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The processing pathway of prelamin A

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SUMMARY

The conversion of mammalian prelamin A to mature lamin A proceeds through the removal of 18 amino acids from the carboxyl terminus. The initial step in this processing is the isoprenylation of a CAAX box cysteine. This proteolytic event is distinctive for prelamin A among the known prenylated mammalian proteins. Since the carboxyl terminus of prelamin A is removed during maturation, it is not obvious that this protein would undergo the two reactions subsequent to prenylation observed in other CAAX box proteins - the endoproteolytic removal of the carboxyl-terminal 3 amino acids and the subsequent methylation of the now carboxyl-terminal cysteine.

To characterize the maturation of prelamin A further, we have developed a CHO-K1 cell line that possesses a dexamethasone-inducible human prelamin A against a genetic background of high mevalonate uptake. Utilizing this cell line in association with antibodies specific to the transgenic prelamin A, we have been able to demonstrate directly in vivo that prelamin A undergoes farnesylation and carboxymethylation prior to conversion to lamin A, as is the case for other prenylated proteins. We have demonstrated previously that in the absence of isoprenylation, conversion of prelamin A to lamin A is blocked, but that unprocessed prelamin A is transported to the nucleus where it can still undergo maturation. Consistent with the implications of these prior studies, we now demonstrate the presence of both subunits of farnesyl-protein transferase in the nucleus.

Key words: lamin A, farnesylation, over-expression

INTRODUCTION

A number of mammalian proteins have been demonstrated to be post-translationally modified by a farnesyl residue in thioether linkage to a carboxyl-terminal cysteine (Sinensky and Lutz, 1992; Cox and Der, 1992). This modification occurs on the cysteine of proteins terminating in a so-called CAAX box in which A is an aliphatic amino acid and X is generally S, M, C, A, or Q. In three well described examples - p21ras (Hancock et al., 1989), γ-transducin (Fukada et al., 1990) and lamin B (Farnsworth et al., 1989; Sobotka-Briner and Chelsky, 1992) - farnesylation is the first of three sequential post-translational modifications that also include an endoproteolytic removal of the AAX tripeptide and subsequent methylation of the now carboxyl-terminal cysteine. Enzymes that catalyze all three of these steps have been characterized in vitro and have been reported to be cytosolic (Reiss et al., 1992; Ashby et al., 1992; Ma and Rando, 1992; Stephenson and Clarke, 1990).

Prior studies from our laboratory have demonstrated that in some respects the precursor of the nuclear lamin A protein, prelamin A, is a novel prenylated protein. We have found suggestive evidence that prelamin A undergoes its post-translational processing in the nucleus rather than in the cytosol (Lutz et al., 1992). Furthermore, in contrast to other mammalian prenylated proteins, prelamin A undergoes a proteolytic removal of the peptide domain bearing the prenyl group during its maturation to prelamin A (Weber et al., 1989; Beck et al., 1990). For this reason, it has heretofore not been possible to determine experimentally whether prelamin A undergoes a processing pathway comparable to that seen for other prenylated proteins. Indeed, although both the sequence of the prelamin A CAAX box (CSIM) and assignment of prenylation by farnesyl in vitro (Lutz et al., 1992) would argue that prelamin A is farnesylated, it has not been previously possible to definitively demonstrate farnesylation of prelamin A in vivo.

In this manuscript, we utilize a cell line bearing an inducible human prelamin A to characterize the processing pathway for this molecule in more detail and in vivo for the first time. Although the carboxyl terminus of prelamin A is removed during conversion of this molecule to lamin A, we demonstrate that its carboxyl-terminal processing is similar to that of other farnesylated proteins. We predict that this series of reactions would result in the production of a short farnesylated peptide reminiscent of fungal mating factors. Suggestive evidence is presented that this pathway may be unique, in mammalian cells, to prelamin A.

MATERIALS AND METHODS

Cells, plasmids and transfection

The pMMLA construct, derived from the previously described pSP64-MMTV vector (Lutz et al., 1992), bears the cDNA for human prelamin A under control of a dexamethasone-inducible (MMTV) promotor. Human prelamin A cDNA cloned into pUC19 (Fisher et al., 1986), a kind gift from Dr N. Chaudhary (Rockefeller University), was excised by digestion with *Eco*RI and *Bam*HI. This sequence was

inserted into pSP64-MMTV (Sarria et al., 1990) between its *Xba*I and *Bam*HI sites.

