of the U1A protein coprecipitate pp54, pp42, pp34, and pp23 from apoptotic Jurkat cell extracts (Fig. 4 D). Finally, the anti-U1A/U2B” (9A9) monoclonal antibody precipitates all four phosphoproteins from extracts prepared from cells subjected to multiple different apoptotic stimuli but not after engagement of the T cell receptor, and the association of these phosphoproteins with the U−snRNPs is blocked in cells engineered to overexpress bcl-2 (Figs. 5 and 6). Thus, all of the experiments described using SLE sera from our initial report have been replicated using the anti-U1A/U2B” (9A9) monoclonal antibody (19).

It remains to be determined whether phosphoproteins are also associated with the U2− and other U−snRNP complexes during apoptosis. Two human sera and four monoclonal antibodies specific for components of the U3−snRNP complex failed to coprecipitate pp54, pp42, pp34, or pp23 (data not shown). A monoclonal antibody directed against the U2B” protein that uniquely precipitated the U2−snRNA (data not shown) was incapable of precipitating pp54, pp42, pp34, and pp23 in most (>10) experiments. Relatively faint bands migrating at 54, 42, 34, and 23 kD were observed on long exposures (data not shown). While this may represent the direct association of the U2−snRNP and the SR proteins, it is equally plausible that these bands represent the association of SR proteins with U1− and U2−snRNPs in an active spliceosome complex.

The identification of pp54, pp42, pp34, and pp23 as SR proteins is suggested by several observations. The respective SR proteins SRp54, SRp42, SC 35, ASF/SF2, and SRp20 have similar migration patterns on SDS PAGE and are phosphorylated exclusively on serine residues (74). SR proteins also interact with components of the spliceosome and copurify with the U1−snRNA during gel filtration analysis (78, 79) and sucrose gradient centrifugation (our unpublished data). All four proteins (pp54, pp42, pp34, and pp23) comigrate with their respective SR counterparts during two-dimensional gel electrophoresis, and anti-SC3 5 is capable of coprecipitating the U1−snRNA (data not shown). Finally, an identical phosphoprotein complex is precipitated by two monoclonal antibodies specific for the phosphorylated forms of SR proteins (Fig. 7; reference 28, 80). A much more difficult question is whether these proteins are stable components of the U1−snRNP that are phosphorylated de novo after an apoptotic stimulus, or rather are recruited to the U1−snRNP complex during apoptosis. SR proteins have few methionine and cysteine residues (two SR proteins have no methionines other than the initiator), perhaps explaining why bands corresponding to these proteins are not consistently observed when immunoprecipitates are prepared from 35S-labeled Jurkat cells. Although the de novo phosphorylation model is favored by the identification of at least three kinases capable of phosphorylating SR domain-containing proteins (40, 73–77), we cannot exclude the possibility that a small fraction of the phosphorylated forms of pp54, pp42, pp34, and pp23 (i.e., an amount below the level of detection obtained by metabolic labeling with [35S]methionine and cysteine) are recruited to the U1−snRNP complex during apoptosis. The answer to this important question awaits the development of other reagents, including anti-SR antibodies that recognize non-phosphorylated SR proteins and epitope-tagged SR proteins for use in transfection experiments.

The inability of the U1−70 kD monoclonal antibody to coprecipitate pp54, pp42, pp34, and pp23 appeared to contradict our argument that these phosphoproteins are associated with the U1−snRNP during apoptosis. This prompted us to test monoclonal antibodies specific for other components of the U1−snRNP for their ability to precipitate this phosphoprotein complex (Fig. 4 D). We hypothesize that only a subfraction of the U1−snRNP complexes present in a cell is associated with SR proteins. In this model, the U1A/U2B” (9A9), anti-Sm (Y12), and anti-U1A monoclonal antibodies recognize this population, while the U1−70 kD monoclonal antibody recognizes a different population that is incapable of interacting with SR proteins, perhaps by a steric hindrance. It is also possible that caspase-mediated cleavage of U1−70 kD during apoptosis disrupts the interaction of U1−70 kD with other SR proteins, an event that may explain the observation that overexpression of the COOH terminus of U1−70 kD (which contains tandem SR domains that are separately caspase cleavage; reference 81) acts as a dominant negative suppressor of RNA splicing and RNA transport (82). Several reports identifying a direct interaction between U1−70 kD and SR proteins support both possibilities (61, 62).

