Characterization of Two Populations of Statin and the Relationship of Their Syntheses to the State of Cell Proliferation

Gee Ching* and Eugenia Wang*‡

*The Rockefeller University, New York 10021; and ‡The Bloomfield Centre for Research in Aging,
Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital and
the Departments of Medicine and Anatomy, McGill University, Montreal, Quebec, Canada

Abstract. Statin has previously been identified to be a 57-kD protein present in the nuclei of quiescent and senescent human fibroblasts, but not in their replicating counterparts (Wang, E. 1985. *J. Cell Biol.* 100: 545–551). In the present report we demonstrate by immunoprecipitation analysis of fractionated cellular extracts the existence of two populations of statin. The Triton X-100–soluble statin is found in replicating sparse cultures as well as in quiescent confluent cultures and quiescent serum-starved cultures of young human fibroblasts, but the Triton X-100–insoluble, nuclear envelope–localized statin is present only in the quiescent cultures. Two-dimensional gel analysis of the immunoprecipitated cellular fractions reveals that both populations of statin have an isoelectric point of 5.3. Pulse–chase experiments show that statin is synthe-

sized as a 57-kD polypeptide and is not processed from a precursor of different molecular mass. Experiments on serum stimulation of quiescent cells show that synthesis of the Triton X-100–insoluble statin decreases rapidly during the transition from the G0 to S phase, and that this decrease is accompanied by a slower reduction in synthesis of the Triton X-100–soluble statin. These results suggest that the cellular expression of the two populations of statin may be associated with the mechanisms controlling the transition between the growing state and the quiescent state and confirm the previous finding that the Triton X-100–insoluble, nuclear envelope–localized statin could be used as a marker for cells arrested at the G0 phase of the cell cycle.

STATIN is a nuclear protein previously identified by a mAb (S-30) that is produced by immunizing a mouse with the detergent-insoluble extract of senescent human fibroblasts (20). Immunofluorescence staining with the antibody shows that statin is present in the nuclei of senescent and quiescent young human fibroblasts but not in the nuclei of replicating young fibroblasts. Immunoprecipitations of total cellular extracts with the antibody demonstrate that statin is a 57-kD protein present only in nonproliferating human fibroblasts. Immunogold labeling localizes the protein at the nuclear envelope of the nonproliferating cells (22). However, extensive biochemical characterization of the protein has not been carried out. Questions as to whether statin is synthesized as a precursor of different molecular mass or present in cellular compartments other than the nuclear envelope remain unanswered.

The nuclear presence of statin is not only characteristic of senescent and quiescent young fibroblasts, but also of tissues comprised of cells that are at the final stage of differentiation

(24). As it is the case for replicating young fibroblasts in vitro, statin is noticeably absent in tissues such as epithelium of the gastrointestinal tract and kidney whose cells still have the potential to divide. While the function of statin is unknown, the nuclear expression of the protein has been shown to be correlated with the state of cell proliferation. Nuclear expression of statin can be induced in young human fibroblasts arrested at the G0/G1 phase of the cell cycle by serum starvation or growth–contact inhibition (20, 22, 25). The induced statin disappears rapidly from the nuclei when the cells are promoted to leave the quiescent state upon serum stimulation (25) or removal of growth–contact inhibition by subculturing to sparse cell density (22). The nuclear disappearance of this protein in the cells reentering the S phase of the cell cycle precedes initiation of DNA synthesis (25). Although a correlation between the nuclear expression of statin and the nonproliferative state of a cell has been demonstrated, similar kinds of studies on the total cellular expression of the protein have not been done. It will be important to know if the nuclear disappearance of statin in cells reentering the S phase of the cell cycle is accompanied by a loss in the total cellular level of the protein.

In this paper, we report a biochemical characterization of the cellular distribution of statin and of its synthesis. The relationship between the total cellular expression of the protein and the state of cell proliferation is also determined.

Gee Ching's present address is Department of Pathology, Columbia University, College of Physicians and Surgeons, New York 10032. Address reprint requests to Dr. Eugenia Wang, The Bloomfield Centre for Research in Aging, Lady Davis Institute, Sir Mortimer B. Davis-Jewish General Hospital and McGill University, 3755 Chemin Côte Ste.-Catherine, Montreal, Quebec, Canada H3T 1E2.