The recipient cell used for transfection with this construct was the CHO-K1-derived met-18b2 cell line (Faust and Krieger, 1987), which expresses a mevalonate transporter (Kim et al., 1992). Cells were transfected by the Lipofectin method, as described by the manufacturer (Gibco/BRL). Stable transfectants expressing human prelamin A were selected for G418 (Sigma) resistance after co-transfection with pSV2-neo (Southern and Berg, 1982) and then screening of individual clones by indirect immunofluorescence with human species-specific antibody (Loewinger and McKeon, 1988). A high expressor (MMLA) was chosen for further study. Optimal induction of lamin A expression was obtained by treatment of the MMLA cells with 10^{-7} M dexamethasone.

Rat 2 cells are a rat fibroblast line (ATCC CRL1764) and were a kind gift from Dr Steven Nordeen (University of Colorado Health Sciences Center).

All cells were grown in Ham's F12 supplemented with 5% fetal calf serum.

Indirect immunofluorescence

Cells, plated on glass coverslips, are rinsed with phosphate buffered saline (PBS) and then fixed with 3% formaldehyde in PBS for 15 minutes at $-20°C$. Following two washes in 5 mM NH_4Cl in PBS, the cells are permeablized to antibody with 0.05% Triton X-100 in PBS for 10 minutes at $-20°C$.

Antibodies and their dilutions for immunofluorescence were: 1E4 (Loewinger and McKeon, 1988), an anti-human lamin A(1/1000), a kind gift from Dr Frank McKeon (Harvard University); bovine farnesyl-protein transferase α(1/50) and β(1/100) subunit antibodies (Omer et al., 1993), kind gifts from Drs C. Omer and J. Gibbs (Merck, Sharp and Dohme), rat farnesyl-protein transferase α-subunit antibody (1/50), a kind gift from Dr M. Brown (Southwestern Medical Center) (Reiss et al., 1991).

Secondary antibodies were, for 1E4, rhodamine-conjugated sheep anti-mouse, diluted 1/25 (Cappel Laboratories); for farnesyl-protein transferase antibodies, rhodamine-conjugated goat anti-rabbit, diluted 1/200 (Biomeda).

Radioimmune precipitations

Antibodies used were 1E4 (1:500 dilution) for A-lamins, an anti-lamin B rabbit antiserum (1:100 dilution) (a kind gift from Dr Nilabh Chaudhary) (Cance et al., 1992), and a rabbit polyclonal anti-human prelamin A-specific antiserum (1:200 dilution) that was generated against the prelamin A-specific peptide CLLGNSSPRTQSPQN and that will be described in detail elsewhere. It's suitability for specific radio-immunoprecipitation of prelamin A is shown in Fig. 8C.

MMLA cells (1×10^7/100 mm Petri dish) were labeled either overnight with [*methyl-*^{3}H]methionine (100 μCi/ml, specific activity 85 Ci/mmol; Amersham) or 6 hours with [^{35}S]methionine (100 μCi/ml, specific activity ≥1000 Ci/mmol; DuPont-New England Nuclear) or for 2 hours with 5-R,S-[^{3}H]mevalonate (200 μCi/ml, specific activity 30 Ci/mmol; American Radiochemical). MMLA cells were treated with lovastatin (8 μg/ml) during mevalonate labeling. Radioactive lamin proteins were bound to anti-lamin antibodies by incubation, with shaking, overnight at $4°C$, precipitated by means of Protein A/Sepharose beads and analyzed by SDS-PAGE as previously described (Beck et al., 1990).

Base release assay

After visualization of the proteins of interest by fluorography, the corresponding bands were cut out of the dried gel and placed into 0.5 ml microfuge tubes and rehydrated with 100 μl of distilled water. Then 200 μl of 1.0 M NaOH was added to the tubes, which were then incubated at $37°C$ for 24 hours in capped, parafilm-sealed scintillation vials containing 2.5 ml of scintillation fluid. The tubes were removed and the base-releasable counts, which derive from methy-

lated carboxyl groups (Chelsky et al., 1987), determined after addition of another 7.5 ml of scintillation fluid. The unreacted material was also determined by liquid scintillation counting.

Assignment of prenyl substituent for prelamin A

Immunoprecipitated [^{3}H]mevalonate-labeled prelamin A was isolated by SDS-PAGE and subjected to Raney nickel reductive cleavage followed by gas-liquid radiochromatographic analysis as previously described (Lutz et al., 1992).

