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The role of isoprenylation in membrane attachment of nuclear lamins

A single point mutation prevents proteolytic cleavage of the lamin A precursor and confers membrane binding properties

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SUMMARY

Mature A- and B-type lamins differ in the extent to which they interact with the nuclear membrane and thus represent an interesting model for studying the role of isoprenylation and carboxyl-methylation in membrane attachment. Both A- and B-type lamins are isoprenylated and carboxyl-methylated shortly after synthesis, but A-type lamins undergo a further proteolytic cleavage which results in the loss of the hydrophobically modified C terminus. Here, we have constructed mutants of chicken lamin A that differ in their abilities to serve as substrates for different post-translational processing events occurring at the C terminus of the wild-type precursor. In addition to studying full-length proteins, we have analyzed C-terminal end domains of lamin A, either alone or after fusion to reporter proteins. Mutant proteins were expressed in mammalian cells, and their membrane association was analyzed by immunofluorescence microscopy and subcellular fractionation. Our results provide information on the substrate specificity and subcellular localization of the lamin-A-specific protease. Moreover, they indicate that hydrophobic modifications of the C-terminal end domains account for the differential membrane-binding properties of A- and B-type lamins. Thus, some of the integral membrane proteins implicated in anchoring B-type lamins to the membrane may function as receptors for the isoprenylated and carboxyl-methylated C terminus.

Key words: lamin A, farnesylation, membrane attachment, precursor processing, proteolysis, lamin receptor

INTRODUCTION

The C-terminal sequence motif CAAX (C, cysteine; A, aliphatic amino acid; X, any amino acid) has been identified as a major determinant for isoprenylation and carboxyl-methylation of a large number of proteins, many of which are implicated in signal transduction processes (for recent review see Marshall, 1993). Proteins shown to be isoprenylated and carboxyl-methylated include yeast mating factors, $p21^{ras}$ and *ras*-related guanine nucleotide binding proteins, γ subunits of heterotrimeric G proteins, phosphodiesterase subunits, rhodopsin kinase and nuclear lamins (reviewed by Clarke, 1992; Schafer and Rhine, 1992; Cox and Der, 1992). The CAAX motif is subject to three subsequent modifications, i.e. attachment, via a thioether bond, of an isoprenyl group to the cysteine, proteolytic removal of the last three (AAX) amino acids, and carboxyl-methylation of the isoprenylated, C-terminal cysteine (for reviews see Maltese, 1990; Glomset et al., 1990; Casey, 1992). Largely depending on the identity of the X residue in the CAAX box, two different types of isoprenyl groups are transferred by structurally distinct enzymes: a C_{15} farnesyl group is attached if X is methionine, serine or alanine, a C_{20} geranylgeranyl group if X is leucine (Kinsella et al., 1991; Seabra et al., 1991; Moores et al., 1991). For reasons that are not yet clear, farnesyl- and geranylgeranyl-modifications are not functionally equivalent (e.g. see Cox et al., 1992). The function(s) of isoprenylation and carboxyl-methylation remain to be fully understood. These modifications substantially increase protein hydrophobicity (Black, 1992), and thus may function in membrane attachment. In support of this view, many isoprenylated proteins are associated with membranes, and mutations at the isoprenylation sites inhibit their membrane association (for review see Clarke, 1992). However, in the case of ras protein, it was shown that stable association with the plasma membrane required not only farnesylation and carboxyl-methylation, but additional palmitoylation of a second cysteine residue, or a stretch of basic amino acids in close proximity to the C terminus (Hancock et al., 1990, 1991). Likewise, farnesylation or geranylgeranylation were not sufficient to confer membrane attachment when CAAX boxes were transferred to heterologous reporter proteins (Hancock et al., 1991; Butrynski et al., 1992). Finally, recent work on signal transduction by guanine nucleotide binding proteins suggests that isoprenylation may be important for protein-protein rather than protein-lipid interactions (for review see Marshall, 1993). Similarly, isoprenylated proteins such as rhodopsin kinase, functioning in visual signal transduction pathways (Inglese et al., 1992), or small GTP-binding

proteins implicated in vesicle trafficking (reviewed by Goud, 1992), are only temporarily associated with membranes, suggesting that the affinity of isoprenyl groups for membranes can be regulated. One interesting model for studying the role of isoprenylation and carboxyl-methylation is provided by the nuclear lamins. These proteins are the major components of the nuclear lamina, a protein meshwork underlying the inner nuclear membrane (Aebi et al., 1986; for reviews see Gerace and Burke, 1988; Nigg, 1989, 1992; McKeon, 1991; Paddy et al., 1992). Furthermore, they may form supramolecular structures also in the interior of nuclei, at least at early stages of the cell cycle (Bridger et al., 1993). Lamins display a tripartite organization with a central α-helical rod domain characteristic of intermediate filament (IF) proteins (McKeon et al., 1986; Fisher et al., 1986). Hydrophobic modifications of the CAAX box are required for targeting newly synthesized lamins to the nuclear membrane (Holtz et al., 1989; Krohne et al., 1989; Kitten and Nigg, 1991), but it is not known whether these modifications are sufficient for mediating persistent membrane attachment of mature lamins (for discussion see Holtz et al., 1989; Worman et al., 1990; Nigg et al., 1992). Like $p21^{ras}$ (Hancock et al., 1989; Casey et al., 1989), lamins carry a farnesyl group (Farnsworth et al., 1989; Lutz et al., 1992), but they lack the additional palmitoylation site or polybasic domain implicated in membrane attachment of ras proteins (Hancock et al., 1990, 1991). According to primary structure, lamins can be classified as A- or B-type (Krohne et al., 1987; Vorburger et al., 1989b; Höger et al., 1990). These differ in the extent to which they interact with the nuclear membrane: when the nuclear lamina disassembles during mitosis, A-type lamins are solubilized, but B-type lamins remain associated with membraneous structures (Gerace and Blobel, 1980; Stick et al., 1988). Two different models have been proposed to explain the strikingly distinct membrane-binding properties of A- and B-type lamins. According to one model, B-type lamins may specifically interact with 'lamin B receptors' embedded within the inner nuclear membrane. In support of this view, several integral membrane proteins were shown to interact with lamins, either directly or indirectly (Worman et al., 1988, 1990; Senior and Gerace, 1988; Powell and Burke, 1990; Padan et al., 1990; Bailer et al., 1991; Simos and Georgatos, 1992; Foisner and Gerace, 1993). Alternatively, the possibility has been considered that membrane association of lamins may depend primarily on farnesylation and carboxyl-methylation of the C-terminal CAAX box. Although both A- and B-type lamins are farnesylated and carboxyl-methylated shortly after synthesis, lamin A undergoes an additional proteolytic cleavage, which results in the loss of a C-terminal peptide that comprises the entire modified CAAX motif (Laliberté et al., 1984; Gerace et al., 1984; Dagenais et al., 1985; Lehner et al., 1986; Beck et al., 1990; Vorburger et al., 1989a; Weber et al., 1989). Thus, it is attractive to causally relate the diminished membrane binding ability of mature lamin A to the absence of a hydrophobically modified C terminus (for discussion see Weber et al., 1989, Vorburger et al., 1989a). At present, there is no direct evidence to support or refute either of these models, and we emphasize that they are not mutually exclusive (see Discussion). The protease responsible for the lamin-A-specific cleavage has not yet been identified, although there is evidence that it may be associated with the inner nuclear membrane (Lehner et al., 1986; see also, however, Holtz et al., 1989). Likewise, its substrate site specificity has not been determined directly, but the determination of the C terminus of mature lamin A affords a reasonable prediction of the most likely primary cleavage site (Weber et al., 1989). Of particular importance to the present study, the action of the lamin-A-specific protease was shown to be strictly dependent on prior farnesylation of the precursor's CAAX box (Beck et al., 1990). To study the contribution of C-terminal isoprenylation to the membrane attachment of lamins, we have asked whether or not it would be possible to influence the membrane binding properties of lamin A by interfering with the lamin-A-specific cleavage event. We found that this processing step could be prevented by substituting a single residue within the putative cleavage site of chicken lamin A. When expressed in mammalian cells, the non-cleavable chicken lamin A assembled normally during interphase, demonstrating that its CAAX box had been processed properly. In further experiments, we also analyzed the subcellular localization and biochemical properties of isolated C-terminal end domains of lamin A. We found that these domains contain all the information necessary for processing of the CAAX box and subsequent cleavage by the lamin-A-specific protease. Moreover, we show that the presence of a CAAX box is sufficient to confer membrane-binding properties either on C-terminal end domains of lamin A or on hybrid reporter proteins. These results indicate that the differential membrane association of mature A- and B-type lamins is determined primarily by the presence or absence of a hydrophobically modified C-terminal end domain. With regard to the postulated role of integral membrane proteins in the membrane attachment of B-type lamins (e.g. see Worman et al., 1988, 1990), our present observations suggest that the farnesylated and carboxyl-methylated C terminus may constitute a major determinant for lamin recognition by some of these proteins.

