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Modification of nuclear lamin proteins by a mevalonic acid derivative occurs in reticulocyte lysates and requires the cysteine residue of the C-terminal CXXM motif

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The C-terminus of nuclear lamins (CXXM) resembles a C-terminal motif (the CAAX box) of fungal mating factors and *ras*-related proteins. The CAAX box is subject to different types of post-translational modifications, including proteolytic processing, isoprenylation and carboxyl methylation. By peptide mapping we show that both chicken lamins A and B_2 are processed proteolytically *in vivo*. However, whereas the entire CXXM motif is cleaved from lamin A, at most three C-terminal amino acids are removed from lamin B_2. Following translation of cDNA-derived RNAs in reticulocyte lysates, lamin proteins specifically incorporate a derivative of [^{14}C]mevalonic acid (MV), i.e. the precursor of a putative isoprenoid modification. Remarkably, no MV is incorporated into lamin B_2 translated from a mutant cDNA encoding alanine instead of cysteine in the C-terminal CXXM motif. These results implicate this particular cysteine residue as the target for modification of lamin proteins by an isoprenoid MV derivative, and they indicate that isoprenylation is amenable to studies in cell-free systems. Moreover, our observations suggest that C-terminal processing of newly synthesized nuclear lamins is a multi-step process highly reminiscent of the pathway elaborated recently for *ras*-related proteins.

Key words: CAAX box/isoprenylation/lamin/post-translational modification/*ras*

Introduction

Nuclear lamins are intermediate filament-type proteins forming a karyoskeletal structure at the nucleoplasmic surface of the inner nuclear membrane (Aebi *et al.*, 1986; for reviews see Franke, 1987; Gerace and Burke, 1988; Nigg, 1988, 1989). They are presumed to be important for the architecture of the nuclear periphery and for stabilizing the nuclear envelope (Gerace and Burke, 1988; Nigg, 1988, 1989). Lamin proteins are subject to multiple types of post-translational modifications, including phosphorylation (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985), carboxyl methylation (Chelsky *et al.*, 1987, 1989) and isoprenylation (Beck *et al.*, 1988; Wolda and Glomset, 1988). Although the functions of these modifications are not yet fully understood, it is likely that they are important for modulating the properties of the nuclear lamina. A strong correlation has been established between cell cycle-dependent changes in the polymeric state of the nuclear lamina and both phosphorylation (Gerace and Blobel, 1980; Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985) and carboxyl methylation (Chelsky *et al.*, 1987), suggesting that these modifications may control the reversible disassembly of the nuclear lamina during mitosis. The role of isoprenylation of lamin proteins has not yet been determined, but it is possible that hydrophobic isoprene moieties may play a role in mediating the association of newly synthesized lamins with the nuclear membrane (Beck *et al.*, 1988; Wolda and Glomset, 1988).

Based on biochemical and structural properties, lamin proteins are generally classified into A- and B-type isoforms (for review see Nigg, 1989). B-type lamins remain associated with the nuclear membrane throughout the cell cycle, whereas A-type lamins are solubilized during mitosis (Gerace *et al.*, 1984; Burke and Gerace, 1986; Stick *et al.*, 1988). This finding suggests that B-type lamins may interact more intimately with the nuclear membrane than A-type lamins, a notion supported by the recent identification of an integral membrane protein of the nuclear envelope as a candidate lamin B receptor (Worman *et al.*, 1988; see also Senior and Gerace, 1988). Whereas B-type lamins are expressed in almost all cell types examined, the expression of A-type lamins is more restricted, raising the possibility that changes in the composition of the nuclear lamina may relate to changes in nuclear organization (Lehner *et al.*, 1987; Stewart and Burke, 1987; Röber *et al.*, 1989; for review see Nigg, 1989). Consistent with this notion, A-type lamins appear to interact more directly with chromatin than B-type lamins (Burke and Gerace, 1986).

During *in vivo* biogenesis of the vertebrate nuclear lamina, A-type lamins are synthesized as higher molecular mass precursors (Gerace *et al.*, 1984; Dagenais *et al.*, 1985; Lehner *et al.*, 1986a). Although processing of these precursors was proposed to involve proteolytic cleavage of a C-terminal peptide from lamin A (e.g. Lehner *et al.*, 1986a), alternative explanations have not been excluded. A short-lived precursor has also been detected in the case of chicken lamin B_2 (Lehner *et al.*, 1986a; Vorburger *et al.*, 1989), but the nature of the modification(s) causing a change in mol. wt upon maturation of lamin B_2 has not yet been determined.

Structurally, all lamins display a tripartite organization typical of intermediate filament proteins. They contain a central α-helical rod domain, flanked by non-α-helical head and tail domains (for reviews see Osborn and Weber, 1986; Franke, 1987; Conway and Parry, 1988; Steinert and Roop, 1988; Nigg, 1989). However, the C-terminal tail domains of most A-type lamins are considerably longer than those of B-type lamins, and a number of characteristic sequence motifs distinguish the two lamin subfamilies (Krohne *et al.*, 1987; Höger *et al.*, 1988; Peter *et al.*, 1989). One intriguing feature emerging from sequence analyses of both A- and B-type lamins is the presence of a conserved C-terminal tetrapeptide motif consisting of a cysteine residue followed by two aliphatic amino acids and a terminal methionine

