

# An Antibody Which Specifically Recognizes Prelamin A but not Mature Lamin A: Application to Detection of Blocks in Farnesylation-dependent Protein Processing<sup>1</sup>

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## ABSTRACT

A polyclonal antibody [anti-prelamin A antibody ( $\alpha$ -PA)] has been obtained against the peptide LLGNSSPRTQSPQN which is proteolytically removed during the farnesylation-dependent processing of prelamin A to mature lamin A. We tested the ability of this antibody to detect inhibition of farnesylation-dependent protein processing of prelamin A. The  $\alpha$ -PA antibody was shown to immunoprecipitate prelamin A from lovastatin-treated HeLa cells but not mature lamin A from untreated cells. Further studies were performed after antigen-affinity chromatographic purification of the antibody. Western blotting of lovastatin-treated HeLa cell extract demonstrated that the purified  $\alpha$ -PA antibody recognizes prelamin A. Furthermore, this signal could be competed away by incubation with the peptide. Indirect immunofluorescence helped detect nuclear accumulation of the antigen in response to treatment of HeLa cells with lovastatin or in Chinese hamster ovary K1 cells transiently transfected with a prelamin A mutant blocked in farnesylation. This antibody should be useful for screening compounds that may block any of the three common steps in the farnesylation-dependent processing of proteins (farnesylation, endoproteolysis, and carboxymethylation) since it appears that prelamin A undergoes all of these reactions prior to removal of the antigenic peptide. Inhibitors of these reactions have been proposed as potential anticancer drugs, since they would be expected to block the biological activity of oncogenic p21<sup>ras</sup> proteins. Since such screening would be performed most efficiently by enzyme-linked immunosorbent assays, we can detect the accumulation of prelamin A after treatment with lovastatin by performing this procedure as well. Application of  $\alpha$ -PA in an enzyme-linked immunosorbent assay, which demonstrates the activity of a peptidomimetic farnesyltransferase inhibitor, supports the use of this antibody in large scale screening for inhibitors of farnesylation-dependent protein processing.

## INTRODUCTION

A small number of proteins have been identified that are posttranslationally modified with a farnesyl group in thioether linkage to a carboxyl-terminal cysteine (1). In mammalian cells the proteins which have been identified as possessing this modification are p21<sup>ras</sup> proteins (H, N, and K) (2, 3), lamin B (4) and prelamin A (5) (the nuclear lamina proteins), cyclic GMP phosphodiesterase  $\alpha$  (6), rhodopsin kinase (7), and  $\gamma$ -transducin (8). The farnesylation reaction entrains a pathway that for all of these proteins involves two more sequential steps as illustrated in Fig. 1.

Finding specific inhibitors of these reactions that would be active in whole cells has been of considerable pharmacological interest because of their possible use in blocking the activity of oncogenic p21<sup>ras</sup> proteins (9). Until this point, the major target of drug development has been the farnesyltransferase reaction (10). Two approaches to screening for such inhibitors have been described: (a) *in vitro* screening based on assay of the farnesyltransferase (11); and (b) *in vivo* screening in *Saccharomyces cerevisiae* based on rescue of so-called *gpa1*

mutants defective in the  $\alpha$  subunit of the yeast heterotrimeric G-protein (12).

Both of these screening methods have inherent limitations. *In vitro* assay does not test the ability of compounds to be taken up by whole cells or, if taken up, to be nonspecifically cytotoxic. Assay of effects on *Saccharomyces* may not be relevant to mammalian cells. In this paper, we describe an *in vivo* mammalian cell culture method for detecting inhibition of farnesylation-dependent protein processing based on the unique endoproteolytic removal of the farnesylated carboxyl-terminal peptide from prelamin A.

## MATERIALS AND METHODS

**Cells.** Chinese hamster ovary K1 and HeLa cells were cultured in Ham's F-12 medium supplemented with 5% fetal calf serum. Transient transfection with wild-type human lamin A and the C661:M mutant lamin A in the pECE expression vector [a kind gift of Dr. Frank McKeon, Harvard University (13)] was by the Lipofectin method as described by the manufacturer (GIBCO/BRL).

