

Proteins Phosphorylated during Stress-induced Apoptosis Are Common Targets for Autoantibody Production in Patients with Systemic Lupus Erythematosus

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Summary

Proteins cleaved by interleukin-1 β converting enzyme family proteases during apoptosis are common targets for autoantibody production in patients with systemic lupus erythematosus (SLE). We have tested the possibility that proteins phosphorylated in cells undergoing apoptosis are also targets for autoantibody production in patients with autoimmune disease. Sera from 9/12 patients containing antinuclear antibodies (10/12 meeting diagnostic criteria for SLE or a lupus overlap syndrome), precipitated new phosphoproteins from lysates derived from Jurkat T cells treated with apoptotic stimuli (i.e., Fas-ligation, gamma irradiation, ultraviolet irradiation), but not with an activation (i.e., CD3-ligation) stimulus. Sera derived from individual patients precipitated different combinations of seven distinct serine-phosphorylated proteins. None of these phosphoproteins were included in precipitates prepared using sera from patients with diseases that are not associated with autoantibody production or using serum from rheumatoid arthritis patients. Protein phosphorylation precedes, or is coincident with, the induction of DNA fragmentation, and is not observed when apoptosis is inhibited by overexpression of bcl-2. Serum from four patients precipitated a serine/threonine kinase from apoptotic cell lysates that phosphorylates proteins of 23-, 34-, and 46-kD in *in vitro* kinase assays. Our results suggest that proteins phosphorylated during apoptosis may be preferred targets for autoantibody production in patients with SLE.

A common feature of autoimmune diseases such as systemic lupus erythematosus (SLE)¹, systemic sclerosis, Sjögren's disease (SD), and mixed connective tissue disease is the breakdown of tolerance to self antigens. A consequence of this immune dysfunction is the production of antibodies reactive with multiple self proteins (1). Remarkably, the self proteins recognized by these antibodies are culled from a relatively small subset of total cellular proteins. Protein targets for autoantibody production can be grouped into distinct classes sharing structural and/or functional properties. One such class is the ribonucleoprotein (RNP) particles involved in the regulation of RNA metabolism. Autoantigens belonging to this class include heterogeneous nuclear RNPs, small nuclear RNPs, the Th/To

RNP complex, and the Ro complex (1–5). It is not known why tolerance to RNP particles is commonly circumvented in patients with autoimmune disease.

It was recently reported that substrates for IL-1 β converting enzyme (ICE) family proteases that are cleaved during apoptosis comprise a second class of proteins that are commonly recognized by antibodies found in the serum of patients with autoimmune disease. Autoantigens belonging to this class include poly (A) ribose polymerase, U1 70-kD snRNP, DNA-dependent protein kinase (DNA-PK), nuclear mitotic apparatus protein, and lamin B (6–10). ICE family proteases function in the effector phase of apoptotic cell death. Their substrates are commonly proteins involved in cellular repair processes, suggesting that they may function to ensure the irreversibility of the programmed cell death program. Although proteolysis has the potential to create novel epitopes in protein substrates, most autoantibodies recognize both native and processed substrates (1). Moreover, only a small subset of the over 100 autoantigens that have been described are known to undergo proteolysis during apoptosis, suggesting that other mechanisms contribute to the immunogenicity of these proteins (7, 9). Interest-

¹Abbreviations used in this paper: ANA, antinuclear antibodies; DNA-PK, DNA-dependent protein kinase; HI-FCS, heat-inactivated FCS; ICE, IL-1 β converting enzyme; NP40, nonidet P40; PVDF, polyvinylidene difluoride; RA, rheumatoid arthritis; RAM, rabbit anti-mouse; RNP, ribonucleoprotein; SAP, stress-activated protein; SD, Sjögren's disease; SLE, systemic lupus erythematosus.

ingly, several proteins, including the U1 70-kD protein, have been shown to translocate from the nucleus to large "apoptotic blebs" at the surface of cultured keratinocytes after UV irradiation (8, 11). This and other observations raise the intriguing possibility that cells undergoing apoptosis are uniquely suited to present modified self proteins to the immune system in such a way that overcomes normal mechanisms of peripheral tolerance (12, 13).

The possibility that cells undergoing apoptosis might be reservoirs of autoantigens led us to examine the possibility that proteins selectively phosphorylated during apoptosis might also be commonly recognized by autoantisera derived from patients with autoimmune disease. Recent results have established that inflammatory cytokines (e.g., TNF- α , Fas-ligand) and environmental stress (e.g., heat shock, UV light, and x irradiation) are potent triggers of apoptotic cell death (14–20). Stress-induced apoptosis requires the activation of a cascade of stress-activated protein (SAP) kinases that phosphorylate their specific substrates on serine or threonine residues (14–20). Although the relevant substrates for these kinases are largely unknown, and neither the kinases nor their substrates have been implicated in the etiology of autoimmune disease, we found that autoimmune sera from patients with SLE and lupus overlap syndromes commonly recognize proteins that are phosphorylated during apoptosis. In addition, serum from four of five patients with SLE or a lupus overlap syndrome were found to selectively precipitate a serine/threonine kinase from apoptotic cell extracts. Our results implicate a SAP kinase in phosphorylation of autoantigens during apoptosis and link a common protein modification to autoantibody production in patients with SLE.

Materials and Methods

Cell Culture. Jurkat cells were grown in 5% CO₂ at 37°C using RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% heat-inactivated FCS (HI-FCS; Tissue Culture Biologicals, Tulare, CA) and penicillin and streptomycin (Mediatech, Inc., Herndon, VA). Cells were grown and harvested at mid-log phase. Jurkat T cells overexpressing bcl-2 (or empty vector), a gift from John Reed (The La Jolla Cancer Research Foundation, La Jolla, CA), were grown in RPMI medium as described above, and supplemented with G418 (GIBCO BRL, Gaithersburg, MD) at a final concentration of 500 μ g/ml. Protein overexpression was confirmed by Western blotting before each experiment.

Metabolic Labeling. Jurkat cells were incubated at a density of 2×10^6 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% HI-FCS, and 5% HI-FCS that had been dialyzed to equilibrium against 10 mM Hepes buffer (Sigma Chemical Co., St. Louis, MO). ³²P-labeled orthophosphate or ³⁵S-labeled methionine and cysteine (Dupont–New England Nuclear, Boston, MA) was added at a concentration of 0.1 mCi/ml. Cells were incubated at 37°C for 10–12 h to allow the cells to reach a steady state before each treatment, unless otherwise indicated.

Cell Lysis. Lysis of cells was performed using nonidet P40

(NP40) (Sigma Chemical Co.) lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris, pH 7.8, 1 mM EDTA). NP40 lysis buffer was supplemented immediately before use with 1 mM sodium vanadate (Sigma Chemical Co.) and a 1:100 dilution of a 100 \times protease inhibitor cocktail prepared by dissolving 10 mg chymostatin, 1.5 mg leupeptin, 7 mg pepstatin A, 850 mg phenylmethylsulfonyl fluoride, 500 mg benzamide, and 5 mg aprotinin in 50 ml of ethanol by stirring overnight. The solution was sterilized by filtration and stored at room temperature (21). 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 the supernatant was immediately used for each experiment.

