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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, cysteinyl 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 Tyr^{657} and Leu^{658}. Endoproteolytic cleavage requires the *S*-prenylated cysteine methyl ester and, in agreement with transfection studies, is more active with the farnesylated than geranylgeranylated cysteinyl 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 CAA*X* consensus sequence at their carboxyl terminus undergo serial post-translational modifications of the cysteinyl residue (1, 2). These modifications include derivitization of the cysteine sulfhydryl with an isoprenoid moiety followed by the endoproteolytic removal of the -AA*X* 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 in thioether linkage to the cysteine (3), whereas when *X* is a leucine, a 20-carbon geranylgeranyl 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 CAA*X* 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 CAA*X* proteins (11). Experiments with mutants, in which the cysteine of the CAA*X* box is replaced by another amino acid, demonstrate that farnesylation is required for the maturation of prelamin A (12). These nonprenylated CAA*X* 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 CAA*X* 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 (Tyr^{657}) and a leucine (Leu^{658}) 18 amino acid residues upstream from the carboxyl terminus of the prelamin A molecule (16). Consistent with this expectation, mutation of Leu^{658} 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 Tyr^{657} and Leu^{658}. 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 "-AA*X*" 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 Radioimmunoprecipitation—HeLa cells were cultured in Ham's F-12 medium supplemented with 10% fetal

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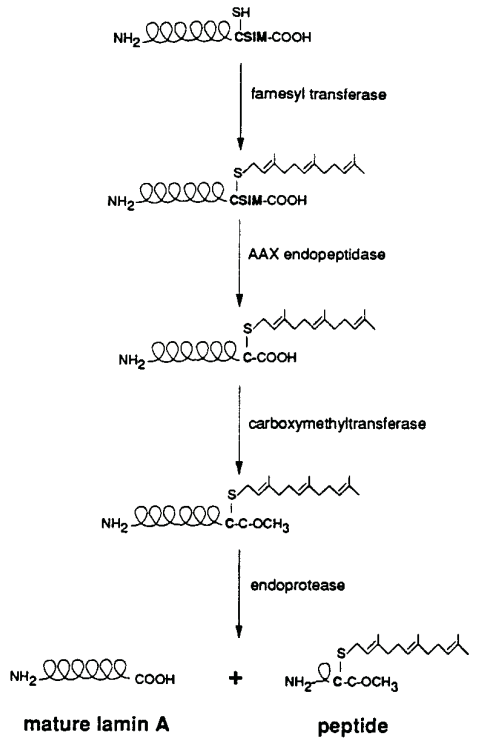


FIG. 1. **The processing pathway of prelamin A.** The last reaction in the sequence is catalyzed by the putative prelamin A endoprotease.

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 carboxylate 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 [^{3}H]mevalonate and [^{35}S]methionine, radioimmunoprecipitation of prelamin A, and mature lamin A and treatment of cells with the farnesyl protein transferase inhibitor, BZA-5B, have all been described elsewhere (15). As in the previous study, prelamin A was immunoprecipitated with the human species specific prelamin A antibody α-PA (14), whereas total lamin A was immunoprecipitated with a panspecies-specific lamin A antibody (a kind gift of Dr. Nilabh Chaudhary, Triplex Pharmaceuticals).

Peptide Synthesis—The polypeptide H_2N-RSYLLGNSSPRTQSPQNC-OCH3 (prelamin A peptide) was synthesized by stepwise solid phase, Fmoc[1]/*tert*-butyl-based chemistry. An ABI-431-A synthesizer (Perkin-Elmer) programmed with the manufacturer's standard single coupling protocol was used to assemble the sequence. The synthesis was initiated with Fmoc-Cyss(Trt)-Wang derivatized polystyrene resin, and all subsequent couplings were carried out with preformed HOBt esters. Following removal of the N-terminal Fmoc group, the peptide resin was cleaved and deprotected by treatment with a mixture of trifluoroacetic acid:thioanisole:β-mercaptoethanol:water:phenol (80:5:5:5) for 4 h. The suspension was filtered and the resulting filtrate was concentrated under reduced pressure. The crude ether precipitate was applied to a preparative Vydac C-4 column and eluted with a linear 0.1% trifluoroacetic acid, acetonitrile gradient system, and the fractions with the best analytical profile were pooled and lyophilized. Conversion to the methyl ester was accomplished in 50% yield by stirring the free peptide in a 5% HCl, methanol solution for 4 h at room temperature. The product was then isolated by utilizing the same HPLC conditions as for the free peptide. Results of amino acid and electrospray mass spectrometry analyses corresponded closely with expected values.