RESULTS

Dexamethasone-inducible over-expression of human prelamin A

In order to analyze the pathway of prelamin A processing, we needed to create conditions in which the intermediates in this processing pathway accumulate to levels that permit biochemical characterization. We attempted to over-express prelamin A by isolation of transfectants in which prelamin A transcription was driven by the SV40 promotor, but were unsuccessful. Reasoning that such over-expression might be lethal, we chose to utilize expression of prelamin A under the control of the dexamethasone-inducible MMTV promotor. To do this, we prepared the construct pMMLA (see Materials and Methods) and isolated a stably transfected clone (MMLA) of met-18b-2 cells, which could be demonstrated to express a dexamethasone-inducible human lamin A as indicated by radioimmunoprecipitation (Fig. 1), immunoblot (Fig. 2) and immunofluorescence (Fig. 3) with the human lamin A species-specific antibody 1E4. These results demonstrate that MMLA cells are inducible for the synthesis of human lamin A, which is processed and assembled correctly into the host cell lamina. The met-18b-2 cell line, derived from CHO-K1, was chosen because of its previously described high rate of mevalonate uptake - some 50-fold greater than wild-type cells (Faust and Krieger, 1987). Based on radioimmunoprecipitation studies, we estimate that MMLA cells have approximately a 4-fold elevated rate of synthesis of lamin A compared to the parental cell line (data not shown).
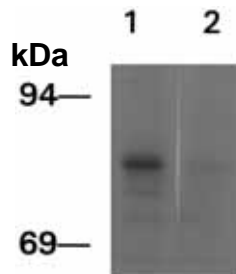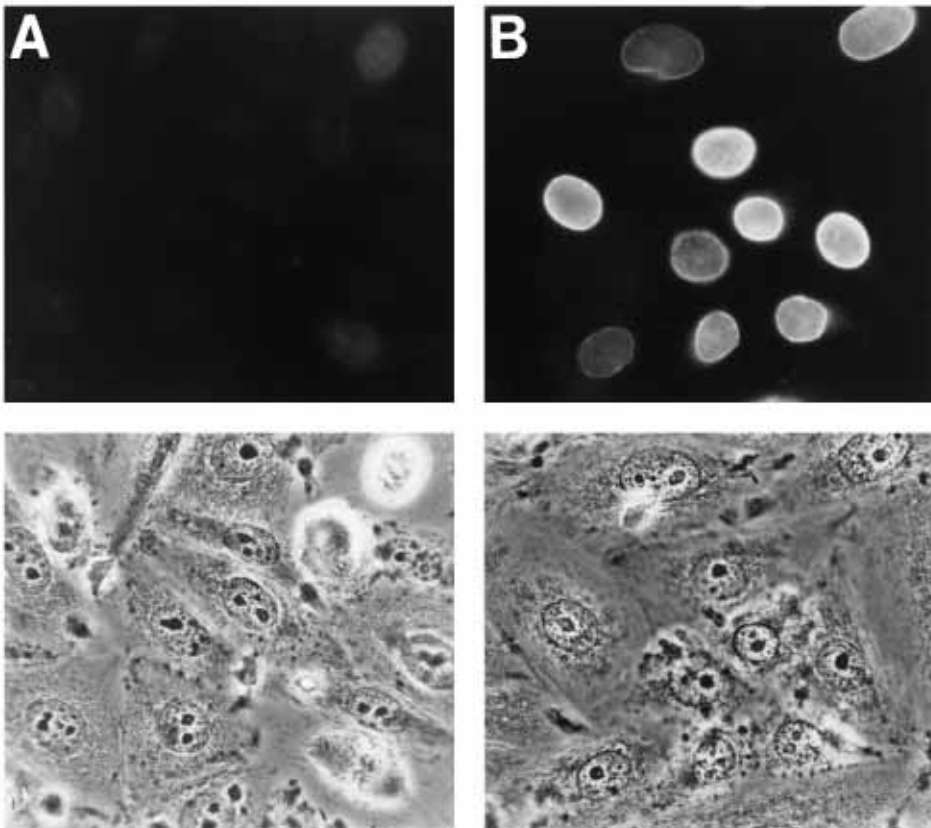

