Regulation of prelamin A endoprotease activity by prelamin A

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Abstract The maturation of lamin A is completed by the endoproteolytic cleavage of its farnesylated precursor protein, prelamin A. In the absence of this cleavage, prelamin A can neither give rise to lamin A nor assemble into the nuclear lamina. We call the enzyme which catalyzes this endoproteolytic step the 'prelamin A endoprotease'. In this study, we begin characterization of the regulation of prelamin A endoprotease. In particular, we address the question as to whether prelamin A endoprotease activity is constitutive in cells or responds to expression of prelamin A. To do this, we compared the activity of this novel endoprotease in cells which express prelamin A with those that do not. Our data shows that the enzymatic activity of prelamin A endoprotease is enhanced by the expression of prelamin A.

Key words: Prelamin A endoprotease; Prelamin A; HeLa cell; Promyelocytic leukemia cell (HL60); Teratocarcinoma cell (F9)

1. Introduction

The nuclear lamina is composed of three major, intermediate filament proteins lamin A, B and C [1,2]. Lamin A is synthesized as a precursor protein, prelamin A [3]. Prelamin A and lamin B, but not lamin C, possess a 'CAAX' consensus sequence at their carboxyl-terminal [4,5]. There are three sequential post-translational modifications of the carboxyl terminal 'CAAX' motif [6]. These three steps are farnesyl modification of the cysteine residue, endoproteolytic removal of the AAX group, and methylation of the carboxyl group of the now carboxyl terminal farnesylated cysteine residue [7]. Following these post-translational modifications, prelamin A undergoes a further endoproteolytic cleavage to complete the formation of mature lamin A [8–11] which is now competent for assembly into the nuclear lamina [1,12]. This cleavage occurs between $\mathbf{Y}^{657}$ and $\mathbf{L}^{658}$ of prelamin A.

In a prior report [8], we have pointed out that there is a consensus sequence in the prelamin A molecule, $\mathrm{RSY}^{657}\mathbf{L}^{658}\mathrm{LG}$, conserved across several species, around this cleavage site which does not occur in any other known protein. This simple hexapeptide was demonstrated to be a substrate for the prelamin A endoprotease, confirming its functional significance in conferring specificity. These observations are consistent with the hypothesis that prelamin A endoprotease is a novel enzyme, which may not be active with other naturally occurring substrates. If so, it might be expected that the activity of the prelamin A endoprotease may be correlated with expression of prelamin A.

In this paper, we initiate the analysis of the regulation of prelamin A endoprotease and test this hypothesis. It has been observed that prelamin A is not expressed in embryonic cells [13] or in a number of rapidly proliferating tissues such as lymphoid tissue, and epithelial cells of lung, kidney, liver and intestine [14]. Here, we compare the prelamin A endoprotease activity of hemopoietic, HL60 promyelocytic leukemia cells and F9 teratocarcinoma cells which lack expression of A/C lamins [15,16], with HeLa cells that expresses high levels of A/C lamins. We then go on to examine the effect of expression of prelamin A in F9 cells on the prelamin A endoprotease activity of such cells. Our results indicate that the activity of this enzyme is enhanced by the expression of prelamin A.

2. Materials and methods

2.1. Cells and antibodies

The cells used in these studies were HeLa (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), F9 mouse teratocarcinoma cells (grown in F12 supplemented with 15% FCS) and HL60 promyelocytic leukemia cells (RPMI supplemented with 20% FCS). F9LA and F9Δ21 are F9 derived cell lines which express human prelamin A or mature lamin A under the control of the control of the mouse mammary tumor virus promoter [17,18]. F9LA and F9Δ21 cells were treated with 1×10^{-7} M dexamethasone to induce the expression of human prelamin A or lamin A [17,18].

A rabbit polyclonal anti-human prelamin A specific antiserum (α-PA) which was generated against a synthetic peptide, CLLGNSSPRTQSPQN [19]; α-A/C, a non-species specific polyclonal antibody specific for A/C lamin (kind gift of Dr. Nilabh Chaudhary, Triplex Pharmaceutical); and 1E4(20) a species specific anti-human lamin A antibody (kind gift of Dr. F. McKeon, Harvard Medical School) were used in this study.

