In Vitro Assay and Characterization of the Farnesylation-dependent Prelamin A Endoprotease*

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The 72-kDa nuclear lamina protein lamin A is synthesized as a 74-kDa farnesylated precursor. Conversion of this precursor to mature lamin A appears to be mediated by a specific endoprotease. Prior studies of overexpressed wild-type and mutant lamin A proteins in cultured cells have indicated that the precursor possesses the typical carboxyl-terminal S-farnesylated cysteine methyl ester and that farnesylation is required for endoproteolysis to occur. In this report, we describe the synthesis of an S-farnesyl, cysteiny1 methyl ester peptide corresponding to the carboxyl-terminal 18 amino acid residues of human prelamin A. This peptide acts as a substrate for the prelamin A endoprotease in vitro, with cleavage of the synthetic peptide at the expected site between Tyr657 and Leu658. Endoproteolytic cleavage requires the S-prenylated cysteine methyl ester and, in agreement with transfection studies, is more active with the farnesylated than geranylgeranylated cysteiny1 substrate. N-Acetyl farnesyl methyl cysteine is shown to be a noncompetitive inhibitor of the enzyme. Taken together, these observations suggest that there is a specific farnesyl binding site on the enzyme which is not at the active site.

Proteins with a CAXX consensus sequence at their carboxyl terminus undergo post-translational modifications of the cysteiny1 residue (1, 2). These modifications include derivitization of the cysteine sulphydryl with an isoprenoid moiety followed by the endoproteolytic removal of the -AXX tripeptide and methylation of the cysteine α-carboxyl group. When the X amino acid is S, C, Q, or M, a 15-carbon farnesyl residue is attached to thiocysteinyl linkage to the cysteine (3), whereas when X is a leucine, a 20-carbon geranylgeranylated cysteiny1 residue is found instead (4).

The nuclear lamina is a thin, fibrous structure that lines the inner nuclear membrane and is believed to function in maintaining nuclear shape and volume (5) and may also be involved in the organization of chromatin in the interphase nucleus (6). In most mammalian cells, it consists of three class V intermediate filament proteins, lamins A, B, and C (5, 6). Prelamin A is the 74-kDa precursor of the 72-kDa nuclear lamin A protein (7).

It possesses a CAXX box sequence (CSIM) (8, 9) and has been shown to be farnesylated in vitro (10) and in vivo (11). Despite the loss of the carboxyl-terminal 18 amino acids of prelamin A in its proteolytic conversion to lamin A, it nevertheless undergoes all of the reactions characteristic of other CAXX proteins (11). Experiments with mutants, in which the carboxyl terminus of the CAXX box is replaced by another amino acid, demonstrate that farnesylation is required for the maturation of prelamin A (12). These nonprenylated CAXX box mutants of prelamin A enter the nucleus, yet are not proteolytically processed and are not incorporated into the nuclear lamina. Similar results have been obtained with nonprenylated prelamin A produced by treating cultured mammalian cells with mevinolin (13, 14) or inhibitors of protein farnesylation (15).

Prelamin A is quantitatively converted to mature lamin A in mammalian cell nuclei, consistent with a direct precursor-product relationship and, hence, with a second endoproteolytic cleavage after the canonical CAXX box modifications (13). Based on a comparison of the predicted sequence for human prelamin A from its cDNA, and direct sequencing of the carboxyl terminus of the mature lamin A molecule, this second endoproteolysis is expected to be between a tyrosine (Tyr657) and a leucine (Leu658) 18 amino acid residues upstream from the carboxyl terminus of the prelamin A molecule (16). Consistent with this expectation, mutation of Leu658 to arginine prevents conversion of prelamin A to mature lamin A (17). These observations argue against the sequential action of multiple proteolytic cleavages in conversion of the methylated and farnesylated intermediate to mature lamin A. Rather, they support the hypothesis that there is a single endoprotease that cleaves this intermediate between Tyr657 and Leu658. We refer to this activity as the “prelamin A endoprotease.” A schematic diagram of the prelamin A processing pathway concluding with the reaction catalyzed by the prelamin A endoprotease is shown in Fig. 1.