Fig. 1. Dexamethasone-inducible human lamin A synthesis in MMLA cells. MMLA cells were labeled for 6 hours with [^{35}S]methionine in the presence (1) or absence (2) of 10^{-7} dexamethasone and the incorporation into lamin A visualized by radio-immunoprecipitation, SDS-PAGE and fluorography.
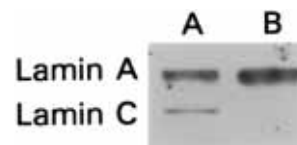

Fig. 2. Immunoblot of human lamin A of MMLA cells. The molecular masses of human lamin A from HeLa cells (A) and MMLA cells are compared (B) by SDS-PAGE followed by antibody visualization. MMLA cells were treated with dexamethasone overnight. Blotting was with the 1E4 antibody.

Fig. 3. Dexamethasone-inducible lamin A of MMLA cells: immunofluorescence - human lamin A of MMLA cells is visualized in the absence (A) or presence (B) of 10^{-7} M dexamethasone overnight. Phase-contrast images of these fields are below the immunofluorescent images.
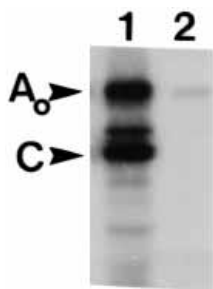

Fig. 4. Radio-immunoprecipitation of [^{3}H]mevalonate-labeled human prelamin A from MMLA cells. The immunoprecipitated labeled prelamin A from MMLA cells (lane 2) is compared to ^{35}S-labeled lamin proteins (lane 1) immunoprecipitated from HeLa cells treated with lovastatin (5 μg/ml). Prelamin A (A_o) and lamin C are indicated. Immunopreciptiations were with the 1E4 antibody.

## Demonstration that prelamin A is farnesylated in MMLA cells

The elevated expression and mevalonate uptake of MMLA cells make them good candidates for the definitive demonstration of the expected farnesylation of this protein. Radioimmunoprecipitation of prelamin A from these cells after labeling with [^{3}H]mevalonate for 2 hours gave rise to a band that comigrated with immunoprecipitated prelamin A from HeLa cells (Fig. 4). The time-course of mevalonate labeling of MMLA cells established that 2 hours was the optimum period for the accumulation of mevalonate-labeled prelamin A (data not shown).

After mevalonate labeling, immunoprecipitated prelamin A was isolated by SDS-PAGE, visualized by fluorography, electroeluted from the rehydrated gel and the isoprenoid substituent assigned by in-line radio-gas liquid chromatography. The results (Fig. 5) clearly demonstrate that prelamin A is farnesylated as would be expected from the sequence of the CAAX
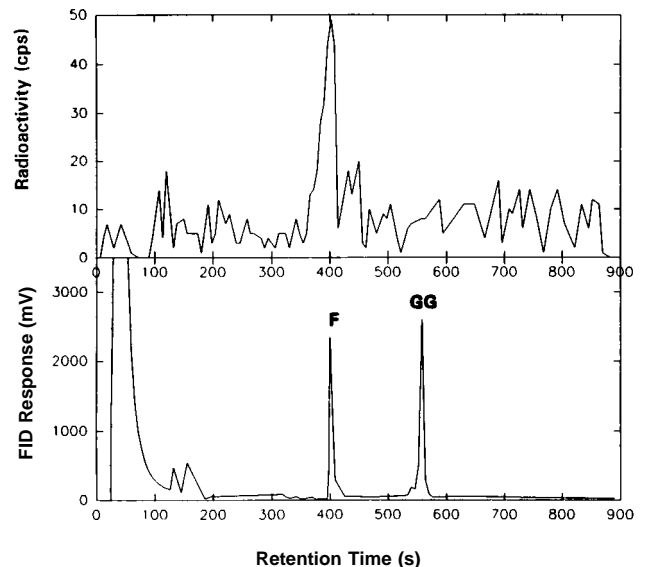
Fig. 5. Prelamin A is farnesylated. The SDS-PAGE band arising from [^{3}H]mevalonate-labeled prelamin A as shown in Fig. 4 was excised, rehydrated and removed from the gel by electroelution. The prenyl substituent on prelamin A was removed by Raney nickel cleavage and identified by co-migration in radio-gas liquid chromatography with bona fide mass standards (F, farnesyl; GG, geranylgeranyl).

