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

By Paul J. Utz,* Maria H. Hottelet,* Walther J. van Venrooij,‡ and Paul Anderson*

From the *Department of Medicine, Division of Rheumatology, Immunology, and Allergy, Brigham & Women’s Hospital, Boston, Massachusetts 02115; and the ‡Department of Biochemistry, University of Nijmegen, 6500 HB Nijmegen, The Netherlands

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–snRNP) 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 (R NPs) such as Ro, La, heterogeneous nuclear (hnRNP), and small nuclear (snRNP) are commonly recognized by autoantibodies found in the serum of patients with autoimmune diseases (1–4). The 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 (PAR; 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), topoisomerase I 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

1Abbreviations used in this paper: DNA-PK, DNA-dependent protein kinase; HIFCS, heat-inactivated FCS; hnRNP, heterogeneous nuclear RNP; MCTD, mixed connective tissue disease; NuMA, nuclear mitotic apparatus protein; PAR, 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 formation (19). We previously identified seven phosphoproteins (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 immunoprecipitates prepared from apoptotic Jurkat cell extracts using sera from patients with SLE and SLE overlap syndromes. 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 similar approach to identify autoantigens that are selectively phosphorylated during apoptosis. Although most of the sera did not precipitate phosphoproteins from radiolabeled apoptotic lysates, five sera known to recognize the U1–snRNP complex precipitated phosphoproteins migrating with apparent molecular masses of 54, 42, 34, and 23 kD by SDS-PAGE. A series of human autoimmune sera directed against the U1–snRNP, but not the U2–snRNP, also coprecipitated this same phosphoprotein complex. Identical results were obtained using anti-U1A human variable domain antibody 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 factors, we used antibodies reactive with SR proteins to precipitate phosphoproteins from apoptotic lysates. A monoclonal antibody specific for a phosphopeptidome common to all SR proteins (mAb104) and a monoclonal antibody specific for the phosphorylated form of the SR protein SC35 precipitated a similar phosphoprotein complex from these lysates. The identification of SR proteins as potential substrates for a serine kinase that is activated during apoptosis has important implications for understanding cell death pathways, 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 using RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supplemented with 9% heat-inactivated FCS (H-I-FCS; Tissue Culture Materials). These cells were maintained in RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supplemented with 45% RPMI 1640 lacking either phosphate (GIBCO BRL) or methionine and cysteine (GIBCO BRL), 2 mM glutamine (Mediatech, Inc.), 5% HI-FCS, and 0.15 mM NaCl (Sigma Chemical Co., St. Louis, MO), 0.15 mM NaCl (Sigma Chemical Co., St. Louis, MO), 2 mM glutamine, 10% dialyzed HI-FCS, and 0.15 mM NaCl (Sigma Chemical Co., St. Louis, MO).

Methylation. Labeling was performed as described (19). Briefly, Jurkat cells were incubated at a density of 2 × 10⁶ cells/ml in labeling medium containing the following: 45% RPMI 1640, 45% RPMI 1640 lacking either phosphate (GIBCO BRL) or methionine and cysteine (GIBCO BRL), 2 mM glutamine (Mediatech, Inc.), 5% H-I-FCS, and 140 mM NaCl (Sigma Chemical Co., St. Louis, MO). [35S]orthophosphate or [32P]orthophosphate (Dupon, NEN, Boston, MA) was added at a concentration of 0.1 mCi/ml. Cells were incubated at 37°C for 10–16 h to allow the cells to reach steady state before each treatment, unless otherwise indicated. Form two-dimensional tryptic phosphopeptide mapping experiments, cells were labeled for 2 h, followed by a 3-h stimulation with anti-Fas antibodies (7C11) in labeling media composed of 90% RPMI 1640 lacking phosphate, 2 mM glutamine, 10% dialyzed HI-FCS, and 0.15 mCi/ml [35S]orthophosphate.

Cell Lysis. Lysis of cells was performed using N-P-40 (Sigma Chemical Co.) lysis buffer (1% N-P-40, 150 mM NaCl, 50 mM Tris, pH 7.8, and 1 mM EDTA). N-P-40 lysis buffer was supplemented immediately before use with 1 mM sodium vanadate (Sigma Chemical Co.) and a 100× protease inhibitor cocktail prepared by dissolving 10 mg chymostatin, 1.5 mg leupeptin, 7 mg pepstatin A, 850 mg phenylmethylsulfonyl fluoride, 500 mg benzamidine, and 5 mg aprotinin in 50 ml of ethanol by stirring overnight (20). The solution was sterilized by filtration and stored at room temperature. All chemicals were purchased from Sigma Chemical Co. After addition of 1 ml lysis buffer, the lysate was incubated on ice for 30 min, centrifuged in a refrigerated microfuge (5402; Eppendorf Inc., Hamburg, Germany) at 14,000 rpm for 15 min, and then the supernatant was used immediately for each experiment.