UV Irradiation. Labeled Jurkat cells were placed on 100 \times 15 mm polystyrene petri dishes (Nunc, Thousand Oaks, CA) at a concentration of 2×10^6 cells/ml and irradiated (Stratalinker 2400; Stratagene Corp., La Jolla, CA) at a distance of 9 cm for 12 s. After irradiation, cells were incubated at 37°C for the indicated times before harvesting.

Gamma Irradiation. Labeled cells were placed in a 50-ml conical tube and irradiated at a dose of 3,300 rad from a Cesium 137 source using an irradiator (Gammacell 1000; 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 Michael 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 μ g/ml followed by goat anti-mouse antibody (Jackson ImmunoResearch Labs., 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 μ l of a 50% solution of protein A–Sepharose (Pharmacia, Uppsala, Sweden) in PBS and 5 μ g rabbit anti-mouse (RAM) IgG (Jackson ImmunoResearch Labs., West Grove, PA) for 1 h, followed by two preclears with protein A–Sepharose overnight. Mouse monoclonal antibodies (5 μ g) and 5 μ g RAM, or 3.5–5 μ l patient serum alone were used in precipitation experiments. Serum from all Brigham and Women's Hospital Arthritis Center (Boston, MA) patients who had a serum sample submitted to the Brigham and Women's Hospital Clinical Immunology Laboratory over an 8-mo-period was collected and stored at –20°C until used. Serum from healthy control patients was a gift from P. Fraser (Brigham and Women's Hospital). Diagnoses and serum characterization were confirmed by chart review by P.J. Utz. Immunoprecipitations were performed after addition of 1% BSA (Intergen Co., Purchase, NY) in PBS to a total volume of 500 μ l, 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 NP40 lysis buffer supplemented with protease inhibitor cocktail, resuspending in SDS loading buffer with 9% 2-mercaptoethanol, boiling for 5 min, and electrophoresing on SDS–polyacrylamide gels as described (22). Proteins were transferred to nitrocellulose (Schleicher & Scheull, Keene, NH) for Western blotting experiments or to polyvinylidene difluoride (PVDF), (Dupont–New England Nuclear) for phosphoaminoacid analysis, and either exposed for autoradiography or subjected to Western blot analysis as indicated (23). The

Table 1. Characterization of Autoimmune Sera

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ANA	1:2,560	+	1:640	1:640	1:640	1:20	+	1:640	1:640	1:40	1:2560	1:160	ND	–
Pattern	D/C	S/D	P/D	P/D	S/D	ND	S/D	D	S	D/N	Nu	S/D	ND	–
RF	ND	ND	ND	ND	ND	–	ND	ND	–	–	–	ND	ND	ND
Ro	+	–	–	–	+	+	+	+	–	–	+	–	ND	ND
La	–	–	–	–	–	–	–	–	–	–	+	–	ND	ND
Sm	–	–	–	–	–	–	–	–	–	–	–	–	ND	ND
dsDNA	+	+	–	–	–	–	+	+	–	–	–	–	ND	ND
ssDNA	+	–	–	+	+	+	+	+	–	–	+	+	ND	ND
RNP	+	–	–	–	–	–	–	–	–	–	+	–	ND	ND
APLA	ND	+	+	ND	–	ND	–	–	–	ND	ND	ND	ND	ND
Comp	↓	NI	↓	↓	NI	↑	NI	NI	ND	NI	NI	NI	ND	ND
Disease	SLE	SLE	SLE	SLE	Over	SD	Over	Over	UCTD	SLE	SLE	SLE	RA	Fib
Bands	pp200 pp54 pp17	pp54	pp54 pp42 pp34 pp23	pp54 pp17	pp17		pp46	pp54 pp42 pp34 pp23 pp17			pp54 pp42 pp34 pp23 pp17	pp54 pp42 pp34		

Individual patient sera are identified by the numbers above each column. ANA, antinuclear antibody titer; *Pattern*, immunofluorescence staining pattern using Hep 2 cells as substrate (*P*, peripheral; *D*, diffuse or homogeneous; *C*, cytoplasmic; *N*, homogeneous nuclear; *S*, speckled; *Nu*, nucleolar); *RF*, rheumatoid factor; *Ro*, RNA binding protein Ro; *La*, RNA binding protein La; *Sm*, Smith antigen; *dsDNA*, double-stranded DNA; *ssDNA*, single-stranded DNA; *APLA*, antiphospholipid antibody, determined by anticardiolipin ELISA assay; *Comp*, complement determined by CH 50 assay. Test results are labeled as positive (+); negative (–); normal (NI); not done (ND); increased (↑); or decreased (↓). Over, SLE overlap syndrome; UCTD, undifferentiated connective tissue disease; Fib, fibrositis. The relative migration of phosphoproteins precipitated using sera derived from individual patients (derived from Fig. 1 A) are as indicated.

mouse monoclonal antibody 4D7, anti-bcl-2 (PharMingen, San Diego, CA) was used for blotting studies at a dilution of 1:1,000. Nitrocellulose blots were blocked with 3% BSA in PBS overnight at 4°C. Bands were visualized using RAM conjugated to horse radish peroxidase (Amersham Corp., Arlington Heights, IL) at a dilution of 1:7,500 in 1% BSA in PBS, and developed using enhanced chemiluminescence performed according to the manufacturer's instructions (Amersham Corp.).

Phosphoaminoacid Analysis. Immunoprecipitates that had been electrophoresed and transferred to PVDF were rinsed thoroughly with water, exposed for radiography, and appropriate bands excised with a razor blade. The radiolabeled bands were then subjected to acid hydrolysis as described (24), with the exception that two-dimensional electrophoresis was performed at 14°C rather than at 4°C.

DNA Fragmentation. Unlabeled Jurkat cells were induced to undergo apoptosis using the above triggers in parallel experiments to those using radiolabeled cells. Cells were collected at the indicated times and centrifuged for 5 min at 1,000 rpm. The cell pellet was lysed by adding 500 μ l DNA lysis buffer (20 mM Tris, pH 7.4, 5 mM EDTA, and 0.4% Triton X-100) and incubating on ice for 15 min, mixing several times. After centrifuging at 4°C at 14,000 rpm for 5 min, supernatants were extracted with a 25 phenol:24 chloroform:1-isoamyl alcohol mixture (GIBCO BRL). Next, 100 μ l 5 M NaCl and 500 μ l isopropanol were added to each tube before incubating overnight at –70°C. Samples were thawed and centrifuged at 14,000 rpm for 5 min, washed once with 70% ethanol, and dried in a Speed-Vac. Pellets

were resuspended in 30 μ l of Tris-EDTA buffer containing 0.1 mg/ml RNase A (Sigma Chemical Co.) and incubated at 37°C for 30 min. After the addition of 10 μ l loading buffer, 10 μ l of each sample, corresponding to 1 million cells per lane, was separated on 0.8% agarose gels and visualized by ethidium bromide staining under UV light.