2.2. Prelamin A endoprotease assay

The prelamin A endoprotease assay was performed essentially as previously described [8]. Briefly, cell pellets were resuspended to a final density of 4×10^8 cells/ml in ice-cold lysis buffer (0.01 M Tris-HCl, pH 7.0, 0.01 M NaCl, 3 mM $MgCl_2$, 0.4% NP-40). Nuclei were isolated after two more washes, in the same buffer, and pelleting by centrifugation in a Sorvall HB-4 rotor for 10 min at $365\times g$. The nuclei were resuspended in the same buffer without NP-40. Protein concentration was obtained by means of the Micro BCA Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL).

The endoprotease reaction was initiated by the addition of ^{125}I-RSY* LLG to the nuclear extract in a final volume of 20 μl in 10 mM MES, pH 6.0. The reaction was run for 20 min at 37°C and 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, Inc. Newark, DE). TLC plates were developed in 10% acetonitrile in water and the spots visualized by autoradiography. The reaction product was identified by its co-migration with a synthetic, iodinated RSY* peptide standard. The amount of labeled RSY formed in the assay was determined by scraping the appropriate spots into tubes and quantitation of radioactivity with a gamma counter.

2.3. Western blotting

F9 and F9LA cell lysates were resolved on 4–20% linear gradient SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) mem-

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branes and probed with 1:200 diluted α-A/C antibody. After washes in TBS containing 0.1% Tween 20, the membrane were incubated with horseradish peroxidase-conjugated anti-rabbit (Amersham Corp.) IgG (1:5000 dilution) for 1 h. Detection of the immunosignal was done using ECL Western blotting detection system (Amersham Corp.).

2.4. Immunofluorescence

Cells were plated on coverslips and incubated overnight. They were then fixed in 3% formaldehyde in PBS for 15 min and permeabilized with 0.05% Triton X-100 in PBS for 10 min at room temperature. The coverslips were then washed three times in 5 mM NH_4Cl in PBS. Prelamin A or lamin A were visualized by incubating for 1 h with either α-PA or 1E4 at 1:200 dilution followed by Texas red-conjugated goat anti-rabbit (Biomeda; 1:500) IgG (H+L) or sheep anti-mouse (Cappell, diluted 1:25) respectively.

3. Results

3.1. Enzymatic activity of prelamin A endoprotease in HeLa, F9 and HL60 cells

As we previously described [8], the hexapeptide, RSYLLG behaves as an appropriate substrate for the prelamin A endoprotease. Therefore in this study, the endoproteolytic activity of prelamin A endoprotease was assayed in vitro by reacting