MATERIALS AND METHODS

Antibodies and immunochemical techniques

For detection of ectopically expressed chicken lamin A in mammalian cells, the species-specific mAbs P-2 and P-5 (Peter et al., 1989) were used for immunofluorescence microscopy and immunoblotting, respectively. Lamin B_2 was detected using the mAb L-20 (Kitten and Nigg, 1991), and fusion proteins tagged with a human c-*myc* epitope (Munro and Pelham, 1987) were visualized using the mAb 9E10 (Evan et al., 1985). Secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse Ig antibodies (Promega) and rhodamine-conjugated goat anti-mouse Ig (Pierce). Cells were prepared for immunofluorescence microscopy exactly as described by Gallant and Nigg (1992), and immunoblotting was performed according to Krek and Nigg (1989).

Cell culture and transfection

HeLa and P19 cells were cultured as described by Krek and Nigg (1989) and Peter and Nigg (1991), respectively. Transfection experiments were carried out using the calcium phosphate method (Chen and Okayama, 1987), as described previously (Krek and Nigg, 1991). In time-course experiments, time zero was defined as the moment when the Ca^{2+}-DNA precipitate was removed from cells.

Construction of lamin A mutants

All DNA manipulations were carried out according to standard procedures (Sambrook et al., 1989), and all mutations and constructions

were verified by DNA sequencing. For site-directed mutagenesis, a cDNA encoding the full-length chicken lamin A precursor (Peter et al., 1989) was inserted into the phage M13mp19. The following point mutations were created, using appropriate oligonucleotides and a mutagenesis kit (Bio-Rad): cleavage(–) mutant, V637 to R (GTG to CGG); mature lamin A, V637 to stop (GTG to TAA); CAAX(–) mutant, S655 to stop (AGC to TAA). The wild-type lamin A precursor and the various mutants were then cloned into the eukaryotic expression vector pD5neo (Peter and Nigg, 1991). For construction of the myc-tagged lamin A tails, *Bsm*AI fragments excised from the cDNAs encoding wild-type and mature lamin A, respectively, were cloned into a Bluescript plasmid (Stratagene) into which a DNA fragment encoding the human c-*myc* tag had previously been inserted (Schmidt-Zachmann and Nigg, 1993). The tail constructs carrying cleavage(–) and CAAX(–) mutations were created by exchanging *Nar*I/*Bam*HI fragments excised from the respective full-length mutants for the corresponding fragment in the wild-type tail plasmid. To generate wild-type and cleavage(–) version tail proteins without nuclear localization signal (NLS), *Nar*I/*Xba*I fragments were excised from the corresponding full-length cDNAs and cloned directly into the *myc*-tag-containing Bluescript plasmid. cDNAs encoding lamin A tails were also fused to the 3′ end of a cDNA coding for *myc*-tagged chicken pyruvate kinase (myc-PK; Schmidt-Zachmann and Nigg, 1993). To this end, *Eco*RI/*Bam*HI fragments were excised from the tail constructs described above, and cloned in-frame behind myc-PK. Fusions of lamin A tails to B-type lamins were done as follows: a *myc*-tagged B_1 tail was constructed by inserting a *Hae*III/*Acc*I fragment isolated from a lamin B_1 cDNA (Peter et al., 1989) into the *myc*-tag-containing Bluescript plasmid. The resulting cDNA encodes a hybrid protein with the N-terminal sequence MEQKLISEEDLN-MNSCSPP^{393}SSRV-, where P^{393} refers to the position in the wild-type lamin B_1 sequence (Peter et al., 1989). From this cDNA, a *Ban*II fragment was isolated and cloned into the *Xma*III site of the lamin A tail mutants. This resulted in replacement of the extreme C terminus of lamin B_1 by the last 24 amino acids of lamin A (i.e. the lamin B_1 C-terminal sequence -QGSPRKPERSCVVM was changed to -QGGRSY(V/R)LGGAGPRRQAPAPQGCSIM in the chimeric proteins). To replace the CAAX box of the full-length lamin B_2 protein by lamin A tails, a *Xma*III fragment (encoding the C-terminal end domain of lamin A) was inserted into a *Xho*I site in the lamin B_2 cDNA. This resulted in hybrid proteins in which the CAAX box of lamin B_2 was replaced by the oligopeptide RSY(V/R)LGGAG-PRRQAPAPQGCSIM. All cDNAs described above were subcloned into the eukaryotic expression vector pCMVneo (Krek and Nigg, 1991).