In Vitro Kinase Assays. Individual immunoprecipitates were washed three times in NP40 lysis buffer, then once with TBS (150 mM NaCl, 20 mM Tris, pH 7.6) before resuspending in 30 μ l kinase buffer (20 mM Tris, pH 7.6, 10 mM MgCl₂, 2 mM MnCl₂, and 20 μ Ci [³²P]-gamma ATP [Dupont–New England Nuclear] 150 mCi/ml) for 30 min at 30°C. The reactions were terminated by addition of sample buffer and boiling for 5 min. Proteins were separated on an SDS-PAGE gel before transfer to PVDF and autoradiography for 2–5 min (25).

Results

Autoimmune Sera Recognize Proteins Phosphorylated during Stress-Induced Apoptosis. Serum from 12 random patients with positive tests for antinuclear antibodies (ANA; defined as \geq 1:20 titer on immunofluorescence staining using Hep2 cells as a substrate), as well as serum from 10 healthy control patients, 5 rheumatoid arthritis patients, and 15 patients with diseases considered to be unassociated with autoantibodies (including fibrositis, tendonitis, bursitis, chronic fa-

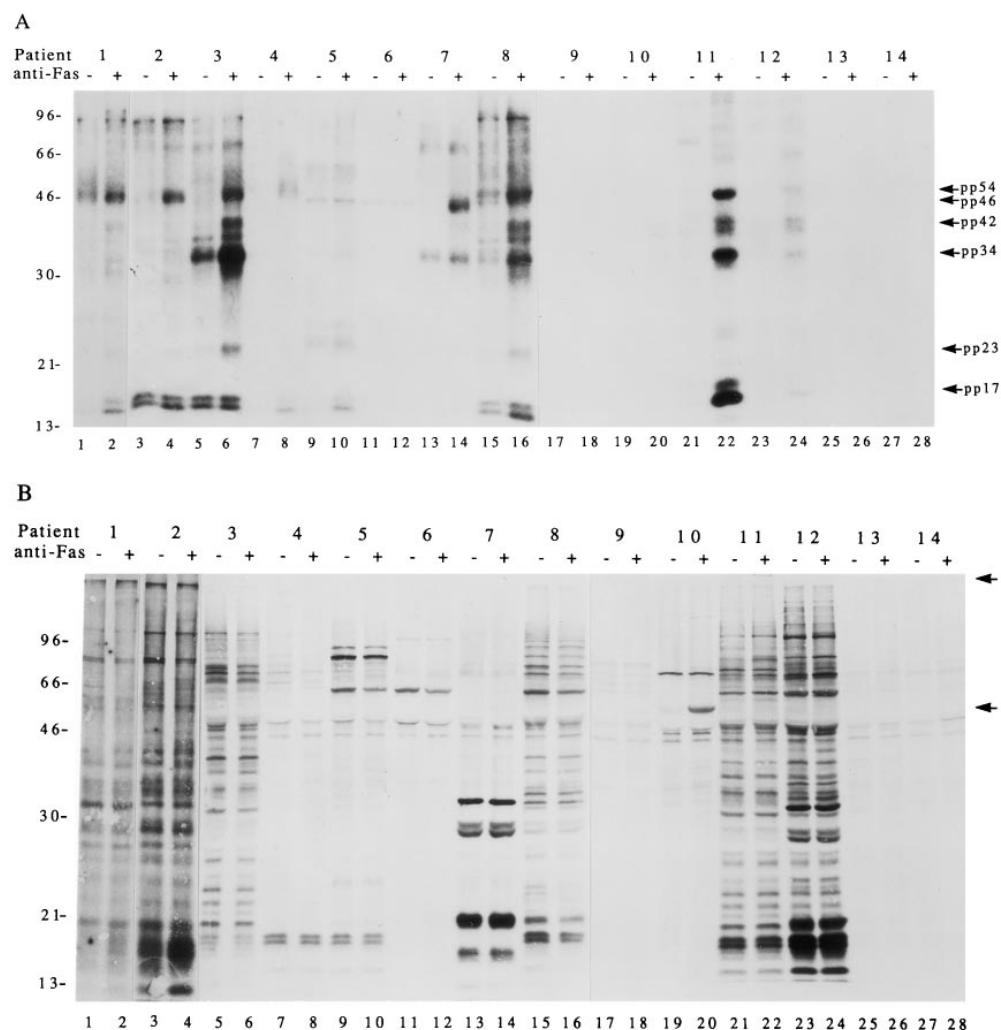


Figure 1. Human autoimmune sera precipitate phosphoproteins from apoptotic Jurkat cell lysates. (A) Jurkat cells were labeled with ^{32}P -orthophosphate, treated with the anti-Fas monoclonal antibody 7C11, and lysed either before (odd numbered lanes) or 2.5 h after (even numbered lanes) the addition of antibodies. Proteins were then precipitated using the indicated autoimmune serum, separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and exposed for autoradiography. Arrows point to new phosphoproteins in the anti-Fas-treated lanes. (B) The identical experiment with ^{35}S -labeled cells. Patient numbers are located above each figure and correspond to those in Table 1. Lane numbers appear beneath the corresponding lane. The relative migration of molecular size markers in kilodaltons are indicated on the left side of the gel.

tigue syndrome, carpal tunnel syndrome, and osteoarthritis), were chosen from the sera collected as described in Materials and Methods. All patients with a positive ANA test were further screened by ELISA for antibodies against DNA, the Smith complex, Ro, La, and RNP, and the patients' charts were reviewed to obtain clinical data sufficient to establish a diagnosis (1). As summarized in Table 1, most patients (10/12) met published criteria for either SLE or lupus in association with a second inflammatory condition (referred to as SLE overlap syndrome; 26). Other conditions were also represented, including SD (patient 6), and undifferentiated connective tissue disease (patient 9). One patient with rheumatoid arthritis (RA; patient 13) and a patient with fibrositis (patient 14) are also presented for comparison.

Jurkat cells metabolically labeled with ^{32}P -orthophosphate were cultured for 2.5 h in the absence or presence of a monoclonal antibody reactive with Fas (anti-7C11), solubilized in NP40 lysis buffer, and immunoprecipitated using the indicated autoimmune or control sera. Immunoprecipitates were separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and subjected to autoradiography.