(Krohne *et al.*, 1987; Höger *et al.*, 1988; Peter *et al.*, 1989; Vorburger *et al.*, 1989). This structure (CXXM) is found in all lamins sequenced so far, except for human lamin C (Fisher *et al.*, 1986; McKeon *et al.*, 1986), and it closely resembles a C-terminal motif (termed the CAAX box) found in *ras*-related proteins and in a number of fungal mating factors (reviewed in Clarke *et al.*, 1988; Magee and Hanley, 1988). As shown for both fungal mating factors (Ishibashi *et al.*, 1984; Akada *et al.*, 1987; Anderegg *et al.*, 1988) and *ras* proteins (Clarke *et al.*, 1988; Gutierrez *et al.*, 1989; Hancock *et al.*, 1989), the CAAX box is the target of a complex series of post-translational modifications. Specifically, the cysteine residue of the CAAX box is modified covalently not only by polyisoprenylation, but, following proteolytic cleavage of the last three amino acids, also by carboxyl methylation (Clarke *et al.*, 1988; Deschenes *et al.*, 1989; Gutierrez *et al.*, 1989). For some time, the same motif had also been implicated in palmitoylation of *ras* proteins (Willumsen *et al.*, 1984; Chen *et al.*, 1985), but more recent work has demonstrated that addition of palmitate occurs on cysteine residues located in close proximity, N-terminal to the CAAX box (Hancock *et al.*, 1989).

In the case of a yeast mating factor, the polyisoprenoid moiety was identified by mass spectroscopy as farnesyl (Anderegg *et al.*, 1988), and it appears likely that the lipophilic isoprene moiety attached to *ras* proteins (Hancock *et al.*, 1989) and nuclear lamins (Beck *et al.*, 1988; Wolda and Glomset, 1988) has a similar structure. The functional significance of post-translational processing of the CAAX box remains to be fully explored, but in the case of $p21^{ras}$, isoprenylation was shown to be essential for both plasma membrane association and biological activity (Gutierrez *et al.*, 1989; Hancock *et al.*, 1989; see also Willumsen *et al.*, 1984).

Here we demonstrate that newly synthesized chicken lamins A and B_2 are both processed proteolytically at their carboxy termini *in vivo*, and we provide evidence that the C-terminal CXXM motif of lamin proteins is essential for isoprenylation. In particular, we show that *in vitro* translated lamin proteins specifically incorporate a derivative of mevalonic acid, i.e. the metabolic precursor of isoprene moieties. By site-directed mutagenesis, we further demonstrate that this modification depends on the presence of the cysteine residue within the C-terminal CXXM motif. These results suggest that the evolutionary conservation of the CXXM motif in nuclear lamins is functionally significant, and they provide the basis for considering mechanistic and functional aspects of C-terminal processing of lamin proteins in relation to recent findings reported for *ras*-related proteins.

Results

Proteolytic processing of the chicken lamin A precursor removes a C-terminal peptide of ~2–3 kd

To unequivocally establish that processing of the lamin A precursor involves proteolysis we have compared the gel electrophoretic migration of specifically labeled peptides generated by selective cleavage of lamin A precursor and product respectively (Figure 1). For these experiments, proteins were labeled using either [^{35}S]methionine (Met) or [^{35}S]cysteine (Cys). Mature lamin A was isolated by immunoprecipitation from cultured cells, whereas labeled lamin A precursor was produced by *in vitro* translation of