Subcellular fractionation of transfected cells

Transfected cells in 6 cm culture dishes were washed once with ice-cold PBS and once with ice-cold TKM (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM KCl, 6 mM 2-mercaptoethanol, 0.27 TIU/ml aprotinin, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin), before they were scraped into 500 μl of TKM. After a 10 minute incubation on ice, cells were homogenized by sonication (Branson sonifier, 10 seconds, position 1, continuous cycle), and complete breakage of cells was controlled by phase-contrast microscopy. A 60 μl sample of 2.3 M sucrose in TKM and 18 μl of 1 M Na_2SO_3 were added to the suspension, and two 300 μl samples were removed. To these, 30 μl of either water or 10% Triton X-100 was added. After a 30 minute incubation at 4°C with shaking, the samples were centrifuged for 15 minutes at 14,000 ***g***. The resulting pellets were washed once with 150 μl of the TKM/sucrose/sodium sulphite solution and centrifuged again. Then, the pellets were taken up in 30 μl of SDS-PAGE sample buffer and boiled for 8 minutes. The combined supernatants from the two centrifugations were precipitated for one hour with 15% (final concentration) trichloroacetic acid (TCA), and precipitates were recovered by centrifugation for 15 minutes at 14,000 ***g***. Precipitates were washed twice with 10% TCA, twice with 90% acetone and once with 100% acetone, before being taken up and boiled in 30 μl of SDS-PAGE sample buffer.

RESULTS

Construction and expression of a non-cleavable lamin A mutant

Fig. 1 shows a schematic view of the lamin A precursor protein. The amino acid sequence of the C terminus of the wild-type protein (wt) is shown enlarged, in the single letter code. The putative cleavage site of the lamin-A-specific protease, as inferred from the sequencing of the mature murine lamin A protein (Weber et al., 1989), is marked by an arrow. Also indicated are the C-terminal sequences of three lamin A mutants: in the cleavage (–) mutant, the valine at the P^{+1} position of the putative cleavage site was changed to arginine; in the CAAX(–) mutant, a stop codon was introduced immediately after the cysteine residue, thus preventing its farnesylation (in analogy to previous results obtained with lamin B_2; see Kitten and Nigg, 1991); and in the mature version of lamin A, a stop codon was introduced after the tyrosine of the cleavage site, to mimic the mature, fully processed protein. To determine whether these wild-type and mutant chicken lamin A proteins could be incorporated into the nuclear lamina in vivo, they were expressed in undifferentiated murine P19 embryonal carcinoma cells. These cells were chosen because they do not express any endogenous lamin A (Peter and Nigg, 1991; Horton et al., 1992), thereby eliminating the possibility that exogenous mutant proteins might be targeted to the nuclear lamina via co-polymerization with endogenous wild-type lamin A (e.g. see Loewinger and McKeon, 1988). Two and 24 hours after transfection, the subcellular localization of the expressed chicken lamin A proteins was determined by indirect immunofluorescence microscopy, using a species-specific monoclonal anti-lamin antibody (mAb P-5; Peter et al., 1989). As shown in Fig. 2, both wild-type lamin A and the cleavage(–) mutant were associated with the nuclear envelope (as inferred from a typical ring-like staining at the nuclear periphery), regardless of whether they were studied 2 or 24 hours after

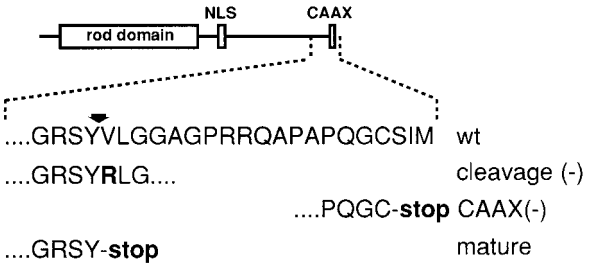

Fig. 1. Schematic representation of wild-type and mutant lamin A precursors. The schematic drawing represents the wild-type lamin A precursor; the positions of the α-helical rod domain, the nuclear localization signal (NLS) and the CAAX box are indicated by boxes. An expanded view of the sequence at the extreme C terminus is provided using the single letter code. The putative cleavage site of the lamin-A-specific protease, as deduced from the sequencing of the C terminus of mature lamin A (Weber et al., 1989), is indicated by an arrow. Below the wild-type (wt) sequence, the structures of three mutants are indicated. The specific point mutations are marked in boldface.

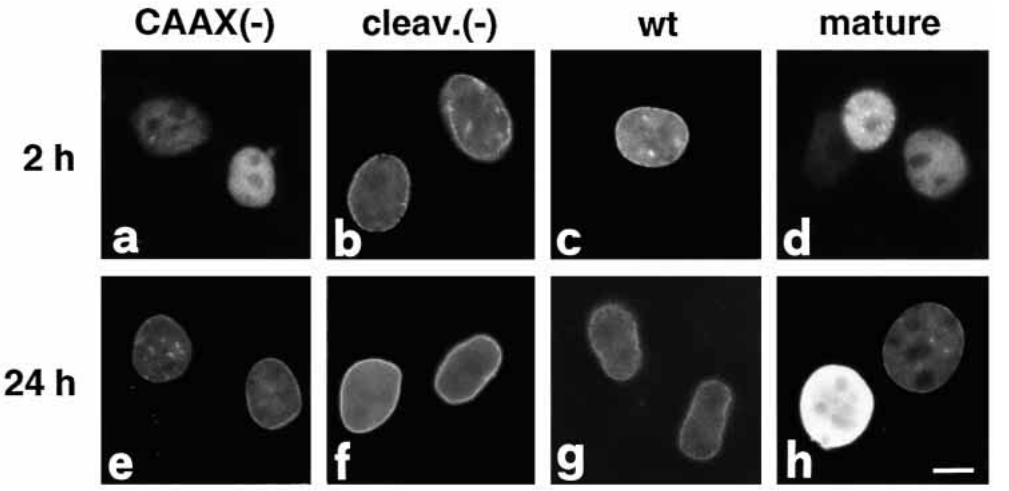

Fig. 2. Localization of lamin A precursors in transfected P19 embryonal carcinoma cells. P19 cells lacking endogenous A-type lamins (Peter and Nigg, 1991; Horton et al., 1992), were transfected with the lamin A precursor cDNAs depicted in Fig. 1. Two hours (a-d) or 24 hours (e-h) after transfection, cells were stained for the ectopically expressed lamin A proteins, using a species-specific mAb (P-5). a and e, CAAX(–) mutant; b and f, cleavage(–) mutant; c and g, wild-type lamin A precursor; d and h, mature lamin A. Bar in h, 10 μm.