Fig. 1 A shows that 9/12 ANA+ autoimmune sera, representing 9/10 SLE or SLE overlap patients, precipitated at least one new phosphoprotein from cells undergoing Fas-mediated apoptosis compared to untreated cells. 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 compared on SDS-PAGE gels (data not shown). The individual phosphoproteins precipitated by several of the patient sera is strikingly similar in profile, but variable in intensity of phosphorylation. For example, serum from patients 1, 2, 3, 4, 8, 11, and 12 precipitates a protein of ~ 54 kD (pp54) that is weakly phosphorylated in untreated cell lysates and strongly phosphorylated in lysates from apoptotic cells. Similarly, a 34-kD protein (pp34) was precipitated using serum derived from patients 3, 8, 11, and 12; and a doublet of ~ 42 kD (pp42) was precipitated using serum derived from patients 3, 8, 11, and 12. None of these phosphoproteins were precipitated using ANA (-) sera derived from patients 13 or 14, nor using sera derived from 12 healthy control patients or 4 additional patients with RA (data not shown). The level of

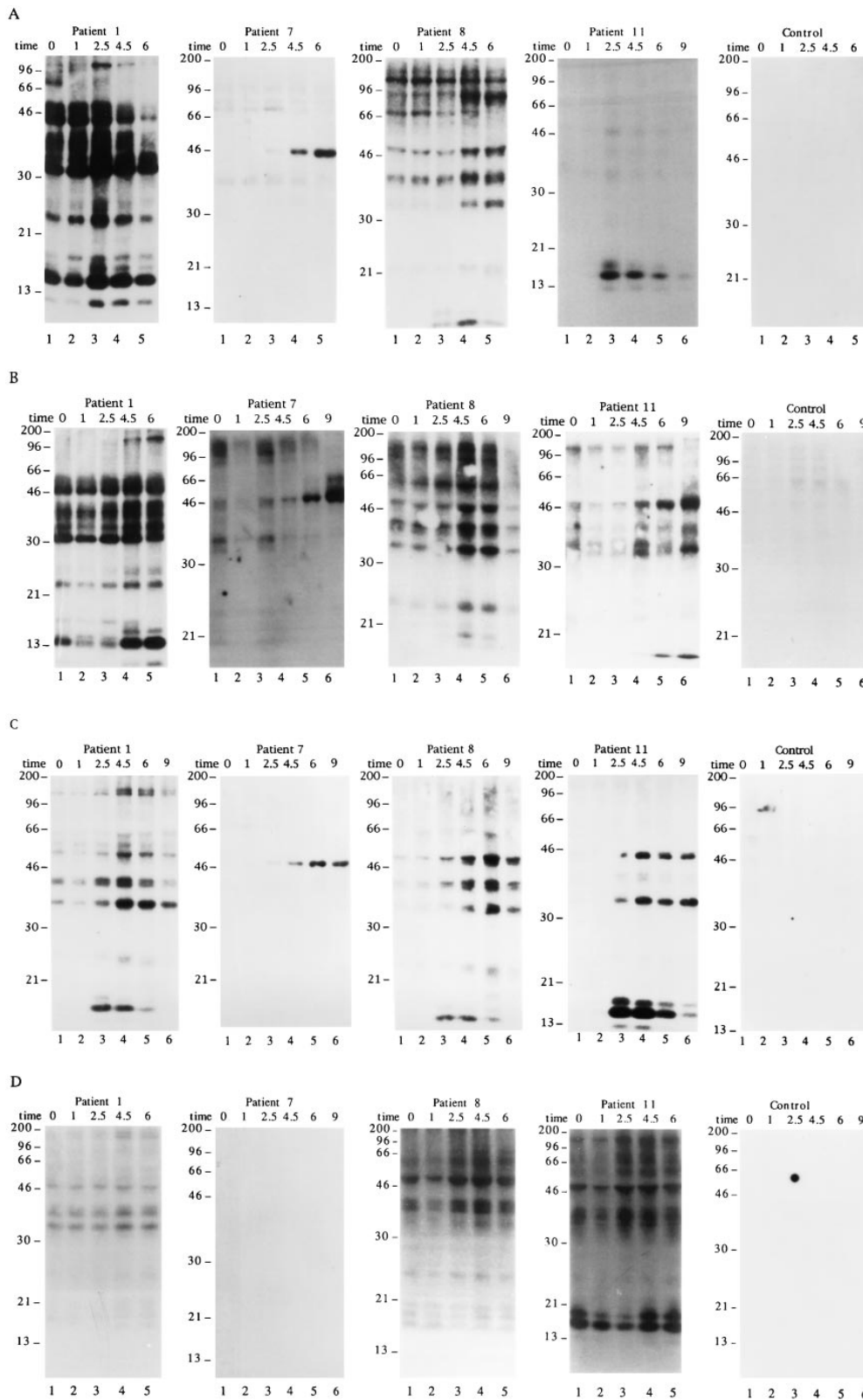


Figure 2. Phosphorylation of autoantigens in response to apoptotic or mitogenic stimuli. Jurkat cells were labeled with ^{32}P -orthophosphate, triggered with apoptotic or mitogenic stimuli, and solubilized using NP40 lysis buffer at the indicated times before immunoprecipitation using sera derived from the indicated patient. Immunoprecipitates were separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and subjected to autoradiographic analysis. (A) Anti-Fas treatment; (B) gamma irradiation; (C) UV irradiation; (D) CD3 cross-linking. Arrows point to new phosphoproteins. The patient number is indicated above each time course. The time, in hours, from initial exposure to each stimulus is indicated at the top of each gel. Lane numbers appear beneath the corresponding lane. The relative migration of molecular size markers in kilodaltons is indicated on the left side of each panel.

phosphorylation of pp42, pp34, and pp17 differed significantly between patients (patients 3, 8, 11, and 12) and was independent of the ANA titer as detected by immunofluorescence (Table 1), suggesting that these phosphoproteins

may be novel and independent of the major proteins responsible for the immunofluorescence detectable as an ANA. In addition to the phosphoproteins described above, three other new phosphoproteins can be seen as bands mi-