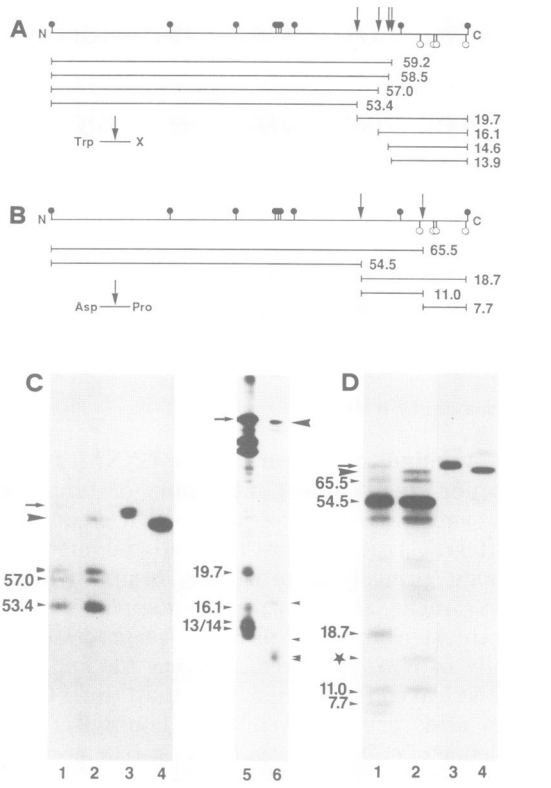

Fig. 1. Peptide mapping localizes lamin A processing to the C terminus. Radiolabeled lamin A precursor protein was produced by *in vitro* translation of RNA, and mature lamin A was immunoprecipitated from metabolically labeled chicken embryo fibroblasts. For visualization of cleavage products, lamin proteins were selectively labeled using either [^{35}S]Met (**C, lanes 1–5**, and **D**) or [^{35}S]Cys (**C, lane 6**). Proteins were eluted from SDS–polyacrylamide gels and subjected to cleavage at either Trp-X (X being any amino acid) or Asp-Pro sites, as outlined in Materials and methods. Cleavage products were analyzed by SDS–PAGE, followed by fluorography. (**A** and **B**) Diagrams illustrating the predicted lengths of the fragments generated by cleavage of chicken lamin A at (**A**) Trp residues (arrows), or (**B**) Asp-Pro sites (arrows). Also indicated are the distributions of the residues used for radioactive labeling, i.e. Met (filled circles) and Cys (open circles), as predicted by the primary structure of the chicken lamin A precursor cDNA (Peter *et al.*, 1989). (**C**) Gel electrophoretic comparison of peptides generated by cleavage of lamin A precursor and mature lamin A at Trp residues. Analysis on 8% gels revealed precise comigration of the predicted large (N-terminal) cleavage products (cf. **lanes 1** and **2**), although, as shown in **lanes 3** and **4**, this gel system readily allowed visualization of the characteristic difference in migration between uncleaved lamin A precursor (arrow) and mature lamin A (large arrowhead). In contrast, analysis on 15% gels revealed a striking difference in the migration of the predicted small (C-terminal) cleavage products, in that peptides derived from mature lamin A (**lane 6**) migrated faster than those derived from the lamin A precursor (**lane 5**). Note that the absence of large cleavage products from **lane 6** is due to the use of [^{35}S]Cys for labeling of this sample, and absence of Cys residues N-terminal to the Trp cleavage sites. (**D**) Gel electrophoretic comparison of peptides generated by cleavage of lamin A precursor and mature lamin A at Asp-Pro sites. Uncleaved proteins and cleavage products were resolved on a 10–17% gradient SDS–polyacrylamide gel. **Lane 1**, cleavage products derived from the lamin A precursor; **lane 2**, cleavage products derived from mature lamin A. For comparison, **lanes 3** and **4** illustrate the migrations of uncleaved lamin A precursor (arrow) and mature lamin A (large arrowhead). Note the absence among the cleavage products derived from mature lamin A (**lane 2**) of C-terminal peptides of 18.7 and 7.7 kd, and the appearance of a faster migrating version of the 18.7 kd peptide (marked by a star). We cannot definitively explain the origin of the band migrating slightly ahead of the 7.7 kd fragment in **lane 1**, but we presume that it may have arisen as an artefact during sample preparation.

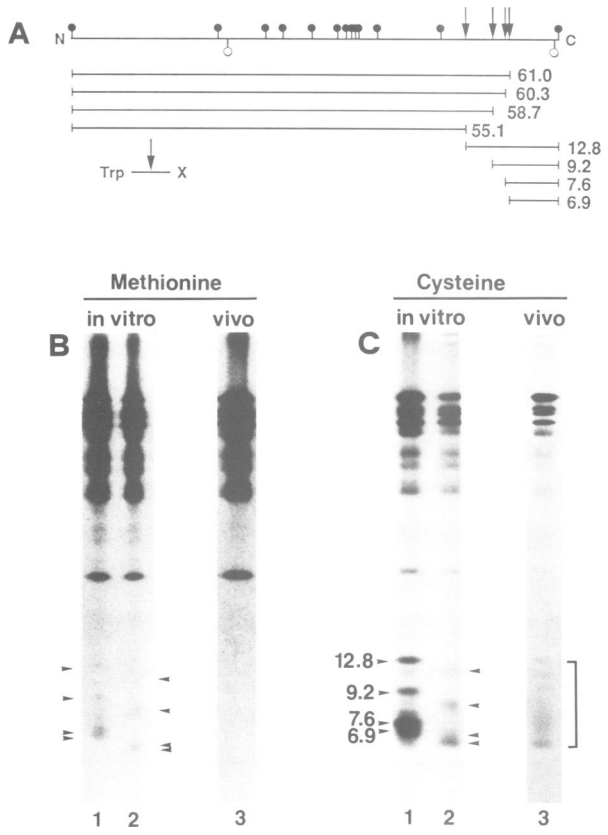

Fig. 2. Comparison by peptide mapping of lamin B_2 processing *in vitro* and *in vivo*. Selective labeling and immunoprecipitation of lamin B_2 as well as cleavage after Trp residues was carried out as described in Materials and methods and the legend to Figure 1. (**A**) Diagram illustrating the predicted lengths of the fragments generated by cleavage of chicken lamin B_2 at Trp residues (arrows). Also indicated are the distributions of the residues used for radioactive labeling, i.e. Met (filled circles) and Cys (open circles), as predicted by the primary structure of the chicken lamin B_2 precursor cDNA (Vorburger *et al.*, 1989). (**B** and **C**) Gel electrophoretic analysis of peptides generated by cleavage of lamin B_2 proteins after labeling with either [^{35}S]Met (**B**) or [^{35}S]Cys (**C**). Lamin B_2 precursor was produced by *in vitro* translation of synthetic RNA, whereas processed forms of lamin B_2 were isolated either after processing in reticulocyte lysates, or after *in vivo* processing in chicken embryo fibroblasts. Following treatment with *N*-chlorosuccinimide, cleavage products were analyzed by electrophoresis on 15% polyacrylamide gels. **Lanes 1**, Cleavage products derived from lamin B_2 precursor. **Lanes 2**, cleavage products derived from lamin B_2 processed *in vitro*; note the faster migration of the small C-terminal peptides generated from the *in vitro* processed form of lamin B_2 (cf. **lanes 2** and **1**, arrowheads). **Lanes 3**, cleavage products derived from mature lamin B_2 after isolation from chicken embryo fibroblasts; note that C-terminal peptides were readily detectable after labeling with [^{35}S]Cys (**C, lane 3**, bracket), but not after labeling with [^{35}S]Met (**B, lane 3**). We also note a difference in electrophoretic mobility between corresponding C-terminal fragments, depending on whether they were generated from lamin B_2 processed *in vitro* (**C, lane 2**, arrowheads) or *in vivo* (**C, lane 3**, bracket).