**Preparation of  $\alpha$ -PA.**<sup>3</sup> The peptide CLLGNSSPRTQSPQN, which contains a sequence found in the peptide removed in the processing of prelamin A to lamin A, was synthesized by standard solid-state methods, purified by reverse phase high-pressure liquid chromatography, and conjugated to keyhole limpet hemocyanin. Rabbits were immunized with the conjugated antigen presented in Freund's adjuvant and given three booster injections each 3 weeks apart with the antigen presented in incomplete Freund's adjuvant. The rabbits were exsanguinated and the serum was isolated. The IgG fraction was purified (14) from a portion of the antiserum by ammonium sulfate precipitation, DEAE chromatography, and affinity chromatography on Sulfolink Gel (Pierce Chemical Co.) conjugated to the peptide antigen.

**Immunoprecipitation and Immunofluorescence.** Immunoprecipitation of A/C lamins with human species-specific antiserum, 1E4 [a kind gift of Dr. Frank McKeon (15)], has been described previously (16). Immunoprecipitation of prelamin A was with nonpurified  $\alpha$ -PA as follows: HeLa cells ( $2 \times 10^6$ /100-mm Petri dish) were labeled overnight with 35  $\mu$ Ci/ml [<sup>35</sup>S]methionine (Amersham); cells were washed four times with ice-cold PBS, harvested by trypsinization, taken up in PBS containing 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, and collected by centrifugation (with two washes of the same buffer) in a clinical centrifuge. The cell pellet was dissolved into 500  $\mu$ l of 111 mM NaCl, 2.2 mM EDTA, and 0.44% SDS in 55 mM triethanolamine (pH 7.5) supplemented with a protease inhibitor mix consisting of 5  $\mu$ g/ml each of leupeptin, pepstatin A, chymostatin, bestatin, antipain, and aprotinin. The cell lysate was heated for 10 min at 68°C, cooled on ice, and cleared of nonspecifically absorbed material and cell debris by the addition of a 1:200 dilution of normal rabbit serum and 100  $\mu$ l of a 1:1 slurry of BSA washed and protein A-Sepharose beads, followed by centrifugation in a microfuge. The nonpurified  $\alpha$ -PA was diluted 1:200 into the supernatant, and the antigen-antibody complex was allowed to form overnight in the cold with shaking. Then, 100  $\mu$ l of protein A-Sepharose beads (1:1 slurry) were added, the mixture was incubated with shaking in the cold for an additional 4 h, and the beads were washed as described (17). The precipitated lamins were removed from the beads into SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis as described previously (17).

Indirect immunofluorescence was performed with antigen-purified  $\alpha$ -PA (1:200). In experiments using 1E4 as the primary antibody, it was diluted

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<sup>3</sup> The abbreviations used are:  $\alpha$ -PA, anti-prelamin A antibody; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TTBS, 0.1% Tween-90 mM NaCl-150 mM Tris base, pH 7.6; ELISA, enzyme-linked immunosorbent assay.