In this report, we describe a cell-free assay for the prelamin A endoprotease and use this assay to characterize its specificity for various substrates. The results indicate that the prelamin A endoprotease has a specific binding site for the farnesyl group and, therefore, is somewhat analogous to the previously described isoprenylated protein “-AXX” endoprotease (18, 19), whose activity is also shown in Fig. 1. It will be seen that these two enzymes differ significantly, however, in that the prelamin A endoprotease is competitively inhibited by nonprenylated peptides, whereas the isoprenylated protein endoprotease is not (18).

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Radioimmunoassay—HeLa cells were cultured in Ham’s F-12 medium supplemented with 10% fetal
calf serum (v/v) 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B. Chinese hamster ovary (CHO)-K1 cells with an up-regulated carbonic anhydrase transporter, Met-18b-2 (20, 21), were cultured in Ham's F-12 medium supplemented with 5% fetal calf serum plus antibiotics as for the HeLa cells.

Labeling of cells with [3H]mevalonate and [35S]methionine, radioimmunoassay, and nuclear run-on assays were performed as described previously (24). Purification was on normal phase HPLC (250 × 4.6 silica gel column) with a mobile phase of hexane/isopropanol (85:15) and a flow rate of 1.5 ml/min (retention time = 4.5 min).

Radioiodinated Polypeptides—Radioiodination of substrate peptides was by the IODO-GEN (Pierce) method as described by Fraker and Speck (25). Briefly, a 1 mg/ml solution of peptide was prepared in a borate buffer; pH 8.2, 6.25 mM borate, 145 mM NaCl, 0.1 mM EDTA. One hundred microliters of this peptide solution was mixed with 300 μCi of Na125I (Amersham Corp.) and incubated in IODO-GEN-lined tubes on ice for 30 min. The reaction was stopped by the addition of 2 μl of 1 M dithiothreitol. The product was separated from unreacted iodine by elution from a P2 desalting column with 10 mM MES (pH = 6.0) buffer containing 2 mM KI and 0.2 mM EDTA. Specific activity of the isolated iodinated peptide was around 0.5 mCi/μmol.

Endoprotease Assay—Nuclei were prepared from HeLa cells as a source of enzyme activity. Cells were harvested by trypsinization and then washed two times with ice-cold phosphate-buffered saline. All subsequent steps were carried out at 4 °C. The cell pellet was resuspended in the same buffer without Nonidet P-40. Protein concentration was obtained by means of the Micro BCA protein assay reagent kit (Pierce).

The endoprotease reaction was initiated by the addition of 125I-labeled peptide (V) at 5 μg for farnesylated peptide) to the nuclear preparation in a final volume of 150 μl in 10 mM MES, pH = 6.0. The reaction was run for various periods of time (linear to 90 min) at 37 °C. The reaction was stopped by the addition of 10 μl of glacial acetic acid and chilling on ice for 10 min. The reaction mix was then cleared by centrifugation at 2,000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was collected and lyophilized, the residue resuspended in 25 μl of water and applied to reverse phase thin layer chromatography plates (Analtech, Inc., Newark, DE). TLC plates were developed in 10% acetonitrile in water, and the spots were visualized by autoradiography. A synthetic, iodinated RSY peptide standard was run on each plate to aid in the identification of the expected product. The amount of labeled RSY formed in the assay was determined by scraping the appropriate counter. The endoprotease activity was expressed as the percentage of the expected product formed.