RNA synthesized from a cloned cDNA (Peter *et al.*, 1989). Two different cleavage procedures were used. In a first experiment, lamin A polypeptides were treated with *N*-chlorosuccinimide (Lischwe and Sung, 1977), a reagent cleaving preferentially after tryptophan (Trp) residues. In a second experiment, they were subjected to mild acid hydrolysis (Shih *et al.*, 1982), a procedure resulting in cleavage between aspartic acid (Asp) and proline (Pro). Figure 1A and B illustrate schematically the fragments expected after cleaving the lamin A precursor at either four closely spaced Trp residues (A) or two Asp-Pro sites (B). Also represented in these schemes are the relative positions of methionines (filled circles) and cysteines (open circles), i.e. the residues used for labeling of lamin A polypeptides.

Figure 1C shows the result of the Trp cleavage procedure. When fragments were analyzed on a low percentage polyacrylamide gel (lanes 1 and 2), three of the four predicted N-terminal fragments were readily resolved but the 59.2 and the 58.5 kd polypeptides comigrated (lanes 1 and 2). Although uncleaved lamin A precursor and product migrated differently in this gel system (lanes 3 and 4), no differences could be detected in the migration of the N-terminal fragments generated by digestion of the two proteins (lanes 1 and 2). In contrast, a clear difference in migration of cleavage products was revealed when analyzing the predicted C-terminal fragments on a high percentage gel (lanes 5 and 6). These results demonstrate that lamin A processing does not change the mobilities of fragments N-terminal to the four Trp residues, but changes the apparent mol. wts of C-terminal peptides.

Figure 1D shows the result of the Asp-Pro cleavage of lamin A precursor (lane 1) and mature lamin A (lane 2). Consistent with the results shown above, two predicted N-terminal fragments at 65.5 and 54.5 kd comigrated, but differences were apparent in the migration of the small C-terminal peptides generated by this procedure. Although an 11 kd fragment, predicted to arise from cleavage at both Asp-Pro sites (Figure 1B), was recovered from both samples and migrated at an invariant position (cf. lanes 1 and 2), expected fragments at 18.7 and 7.7 kd were visible only in the case of the lamin A precursor (lane 1). In contrast, cleavage of mature lamin A produced a band at ~15.7 kd (star), replacing the 18.7 kd fragment, but no band corresponding to the 7.7 kd fragment (lane 2). Considering the distribution of the only two Met residues contributing to the labeling of these C-terminal fragments (Figure 1B), this result demonstrates unequivocally that the loss of the 7.7 kd fragment, as well as the shift in migration of the 18.7 kd fragment, is due to a proteolytic modification of the lamin A precursor. In support of this conclusion, we also note that labeling of the fragments at 15.7 kd (star) and 11.0 kd is less intensive than labeling of the one at 18.7 kd, consistent with the presence of only one versus two Met residues in these fragments (see Figure 1B). Taken together, these results demonstrate that the changes in apparent mol. wts observed in the course of lamin A biosynthesis are due to proteolytic removal of a C-terminal peptide. This cleaved peptide has an estimated molecular mass of ~2–3 kd (Figure 1C and D) and thus would be expected to include the entire CXXM C-terminus.

Proteolytic processing of the chicken lamin B_2 precursor in vivo removes at most three amino acids

Figure 2 summarizes the results of a similar analysis for chicken lamin B_2. Based on our earlier finding that processing of this B-type lamin occurs not only *in vivo* (Lehner *et al.*, 1986a), but also in reticulocyte lysates (Vorburger *et al.*, 1989), it was possible to compare the structure of the precursor synthesized *in vitro* with that of processed forms produced either *in vitro* or *in vivo*. Lamin B_2 polypeptides were labeled using either [^{35}S]Met (Figure 2B) or [^{35}S]Cys (Figure 2C) and cleaved after Trp residues (Figure 2A). When the cleavage products of *in vitro*

synthesized proteins were analyzed on high percentage SDS–polyacrylamide gels, the small C-terminal peptides generated by cleavage of *in vitro* processed lamin B_2 clearly migrated faster than those of the precursor (Figure 2B and C, cf. lanes 2 and 1). No differences were observed in the migration of the large N-terminal cleavage products, a result confirmed more rigorously by analyses on low percentage gels (not shown). Although the differences in the mobilities of the peptides derived from these *in vitro* synthesized and processed proteins might appear to reflect a proteolytic event, this interpretation is not correct. As shown in Figure 2A, only one Met residue contributes to radioactive labeling of the C-terminal peptides generated by Trp cleavage, and, more importantly, this Met residue is the very last C-terminal amino acid predicted by the cDNA sequence. Thus, the fact that we did recover [^{35}S]Met labeled C-terminal peptides from *in vitro* processed lamin B_2 demonstrates that the modification affecting the mobility of this protein did not entail removal of any amino acids from the carboxy terminus.

