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# Subcellular localization and partial purification of prelamin A endoprotease: an enzyme which catalyzes the conversion of farnesylated prelamin A to mature lamin A

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**Abstract The nuclear lamina protein, lamin A is produced by proteolytic cleavage of a 74 kDa precursor protein, prelamin A. The conversion of this precursor to mature lamin A is mediated by a specific endoprotease, prelamin A endoprotease. Subnuclear fractionation indicates that the prelamin A endoprotease is localized at the nuclear membrane. The enzyme appears to be an integral membrane protein, as it can only be removed from the nuclear envelope with detergent. It is effectively solubilized by the detergent *n*-octyl-β-D-glucopyranoside and can be partially-purified (∼1200-fold) by size exclusion and cation exchange (Mono S) chromatography. Prelamin A endoprotease from HeLa cells was eluted from Mono S with 0.3 M sodium chloride as a single peak of activity. SDS-PAGE analysis of this prelamin A endoprotease preparation shows that it contains one major polypeptide at 65 kDa and smaller amounts of a second 68 kDa polypeptide. Inhibition of the enzyme activity in this preparation by specific serine protease inhibitors is consistent with the enzyme being a serine protease.**

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*Key words:* Prelamin A endoprotease; Prelamin A; HeLa cell; Endoprotease purification

## 1. Introduction

Proteins which carry a CAAX (X = methionine, serine, alanine) consensus sequence at their carboxy-terminus are substrates for farnesylation, a 15-carbon isoprenoid, modification, followed by endoproteolytic removal of the AAX tripeptide and carboxyl methylation of the now terminal cysteine [1–5]. Farnesyl modification of proteins is of interest due to its role in the function of a number of growth regulatory proteins, including ras-oncogene products [6], two of the structural proteins of nuclear lamina, prelamin A, a precursor protein for lamin A [7] and lamin B [8], cyclic GMP phosphodiesterase α, rhodopsin kinase and γ-transducin [9]. These post-translational modifications apparently act, in concert with other structural features of the modified proteins, to promote targeting of these proteins to specific cellular compartments. Uniquely, prelamin A undergoes a second endoproteolytic cleavage following the post-translational modifications [10–13]. Despite the loss of the carboxy-terminal 18 amino acids, prelamin A nevertheless undergoes all of the reactions characteristic of other CAAX proteins [14]. We have previously shown that farnesylation-dependent removal of the 18 carboxy-terminal amino acids is a requirement for assembly of lamin A into the nuclear lamina [7]. In the absence of this cleavage, prelamin A can neither give rise to lamin A nor assemble into the nuclear lamina [15]. We call this enzyme, which specifically cleaves the farnesylated carboxy-methylated precursor, the 'prelamin A endoprotease.'

Based on a comparison of the predicted sequence for human prelamin A from its cDNA and direct sequencing of the carboxy-terminus of the mature lamin A molecule, this second endoproteolysis occurs between a tyrosine ($Y^{657}$) and a leucine ($L^{658}$) 18 amino acid residues upstream from the carboxy-terminus of the prelamin A molecule [11]. Also, searches of protein sequence data bases revealed that the $RSY^{657}L^{658}LG$ sequence in the prelamin A molecule which contains the carboxy-terminus of mature lamin A, is unique and highly conserved between species. Recently, we demonstrated [14,16] that this simple hexapeptide is a substrate for the prelamin A endoprotease, confirming its functional significance in conferring specificity. We have also presented some evidence suggesting that the prelamin A endoprotease has prelamin A as its only substrate. Prelamin A is the only nuclear protein that undergoes prenylation-dependent endoproteolysis. Furthermore, we have shown that the activity of prelamin A endoprotease is significantly higher in cells that express prelamin A compared to those that do not [16]. In this context, it becomes of interest to determine whether the nucleus is the only cellular compartment in which this endoproteolytic activity resides. Detection of this activity in cytosolic and nuclear preparations would suggest that there might be multiple substrates for prelamin A endoprotease. However, if the nucleus is the only cellular compartment for this activity, this would be consistent with the hypothesis that prelamin A is the only substrate for this endoprotease.