Materials and Methods

Cell Cultures

GM0011, a cell strain of normal human fibroblasts derived from a donor of 8 fetal weeks, was obtained from the Human Genetic Cell Repository (Camden, NJ). Its in vitro lifespan, expressed in cumulative population doubling level (CPDL),[1] has previously been determined (20, 21, 23). Cultures of GM0011 at CPDL less than 15 were used to obtain a fairly homogeneous population of young cells with high replicative capacity.

All cultures were grown on petri dishes of 10-cm diameter in MEM supplemented with 10% FBS and 1% nonessential amino acids at 37°C in a humidified atmosphere of 5% CO_2 as described (20). Cells were either seeded at 7×10^3 cells/cm^2 and grown to a confluent monolayer (6×10^5 cells/cm^2), or seeded at 3×10^3 cells/cm^2 and permitted to grow for 2–3 d to obtain a sparse density (8–9×10^3 cells/cm^2). These replicating sparse cultures and quiescent confluent cultures were then used for the labeling experiments described below.

Preparation of mAb

The mAb S44-1 was prepared and screened as described (20). This antibody was shown by the techniques of immunofluorescence microscopy and immunoprecipitation to react specifically with statin as the mAb S-30 (20) did. It has a higher titer than S-30 and therefore was used in the present study.

Steady-state Metabolic Labeling

Replicating sparse cultures and quiescent confluent cultures were rinsed with methionine-free MEM three times and labeled with 400 μCi [^{35}S]methionine (>1,000 Ci/mmol; New England Nuclear, Boston, MA) in 4 ml of methionine-free MEM supplemented with 0.5 or 10% FBS (for confluent and sparse cultures, respectively) and 0.8 μg/ml of unlabeled L-methionine at 37°C for 16 h.

Pulse–Chase Experiments

Quiescent confluent cultures were rinsed three times with methionine-free MEM and pulse labeled with 800 μCi [^{35}S]methionine (>1,000 Ci/mmol) in 4 ml of methionine-free MEM at 37°C for 30 min. The cultures were then washed twice and incubated in 10 ml of methionine-containing MEM at 37°C for various lengths of time.

Serum Stimulation of Quiescent Cells

Replicating sparse cultures and quiescent confluent cultures grown in serum-supplemented MEM as described above were rinsed with serum-free MEM three times and once, respectively, refed with 10 ml of MEM containing 0.1% serum, and allowed to incubate for 48 h to attain a uniform quiescent state. The resulting quiescent cultures were incubated in 10 ml of MEM containing 10% FBS and 1% nonessential amino acids for designated lengths of time. These cultures were then labeled at 37°C for 2 h in 4 ml of methionine-free MEM containing 500 μCi [^{35}S]methionine (>1,000 Ci/mmol) and 10% dialyzed FBS. A serum-depleted, quiescent sparse culture was also prepared and labeled with [^{35}S]methionine for 2 h in the absence of FBS.

Cell Fractionation by Detergent Solubilization

All steps were done at 0–4°C unless otherwise indicated. [^{35}S]Methionine-labeled cultures were washed four times with PBS. Cells were lysed with 1 ml of RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA) containing 0.5 mM PMSF, 10 μg/ml of aprotinin, 2 μg/ml each of leupetin and pepstatin (all protease inhibitors from Boehringer Mannheim Biochemicals, Indianapolis, IN), scraped into Eppendorf tubes, vortexed, and incubated in ice for 10 min. After a 10-min centrifugation at 15,000 g and 4°C, the detergent-soluble (supernatant) and -insoluble (pellet) fractions were separated. The soluble fraction was further centrifuged at 15,000 g for 1 h to remove any insoluble materials. The detergent-insoluble pellet was washed once with 10 mM Tris–HCl, pH 7.4, and digested with 0.1 mg/ml of DNase I (Sigma Chemical Co., St. Louis, MO) in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl$_2$, 0.5 mM

_____

1. *Abbreviation used in this paper*: CPDL, cumulative population doubling level; RIPA, radioimmunoprecipitation analysis.

PMSF, and 10 μg/ml of aprotinin at 4°C for 1 h with constant mixing. After a 10-min centrifugation, the supernatant was removed, and the pellet was washed once with 10 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, and once with 10 mM Tris–HCl, pH 7.4. The pellet was then sonicated briefly in 300–400 μl of 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.4% SDS (reference 15) containing 0.5 mM PMSF, 10 μg/ml of aprotinin, 2 μg/ml each of leupetin and pepstatin, and boiled for 5 min. The sample was cooled to room temperature and 10 mM Tris–HCl, pH 7.4, containing 20% Triton X-100 was added to a final concentration of 1% Triton X-100. The sample was then centrifuged at 15,000 g and 4°C for 30 min to remove insoluble materials. The resulting supernatant represents the detergent-insoluble fraction of the cellular extract.