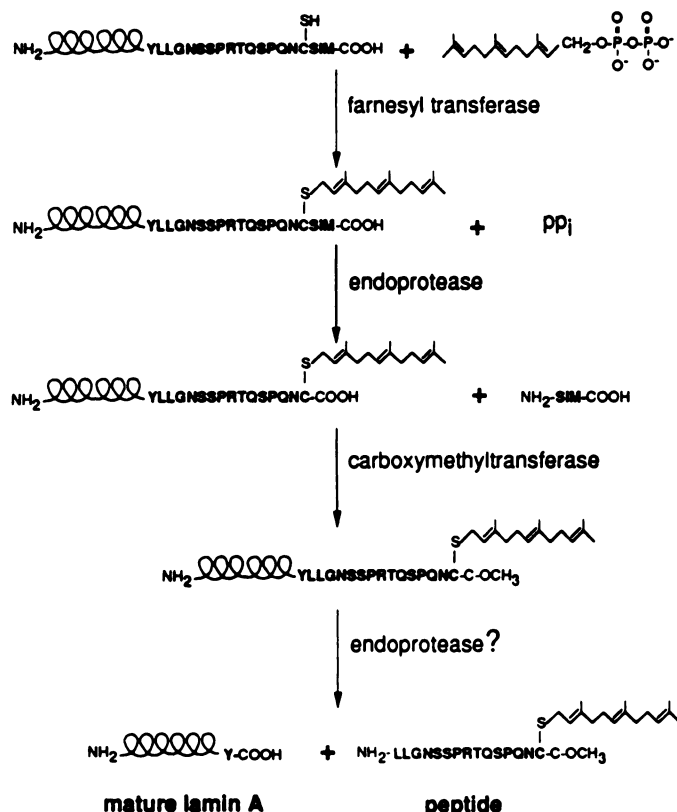


Fig. 1. Pathway of prelaminate A processing. No direct evidence exists for the second endoprotease reaction. The peptide against which  $\alpha$ -PA was prepared is (C)LLGNSSPRTQSPQN.

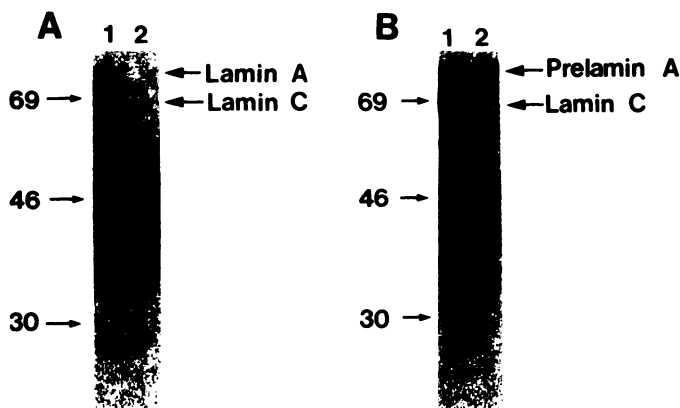


Fig. 2. Radioimmuno-precipitation of lamin proteins from untreated HeLa cells (A) or lovastatin (5  $\mu$ g/ml)-treated HeLa cells (B) with 1E4 (Lane 1) or  $\alpha$ -PA (Lane 2).

1:300. Cells were plated on glass coverslips, rinsed with PBS, and then fixed with 3% formaldehyde in PBS for 15 min at 20°C. The coverslips were washed twice in 5 mM NH<sub>4</sub>Cl in PBS and then permeabilized with 0.05% Triton X-100 in PBS for 10 min at +20°C. Secondary antibodies for 1E4 were: red, rhodamine-conjugated goat anti-mouse (Biomeda; 1:200); blue, fluoroblue-conjugated goat anti-mouse (Biomeda; 1:200); and green, fluorescein isothiocyanate-conjugated goat anti-mouse (Cappel; 1:200). Secondary antibodies for  $\alpha$ -PA were: red, rhodamine-conjugated goat anti-rabbit (Biomeda; 1:200); and green, fluorescein isothiocyanate-conjugated goat anti-rabbit (Cappel; 1:200).

**Western Blotting.** HeLa cells (5 × 10<sup>6</sup>/100-mm Petri dish) were treated for 16 h with lovastatin (5  $\mu$ g/ml), washed three times with PBS, and harvested into 200  $\mu$ l of SDS-polyacrylamide gel electrophoresis loading buffer. The HeLa cell extracts (5  $\mu$ l) were electrophoresed for 2 h at 100 V on a 10% acrylamide gel (Hoefer; Mighty Small gel apparatus). The gels were then soaked in a blotting buffer (Tris-glycine buffer-1% SDS-20% methanol) for 5

min and transferred at 25 V to a nitrocellulose membrane (0.45  $\mu$ m) at 4°C for 15 h. Next, the membrane was blocked with 5% BSA in TTBS for at least 2 h. After blocking, the membrane was washed with TTBS and incubated with  $\alpha$ -PA antibody (1:200) for 1 h at room temperature. For the competition experiments, the  $\alpha$ -PA antibody was incubated with 10–100  $\mu$ g/ml of competitor for 1 h at room temperature prior to probing the membrane. The (C)LLGNSSPRTQSPQN peptide, insulin A chain, and BSA were used as competitors. Following the primary antibody incubation, the membrane was washed with TTBS and incubated with the secondary antibody (goat anti-rabbit IgG coupled to horseradish peroxidase; Bio-Rad Laboratories) at a 1:5000 dilution. Finally, the membrane was washed with TTBS and the immunocomplexes were detected using an enhanced chemiluminescence kit (Amersham).