transfection (b,c and f,g, respectively). In contrast, transfected mature lamin A and the CAAX(–) mutant showed a nucleoplasmic distribution when analyzed at the 2 hour time point (a,d). Some association of these proteins with the nuclear lamina could be seen by 24 hours (see ring-like lamina staining in e,h), but was observed only in transfected cells that had gone through mitosis (as indicated by the formation of characteristic cell doublets, often displaying postmitotic bridges; see also Horton et al., 1992). This latter result supports the notion that the post-mitotic assembly of A-type lamins can occur in the absence of a farnesylated C terminus, and presumably involves some as yet poorly defined interaction with decondensing chromatin (see Horton et al., 1992; Nigg et al., 1992; and Discussion). The above results thus confirm previous studies showing that farnesylation is a prerequisite for the integration of newly synthesized lamins into the pre-existing nuclear lamina during interphase of the cell cycle (Holtz et al., 1989; Krohne et al., 1989; Kitten and Nigg, 1991; Horton et al., 1992). Furthermore, they show that the wild-type lamin A precursor and the cleavage(–) mutant were assembled with comparable kinetics, indicating that both proteins were farnesylated with similar efficiencies, and that proteolytic processing of the lamin A precursor is not a prerequisite for its integration into the lamina. By immunoblotting, we have analyzed the sizes of the ectopically expressed chicken lamin A proteins (data not shown, but see below). We found that the CAAX(–) mutant did not appear to be processed to the mature sized protein, consistent with the absence of farnesylation on this protein (see also Kitten and Nigg, 1991), and confirming the requirement of the lamin-A-specific protease for prior farnesylation of the precursor (Beck et al., 1990). Furthermore, no proteolytic processing was observed in the case of the cleavage(–) mutant, supporting the identification of the cleavage site depicted in Fig. 1 (data not shown, but see Figs 3 and 6).

Membrane association of permanently farnesylated lamin A tails

To determine whether the persistence of the farnesylated C-terminal precursor peptide would confer membrane-binding properties to lamin A, we attempted to biochemically fractionate mitotically arrested cells that had been transfected with full-length wild-type and mutant lamin A proteins. Unfortunately, these experiments were not feasible because of a pronounced sensitivity of P19 cells to the drugs used for cell cycle synchronization (see also Horton et al., 1992). Conversely, transfected HeLa cells could readily be arrested in mitosis, but the interpretation of fractionation results was difficult because of interactions between exogenous mutant and endogenous wild-type lamin A proteins (data not shown). To circumvent these difficulties, we constructed wild-type and mutant versions of epitope-tagged C-terminal end domains (tails) of lamin A (for a schematic representation see Fig. 3A). Previous in vitro assembly studies have shown that C-terminal end

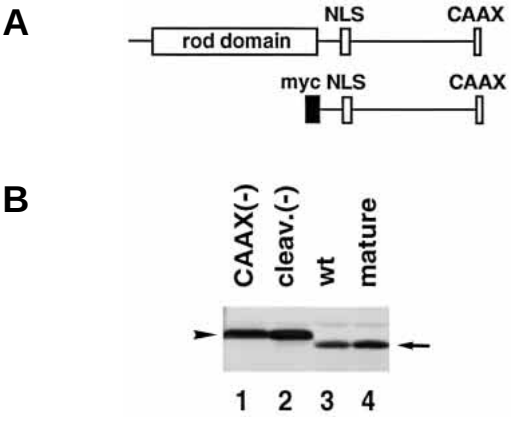

Fig. 3. Proteolytic cleavage of mutant lamin A tails. (A) Schematic representation of myc epitope-tagged lamin A tail constructs. The N-terminal amino acid sequence of these fusion proteins is MEQKLISEEDLNMNSCS^{389}PSPSS-, where S^{389} refers to the position in the wild-type lamin A precursor (Peter et al., 1989). In addition to the wild-type precursor tail, three different mutant tails were constructed, carrying the same point mutations as indicated in Fig. 1. (B) Detection of transfected tail proteins by immunoblotting. HeLa cells were transfected with the tail constructs indicated above each lane, and lysates were probed with the myc-tag-specific mAb 9E10. Note that the CAAX(–) (lane 1) and cleavage(–) (lane 2) mutant tails migrate more slowly than either the processed wild-type (lane 3) or the mature tail (lane 4), consistent with the presence in these mutants of an additional C-terminal peptide of 17 amino acids (see Fig. 1). Calculated molecular masses are 28.3 kDa for the processed tails (arrow), 29.9 kDa for the non-cleaved tails (arrowhead).

domains of lamins are not required for either lamin dimerization or longitudinal polymerization of dimers (Gieffers and Krohne, 1991; Heitlinger et al., 1991), and that head-to-tail polymerization of dimers is actually favored by the absence of C-terminal end domains (Heitlinger et al., 1992). On the basis of these results, we considered it likely that isolated lamin A tails would not undergo extensive interactions with endogenous full-length lamin proteins. HeLa cells were transiently transfected with cDNAs encoding wild-type precursor and mature lamin A tails, as well as CAAX(–) and cleavage(–) tail mutants. The expression of the corresponding proteins was then monitored by immunoblotting, using antibodies against the myc epitope tag (Fig. 3B). All proteins were expressed at comparable levels. As observed for the corresponding full-length lamin A mutants (not shown), tail proteins were found to migrate at two different sizes: the wild-type tail (lane 3) comigrated exactly with the mature protein (lane 4), indicating that it had been correctly processed at the CAAX box and subsequently cleaved by the lamin-A-specific protease. The cleavage(–) tail mutant displayed a reduced electrophoretic mobility (lane 2), showing that a valine to arginine mutation at the P^{+1} position abolished cleavage by the protease. An identically reduced mobility was seen for the CAAX(–) mutant (lane 1), confirming that hydrophobic modifications of the CAAX box are required for subsequent cleavage of the lamin A precursor (Beck et al., 1990). These results demonstrate that the C-terminal end domain of lamin A contains all the information necessary for isoprenylation, carboxyl-methylation, and cleavage of the lamin A precursor. Fig. 4 shows the subcellular localization of the tail proteins 24 hours after transfection into HeLa cells. All four proteins localized to the nucleus, consistent with the presence of a NLS in the C-terminal end domain of lamin A (Loewinger and McKeon, 1988). Homogeneous nucleoplasmic distributions were observed for the CAAX(–) mutant (a), the wild-type protein (c) and the mature tail protein (d), indicating that these tails were unable to interact efficiently with either the pre-existing lamina or the nuclear membrane. In contrast, a striking rim-like staining in the region of the nuclear envelope was seen in the case of the cleavage(–) tail mutant (b). Some association of the cleavage(–) mutant with the perinuclear area was also visible (Fig. 4b), indicating that this protein was not strictly confined to the nucleus. In view of the biochemical data shown below, we consider it likely that this perinuclear staining may reflect an association of the cleavage(–) mutant with endomembranes, particularly the endoplasmic reticulum (ER; see also Holtz et al., 1989), but detailed studies on this phenotype would require the use of immuno-electron microscopy. To corroborate the above immunocytochemical data, we next carried out a biochemical analysis of the membrane association of the various lamin A tail proteins. Following transfection of exponentially growing HeLa cells, these were lysed in the presence or absence of non-ionic detergent (Triton X-100) and fractionated by centrifugation. The partitioning of the lamin A tail proteins between soluble and particulate fractions was then analyzed by immunoblotting (Fig. 5). Those tails that could not possibly be farnesylated because they lacked a CAAX box, i.e. the CAAX(–) mutant and the mature tail, were recovered almost quantitatively in the supernatant fractions (S), regardless of whether fractionations had been carried out in the presence or absence of detergent (Fig. 5). The same result was obtained also for the wild-type tail (Fig. 5), consistent with the previous data showing that the lamin-A-specific protease efficiently removed the modified CAAX box from this tail (see Fig. 3B). In contrast, the cleavage(–) mutant was found predominantly in the particulate fraction (P), provided that cell lysis had been carried out in the absence of detergent (Fig. 5, compare lanes 1 and 2). Addition of Triton X-100 efficiently solubilized the cleavage(–) mutant (Fig. 5, compare lanes lanes 3 and 4), as expected for a protein interacting with membranes. In further experiments, we have also fractionated transfected HeLa cells that had been arrested in mitosis, with indistiguishable results for all four proteins (data not shown). Together with the immunolocalization data shown above (Fig. 4b), this fractionation behavior indicates that the cleavage(–) lamin A tail is able to interact with nuclear membranes.