In addition to transcriptional and translational regulation of apoptosis, our results suggest that a third regulatory mechanism for programmed cell death is at the level of messenger RNA splicing. It has been shown that cells expressing the larger splice variant of the bcl-x gene (bcl-xL) are protected against cell death, while cells expressing the shorter form lacking the highly conserved BH1 and BH2 interaction domains (bcl-xS) have an increased susceptibility.
to cell death (36, 83–85). Similar regulation has been described for the Caenorhabditis elegans ced-4 gene product (86), for the death domain-containing receptor LARD (87), and for caspase 2 (Nedd2/Ich1) in which the protein product of the larger splice variant (Ich1L) is proapoptotic and the shorter variant (Ich1S) is protective (36, 83–85). It has been shown that reversible phosphorylation of SR proteins (e.g., ASF/SF2) can alter their ability to select alternative mRNA splice sites (65, 88, 89). It is tempting to speculate that SR protein phosphorylation may regulate levels of prosurvival factors such as bcl-xL and Ich1S, or of proapoptotic factors such as bcl-xS and Ich1L, thus altering the susceptibility of a particular cell to an apoptotic trigger. Although this is unlikely to be an important mechanism after engagement of a dedicated death receptor such as Fas or the TNF receptor, both of which rapidly activate irreversible caspase cascades, alternative splicing of bcl-x, caspase 2, and other unidentified mRNAs may be a critical checkpoint when cells are subjected to slowly lethal or sublethal stimuli.

Autoantibodies reactive with core components of the U1–snRNP (anti-Sm) are specifically found in patients with SLE. The observation that snRNP particles reside in plasma membrane blebs formed at the surface of cells undergoing apoptosis suggests that antigens presented in this manner might bypass normal mechanisms of tolerance. In addition to its subcellular localization, the U1–snRNP complex undergoes profound structural alterations in cells undergoing apoptosis. These structural alterations could produce novel peptide epitopes to which T cells have not been rendered tolerant. This may be particularly important for the production of autoantibodies reactive with the U1–snRNP complex, which is subject to the phenomenon of “epitope spreading” whereby an immune response to one component of the particle promotes the formation of antibodies reactive with other components of the particle (90). We propose that a T cell response directed against modified components of the U1–snRNP complex (e.g., caspase-cleaved U1-70 kD and/or phosphorylated SR-derived peptides) may promote the formation of antibodies reactive with other components of the complex. By driving the maturation of potentially self reactive B cells specific for components of the U1–snRNP particle, T cells recognizing these neoepitopes could be essential for autoantibody production. It is currently unknown whether human autoantibodies directly recognize SR proteins. With the identification of monoclonal antibodies capable of recognizing pp54, pp42, pp34, and pp23 in apoptotic Jurkat cells, it should now be possible to address these and several other important questions. Are SR proteins stable components of the U1–snRNP complex that are phosphorylated during apoptosis, or are they recruited to the complex during cell death? What is the kinase responsible for SR protein phosphorylation, and what is its role in programmed cell death pathways and RNA splicing? Answers to these important questions and the identification of other posttranslational modifications of autoantigens during apoptosis are certain to yield valuable clues to the pathogenic mechanisms underlying autoimmune diseases such as SLE, scleroderma, MCTD, and Sjögren's Syndrome. Further studies may identify components of this putative kinase pathway as novel molecular targets for pharmacologic therapy of autoimmune disease.
References


560 Serine/Arginine Splicing Factors in Apoptosis