**ELISA.** HeLa cells (5 × 10<sup>5</sup>/35-mm Petri dish) were incubated for 24 h in Ham's F-12 medium supplemented with 5% fetal calf serum. The cells were then treated for 16–18 h with a prenylation inhibitor, B581. B581 was a kind gift of Dr. A. Garcia, Eisai Research Institute, Andover, MA. The dishes were washed with PBS, fixed with 2 ml acetic acid/methanol at –20°C for 10 min, and washed three times with PBS. The fixed cells were then incubated with shaking for 2 h with 3% BSA in TTBS followed by four washes with TTBS. The cells were then incubated with shaking for 2 h with affinity-purified  $\alpha$ -PA (1:200) in 3% BSA-TTBS followed by four washes with TTBS. Next, the cells were incubated with shaking for 2 h with the secondary antibody (blotting grade, affinity-purified goat anti-rabbit IgG coupled to alkaline phosphatase; Bio-Rad Laboratories; diluted 1:1000 in TTBS) followed by four washes with TTBS and two washes with 0.5 mM MgCl<sub>2</sub> in 1 M Tris (pH 9.5). The bound alkaline phosphatase was then assayed by the addition of 1 ml of a 1-mg/ml solution of *p*-nitrophenyl phosphate (Sigma) in 0.5 mM MgCl<sub>2</sub> in 1 M Tris (pH 9.5) followed by a 30 min incubation with shaking. The reaction was stopped by the addition of 1 ml of 0.1 M EDTA in 1 M Tris (pH 9.5). The supernatant was removed and the absorbance was determined at 405 nm. Control experiments established that these conditions were within the linear range with regard to cell number and time of assay.

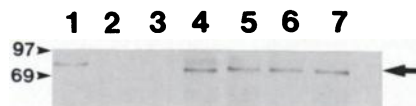


Fig. 3. Western blotting of prelaminate A from HeLa cells treated with lovastatin (5  $\mu$ g/ml) in the absence (Lane 1) and presence (Lanes 2–7) of competitor. Lanes 2 and 3, Western blot done with  $\alpha$ -PA antibody in the presence of 10 and 100  $\mu$ g/ml peptide antigen, respectively. Lanes 4 and 5,  $\alpha$ -PA Western blot done in the presence of 10 and 100  $\mu$ g/ml of insulin A chain, respectively. Lanes 6 and 7,  $\alpha$ -PA Western blot done in the presence of 10 and 100  $\mu$ g/ml of bovine serum albumin, respectively.

