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The inner nuclear membrane protein p58 associates *in vivo* with a p58 kinase and the nuclear lamins

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p58, also referred to as the lamin B receptor, is an intrinsic protein of the inner nuclear membrane that binds *in vitro* to lamin B. Previous studies have demonstrated that p58 is phosphorylated *in vivo* and removal of its phosphate moieties affects lamin B binding. Using affinity-purified antipeptide antibodies, we have now immunoisolated p58 from bird erythrocyte lysates under isotonic, non-denaturing conditions. Analysis of the immunopurified material shows that five distinct proteins are tightly and specifically associated with p58. Two of these polypeptides can be identified as nuclear lamins A and B. The immunoisolate also contains a kinase activity that phosphorylates p58 *in vivo* and *in vitro*, exclusively at serine residues, as indicated by phosphoamino acid analysis and two-dimensional phosphopeptide mapping. Cell fractionation experiments and *in vitro* phosphorylation assays demonstrate that the p58 kinase resides in the nuclear envelope and is distinct from protein kinase A and cdc2 kinase, for both of which p58 is an *in vitro* substrate. These data suggest that p58 is interacting *in vivo* with a p58 kinase and the nuclear lamins.

Key words: immunoisolation/inner nuclear membrane/lamin B receptor/nuclear lamina/p58 kinase

Introduction

The nuclear lamina is a filamentous meshwork underlying the inner nuclear membrane (Gerace and Blobel, 1980; Aebi *et al.*, 1986). In most higher eukaryotic cells, this structure is made of two types of proteins termed type A and type B lamins (Gerace *et al.*, 1978). The lamins belong to the large family of intermediate filament proteins (Aebi *et al.*, 1986; Fisher *et al.*, 1986; McKeon *et al.*, 1986). Their self-assembly is partially understood on the basis of principles that govern the polymerization of cytoplasmic intermediate filaments (IFs) (for a review see Steinert and Roop, 1988). However, it remains unclear how the lamina meshwork is anchored to the inner nuclear membrane. Type B lamins, unlike (mature) lamin A and cytoplasmic IF proteins, may interact directly with the lipid bilayer via a covalently-bound isoprenyl group that they all possess and which is essential for nuclear membrane targeting (Wolda and Glomset, 1988; Krohne *et al.*, 1989; Kitten and Nigg, 1991). Furthermore, it has been proposed that a protein factor may be responsible for the coupling of the B type lamins to the inner nuclear membrane (Gerace and Blobel, 1982; Worman *et al.*, 1988).

Only a few intrinsic components of the nuclear envelope or the inner nuclear membrane have been characterized so far; these include a set of immunochemically interrelated mammalian proteins with molecular masses of 75, 68 and 55 kDa (Senior and Gerace, 1988), a 53 kDa protein in *Drosophila* cells (Harel *et al.*, 1989), a 54 kDa polypeptide in chicken cells (Bailer *et al.*, 1991) and a 58 kDa protein (p58) of the turkey erythrocyte nuclear envelope (Worman *et al.*, 1988). cDNA sequencing demonstrates that p58 contains eight hydrophobic segments that may represent the transmembrane domains of this protein (Worman *et al.*, 1990) and immunoelectron microscopy shows an exclusively inner nuclear membrane localization (J.Yuan, J.-C. Courvalin, G.Blobel and H.J.Worman, in preparation). Based on biochemical and immunochemical data (Bailer *et al.*, 1991), it seems that the 54 and the 58 kDa proteins are closely related, if not identical. Of these polypeptides, p58 has been demonstrated to bind purified lamin B *in vitro* and has been suggested to function as a 'lamin B receptor' in the context of the nuclear envelope (Worman *et al.*, 1988). p58 has been immunochemically identified in tissues other than erythrocytes (Worman *et al.*, 1990; K.D.Radsak and S.D.Georgatos, unpublished observations) and in the yeast *Saccharomyces cerevisiae* (Georgatos *et al.*, 1989).

p58 is phosphorylated *in vivo* exclusively at serine residues and this phosphorylation appears to be essential for lamin B binding (Appelbaum *et al.*, 1990). Furthermore, it can be hyperphosphorylated *in vivo* after stimulation of turkey red blood cells by β-adrenergic agonists (Appelbaum *et al.*, 1990). cDNA sequence analysis further shows the existence of two protein kinase A (PKA) consensus phosphorylation sites and of two sites conforming to known cdc2 kinase substrate motifs at the amino-terminal domain of p58 (Worman *et al.*, 1990).

To identify factors that may modify or interact with p58 *in vivo*, we have attempted to isolate this polypeptide using mild detergent extraction and immunoisolation with site-specific antibodies. We report here that p58 is associated with the nuclear lamins and a specific kinase for which serves as a substrate.

Results

Characterization of the anti-p58 antibodies

To generate site-specific antibodies, we synthesized a number of peptides modelled after the published cDNA sequence of chicken liver p58 (Worman *et al.*, 1990). One of these peptides, R1 (Figure 1A), elicited high affinity polyclonal antibodies (# 168 antiserum) after immunization of rabbits. Following affinity purification over an agarose–R1 column, the # 168 antibodies were tested by immunoblotting and indirect immunofluorescence. Examination of turkey nuclear envelope fractions by immunoblotting shows that the affinity purified antibodies specifically recognize p58 (Figure 1B). No cross-reacting proteins can be detected by the antibodies

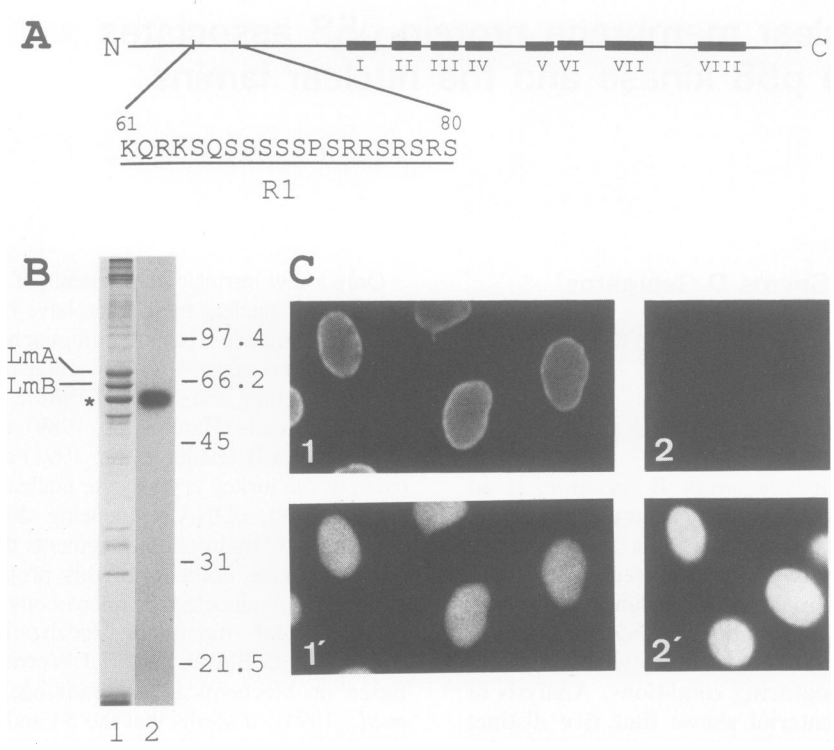

Fig. 1. Characterization of the anti-peptide antibodies against p58. **(A)** Amino acid sequence (one letter code) of the p58 peptide R1 (amino acid residues 61–80), used as antigen for the production of the # 168 antibodies. The relative position of the peptide in the p58 molecule is also schematically indicated. Black boxes along the p58 sequence and roman numerals represent hydrophobic amino acid stretches that can form potential transmembrane domains (Worman *et al.*, 1990). **(B)** SDS–PAGE analysis and Coomassie Blue staining of a sample of turkey erythrocyte nuclear envelopes (lane 1) and autoradiogram of an immunoblot of an identical sample using affinity purified # 168 antibodies and [^{125}I]protein A (lane 2). Protein bands corresponding to lamin A (LmA) and lamin B (LmB) are indicated by bars on the left. The asterisk denotes the position of p58. Bars on the right correspond to molecular masses (in kDa). **(C)** Immunofluorescence microscopy of turkey erythrocytes using the affinity purified # 168 antibodies (panel 1) or the same antibodies mixed with an excess of peptide R1 (panel 2) and staining of the nuclei with DAPI (panels 1′ and 2′ respectively).