The *S*-all-*trans*-farnesyl peptide derivative was synthesized (22) by a base catalyzed reaction with farnesyl bromide (Aldrich). The prelamin A peptide (4.33 μmol) was dissolved in 15 ml of dimethylformamide, H_2O, 0.5 M $KHCO_3$ (5:1:1), and then 6.5 μmol of farnesyl bromide were added in 1.85 ml of dimethylformamide. The reaction was in the dark at room temperature for 20 min and was terminated by the addition of 0.67 ml of 1 M HCl. After the solvent was removed, the residue was redissolved in acetonitrile:water; 1:1, and the product was purified by reverse-phase HPLC on a C-18, 10 × 150 mm, Econosphere column (Alltech/Applied Science, Deerfield, IL). The mobile phase was a linear gradient of 0–30% solvent B in solvent A (solvent A: 0.1% trifluoroacetic acid in H_2O; solvent B: 0.1% trifluoroacetic acid, 99% CH_3CN, 0.9% H_2O). With a flow rate of 1 ml/min, the farnesylated prelamin A peptide eluted at 30 min. Synthesis of the expected product was confirmed by electrospray mass spectrometry (molecular mass = 2227 Da). Demethylation was by base hydrolysis in methanol:water as described previously (23), and purification of the demethylated peptide was by reverse-phase HPLC on the same system used for the methylated peptide (retention time = 27 min).

The *S*-all-*trans*-geranylgeranylated peptide was synthesized (22) by reacting 180 nmol of all-*trans*-geranylgeranyl bromide (American Radiochemical, St. Louis, MO) dissolved in 50 μl of dimethylformamide with 190 nmol of prelamin A peptide dissolved in 350 μl of NaI-saturated triethylamine:dimethylformamide; 1:150. The reaction was for 15 min at room temperature in the dark and stopped by the addition of 16 μl of 1 M HCl. The geranylgeranylated prelamin A peptide was purified on reverse-phase HPLC as for the farnesylated peptide; retention time = 35 min. Electrospray mass spectrometry of the product gave the expected molecular mass of 2296 Da.

N-Acetyl farnesyl methyl cysteine was prepared by acid catalyzed methylation of commercial *N*-acetyl farnesyl cysteine (Calbiochem) 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 Na^{125}I (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. Typical 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 to a final density of 4 × 10^8 cells/ml in a lysis buffer (0.01 M Tris-HCl, pH 7.0, 0.01 M NaCl, 3 mM $MgCl_2$, 0.4% Nonidet P-40) reported to leave nuclei intact (26). 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 × *g*. The nuclei were 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 ^{125}I-labeled peptide (V_{max} at 5 μM 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 spots into tubes and quantitation of radioactivity with a γ counter.

Constructs—Wild-type and nonfarnesylatable mutant (MSIM) prelamin A cDNAs cloned into the *Eco*RI(5′)-*Bam*HI(3′) of the SV-40 based expression vector, pECE, were kind gifts of Dr. F. McKeon (Harvard Medical School) and have been previously described (10, 27). The prelamin A mutant terminating in the CAA*X* sequence CVLL was a kind gift of Dr. Paul Kirschmeier (Schering-Plough Research Institute). This mutant was prepared by means of the pAltered sites mutagnesis kit (Promega, Madison. WI). Wild-type prelamin A cDNA was cloned into pAlter between the *Eco*RI and *Xba*I sites. The mutagnesis 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) dideoxy sequencing method. The mutant cDNA was subcloned into the cytomeg-

[1] The abbreviations used are: Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high pressure liquid chromatography; MES, 4-morpholine-ethanesulfonic acid.

TABLE I
Carboxyl-terminal prelamin A sequences of various species

Sequences homologous to the putative endoprotease site (↓) between Tyr[657] and Leu[658] of human prelamin A were obtained from the Swiss-Prot data base. In this data base, the sequence **RSYLLG** is found only in the proteins shown below.