Here, we initiate the purification of prelamin A endoprotease by subcellular fractionation, thereby localizing it to the nuclear envelope. This observation is consistent with prelamin A being a unique substrate for the prelamin A endoprotease. The activity of this membrane enzyme is successfully solubilized in detergent *n*-octyl-β-D-glucopyranoside (octyl glucoside) and partially-purified.

## 2. Materials and methods

### 2.1. Subfractionation of HeLa nuclei

HeLa cells were cultured in Ham's F12 medium supplemented with 10% fetal calf serum (v/v) (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml amphotericin B.

Cells were washed several times with phosphate-buffered saline

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(PBS), harvested from culture dishes by trypsinization and pelleted by centrifugation in an IEC clinical centrifuge (Damon/IEC, Needham Heights, MA, USA) for 10 minute at $140 \times g$ and then resuspended in five volumes of PBS. Resuspended, washed cells ($2 \times 10^8$/ml) were lysed (500 psi for 10 s) with a nitrogen cavitation bomb (Kontes Glass Company, Millville, NJ, USA). Large particulate material, including nuclei, was collected by centrifugation ($900 \times g$, 10 min) and retained for preparation of the nuclear extract. The post-nuclear supernatant was also assayed for activity. All fractionation steps were carried out at room temperature.

The crude nuclei were resuspended in 10 ml TEA buffer (20 mM triethanol amine-HCl, pH 7.5, 1 mM DTT, 0.1 mM $MgCl_2$, 2 μg/ml DNase, 250 ng/ml RNase) and incubated on ice for 15 min. Nuclear envelopes were prepared by a modification [13] of the procedure of Dwyer and Blobel [17]. The nuclear envelope fraction was treated with 3% octyl glucoside to solubilize the enzyme. Except for the purified enzyme, protein concentrations were measured using the Micro BCA Protein Assay Reagent kit (Pierce Chemical, Rockford, IL, USA).

*2.2. Assay for the activity of prelamin A endoprotease*

This assay was performed as previously described [10,12]. Briefly, the endoprotease reaction was initiated by the addition of [$^{125}$I]RSY*LLG to the cell fractions in a final volume of 20 μl in enzyme assay buffer (10 mM MES, pH 5.0). After 20 min at 37°C, the reaction was stopped by the addition of 5 μl of glacial acetic acid and chilling on ice for 10 min. The reaction mixture was applied to reverse phase thin layer chromatography (TLC) plates (Analtech, Newark, DE, USA), which were developed with 10% acetonitrile in water. The product spots were visualized by autoradiography. The identity of the reaction product was confirmed by its co-migration with a synthetic, iodinated RSY* peptide standard. Amounts of labelled RSY formed were determined by scraping the appropriate spots into tubes and quantitation of radioactivity with a gamma counter.

*2.3. Purification of prelamin A endoprotease*

Nuclear envelope extract from $\sim 10^{10}$ HeLa cells was concentrated approximately 4-fold by an Amicon CentriPrep-10 concentrator. One-half of the concentrated nuclear envelope extract (a volume of 200 μl) was loaded onto a Sephacryl S-200-HR ($1 \times 60$ cm) column (Pharmacia Fine Chemicals) that had been equilibrated in 10 mM MES, pH 5.5, containing 0.1% octyl glucoside. Under these conditions, blue dextran eluted at 60 min indicating a void volume of 15 ml. The flow rate was 0.25 ml/min and the material eluting after the void volume was collected in 0.5 ml fractions. Column calibration was based on the size standards IgG, albumin, trypsinogen and cytochrome *c*.

The enzymatically active fractions from the gel filtration column (70–82) were pooled and concentrated approximately 2-fold by an Amicon CentriPrep-10 concentrator prior to loading on a Mono S (HR 5/5 cation exchange) column (Pharmacia Fine Chemicals). The Mono S column was equilibrated with 10 mM MES, pH 5.5, containing 0.1% octyl glucoside and 0.2% $NaN_3$. The flow rate was maintained at 0.75 ml/min during loading and graident elution. After loading the sample, the column was washed with equilibration buffer for 10 min, followed by development with a 30 min linear gradient from 0 to 0.6 M NaCl in the equilibration buffer. The column was then washed with buffer containing 1 M NaCl for 10 min and re-equilibrated for 20 min. Fractions were collected at 1 min intervals for the entire 70 min elution program. The active fractions from Mono S run were pooled and stored at −80°C.