in the plasma membrane or cytosolic fractions (data not shown). Figure 1C, also shows that the # 168 antibodies stain the nuclear periphery of turkey red blood cells and that this decoration is abolished when the antibody is mixed with an excess of soluble R1 peptide.

Identification of p58 partners

Immunoprecipitation of p58 from Triton X-100 lysates of whole erythrocytes (electrophoretic profile of this material is shown in Figure 2A, lane 1) was performed using the affinity-purified # 168 antibodies. Analysis of the immunoisolate by SDS–PAGE and staining with Coomassie Blue reveals the presence of a 58 kDa band (Figure 2A, lane 2) that is not immunoprecipitated when the antibodies are mixed with an excess of R1 peptide (Figure 2A, lane 3). This protein can be identified as p58 by probing blots of the immunoprecipitate with # 168 antibodies (Figure 2B, lane 1) or with other anti-p58 antibodies (not shown). Silver staining further shows the presence in the immunoprecipitate of several quantitatively minor bands at 150, 70, 66, 34 and 18 kDa of roughly equivalent molar amounts (Figure 2A, lane 4). None of these polypeptides is detected when the immunoprecipitation is carried out in the presence of an excess of the R1 peptide (Figure 2A, compare lanes 4 and 5). From these data, we conclude that at least a fraction of p58 is solubilized (under isotonic, non-denaturing conditions) as a multicomponent 'complex'.

p58 is associated with the nuclear lamins and a p58 kinase

To examine whether any of the proteins coimmunoprecipitated with p58 represent the nuclear lamins, precipitated material was analysed by SDS–PAGE and immunoblotting with specific antibodies. Figure 2B, lane 2, demonstrates that the 70 and 66 kDa polypeptides specifically react with affinity-purified antibodies (aLI) recognising conserved features of type A and B lamins. The identity of the lower lamin band as lamin B2 can be confirmed by probing replica blots with a monoclonal antibody (E3) specific for avian lamin B2 (Figure 2B, lane 3). Arguing for a specific interaction between the lamins and the other components of the 'complex' is the fact that neither of the lamins is precipitated when the experiment was carried out in the presence of an excess of R1 (Figure 2C, lanes 1 and 2), when the anti-p58 antibodies are substituted by non-immune rabbit IgG (Figure 2C, lane 5) or when no antibodies are added (Figure 2C, lane 6). Interestingly, when the solubilization and the immunoprecipitation are carried out in the presence of ATP, an ATP-regeneration system and a phosphatase inhibitor (β-glycerophosphate), the amount of coimmunoprecipitated lamin B increases significantly (Figure 2C, lanes 3 and 4).

Incubation of immunoisolated p58 complexes with [γ-^{32}P]ATP and analysis of the reaction mixtures by SDS–PAGE and autoradiography reveal that p58 can be

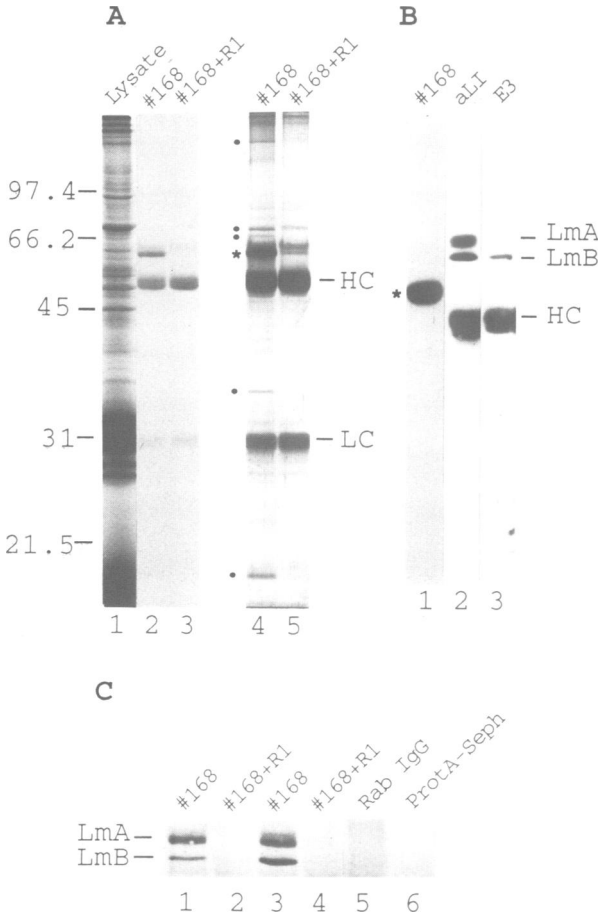

Fig. 2. p58 associates with the nuclear lamins and three other proteins. **(A)** SDS−PAGE and Coomassie Blue staining of turkey erythrocyte Triton X-100 lysates (lane 1), immunoprecipitated proteins using the affinity-purified # 168 antibodies (lane 2) and immunoprecipitated proteins with the same antibodies in the presence of peptide R1 (lane 3). Lanes 4 and 5 correspond to lanes 2 and 3, respectively, except that the gels were stained with silver. Dots mark the proteins that specifically coimmunoprecipitate with p58 (p58 is denoted by the asterisk). HC, IgG heavy chain; LC, IgG light chain. Numbered bars indicate molecular masses (in kDa). **(B)** Autoradiograms of blots of proteins immunoprecipitated by the affinity-purified # 168 antibodies. Replica blots were incubated with anti-p58 (# 168) antibodies (lane 1), anti-lamin (aLI) antibodies (lane 2) or anti-lamin B2 (E3) antibodies (lane 3) and [^{125}I]protein A. The IgG heavy chain (HC) band shows up in lanes 2 and 3 due to longer exposure times. Lamin A, lamin B and IgG heavy chain are marked by bars. The p58 is marked by an asterisk. **(C)** Immunoprecipitated proteins analysed by immunoblotting with an anti-lamin antibody (aLI). Immunoprecipitations were carried out using the affinity purified # 168 antibodies (lane 1), # 168 antibodies mixed with peptide R1 (lane 2), rabbit non-immune IgG (lane 5) or protein A−Sepharose alone (lane 6). Lanes 3 and 4: immunoprecipitation was carried out as in lanes 1 and 2 but the lysis buffer was supplemented with ATP, an ATP-regenerating system and β-glycerophosphate. The blots were stained using an alkaline phosphatase conjugated goat anti-rabbit antibody. Only the relevant parts of the blots are shown. Note that the band comigrating with lamin A in lane 5 is an unrelated protein contaminant from the IgF preparation.

heavily phosphorylated under these conditions (Figure 3A, compare lane 2 with lanes 1 and 3). This suggests the presence in the immunoprecipitate of a p58 kinase activity that is associated directly or indirectly with p58. Incubation of the immunoprecipitates with [γ-^{32}P]GTP under the same