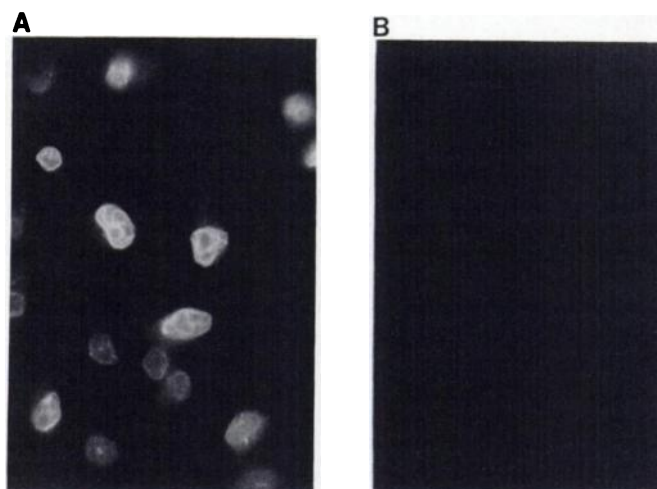
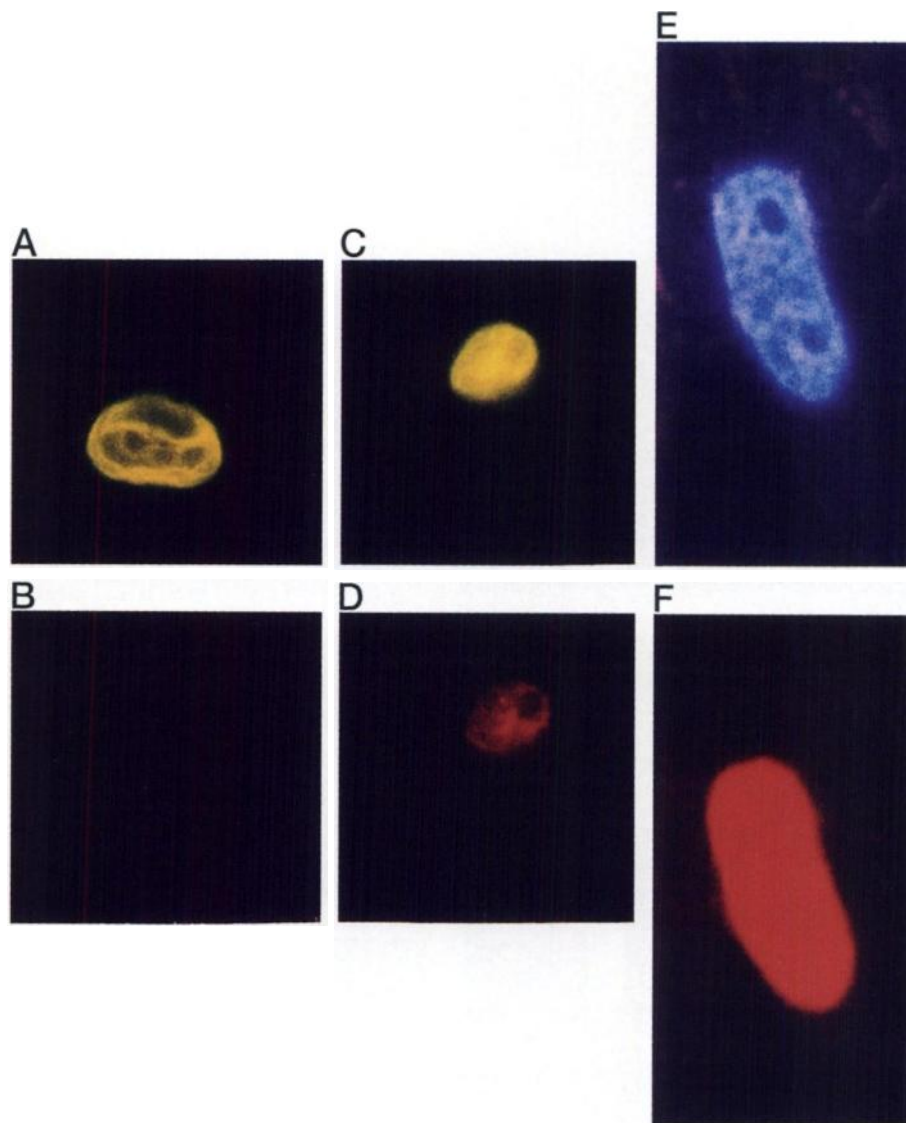


Fig. 4. Immunofluorescent visualization of prelaminate A by  $\alpha$ -PA in lovastatin treated HeLa cells (A). No prelaminate A is detected by  $\alpha$ -PA in untreated cells (B), since prelaminate A is present in significant quantities only when farnesylation-dependent prelaminate A processing is blocked.