Constructs—Wild-type and nonfarnesylatable mutant (MSIM) prelamin A CDNAs cloned into the EcoRI(5’)-BamHI(3’) of the SV-40 based expression vector, pECE, were kindly gifts of Dr. F. McKeon (Harvard Medical School) and have been previously described (10, 27). The prelamin A mutant terminating in the CAA sequence CVLL was a kind gift of Dr. Paul Kirschmeier (Schering-Plough Research Institute). This mutant was prepared by means of the pAltered sites mutagene kit (Promega, Madison, WI). Wild-type prelamin A cDNA was cloned into pBluescript II KS(−) at the EcoRI and XbaI sites. The mutagenesis protocol was that described by Kramer et al. (28) as modified by Promega and carried out according to the manufacturer. Sequence verification in the mutant was by the Sequenase (U. S. Biochemical Corp., Cleveland, OH) diodeoxy sequencing method. The mutant cDNA was subcloned into the cytomeg-
alovirus promotor-based expression vector pcDNA3 (Invitrogen, San Diego, CA) between the EcoRI and XhoI sites within the polylinker.Transient transfections were by the Lipofectin method (Life Technologies, Inc.) as described previously (10).

RESULTS

Design of a Peptide Substrate for the Prelamin A Endoprotease—As described above, experiments in other laboratories (16, 17) indicate that human prelamin A is endoproteolytically cleaved between Tyräs and Leuäs. In an effort to determine what other features of the prelamin A primary sequence might play a role in substrate determination, we compared the prelamin A sequences reported for several vertebrate species. The results of such a comparison (Table I) suggest that at least three amino acid residues on either side of the cleavage site, the amino acid sequence RSYLLG, may be conserved across species lines. A search on the Swiss-Prot data base (release 33) for the sequence RSYLLG did not reveal this sequence in any protein except prelamin A. This observation reinforced the proposition that this sequence is important for recognition by the prelamin A endoprotease.

Our prior studies (11) identifying intermediates in the prelamin A processing pathway (Fig. 1), also suggested that the endoproteolysis substrate possessed a farnesylated and methylated cysteine at the carboxyl terminus. We thus predicted that peptide I (Structure 1) would be a suitable substrate for the human prelamin A endoprotease where \( \text{Y}^* \) is a radioiodinated tyrosine. If this were an appropriate substrate for the prelamin A endoprotease, the tripeptide RSY* would be released. Synthesis of the methylated apopeptide was achieved by solid state methods followed by carboxyl-terminal methylation with 5% HCl, MeOH as described under “Experimental Procedures.” After HPLC purification of the apopeptide, the cysteine was farnesylated by reaction with farnesyl bromide under mild basic conditions and repurified by reverse phase HPLC. Synthesis of the farnesylated peptide was confirmed by electrospray mass spectrometry (data not shown), and the peptide was then radioiodinated by the IODO-GEN method.

The radioiodination was expected to permit quantitative monitoring of product formation for in vitro enzyme assay. Utilizing crude nuclear extracts from HeLa cells as a source of enzyme, formation of the expected RSY* product was detected by reverse phase thin layer chromatography. No product was formed when heat treated nuclear extracts were used (Fig. 2). The co-migration of the proteolytic product with synthetic radiolabeled RSY was observed to be the standard assay conditions described under “Experimental Procedures.”
prenylated, whereas the CVLL sequence has been shown to produce geranylgeranylation of other proteins (29). Geranylgeranylation of the CVLL-prelamin A was confirmed by demonstrating efficient [3H]mevalonate labeling of the protein (Fig. 7) in the presence of the farnesyl protein transferase inhibitor, BZA-5B (30). We have previously reported that BZA-5B effectively inhibits the incorporation of [3H]mevalonate into the farnesyl substituent of wild-type prelamin A (15).

We next compared the proteolytic conversion of the wild-type and mutant prelamin A. As expected, there was no prelamin A detected in cells transfected with the wild-type construct whereas, as previously reported (10), the nonprenylated prelamin A cannot be processed to the mature protein. In contrast to these proteins, the geranylgeranylated mutant could be converted to mature lamin A, but less efficiently than the wild-type protein, as indicated by the large amount of prelamin A which accumulates in these cells (Fig. 8). The accumulation of prelamin A in cells transfected with the CVLL mutant was also confirmed by immunoprecipitation and indirect immunofluorescence with a prelamin A-specific antibody (data not shown).