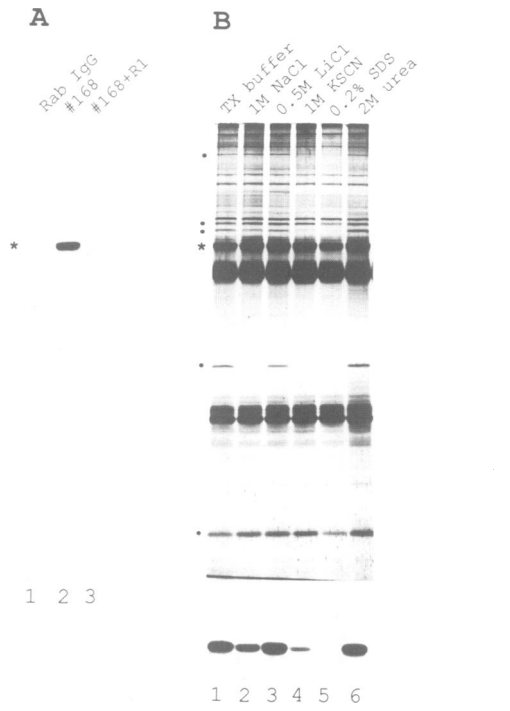

Fig. 3. The p58 partners are tightly associated and include a protein kinase that phosphorylates p58. **(A)** Material immunoprecipitated by non-immune rabbit IgG (lane 1), affinity-purified # 168 antibodies (lane 2) or affinity-purified # 168 antibodies plus R1 (lane 3), was incubated with [γ-^{32}P]ATP. The samples were analysed by SDS−PAGE and autoradiography. The position of p58 is marked by an asterisk. **(B)** Material immunoprecipitated by affinity-purified # 168 antibodies was washed with 0.1% Triton buffer (lane 1) or with Triton buffer containing 1 M_r NaCl (lane 2), 0.5 M LiCl (lane 3), 1 M KSCN (lane 4), 0.2% SDS (lane 5), 2 M urea (lane 6) and incubated with [γ-^{32}P]ATP. The samples were analysed by SDS−PAGE and either silver-stained (upper panel) or autoradiographed (lower panel; only the area corresponding to p58 is shown).

conditions does not result in p58 phosphorylation, indicating that the p58-associated kinase exclusively utilizes ATP as a phosphate donor (data not shown).

To assess the stability of the immunoisolated material under various conditions, immunoprecipitates were washed with buffers containing high salt, low concentrations of urea or chaotropic and denaturing agents such as KSCN and SDS. After further washing with isotonic 0.1% Triton buffer, the immunoprecipitates were incubated with [γ-^{32}P]ATP and analysed by SDS−PAGE and autoradiography (Figure 3B). The composition of the p58-associated proteins and the activity of the p58 kinase are not affected by treatment with 0.5 M LiCl or 2 M urea (Figure 3B, compare lanes 1, 3 and 6). However, treatment with higher salt (1 M NaCl) dissociates the 34 kDa protein and leads to a reduction of the p58 kinase activity (Figure 3B, compare lanes 1 and 2). Exposure of the immunoisolated material to 1 M KSCN or 0.2% SDS results in almost complete removal of all of the p58-associated proteins with the notable exception of the 18 kDa component (Figure 3B, compare lanes 1, 4 and 5). These results suggest a tight and specific association between the coimmunoprecipitated proteins. This association can only be disrupted by chaotropic and denaturing agents.

To examine the extent to which the association of p58 with its partners pre-existed *in vivo*, we repeated the

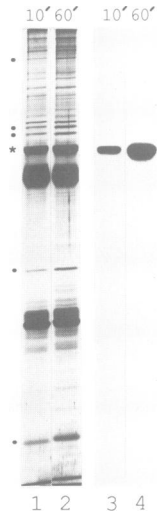

Fig. 4. The association of p58 with its partners can be detected within 10 min after preparation of erythrocyte extracts. Triton extracts (prepared by a 5 min extraction of red cell ghosts) were incubated with affinity-purified # 168 antibodies for either 10 (lanes 1 and 3) or 60 min (lanes 2 and 4). After immunoprecipitation, a set of samples was analysed by SDS−PAGE and silver staining (lanes 1 and 2). Alternatively, the immunoprecipitated material was first incubated with [γ-^{32}P]ATP and then analysed by SDS−PAGE and autoradiography (lanes 3 and 4). Asterisk indicates the position of p58, whereas dots denote the specifically co-immunoprecipitated proteins. Note that the stoichiometry of the p58 complex does not change upon shorter (10 min) or longer (60 min) sample processing (compare also with Figure 2A, lanes 4 and 5).

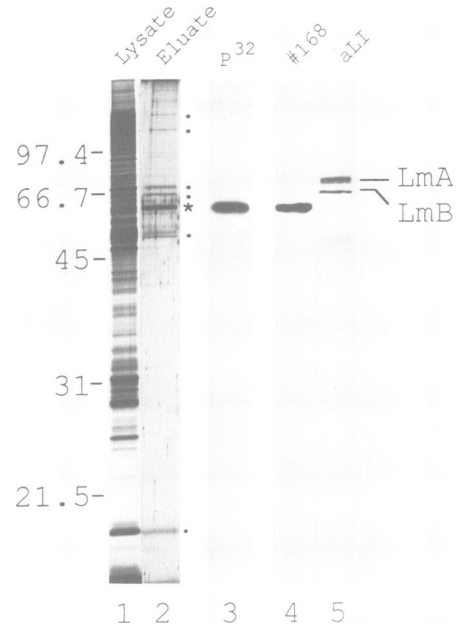

Fig. 5. Isolation of p58 and p58-associated proteins by immunoaffinity chromatography. Erythrocyte ghosts were extracted with 1% Triton buffer containing 20 mM β-glycerophosphate and the extract was applied to a protein A− # 168 antibody column. Bound proteins were eluted with 1 mg/ml peptide R1 in 0.1% Triton buffer. SDS−PAGE and silver staining of the Triton lysate is shown in lane 1 and of the eluted material in lane 2. Lane 3, SDS−PAGE and autoradiography of a sample of the column eluate incubated with [γ-^{32}P]ATP. Lanes 4 and 5, immunoblotting of the eluted proteins with anti-p58 (# 168) antibodies (lane 4) or anti-lamin (aLI) antibodies (lane 5). Proteins that copurify with p58 are marked by dots and the asterisk denotes the position of p58.

immunoisolation in a time-course fashion. Extraction with detergent was performed for 5 min on ice and the extract was immunoprecipitated for various time intervals. Consistent with the idea of an *in vivo* interaction, the same set of proteins are coimmunoprecipitated even after a 10 min incubation of the extract with the antibodies. In addition, the relative amounts of the coimmunoprecipitated proteins do not seem to vary with sample processing time (Figure 4).

To explore another method of immunoisolation, we proceeded as follows: washed erythrocyte ghosts (obtained by lysing the red blood cells in hypotonic media) were extracted with Triton X-100 under isotonic conditions and in the presence of β-glycerophosphate (a phosphatase inhibitor) (SDS−PAGE profile of the Triton lysate is shown in Figure 5, lane 1). This extract was passed through a protein A−Sepharose− # 168 antibody affinity column and after appropriate washings, the column-bound material was eluted by soluble R1 peptide. SDS−PAGE and silver staining (Figure 5, lane 2) demonstrate that the R1-eluted fraction contains several polypeptides (in addition to p58). Four of these proteins match the proteins found in the p58 immunoprecipitates. However, their mass ratios in relation to p58 are detectably increased (compare with Figure 2A, lane 4). The 34 kDa protein, which is not present in the R1 eluate under the conditions described above, is detected when β-glycerophosphate is omitted from the media (data not shown). The presence of nuclear lamins and the p58-associated kinase in the R1 eluate can be confirmed by immunoblotting (Figure 5, lane 5) and *in vitro* phosphorylation assays (Figure 5, lane 3).

