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In Vivo Phosphorylation of the Lamin B Receptor

BINDING OF LAMIN B TO ITS NUCLEAR MEMBRANE RECEPTOR IS AFFECTED BY PHOSPHORYLATION*

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Previous studies have shown that the nuclear envelope of avian erythrocytes contains a 58-kDa integral membrane protein (p58) which serves as a receptor for the karyoskeletal protein lamin B (Worman, J. H., Yuan, J., Blobel, G., and Georgatos, S. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 8531–8534). We now demonstrate that p58 is phosphorylated *in vivo* at serine residues and that its phosphorylation is stimulated by isoproterenol in a dose-dependent fashion. We further show that dephosphorylation of p58 reduces significantly its binding to lamin B. These data suggest that phosphorylation may constitute one of the major mechanisms regulating the lamina-nuclear membrane interactions.

The nuclear envelope of eukaryotic cells contains a family of peripheral membrane proteins, termed *lamins* (1). These proteins can be divided into two classes, the type A and type B lamins (1, 2), and share structural organization principles with cytoplasmic intermediate filament proteins (3–5). The lamins, like other intermediate filament subunits, polymerize homotypically and heterotypically (2, 6) and form the *nuclear lamina*, a karyoskeletal structure interposed between the heterochromatin and the inner nuclear membrane (1, 5, 7).

At interphase, the lamins maintain a strong association with the inner nuclear membrane and are resistant to high salt or detergent extraction (1, 8, 9). At the onset of mitosis, the nuclear lamina is depolymerized (8). Both types of lamins become hyperphosphorylated (8, 10, 11), but they disassemble in two distinct manners; the type A lamins diffuse in the cytoplasm in the form of small oligomers, whereas the type B lamins remain associated with a population of vesicles (8, 12), presumably derived from the breakdown of the nuclear membrane. At telophase, the above sequence of events is reversed; the lamins become dephosphorylated and reassemble, while the nuclear membrane is reformed, perhaps by fusion of (mitotic) vesicles (8, 12). These observations indicate that the type B lamins and the nuclear membrane remain intimately associated throughout the entire cell cycle.

The precise mechanism via which the type B lamins attach to the nuclear membrane is not clear. Initial data, concerning the properties of the lamins, have been explained differently, giving rise to two interpretations. Because type B lamins are rather insoluble in aqueous solutions and post-translationally modified (*i.e.* isoprenylated, see Ref. 13), it has been proposed that they may associate directly with the lipid bilayer (14). Alternatively, others have suggested a putative linkage via a protein "receptor" (1). In order to address this question we previously studied the binding of isolated lamins to lamin-depleted nuclear envelopes from avian erythrocytes (15). We found that the type B lamins (but not the type A lamins) bind saturably and with high affinity to an integral nuclear membrane protein with a molecular mass of 58,000 (p58). Preliminary experiments show that p58 is widespread among higher eukaryotic cells[1] and that a p58 analogue exists in yeast (16).

Given the dramatic changes in the structure of the nuclear lamina during the cell cycle and the apparent involvement of phosphorylation in these processes, we examined the phosphorylation state of p58 during interphase and the influence of phosphorylation on its binding to type B lamins. We show here that p58 is a major phosphoprotein of the nuclear envelope and that phosphorylation may affect its function.

MATERIALS AND METHODS

Cell Fractionation—Plasma membranes and nuclear envelopes were isolated from turkey erythrocytes as previously described (17). The nuclear envelopes were subfractionated by urea extraction as specified in Ref. 15.