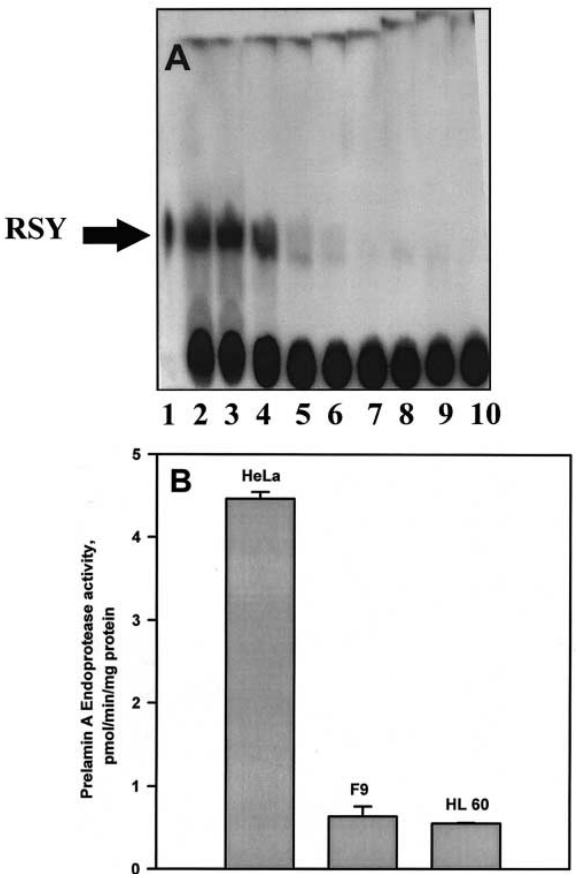

Fig. 1. Assay of prelamin A endoprotease activity. A: Autoradiographic visualization of reverse-phase TLC separation of reaction products. Lane 1: synthetic radio-iodinated RSY*; lanes 2–4: HeLa nuclei; lanes 5–7: F9 nuclei; and lanes 8–10: HL60 nuclei are assayed for endoprotease activity by formation of radio-iodinated RSY as described under Section 2. The radio-labeled material at the solvent front co-migrates with iodotyrosine. The nuclei used in these assays corresponded to 3 μg of protein. B: The quantitation of the reaction product RSY*. The activity of prelamin A endoprotease is calculated following the detection of the reaction product on the film. The results are the average of 3 determinations.

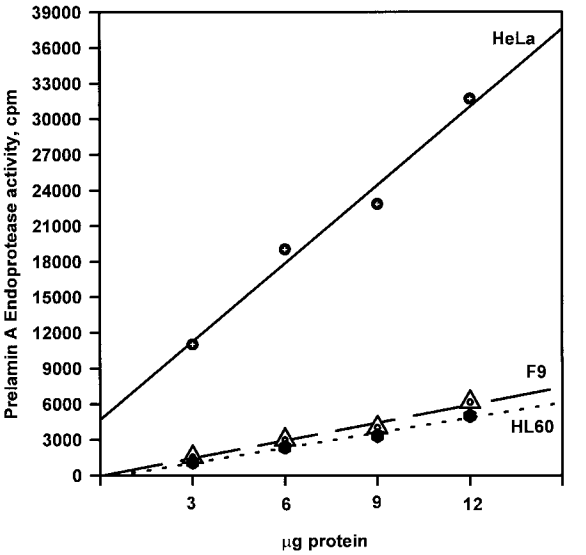

Fig. 2. The prelamin A endoprotease assay in HeLa, F9 and HL60 cells is linear with protein. The endoprotease assay is performed with 3, 6, 9 and 12 μg nuclear protein from HeLa (circle with plus), F9 (triangle with circle) and HL60 (filled hexagon) cells. Each point is the average of two determinations.

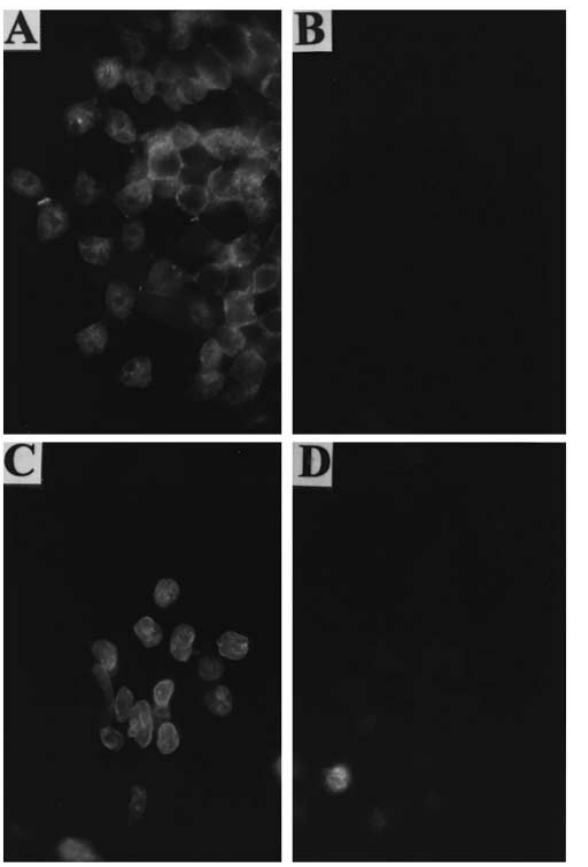

Fig. 3. Dexamethasone-inducible expression of human prelamin and lamin A in F9LA cells. Immunofluorescent visualization of prelamin A in mevinolin-treated F9LA cells in the presence (A) or absence (B) of 10^{-7} M dexamethasone treatment overnight. When F9LA cells were fixed and stained with a human specific anti-lamin A antibody (1E4) dexamethasone-inducible expression of human lamin A could be detected (C).There was no staining in the absence of dexamethasone (D).