In previous studies using two-dimensional gel electrophoresis, we had been unable to detect any differences in the migration of uncleaved mature lamin B_2 proteins, irrespective of whether these had been processed *in vivo* or *in vitro* (Vorburger *et al.*, 1989). Therefore, we were surprised to find that no [^{35}S]Met-labeled C-terminal peptides could be recovered after cleavage of mature lamin B_2 isolated from cultured cells (Figure 2B, lane 3). Thus, although the *in vitro* studies showed that the mobility shift accompanying lamin B_2 processing could not be attributed to proteolysis, some type of C-terminal proteolytic cleavage did occur *in vivo*. To examine this proteolytic processing in more detail, we isolated and cleaved *in vivo* processed lamin B_2 after labeling with [^{35}S]Cys. In this case, [^{35}S]Cys labeled C-terminal peptides could readily be recovered (Figure 2C, lane 3). This result is highly informative because the only Cys residue present in C-terminal peptides is the one located four residues from the carboxy terminus (Figure 2A). Taken together, the results shown in Figure 2 demonstrate that proteolytic processing of lamin B_2 did occur *in vivo*, but not *in vitro*. Moreover, they show that *in vivo* proteolysis of lamin B_2 removed the last C-terminal Met residue but not the Cys residue forming part of the CXXM motif, and thus concerned at most three amino acids.

We note a slight but significant difference in the migration behaviour of [^{35}S]Cys-labeled C-terminal peptides, depending on whether lamin B_2 had been processed *in vitro* or *in vivo* (Figure 2C, cf. lanes 2 and 3). This observation may indicate that, in addition to C-terminal cleavage of up to three amino acids, *in vivo* processing of lamin B_2 involves yet another step that is not reproduced in the reticulocyte lysates used here. We believe that this additional processing may be carboxyl methylation of a newly exposed C-terminal cysteine residue (see Discussion).

Lamin proteins translated in reticulocyte lysates specifically incorporate a derivative of [^{14}C]mevalonic acid

Prompted by the recent finding that lamin proteins are isoprenylated (Beck *et al.*, 1988; Wolda and Glomset, 1988) and the demonstration that the Cys residue of the CAAX box functions as the acceptor for isoprenoid moieties in *ras* proteins and yeast mating factors (for references see Hancock *et al.*, 1989), we decided to examine a possible relationship between C-terminal processing of lamin proteins and isoprenylation. For this purpose we translated RNAs encoding all three known chicken lamin isoforms in reticulocyte lysates in the presence of [^{14}C]mevalonic acid (MV), the biosynthetic precursor of isoprene derivatives (Schmidt *et al.*, 1984). As shown in Figure 3, all three lamin proteins readily incorporated a derivative of MV in a cell-free reaction (lanes 2, 4 and 6). For comparison, Figure 3 also shows the migration of the three lamin proteins after translation in the presence of [^{35}S]Met (lanes 1, 3 and 5). Of particular interest, only the mature form of lamin B_2 incorporated [^{14}C]MV, whereas the precursor form was not modified (Figure 3 cf. lanes 5 and 6). Creatine kinase, translated for control, was readily visualized by labeling with [^{35}S]Met (lane 7), but did not incorporate any [^{14}C]MV (lane 8), attesting to the specificity of the MV modification.

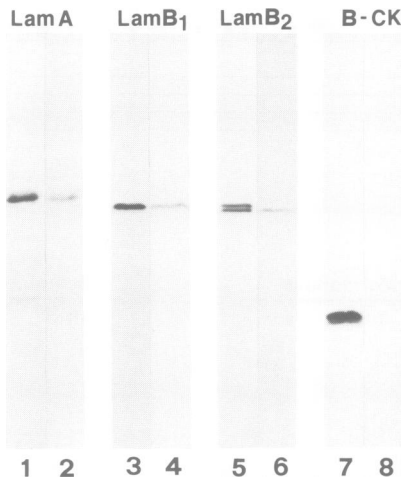

Fig. 3. Specific *in vitro* modification of lamin proteins by a derivative of MV. Synthetic cDNA-derived RNAs were translated in reticulocyte lysates, in the presence of either [^{35}S]Met (**lanes 1, 3, 5** and **7**) or R-[2-^{14}C]MV lactone (**lanes 2, 4, 6** and **8**). The duration of translation (90 min) was chosen such as to yield about equal amounts of lamin B_2 precursor and product, as a result of *in vitro* processing (Vorburger *et al.*, 1989). Proteins labeled with [^{35}S]Met were analyzed directly, whereas proteins modified by an MV derivative were concentrated by immunoprecipitation. Samples were analyzed by 8% SDS–PAGE and fluorography. **Lanes 1** and **2**, lamin A; **lanes 3** and **4**, lamin B_1; **lanes 5** and **6**, lamin B_2; note that only into the processed (i.e. faster migrating) form of lamin B_2 is labeled by [^{14}C]MV (compare **lanes 6** and **5**); **lanes 7** and **8**, creatine kinase (brain type).

