

Dephosphorylation of autoantigenic ribosomal P proteins during Fas-L induced apoptosis: a possible trigger for the development of the autoimmune response in patients with systemic lupus erythematosus

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Abstract

Objectives—Autoimmune diseases are characterised by the production of autoantibodies against various autoantigens. In the past few years data have been published on a possible role of apoptosis in the development of autoimmunity. These include the finding that several autoantigens become modified (for example, by cleavage) during apoptosis, and the observation that these modified antigens are translocated to the cell surface. When the normal clearance of apoptotic cells somehow is disturbed, such modified antigens might become exposed to the immune system. Because acidic ribosomal P (phospho-) proteins targeted by autoantibodies in systemic lupus erythematosus (SLE) are also concentrated at the surface of apoptotic cells, this study aimed at investigating what modifications occur on these antigens during apoptosis.

Methods—Apoptosis in Jurkat cells was induced by Fas ligand (Fas-L), and the fate of autoantigenic P proteins was analysed in both normal and apoptotic total cell extracts.

Results—The autoantigenic P proteins were not cleaved but dephosphorylated during Fas-L induced apoptosis. This dephosphorylation was prevented when caspase activity was inhibited.

Conclusions—As has been shown for other autoantigens targeted by autoantibodies in SLE, P proteins also are modified during apoptosis. P1 and P2 are completely dephosphorylated while P0 is partly dephosphorylated. Because the epitope targeted by autoantibodies normally is phosphorylated, it is possible that the apoptotic dephosphorylation of the antigen might be the trigger for the development of the autoimmune response against P proteins.

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Systemic autoimmune diseases are characterised by the production of autoantibodies against a wide variety of nuclear and cytoplasmic antigens. Most of these autoantibodies have an important role as diagnostic marker,

and in some cases their presence in patient serum samples has a prognostic value for the development of the disease. In the past few years it has been shown that during apoptosis several autoantigens are relocated from their normal cellular sites to the plasma membrane, where they become clustered and concentrated in characteristic surface structures: small blebs and apoptotic bodies.¹ The small blebs contain fragmented endoplasmic reticulum, ribosomes (including ribosomal P proteins), and the Ro 52 kDa autoantigen, which are normally located in the cytosol; in the apoptotic bodies nuclear antigens, nucleosomal DNA, Ro 60 kDa, La, and the small nuclear ribonucleoproteins, are clustered. Some of the clustered autoantigens (that is, U1 70 kDa, DNA topoisomerase I, α fodrin, lamin B) have been cleaved by a family of proteolytic enzymes called caspases, cysteine proteases that cleave after aspartic acid.² The caspases are activated by several apoptotic stimuli such as ultraviolet irradiation or activation of death receptors (Fas or tumour necrosis factor receptor). Other autoantigens, including fibrillarin, CENP-B, alanyl tRNA synthetase, Ku-70, and RNA polymerase I and II, are cleaved during cytotoxic T lymphocyte mediated apoptosis by granzyme B, a serine protease which is contained in the cytotoxic T lymphocyte granules.²

However, not all clustered autoantigens are proteolytically modified; some of them undergo other post-translational modifications, such as phosphorylation, transglutamination, and dephosphorylation (see review by Utz *et al*³). It has also been shown that, particularly in systemic lupus erythematosus (SLE), the clearance of apoptotic cells is less efficient,⁴ supporting the idea that such post-translationally modified autoantigens clustered at the cell surface might become exposed to the immune system. Among the antigens that are clustered in small apoptotic blebs are the acidic ribosomal P (phospho-) proteins, which are targeted by autoantibodies in about 15% of patients with SLE. These autoantibodies are generally not detected in patients with autoimmune diseases other than SLE or in healthy subjects, which indicates that they are specific for SLE. They are mainly detected in patient serum samples during the active phase of