Species	Sequence
Human	LVT**RSY** ↓ **LLG**NSSPRTQSPQNCSIMCOOH
Rat	LVT**RSY** **LLG**NSSPRTQ…
Mouse	LVT**RSY** **LLG**NSSPRSQ…
Chick	LLG**RSY** **VLG**GAGPRRQ…

alovirus promotor-based expression vector pcDNA3 (InVitrogen, San Diego, CA) between the *Eco*RI and *Xba*I 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 Tyr[657] and Leu[658]. 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 **Y*** is a radioiodinated tyrosine. If this were an appropriate substrate for the prelamin A endoprotease, the tripeptide RS**Y*** 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 mildly 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 RS**Y*** 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 radioiodinated RSY on thin layer was also confirmed by HPLC. The putative RS**Y*** was eluted from the TLC plate and mixed with unlabeled RSY. These samples were subjected to reverse phase HPLC and co-migration of the radiolabeled proteolysis product and the mass standard was demonstrated (Fig. 3). These observations indicated that an assay for the prelamin A endoprotease utilizing **I** as a substrate was feasible. Release of RS**Y*** was time dependent (Fig. 4). We examined the temperature (optimum = 37 °C) and pH dependence (optimum = 6) of the reaction as well, leading to the standard assay conditions described under "Experimental Procedures."

STRUCTURE 1

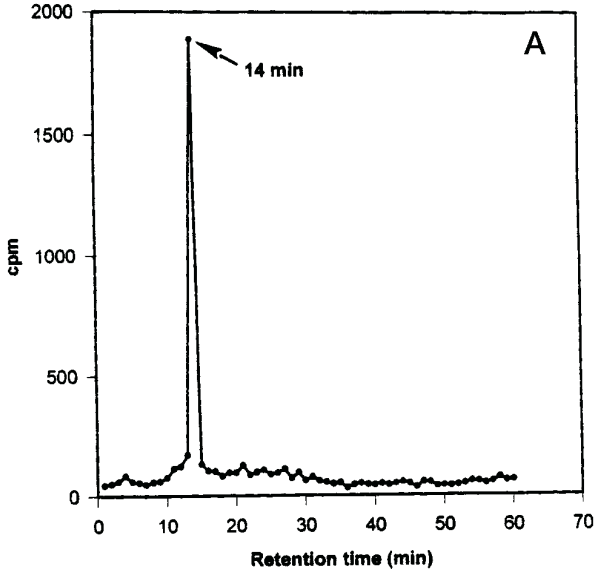
FIG. 2. **Enzyme catalyzed formation of RSY from synthetic substrate (Structure 1).** *Lane 1*, Synthetic radioiodinated RSY; *lane 2*, boiled (5 min) nuclei are assayed for activity as described under "Experimental Procedures"; *lane 3*, nuclei are assayed for endoprotease activity by formation of radioiodinated RSY (*arrow*), as described under "Experimental Procedures." The radiolabeled material at the solvent front co-migrates with iodotyrosine. The nuclei used in these assays corresponded to 175 μg of protein.

Kinetic Behavior of the Prelamin A Endoprotease—We now sought to determine whether the proteolytic release of RSY from **I** was well behaved kinetically and correlated with *in vivo* observations on prelamin A endoproteolysis. Examination of the substrate dependence of the reaction (Fig. 5*A*) indicated that the reaction exhibited classical Michaelis-Menten kinetics with a K_m of 0.67 μM and a V_{max} of 6.5 pmol/min/mg of protein. Under V_{max} conditions, with crude nuclear extract as a source of enzyme, the assay was linear with enzyme protein from 20 to 175 μg/assay (Fig. 5*B*). These results indicate that the enzyme assay for the putative prelamin A endoprotease is well behaved and is suitable for further study of the activity.

To examine the specificity of the protease for the isoprenoid moiety, we synthesized the geranylgeranylated analogue of **I** from the prelamin A apopeptide and geranylgeranyl bromide, as described under "Experimental Procedures." Preliminary experiments demonstrated that the radioiodinated, geranylgeranylated prelamin A peptide gave rise to RSY in endoprotease assays. That these two substrates were being proteolyzed by the same enzyme was demonstrated by determining that the geranylgeranylated peptide is a competitive inhibitor for cleavage of the farnesylated peptide substrate (Fig. 6).