In vitro modification of lamin B_2 by a MV derivative requires the cysteine residue of the CXXM C terminus

Next, we asked which parts of lamin proteins might be essential for cell-free modification by MV derivatives. In light of recent results obtained with *ras* proteins (Hancock *et al.*, 1989) and yeast mating factors (Anderegg *et al.*, 1988), we expected cysteines to serve as acceptors for MV derivatives, and therefore, chose lamin B_2 for further analysis. This lamin isoform contains only two cysteine residues (Vorburger *et al.*, 1989), one located in coil 1b of the rod domain (position 191), the other in the C-terminal CXXM motif (position 597). By site-directed mutagenesis of a lamin B_2 cDNA, both of these residues were converted to alanines (Ala), either individually or in combination. The mutated lamin proteins were then translated from the respective cDNA-derived synthetic RNAs, and examined for

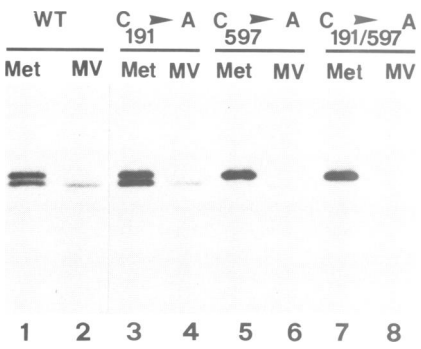

Fig. 4. *In vitro* processing of lamin B_2 mutants. Synthetic RNAs were transcribed from plasmids encoding either wild-type (WT) or mutant lamin B_2 cDNA constructs. Mutations concerned amino acids at either position 191 or position 597, or both, and consisted in converting the corresponding Cys (C) codons to Ala (A) codons. RNAs were translated for 90 min in reticulocyte lysates, in the presence of either [^{35}S]Met (**lanes 1, 3, 5** and **7**) or R-[2-^{14}C]MV lactone (**lanes 2, 4, 6** and **8**). Proteins labeled with [^{35}S]Met were analyzed directly, whereas proteins modified by an MV derivative were concentrated by immunoprecipitation. Samples were analyzed by 8% SDS–PAGE and fluorography. **Lanes 1** and **2**, wild-type lamin B_2; **lanes 3** and **4**, lamin B_2 having Cys191 converted to Ala; **lanes 5** and **6**, lamin B_2 having Cys597 converted to Ala; **lanes 7** and **8**, lamin B_2 having both Cys191 and Cys597 converted to Ala. Note, first, that only fast migrating forms of lamin B_2 are labeled by an MV derivative (**lanes 1–4**), and, second, that lack of incorporation of this derivative into the Cys597 mutants correlates with lack of conversion of the primary lamin B_2 translation product to the faster migrating form (**lanes 5–8**).

their abilities to be processed to the faster migrating form, and for their abilities to incorporate [^{14}C]MV in the reticulocyte lysate (Figure 4). All translations were carried out in the presence of either [^{14}C]MV (lanes 2, 4, 6 and 8) or, for comparison, [^{35}S]Met (lanes 1, 3, 5 and 7). With repect to both of the above criteria, the mutant having only Cys191 (i.e. the residue in the rod domain) replaced by Ala was processed in the same way as the wild-type lamin B_2 protein (Figure 4, cf. lanes 3 and 4 with lanes 1 and 2). In contrast, the mutant lamins having Cys597 (i.e. the residue forming part of the CXXM motif) converted to Ala, either singly or in combination with a mutation at Cys191, failed to be processed to a faster migrating form (Figure 4, lanes 5 and 7). Concomitantly, they did not incorporate the MV derivative (Figure 4, lanes 6 and 8). From these results we conclude that, as assayed *in vitro*, the Cys residue of the C-terminal CXXM motif is essential for the processing event inducing the mobility shift characteristic of lamin B_2 maturation, and, concomitantly, is required for incorporation of an MV derivative into lamin proteins.

Discussion

In vitro modification of lamin proteins by derivatives of MV

A first major result emerging from this study is that, following translation in reticulocyte lysates, all three chicken lamin isoforms could specifically be modified by an MV derivative. Although lamin proteins were previously shown to incorporate a derivative of MV *in vivo* (Beck *et al.*, 1988; Wolda and Glomset, 1988), there was no evidence to suggest that such a modification might occur *in vitro*. The MV derivative incorporated into lamin proteins *in vivo* is presumed to be of an isoprenoid nature, but its precise structure has not yet been determined. In the absence of direct structural information, we cannot address the question as to what extent the *in vitro* modification reported here mimics the isoprenylation occurring *in vivo*. Despite this uncertainty, our unexpected finding suggests that certain enzymological and structural aspects of isoprenylation are amenable to study in cell-free systems.

By site-directed mutagenesis we show that *in vitro* modification of lamin B_2 by an MV derivative depends on the presence of the Cys residue in the C-terminal CXXM motif. This finding attests to the specificity of the observed reaction. Particularly, it provides a highly provocative link between processing of nuclear lamins and that of *ras*-related proteins. In fact, a recent study examining the structural requirements for *in vivo* isoprenylation of *ras* proteins also identifies the Cys residue of the C-terminal CAAX box as being essential for modification by MV derivatives (Hancock *et al.*, 1989).