Chromatographic procedures were performed with an intermediate pressure protein liquid chromatography system (Waters 625LC system). SDS-PAGE was carried out as described by Laemmli [18] and the gels were calibrated with broad range SDS-PAGE standards (Life Technology). The protein content of purified endoprotease was estimated by Coomassie blue R-250 staining of a 10% SDS-PAGE in which known amounts (0.1–0.5 μg) of broad range (Life Technology) SDS-PAGE standard proteins were used as a reference.

## 3. Results

*3.1. Subcellular distribution of prelamin A endoprotease in HeLa cells*

In this study, the synthetic substrate, [$^{125}$I]RSY*LLG [14,16] was used to assay the prelamin A endoprotease. The RSY* product was isolated by reverse phase TLC, visualized by autoradiography and identified by co-migration with radio-iodinated RSY* synthetic peptide, as described before [14].

Previous studies [15] from our laboratory and others [12] on whole cells have established that endoproteolytic processing of prelamin A takes place in the nucleus. Consistent with this observation, no endoprotease activity was detected in post-nuclear supernatants but was detected in crude nuclei (Fig. 1). Furthermore, the enzyme activity was enriched in the nuclear envelope relative to the nucleoplasm. These observations are in agreement with the hypothesis that prelamin A endoprotease is associated with nuclear envelopes.

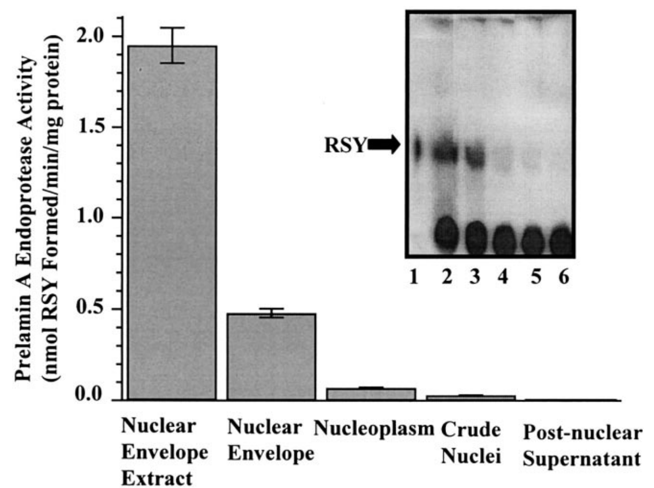


Fig. 1. Subcellular distribution of prelamin A endoprotease in HeLa cells. The prelamin A endoprotease was assayed in various subcellular fractions and the RSY* formed followed by autoradiographic visualization after reverse-phase TLC separation of the reaction products. The specific activity of prelamin A endoprotease is determined following the detection of the reaction product on the film. The results are the average of three determinations. A typical radiochromatogram is shown. Lane 1, synthetic radio-iodinated RSY*; lane 2, HeLa nuclear envelope extract; lane 3, nuclear envelope; lane 4, nucleoplasm; lane 5, crude nuclei and lane 6, post-nuclear supernatant are assayed for endoprotease activity by formation of radio-iodinated RSY as described under Section 2. The radio-labelled material at the solvent front co-migrates with iodotyrosine. There was 40 μg protein used per assay.

*3.2. Solubilization of endoprotease activity*

Initial experiments were aimed at determining whether the protease behaved as an integral membrane protein. The HeLa nuclear envelope was resuspended in 10 mM MES buffer at pH 5.0 with a hand-held homogenizer and incubated at 4°C for 45 min. The membranes were pelleted by centrifugation at $100\,000 \times g$ for 1.5 h. All of the original activity remained associated with the pellets, which were then subjected to other solubilzing agents.