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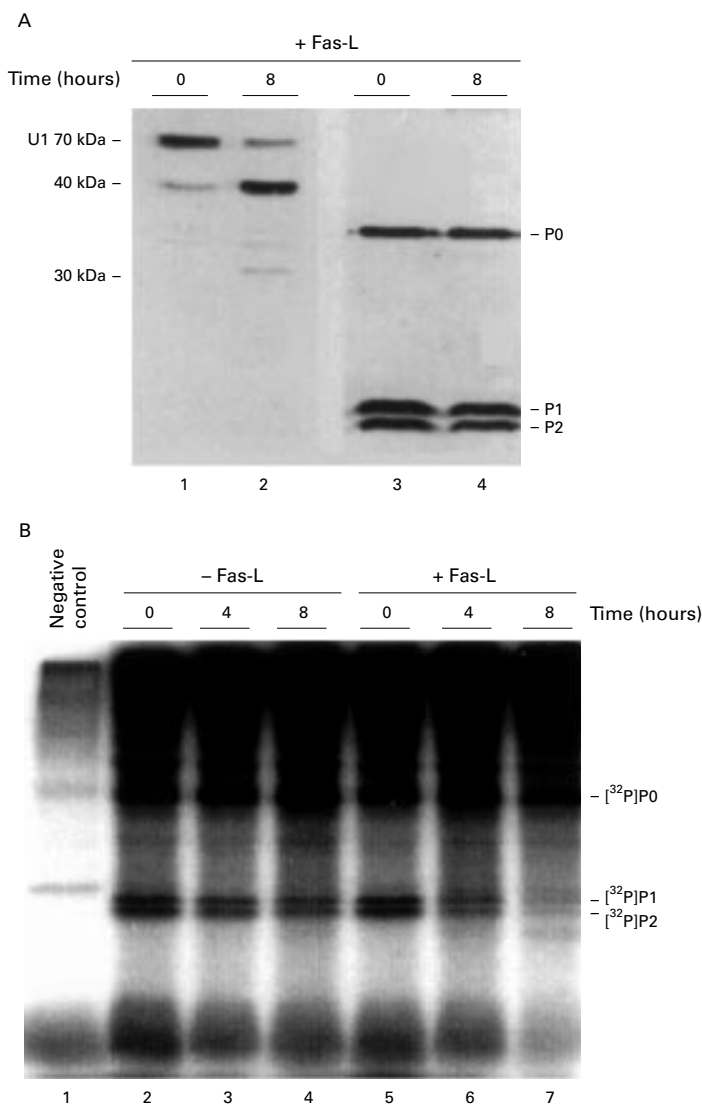


Figure 1 Fas-L induced apoptotic modification of the ribosomal P proteins. (A) Jurkat cells were incubated for 0 and 8 hours with Fas-L. Total protein extracts were analysed by 15% SDS-PAGE and western blotting using anti-P serum (patient 10026) as well as anti-U1 70 kDa patient serum (patient H42). The P proteins (P0, P1, and P2 indicated on the right) are not detectably cleaved (lanes 3, 4). The position of U1 70 kDa (lane 1) and its 40 kDa cleavage product (lane 2) are indicated on the left. The band visible in lane 2 at approximately 30 kDa corresponds to an additional apoptotic cleavage product of the U1 70 kDa protein. (B) ³²P labelled Jurkat cells were incubated for 0, 4, and 8 hours with or without Fas-L. ³²P labelled P proteins were immunoprecipitated from cell extract with an anti-P serum (lanes 2–7) from a patient with SLE and subjected to autoradiography after 15% SDS-PAGE. Lane 1 represents the negative control (non-specific binding of proteins to protein-A agarose beads).

disease in association with lupus nephritis,⁵ hepatitis,⁶ and with the development of central nervous system disease,⁷ so that the anti-P antibodies can be considered as good markers for the diagnosis of SLE. They are directed towards three ribosomal proteins called P0, P1, and P2 (38, 19, and 17 kDa, respectively). The P proteins are associated with the eukaryotic 60S ribosomal subunit as a pentameric complex (one copy of P0 and two copies each of P1 and P2). This complex interacts with the 28S rRNA molecule forming a GTPase domain which is active during the elongation step of protein translation.⁸ The anti-P autoantibody recognises in all three P proteins the 22 amino

acid long C terminal end (C-22), which contains two possible phosphorylation sites (¹⁰²S and ¹⁰⁵S) for casein kinase II.⁹ In vitro both sites can be phosphorylated, but in vivo only the ¹⁰⁵S site seems to be used by the kinase.^{8–9}

Because several other autoantigens targeted by autoantibodies in SLE appear to be cleaved or modified during apoptosis,³ this study aimed at investigating whether the ribosomal P proteins are modified during Fas ligand (Fas-L) induced apoptosis.