Proteolytic processing of A- and B-type lamin precursors

The second major result of the present study is that both A- and B-type lamin proteins are processed proteolytically at their carboxy termini. In the course of proteolysis of chicken lamin B_2, the C-terminal Met is removed, but the Cys residue of the CXXM motif remains. Although we have no direct information on the fate of the two amino acids between the Cys and the Met, in analogy to the processing of *ras* proteins and yeast mating factors (for references see Magee and Hanley, 1988; Hancock *et al.*, 1989), we predict that they are removed from newly synthesized lamins, leaving a C-terminal (isoprenylated) Cys residue. In further analogy to results obtained with *ras* proteins and yeast mating factors (for references see Clarke *et al.*, 1988), we also predict that this Cys residue represents the target for subsequent carboxyl methylation, a modification known to occur in mature B-type lamins (Chelsky *et al.*, 1987).

At present we cannot definitively explain why processing of lamin B_2 changes the electrophoretic mobility of this protein both *in vivo* (Lehner *et al.*, 1986a) and *in vitro* (Vorburger *et al.*, 1989). However, we emphasize that the increase in gel electrophoretic mobility that accompanies the maturation of this protein cannot be attributed to proteolysis. This conclusion is based on the finding that the fast-migrating form of lamin B_2 produced by processing in a reticulocyte lysate still contains the C-terminal Met residue. It is interesting, therefore, that only the mature form of lamin B_2 was labeled by [^{14}C]MV, whereas the precursor form was not, and that mutational analysis revealed a correlation between the incorporation of [^{14}C]MV into lamin B_2 and the change in its electrophoretic mobility (Figures 3 and 4). The most straightforward interpretation of this result is that the addition of an MV derivative itself causes a faster migration of lamin B_2 on SDS gels. However, we cannot exclude the existence of yet another, unidentified modification that might both cause the observed change in electrophoretic mobility of lamin B_2 and represent a prerequisite for subsequent isoprenylation. Whatever the correct interpretation, lamin B_2 is unique in displaying a mobility shift upon incorporation of [^{14}C]MV, in that neither lamin A nor lamin B_1 show such a behavior.

In contrast to the limited proteolysis of lamin B_2, affecting at most three C-terminal amino acids, lamin A is subject to a further proteolytic processing event. This additional processing occurs after association of newly synthesized lamin A with the nuclear envelope (Gerace *et*

al., 1984; Lehner *et al.*, 1986a) and results in removal of a peptide containing the isoprenylated Cys residue (Beck *et al.*, 1988). At present, we do not know the precise site of cleavage of chicken lamin A, and direct protein sequence analysis will be required to determine the C-terminus of mature lamin A.

Comparison of lamin and ras precursor processing: functional implications

Based on our present findings, and in analogy to recent results on the modifications affecting the CAAX box of *ras* proteins (Clarke *et al.*, 1988; Hancock *et al.*, 1989), we propose the following model for post-translational processing of newly synthesized nuclear lamins: we envisage that all lamin proteins containing a conserved C-terminal CXXM motif are isoprenylated shortly after synthesis on free ribosomes (Lehner *et al.*, 1986a). As demonstrated clearly by our present results (Figure 2), isoprenylation is then followed by proteolytic removal of (presumably) three C-terminal residues, leaving an isoprenylated C-terminal Cys. In strict analogy to *ras* proteins and yeast mating factors (Clarke *et al.*, 1988; Gutierrez *et al.*, 1989; Hancock *et al.*, 1989), we predict that this Cys residue then represents the target for carboxyl methylation. As suggested previously, further proteolytic cleavage of A-type lamins occurs after import into the nucleus (Gerace *et al.*, 1984; Lehner *et al.*, 1986a). This final processing, restricted to A-type lamins, leads to removal of a 2–3 kd C-terminal peptide including the entire (modified) CXXM motif.

Although several individual steps in the pathway depicted here must await direct experimental verification, we emphasize that the model is consistent with several lines of evidence. First, it readily explains why the lamin A precursor is isoprenylated, but mature lamin A is not (Beck *et al.*, 1988). Second, it explains why only B-type lamins are carboxyl methylated but A-type lamins are not (Chelsky *et al.*, 1987).

In the case of *ras* proteins, isoprenylation was shown to be essential for subsequent palmitoylation of nearby Cys residues, and for mediating the association of *ras* proteins with the plasma membrane (Hancock *et al.*, 1989). In turn, membrane association was shown to be important for biological activity of both normal and transforming *ras* gene products (Willumsen *et al.*, 1984; Buss and Sefton, 1986; Hancock *et al.*, 1989). B-type lamin sequences do not predict Cys residues in close proximity with the isoprenylated C-terminus (Krohne *et al.*, 1987; Höger *et al.*, 1988; Peter *et al.*, 1989; Vorburger *et al.*, 1989), and there is no evidence for palmitoylation of lamin proteins. However, it remains an attractive possibility that isoprenylation may by itself play a major role in mediating nuclear envelope association of newly synthesized lamin proteins. Consistent with this idea, proteolytic removal of a 2–3 kd C-terminal peptide from lamin A occurs subsequent to association of this protein with the nuclear envelope (Gerace *et al.*, 1984; Lehner *et al.*, 1986a) and, remarkably, this processing step is not observed under conditions inhibiting lamin isoprenylation (Beck *et al.*, 1988).

To examine the possibility that isoprenylation may be required for proper interaction of newly synthesized lamins with the inner nuclear membrane, we are in the process of transfecting mutated chicken lamin proteins into cultured mammalian cells. The availability of cell lines expressing mutated lamin proteins should allow us to obtain further information on the functional significance of lamin isoprenylation.