In order to further evaluate the biological relevance of the in vitro results, the activity of the nonfarnesylated analogue of Structure 1 was examined as a substrate. Transfection studies from our laboratory (10) and others (12), with nonfarnesylatable prelamin A mutants, have demonstrated that conversion of prelamin A to mature lamin A will not occur in such mutants. Consistent with these results, our standard assay did not indicate any formation of the RSY product from the nonfarnesylated substrate (data not shown). We also examined the activity of base-demethylated I as a substrate and again observed no formation of RSY (data not shown). This finding demonstrates that in addition to farnesylation, the substrate cysteine must be methylated to be active as a substrate. This result is consistent with a prior report from our laboratory demonstrating that the maturation of prelamin A proceeds through a farnesylated and methylated cysteine intermediate (11).

Inhibitor Studies—The studies, described above, indicate at least two critical chemical features of the prelamin A peptide which render it active as a substrate: the putative RSYLLG endoprotease cleavage site and a carboxyl-terminal farnesylated methylated cysteine. Therefore, we examined the effect of N-acetyl farnesyl methyl cysteine (Fig. 9A) and RSYLLG (Fig. 9B) on the in vitro formation of RSY from I. The results indicate that both compounds can inhibit formation of RSY.

The Prelamin A Endopeptidase

**Figure 3.** Co-migration of iodinated cleavage product of prelamin A peptide endoproteolysis with synthetic RSY. Material comigrating with RSY on TLC was eluted with CH$_3$CN (99%), H$_2$O (0.9%), trifluoroacetic acid (0.1%). To this was added bona fide synthetic RSY mass standard (0.3 µg). The sample was then analyzed by reverse phase HPLC on the same system used for the prenylated peptides at a flow rate of 1 ml/min. The radioactive material was monitored by collecting fractions every minute and counting them in a γ-counter (panel A), whereas the mass RSY standard was monitored by in-line absorbance measurement at 210 nm (panel B).

**Figure 4.** Time dependence of prelamin A peptide endoproteolysis. Radiiodinated RSY formed in the prelamin A endopeptidase assay described under “Experimental Procedures” was monitored by reverse phase TLC and γ-counting as a function of incubation time. The results shown are the average of two determinations. There were 175 µg of protein used per assay. I was used as substrate at a concentration of 5 µM.
we also examined the activity of radioiodinated RSYLLG as a substrate in the endoprotease reaction. In contrast to the non-farnesylated prelamin A peptide, RSYLLG is, indeed, efficiently hydrolyzed by the endoprotease \((K_m = 0.27 \mu M; V_{max} = 14.2 \text{ pmol/min/mg of protein})\).

In order to get preliminary information on the catalytic nature of the prelamin A endoprotease we examined class inhibitors of aspartic proteinases (pepstatin), metalloproteases (EDTA, EGTA), cysteine proteases (leupeptin, E-64), and serine proteases (aprotinin, 3,4-dichloroisocoumarin, chymostatin, phenylmethylsulfonyl fluoride). Inhibition was only obtained with the serine protease inhibitors (Fig. 10). The negative results for the other classes of protease inhibitors tested are not shown.

**DISCUSSION**

We (10, 13) and others (12) have reported that prelamin A does not undergo processing or assembly into the nuclear lamina in the absence of farnesylation. Farnesylated prelamin A mutated in the endoprotease site (RSYLLG→RSYRLG) has been reported by Hennekes and Nigg (17) to localize to the nuclear periphery but not undergo endoproteolysis to mature lamin A. Based on this finding, these workers suggested that one function of farnesylation of prelamin A is localization to the nuclear envelope.