Materials and methods

CELL CULTURE

Jurkat suspension cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% heat inactivated fetal calf serum, 200 µg/ml G418 (Gibco BRL), sodium pyruvate (1 mM), penicillin (1 mM), and streptomycin (1 mM).¹⁰ Murine Neuro2A cells transfected with murine Fas-L or with the empty transfection vector (mock) were cultured and the murine Fas-L supernatant was obtained as described previously.¹¹

INDUCTION OF CELL DEATH AND ANALYSIS OF APOPTOTIC CELL EXTRACT

Jurkat cells were incubated for the indicated time period with murine Fas-L or mock supernatant as described previously.¹¹ After the induction of apoptosis, cells were washed twice with phosphate buffered saline and lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5% NP-40, 100 mM KCl, 1 mM EDTA, 1 mM DTE, 1 × protease inhibitor cocktail from Boehringer Mannheim) on ice for 15 minutes (100 × 10⁶ cells/ml). Cell lysates were centrifuged (15 minutes, 4°C, 13 000 rpm) and the supernatants were used for analysis. In caspase inhibitor experiments, Jurkat cells were preincubated for one hour at 37°C with 5 and 20 µM z-VAD-FMK (irreversible general caspase inhibitor, Calbiochem) or in dimethyl sulphoxide as control, before induction of apoptosis. The apoptotic status of the cells was checked by immunoblotting using an anti-U1 70 kDa positive human serum.¹¹ Immunoblotting procedures using anti-P or anti-U1 70 kDa positive patient serum samples were as described previously.^{10–11} Serum samples were provided by 10 patients with SLE who satisfied the revised criteria of the American College of Rheumatology for SLE.¹²

IN VIVO METABOLIC ³²P LABELLING OF JURKAT CELLS AND IMMUNOPRECIPITATION

³²P labelling was performed as previously described¹⁰ using normal and apoptotic Jurkat cells. To immunoprecipitate radiolabelled P proteins, 20 µl of protein A-agarose beads were incubated with 1 µl of an anti-P positive patient serum (overnight at 4°C) in 500 µl IPP150 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% NP-40), followed by three washes with the same buffer. The ³²P labelled proteins of 2 × 10⁶ cells were then allowed to bind antibody-bound beads during two hours rotating incubation at 4°C in IPP150. After three washes with