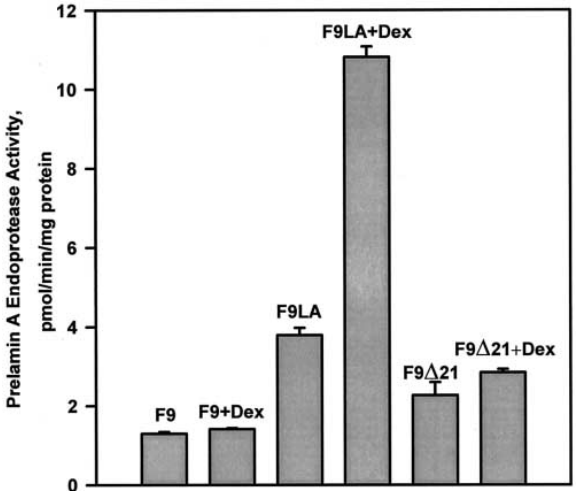

Fig. 4. Expression of the lamin A precursor up regulates the activity of prelamin A endoprotease. The prelamin A endoprotease is assayed in F9 cells which are permanently transfected with dexamethasone inducible constructs expressing lamin A (F9Δ21) and its precursor protein (F9LA). Control experiments demonstrate that dexamethasone treatment has no effect on prelamin A endoprotease activity in the parental F9 cells. The results shown are the average of 3 determinations.

the radioiodinated synthetic peptide ^{125}I-RSY* LLG with the crude nuclei from three cell lines: HeLa which express prelamin A, and HL60 and F9 cells which do not express prelamin A. The reaction mixture was resolved by reverse-phase TLC and visualized by autoradiography (Fig. 1A). The cleavage product of prelamin A endoprotease was followed by its co-migration with radio-iodinated RSY* synthetic peptide, as described before [8]. After quantitation of the amount of RSY* formed by means of γ-counting, the enzymatic activity of prelamin A endoprotease in HL60 and F9 cells was found to be 9 times less than the activity in HeLa cells (Fig. 1B). We also confirmed the linearity of the prelamin A endoprotease assay with protein in these cells (Fig. 2). These observations are in agreement with the hypothesis that prelamin A endoprotease activity may be regulated by prelamin A expression.

3.2. *Expression of lamin A precursor enhances the activity of prelamin A endoprotease*

At this point, it was not obvious whether the variation in the prelamin A endoprotease activity occurred as a result of cell type or the expression of prelamin A. To distinguish between these possibilities, we utilized F9 cells permanently transfected with human prelamin A (F9LA) or lamin A (F9Δ21) constructs [17,18], which permits dexamethasone induction of prelamin or lamin A.

The dexamethasone induction of lamin A or prelamin A in F9LA cells is shown in Fig. 3. Following its synthesis, prelamin A continuously gives rise to mature lamin A. As we have demonstrated previously [11,18], prelamin A accumulates in the nuclei of cells treated with lovastatin, an inhibitor of mevalonate biosynthesis and, hence, the farnesylation dependent processing of prelamin A. Nuclear staining with α-PA is observed in lovastatin-treated F9LA cells only in the presence of dexamethasone (Fig. 3A,B). To demonstrate that this material was of transgenic origin, F9LA cells were also stained with human specific anti-lamin A (1E4). Staining was observed only in cells treated with dexamethasone (Fig. 3C,D).

Parental F9, F9LA and F9Δ21 cells were assayed for prelamin A endoprotease activity in the presence or absence of dexamethasone supplementation. Only in the F9LA cells, which express prelamin A, was a significant response of prel-