Fig. 5. Double immunofluorescent visualization of transiently transfected human prelamins A. Wild-type mature lamin A is detected with 1E4 (A, green), but not  $\alpha$ -PA (B, red); whereas, when an identical experiment is done on lovastatin-treated cells (5  $\mu$ g/ml), the prelamins A that accumulate are detected by both 1E4 (C, green) and  $\alpha$ -PA (D, red). A mutant prelamins A (C661:M), which cannot be farnesylated, is also recognized by both 1E4 (E, blue) and  $\alpha$ -PA (F, red).



## RESULTS

**Detection of Inhibition of Prelamin A Processing by Lovastatin with  $\alpha$ -PA.** It has been well established that  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA reductase inhibitors, such as lovastatin, block the prenylation of proteins through inhibition of synthesis of the polyisoprenoid precursors. The consequent blockage of prelamins A processing by lovastatin has been well demonstrated previously in our laboratory (17). We examined the effects of lovastatin treatment on detection of prelamins A by immunoprecipitation. Treatment of HeLa cells with 5  $\mu$ g/ml of lovastatin results in the immunoprecipitation of  $M_r$  74,000 protein that comigrates with a protein immunoprecipitated by a previously described anti-lamin A/C antiserum (1E4). This protein, which accumulates in lovastatin treated cells, has demonstrated previously to have been prelamins A (16, 17). To determine if  $\alpha$ -PA recognizes prelamins A, but not mature lamin A, we radioimmunoprecipitated the A/C lamins with 1E4 or  $\alpha$ -PA from HeLa cells untreated (Fig. 2A) or treated (Fig. 2B) with lovastatin. A protein that is immunoprecipitated from lovastatin-treated cells, but not untreated cells, comigrates precisely with the band immunoprecipitated by 1E4 previously shown to be prelamins A (16, 17). These results demonstrate specific recognition of prelamins A, but not mature lamin A, by  $\alpha$ -PA. Such specific detection of prelamins A validates the concept that

$\alpha$ -PA can be used to detect inhibitors of farnesylation-dependent protein processing.

The specific detection of prelamins A by the  $\alpha$ -PA antibody was further examined by Western blotting (Fig. 3). The  $\alpha$ -PA antibody recognizes one major band with a molecular weight of 74,000 in extracts from HeLa cells which have been treated with lovastatin (Fig. 3, Lane 1). When the antibody is incubated with the peptide antigen, (C)LLGNSSPRTQSPQN, this specific band is no longer detected (Fig. 3, Lanes 2 and 3). In contrast, neither insulin A chain nor BSA can compete away the signal (Fig. 3, Lanes 4–7). This suggests that the recognition of prelamins A by  $\alpha$ -PA is specific.

Specific detection can also be demonstrated in indirect immunofluorescence. When HeLa cells are treated with lovastatin, their nuclei can be visualized by  $\alpha$ -PA, which gives little or no signal in untreated cells (Fig. 4). Confirmation that this result is due to the accumulation of prelamins A can be obtained by expression in Chinese hamster ovary K1 cells of a human mutant prelamins A that are defective in farnesylating and, hence, processing, by virtue of a substitution of a methionine for the cysteine that undergoes farnesylation [C661:M; (13)]. Visualization of transfected human wild-type (Fig. 5, A and B) and C661:M (Fig. 5, E and F) prelamins A by double immunofluorescence reveals that cells expressing the wild-type protein are not

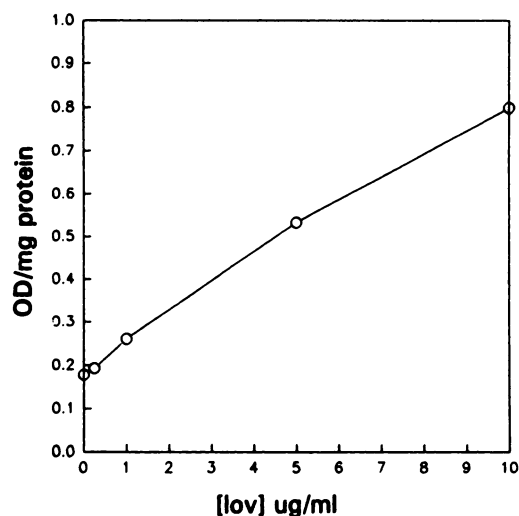


Fig. 6. Effect of various doses of lovastatin on the accumulation of prelamins A in HeLa cells as measured with an ELISA. Cells are treated overnight with lovastatin prior to the ELISA. Points represent the mean of duplicate experiments.