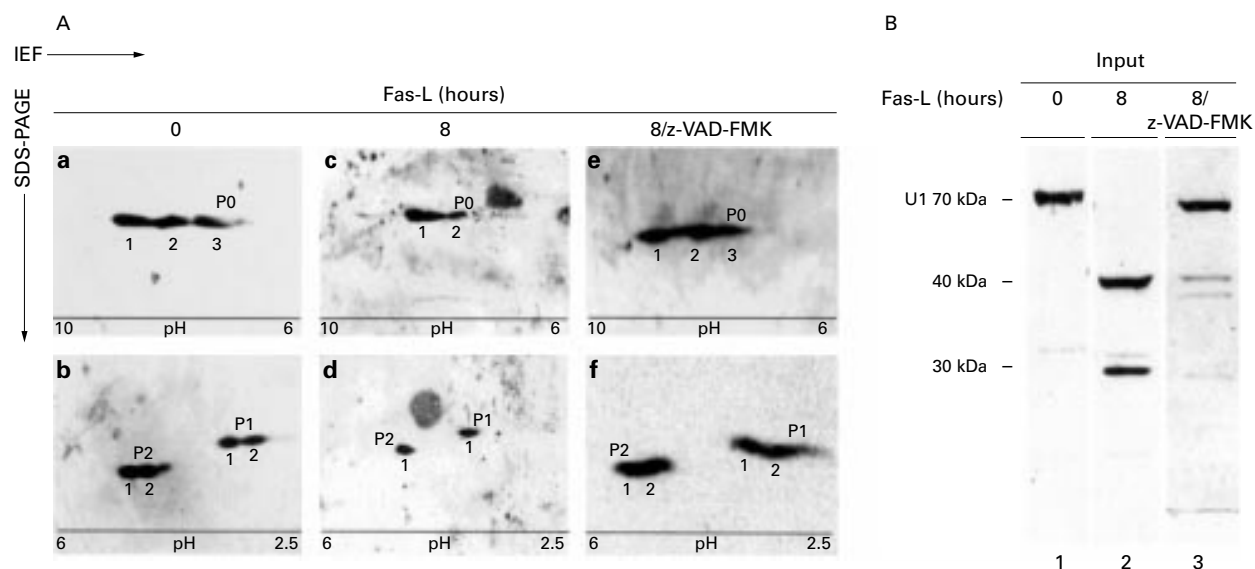


Figure 2 Two dimensional IEF/SDS-PAGE analysis of P proteins from normal and apoptotic Jurkat cells. (A) Jurkat cells were incubated for the indicated time period with Fas-L or pretreated with z-VAD-FMK (general caspase inhibitor). Total cell extracts from 2×10^6 cells were fractionated by IEF using a linear gradient of ampholytes (given at the bottom of the figures) followed by 15% SDS-PAGE in the second dimension. Spots of P0, P1, and P2 were detected by immunoblotting using anti-P patient serum 10026. (B) The one dimensional blot was also probed with anti-U1 70 kDa positive patient serum H42 to test the apoptotic status of the cells before the 2D-PAGE analysis (lanes 1, 2, and 3).

IPP150, the beads were resuspended in 20 μ l of sample buffer¹⁰ and the immunoprecipitated proteins were subsequently analysed by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

TWO DIMENSIONAL GEL ELECTROPHORESIS

Total cell extracts (2×10^6 cells) from normal and eight hours' Fas-L induced apoptotic cells, either treated or untreated with the z-VAD-FMK caspase inhibitor, were analysed by two dimensional PAGE (2D-PAGE) using isoelectric focusing (IEF) in the first dimension and 15% SDS-PAGE in the second dimension.¹³ Pharmalyte ampholytes (Pharmacia) with pH ranges 2.5–5/5–8 and 3.5–10 for IEF gels were used in a ratio of 3:2. After the 2D-PAGE, the proteins were transferred to nitrocellulose membranes and the immunoblots were probed with an anti-P positive patient serum.

Results

Fas-L induced apoptosis was monitored by immunoblotting of normal and apoptotic total Jurkat cell extracts probed with the anti-U1 70 kDa positive serum.¹¹ During apoptosis the U1 70 kDa antigen is specifically cleaved by caspase-3, with the subsequent production of a 40 kDa truncated protein which is recognised by U1 70 kDa positive serum samples. Treatment of Jurkat cells with murine Fas-L¹¹ induced apoptosis as indicated by the almost complete disappearance of the U1 70 kDa autoantigen and appearance of the typical 40 kDa apoptotic cleavage product (fig 1A, lanes 1 and 2). No cleavage products could be detected on the same blot probed with the anti-P positive serum (fig 1A, compare lanes 3 and 4), indicating that the autoantigenic P proteins were not detectably cleaved by caspases during

Fas-L induced apoptosis. This experiment was repeated with several anti-P positive serum samples with the same negative result.

Figure 1B shows ³²P labelled P proteins from normal (lanes 2–4) and Fas-L induced (lanes 5–7) apoptotic cells immunoprecipitated by the patient anti-P autoantibodies. The control (lane 1) shows the non-specific binding of proteins to uncoated beads (no serum added). The ³²P phosphate signal of the P1 and P2 proteins had decreased already after four hours of Fas-L induced apoptosis and was barely detectable after eight hours, suggesting that the phosphorylated status of these P proteins diminishes during the apoptotic process. The ³²P signal of the P0 protein seemed to decrease as well, but much less than the P1 and P2 proteins.

Because a change in the phosphorylation status of a protein will alter its isoelectric point, we also analysed the P proteins by 2D-PAGE (fig 2A) after 0 and 8 hours' Fas-L induced apoptosis. The P0 protein was resolved in three major isoforms (fig 2A-a, numbered 1–3) on the blot of normal cell extracts and shifted towards a more basic pH after eight hours of Fas-L induced apoptosis, indicating that there was a decrease in the amount of the most acidic components (that is, components 2 and 3) owing to a partial dephosphorylation of the protein (fig 2A-c). The P1 and P2 proteins could each be resolved into two isoforms on blots of normal cell extracts (fig 2A-b) and these isoforms shifted clearly towards one isoform after induction of apoptosis (fig 2A-d). The loss of the more acidic components of P1 and P2 (numbered 2) is related to the dephosphorylation that occurs during apoptosis as can also be concluded from the in vivo ³²P labelling experiments (fig 1B). The dephosphorylation of the P proteins is specifically related to the caspase-executed apoptotic process as is shown in figs 2A-e and 2A-f. In this experiment, cells