Substrate requirements and subcellular localization of the lamin-A-specific protease

To obtain further information on the subcellular localization and substrate specificity of the lamin-A-specific protease, we next studied the processing of lamin A tails in mutants lacking a NLS (Fig. 6) and in different chimeric proteins (Figs 7 and 8). In the first experiment, we constructed epitope-tagged lamin A tails lacking a NLS (Fig. 6A). Following transfection of

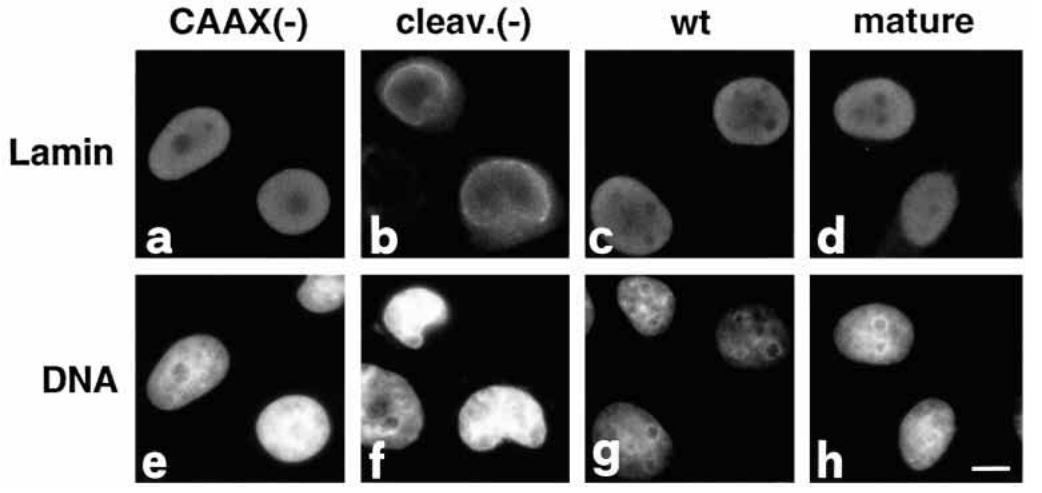

Fig. 4. Immunocytochemical evidence for membrane association of the non-cleavable mutant lamin A tail. Lamin A tail proteins were expressed in HeLa cells. 24 hours after transfection their subcellular localization was determined by immunofluorescence microscopy, using the myc-tag-specific mAb 9E10. Cells were transfected with the tail constructs indicated above (a-d). (e-h) The corresponding cells stained for DNA with Hoechst dye. Note the pronounced rim-staining of nuclear envelopes in b. Bar in h, 10 μm.

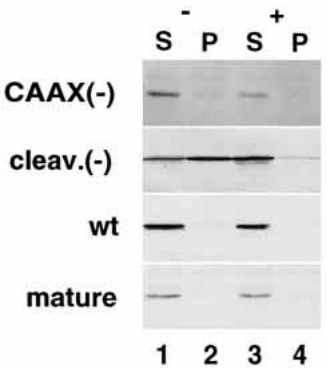

Fig. 5. Biochemical evidence for membrane association of the non-cleavable mutant lamin A tail. Lamin A tail proteins were expressed in HeLa cells. 24 hours after transfection cells were fractionated in the presence (+) or absence (–) of Triton X-100, as described in Materials and Methods. The partitioning of lamin A tail proteins between soluble (S) and pelletable (P) fractions was determined by immunoblotting with the myc-tag-specific mAb 9E10.

wild-type precursor and cleavage(–) versions of such tails into HeLa cells, their processing was analyzed by immunoblotting (Fig. 6B) and their subcellular localization determined by immunofluorescence microscopy (Fig. 6C). The wild-type tail displayed a faster electrophoretic mobility than the cleavage(–) mutant tail (Fig. 6B, lanes 1 and 2), indicating that a NLS was not required for proteolytic processing. We were surprised to find that the processed wild-type lamin A tail protein was localized to the nucleus, although it lacked a NLS (Fig. 6C,a). However, considering the small size of this tail protein, it seems plausible that its nuclear accumulation might reflect passive entry into the nucleus by diffusion, followed by binding to chromatin (as proposed by Höger et al., 1991), or other intranuclear components. In contrast to the wild-type tail, the cleavage(–) mutant was unable to enter the nucleus and instead accumulated in a perinuclear area (Fig. 6C,b). In view of the results shown above (Figs 4 and 5), we consider it likely that this mutant was prevented from entering the nucleus because of interactions with cytoplasmic endomembranes. Consistent with this interpretation, the mutant tail frequently showed a non-homogeneous, reticular distribution in the perinuclear area (Fig. 6C,b). The results obtained so far indicated that all specificity determinants for the lamin-A-specific protease were contained within a segment of lamin A extending from residue 423 (C-terminal to the NLS) to 657, the last C-terminal amino acid. To define further the specificity of this enzyme, we transferred the extreme C terminus (starting at residue 634) of both the wild-type lamin A precursor and the cleavage(–) mutant to either a full-length lamin B_2 protein or the tail of lamin B_1. The resulting chimeric proteins contain the lamin A cleavage site (including N-terminal residues up to position P^{-4}) in the context of the C-terminal end domains of B-type lamins (for schematic representations see Fig. 7). When these chimeric proteins were expressed in HeLa cells, wild-type and cleavage(–) versions were found to display identical electrophoretic mobilities (Fig. 7), regardless of whether lamin A tails had been fused to lamin B_2 (Fig. 7A) or B_1 (Fig. 7B). The absence of cleavage of these proteins suggests that the lamin-A-specific protease requires not only a modified CAAX