Protein Chemical Procedures—*In vivo* phosphorylation of turkey erythrocytes was performed according to Beam *et al.* (18) by incubating washed erythrocytes (at a hematocrit of 10%) with [^{32}P]orthophosphate at 37 °C for various periods of time, as specified in the figure legends. For isoproterenol stimulation, the cells were preincubated with [^{32}P]orthophosphate for 3.5 h and then for an additional 20 min with different concentrations of isoproterenol. Phosphoamino acid analysis was performed by a modification of the procedure of Hunter and Sefton (19). Briefly, the region containing the 58-kDa phosphoprotein band was excised from SDS[2]-polyacrylamide gels, and the gel slice was washed with distilled water and 10% methanol and incubated with α-chymotrypsin (75 μg/ml in 50 mM ammonium bicarbonate, pH 8.0) at 37 °C for 18 h. The material recovered in the supernatant was lyophilized, hydrolyzed with 6 N HCl at 100 °C for 60 min under N_2, lyophilized again, dissolved in 10% acetic acid and 1% formic acid (pH 1.8), and relyophilized. This last step was repeated twice more. Finally, the sample was applied to cellulose thin-layer chromatography plates and analyzed together with phosphoamino acid standards (*O*-phospho-DL-serine (Sigma), *O*-DL-threonine (Sigma), and *O*-L-tyrosine (Sigma). Phosphoamino acids were visualized by ninhydrin staining and autoradiography. *In vitro* dephosphorylation of p58 was performed as follows. Polypeptides of urea-extracted nuclear envelopes were fractionated by SDS-PAGE (see below) and transferred to nitrocellulose sheets. Replica blots were washed first with 155 mM NaCl, 15 mM Tris-HCl, and 0.1% Tween 20, pH 7.35 (washing buffer), and then with washing buffer containing 0.1% gelatin for 3 h. One replica was incubated with a mixture of alkaline phosphatases (consisting of 100 units/ml bovine kidney (Sigma) and calf intestine (Boehringer Mannheim) alkaline phospha-

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This paper is dedicated to Eva Applebaum and Elias Brountzos.

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[1] S. Georgatos and K. Radsak, unpublished observations.

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

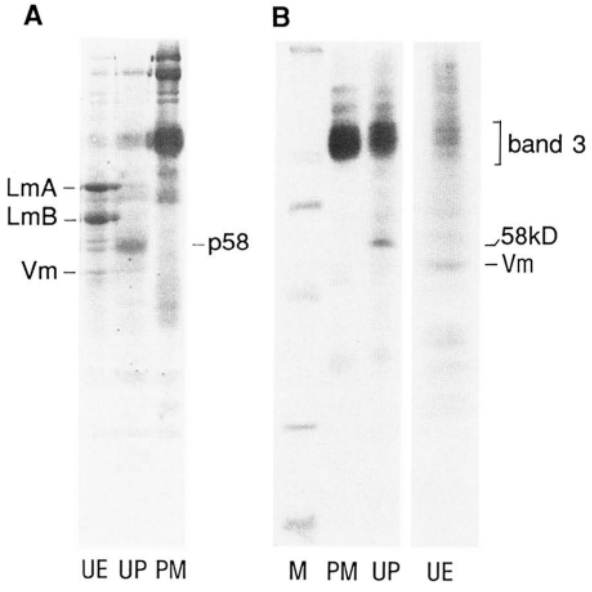

FIG. 1. *A*, electrophoretic profiles of turkey erythrocyte fractions. Circulating turkey erythrocytes were subcellularly fractionated, and the resulting nuclear envelopes and plasma membrane were extracted with 8 M urea as described under "Materials and Methods." Samples were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) on 10.5% acrylamide gels and stained with Coomassie Blue. *UE*, urea extract of nuclear envelopes; *UP*, urea-extracted nuclear envelopes (residue); *PM*, urea-extracted plasma membranes. The positions of lamin A (*LmA*), lamin B (*LmB*), vimentin (*Vm*), and lamin B receptor (*p58*) are indicated. *B*, autoradiographic profiles of a urea extract of nuclear envelopes (*UE*), urea-extracted nuclear envelopes (*UP*), and urea-extracted plasma membranes (*PM*) that were obtained after incubation of intact erythrocytes with $^{32}P_i$ (3.5 h) and analysis of the resulting fractions by SDS-PAGE. The positions of band 3 (originating from plasma membrane contamination), vimentin (*Vm*), and a major 58-kDa phosphoprotein of the nuclear envelope are indicated. Note the partitioning of the latter polypeptide with the urea-unextractable material. *M* denotes ^{14}C-labeled molecular weight markers (from *top* to *bottom*: phosphorylase *b*, M_r = 97,400; bovine serum albumin, M_r = 66,200; hen ovalbumin, M_r = 42,699; bovine carbonic anhydrase, M_r = 31,000).