To investigate further whether the p58 'complex' can be isolated in a different way, a similarly prepared Triton extract

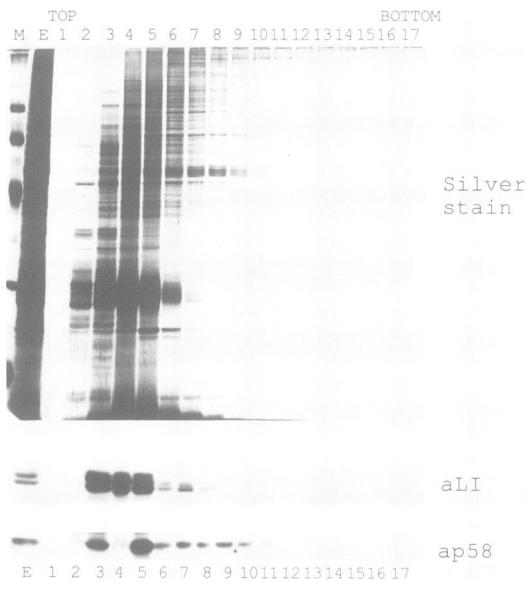

Fig. 6. Fractionation of a Triton X-100 extract of erythrocyte ghosts by centrifugation through a 5−20% sucrose gradient. SDS−PAGE and silver staining of the sucrose gradient fractions is shown in the top panel. Each fraction was TCA-precipitated and analysed by immunoblotting using anti-lamin (aLI) antibodies (middle panel) or anti-p58 (ap58) antibodies (bottom panel). M, molecular weight markers; E, material loaded on the gradient; 1−17, fraction number (top of gradient to the left).

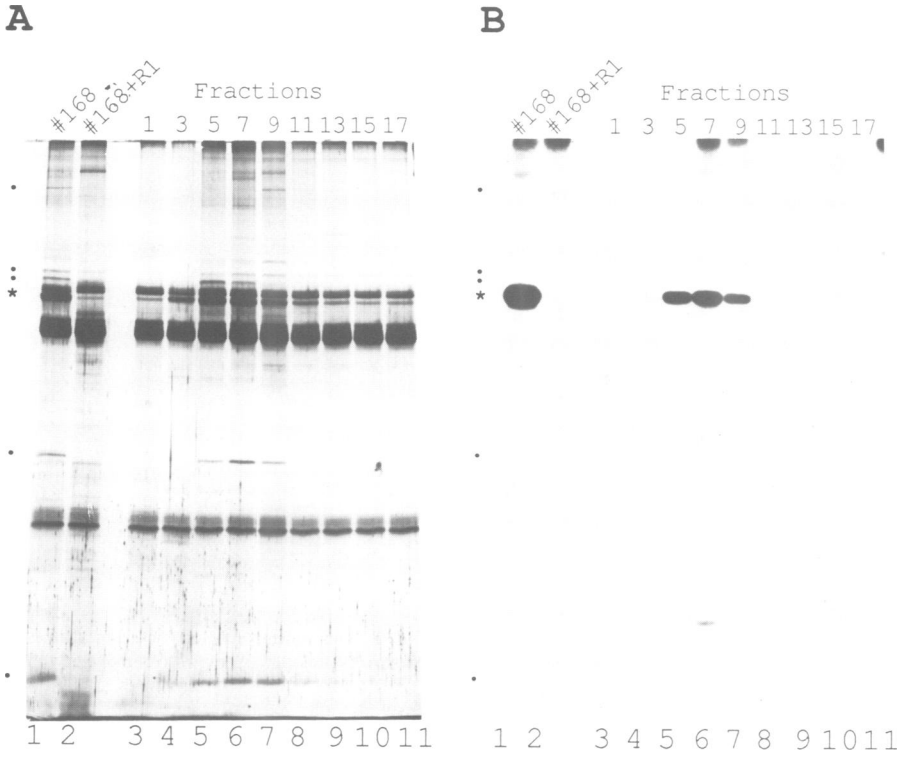

Fig. 7. Immunoprecipitation from sucrose gradient fractions using the affinity-purified # 168 antibodies. **(A)** SDS−PAGE and silver staining of proteins immunoprecipitated from erythrocyte ghost lysates using the affinity purified # 168 antibodies (lane 1) or the same antibodies mixed with peptide R1 (lane 2) and of proteins immunoprecipitated from individual sucrose gradient fractions (lanes 3−11). Fraction number is shown on top of each lane. Dots mark proteins that specifically coimmunoprecipitate with p58. The asterisk denotes the position of p58. **(B)** Autoradiography of the same samples as in (A) after incubation of the immunoprecipitates with [γ-^{32}P]ATP.

of erythrocyte ghosts was fractionated by centrifugation through a 5−20% isokinetic sucrose gradient (SDS−PAGE profile of the corresponding fractions depicted in Figure 6, top panel). Immunoblotting of the gradient fractions with anti-p58 antibodies reveals two different p58 peaks (Figure 6, bottom panel); probing of the gradient fractions with anti-lamin antibodies shows that the lamins migrate as a broad asymmetric band, overlapping with both p58 peaks (Figure 6, middle panel). Immunoprecipitation by # 168 antibodies reveals that the fast sedimenting p58 (recovered from fractions 5−9) is complexed with the lamins, the 34 and 18 kDa proteins (Figure 7A, lanes 5−7), and p58 kinase (Figure 7B, lanes 5−7). Conversely, the immunoprecipitate from the slow sedimenting p58 (fraction 3) is devoid of any other proteins and does not include the p58 kinase activity (Figure 7A and B, lane 4). The simplest interpretation of these results is that the slow migrating form of p58 represents dissociated material, whereas the fast migrating population constitutes the native p58 'complex'. Interestingly, the 150 kDa protein is only detected in the immunoprecipitate from fraction 9 and not from fractions 5 or 7, suggesting the existence of partial 'complexes' with different sedimentation velocities.

The p58-associated kinase resides in the nuclear envelope and is responsible for the in vivo phosphorylation of p58

Knowing that p58 is exclusively localized in the nuclear envelope (Worman *et al.*, 1988, 1990), we reasoned that the p58-associated kinase may also reside in the same cellular compartment. To investigate this question, we incubated salt-washed turkey erythrocyte nuclear envelopes (electrophoretic profile shown in Figure 8, lane 1) with [γ-^{32}P]ATP. Electrophoretic analysis and autoradiography show that p58 is significantly phosphorylated under these conditions (Figure 8, lane 5). The same figure shows that the turkey erythrocyte lamins A and B do not incorporate any detectable amount of phosphate, whereas a subset of histones are apparently labelled. The identity of the phosphorylated band as p58 can be confirmed by immunoprecipitation (see below). In support of this, microsequencing of the 58 kDa phosphoprotein yields the N-terminal sequence PN(X)KYAD, corresponding to the reported N-terminal sequence PNRKYAD of chicken liver p58 (Worman *et al.*, 1990).