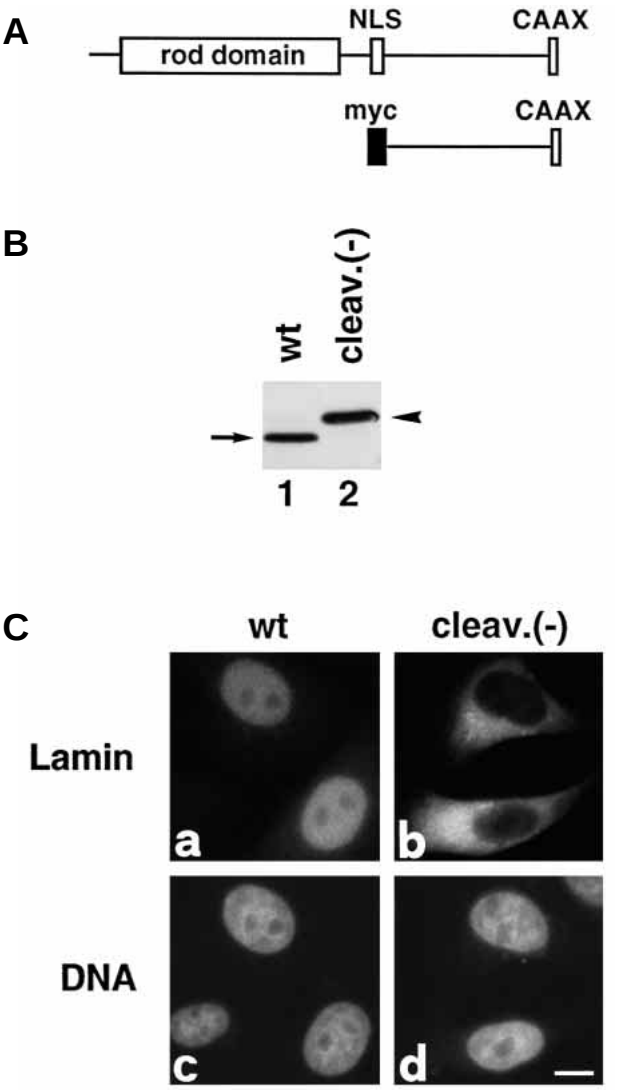

Fig. 6. Proteolytic cleavage and subcellular localization of lamin A tails without NLS. (A) Schematic representation of myc epitope-tagged lamin A tails lacking a NLS. The N-terminal amino acid sequence of these fusion protein is MEQKLISEEDLNMNSR^{415}LEDGE-, where R^{415} refers to the position in the wild-type lamin A precursor (Peter et al., 1989). In addition to the wild-type precursor tail, a cleavage(–) mutant was analyzed; this mutant carried the same arginine-for-valine replacement as indicated in Fig. 1. (B and C) The fate of tail proteins in transfected HeLa cells was analyzed by immunoblotting (B) and immunofluorescence microscopy (C), exactly as described in the legends to Figs 3 and 4. (B) Note the different migrations of the proteolytically cleaved wild-type tail (lane 1) and the cleavage(–) tail mutant (lane 2). Calculated molecular masses are 25.7 kDa for the processed wild-type tail (arrow), and 27.3 kDa for the non-cleaved tail (arrowhead). (C) Proteolytically cleaved wild-type tail localized exlusively to the nucleoplasm (a), whereas the cleavage(–) tail was retained in the cytoplasm (b). (c and d) The same cells as in a and b, after staining of DNA with Hoechst dye. Bar in d, 10 μm.

box for recognition of the lamin A precursor, but also some lamin-A-specific determinant(s) located between the NLS and the P^{-4} position of the cleavage site. Most likely, these determinants are encoded within a lamin-A-specific exon (see Stick,

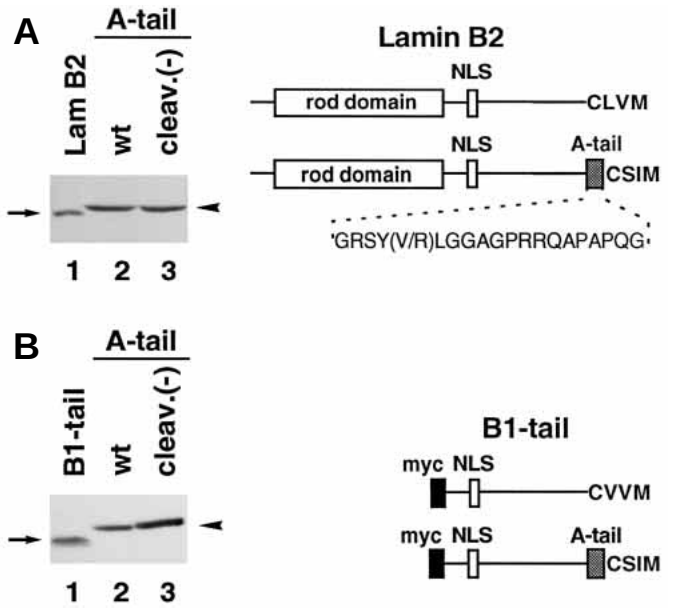

Fig. 7. Transfer of lamin A tails onto B-type lamins. Tails corresponding to the C-terminal 24 amino acids of the wild-type or cleavage(−) lamin A precursors were transferred onto B-type lamins. The sequences of the transferred tails are indicated by shaded boxes and shown in the single letter code in A. In the case of lamin B_2 (A), chimeric proteins begin at the N terminus of lamin B_2 and lamin A tails replace the last 4 residues (i.e. the CAAX box). In the case of the myc-tagged lamin B_1 tail (B), chimeric proteins begin at residue 393 of lamin B_1 and lamin A tails replace the last 12 amino acids. To monitor proteolytic processing of the chimeric proteins, extracts of transfected HeLa cells were separated on a 8% (A) or a 14% (B) SDS-polyacrylamide gel, and probed by immunoblotting with mAbs specific for lamin B_2 (L-20; A) or the myc-tag (9E10; B). (A) Lane 1, the migration of endogenous lamin B_2 in chick embryo fibroblast extract; lane 2, transfected lamin A wild-type tail fused to lamin B_2; lane 3, transfected lamin A cleavage(−)-tail fused to lamin B_2. Note that both fusion proteins comigrate (arrowhead), but show a slower electrophoretic mobility than lamin B_2, analyzed for comparison (arrow). Calculated molecular masses are 67.6 kDa for lamin B_2 (lane 1) and 69.5 kDa for the chimeric proteins (lanes 2 and 3) (B) Lane 1, transfected myc-tagged lamin B_1-tail; lane 2, lamin A wild-type tail fused to myc-tagged lamin B_1 tail; lane 3, lamin A cleavage(−) mutant fused to myc-tagged lamin B_1 tail. Note that both fusion proteins comigrate (arrowhead), but show a slower electrophoretic mobility than the myc-tagged lamin B_1 tail, analyzed for comparison (arrow). Calculated molecular masses are 22.9 kDa for the myc-tagged lamin B_1 tail (lane 1) and 23.9 kD for the chimeric proteins (lanes 2 and 3).