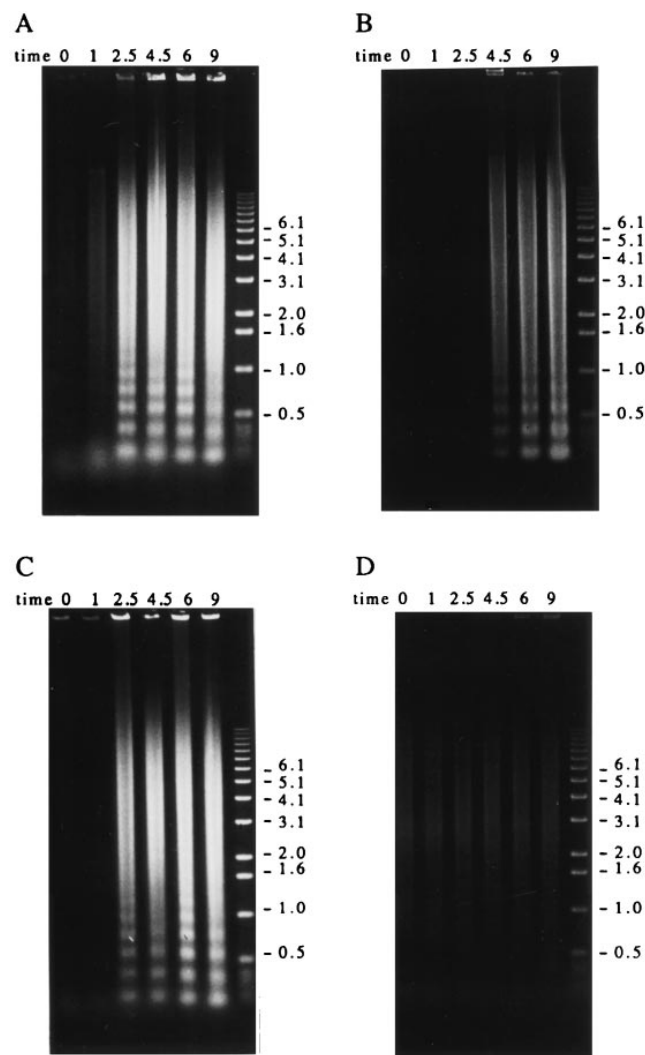


Figure 3. Autoantigen phosphorylation coincides with or precedes the onset of DNA fragmentation in apoptotic Jurkat cells. Jurkat cells were triggered to undergo apoptosis and harvested at the indicated times. Each time point represents a total of 1 million cells. The DNA was prepared as described in the Materials and Methods, separated on a 0.8% agarose gel and visualized by staining with ethidium bromide before UV exposure. (A) Anti-Fas treatment; (B) gamma irradiation; (C) UV irradiation; (D) anti-CD3 treatment. The time, in hours, from initial exposure to each stimulus is indicated at the top of each lane. The relative migration of molecular size markers in kilobases is indicated on the right side of each panel.

grating at the following positions: 17 kD doublet (pp17; patients 1, 4, 5, 8, and 11), 23 kD (pp23; patients 3, 8, and 11), and 46 kD (pp46; patient 7). A seventh protein migrating between 96 and 200 kD (pp200) was observed for patient 1 (Fig. 2, A–C).

The preferential inclusion of phosphoproteins in precipitates prepared from apoptotic versus nonapoptotic lysates could result from de novo phosphorylation of autoantigens, increased extractability of the phosphoproteins during the detergent lysis, or recruitment of preexisting or new phosphoproteins to the autoantigen complex during apoptosis. To differentiate between these three possibilities, the ex-

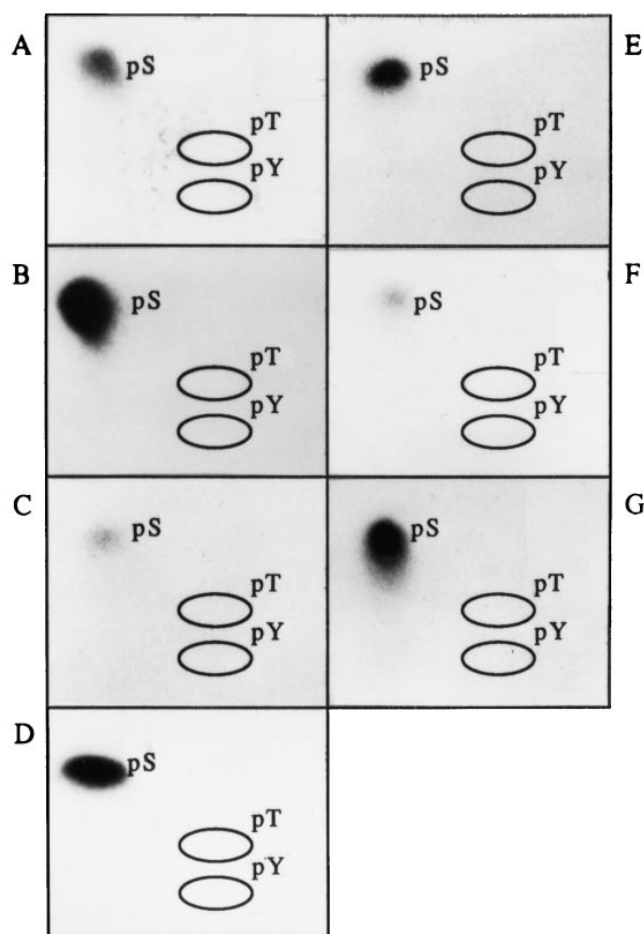


Figure 4. Autoantigens are phosphorylated exclusively on serine residues during Fas-mediated apoptosis. Jurkat cells were labeled with ^{32}P -orthophosphate, treated with the anti-Fas monoclonal antibody 7C11, and solubilized using NP40 lysis buffer after 2.5 h. Proteins were then precipitated with autoimmune serum, separated on a 12% SDS-polyacrylamide gel, transferred to PVDF, and exposed for autoradiography. Individual phosphoproteins were localized on the membrane, excised, and subjected to acid hydrolysis. Phosphoamino acids were separated by two-dimensional electrophoresis in pH 1.9 buffer in the horizontal dimension, followed by pH 3.5 buffer in the vertical dimension before autoradiographic analysis. Individual proteins correspond to those described in Table 1 as follows: (A) patient 1, pp200; (B) patient 1, pp54; (C) patient 7, pp46; (D) patient 11, pp42; (E) patient 3, pp34; (F) patient 8, pp23; and (G) patient 11, pp17. Migration of phosphoaminoacid standards are labeled with circles as follows: phosphoserine (pS), phosphothreonine (pT), phosphotyrosine (pY).

periment shown in Fig. 1 B was performed using cells that were metabolically labeled with ^{35}S -methionine and cysteine in a manner identical to the experiment depicted in Fig. 1 A, which used cells labeled with ^{32}P -orthophosphate. In most cases, immunoprecipitates prepared from apoptotic and nonapoptotic lysates contained similar ^{35}S -labeled proteins. Two exceptions were observed. A 60-kD protein and a >200-kD protein were included in immunoprecipitates prepared from apoptotic, but not nonapoptotic lysates using sera derived from patients 10 (Fig. 1 B, lane 20), and 11 (Fig. 1 B, lane 22), respectively (indicated with arrows on the right side of the panel). Although neither of these