The studies presented in this report are consistent with the hypothesis that another possible function for farnesylation is binding of the farnesylated and methylated prelamin A to the prelamin A endoprotease. Several observations particularly pertain to this point. The specificity of the endoprotease for farnesylation over geranylgeranylation in vitro and in whole cells is consistent with recognition of the prenyl substituent by the enzyme. Noncompetitive inhibition by N-acetyl farnesyl...
methyl cysteine is also consistent with a binding site on the endoprotease for the prenyl group, albeit at a site other than the active site.

The lack of cleavage of the nonfarnesylated prelamin A peptide substrate in vitro, and the similar lack of cleavage of the mature lamin A (A) proteins were immunoprecipitated with the human species specific lamin A antibody 1E4 and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Molecular mass markers (in kDa) are indicated on the right.

The CVLL prelamin A mutant is processed to mature lamin A but less efficiently than wild-type prelamin A. CHO-K1 cells (1 × 10⁶) were transfected with human prelamin A (CSIM) (lanes 1 and 2), a nonprenylatable prelamin A mutant (MSIM) (lanes 3 and 4) and the CVLL prelamin A mutant (lanes 5 and 6) and then labeled with 35 μCi/ml [35S]methionine for 16 h. The transgenic prelamin A (A) and mature lamin A (A) proteins were immunoprecipitated with the human species specific lamin A antibody 1E4 and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Molecular mass markers (in kDa) are indicated on the right.

FIG. 8. The CVLL prelamin A mutant is processed to mature lamin A but less efficiently than wild-type prelamin A. CHO-K1 cells (1 × 10⁶) were transfected with human prelamin A (CSIM) (lanes 1 and 2), a nonprenylatable prelamin A mutant (MSIM) (lanes 3 and 4) and the CVLL prelamin A mutant (lanes 5 and 6) and then labeled with 35 μCi/ml [35S]methionine for 16 h. The transgenic prelamin A (A) and mature lamin A (A) proteins were immunoprecipitated with the human species specific lamin A antibody 1E4 and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Molecular mass markers (in kDa) are indicated on the right.

FIG. 9. Eadie-Hofstee analysis of inhibition of the prelamin A peptide endoprotease by N-acetyl farnesyl methyl cysteine (A) or RSYLLG (B). A, the endoprotease assay was performed in the absence (●) or presence of 1 μM (×) or 2 μM (●) N-acetyl farnesyl methyl cysteine and various concentrations of the prelamin A peptide substrate. The parallel lines observed for the two concentrations of inhibitor are diagnostic for noncompetitive inhibition. B, the endoprotease reaction was performed in the absence (●) or presence of 1 μM (×) or 2 μM (●) RSYLLG and various concentrations of the prelamin A peptide substrate. The intersection of the lines on the y axis is diagnostic of competitive inhibition.

FIG. 10. Inhibition of prelamin A peptide endoproteolysis by serine protease inhibitors. Nuclei were taken up in assay buffer, preincubated for 10 min with various serine protease inhibitors (0.8 mM) and then the radioiodinated prelamin A peptide was added. Incubation was continued for an additional hour and labeled RSY formed (arrow) detected by autoradiography. Lane 1, radioiodinated RSY standard; lane 2, aprotinin; lane 3, 3,4-dichloroisocoumarin; lane 4, chymostatin; lane 5, Phenylmethylsulfonyl fluoride; lane 6, untreated control.

FIG. 11. A model for the prelamin A endoprotease. The RSYLLG endoprotease site in prelamin A is hypothesized to be masked in a higher order structure making it inaccessible to the endoprotease active site (panel A). Farnesylation is then suggested to result in binding of the substrate by an allosteric site on the endoprotease facilitating recognition of the RSYLLG sequence at the active site (panel B). prelamin A molecule in whole cells, stands in contrast to the efficient cleavage of the hexapeptide substrate, RSYLLG. We would speculate the basis of these observations is that the RSYLLG sequence, as a part of the prelamin A molecule, cannot be presented to the active site of the endoprotease, perhaps because of secondary structural constraints. Specific binding of the farnesylated and methylated cysteine would, thus, direct the RSYLLG sequence to the proteolytic cleavage site. That methylation is also important for substrate reactivity is indicated by the lack of cleavage of the demethylated prelamin A peptide. Higher order structure in the C terminus of nonfarnesylated prelamin is consistent with the previous finding from our laboratory that the prelamin A peptide domain is inhibitory for prelamin A assembly into the lamina (10). An illustration of our hypothesis for the role of farnesylation in prelamin A endoproteolysis is shown in Fig. 11.