Extraction of the nuclear envelopes with Triton X-100 releases a substantial amount of p58 (Figure 8, lanes 3 and 4). Incubation of the Triton-solubilized material or the detergent-insoluble material with [γ-^{32}P]ATP and analysis by SDS−PAGE and autoradiography reveal the existence of phosphorylated p58 in both of these fractions (Figure 8, lanes 7 and 8). Immunoprecipitation of the Triton-extracted p58 with # 168 antibodies and addition of [γ-^{32}P]ATP to the immunoprecipitate, results in the phosphorylation of p58, exactly as shown in Figure 3A, lane 2 (data not shown). Mixing of erythrocyte plasma membranes with a Triton X-100 extract of nuclear envelopes and *in vitro* phosphorylation does not result in enhancement of p58 phosphorylation, ruling out the possibility that the p58 kinase originates from plasma membrane contamination (Figure 8, lanes 9−11). These data demonstrate the existence of a nuclear envelope-bound kinase that can be partially coextracted with p58 and which modifies p58 *in vitro*.

To examine the relationship between the kinase activity that is coisolated with p58 from whole cell lysates, the envelope-bound p58 kinase and the kinase that modifies p58 *in vivo*, we performed the following experiments. First, turkey erythrocytes were labelled by incubation with $^{32}P_i$ and the *in vivo* phosphorylated p58 was immunoprecipitated by # 168 antibodies. Secondly, p58 was immunoisolated from whole erythrocyte lysates using the same antibodies and then phosphorylated *in vitro* (as shown in Figure 3A). Thirdly, nuclear envelope-bound p58 was phosphorylated *in vitro* and then immunoprecipitated by # 168 antibodies. Samples from these three preparations were analysed by SDS–PAGE, blotted onto nitrocellulose filters and the corresponding 58 kDa bands were excised and processed for two-dimensional phosphopeptide mapping (for details see Materials and methods).

Figure 9A demonstrates that *in vivo* labelled p58 yields three major (peptides designated 1, 5 and 9) and nine minor phosphopeptides. The pattern of the phosphopeptides is virtually identical to the patterns produced by the two *in vitro* phosphorylated preparations (Figure 9B and C). The only differences between the three maps are that two or three of the minor *in vivo* phosphopeptides are not detectable in *in vitro* labelled material and that the relative intensity of labelling of the major phosphopeptides differs. Such differences may be due to the fact that the accessibility of certain p58 sites to the p58 kinase changes during the fractionation or the isolation procedure. The identity of the common phosphopeptides in the *in vivo* and *in vitro* labelled p58 is confirmed by mixing equal counts per min of the two tryptic digests prior to mapping analysis (Figure 9D). Phosphoamino acid analysis (not shown) of the *in vitro* phosphorylated p58 demonstrates that the protein is modified exclusively at serine residues, exactly as reported for the *in vivo* phosphorylated p58 (Appelbaum *et al.*, 1990). These data indicate that all of the major p58 phosphorylation sites that are modified *in vivo* are also recognized by the 'complex'-associated and the nuclear envelope-bound p58 kinase. Thus, it can be concluded that the p58-associated kinase is responsible for the modification of p58 both *in vivo* and *in vitro*.

The p58 kinase is distinct from PKA, cdc2 and other known kinases

In parallel studies (G.Simos and S.D.Georgatos, manuscript in preparation), we have observed that p58 can be *in vitro* phosphorylated by purified PKA and cdc2 kinase, as predicted from analysis of its sequence motifs (Worman *et al.*, 1990). To examine whether the p58 kinase relates to any of these enzymes, we performed *in vitro* phosphorylation experiments employing specific protein kinase inhibitors. PKI, the active peptide fragment of the heat-stable inhibitor of PKA, inhibits the PKA-mediated phosphorylation of p58, but does not affect the phosphorylation mediated by the nuclear envelope-bound p58 kinase (Figure 10, top and bottom panels). The same holds for LI, a specific peptide inhibitor of cdc2 (see Materials and methods), which only inhibits the phosphorylation of p58 by the corresponding enzyme (Figure 10, middle and bottom panels). The p58 kinase is not affected by a known inhibitor of protein kinase C (PKC) (Figure 10, bottom panel, lane 3). In the experiments shown, PKA or cdc2 were added to the nuclear envelope fractions after inactivating the endogenous kinase by heating (see below).

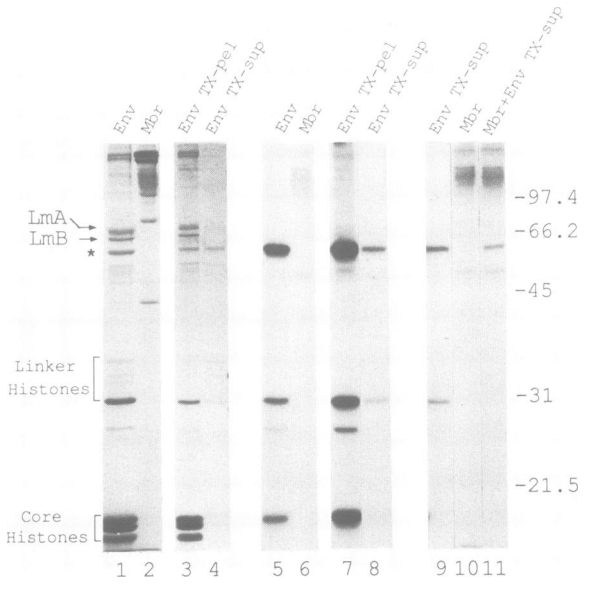

Fig. 8. The nuclear envelopes contain a kinase activity that phosphorylates p58. Samples of turkey erythrocyte nuclear envelopes (lanes 1 and 5), plasma membranes (lanes 2 and 6), residue of nuclear envelopes after extraction with 1% Triton X-100 (lanes 3 and 7) and Triton X-100 extract of nuclear envelopes (lanes 4 and 8) were incubated with [γ-^{32}P]ATP, analysed by SDS–PAGE on 12% gels and stained with Coomassie Blue (lanes 1–4) or autoradiographed (lanes 5–8). Arrows indicate the positions of lamin A (LmA) and lamin B (LmB). p58 is indicated by an asterisk. Lanes 9–11: autoradiography of a sample of the Triton X-100 extract of the nuclear envelopes (lane 9) and a sample of plasma membranes (lane 10) incubated separately or as a mixture (lane 11) with [γ-^{32}P]ATP, β-glycerophosphate and EGTA. Compare the level of phosphorylation of the p58 band in lanes 9 and 11. Numbered bars on the right indicate molecular masses (in kDa).

To examine further the relationship of the p58 kinase with other known kinases, nuclear envelopes or a Triton X-100 extract of nuclear envelopes were incubated with either casein (0.4 mg/ml) or 0.5 mM of the casein kinase II-specific peptide substrate, RRREEETEEE (Kuenzel and Krebs, 1985). We found that neither casein, nor the casein kinase II substrate inhibit the phosphorylation of p58 by the endogenous kinase or become significantly phosphorylated (data not shown), suggesting that the nuclear envelope-associated kinase is not a casein kinase II-related enzyme. Furthermore, addition of EGTA or Ca^{2+} in the phosphorylation assay does not affect the p58 kinase activity. Thus, it would appear that the p58 kinase is not a Ca^{2+}-dependent enzyme. On the contrary, heating at 60°C for 10 min, addition of EDTA, or addition of 5′-(4-fluorosulfonyl-benzoyl)adenosine (FSBA), an irreversible inhibitor of protein kinases, abolish the p58 kinase activity (data not shown). In combination, these results indicate that p58 is phosphorylated by a nuclear envelope-associated enzyme which is distinct from other major known protein kinases.