Immunoprecipitations and Gel Electrophoresis

All steps were done at 4°C unless otherwise indicated. Immunoprecipitations were carried out overnight in a final volume of 0.5 ml of RIPA buffer containing 1×10^6 acid-precipitable cpm of [^{35}S]methionine-labeled cellular fractions, 3 μl of S44-1 ascite fluid, 0.5 mM PMSF, 10 μg/ml of aprotinin, 2 μg/ml each of leupetin and pepstatin with constant mixing. The mixtures were incubated first with 20 μg of affinity-purified rabbit anti–mouse IgG (Cappel Laboratories, Malvern, PA) for 2 h, and then 50 μl of a 10% suspension of protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) for 1 h with constant mixing. The beads were pelleted in a microcentrifuge and washed three times with RIPA buffer and once with 10 mM Tris–HCl, pH 7.4. The beads and bound proteins were boiled in 30 μl of twice-concentrated SDS sample buffer, and the eluted proteins were analyzed by SDS-PAGE (11) in 10% polyacrylamide gels, followed by autoradiography. For the studies on serum stimulation of quiescent cells, the autoradiograms were scanned by laser densitometer (ultroscan XL; LKB Instruments Inc., Gaithersburg, MD) for quantitative measurements of the relative amounts of statin. For analysis of the immunoprecipitates by two-dimensional PAGE (14), the bound proteins were eluted from the beads by a 15-min incubation in 9.5 M urea, 2% (vol/vol) NP-40, 2% (vol/vol) Ampholines (1.6% pH range 5–7; 0.4% pH range 3–10), and 5% (vol/vol) 2-mercaptoethanol at room temperature. The eluted proteins were separated by IEF (1.6% pH range 5–7, 0.4% pH range 3–10) in the first dimension and SDS-PAGE in the second dimension.

[Methyl-^{3}H]Thymidine Incorporation

Parallel sets of sparse cultures and confluent cultures that were made quiescent by serum depletion as already described were refed with MEM supplemented with 10% FBS and 1% essential amino acids and allowed to incubate for designated lengths of time. [Methyl-^{3}H]thymidine (6 Ci/mmol; New England Nuclear) was added to a final concentration of 10 μCi/ml at 0, 3, 7, 14, 22, or 26 h after serum stimulation, the cells were incubated for an additional 1 h. DNA synthesis was measured by determining the incorporation of [methyl-^{3}H]thymidine into acid-precipitable materials as described (9) with minor modifications. Cells were rinsed three times with PBS, fixed in 10% TCA for 10 min, and washed again with PBS. The manipulations were carried out at 0°C. The cells were solubilized in 4 ml of 0.2 N NaOH at 70°C for 10 min, and then neutralized with 0.4 ml of 2 N HCl. To measure the radioactivity, 0.5 ml of the neutralized sample was taken out for scintillation counting.