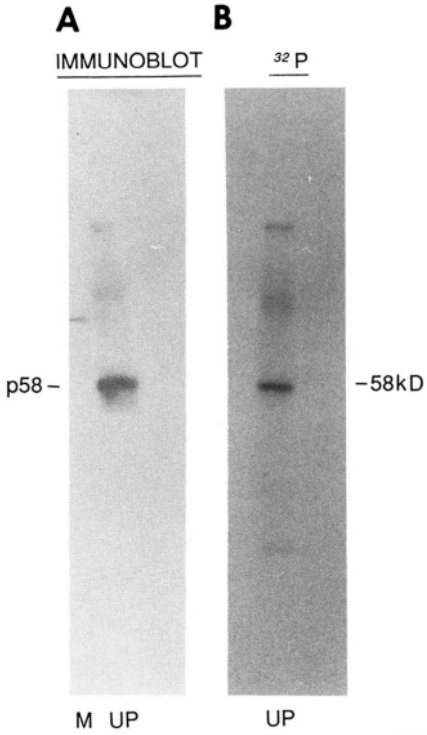

FIG. 2. **One-dimensional comparison of the electrophoretic mobility of the 58-kDa phosphoprotein and the lamin B receptor (*p58*).** *A*, urea-extracted nuclear envelopes (*UP*) were prepared, analyzed by SDS-PAGE, and the proteins contained in this fraction were transferred to nitrocellulose. The sample (together with a sample of molecular weight markers (*M*)) was then immunoblotted by anti-p58 antibodies (diluted 1:500). The position of the p58 antigen is indicated (*p58*). *B*, urea-extracted nuclear envelopes were prepared from cells incubated with $^{32}P_i$ (18 h). This fraction was analyzed in parallel (adjacent lane) with the sample shown in *A*, transferred to nitrocellulose, and directly autoradiographed. Note the comigration of the 58-kDa phosphoprotein and the p58 antigen.

tases) in a buffer containing 100 mM Tris-HCl, 5 mM $MgCl_2$, 0.1% Tween 20, and 0.5 mM phenylmethylsulfonyl fluoride, pH 8.0, for 2 h at 37 °C. The other replica was treated similarly, except in the absence of phosphatase. The blots were processed for lamin B binding as specified (15).

To control for proteolysis during alkaline phosphatase treatment, urea-extracted nuclear envelopes were fractionated by SDS-PAGE, transferred to nitrocellulose sheets and either incubated with an alkaline phosphatase solution (see above) or treated with buffer. Thereafter, the blots were stained with 0.1% India ink, the corresponding p58 bands excised, and the protein was eluted from the nitrocellulose by 8 M urea, 1 M NaCl, 4 mM EDTA, 1 mM dithiothreitol, 0.5% SDS, and 0.5 mM phenylmethylsulfonyl fluoride, pH 10.5, for 1 h at room temperature. The eluted protein was then re-electrophoresed and tested by immunoblotting using anti-p58 antibodies (15).

Other Methods—Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (20). Transfer of proteins to nitrocellulose sheets and immunoblotting were done as previously described (15). Protein concentrations were estimated using the Bio-Rad protein determination kit.

RESULTS AND DISCUSSION

Identification of a 58-kDa Integral Nuclear Membrane Phosphoprotein—To investigate whether p58 is phosphorylated *in vivo*, we incubated turkey erythrocytes with $^{32}P_i$ and isolated nuclear envelopes and plasma membranes. The nuclear envelopes were subsequently extracted with 8 M urea (which removes the nuclear lamins but not the lamin B receptor, see Ref. 15), and the resulting fractions were analyzed by SDS-PAGE (Fig. 1*A*) and autoradiography (Fig. 1*B*). Fig. 1*B* shows that the nuclear envelope fraction contains three major phosphoproteins at approximately 52, 58, and 100 kDa (*lanes UE* and *UP*). The 100-kDa band represents the erythrocyte anion transporter (band 3) and originates from (variable) contamination with plasma membranes. Upon urea extraction of the nuclear envelopes, the 52-kDa polypeptide is recovered in the supernatant (Fig. 1, *A* and *B*, *lanes UE*), whereas the 58-kDa polypeptide is seen exclusively in the membrane residue of the urea-extracted envelopes (*lanes UP*). Judging from its molecular weight and solubility properties, the 52-kDa phosphoprotein appears to be vimentin, a subunit of intermediate filaments known to be phosphorylated in these cells (21). Comparison of the above profiles with electrophoretograms generated using plasma membrane fractions (Fig. 1, *A* and *B*, *lanes PM*) confirms that the band seen at 100 kDa represents the anion transporter (band 3), which is known to be phosphorylated (22). The 58-kDa phosphoprotein, however, appears to partition exclusively with the nuclear fraction and to be an integral component of the nuclear membrane (*lanes UP*).