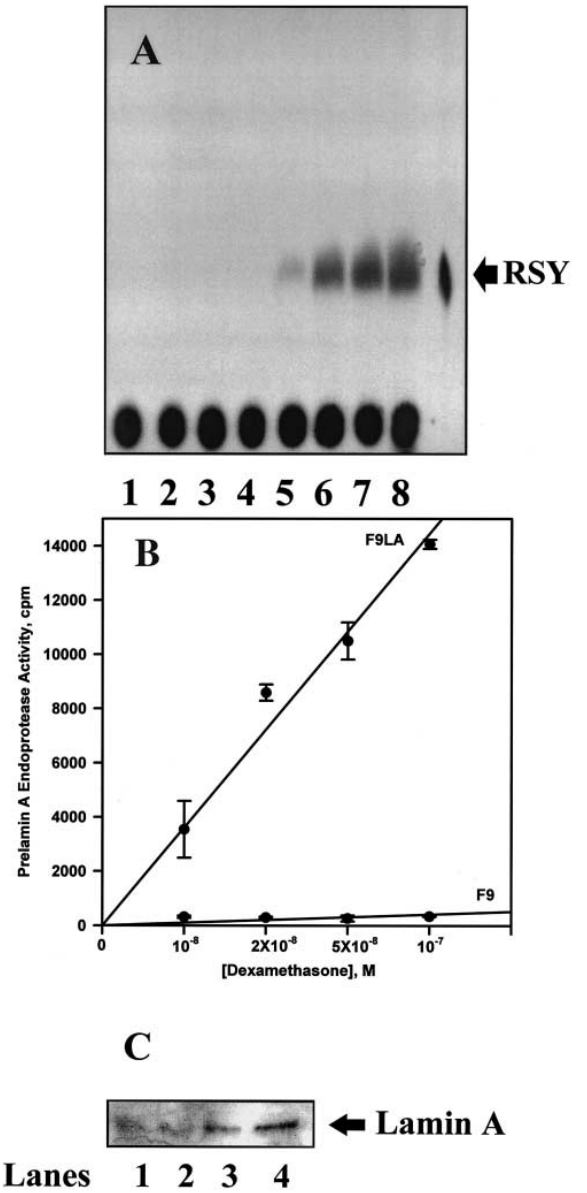

Fig. 5. Dose–response of prelamin A endoprotease and prelamin A to dexamethasone in F9 and F9LA cells. A: Autoradiographic visualization of reverse-phase TLC isolation of RSY peptide formed in response to various concentrations of dexamethasone. Cells were treated with 10^{-8}, 2×10^{-8}, 5×10^{-8} or 10^{-7} M dexamethasone overnight and the nuclear extracts were prepared for in vitro assay. Lanes 1–4: F9 nuclei; lanes 5–8: F9LA nuclei are assayed for endoprotease activity by formation of radio-iodinated RSY as described under Section 2. B: The quantitation of the reaction product RSY*. The activity of prelamin A endoprotease is calculated following the detection of the reaction product on the film. C: Concentration dependent dexamethasone-inducible lamin A expression in F9LA cells. F9LA cells were treated with 10^{-8}, 2×10^{-8}, 5×10^{-8} or 10^{-7} M dexamethasone (lanes 1–4) overnight. The cell lysates were run on SDS-PAGE and lamin A is visualized by immunoblotting with lamin A/C antibody (α-A/C).

amin A endoprotease activity observed to dexamethasone (Fig. 4). To more clearly correlate prelamin A endoprotease activity with the extent of expression of prelamin A, F9 and F9LA cells were treated with various concentrations of dexamethasone. The results indicate that prelamin A endoprotease activity is dose-responsive to dexamethasone treatment (Fig. 5A,B) in the same concentration range (10^{-8}–10^{-7} M) as is prelamin A expression (Fig. 5C).

4. Discussion

Following farnesylation and the other canonical CAAX box post-translational modifications, the lamin A precursor must 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 [3,8,19,20]. However, before this study, no data was available regarding possible regulation of the endoprotease.

To understand the effect of the expression of lamin A precursor on the enzymatic activity of prelamin A endoprotease, we assayed this enzyme in cell lines which do and do not express the lamin A precursor. It has previously been noted that expression of prelamin A in embryonal carcinoma cells which do not express the lamin A gene still results in the assembly and processing of the precursor [17,21,22]. We found large differences in prelamin A endoprotease activity between cells that normally express or do not express lamin A. Our experiments suggest that the ability of cells to process transgenic prelamin A depends, in part, on up-regulation of the prelamin A endoprotease, which, in turn, appears to be regulated by the level of prelamin A expression.

These results may also be relevant to the question of substrate specificity of the prelamin A endoprotease. Based on the primary structural constraints for recognition of substrate by the prelamin A endoprotease that we have previously described and the apparent uniqueness of those features (farnesylation and the RSYLLG motif) to prelamin A, it would appear plausible that this enzymes functions solely to process prelamin A. The up-regulation of this enzyme by prelamin A that we report here is also consistent with this specificity for prelamin A.

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