Results

To determine whether statin was present in cellular compartments other than the nuclear envelope, replicating sparse culture and quiescent confluent culture of young human fibroblasts (CPDL = 10) labeled with [^{35}S]methionine for 16 h were lysed with RIPA buffer containing 1% Triton X-100, and the resulting detergent-soluble and -insoluble fractions of the cell extracts were immunoprecipitated with the mAb specific for statin. Since previous studies have indicated that statin is absent in the nuclei of cells that are in the replicative state (20, 24), the metabolic labeling of the quiescent confluent culture was done in the presence of 0.5% instead of 10% FBS to avoid any mitogenic effect caused by serum stimulation during the steady-state labeling. The replicating sparse culture was labeled in the presence of 10% FBS.
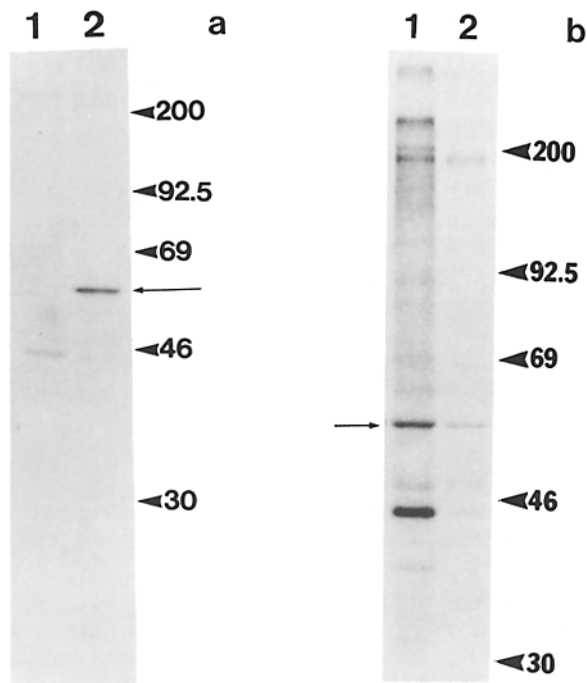
Figure 1. Detection of statin in sparse and confluent cultures of young human fibroblasts. Replicating sparse culture (*a*) and quiescent confluent culture (*b*) of young fibroblasts (CPDL = 10) labeled with [^{35}S]methionine for 16 h in the presence of 10 and 0.5% serum, respectively, were extracted with RIPA buffer and separated into the Triton X-100–insoluble (lane *1*) and -soluble (lane *2*) fractions. The cell fractions were immunoprecipitated by the antistatin antibody and the resulting immunoprecipitates were analyzed by SDS-PAGE. Statin is indicated by an arrow. The positions of molecular mass markers are shown by numbers (given in kD). The band at 43-kD represents actin nonspecifically precipitated.
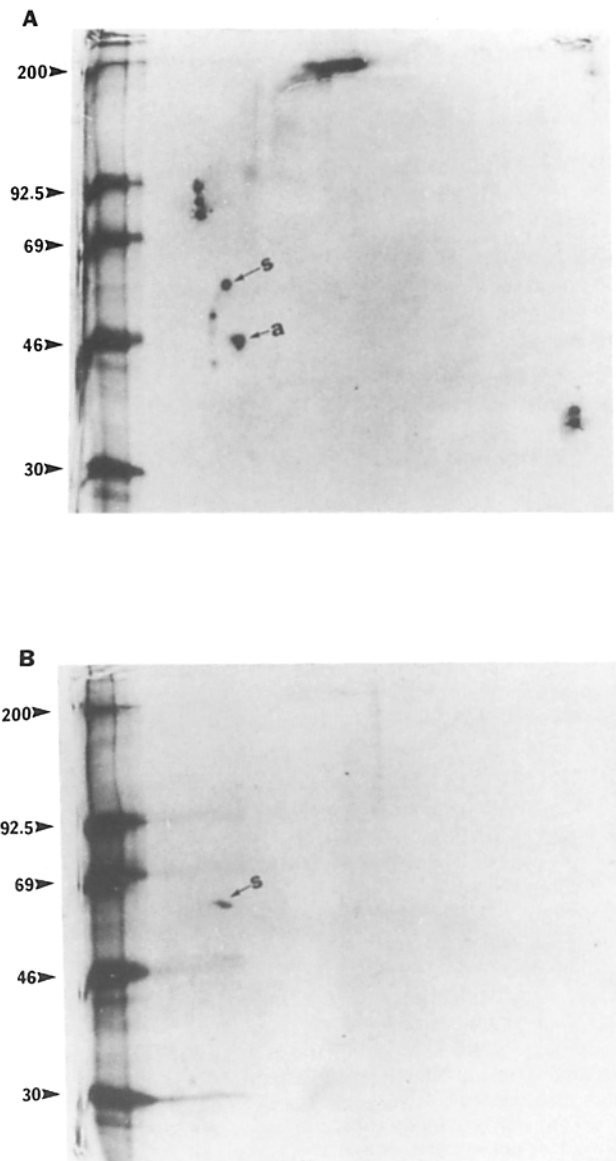
Figure 2. Two-dimensional gel analysis of statin. The Triton X-100–insoluble (*A*) and -soluble (*B*) fractions from the quiescent confluent culture of young fibroblasts (CPDL = 10) labeled with [^{35}S]methionine for 16 h were immunoprecipitated with the antistatin antibody. The immunoprecipitates were subjected to IEF in the first dimension (acidic end starts from the left) and SDS-PAGE in the second dimension (positions of molecular mass markers are shown by numbers). Statin (*S*) and nonspecifically precipitated actin (*A*) are indicated by arrows. In *a*, the three spots at the upper left corner and two spots at the lower right corner are position marks put on the dried SDS–polyacrylamide gel to make alignment of the gel with the autoradiogram easier.