1992, and Discussion). In the next series of experiments, we fused wild-type and cleavage(−) versions of lamin A tails, with or without NLS, to a cytoplasmic reporter protein, i.e. an epitope-tagged pyruvate kinase (for schematic representations see Fig. 8). These chimeric proteins were expected to be of sufficient size to prevent their passive entry into nuclei, and nuclear accumulation should thus be dependent on the lamin's NLS. When extracts of transfected HeLa cells were analyzed by immunoblotting (Fig. 8B), we found that processing of wild-type lamin A tails had occurred (lanes 1 and 3), regardless of whether or not the chimeric proteins contained a NLS. This could be inferred from the reduced electrophoretic mobilities of the corresponding cleavage(−) mutants (lanes 2 and 4). Processing of the wild-type tails was quantitative in the absence of a NLS (lane 3), but only partial in its presence (lane 1). Fig. 8C illustrates the subcellular localizations of the various chimeric proteins. Both proteins containing the lamin's NLS were predominantly nuclear (a and b), but those lacking a NLS were cytoplasmic, as expected in view of the fact that their sizes prevented diffusion through nuclear pores (c and d). However, whereas the proteins containing wild-type lamin A tails were diffusely distributed (a and c), the cleavage(−) mutants showed distinctly non-homogeneous distributions. In the cleavage(−) mutant with NLS, a rim-staining of the nuclear envelope could be discerned, in addition to staining of some cytoplasmic patches (b), whereas in the cleavage(−) mutant without NLS, the cytoplasmic staining was non-homogeneous and reticular, reminiscent of the staining of membraneous structures (d; see also Fig. 6C,b; and Holtz et al., 1989).

DISCUSSION

In this study, we describe the construction and analysis of several lamin A mutants, including a lamin A precursor that can no longer be cleaved by the lamin-A-specific protease. Immunocytochemical and biochemical analyses of transiently expressed full-length lamin A proteins, as well as the corresponding mutant tail constructs, allowed us to evaluate the contribution of CAAX box modifications to the membrane attachment of lamins, and to obtain information on the substrate specificity and subcellular localization of the lamin-A-specific protease. As with all mutational analyses of proteins, our conclusions are subject to the reservation that misfolding of individual mutant proteins might complicate the interpretation of results. However, the problem appears to be minimal in the present study, since internally consistent results were obtained with multiple mutant proteins.

Assembly of permanently isoprenylated lamin A into the nuclear lamina

We found that a single (arginine for valine) substitution at position P^{+1} of the putative cleavage site generated a lamin A precursor protein that could no longer be cleaved by the lamin-A-specific protease. This observation argues against sequential actions of endo- and exoproteases, and instead suggests that the C terminus of mature lamin A is most probably generated by a single endoprotease, cutting the lamin A precursor between an aromatic and an apolar residue (see Fig. 1; and Weber et al., 1989). When mature lamin A and CAAX(−) mutant proteins were expressed in P19 embryonal carcinoma cells (i.e. cells lacking endogenous lamin A), they did not assemble at the nuclear envelope, unless transfected cells were allowed to pass through mitosis (Fig. 2). A similar observation has recently been reported for mammalian lamin C, a lamin A-related isoform lacking a CAAX box (Horton et al., 1992). These results confirm that newly synthesized lamins require farnesylation for association with the nuclear membrane in interphase cells (Holtz et al., 1989; Krohne et al., 1989; Kitten and Nigg, 1991; Beck et al., 1990; Lutz et al., 1992), and that a distinct, farnesylation-independent pathway, presumably based on direct or indirect interactions between lamins and

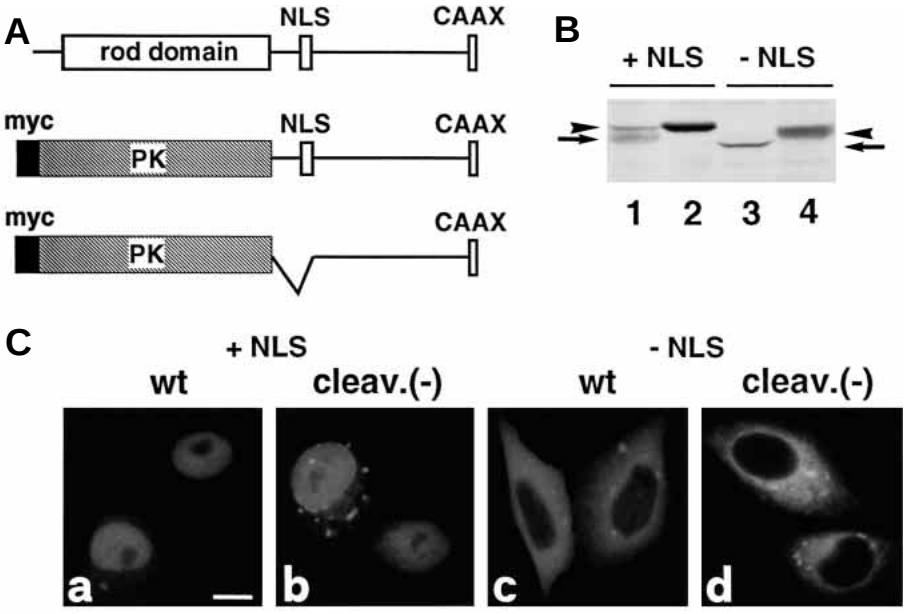

Fig. 8. Analysis of pyruvate kinase-lamin A tail fusion proteins. (A) Schematic representation of chimeric proteins consisting of lamin A tails fused to myc-tagged chicken pyruvate kinase (PK). All protein have an identical N terminus, beginning with the sequence MEQKLISEEDLNMNSNSL^{17}HAAM-. The precise structure at the junctions between PK and lamin A tails is -GYTNT^{523}MGCRNSISSLSNSCS^{389}PSPSS- for constructs containing the lamin NLS, and -GYTNT^{523}MGCRNSISSLSNSR^{415}LEDGE- for constructs lacking the lamin NLS. Italic and arabic numbers denote amino acid positions in wild-type PK (Kalderon et al., 1984) and lamin A (Peter et al., 1989), respectively. (B) Extracts were prepared from HeLa cells 24 hours after transfection and separated on a 8% SDS-polyacrylamide gel. The sizes of the fusion proteins were determined by immunoblotting, using the myc-tag-specific mAb 9E10. Fusion proteins contained the following lamin A tails: lane 1, wild-type tail with NLS; lane 2, cleavage(–) tail with NLS; lane 3, wild-type tail without NLS; lane 4, cleavage(–) tail without NLS. Arrowheads indicate the migrations of proteins that had not been cleaved by the lamin-A-specific protease, whereas arrows point to the cleaved proteins. Calculated molecular masses are: lane 1 (processed form), 85.3 kDa; lane 2, 86.9 kDa; lane 3, 82.6 kDa; lane 4, 84.2 kDa. (C) Subcellular localization of PK-lamin A tail fusion proteins 24 hours after transfection of HeLa cells. Immunofluorescence staining was carried out using the myc-tag-specific mAb 9E10. Fusion proteins contained the following lamin A tails: (a) wild-type tail with NLS; (b) cleavage(–) tail with NLS; (c) wild-type tail without NLS; (d) cleavage(–) tail without NLS. Bar in a, 10 μm.

chromatin (Burke and Gerace, 1986; Foisner and Gerace, 1993), contributes to lamina reassembly at the end of mitosis (Horton et al., 1992; Nigg et al., 1992). Wild-type and non-cleavable lamin A precursors were assembled with indistinguishable kinetics (Fig. 2), indicating that both proteins were farnesylated with similar efficiencies. Contrary to a recent postulate (Lutz et al., 1992), this shows also that proteolytic removal of the hydrophobically modified C-terminal precursor peptide does not represent a prerequisite for assembly of newly synthesized lamin A at the nuclear envelope.