UV Irradiation. Labeled Jurkat cells were plated on 100 × 15-mm polystyrene petri dishes (Nunc, Thousand Oaks, CA) at a concentration of 2 × 10⁶ cells/ml and irradiated in a Stratalinker 2400 (Stratagene, La Jolla, CA) at a distance of 9 cm for 18 s. After irradiation, cells were incubated at 37°C for the indicated times before harvesting.

Gamma Irradiation. Labeled cells were in a 50 ml conical tube and irradiated at a dose of 3,300 rad from a Cesium 137 source using an irradiator (GammaCell 1500; Nordion International, Kanata, Ontario, Canada). After irradiation, cells were placed in culture dishes at 37°C and incubated for the indicated times before harvesting.

Cellular Activation. Labeled Jurkat cells were treated with the following antibodies: anti-Fas antibody 7C11 (provided by M. Ichael Robertson, Indiana University, Bloomington, IN) from hybridoma supernatant at a final dilution of 1:500; and anti-CD3 antibody (Coulter Immunology, Hialeah, FL) at a concentration of 5 μg/ml followed by goat anti–mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at the same concentration. Cells were incubated at 37°C for the indicated times before 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 PBS and 5 μg rabbit anti–mouse (RAM) IgG (Jackson ImmunoResearch Laboratories) for 1 h, followed by two precipitates with protein A–Sepharose overnight. Mouse monoclonal antibodies (5 μg) and 5 μg RAM (IgG or IgM), or 2 μl patient serum alone were used in precipitation experiments. Immunoprecipitation experiments using anti-U1A human antibody fragments were performed as described (21). The following human polyclonal antibodies were stored at −70°C until before harvesting.
used: anti-Ro, anti-La, anti-Smith complex (Sm), anti-Jo-1, anti-nucleolar, anticientromere, 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. Medgeder and N. Fertig, University of Pittsburgh School of Medicine, Pittsburgh, PA); two different anti-U1/U2 monoclonal sera (Sera Y and G; reference 22) and anti-SR P (J. Craft, Yale University School of Medicine, New Haven, CT); anti-NuMA (serum AS), and anti-U BF (serum JO; E. Tan and C. Casano, The Scripps Institute, La Jolla, CA); anti-RNA polymerase I/I1 and II (serum KA), anti-polymerase I/I1 (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/URNP (Immunovation Inc., Springdale, AZ); anti-U1-70 kD snRNP 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–snNCP complex (K83, B125, B175, H34, H165, K4, and L41) and a control serum specific for both U1 and U2–snNPs (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 pp54, pp42, pp34, and pp23 (corresponding to patients 1, 8, 11, and 12) were described previously (19). Serum from a fifth patient (patient 3) also coprecipitated these proteins but was unavailable in sufficient quantity to complete the studies described below (19). The following mouse monoclonal antibodies were stored at −70°C until used: anti-lamin B (E3), and anti-lamin A + B (E6; E.A. Nigg, University of Geneva, Geneva, Switzerland); anti-lamin B (Calbiochem-NovaBiochem Corp., San Diego, CA); anti–proliferating cell nuclear antigen (PCNA; Zymed Laboratories, Inc., South San Francisco, CA); anti–DNA-PK (D. Weaver, Dana Farber Cancer Institute, Boston, MA); seven human monoclonal antibodies reactive with Fas (anti-7C11), soluble and rotation in a 4°C solution containing 50 μg/ml proteinase K (Sigma Chemical Co.), 10 mM Tris, pH 7.8, 10 mM EDTA, and 0.5% SDS. The RNA was isolated after two extractions with a phenol/chloroform/isoamyl alcohol (25:24:1) mixture (GIBCO BRL). The RNA was precipitated overnight at −70°C after the addition of 20 μl 3 M sodium acetate, 400 μl ethanol, and 10 μg transfer RNA (Sigma Chemical Co.) as a carrier. The RNP 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, NJ) 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 Jurkat extracts and immunoprecipitates prepared as above, to confirm that the well-characterized sera precipitated the expected target antigen. Most of the sera were derived from patients with autoimmune disease. In addition, six murine monoclonal antibodies reactive with known autoantigens (Ku, DNA-PK, lamin A and B, Ki67, and PCNA) were included in this screen.