Discussion

Molecular associations of p58

In this study, we have used mild detergent solubilization in combination with conventional immunochemical methods to isolate bird erythrocyte p58 under isotonic, non-denaturing conditions. By analysing the immunopurified material, we

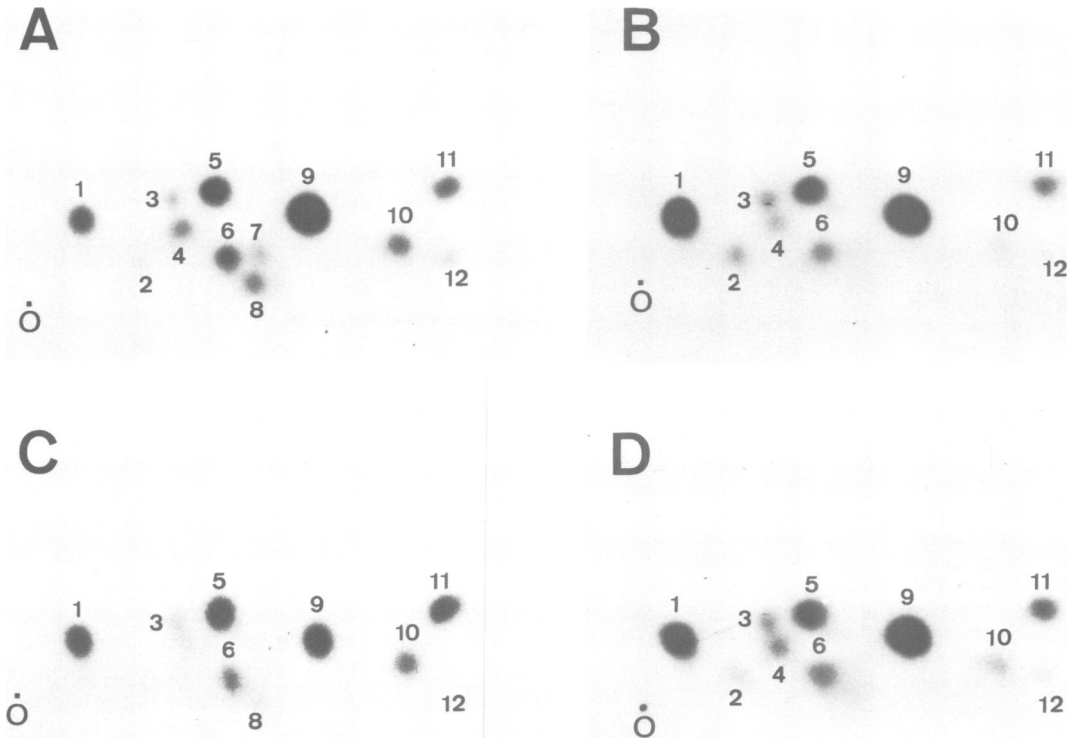

Fig. 9. Tryptic phosphopeptide analysis of p58. Phosphorylated p58 was transferred to nitrocellulose and digested with trypsin. The eluted phosphopeptides were separated by electrophoresis at pH 8.9 (horizontal direction; cathode to the right) and by ascending chromatography. Origins of sample application are marked by 'O'. **(A)** Turkey erythrocytes were labelled with $^{32}P_i$ and *in vivo* phosphorylated p58 was isolated by immunoprecipitation. **(B)** p58 was immunoprecipitated from erythrocyte lysates and phosphorylated *in vitro* by incubating the immunoprecipitate with [γ-^{32}P]ATP. **(C)** Turkey erythrocyte nuclear envelopes were incubated with [γ-^{32}P]ATP and *in vitro* phosphorylated p58 was isolated by immunoprecipitation. **(D)** Equal Cerenkov counts of digests of *in vivo* (A) and *in vitro* (B) phosphorylated p58 were mixed and analysed.

have shown that p58 is associated *in vivo* with the nuclear lamins A and B, a p58 kinase and other, as yet uncharacterized polypeptides. The specific nature of the p58 interactions has been demonstrated by showing that: (i) the same set of proteins are coisolated by either immunoaffinity chromatography or immunoprecipitation with affinity-purified anti-p58 antibodies, (ii) none of the p58-associated proteins is precipitated in the presence of R1, which inhibits the binding of p58 to the # 168 antibodies, or by non-immune rabbit IgG, and (iii) all of these components comigrate and remain associated upon fractionation of erythrocyte lysates by velocity sedimentation on sucrose gradients.

Inspection of the electrophoretic profile of a sample immunoprecipitated by anti-p58 antibodies from whole erythrocyte Triton-lysates indicates that most of the p58-associated proteins occur in roughly stoichiometric quantities. This does not apply to p58 itself, because the antibodies do not differentiate between 'complexed' and 'free' p58. A population of free p58 is indeed observed upon fractionation of lysates on a sucrose gradient (Figures 6 and 7). This population may arise either by solubilization of p58 that is uncomplexed *in situ* or by dissociation of the detergent soluble p58 from its partners during the extraction procedure. Favouring the second possibility, removal of the erythrocyte cytosol by hypotonic lysis prior to Triton solubilization and subsequent isolation of the p58 and its partners by immunoaffinity chromatography in the presence of β-glycerophosphate result in a quantitative enrichment of the non-p58 proteins. It is also noteworthy that under these conditions, one of the proteins that coimmunoprecipitate with p58 (the 34 kDa polypeptide) is not recovered. In any case, it is currently unclear whether p58 forms a distinct complex or whether it associates with its partners at different stoichiometries. The precise assessment of the stoichiometries among the p58-associated proteins would require determination of the relative quantities of each of the proteins in the starting material (lysate) and knowledge of their aggregation state, i.e. whether they occur as monomers, dimers, trimers etc. Such questions will be addressed as soon as appropriate antibodies against all of these polypeptides become available and p58 is purified to homogeneity.

At present, we do not know which of the p58-associated proteins are directly interacting with p58. However, based on previous *in vitro* work (Worman *et al.*, 1988; Appelbaum *et al.*, 1990) and on recent results demonstrating a 'receptor–ligand' relationship between p58 and lamin B by anti-idiotypic antibodies (Lassoued *et al.*, 1991), we can suggest that at least lamin B is directly bound to p58. On the other hand, lamin A may be indirectly connected to p58 via lamin B (for lamin A–lamin B binding see Georgatos *et al.*, 1988; Krohne *et al.*, 1987), or bind to p58 independently of lamin B.

Potential roles of the p58–lamin interaction

Based on the fact that the lamins are peripheral membrane proteins, whereas p58 is thought to transverse the inner nuclear membrane, it can be suggested that the p58 connects the lamina network to the nuclear membrane. In this sense,

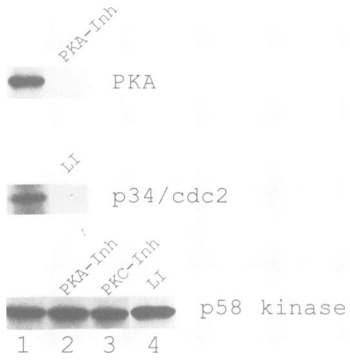

Fig. 10. The p58 kinase is distinct from PKA and cdc2 kinase. **Upper panel.** Phosphorylation of p58 after incubation of the nuclear envelopes with PKA in the absence (lane 1) or in the presence (lane 2) of 1 μM PKA inhibitor. **Middle panel.** p58 phosphorylation in the nuclear envelopes by $p34^{cdc2}$ kinase in the absence (lane 1) or in the presence (lane 2) of 250 μM of peptide LI. **Lower panel.** phosphorylation of p58 by the nuclear envelope associated p58 kinase in the absence (lane 1) or in the presence of 1 μM PKA inhibitor (lane 2), 10 μM PKC inhibitor (PKC 19–36, lane 3) and 250 μM of peptide LI (lane 4). The samples in the upper two panels were heated at 60°C for 10 min before the phosphorylation assay to inactivate the endogenous p58 kinase. Only the relevant parts of the autoradiographed SDS gels are shown.

p58 may indeed behave as a 'lamin receptor', i.e. as a protein whose main function is to couple the karyoskeleton to the lipid bilayer. Alternatively, the interaction of p58 with the nuclear lamina may provide means for anchoring the p58 at the inner nuclear membrane. In such a case, the lamins would operate as 'p58 receptors' (for a discussion see Nigg, 1992).