box and is consistent with previous in vitro studies (Lutz et al., 1992).
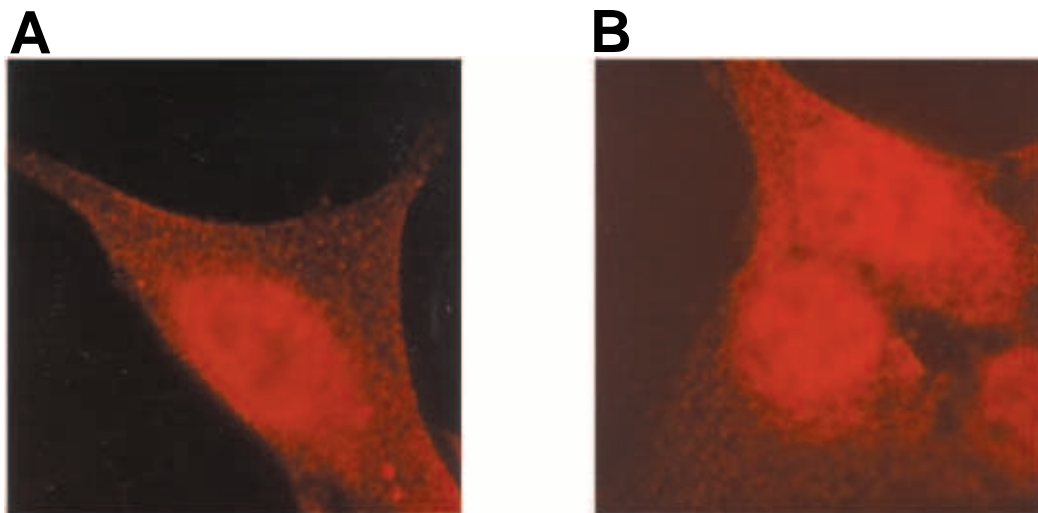
Fig. 6. Presence of farnesyl transferase in the nucleus of 3T3 cells. (A) visualization with antiserum against α-subunit; (B) visualization with antiserum against β-subunit.

## Farnesyl-protein transferase can be found in the nucleus of mammalian cells

We have demonstrated previously that treatment of cells with lovastatin results in the accumulation of unprenylated prelamin A in the nucleus (Beck et al., 1990; Lutz et al., 1992). Relief of the lovastatin block resulted in the rapid conversion of the prelamin A to lamin A. These data argued that processing of prelamin A could occur in the nucleus. With the above demonstration that prelamin A is farnesylated, we would, therefore, predict that farnesyl-protein transferase can be found in the nucleus.

We sought to test this hypothesis by indirect immunofluorescence. We obtained antibodies to the α and β subunits from others and visualized the sub-cellular distribution of these proteins in 3T3 cells (Fig. 6A,B). We also visualized the distribution of the α subunit in Rat 2 fibroblasts (Fig. 7A), since we expected greater specificity in these cells as the α-subunit antibody was raised against the rat protein. To ensure that specific recognition of the α subunit was being observed in the Rat 2 cells, we determined that the antibody-binding to these cells was inhibited by recombinant human farnesyl-protein transferase (Fig. 7B). The addition of 100 μg/ml of bovine

serum albumin during antibody-binding had no effect (data not shown). Presence of α-subunit in the nucleus of Rat 2 cells was also confirmed by confocal microscopy (Fig. 7C). The results demonstrate clearly the specific recognition of farnesyl-protein transferase in the nucleus as well as in the cytosol. The presence of the enzyme in the cytosol is expected since it has a number of cytosolic protein substrates, e.g. p21ras (Hancock et al., 1989), γ-transducin (Fukada et al., 1990), rhodopsin kinase (Inglese et al., 1992), and is found in 100,000 *g* supernatants of cell-free homogenates (Reiss et al., 1991).