To examine whether the 58-kDa phosphoprotein and p58 were related, ^{32}P-labeled and nonlabeled polypeptides of urea-extracted nuclear envelopes were fractionated by SDS-PAGE, replica gels were transferred to nitrocellulose filters, and one sample (the labeled) was directly autoradiographed, whereas the other (the nonlabeled) was probed with specific anti-p58 antibodies (15). Fig. 2 shows that the p58 antigen and the

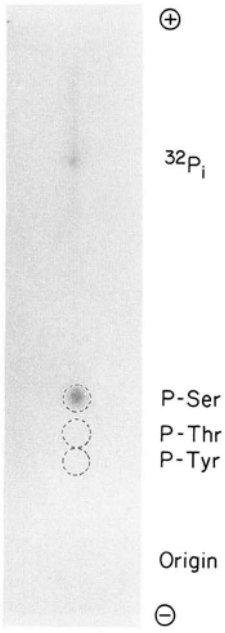

FIG. 3. **Phosphoamino acid analysis of the 58-kDa protein of the nuclear envelopes.** Erythrocyte proteins were labeled, by incubating turkey erythrocytes with $^{32}P_i$ (18 h), and then analyzed by SDS-PAGE. The 58-kDa band was excised from the gel and then processed for amino acid analysis as specified under "Materials and Methods." The autoradiographic profile of the digest is shown in comparison with the migration of co-electrophoresed phosphoamino acid standards: *P-Ser*, phosphoserine; *P-Thr*, phosphothreonine; *P-Tyr*, phosphotyrosine. Note the co-migration of the 58-kDa phosphoamino acids exclusively with the phosphoserine marker.

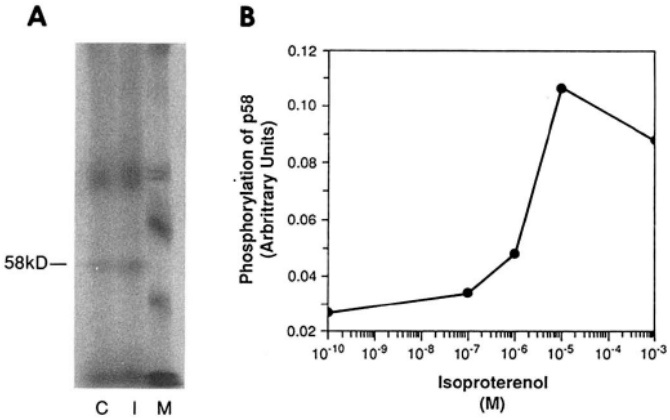

FIG. 4. **Isoproterenol stimulation of the phosphorylation of the 58-kDa protein of the nuclear envelopes.** *A*, Autoradiographic profiles of urea-extracted nuclear envelopes from cells incubated with $^{32}P_i$ (3.5 h) in the absence (*C*) and in the presence (*I*) of 10^{-6} M isoproterenol (20 min). *M* is a sample of ^{14}C-labeled molecular weight markers. Notice the higher incorporation of $^{32}P_i$ in *I* compared with *C*; *B*, dose dependence of the stimulatory effect of isoproterenol on the phosphorylation of the 58-kDa protein. Erythrocyte proteins were labeled with $^{32}P_i$ as specified under "Materials and Methods" in the presence of increasing concentrations of isoproterenol. After SDS-PAGE and autoradiography, the autoradiograms were scanned by a laser scanner, and the integrated peak area of the 58-kDa band was plotted as a function of isoproterenol concentration (in molar units).

phospholabeled 58-kDa polypeptide exactly comigrate in one dimension. Since in our previous studies (15) we have defined p58 (and raised antibodies against it) using one-dimensional criteria, these data suggest that the 58-kDa phosphoprotein is contained within the p58 antigen "band". Other observations (presented below) further support this interpretation.