We then examined the activity of radioiodinated geranylgeranyl prelamin A peptide as a substrate in detail. A comparison of the kinetic parameters of the farnesylated and geranylgeranylated peptides is shown in Table II. Since these assays are performed with identical amounts of the same crude enzyme preparation, the ratio V_{max}/K_m gives a measure of the relative efficiency with which the endoprotease can utilize the two substrates. It thus appears that the farnesylated substrate is approximately 19-fold more reactive with the prelamin A endoprotease than the geranylgeranylated substrate.

Lower reactivity with the geranylgeranylated substrate was also demonstrated in whole cells by transfection studies. Chinese hamster (CHO-K1) cells were transiently transfected with CAA*X* box mutant (MSIM or CVLL) or wild-type (CSIM) human prelamin A constructs. The MSIM sequence cannot be



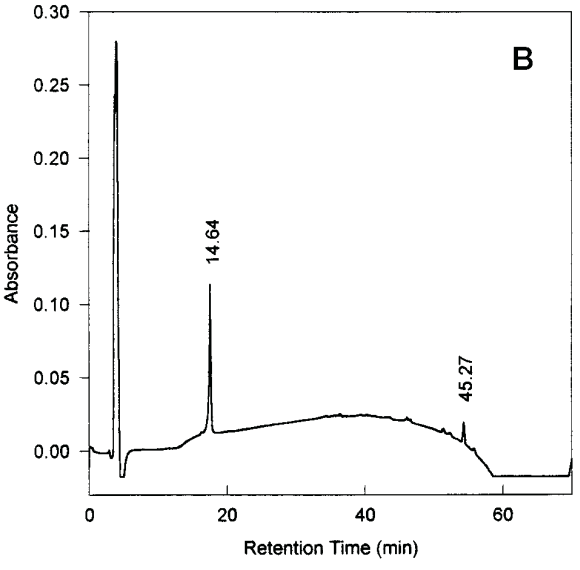


FIG. 3. **Co-migration of iodinated cleavage product of prelamin A peptide endoproteolysis with synthetic RSY.** Material comigrating with RSY on TLC was eluted with CH_3CN (99%), H_2O (0.9%), trifluoroacetic acid (0.1%). To this was added bona fide synthetic RSY mass standard (0.3 μg). The sample was 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*).

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 [^{3}H]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 [^{3}H]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 pre-

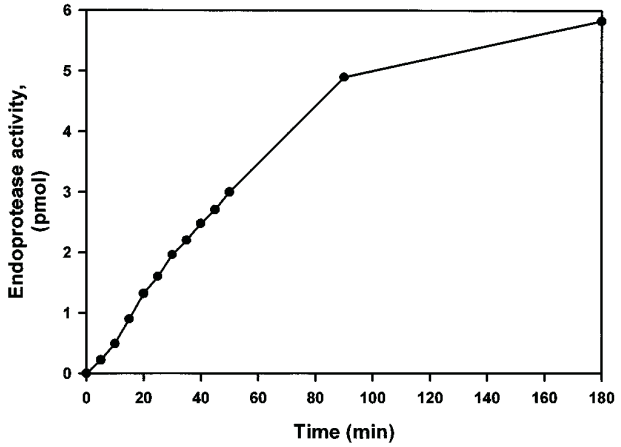


FIG. 4. **Time dependence of prelamin A peptide endoproteolysis.** Radioiodinated RSY formed in the prelamin A endoprotease assay described under "Experimental Procedures" was monitored by reverse phase TLC and γ-counting as a function of incubation time. The results shown are the average of two determinations. There were 175 μg of protein used per assay. **I** was used as substrate at a concentration of 5 μM.