Demonstration that prelamin A, but not lamin A, is carboxymethylated in MMLA cells

Other well characterized farnesylated proteins such as p21ras (Casey et al., 1989; Hancock et al., 1991) and lamin B (Farnsworth et al., 1989; Sobotka-Briner and Chelsky, 1992) undergo endoproteolytic removal of the carboxy-terminal 3 amino acids of the CAAX box followed by methyl esterification of the now carboxy-terminal cysteine. Since in prelamin A processing the whole carboxy terminus of the protein is removed, it is not at all obvious that prelamin A would necessarily undergo these intermediate steps during formation of
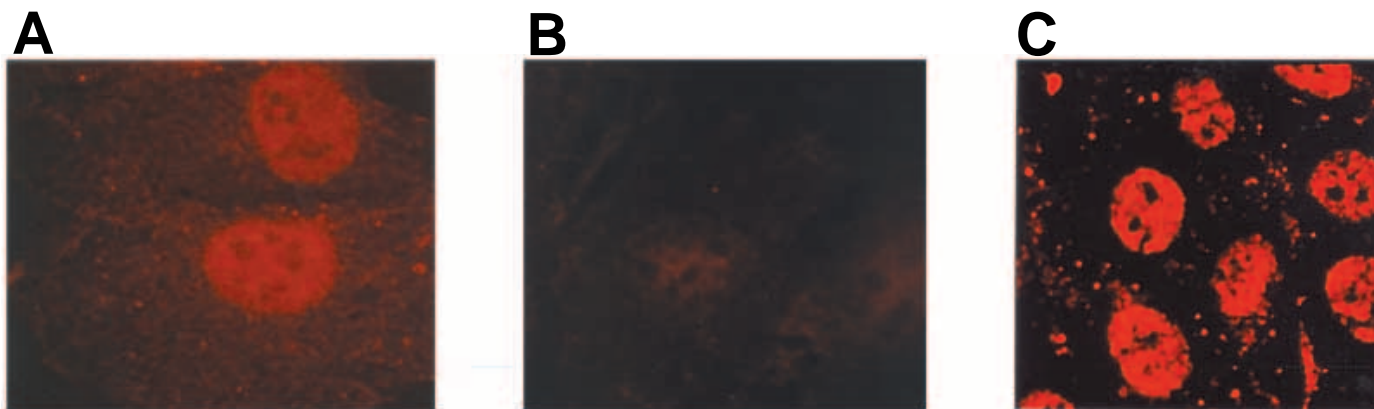
Fig. 7. Presence of α-subunit of farnesyl transferase in the nucleus of Rat 2 cells. (A) Visualization with antiserum against α-subunit; (B) same as (A) with the addition of 100 μg/ml of recombinant farnesyl transferase; (C) confocal image of a section through the nucleus of cells from the slide shown in (A).

mature lamin A. To determine whether prelamin A becomes carboxymethylated during its conversion to mature lamin A, we utilized the following strategy.

MMLA cells were induced with dexamethasone and incubated overnight with [*methyl*-³H]methionine. Tritium from this molecule has been well demonstrated to be incorporated into proteins either as methionine residues or, through metabolism to S-adenosyl methionine, into methyl substituents of carboxyl acceptors (Chelsky et al., 1987). To quantitatively determine carboxymethylation of prelamin A, we first sequentially immunoprecipitated prelamin A, lamin A and lamin B with specific antibodies for each of these proteins and isolated them by SDS-PAGE, followed by fluorographic visualization (Fig. 8A). Regions of the dried gel bearing these proteins were excised, re-hydrated and treated with base for vapor-phase transfer of radioactive methanol to scintillation fluid for determination of the counts incorporated as carboxymethyl ester. After completion of the decarboxylation reaction, the counts incorporated into the protein in non-base releasable form were determined by transfer of the reaction mix to a separate scintillation vial. Since the amino acid sequences of lamin A, prelamin A and lamin B are known, the carboxymethylation can be normalized to the amount of each protein synthesized by correction for the number of methionines in each of these proteins.

The results (Fig. 8B) confirm the carboxyl-terminal methylation of lamin B that has previously been well documented (Sobotka-Briner and Chelsky, 1992). The nearly 10-fold greater incorporation of ³H as base-releasable counts in prelamin A compared to lamin A strongly argues that prelamin A processing involves a terminally carboxymethylated intermediate. Removal of the carboxyl terminus of prelamin A during its conversion to mature lamin A would result in the observed lack of carboxymethylation of mature lamin A - a result that has been reported previously (Chelsky et al., 1987). The lower level of carboxymethylation in prelamin A than in lamin B is probably due to the carboxymethylated prelamin A being only one of four kinetic intermediates in the formation of mature lamin A. The other intermediates would be recognized by the prelamin A antibody and thus contribute protein counts but not methyl counts to the determination. The specificity of the prelamin A antibody for immunoprecipitation is demonstrated in Fig. 8C. Although the experiments in Fig. 8 do not directly demonstrate the existence of a farnesylated, methylated intermediate in prelamin A maturation the presence of both of these modifications in prelamin A and their absence in mature lamin A strongly suggests that such an intermediate exists.