To determine the specific amino acid residues that bear the phosphate groups in the 58-kDa phosphoprotein, we prepared ^{32}P-labeled nuclear envelopes, and the 58-kDa band was excised, digested with chymotrypsin, the digest acid-hydrolyzed, and the hydrolysate subjected to high voltage electrophoresis. The results of this analysis are shown in Fig. 3 and demonstrate that the phosphate groups in 58-kDa phosphoprotein occur exclusively at serine residues.

Isoproterenol Stimulates the Phosphorylation of the 58-kDa Phosphoprotein—Because previous studies had demonstrated that a number of structural proteins of the turkey erythrocyte (including ankyrin, vimentin, etc., see Refs. 18, 21, and 22) are phosphorylated by cAMP-dependent protein kinases at serine residues and that their phosphorylation is stimulated by β-adrenergic agonists, we investigated the effects of isoproterenol on the phosphorylation of the 58-kDa phosphoprotein. As shown in Fig. 4*A*, isoproterenol (at a concentration of 10^{-6} M) significantly enhances phosphorylation (compare *lanes C* and *I*). Repeating the experiment with different concentrations of the isoproterenol, we found that phosphorylation is dose-dependent (Fig. 4*B*). The phosphorylation of vimentin and ankyrin is also stimulated by isoproterenol, as has been previously reported (data not shown).

Phosphorylation of p58 Affects Its Binding to Lamin B—If the 58-kDa phosphoprotein represents the authentic lamin B receptor (p58), its phosphorylation may somehow affect the binding to lamin B. To investigate the influence of phosphorylation on p58-lamin B interactions, we proceeded with *in vitro* binding experiments. Nuclear envelopes were prepared from turkey erythrocytes, extracted with 8 M urea, and the resulting urea-"stripped" membranes (containing the p58) were fractionated by SDS-PAGE. After transfer to nitrocellulose filters, the nuclear membrane polypeptides were dephosphorylated *in situ* by incubating the nitrocellulose filter with calf intestine and bovine kidney alkaline phosphatases (other experiments with ^{32}P-labeled p58 had demonstrated that this treatment removes the bulk of the phosphate). Replica blots with alkaline phosphatase-treated samples or mock treated samples were then incubated with isolated ^{125}I-lamin B as previously described (15). Fig. 5*B* shows that removal of phosphate from p58 results in a dramatic decrease of lamin B binding (compare *lanes NT* and *AP*). This finding demonstrates that the lamin B-p58 interaction is affected by the phosphorylation state of p58. It also suggests that the phosphoprotein migrating at 58 kDa represents the lamin B receptor.

To rule out the possibility that lamin B binding to p58 is reduced after alkaline phosphatase treatment because of proteolysis (the enzyme preparations could conceivably be contaminated with proteases), we treated replica blots of turkey erythrocyte urea-extracted nuclear envelopes either with buffer or alkaline phosphatase. The bands corresponding to p58 were then excised, the protein eluted by a mixture of urea and SDS, and the samples re-electrophoresed. Immunoblotting with anti-p58 antibodies (Fig. 5*C*) showed no significant degradation in both cases. Thus, the reduced binding of ^{125}I-lamin B to alkaline phosphatase-treated p58 does appear to be the result of specific dephosphorylation of this protein.

In conclusion, we show here that the avian erythrocyte nuclear envelope contains a major 58-kDa phosphoprotein that is phosphorylated at serine residues. Phosphorylation is increased by β-adrenergic agents. This polypeptide, like p58, represents an integral (nuclear) membrane protein. At this point, we cannot formally rule out the possibility the p58 and the 58-kDa phosphoprotein are distinct proteins. As our anti-p58 antibodies were raised against the p58 "band," they would