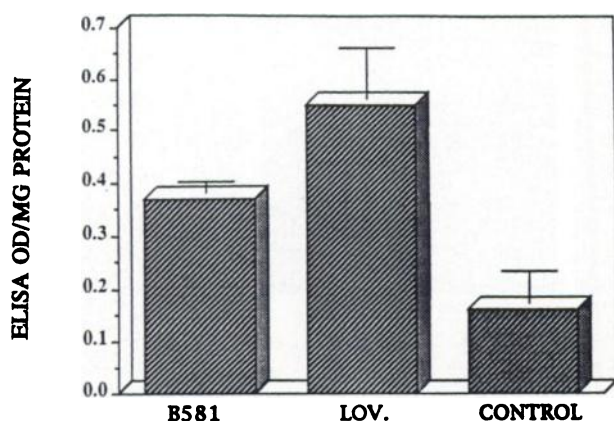


Fig. 7. An ELISA with  $\alpha$ -PA detects the accumulation of prelamins A produced by the protein farnesyltransferase inhibitor B581 (200  $\mu$ M) or with lovastatin (5  $\mu$ g/ml). Cells are treated overnight with inhibitor prior to the ELISA. Points represent the average of three separate experiments  $\pm$  SD (bars).

recognized by  $\alpha$ -PA, whereas cells which accumulate the mutant prelamins A are recognized by  $\alpha$ -PA. Visualization of transfected wild-type prelamins A can be produced, as expected, by treating the transfected cells with lovastatin (Fig. 5, C and D).

Although these results clearly demonstrate the specificity of  $\alpha$ -PA in permitting detection of prelamins A accumulation, a high throughput screening assay would be best performed by ELISA methods since these procedures lend themselves well to automation. To test the feasibility of such a method, we first developed a procedure for detecting prelamins A by ELISA in cells treated with various amounts of lovastatin. The results of such an experiment (Fig. 6) clearly indicate that an ELISA based on  $\alpha$ -PA can be used to detect inhibition of farnesylation-dependent protein processing. To confirm this conclusion, we also examined a peptidomimetic farnesyl protein transferase inhibitor, B581 (18), which has previously been reported to partially block processing of both prelamins A and p21<sup>ras</sup>. In immunofluorescent experiments, we found that B581 produced significant accumulation of prelamins A (data not shown) when cells were treated overnight with an inhibitor concentration of 200  $\mu$ M. As expected,

overnight treatment of HeLa cells with 200  $\mu$ M B581 also produced accumulation of prelamins A detectable by our ELISA method (Fig. 7).

## DISCUSSION

Unlike other known isoprenylated proteins of mammalian cells, farnesylation of prelamins A results in removal of the peptide shown in Fig. 1. Since removal of this peptide requires that the prior reactions take place, inhibitors that block these reactions would be expected to block the removal of the prelamins A peptide as well. All of these processing reactions, which are common to the farnesylation-dependent processing of p21<sup>ras</sup> proteins, are potential targets of anticancer drugs.

In this paper, we have described a facile immunoassay for the screening for such compounds in intact mammalian cells using an antibody against the prelamins A peptide. Screening with mammalian cells as targets provides a more physiological model than previously described *in vitro* or in yeast growth screens, as well as being suitable for searches for inhibitors for all three processing reactions in one screen. Although the antibody we have prepared is potentially limiting since it was prepared as a rabbit polyclonal, it should be possible to prepare monoclonal antibodies raised against the same peptide. With a greater source of antibody, the ELISA assay described above can be modified to be run with microtiter plates and automated plate readers to provide extremely rapid screening of large numbers of compounds. It is hoped that this procedure will be useful in identifying pharmaceuticals that antagonize the function of p21<sup>ras</sup> proteins.

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