Statin was present in large amounts in the Triton X-100–insoluble fraction of the quiescent confluent culture (Fig. 1 *b*, lane *1*), but not in that of the replicating sparse culture (Fig. 1 *a*, lane *1*). These results are consistent with the previous studies that determined statin was present in the nuclear envelope of density-arrested young fibroblasts and absent in the nuclear envelope of replicating young fibroblasts (20, 22). DNase treatment of the Triton X-100–insoluble fractions did not release statin (data not shown). Actin, a 43-kD major protein in the Triton X-100–insoluble fraction, was coprecipitated by the antistatin antibody (Fig. 1, *a* and *b*, lanes *1*). The nonspecific immunoprecipitation of actin has also been seen with other antibodies (12). Statin was also found in the Triton X-100–soluble fractions of both the replicating sparse culture and quiescent confluent culture (Fig. 1, *a* and *b*, lanes *2*). Since integral membrane proteins of nuclear envelope are Triton X-100 insoluble, the presence of statin in the Triton X-100–soluble fractions of these cultures indicates that a population of the protein is located in other cellular compartment(s) besides the nuclear envelope. These results, taken together, demonstrate that two populations of statin exist: the Triton X-100–insoluble population that is an integral protein of the nuclear envelope, and the Triton X-100–soluble population that is located in the other cellular compartment(s). The Triton X-100–insoluble statin is pres-

ent specifically in the quiescent confluent culture, whereas the Triton X-100–soluble statin is present in the replicating sparse culture as well as in the quiescent confluent culture of young human fibroblasts.

The Triton X-100–insoluble and -soluble fractions from the quiescent confluent culture of young human fibroblasts (CPDL = 10) labeled with [^{35}S]methionine for 16 h were immunoprecipitated with the antistatin antibody and analyzed by two-dimensional gel electrophoresis. Fig. 2 shows
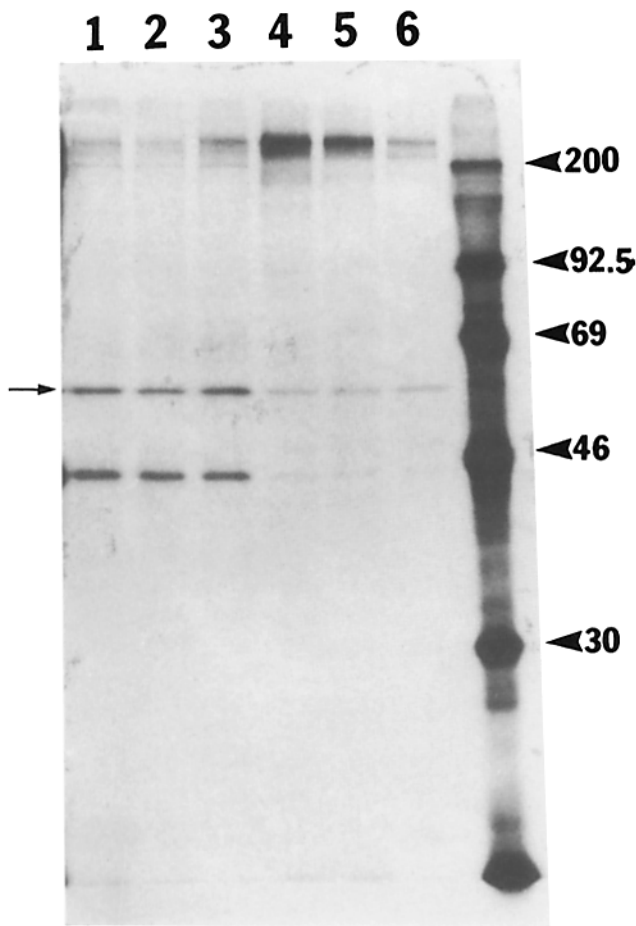
Figure 3. Pulse–chase analysis of statin in young fibroblasts. Quiescent confluent cultures of young fibroblasts (CPDL = 10) were pulse labeled with [³⁵S]methionine for 30 min (lanes *1* and *4*) and chased in serum-free medium containing unlabeled L-methionine for 30 (lanes *2* and *5*) or 90 min (lanes *3* and *6*). The Triton X-100–insoluble (lanes *1–3*) and -soluble (lanes *4–6*) fractions obtained from these cells by RIPA buffer extraction were immunoprecipitated with the antistatin antibody. The immunoprecipitates were subjected to SDS-PAGE. Statin is indicated by an arrow (*left*). The band at 43 kD in lanes *1–3* represents actin nonspecifically precipitated.

that statin in both fractions exhibited an isoelectric point of 5.3 and appeared to exist as single isoform since longer exposure of the autoradiograms did not reveal more than one 57-kD species.