DISCUSSION

Our studies on the farnesylation-dependent processing of prelamin A indicate that the sequential modifications that this molecule undergoes are similar to those previously described for other farnesylated proteins with two notable exceptions: the processing events for prelamin A can take place entirely in the nucleus and the farnesylated, methylated cysteinyl carboxyl-terminal peptide of the prelamin A molecule is removed from this protein.

The presence in the nucleus of a key activity required for
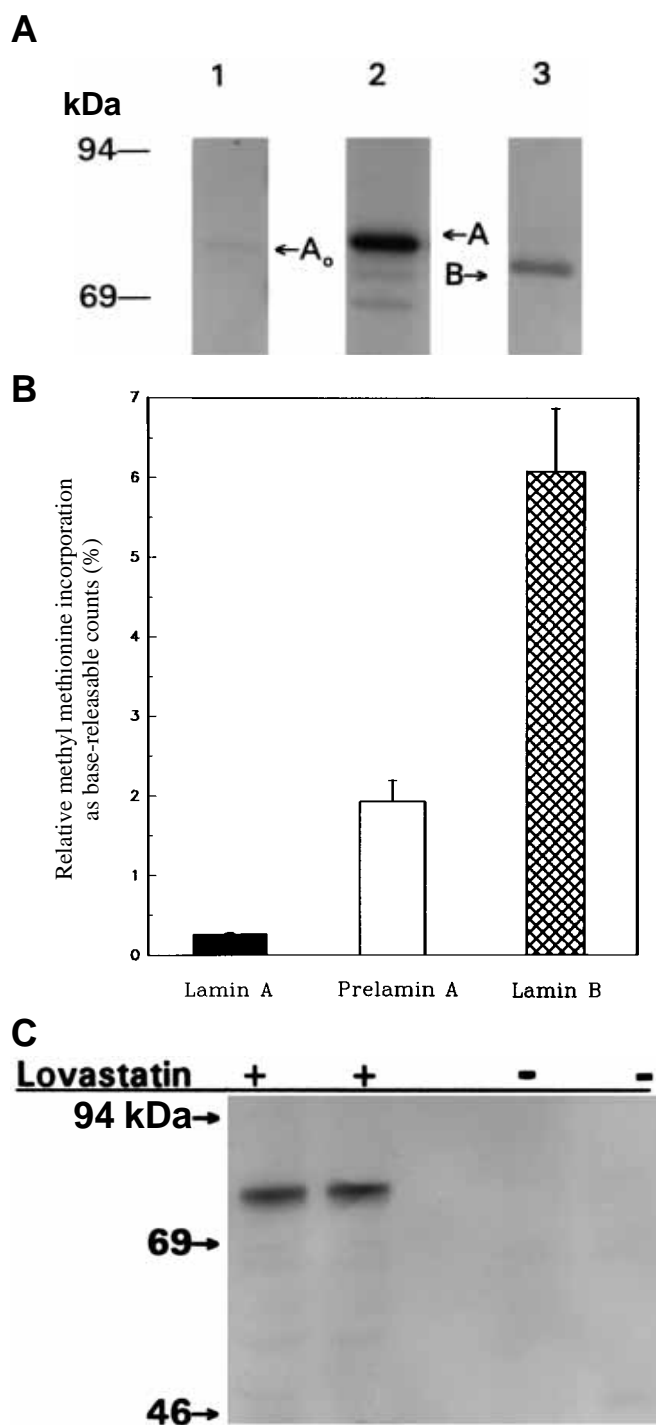
Fig. 8. Carboxymethylation of prelamin A. (A) After labeling of MMLA cells with [*methyl*-³H]methionine, prelamin A (lane 1), lamin A (lane 2) and lamin B (lane 3) are sequentially immunoprecipitated and isolated by SDS-PAGE. (B) Relative incorporation of label into carboxymethyl moieties is determined by base-release assay (see Materials and Methods). (C) Specificity of the prelamin A anti-sera in the immunoprecipitation of prelamin A in MMLA cells. The antiserum only immunoprecipitates a 74 kDa protein when prelamin A conversion to lamin A is blocked by lovastatin. Cells (4×10^6/100 mm plate) were incubated overnight with 35 µCi/ml [³⁵S]methionine (Trans-label, Amersham) and 10^{-7} M dexamethasone in the presence (+) or absence (−) of 10 µg/ml lovastatin.