Urea (4 M), which can solubilize the nuclear lamin proteins [19], and a variety of detergents were tested. After extraction, insoluble material was collected by ultracentrifugation and both pellets and supernatant were assayed for the endoprotease activity. The supernatant from the 4 M urea treatment was dialyzed against 10 mM MES and assayed but no appreciable proteolytic activity was found in the supernatant. That treatment with 4 M urea did not solubilize the enzyme activity

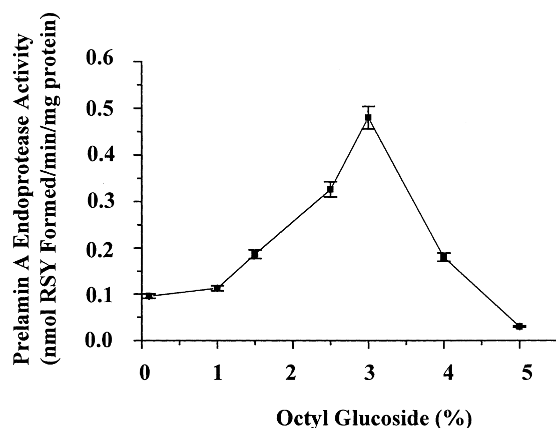


Fig. 2. Octyl glucoside solubilization of prelamin A endoprotease activity. The HeLa nuclear envelope was suspended at 4°C to 4 mg of protein/ml in 10 mM MES, pH 5.0. 100 μl sample in each tube was mixed with 100 μl of octyl glucoside solutions in the same buffer to give the final concentrations shown and the samples were kept on ice for 30 min. After centrifugation at 100 000 × $g$ for 1.5 h, the supernatants were assayed for prelamin A endoprotease activity. The results are the average of three determinations.

is consistent with the notion that prelamin A endoprotease from the HeLa nuclear envelope is an integral membrane protein rather than associated with the nuclear lamina. Consistent with nuclear membrane association of the prelamin A endoprotease, extraction with non-ionic detergents (NP-40 or octyl glucoside), a treatment which is known to solubilize nuclear membranes but leaves behind an insoluble nuclear pore-lamina complex [10,17], substantially solubilized the enzyme and increased its specific activity (Fig. 1).

For solubilization of activity for enzyme purification, we chose to use octyl glucoside, as it effectively released 90% of the endoprotease activity from the nuclear envelope under optimum conditions. Optimal solubilization was obtained at 3% octyl glucoside (Fig. 2). Moreover, octyl glucoside does not interfere with the monitoring of columns at 280 nm.

By three criteria, the enzyme was successfully solubilized by 3% octyl glucoside: (a) After centrifugation at 100 000 × $g$ for 1.5 h, the endoprotease activity was maintained in the supernatant, (b) 100% of the octyl glucoside-solubilized endoprotease activity passed through a 0.22 μm filter (Millipore), (c) after size exclusion chromatography of the nuclear envelope extract, prelamin A endoprotease activity was detected between the void volume and the column volume.

### 3.3. Partial purification of prelamin A endoprotease

The solubilized HeLa nuclear envelope was chromatographed on a Sephacryl S-200-HR column. Protein elution was monitored at 280 nm and fractions were assayed for activity. The major activity peak (Fig. 3A) corresponded to a size of approximately 65 kDa. Most of the protein applied was excluded from the column (data not shown). Peak activity fractions were pooled and concentrated approximately 2-fold by an Amicon CentriPrep-10 prior to chromatography on a Mono S column. Fig. 3B shows the elution profile of prelamin A endoprotease from the ion exchange column. The majority of the protein and approximately half of the activity did not bind to this column. The activity retained on the column (second peak) was eluted at approximately 0.3 M sodium