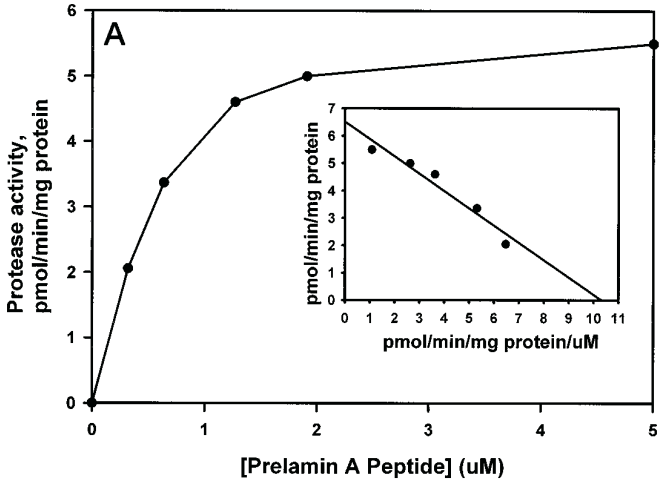
lamin A cannot be processed to the mature protein. In contrast to these proteins, the geranylgeranylated mutant could be converted to mature lamin A, but less efficiently than the wild-type protein, as indicated by the large amount of prelamin A which accumulates in these cells (Fig. 8). The accumulation of prelamin A in cells transfected with the CVLL mutant was also confirmed by immunoprecipitation and indirect immunofluoresence 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 acetyl farnesyl cysteine methyl ester (*N*-acetyl farnesyl methyl cysteine) (Fig. 9*A*) and RSYLLG (Fig. 9*B*) on the *in vitro* formation of RSY from **I**. The results indicate that both compounds can inhibit formation of RSY.

N-Acetyl farnesyl methyl cysteine inhibits the prelamin A endoprotease, noncompetitively, with an apparent K_i of approximately 17 μM. We interpret this result to be consistent with a farnesyl cysteine recognition domain in the prelamin A endoprotease which is distinct from the endoproteolysis site. On the other hand, RSYLLG exhibits competitive inhibition of the prelamin A endoprotease as would be expected if this sequence binds to the active site of the enzyme. The observed K_i for RSYLLG is 0.9 μM, which is quite similar to the K_m of the farnesylated prelamin A peptide.

Since RSYLLG can act as an efficient competitive inhibitor,



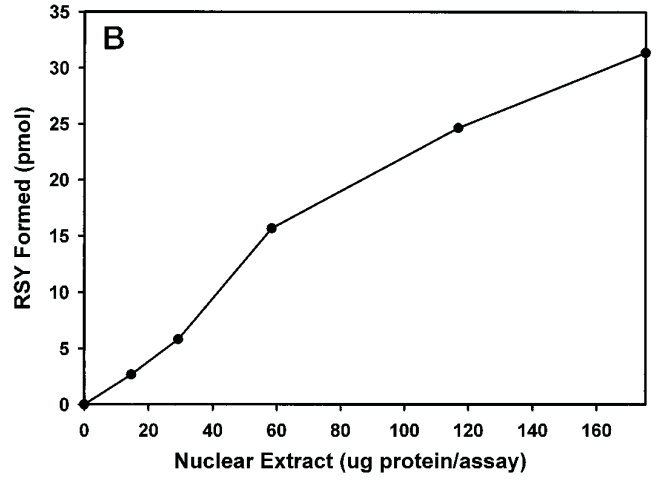


FIG. 5. **Substrate and protein dependence of the prelamin A peptide endoprotease activity.** Assays were done at various substrate and protein concentrations as shown, otherwise following the protocol described under "Experimental Procedures." Each point is the average of two determinations. *A*, substrate dependence. The *inset* shows an Eadie-Hofstee plot of the data from which the kinetic constants were derived. There were 175 μg of protein used per assay. *B*, protein dependence.

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 μM; V_{max} = 14.2 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 → RSY**R**LG) 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.

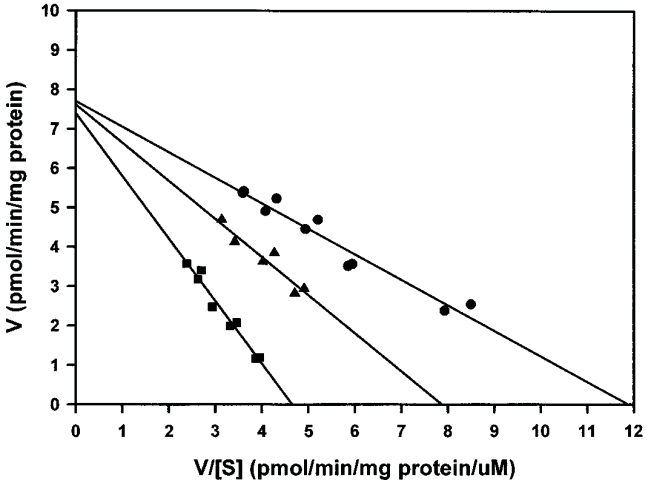


FIG. 6. **Geranylgeranylated prelamin A peptide is a competitive inhibitor of the endoproteolysis of farnesylated prelamin A.** The endoproteolysis of **I** (0.3, 0.6, 0.9, 1.2, and 1.5 μM) was assayed in the absence (●) or presence of 2 μM (▲) or 4 μM (■) geranylgeranylated peptide and the data analyzed as an Eadie-Hofstee plot. The intersection of the lines on the *y* axis is diagnostic of competitive inhibition.