prelamin A processing - the farnesyl-protein transferase - is consistent with studies that we have previously reported (Lutz et al., 1992) as well as studies of lamin carboxymethylation (Sobotka-Briner and Chelsky, 1992). Also consistent with nuclear localization of prelamin A processing is a recent report that microinjected prelamin A localizes to the nucleoplasm prior to assembly into the lamina (Goldman et al., 1992). We have obtained similar results by immunofluorescent localization of non-farnesylated prelamin A and have argued that the proteolytic removal of the prelamin A carboxyl terminus is required for assembly of lamin A into the lamina (Lutz et al., 1992). We have not yet determined whether B-lamins, which also undergo farnesylation-dependent carboxyl-terminal processing (but retain the modified cysteine in the mature protein) are also farnesylated in the nucleus, but this would seem likely.

The presence of farnesyl-protein transferase in the mammalian cell nucleus immediately raises the question of the mechanism of its localization to that organelle. There is no obvious nuclear localization signal, of the polybasic SV40 T antigen type, in the published sequences of either the α or β subunits of this enzyme (Omer et al., 1993). Current models of nuclear transport require the presence of such a signal or the binding to another protein with a nuclear localization signal to permit uptake. Perhaps farnesyl-protein transferase can bind such a transporter peptide, or perhaps it possesses a distinct mechanism to facilitate the nuclear uptake. Binding to a transporter peptide could explain the dual localization of the farnesyl-protein transferase in cytosol and nuclei, since such a mechanism could result in equilibration of proteins across the nuclear membrane. Proteins possessing a nuclear localization signal not only exhibit enhanced uptake into the nucleus, but also accumulate to much higher levels there relative to other cellular compartments. Whatever mechanism exists for transport must also permit the observed distribution of this enzyme in cytosol as well as nuclei.

An attractive hypothesis is that the nuclear farnesyl-protein transferase is in some way chemically distinct from the cytosolic enzyme. Preliminary studies comparing the sensitivity of Triton-X-100-soluble proteins versus Triton-insoluble proteins to farnesyl-protein transferase inhibitors (James et al., 1993) suggests that farnesylation of the soluble proteins is differentially sensitive to benzodiazopine peptidomimetics. Since the major Triton-insoluble proteins are those of the nuclear lamina, this finding, if borne out by more complete studies, would be consistent with a chemical difference between the cytosolic and nuclear farnesyl-protein transferase.

Given that the end result of prelamin A processing is a protein missing its carboxyl-terminal 18 amino acids, it is not obvious that the maturation of this molecule would proceed through a carboxymethylated cysteine intermediate. Highly analogous farnesylation-dependent processing pathways are well described for certain fungal peptides, which act as mating factors. The most similar situation to that seen for prelamin A is the mating factor of the basidomycete *Tremella mesenterica*, which arises from a 26 kDa precursor protein by carboxyl-terminal modification and subsequent endoproteolytic release of the 2 kDa biologically active farnesylated peptide (Miyakawa et al., 1985).

The demonstration of a carboxymethylated farnesylated cysteinyl intermediate in prelamin A maturation is also highly similar to the steps involved in the maturation of *Saccha-romyces cerevisiae* mating pheromone **a**-factor (Marcus et al., 1990). This peptide pheromone also terminates in a farnesylated, carboxymethylated cysteine and is generated from a larger precursor peptide by an endoproteolytic clip. It has recently been reported (Chen et al., 1992) that the endoproteolytic reaction that is required for **a**-factor maturation is sensitive to mutations in the precursor peptide, which flank the junction of the amino-terminal extension and mature **a**-factor. This result suggests that the endoprotease involved in **a**-factor maturation recognizes a sequence of amino acid residues around the cleavage site. If a similar endoproteolytic mechanism were operative in the case of prelamin A, it might be expected that an unusual and conserved amino acid sequence might be found in prelamin A flanking the lamin A carboxyl terminus. Computer data base searches reveal that the hexapeptide sequence flanking the putative endoproteolysis site (RSY↓LLG) is unique to prelamin A in the current protein sequence data base, as well as being highly conserved across species. This sequence, therefore, stands as a candidate for the recognition site of a prelamin A-specific endoprotease.

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