were preincubated with z-VAD-FMK, a general caspase inhibitor, before the induction of apoptosis. The total cell extract was then analysed by 2D-PAGE assay using the anti-P positive serum as probe. A comparison of the two dimensional patterns obtained from the z-VAD-FMK treated cells and from the untreated apoptotic cells (compare e and f with c and d in fig 2), clearly showed that dephosphorylation of the P proteins did not occur when caspase activity was inhibited. In the control lanes (fig 2B) the apoptotic induced cleavage of the U1 70 kDa antigen and its complete inhibition by the z-VAD-FMK are shown. From these results which were reproducibly obtained with different patient serum samples, we conclude that the dephosphorylation of the P proteins is an apoptotic process, probably mediated by a caspase induced phosphatase.

Discussion

During cell death a number of key cellular components become modified, either by proteolytic/nucleolytic cleavage or by amino acid/nucleotide modification.³ It is suggested that, under some conditions, these modified self components can be exposed to the immune system, leading to a normal immune response. In genetically susceptible subjects, however, this process may in some cases evolve into a full-blown autoimmune response, leading to the production of autoantibodies directed to the whole (unmodified) antigen.

Data have been published on the cleavage and other post-translational modifications of autoantigens during apoptosis,¹⁻³ and among those targeted by autoantibodies in rheumatic autoimmune diseases, only the La protein has been shown to be dephosphorylated.¹⁰ In this paper we demonstrate that the ribosomal P proteins, a group of three SLE-specific autoantigens, undergo a similar modification. The phosphorylation status of the P proteins is important for their binding to the large subunit of the eukaryotic ribosome and is essential for the functional activity of the ribosome during the elongation step of protein synthesis.^{8,14} Dephosphorylation of the P complex, or one of its components, thus not only blocks the interaction of these proteins with the ribosome,¹⁴ in the apoptotic cells they are also translocated to small blebs at the periphery of the cell, facilitating their recognition as non-self by the immune system.¹ Our data show that the dephosphorylation of P0 is not as complete as that of the P1 and P2 proteins. In the P0 sequence there are more predicted phosphorylation sites (<http://www.cbs.dtu.dk/services/NetPhos>) but the P proteins have the phosphorylation sites at the C-22 terminus, the major epitope recognised by the autoantibodies, in common. In vivo primarily the site at ¹⁰⁵S is phosphorylated.^{8,9} It has been shown by two dimensional analysis¹⁵ that dephosphorylation of ribosomal P proteins after alkaline phosphatase (AP) treatment in vitro results in a cathodal shift of both P1 and P2, leading to a resolution into single spots. The P0 is not

completely resolved and appears as a "streak" after AP treatment.¹⁵ In our two dimensional assays after Fas-L incubation the P1 and P2 proteins are also reduced to single spots and the streak of P0 shifts towards a more basic pH in apoptotic cells because of the loss of the most acidic isoform. These patterns thus are similar to those obtained after AP treatment, suggesting the action of an apoptotically regulated phosphatase. The observed decrease in the ³²P labelling of the autoantigenic P proteins during apoptosis (fig 1B) supports this conclusion. The dephosphorylation is clearly dependent on caspase because it does not occur when caspase activity is blocked by inhibitors. As in the case of the dephosphorylation of the La autoantigen¹⁰ we assume that the phosphatase is activated by caspases during the apoptotic process. Because the P1 and P2 proteins resolve into single spots after AP treatment and during apoptosis, it is likely that in these proteins the C terminal phosphorylation site at the ¹⁰⁵S is dephosphorylated. This part of the P proteins, which normally is phosphorylated, also contains the major B cell epitope. Possibly, the dephosphorylation alters the conformation of these proteins in such a way that the C-22 terminal part of these proteins becomes a non-self or neo-epitope which can trigger an immune response. There is some evidence that anti-P antibodies in patients with SLE recognise both the phosphorylated and the non-phosphorylated form of the C-22 terminal peptide, suggesting that in patients there are at least two different populations of anti-P antibodies.¹⁵ Further studies are needed to show whether the apoptotically modified non-phosphorylated form of P proteins can trigger an autoimmune response.

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