TABLE II
Comparison of the kinetic parameters of the farnesylated and geranylgeranylated prelamin A peptide substrates

K_m and V_{max} for each substrate was determined by Eadie-Hofstee analysis.

Substrate[a]	K_m	V_{max}	V_{max}/K_m
	μM	*pmol/min/mg protein*	min^{-1}
Farnesylated peptide	0.67	6.5	9.7
Geranylgeranylated peptide	2.0	1.0	0.5

[a] These substrates are iodinated. Therefore, it is possible that the kinetic parameters of the natural substrates could be different. However, it is the relative values of these parameters for the two substrates that are of interest.

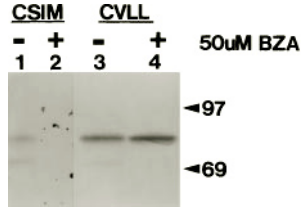


FIG. 7. **The CVLL-prelamin A mutant is geranylgeranylated *in vivo*.** Met-18b-2 cells (1×10^6) were transiently transfected with wild-type human prelamin A (*lanes 1* and *2*) or the CVLL- prelamin A mutant (*lanes 3* and *4*) and then treated with or without BZA-5B (50 μM) for 2 h prior to labeling with 100 μCi/ml [^{3}H]mevalonate. The cells were then incubated for an additional 16 h. The transgenic prelamin A was immunoprecipitated with human species specific anti-prelamin A and analyzed by SDS-polyacrylamide gel electrophoresis and flourography. Molecular mass markers (in kDa) are on the *right*. The *upper band* in these immunoprecipitations migrates at 74 kDa, corresponding to prelamin A.

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

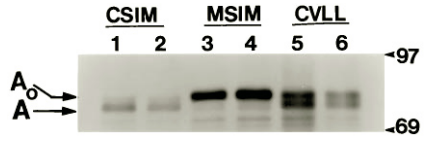
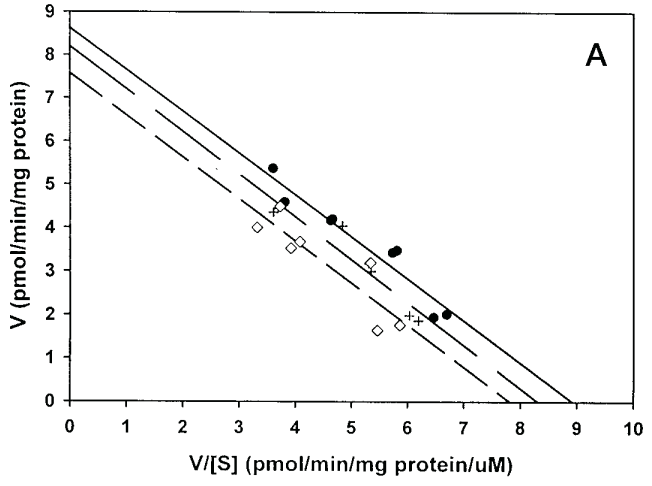


FIG. 8. **The CVLL prelamin A mutant is processed to mature lamin A but less efficiently than wild-type prelamin A.** CHO-K1 cells (1×10^6) were transfected with human prelamin A (*CSIM*) (*lanes 1* and *2*), a nonprenylatable prelamin A mutant (*MSIM*) (*lanes 3* and *4*) and the CVLL prelamin A mutant (*lanes 5* and *6*) and then labeled with 35 μCi/ml [^{35}S]methionine for 16 h. The transgenic prelamin A (A_o) 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 flourography. Molecular mass markers (in kDa) are indicated on the *right*.