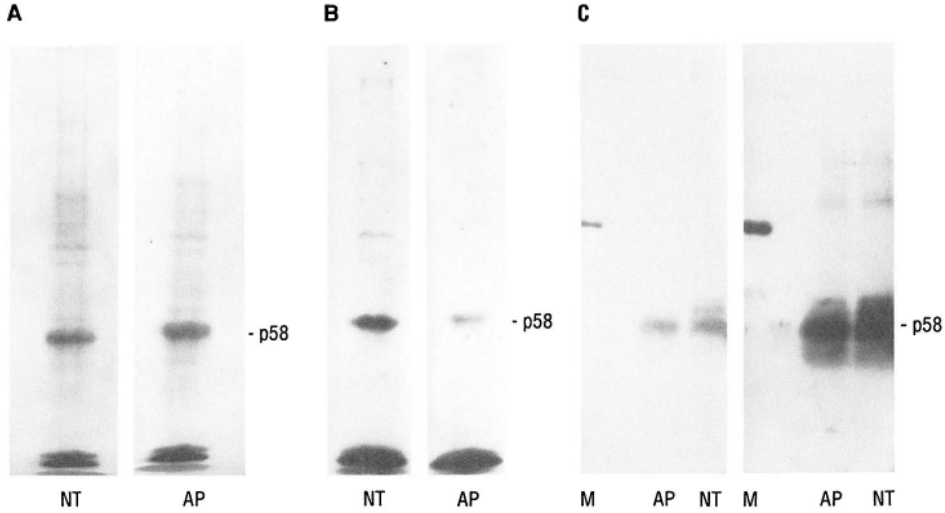

FIG. 5. **Effect of phosphorylation on the binding of lamin B to the lamin B receptor (*p58*).** Urea-extracted nuclear envelopes were prepared and the polypeptides contained in this fraction were analyzed by SDS-PAGE (7.5% acrylamide gel). Two adjacent lanes, loaded with exactly the same amount of material, were then transferred to nitrocellulose. One strip (*AP*) was treated with alkaline phosphatase (as specified under "Materials and Methods") whereas the other (*NT*) was treated with buffer alone. After extensive washings, the two blots were probed with ^{125}I-rat liver lamin B. *A*, India ink-stained blots of NT and AP. *B*, autoradiograms of the same blots after probing with ^{125}I-lamin B. Note the reduced binding of ^{125}I-lamin B to the p58 in the replica that was treated with alkaline phosphatase. *C*, effect of alkaline phosphatase treatment on the integrity of the lamin B receptor (*p58*). Samples of urea-extracted nuclear envelopes were treated as in *A*, (*AP*, incubated with alkaline phosphatase; *NT*, treated with buffer alone). The two blots were then washed and the nitrocellulose filters stained with 0.1% India ink. The p58 bands from the two samples were excised and the protein eluted by a mixture of urea and SDS at alkaline pH (see "Materials and Methods"). The eluted material was then re-electrophoresed and probed with anti-p58 antibodies (1:500). Autoradiograms are shown after exposure of the film for 2 h (*left*) and 24 h (*right*) at −70 °C. *M* is a lane containing molecular weight markers (see Fig. 1) showing background reactivity with phosphorylase *b*. Note the absence of significant degradation in the AP sample (by comparison with the NT sample).

not be suitable to distinguish between different protein species that comigrate in one dimension.

To demonstrate, however, that the lamin B receptor and the 58-kDa phosphoprotein are related, we used alkaline phosphatase treatment of the p58 and then examined the effects on the binding to lamin B. These data, presented in Fig. 5, complement the data in Figs. 1 and 2 and suggest that the lamin B receptor is indeed a phosphoprotein.

These results implicate phosphorylation in nuclear lamin-nuclear membrane interactions and indicate that constitutive phosphorylation of p58 may be among the mechanisms that contribute to a stable lamin B-nuclear membrane attachment. Although it is not clear whether the same sites on p58 are phosphorylated in the absence and in the presence of adrenergic stimulation, the fact that isoproterenol induces p58 phosphorylation suggests that the lamin B-nuclear membrane interactions may be subjected to hormonal control. It seems interesting that adrenergic stimulation results in hyperphosphorylation of several cytoskeletal and karyoskeletal components as, for example, ankyrin, vimentin, and lamin B receptor. One highly speculative functional implication concerning this *en bloc* modification could be that the molecular interactions along the plasma membrane-intermediate filament-nuclear lamina axis (23–26) are coordinately regulated by humoral factors and in particular by β-adrenergic agents.

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