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 N-P-40 lys buffer, and immunoprecipitated using patient serum or monoclonal antibodies as previously described (17). Sera from patients with SLE and 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 pp54, pp42, pp34, and pp23 (corresponding to patients 1, 8, 11, and 12) were described previously (19). Serum from a fifth patient (patient 3) also coprecipitated these proteins but was unavailable in sufficient quantity to complete the studies described below (19). The following mouse monoclonal antibodies were stored at −70°C until used: anti-lamin B (E3), and anti-lamin A + B (E6; E.A. Nigg, University of Geneva, Geneva, Switzerland); anti-lamin B (Calbiochem-NovaBiochem Corp., San Diego, CA); anti–proliferating cell nuclear antigen (PCNA; Zymed Laboratories, Inc., South San Francisco, CA); anti–DNA-PK (D. Weaver, Dana Farber Cancer Institute, Boston, MA); seven human monoclonal antibodies reactive with Fas (anti-7C11), soluble and rotation in a 4°C solution containing 50 μg/ml proteinase K (Sigma Chemical Co.), 10 mM Tris, pH 7.8, 10 mM EDTA, and 0.5% SDS. The RNP was isolated after two extractions with a phenol/chloroform/isoamyl alcohol (25:24:1) mixture (GIBCO BRL). The RNA was precipitated overnight at −70°C after the addition of 20 μl 3 M sodium acetate, 400 μl ethanol, and 10 μg transfer RNA (Sigma Chemical Co.) as a carrier. The RNP 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.

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A, B, B, and C are indicated on the right side of the panel. The relative migration of molecular mass markers in kilodaltons is indicated on the left side of each panel. Bands corresponding to the U–snRNP proteins are described in detail in Materials and Methods. Immunoprecipitates were separated on a 12% SDS–polyacrylamide gel, transferred to nitrocellulose, and then subjected to autoradiographic analysis. Individual sera are described in detail in Materials and Methods. Immunoprecipitates were separated on a 12% SDS–polyacrylamide gel, transferred to nitrocellulose, and exposed for autoradiography. Most sera did not precipitate unique phosphoproteins from apoptotic lysates. For example, sera reactive with nuclear proteins implicated in DNA replication, binding, or repair (i.e., Ku, DNA-PK, PCNA, Scl-70, and histone) failed to reproducibly precipitate new phosphoproteins from apoptotic Jurkat cell extracts (data not shown). Similarly, proteins present in the nuclear matrix or involved in mitosis (i.e., lamins A and B, centromere A and B, Ki-67, NuMA, sp140, and sp100), the nucleolus (i.e., Tef/To, UBF/NOR-90, RNA polymerase I, II, and III, and U3-snRNPs), or cytoplasmic components of the translational apparatus (i.e., J0-1, PI-7, PI-12, SRP, and ribosomal P) were unmodified in these experiments (data not shown). In contrast, several sera specific for U–snRNP complexes precipitated phosphoproteins of 54, 42, 34, and 23 kD from apoptotic Jurkat cell extracts (Fig. 1 A). The phosphorylation of these proteins did not result from a nonspecific, general increase in kinase activity after Fas engagement, as $^{32}$P-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, Ro and La) was unaltered in cells undergoing apoptosis.

**Figure 1.** Human autoimmune sera specific for U–snRNP complexes precipitate phosphoproteins from apoptotic Jurkat cell lysates. (A) Jurkat cells were labeled with $^{35}$S methionine 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 exposed for autoradiography. Individual sera are described in detail in Materials and Methods. Immunoprecipitates were separated on a 12% SDS–polyacrylamide gel, transferred to nitrocellulose, and subjected to autoradiographic analysis. The relative migration of molecular mass markers in kilodaltons is indicated on the left side of the gel. (B) Immunoprecipitation from $^{35}$S-labeled Jurkat cell lysates 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 then subjected to autoradiographic analysis. The relative migration of molecular mass markers in kilodaltons is indicated on the left side of each panel. Bands corresponding to the U–snRNP proteins A, B, B, and C are indicated on the right side of the panel.
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metabolically labeled with $^{32}$P-orthophosphate were 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. R. 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 (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. R. Rosen).

5S rRNA

snRNA U2

snRNA U1

snRNA U4

5S rRNA

snRNA U5

snRNA U6

tRNA

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. The relative migration of the U1–snRNP complex 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.

U1 snRNA

U2 snRNA

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precipitated either the U-snRNPs 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. Bands corresponding to 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/U2B99 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. Bands corresponding to pp54, pp42, pp34, and pp23 are shown on the left side of the panel.