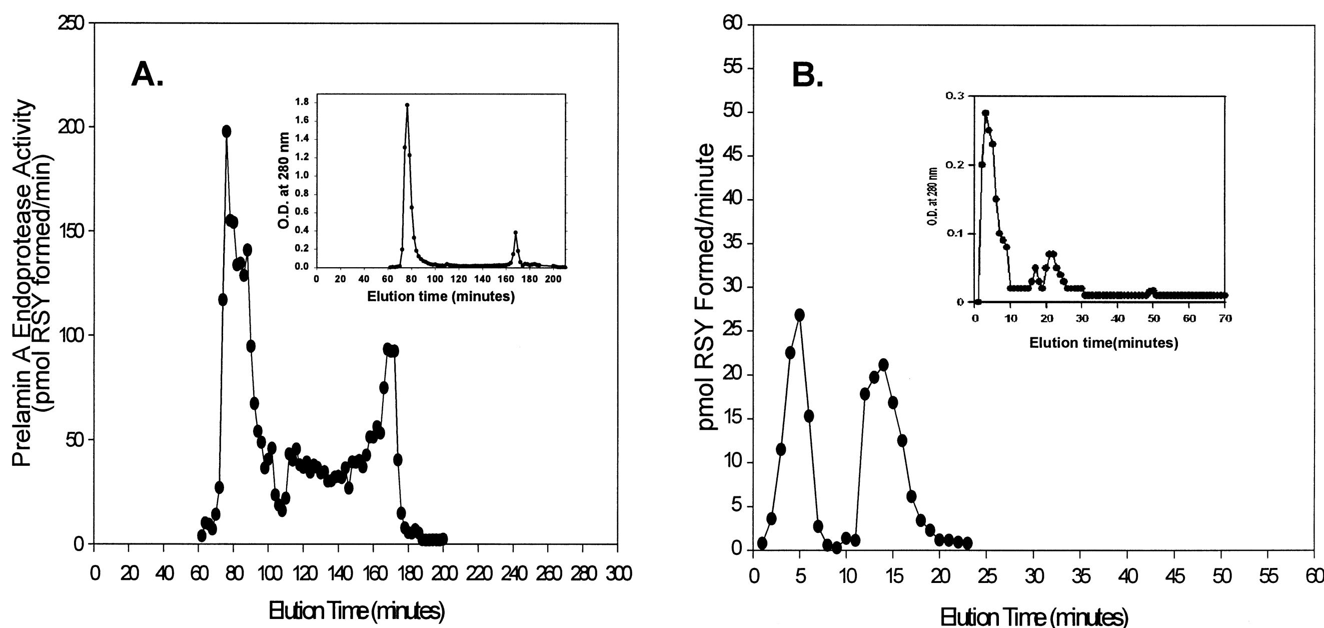


Fig. 3. Chromatographic purification of prelamin A endoprotease. A: Sephacryl S-200-HR chromatography: HeLa nuclear envelope extract was prepared and concentrated as described under Section 2. A 200 μl sample (1.068 mg of protein) was chromatographed on a Sepharose 200 column equilibrated in reaction buffer and eluted as described in Section 2. The protein content of each fraction (inset) was estimated from the absorbance at 280 nm. An aliquot (20 μl) of each fraction was assayed for prelamin A endoprotease activity. B: Mono S chromatography fractions eluting between 74 and 104 min from S200 gel filtration chromatography were pooled and concentrated to a volume of 4.5 ml (256 μg of protein) and applied to a Mono S 5/5 column equilibrated in 10 mM MES, 0.1% octyl glucoside, pH 5.0. The column was run at a flow rate of 0.75 ml/min and fractions were collected at 1 min intervals. The loading and NaCl gradient elution conditions were as described in Section 2. The protein content of each fraction was estimated from the absorbance at 280 nm (inset). An aliquot (20 μl) of each fraction was assayed for prelamin A endoprotease activity.

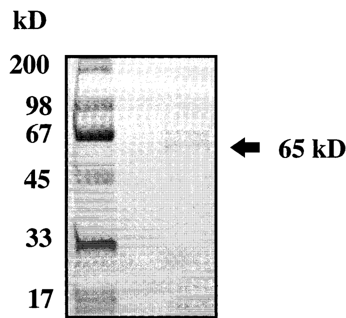


Fig. 4. Partially-purified prelamin A endoprotease on SDS-PAGE. The active fractions (12–19) eluted from Mono S under the gradient were pooled and concentrated to 60 μl. This partially-purified material was subjected to electrophoresis on a 10% SDS-PAGE mini gel, the protein bands were detected by Coomassie blue R-250 staining. The gel was calibrated with the indicated protein molecular weight standards. These preparations gave two bands on SDS-PAGE, the major band having a molecular weight of 65 kDa.

chloride. This peak (↓) from the Mono S column was collected, concentrated and re-analyzed on a S200 gel filtration column. A peak of activity from this fraction was eluted around 65 kDa (data not shown). SDS-PAGE of the fractions that emerged from the final 0.3 M sodium chloride Mono S column revealed two peptides with molecular masses of 68 and 65 kDa (Fig. 4).