Materials and methods

Cell culture, metabolic labeling and immunoprecipitation

Fibroblasts were obtained from the skins of 10–11 day old chicken embryos and cultured as described previously (Lehner *et al.*, 1986b). For metabolic labeling, monolayers were washed and incubated in Met- or Cys-free media for 30 min. Then, cells were incubated in the same media supplemented with 4% fetal calf serum and [^{35}S]Met or [^{35}S]Cys (Amersham) at 50 μCi/ml for 5 h.

For immunoprecipitation, cells were solubilized in 0.8 ml RIPA (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 0.02 M Tris–HCl pH 7.2). To remove insoluble material, the lysates were centrifuged for 10 min at 17 000 *g*. Aliquots of the supernatants (0.2–0.4 ml) were then incubated for 1 h at 4°C with 5 μl of a rabbit anti-lamin A+B_2 serum (Lehner *et al.*, 1986c). Immune-complexes were adsorbed to Pansorbin (Calbiochem) by an additional 1 h incubation on ice, and then washed four times with RIPA and once with 50 mM Tris–HCl pH 7.5, by centrifugation for 2 min at 17 000 *g*. Antigens were released from the final pellets by 5 min boiling in gel sample buffer (Laemmli, 1970) and separated by SDS–PAGE for peptide mapping (as described below) or stored at −20°C.

Preparation and in vitro translation of synthetic RNA

Synthetic RNAs were transcribed *in vitro* from cloned cDNAs as described previously (Vorburger *et al.*, 1989). *In vitro* translations were carried out in reticulocyte lysates for 90 min according to the instructions provided by Promega Biotec. Approximately 400 ng of synthetic RNA were used in 50 μl reactions. Translated proteins were labeled using either [^{35}S]Met or [^{35}S]Cys. Translation reactions in the presence of R-[2-^{14}C]MV lactone (56.7 mCi/mmol; Amersham, CFA.660) were carried out by using 25 μCi/ml of the radioactive compound. Prior to use, the radioactive MV lactone (supplied in benzene) was dried at 50°C under a stream of nitrogen and dissolved in ultrapure H_2O. After translation, detergents were added to adjust RIPA conditions and the inhibitors PMSF (1 mM), aprotinin (50 μg/ml) and iodoacetamid (5 mM) were added. Following immunoprecipitation, labeled proteins were separated by SDS–PAGE and visualized by fluorography. For peptide mapping of lamin proteins, gels were processed as described below.

Peptide mapping

N-chlorosuccinimide cleavage of lamin proteins after Trp residues was performed essentially as described by Lischwe and Sung (1977). Radioactively labeled lamin proteins were isolated by immunoprecipitation and subjected to electrophoresis on thin (0.75 mm) 8% SDS–polyacrylamide gels. Gels were washed in water for 10 min, dried, and exposed to Fuji X-ray film at −70°C. Lamin bands were cut out from dried gels and rehydrated in a 0.1% solution of urea in acetic acid:water (1:1). Gel slices were then exposed for 20 min to 40 mM *N*-chlorosuccinimide (Fluka, purum) in the above rehydration solution. After several washes in rehydration solution, gel slices were incubated in 0.5 M Tris–HCl pH 6.8, 0.4% SDS, 0.02% bromophenol blue for 20 min. Subsequently, they were equilibrated in gel sample buffer and loaded directly onto 8 or 15% SDS–polyacrylamide gels. Cleavage products were visualized by fluorography for 30–60 days.

Formic acid cleavage of lamin proteins at Asp-Pro was carried out essentially as described by Shih *et al.* (1982). Immunoprecipitated lamins were resolved by SDS–PAGE and visualized by exposing dried gels to X-ray film. Lamin bands were excised, rehydrated in 500 μl of 50 mM NH_4HCO_3, 0.1% SDS, 1% β-mercaptoethanol, 1 mM PMSF, 1% Trasylol. Gel slices were mashed and subsequently boiled for 5 min in the above rehydration solution. Lamin proteins were eluted by constant rocking at room temperature for 2 h. Gel pieces were then sedimented by centrifugation in an Eppendorf centrifuge (17 000 *g*, 5 min) and the pelleted gel pieces were re-extracted using an additional 500 μl of ammonium bicarbonate solution for 1 h. The two samples containing eluted lamins were pooled and 20 μg of RNase A (Boehringer, Mannheim) added. Proteins were precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 15% and incubation on ice for 90 min. After centrifugation (17 000 *g*, 10 min), pellets were washed once with 10% TCA and once with a 1:1 mixture of ethanol:ether. Finally, pellets were dried by lyophilization. For Asp-Pro cleavage, samples containing ~20 000 c.p.m. were dissolved in 500 μl of 70% (v/v) formic acid and incubated at 50°C for 8 h. The formic acid was removed by lyophilization and the cleavage

products were taken up in 25 μl of gel sample buffer, separated on 10–17% gradient SDS–polyacrylamide gels and visualized by fluorography for 10–14 days.

Site-directed mutagenesis

All mutations in lamin B_2 cDNAs were constructed by oligonucleotide-directed mutagenesis (Kunkel *et al.*, 1987), using the muta-gene kit provided by BioRad, and confirmed by sequencing of the mutated cDNAs. A detailed desription of these mutants will be presented elsewhere. For subsequent transcription–translation studies, mutated cDNAs were subcloned into pGEM-3Zf (+/–) vectors (Promega Biotec).

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