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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 R060, R052, La, and the anti-T cell intracellular antigen-related protein (TIA1 antibody 6E3 (33)). As shown in Fig. 4 A, 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 TIA1 (6E3, Fig. 4, A and B, lane 10), R060 (lane 12), R052 (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. 4 B) demonstrates no difference between immunoprecipitates prepared from apoptotic and nonapoptotic cell extracts, consistent with the results shown in Fig. 1 B. 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. 4 C).

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. 4 D, 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. 4 D, 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.

Association of pp54, pp42, pp34, and pp23 with U-snRNPs. A company of apoptosis but not T Cell Receptor 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 Apoptosis and Precipitation of pp54, pp42, pp34, and pp23. Next, we asked whether the precipitation of these phos-
5–8) were labeled with 32P-orthophosphate and subjected to gamma irradiation in Jurkat cells overexpressing bcl-2. Jurkat (neo) control transfomants (lanes 1–4) or Jurkat (bcl-2) transfomants (lanes 5–8) were labeled with 32P-orthophosphate, subjected to gamma irradiation, solubilized in NP-40 lysis buffer, precipitated using anti-U1A/U2B 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.

Figure 6. In vivo phosphorylation of U1–snRNP components is inhibited in gamma-irradiated Jurkat cells overexpressing bcl-2. Jurkat (bcl-2) transfomants (lanes 1–4) or Jurkat (neo) control transfomants (lanes 5–8) were labeled with 32P-orthophosphate, subjected to gamma irradiation, solubilized in NP-40 lysis buffer, precipitated using anti-U1A/U2B 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 Lα 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 T/H T0 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 NH2-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 U1 snRNP complexes are a group of related nuclear particles containing a unique, uridine-rich, structural RNA (termed the U1 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, U1 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 complexity of this complex process is facilitated by other splicing factors that transiently associate with the U1 snRNP complexes, particularly the U1 and U2 snRNP proteins (55, 56). Splicing factors belonging to the SR family are highly conserved proteins containing one or more RNA recognition motifs (RRMs) at their NH2 termini and a SR domain highly conserved proteins containing one or more RNA (55, 56). Splicing factors belonging to the SR family are U1–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 nucleus.

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 demonstrates 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, Cik/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 mRNA splicing, are in progress.

The evidence suggesting that pp54, pp42, pp34, and pp23 are components of the U 1--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 (Y 12 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-U 1A/U 2B*+ (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-U 1A/U 2B*+ (9A9) monoclonal antibody (19).

It remains to be determined whether phosphoproteins are also associated with the U 2-- 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. Rarely, 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 U 2--snRNP and the SR proteins, it is equally plausible that these bands represent the association of SR proteins, U 1-- and U 2--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 SR p54, SR p42, SC 35, ASF/SF2, and SR p20 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 U 1--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-SC35 is capable of coprecipitating the U 1--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 U 1--snRNP that are phosphorylated de novo after an apoptotic stimulus, or rather are recruited to the U 1--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 U 1--snRNP complex during apoptosis. The answer to this important question awaits the development of other reagents, including anti-SR antibodies that recognize nonphosphorylated SR proteins and epitope-tagged SR proteins for use in transfection experiments.

The inability of the U 1-70 kD monoclonal antibody to coprecipitate pp54, pp42, pp34, and pp23 appeared to contradict our argument that these phosphoproteins are associated with the U 1--snRNP during apoptosis. This prompted us to test monoclonal antibodies specific for other components of the U 1--snRNP for their ability to precipitate this phosphoprotein complex (Fig. 4 D). We hypothesize that only a subfraction of the U 1--snRNP complexes present in a cell is associated with SR proteins. In this model, the U1A/U2B*+ (9A9), anti-Sm (Y 12), and anti-U1A monoclonal antibodies recognize this population, while the U 1-70 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 U 1-70 kD during apoptosis disrupts the interaction of U 1-70 kD with other SR proteins, an event that may explain the observation that overexpression of the COOH terminus of U 1-70 kD (which contains tandem SR domains that are separated after caspase cleavage; reference 81) acts as a dominant negative suppressor of RNA splicing and RNA transport (82). Several reports identifying a direct interaction between U 1-70 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 short 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 (Need2/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.

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Address correspondence to Paul J. Utz, Department of Medicine, Division of Rheumatology, Immunology, and Allergy, Brigham & Women’s Hospital, Smith Bldg., Rm. 608, 75 Francis St., Boston, MA 02115. Phone: 617-525-1216; Fax: 617-525-1310; E-mail: pjutz@rics.bwh.harvard.edu

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