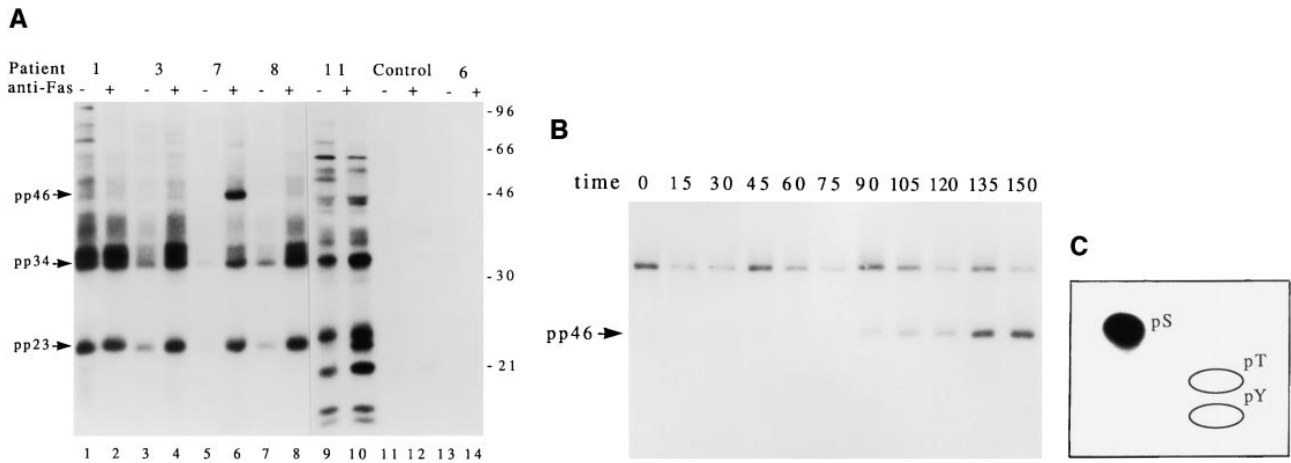


Figure 5. Autoimmune serum precipitates a serine kinase activity from apoptotic Jurkat lysates. Jurkat cells cultured in the absence (odd numbered lanes) or presence (even numbered lanes) of anti-Fas were solubilized in NP40 lysis buffer after 2.5 h, and precipitated using 3.5 μ l of serum derived from the indicated patient. Individual precipitates were subjected to an in vitro kinase reaction at 30°C for 30 min, separated on an SDS-polyacrylamide gel, transferred to nitrocellulose, and subjected to autoradiographic exposure for 2 min. (A) In vitro kinase reaction. Serum derived from the patient number indicated at the top of the figure corresponds to patients described in Table 1. The relative migration of molecular size markers in kilodaltons is indicated on the right side of the panel. (B) Kinetics of kinase activation after Fas ligation as measured using the in vitro kinase reaction performed on immunoprecipitates prepared using serum derived from patient 7. The time in minutes from initial exposure to anti-Fas is indicated at the top of each lane. The position of pp46 is indicated with an arrow on the left side of the panel. (C) Phosphoamino acid analysis of the in vitro phosphorylated 46-kD protein. Migration of phosphoamino acid standards are labeled with circles as follows: phosphoserine (pS), phosphothreonine (pT), phosphotyrosine (pY).

proteins clearly corresponded to the phosphoproteins identified in Fig. 1 A, 35 S-labeled proteins (Fig. 1 B) migrating similarly to the phosphoproteins identified in Fig. 1 A were observed in all cases. Taken together, these results are most consistent with de novo phosphorylation of autoantigens during apoptosis.

Phosphorylation of Autoantigens Accompanies Apoptosis, but Not T Cell Receptor Stimulation. The results shown in Fig. 1 indicate that autoimmune sera preferentially precipitate proteins phosphorylated in response to Fas ligation. To determine whether these proteins are also phosphorylated during apoptosis triggered by stimuli other than Fas ligation, selected patient sera were used to precipitate 32 P-labeled Jurkat lysates prepared from cells subjected to apoptotic stimuli or an activation stimulus for various times. This kinetic analysis reveals that phosphorylation of autoantigens is induced between 1 and 2.5 h after Fas ligation (Fig. 2 A), between 2.5 and 4.5 h after gamma irradiation (Fig. 2 B), and between 1 and 2.5 h after UV irradiation (Fig. 2 C). Individual autoantisera precipitate a similar cadre of phosphoproteins regardless of the apoptotic trigger. 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 new protein phosphorylation, nor DNA fragmentation over the course of this experiment (Figs. 2 D and 3 D). Control sera derived from an individual without autoimmune disease did not precipitate phosphoproteins from apoptotic lysates, nor from lysates prepared from CD3-stimulated cells (Fig. 2, A–D, right). The kinetics of DNA fragmentation induced by apoptotic or activation stimuli was also determined. As shown in Fig. 3, A–D, the onset of DNA fragmentation is approximately

coincident with the phosphorylation of autoantigens regardless of the apoptotic stimulus.

In addition to the phosphorylation of autoantigens during apoptosis, selected phosphoproteins appear to be rapidly dephosphorylated, and then rephosphorylated in a reproducible manner over the course of the kinetic assay (pp17 and pp23; Fig. 2 B, lanes 1–4). The level of basal phosphorylation of several autoantigens, particularly pp34, pp23, and pp17 was somewhat variable in each experiment (e.g., patient 1, Fig. 2, A–D), and appeared to be related to the initial density of the cells at the time of labeling, with less dense (and presumably more active) cells labeling more uniformly (our unpublished observations).

Phosphoaminoacid Analysis of Autoantigens. Since both tyrosine kinases and serine/threonine kinases have been implicated in signaling Fas-mediated apoptosis (16, 17, 19, 25, 27–30), we subjected all seven phosphoprotein autoantigens to phosphoaminoacid analysis. In each case, phosphorylation was restricted to serine residues (Fig. 4, A–G), implicating one or more serine/threonine protein kinases in the phosphorylation of these autoantigens.

A Protein Kinase Activity Is Precipitated from Apoptotic Lysates Using Selected Patient Sera. A cascade of stress-activated serine/threonine kinases has been implicated in signaling apoptotic cell death (16, 17, 19, 25, 31). Individual kinases within this cascade are regulated, in part, by phosphorylation. It is therefore possible that stress-activated kinases may be recognized directly by sera derived from patients with autoimmune disease, or may be recruited during apoptosis to preexisting complexes. To test this possibility, lysates from untreated or anti-Fas-treated Jurkat cells were precipitated with individual patient sera, and subjected to an in vitro kinase assay as described (25). Five sera were chosen to en-