Independently of these scenarios, the fact that p58 associates with the lamins implies that the p58 may be involved in post-mitotic nuclear envelope reassembly. As type A lamins have an affinity for both mitotic chromosomes (Burke, 1990; Glass and Gerace, 1990) and for type B lamins (Georgatos *et al.*, 1988), it is plausible to speculate that mitotic vesicles carrying the p58 and its partners are first targeted onto the chromosomes and then serve as 'nucleation sites' for lamin and membrane assembly. A chromatin-anchorage function can also be suggested for the p58 'complex' during interphase, taking into account that lamin A has been found to interact with components of interphase chromatin (Höger *et al.*, 1991; Yuan *et al.*, 1991).

Regulation of p58 function by phosphorylation

In previous studies, we have found that p58 is phosphorylated *in vivo* during interphase (Appelbaum *et al.*, 1990). The data presented here confirm and extend these earlier findings by demonstrating the existence of a p58 kinase that is strongly associated with p58 and resides at the nuclear envelope. The p58 kinase represents the first example of an enzyme that selectively modifies an integral nuclear membrane protein in a constitutive fashion. In previous studies, a nuclear lamina-associated protein kinase of Ehrlich ascites tumour cells has been reported to phosphorylate a 52 kDa polypeptide of the nuclear envelope (Dessev *et al.*, 1988). However, since this activity also modifies the nuclear lamins, it is unlikely that it represents an enzyme similar to the one we report on.

Because the p58 kinase appears to be distinct from PKA and cdc2 kinase (this report and our unpublished data), we think that p58 may be subject to multiple regulation. More specifically, assuming that the PKA-mediated phosphorylation of p58 occurs *in vivo* and also assuming that the hyperphosphorylation of p58 after stimulation with β-adrenergic agonists (Appelbaum *et al.*, 1990) is mediated by PKA, one can postulate two modes of p58 modification: a constitutive one due to the p58 kinase and an inducible one that is mediated by PKA. There remains to be examined whether the cdc2-mediated phosphorylation of p58, which readily occurs *in vitro*, can also occur *in vivo*. In such a case, the p58 molecule would also be subject to cell cycle-dependent control.

The physiological significance of p58 phosphorylation can be correlated with two processes: the regulation of its binding to lamin B and its transport and retention at the inner nuclear membrane. With regard to the first question, *in vitro* work has previously shown that p58 dephosphorylation abolishes binding to lamin B (Appelbaum *et al.*, 1990). This finding is supported by our preliminary data showing that detectably higher amounts of lamins copurify with p58 when immunoprecipitation is carried out in the presence of ATP and an ATP-regenerating system. As far as the second problem is concerned, it seems reasonable to assume that the stabilization of p58 at the inner nuclear membrane relies on anchorage to the nuclear lamina and is dependent on its phosphorylation state.

Finally, in preliminary experiments we have observed that the p58 kinase modifies primarily sites located in the N-terminal domain of p58. It is therefore noteworthy that three different kinases, i.e. p58 kinase, PKA and cdc2, can modify the N-terminal domain of p58. If this domain is exposed to the nucleoplasm, it may participate in the various interactions between p58 and its partners in a phosphorylation-dependent manner.

Materials and methods

Reagents

The purified catalytic subunit of bovine heart PKA and the active peptide fragment of the heat-stable inhibitor of PKA (Cheng *et al.*, 1986) were purchased from Sigma (Sigma Chemical Co., Deisenhofen, Germany). The cdc2 kinase, purified from nocodazole-treated HeLa cells (Brizuela *et al.*, 1989) and the casein kinase II substrate peptide were kindly provided by G.Draetta (EMBL). The peptide inhibitor of kinase C (PKC 19–36, House and Kemp, 1987) was obtained from GIBCO BRL (Life Technologies, Inc., Gaithersburg, MD, USA). The synthetic peptide LI , corresponding to residues 1–32 of human lamin A/C (Fisher *et al.*, 1986), was synthesized at the Biopolymer Facility of Rockefeller University (New York, USA). The LI peptide contains a well characterized site for direct cdc2 phosphorylation (S^{21}PTR; Ward and Kirschner, 1990) and it has been previously shown to be a substrate as well as a competitive inhibitor of cdc2-dependent phosphorylation of nuclear lamins (Peter *et al.*, 1990). The polyclonal anti-lamin antibody aLI has been developed in rabbits using as an antigen peptide LI (Djabali *et al.*, 1991) and was used after affinity purification. The polyclonal antibodies against p58 were previously characterized (Worman *et al.*, 1988). The monoclonal anti-chicken lamin B2 antibody (E3) was kindly provided by E.A.Nigg (ISREC, Switzerland) (Lehner *et al.*, 1986). Peptide R1, corresponding to residues 61–80, of the chicken liver p58 (Worman *et al.*, 1990), was made at the Protein Sequencing and Peptide Synthesis Facility of EMBL (Heidelberg, Germany).

Immunochemical procedures

For immunization, the synthetic peptides were coupled to keyhole limpet hemocyanin (Sigma) using gluraraldehyde as a cross-linker. The coupled peptides were injected in the thigh lymph nodes and subcutaneously in rabbits (200–300 μg per animal, in complete Freunds adjuvant) at day 0. The animals were boosted at day 21 (same amount of peptide in incomplete Freunds adjuvant, subscapularly) and sera were collected 1 week later. Subsequent boosts were administered at least 3 weeks after each bleed. The