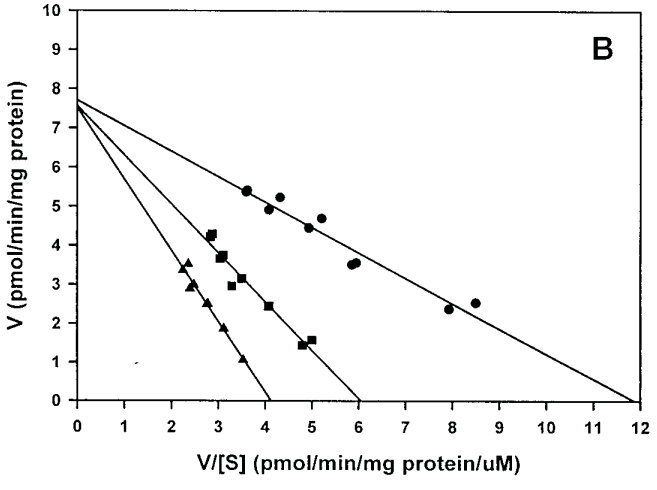


FIG. 9. **Eadie-Hofstee analysis of inhibition of the prelamin A peptide endoprotease by *N*-acetyl farnesyl methyl cysteine (*A*) or RSYLLG (*B*).** *A*, the endoprotease assay was performed in the absence (●——●) or presence of 1 μM (×——×) or 2 μM (□——□) *N*-acetyl farnesyl methyl cysteine and various 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.

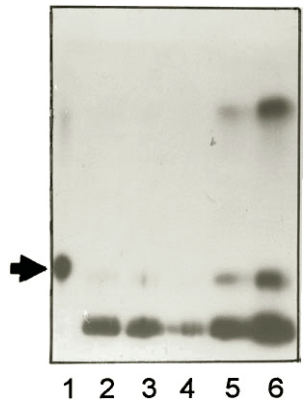


FIG. 10. **Inhibition of prelamin A peptide endoproteolysis by serine protease inhibitors.** Nuclei were taken up in assay buffer, preincubated for 10 min with various serine protease inhibitors (0.8 mM) and then the radioiodinated prelamin A peptide was added. Incubation was continued for an additional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

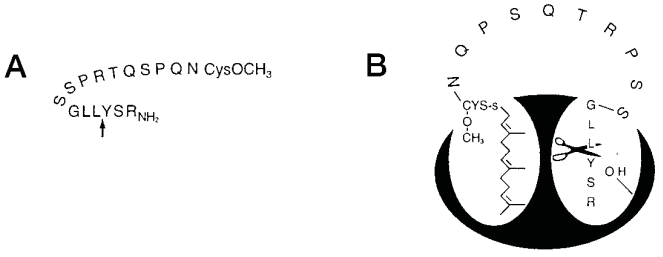


FIG. 11. **A model for the prelamin A endoprotease.** The RSYLLG endoprotease site in prelamin A is hypothesized to be masked in a higher order structure making it inaccessible to the endoprotease active site (*panel A*). Farnesylation is then suggested to result in binding of the substrate by an allosteric site on the endoprotease facilitating recognition of the RSYLLG sequence at the active site (*panel B*).

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 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 cysteinyl 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 K_i 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 K_i for the noncompetitive inhibition of the P-glycoprotein ATPase (31) by *N*-acetyl farnesyl methyl cysteine. It is also comparable to to the K_m values for two other farnesylated substrates for other enzymes. These are the "prenyl cysteine-directed α-carboxymethyl transferase (32)," which has a K_m 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 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-carboxymethyl transferase 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-carboxymethyl transferase (22, 32) and the isoprenylated protein endoprotease (33).

Similarly, functional specificity of farnesylation relative to geranylgeranylation has been demonstrated for mammalian $p21^{ras}$ 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.