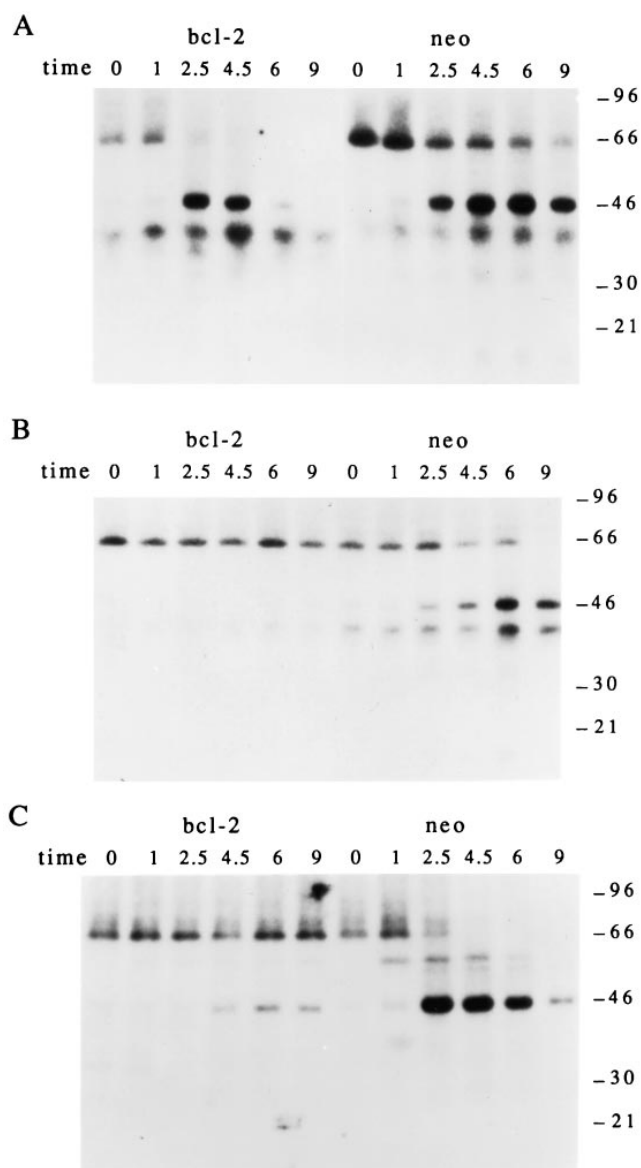


Figure 6. In vivo phosphorylation of pp46 correlates with the induction of apoptosis and is inhibited in Jurkat cells overexpressing bcl-2. Jurkat transformants (*bcl-2*, left) or Jurkat control transformants (*neo*, right) were labeled with ^{32}P -orthophosphate, subjected to the indicated apoptotic stimulus, solubilized in NP40 lysis buffer, and precipitated using serum derived from patient 7 before electrophoretic separation. (A) Anti-Fas treatment; (B) gamma irradiation; (C) UV irradiation. The relative migration of molecular size markers in kilodaltons is indicated on the right side of each panel. The time, in hours, from initial exposure to each stimulus is indicated at the top of each lane.

compass all seven phosphoproteins that had been identified in the initial screen using in vivo-labeled apoptotic Jurkat cells (Fig. 1 A and Table 1). In addition, sera from a healthy control patient and patient 6, whose serum is monospecific for the Ro protein, were included for comparison. Fig. 5 A shows that 4/5 ANA + patient sera (i.e., patients 3, 7, 8, and 11) precipitate a kinase whose activity is increased in apoptotic cell extracts compared to untreated cell extracts. The healthy control patient and patient 6 were devoid of

kinase activity in this assay. Phosphoproteins migrating at 34 kD (Fig. 5, lanes 4, 6, 8, and 10), 23 kD (lanes 4, 6, 8, and 10), and 46 kD (lane 6) were identified in this assay. The relative migration of these phosphoproteins is similar to that of prominent phosphoproteins identified in the in vivo phosphorylation assay shown in Fig. 1 A. The kinetics with which the kinase (precipitated using serum from patient 7) was activated after Fas ligation was correlated with the induction of DNA fragmentation in the experiment shown in Fig. 5 B. In this experiment, Jurkat cells were cultured in the presence of anti-Fas monoclonal antibodies for the indicated times before processing for DNA fragmentation and in vitro kinase activity. The first appearance of pp46 in the in vitro kinase assay was observed at 90 min (Fig. 5 B), while DNA fragmentation was first observed 120 min after Fas ligation (data not shown). Phosphoamino acid analysis of pp46 showed that the in vitro phosphorylation of pp46 is restricted to serine residues (Fig. 5 C), consistent with the in vivo results shown in Fig. 4 C. A similar kinetic analysis targeting pp34 and pp23 using serum from patient 11 (Fig. 5 A, lanes 9 and 10) gave similar results (data not shown). These results are consistent with the less rigorous time courses presented in Figs. 2 and 3, and suggest that a serine/threonine kinase activated by Fas stimulation is present in the immunoprecipitates from patients 7 and 11 at a time that precedes the onset of DNA fragmentation.

***Bcl-2* Overexpression Blocks Apoptosis and Phosphorylation of pp46.** We next asked whether the phosphorylation of pp46 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 and UV irradiation (32–35). In Fig. 6, Jurkat T cells stably transformed with either bcl-2 (left) or empty vector (right) were labeled with ^{32}P -orthophosphate and subjected to Fas ligation, gamma irradiation, or UV irradiation. Cells were solubilized at the indicated times and lysates were precipitated using serum derived from patient 7. While phosphorylation of pp46 is rapidly induced in Jurkat (*neo*) control cells in response to gamma irradiation (Fig. 6 B, right), pp46 is absent from Jurkat (*bcl-2*) transformants treated with this same stimulus (Fig. 6 B, left). Qualitatively similar results are seen with UV irradiation (Fig. 6 C), although a small amount of pp46 is observed in Jurkat (*bcl-2*) transformants beginning at 4.5 h. Overexpression of bcl-2 effectively inhibited apoptosis in response to these triggers, as judged by the induction of DNA fragmentation (data not shown). In contrast, phosphorylation of pp46 after Fas ligation was relatively unaffected by overexpression of bcl-2 (Fig. 6 A). The induction of DNA fragmentation after Fas ligation was similarly unaffected by overexpression of bcl-2 in these cells (data not shown), supporting the correlation between phosphorylation of pp46 and the induction of apoptosis. A similar inhibitory effect of bcl-2 after gamma and UV irradiation but not anti-Fas treatment, on the phosphorylation of pp54, pp34, and pp17 (Fig. 1 A and Table 1) recognized by serum from patient 11, was also observed (data not shown). Taken together, these results demonstrate that the in vivo phosphorylation of all four autoantigens that were

tested correlated with the induction of apoptosis, and is downstream of the inhibitory effects of bcl-2.

Discussion

SLE is characterized by the production of autoantibodies that recognize a restricted subset of intracellular proteins and nucleic acids (1). Autoantibodies reactive with single- and double-stranded DNA, as well as nuclear RNP complexes are responsible, in part, for the ability of lupus serum to bind to the nuclei of cultured cells (1, 5). Antinuclear antibodies are almost always found in the serum of patients with SLE, and their presence has both diagnostic and pathogenic implications in this disease. Specific autoantibody profiles are associated with disease subsets (e.g., anti-Jo-1 histidyl tRNA synthetase and myositis) and are predictive of future disease manifestations (e.g., anti-Jo-1 and interstitial lung disease; 36, 37). Other autoantibodies have been shown to be directly pathogenic in animal models and in human disease, including anti-DNA antibodies (immune-complex glomerulonephritis), antiphospholipid antibodies (antiphospholipid antibody syndrome, characterized by arterial and venous thromboses and recurrent fetal loss), and anti-Ro antibodies (congenital heart block in the neonatal lupus syndrome) (36–40).