antisera were screened by immunoblotting and indirect immunofluorescence. Anti-p58 antibodies from the #168 serum were purified by affinity chromatography over an R1-Affigel 15 matrix (10 mg of R1/ml of resin). Bound antibodies were eluted by washing the column with 200 mM glycine−HCl pH 2.3, 500 mM NaCl and the collected fractions were immediatelly neutralized by the addition of 1 M Tris base. The fractions containing the antibody were pooled, dialysed against PBS, concentrated by ultrafiltration and stored at −80°C. Immunoblotting was performed as previously described (Georgatos and Blobel, 1987). For the detection of immunoreactive proteins either [^{125}I]protein A or alkaline phosphatase-conjugated goat anti-rabbit antibodies were used. For immunofluoresence microscopy, turkey erythrocytes were washed with PBS, allowed to adhere to Alcian Blue-coated coverslips, fixed with 3.5% formaldehyde in PBS for 10 min at room temperature and permeabilized with 0.4% Triton X-100 for 10 min at room temperature. p58 was localized using affinity-purified #168 antibodies and a secondary FITC-goat anti-rabbit antibody (Cappel Laboratories, West Chester, PA, USA). For immunoprecipitation, turkey erythrocytes were lysed with 1% Triton buffer (1% Triton X-100, 50 mM Tris−HCl pH 7.4, 150 mM NaCl) containing protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml aprotinin and 1 mM PMSF). When required, the buffer was supplemented with 1 mM ATP, 80 μg/ml creatine kinase, 20 mM phosphocreatine and 20 mM sodium β-glycerophosphate. The lysates were kept on ice for 30 min and then centrifuged at 12 000 *g* for 15 min. The supernatant was incubated with the affinity-purified #168 antibodies (5 μg/ml) for 3 h, at 4°C under shaking. After this, protein A−Sepharose was added (10 μl/ml) to the reaction mixtures and incubation continued for 1 h at 4°C. For rapid immunoprecipitation the antibodies were first preabsorbed to protein A−Sepharose beads and then mixed and incubated with the supernatant for various time intervals between 10 and 60 min. Immune complexes were harvested by centrifugation and washed three times with 1% Triton buffer and once with 0.1% Triton buffer. The pelleted beads were resuspended in 0.1% Triton buffer, the appropriate volume of 4×sample electrophoresis buffer (0.25 M Tris−HCl pH 6.8, 9.2% SDS, 40% glycerol, 0.2% bromophenol blue and 100 mM DTT) was added and the samples were heated at 95°C for 3 min and analysed by SDS−PAGE. For *in vitro* phosphorylation, the immunoprecipitates were resuspended in 0.1% Triton buffer containing 10 mM $MgCl_2$ and 50 μM of [γ-^{32}P]ATP (2000 Ci/mol), incubated for 1 h at 30°C and processed for SDS−PAGE and autoradiography. To immunoisolate *in vitro* phosphorylated p58 from nuclear envelope fractions, the envelopes were first solubilized with 2% SDS, 50 mM Tris−HCl pH 7.4 and then 10 vol of 1% Triton buffer were added. Immunoprecipitation was then carried out as described above. For immunoaffinity chromatography, the protein A−Sepharose− #168 antibody column was prepared by incubating 4 ml of #168 immune serum with 1 ml of protein A−Sepharose for 4 h at 4°C and washing non-bound material away with PBS. Turkey erythrocytes (from 10 ml of blood) were hypotonically lysed by stirring in 60 ml of 5 mM Na_3PO_4, 2 mM $MgCl_2$, 1 mM DTT and 1.3 mM PMSF pH 7.6 for 10 min on ice. The erythrocyte ghosts were recovered by centrifugation, washed once more with the same buffer, extracted with 40 ml of 1% Triton buffer containing protease inhibitors and 20 mM sodium β-glycerophosphate for 30 min on ice and centrifuged at 12 000 *g* for 15 min at 4°C. The detergent extract was then applied onto the immunoaffinity column. Non-bound material was removed by washing the column first with 1% and then with 0.1% Triton buffer. Finally, the column bound material was eluted by a solution containing 1 mg/ml peptide R1 in 0.1% Triton buffer. The column eluate was treated with protein A−Sepharose to remove traces of IgG, 'bleeding' from the column and dialysed to remove the peptide.

Cell fractionation, in vivo and in vitro phosphorylation

Turkey erythrocyte nuclear envelopes and plasma membranes were isolated as previously described (Georgatos and Blobel, 1987). When needed, the nuclear envelopes were extracted with 20 mM Tris−HCl pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF and 1% Triton X-100 at 0°C for 10 min. For *in vivo* ^{32}P labelling, red blood cells were labelled with ^{32}P as specified by Appelbaum *et al.* (1990). Briefly, a 200 μl pellet of PBS washed erythrocytes was incubated with 2 ml of 157 mM NaCl, 2.5 mM KCl and 11.1 mM glucose in 10 mM HEPES pH 7.4, 500 units/ml penicillin and streptomycin, and 1 mCi ^{32}P for 18 h. After the end of incubation the cells were washed three times with the same buffer and then lysed in 1 ml of 2% SDS, 50 mM Tris−HCl pH 7.4. The volume of the lysate was then adjusted to 10 ml with 1% Triton X-100, 50 mM Tris−HCl pH 7.4, 100 mM NaCl, 50 mM NaF, 0.1 mM sodium orthovanadate, 2 mM EDTA and protease inhibitors. The final lysate was centrifuged at 12 000 *g* for 10 min and then immunoprecipitation of p58 was carried out as described above. For PKA phosphorylation, 20 units of the catalytic subunit were incubated with 15 μg of nuclear envelopes in a buffer containing 50 μM of [γ-32P]ATP (2000 Ci/mol), 15 mM Tris−HCl pH 7.0, 30 mM NaCl, 0.3 mM $MgCl_2$ and 1mM DTT in a reaction volume of 25 μl. The assay mixture was incubated for 30 min at 30°C and the reaction was stopped by adding appropriate volume of 4×sample electrophoresis buffer and heating at 95°C for 3 min. For cdc2 phosphorylation, 0.1 μl of the cdc2 preparation (activity ~70 pmol/min/μl, using histone H1 as a substrate) were incubated with 15 μg of nuclear envelope in a buffer containing 50 μM [γ-^{32}P]ATP (2000 Ci/mol), 50 mM Tris−HCl pH 7.4, 10 mM $MgCl_2$ and 1 mM DTT in a reaction volume of 25 μl. Samples were incubated for 30 min at 30°C and the reaction was quenched by the addition of 4×sample buffer and boiling. The endogenous kinase activity was assayed under the same conditions.

Sucrose gradient sedimentation

1 ml fractions of 1% Triton extracts of erythrocyte ghosts, prepared as described above, were layered onto 5−20% sucrose gradients (12 ml made in 1% Triton buffer) and centrifuged at 39 000 r.p.m. for 17 h in a Beckman SW40Ti rotor. The gradients were fractionated from the top into 0.8 ml aliquots using a Haake-Buchler AutoDensiflow gradient fractionator.

Microsequencing

To determine the NH_2-terminal sequence of the 58 kDa phosphoprotein, samples of phosphorylated nuclear envelopes were analysed by SDS−PAGE and parallel lanes were either autoradiographed or transferred to ProBlott membranes (Applied Biosystems) and stained by Coomassie Blue. The bands corresponding to the 58 kDa phosphoprotein were excised from the blot, rinsed with distilled water and used for microsequencing. The protein sequence was determined by Edman degradation employing the Applied Biosystems, Model 477A, system.

Phosphopeptide mapping and phosphoamino acid analysis

Proteolytic peptide mapping was performed essentially as described by Luo *et al.* (1991). Briefly, immunoprecipitates of *in vitro* or *in vivo* phosphorylated p58 were run on a SDS−PAGE gel and then transferred to a nitrocellulose sheet. The radioactive p58 bands were excised, soaked in 0.5% PVP-360 (polyvinylpyrrolidone) in 100 mM acetic acid for 1 h at 37°C and washed extensively with water. The protein was digested by trypsin in 50 mM NH_4HCO_3 at 37°C, overnight. The released peptides were dried, resuspended in water and loaded on a cellulose TLC plate (Kodak). Electrophoresis (in the first dimension) was run at pH 8.9 (1% ammonium carbonate) for 1 h at 500 V; ascending chromatography (in the second dimension) was performed using as a solvent a mixture of *n*-butanol−pyridine−glacial acetic acid−water in ratios of 75:50:15:60. For phosphoamino acid analysis, the tryptic digest was treated with 5.7 M HCl at 110°C for 90 min, dried and electrophoresed on cellulose TLC plates at pH 3.5 (pyridine−glacial acetic acid−water 4:40:756) for 1 h at 400 V.

Other methods

SDS−PAGE was performed according to Laemmli (1970), using 10 or 12% gels. Dried gels and blots probed with [^{125}I]protein A were exposed to Kodak X-ray film with intensifying screens. Protein determinations were made using a BioRad protein determination kit (BioRad, Richmond VA, USA).

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