REFERENCES

1. Clarke, S. (1992) *Annu. Rev. Biochem.* **61,** 355–386
2. Zhang, F. L., and Casey, P. J. (1996) *Annu. Rev. Biochem.* **65,** 241–269
3. Reiss, Y., Stradley, S. J., Gierasch, L. M., Brown, M. S., and Goldstein, J. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 732–736
4. Okoyama, K., Goodwin, G. W., Ghomashchi, F., Glomset, J. A., and Gelb, M. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5302–5306
5. Gerace, L. and, Burke, B. (1988) *Annu. Rev. Cell Biol.* **4,** 335–374
6. Nigg, E. (1992) *Curr. Opin. Cell Biol.* **4,** 105–109
7. Beck, L. A., Hosick, T. J., and Sinensky, M. (1988) *J. Cell Biol.* **107,** 1307–1316
8. Fisher, D. Z., Chaudhary, N., and Blobel, G. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 6450–6454
9. McKeon, F. D., Kirschner, M. W., and Caput, D. (1986) *Nature* **319,** 463–468
10. Lutz, R. J., Trujillo, M. A., Denham, K. S., Wenger, L., and Sinensky, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3000–3004
11. Sinensky, M., Fantle, K., Trujillo, M. A., McLain, T. M., Kupfer, A., and Dalton, M. (1994) *J. Cell Sci.* **107,** 61–67
12. Holtz, D., Tanaka, R. A., Hartwig, J., and McKeon, F. D. (1989) *Cell* **59,** 969–977
13. Beck, L. A., Hosick, T. J., and Sinensky, M. (1990) *J. Cell Biol.* **110,** 1489–1499
14. Sinensky, M., Fantle, K., and Dalton, M. (1994) *Cancer Res.* **54,** 3229–3232
15. Dalton, M. B., Fantle, K. S., Bechtold, H. A., DeMaio, L., Evans, R. M., Krystosek, A., and Sinensky, M. (1995) *Cancer Res.* **55,** 3295–3304
16. Weber, K., Plessmann, U., and Traub, P. (1989) *FEBS Lett.* **257,** 411–414
17. Hennekes, H., and Nigg, E. A. (1994) *J. Cell Sci.* **107,** 1019–1029
18. Ma, Y.-T., and Rando, R. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6275–6279
19. Chen, Y., Ma, Y. T., and Rando, R. R. (1996) *Biochemistry* **35,** 3227–3237
20. Faust, J., and Krieger, M. (1987) *J. Biol. Chem.* **262,** 1996–2004
21. Kim, C. M., Goldstein, J. L., and Brown, M. S. (1992) *J. Biol. Chem.* **267,** 23113–23121
22. Stephenson, R. C., and Clarke, S. (1990) *J. Biol. Chem.* **265,** 16248–16254
23. Ghomaschi, F., Zhang, X., Liu, L., and Gelb, M. H. (1995) *Biochemistry* **34,** 11910–11918
24. Tan, E. W., Perez-Sala, D., Canada, F. J., and Rando, R. R. (1991) *J. Biol. Chem.* **266,** 10719–10722
25. Fraker, P. J., and Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80,** 849–857
26. Chelsky, D., Olson, J. F., and Koshland, D. E., Jr. (1987) *J. Biol. Chem.* **262,** 4303–4309
27. Loewinger, L., and McKeon, F. (1988) *EMBO J.* **7,** 2301–2309
28. Kramer, W., Drutsa, V., Jansen, H. W., Kramer, B. Pflugfelder, M., and Fritz, H. J. (1984) *Nucleic Acids Res.* **12,** 9441–9456
29. Cox, A. D., Hisaka, M. M., Buss, J. E., and Der, C. J. (1992) *Mol. Cell. Biol.* **12,** 2605–2615
30. James, G. L., Goldstein, J. L., Brown, M. S., Rawson, T. E., Somers, T. C., McDowell, R. S., Crowley, C. W., Lucas, B. K., Levinson, A. D., and Marsters J. C., Jr. (1993) *Science* **260,** 1937–1942
31. Zhang, L. Sachs, C. W., Fu, H.-W., Fine, R. L., and Casey, P. J. (1995) *J. Biol. Chem.* **270,** 22859–22865
32. Pillinger, M. H., Volker, C., Stock, J. B., Weissman, G., and Philips, M. R. (1994) *J. Biol. Chem.* **269,** 1486–1492
33. Ma, Y.-T., Chaudhuri, A., and Rando, R. (1992) *Biochemistry* **31,** 11772–11777
34. Ma, Y.-T., Gilbert, B. A., and Rando, R. R. (1993) *Biochemistry* **32,** 2386–2393
35. Marshall, C. J. (1993) *Science* **256,** 1865–1866
36. Kuroda, Y., Suzuki, N., and Kataoka, T. (1993) *Science* **259,** 683–686
37. Inglese, J., Koch, W. J., Caron, M. G., and Lefkowitz, R. J. (1992) *Nature* **359,** 147–150
38. Caldwell, G. A., Wang, S. H., Naider, F., and Becker, J. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1275–1279