Evidence continues to mount that defects in apoptosis are at least partially involved in the pathogenesis of SLE. The genetic defects responsible for the lupus-like diseases found in MRL/*lpr/lpr* and C3H/HeJ-*gld/gld* mice have been identified as the genes encoding Fas and Fas ligand, respectively, a receptor–ligand pair required for activation-induced death of lymphocytes (41, 42). Additional evidence linking defects in apoptosis to the pathogenesis of SLE comes from studies showing that proteins cleaved by ICE family proteases during apoptosis are common targets for autoantibody production in patients with SLE. This autoantigenic subset includes several DNA repair enzymes, including poly (A) ribose polymerase and the catalytic subunit of DNA-PK (7, 43, 44). Several autoantigens are concentrated at membrane blebs rimming the surface of keratinocytes undergoing apoptosis. Although the role of these membrane blebs in antigen presentation is not known, these results suggest the intriguing possibility that proteins modified during apoptosis may be preferred targets for autoantibody production (6–8, 11, 43, 44). Further support for this hypothesis comes from two recent studies demonstrating that immunization of mice with apoptotic cells leads to the production of autoantibodies, including antibodies directed against DNA-PK (Zhang, C., and S. Schlossman, unpublished data; and 12, 13). This possibility led us to screen serum from patients with autoimmune disease for antibodies reactive with proteins that are phosphorylated during apoptosis. Our results indicate that substrates of serine/threonine kinases activated during stress-induced apoptosis are commonly included in precipitates from apoptotic cells using serum from patients with SLE. Using a more stringent ANA titer of $\geq 1:160$ as a cutoff value, as opposed to the $\geq 1:20$ titer used in initial patient selection, demonstrates

that at least one new phosphoprotein is observed in 9/10 ANA + sera, and all nine sera from SLE or SLE overlap patients. It should be emphasized, however, that the small number of patients presented in this initial study precludes generalizations about disease associations or prevalence of autoantigen phosphorylation. Future studies using sera from larger numbers of patients with well-defined clinical syndromes and carefully defined serologic characteristics will clarify the importance of autoantigen phosphorylation in autoimmune disease.

Stress-activated serine/threonine kinases (SAP kinases, also referred to as JNK and p38) play an essential role in signaling stress-induced apoptosis (15–17, 30, 31, 45). Several transcription factors (e.g., c-Jun, Elk-1, and ATF-2) are substrates for SAP/JNK/p38, and dominant inhibitory mutations in c-Jun can block stress-induced apoptosis, suggesting that stress-activated kinases influence apoptosis at the transcriptional level. The kinase activity responsible for phosphorylation of pp46 has several similarities to the SAP/JNK kinases, including a requirement for magnesium and manganese but not calcium, resistance to RNase and DNase treatment (data not shown), and identical kinetics of phosphorylation and serine specificity (15–17, 30, 31, 45). Another stress-activated serine/threonine kinase that has been implicated in a signaling cascade leading to apoptosis is FAST kinase (25, 46). FAST kinase phosphorylates TIA-1, but not TIAR, two related RNA-binding proteins that appear to regulate mRNA translation (Kedersha, N., and P. Anderson, unpublished observations) and have been shown to translocate from the nucleus to the cytoplasm during Fas-mediated apoptosis (25, 47). We have identified autoantibodies reactive with TIA-1 and TIAR in the sera of ~2% of patients with a positive ANA (Utz, P.J., and P. Anderson, unpublished observations), consistent with our hypothesis that proteins phosphorylated during apoptosis are preferred targets for autoantibody production. Additional kinases that may be involved in signaling apoptosis include protein kinase C (48), cyclin dependent kinases (49–52), cAMP kinase I (53, 54), casein kinase I (55), Pim-1 (56), Wee-1 (57), and PITSLRE kinases (28). Further characterization of the kinase activities described in this report is ongoing and should allow identification of the responsible kinase(s) and elucidation of their role in autoantibody production and programmed cell death.

The identity of the seven kinase substrates described in this report is not known. Over 100 autoantigens have been identified to date, some of which are known phosphoproteins, including the ribosomal proteins P0, P1, and P2 that are similar in size to the pp17 doublet and pp42 (1). It is possible that these substrates are novel, apoptosis-specific proteins, and perhaps are part of the core apoptotic machinery. A second possibility is that the observed bands may represent proteolytic cleavage products of larger phosphoproteins, as would be predicted for a phosphoprotein such as DNA-PK. The disappearance of the weakly (and variably) phosphorylated 68-kD band precipitated from non-apoptotic cell lysates with serum from patient 7 (Figs. 1 A, 5, and 6) and the subsequent appearance of pp46 in precip-

itates from apoptotic lysates support this possibility, although the marked increase in phosphorylation of pp46 both in vivo and in vitro argues strongly for a new phosphorylation event. Cleavage and phosphorylation are not necessarily mutually exclusive events, and it remains plausible that serine phosphorylation during apoptosis may target these autoantigens or other proteins in a macromolecular complex for cleavage by an ICE-like protease. Comparison of the sizes of the phosphoproteins, presented in Fig. 1 and Table 1, with the published sizes of ICE-like protease cleavage products demonstrates similarities with U1-70 kD (40-kD product) and pp42 (6); nuclear lamin B (68-kD precursor and 45-kD product) and pp46 (10, 58); UBF/Nor-1 (55-, 35-, 32-, and 24-kD products) and pp54, pp34, and pp23, respectively (9); and an unidentified protein fragment of 35-kD (patient RW) and pp34 (7). A focus of future studies will be to identify each of these seven phosphoproteins and to identify their role in apoptosis and autoantibody production.

In vivo autoantigen phosphorylation was inhibited in cells overexpressing bcl-2 after UV or gamma irradiation, but not after Fas stimulation. Programmed cell death, as assayed by DNA fragmentation and characteristic cell morphologic changes, correlated precisely with antigen phosphorylation for each stimulus. This, together with the observation that none of the autoantigens was phosphorylated in response to CD3 stimulation, suggests that the phosphorylation of autoantigens is specifically correlated with the activation of intrinsic cell death pathway(s) and is not an epiphenomenon associated with stress stimuli. Although the molecular mechanisms by which bcl-2 inhibits apoptosis are not known, it appears to act at a signaling step preceding the activation of the protease apopain/CPP32 (32–35). Recent results showing physical interactions between bcl-2 family members and serine/threonine kinases involved in signal-

ing cell growth (e.g., raf-1) suggest that activation of kinase cascades similar to the cascade (s) described in this report might precede the activation of ICE family proteases under some conditions (59–61).

The self antigens recognized by autoantibodies found in lupus serum comprise a small subset of total cellular proteins. It is striking that many of these antigens are substrates for proteases and kinases involved in signaling or execution of apoptotic cell death. Why are proteins modified during apoptosis preferred targets for autoantibody production in patients with SLE? Perhaps a clue comes from the observation that some autoantigens are concentrated at membrane blebs formed at the surface of apoptotic cells (8, 11). Phosphorylation during apoptosis of the protein constituents of macromolecular complexes such as RNPs may produce neoepitopes or may target individual proteins for proteolysis and/or translocation to membrane blebs, as has been observed for the U1-70-kD protein (6, 8). Although intracellular antigens presented on the surface of apoptotic cells might be recognized by self-reactive B lymphocytes, a productive immune response would not be generated in the absence of antigen-specific helper T cells. It is possible, however, that proteolysis or phosphorylation of selected proteins during apoptosis produces neoepitopes to which T cells are not tolerized. In the special case in which the apoptotic cell is also an APC, priming of naive T cells may ensue. T cell recognition of modified self proteins presented by apoptotic APCs could drive the differentiation and expansion of autoreactive B cells, resulting in autoantibody production. This hypothesis requires that APCs from patients with SLE are unusually sensitive to stress-induced apoptosis. This sensitivity could be conferred by a combination of genetic and environmental factors.

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