An important feature of this hypothesis is that we are suggesting the existence of a farnesyl cysteine methyl ester binding site on the prelamin A endoprotease. Studies of other enzymes are also consistent with binding sites for farnesyl cysteine methyl ester. The Kᵢ for noncompetitive inhibition of the prelamin A endoprotease by N-acetyl farnesyl methyl cysteine (17 μM) is essentially identical to that reported for the apparent Kᵢ for the noncompetitive inhibition of the P-glycoprotein ATPase (31) by N-acetyl farnesyl methyl cysteine. It is also comparable to the Kᵢ values for two other farnesylated substrates for other enzymes. These are the “prenyl cysteine-directed a-carboxymethyl transferase (32),” which has a Kᵢ of...
11.6 μM for N-acetyl, S-farnesyl cysteine and the “isoprenylated protein endoprotease (19, 33),” which has a $K_{m}$ of 6 μM for its farnesylated oligopeptide substrate. Prenylation is required for substrate activity with these enzymes consistent with a polyisoprenyl binding site. Extensive structure-activity studies of inhibitors of the isoprenylated protein endoprotease have particularly been interpreted as consistent with a farnesyl cysteine binding site (34). However, this enzyme differs significantly from the prelamin A endoprotease in that nonprenylated peptides do not act as competitive inhibitors (18). It should also be noted that the “isoprenylated protein endoprotease” is not significantly from the prelamin A endoprotease in that nonprenylated protein binding site (34). However, this enzyme differs significantly from the prelamin A endoprotease in that nonprenylated protein endoprotease (19, 33),” which has a $K_{m}$ of 6 μM for its farnesylated oligopeptide substrate. Prenylation is required for substrate activity with these enzymes consistent with a polyisoprenyl binding site. Extensive structure-activity studies of inhibitors of the isoprenylated protein endoprotease have particularly been interpreted as consistent with a farnesyl cysteine binding site (34). However, this enzyme differs significantly from the prelamin A endoprotease in that nonprenylated peptides do not act as competitive inhibitors (18). It should also be noted that the “isoprenylated protein endoprotease” is not affected by serine protease inhibitors (34) and is, therefore, almost certainly distinct from the enzyme described in this report.

It has been postulated (35) that protein prenylation serves as “a mediator of protein-protein interactions” rather than acting as a hydrophobic anchor to lipid bilayer membranes. The data presented here for the prelamin A endoprotease, as well as the prior studies of the S-prenylcysteine α-carboxymethyltransferase and isoprenylated protein endoprotease, are clearly supportive of this hypothesis.

The existence of such a polyisoprenoid recognition domain in various enzymes is also consistent with a discrimination between polyisoprenoid substituents in biological processes. We observe in our current studies a difference in the rate of endoproteolytic cleavage of farnesylated and geranylgeranylated substrates both in whole cells and in vitro. Such dependence of substrate activity on the isoprenoid substituent has also been reported for the S-prenylcysteine α-carboxymethyltransferase (22, 32) and the isoprenylated protein endoprotease (33).

Similarly, functional specificity of farnesylation relative to geranylgeranylation has been demonstrated for mammalian p21<sup>ras</sup> in cell growth (29), yeast RAS2 activation of adenylate cyclase (36), light-regulated association of rhodopsin kinase with ROS membranes (37) and yeast a-factor induction of mating (38). It is, therefore, intriguing to speculate that a general function of the farnesyl residue is to bind to specific sites on other proteins.

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