In multiple preparations of endoprotease purified by the above procedure, we always observed both 68 and 65 kDa bands. Table 1 shows the results of one complete purification procedure for prelamin A endoprotease using the steps outlined above. For consistency, we assayed all of the fractions throughout the purification. The assay of crude nuclei appeared to be inaccurate, since there was a large increase in the total activity upon nuclear envelope preparation. The results indicated that endoprotease had been purified approximately 1200-fold relative to its activity in crude nuclei, with an overall yield of 16.11%. With this procedure, after SDS-PAGE, we were able to isolate approximately 5 μg of purified prelamin A endoprotease from 60 mg of HeLa crude nuclei protein. It should be noted that the protein concentration of the final purified material was estimated from the intensity of the bands observed on Coomassie-stained SDS-PAGE (Fig. 4) and is therefore, along with the final specific activity, only approximate.

Consistent with data that we have previously reported on crude nuclei [14], the purified prelamin A endoprotease activity was inhibited greater than 95% by 1 mM concentrations of serine protease inhibitors, aprotinin, PMSF and isocoumarin (data not shown).

## 4. Discussion

Following farnesylation and the other two-canonical CAAX box post-translational modifications, prelamin A has to undergo a further proteolytic cleavage to give rise to lamin A. The functional significance of prelamin A endoproteolysis in the formation and assembly of lamin A into the nuclear lamina has been described previously by our laboratory and others [10,16,17,20]. The further characterization of the functional significance of prelamin A endoproteolytic processing requires the effective solubilization and purification of the enzyme which catalyzes this reaction, the prelamin A endoprotease. Our studies on subnuclear localization of the activity indicate that it is associated with the nuclear envelope. The enzyme is tightly associated with the nuclear envelope but can be released, in the active form, by treatment of nuclear envelope preparations with detergent. Previously, we showed that prelamin A endoprotease activity is correlated with the level of expression of prelamin A [16]. Now, in this study, its activity is localized to the HeLa nuclear envelope. Since there is no other farnesylated protein with the RSYLLG motif in nuclei, it appears likely that prelamin A is the sole substrate for the prelamin A endoprotease.

Solubilization of the enzyme could be demonstrated with several detergents, although, for reasons stated above, octyl glucoside was used in our studies. We have achieved some success in partially purifying the prelamin A endoprotease obtaining a preparation with an approximately 1200-fold greater specific activity than that of crude nuclei. The purified material gave two bands on SDS-PAGE, the major band having a molecular weight of 65 kDa, consistent with the size of the enzyme as assessed by gel filtration. We, therefore, propose that this polypeptide is the prelamin A endoprotease.

Based on inhibition of activity with aprotinin, PMSF and isocoumarin, we conclude that the enzyme is a serine protease. This is in contrast to the farnesylation-dependent endoprotease which catalyzes the maturation of the yeast a-factor, a reaction similar to prelamin A endoproteolysis. The a-factor endoprotease (Ste24p) appears to be a metalloprotease [21].

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Table 1
Purification data for prelamin A endoprotease (from $\sim 10^{10}$ HeLa cells)

| Step | Fraction | Protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min/mg) | Purification (fold) | Recovery (%) |
|---|---|---|---|---|---|---|
| 1 | Crude nuclei | 60.15 | 2.42 | 0.0402 | | |
| 2 | Nucleoplasm | 10.08 | 0.27 | 0.0264 | | |
| 3 | Nuclear envelope | 42.00 | 20.2 | 0.480 | 1 | 100 |
| 4 | Nuclear envelope extract | 10.44 | 19.1 | 1.825 | 4 | 94.5 |
| 5 | Sephacryl/S200HR | 0.0256 | 7.0 | 27.4 | 57 | 34.8 |
| 6 | Mono S | 0.0056 | 3.2 | 580 | 1208 | 16.1 |

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