Substrate requirements and subcellular localization of the lamin-A-specific protease

Concerning the properties of the lamin-A-specific protease, our results show that both N terminus and rod domain are dispensible for cleavage of the lamin A precursor, suggesting that lamin oligomerization is not required for substrate recognition. Instead, the action of this protease depends on hydrophobic modifications of the CAAX box, confirming previous data (Beck et al., 1990), as well as some unidentified lamin-A-specific determinant(s) located between the NLS and the P^{-4} position of the cleavage site. As B-type lamins cannot provide these latter determinants (see Fig. 7), they are most likely encoded within a lamin-A-specific exon (Stick, 1992). If one assumes evolutionary conservation of the intron-exon structure of the lamin A gene, such determinants may be expected to be encoded within a region spanning (approximately) residues 571 to 633 of the chicken lamin A protein. From the present analysis of chimeric proteins displaying different subcellular distributions, we conclude that the lamin-A-specific protease may be associated with both the nuclear membranes and the ER (see also Lehner et al., 1986; Holtz et al., 1989). We cannot exclude the possibility that ER-associated protease may play a physiological role in lamin A cleavage, but favour the idea that this population represents newly synthesized protease en route to the nuclear envelope. Under physiological conditions, most of the lamin A cleavage is likely to take place at the nuclear envelope, since nuclear uptake of the lamin A precursors occurs with much faster kinetics than precursor cleavage (Lehner et al., 1986; see also Goldman et al., 1992).

Isoprenylation and membrane attachment of lamin A

As shown by immunofluorescence microscopy and subcellular fractionation, isoprenylated and carboxyl-methylated lamin A tails were able to associate with membranes, from where they could be released by the addition of detergent (Figs 4 and 5). This result indicates that oligomerization is unlikely to be required for the membrane association of lamins. Whereas non-cleavable lamin A tails containing a NLS showed a subcellular distribution consistent with nuclear envelope association, corresponding tails lacking a NLS displayed an non-homogeneous cytoplasmic distribution (Figs 4 and 8), suggesting that they might have been associated with cytoplasmic endomembranes (see also Holtz et al., 1989). Finally, it is noteworthy that fully processed lamin A tails accumulated in the nucleus regardless of whether or not they carried a NLS. This may reflect an ability of such tails to bind to chromatin, as proposed previously (Höger et al., 1991). Our results suggest that isoprenylation and carboxyl-methylation constitute major factors in determining whether or not a particular lamin isoform is bound to the nuclear membrane. At first glance, this conclusion may appear to contradict recent evidence indicating that hydrophobic modifications of CAAX boxes are not sufficient for protein binding to the plasma membrane (for references see Introduction). One could argue that differences in the physicochemical properties of nuclear

membranes and plasma membranes might explain their different abilities to interact with farnesyl groups. This is unlikely, however, since isoprenylated proteins interact with many different membranes, and isoprenylation per se does not confer specificity for any particular membrane (e.g. see Chavrier et al., 1991). Also, there is no detailed information on the ability of polyisoprenyl substituents to interact with lipid bilayers, and recent results suggest that these structures may play important roles in mediating protein-protein rather than protein-lipid interactions (for review see Marshall, 1993).

Do integral membrane proteins function as protein-farnesyl receptors?

Several integral membrane proteins that appear to interact with nuclear lamins have been described (Worman et al., 1988, 1990; Bailer et al., 1991; Simos and Georgatos, 1992; Foisner and Gerace, 1993; see also Senior and Gerace, 1988; Padan et al., 1990). Among these, a 54-58 kDa protein called 'lamin B receptor' (Worman et al., 1988) contains several membrane-spanning domains of unknown function (Worman et al., 1990), and it is tempting to speculate that these hydrophobic domains might be able to interact with lamin-associated farnesyl groups. Critical testing of this proposal will require the reconstitution of the purified 'lamin B receptor' into artificial lipid bilayers. On the basis of our present results, we would predict that the farnesylated and carboxyl-methylated CAAX box may constitute a major determinant in the interaction between lamins and at least some of the lamin-binding integral membrane proteins described. Although such proteins would be expected to recognize not only the hydrophobically modified CAAX box but also lamin-specific protein determinants, it may be appropriate to consider them as 'lamin-farnesyl receptors' rather than 'lamin B receptors' (see also Nigg et al., 1992).

The evolution of lamin A processing and chromatin binding

The analysis of intron-exon patterns in lamin genes indicates that the lamin-A-specific C-terminal end domain resides on a single exon, which may have been inserted into a B-type lamin-like ancestoral gene by exon shuffling (Stick, 1992). Co-expression of lamins with different solubility properties has been observed in all vertebrates studied (Krohne and Benavente, 1986; Nigg, 1989), and may also occur in *Drosophila* (Bossie and Sanders, 1993). Most undifferentiated cell types lack A-type lamins (e.g. see Lehner et al., 1987; Stewart and Burke, 1987; Lebel et al., 1987; Wolin et al., 1987), indicating that these isoforms are not required for cell proliferation. However, A-type lamins may conceivably play a role in establishing (Lourim and Li, 1989) or stabilizing nuclear architecture during cell differentiation (for discussion see Nigg, 1989; Röber et al., 1989; Peter and Nigg, 1991). Although it has been reported that lamins may not be required for nuclear envelope assembly at the end of mitosis in all cell types (Newport et al., 1990), it is widely believed that they may contribute to the establishment of specific interactions between chromatin and the nuclear envelope (Burke and Gerace, 1986; Benavente and Krohne, 1986; Glass and Gerace, 1990; Höger et al., 1991; Yuan et al., 1991; Dabauvalle et al., 1991; Ulitzur et al., 1992; Paddy et al., 1992; Foisner and Gerace, 1993). In this context, the solubility of A-type lamins in mitotic cells may be critical, since it would facilitate early interactions with the surface of telophase chromosomes. If future work substantiates the hypothesis that specific lamin-chromatin interactions influence DNA replication and/or tissue-specific gene expression (for discussion see Benavente and Krohne, 1986; Burke and Gerace, 1986; Nigg, 1989; Lourim and Lin, 1989; Glass and Gerace, 1990; Höger et al., 1991; Meier et al., 1991; Peter and Nigg, 1991; Ludérus et al., 1992), the acquistion of an A-type lamin with a proteolytic cleavage site for removal of the hydrophically modified CAAX box may have been an important event during the evolution of metazoan organisms.

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