To investigate whether statin was synthesized as a 57-kD protein or was processed from a precursor of different molecular mass, quiescent confluent cultures of young fibroblasts (CPDL = 10) were pulse labeled with [³⁵S]methionine in the absence of serum for 30 min and chased for various lengths of time in a serum-free medium containing unlabeled methionine. After the pulse labeling or designated periods of chases, cells were lysed with RIPA buffer and separated into Triton X-100–insoluble and -soluble fractions which were then immunoprecipitated with the antistatin antibody. As it is the case for the steady-state labeled quiescent cells (Fig. 1 *b*, lanes *1* and *2*), statin in the cells pulsed labeled for 30 min was synthesized as a 57-kD protein which

was detected in both the Triton X-100–insoluble and -soluble fractions (Fig. 3, lanes *1* and *4*). The same 57-kD protein was detected in the two fractions after 30 and 90 min of chases (Fig. 3, lanes *2* and *5*, and lanes *3* and *6*, respectively). Longer chases for up to 14 h also did not reveal any species other than the 57-kD protein (data not shown).

Previous studies have demonstrated that the nuclear expression of statin is correlated to the nonproliferative state of the cell (20, 22, 24). Since statin is found to be present in the Triton X-100–insoluble and -soluble fractions of the cell extracts (Fig. 1), it is important to further investigate the correlation between the synthesis of total cellular statin and the proliferation state of the cell. To perform such a study, replicating sparse cultures and quiescent confluent cultures of young fibroblasts (CPDL = 13) were made uniformly quiescent by serum depletion, and the resulting quiescent cultures were stimulated with 10% serum for various lengths of time and then labeled with [³⁵S]methionine for 2 h in the presence of 10% serum. Synthesis of statin was determined by immunoprecipitation of the Triton X-100–insoluble and -soluble fractions from the labeled cultures extracted with RIPA buffer, followed by quantitative measurements of the relative amounts of statin by densitometric analysis of the autoradiograms obtained from gel electrophoresis of the immunoprecipitates. To correlate synthesis of statin with the proliferation state of the cells, cellular DNA synthesis after serum stimulation was measured by [methyl-³H]thymidine incorporation into parallel cultures.

Fig. 4 shows that 5 h after the addition of serum to quiescent confluent culture, synthesis of total cellular statin decreased (compare lanes *1* and *5* with lanes *2* and *6*), with newly synthesized statin present at a slightly reduced level (80%) in the soluble fraction (lane *6*) and at a substantially reduced level (48%) in the insoluble fraction (lane *2*). In contrast, synthesis of the coprecipitated actin began to increase (Fig. 4, compare lanes *1* and *2*). By 9 h after serum stimulation, synthesis of the Triton X-100–soluble statin was drastically reduced (to 15%) (Fig. 4, compare lanes *6* and *7*), whereas synthesis of the Triton X-100–insoluble statin remained at approximately the same low rate as that of the 5-h serum stimulation (Fig. 4, compare lanes *2* and *3*). A 16-h serum stimulation did not cause any further change in synthesis of both populations of statin (Fig. 4, lanes *4* and *8*). Synthesis of both populations of statin thus appeared to drop to a low, constant level which probably represents a basal level of the protein in the serum-stimulated confluent culture. It should be noted that the rapid decrease in synthesis of total cellular statin occurred before a substantial increase in DNA synthesis was detected at 14–15 h after addition of serum (Table I).

Fig. 5 *a* shows that in quiescent sparse culture labeled with [³⁵S]methionine for 2 h in the absence of serum, statin was present in both the Triton X-100–insoluble and -soluble fractions. When the culture was labeled for 2 h in the presence of 10% serum, synthesis of the Triton X-100–insoluble statin was turned off rapidly by a 2-h serum stimulation during the labeling, whereas synthesis of the Triton X-100–soluble statin did not change (compare lanes *1* and *2* of Fig. 5 *a* with lanes *1* and *5* of Fig. 5 *b*). However, after 5 h of serum stimulation, a decrease (to 83%) in the synthesis of the Triton X-100–soluble statin began to be detected (Fig. 5 *b*, compare lanes *5* and *6*). It is noteworthy that this decrease occurred
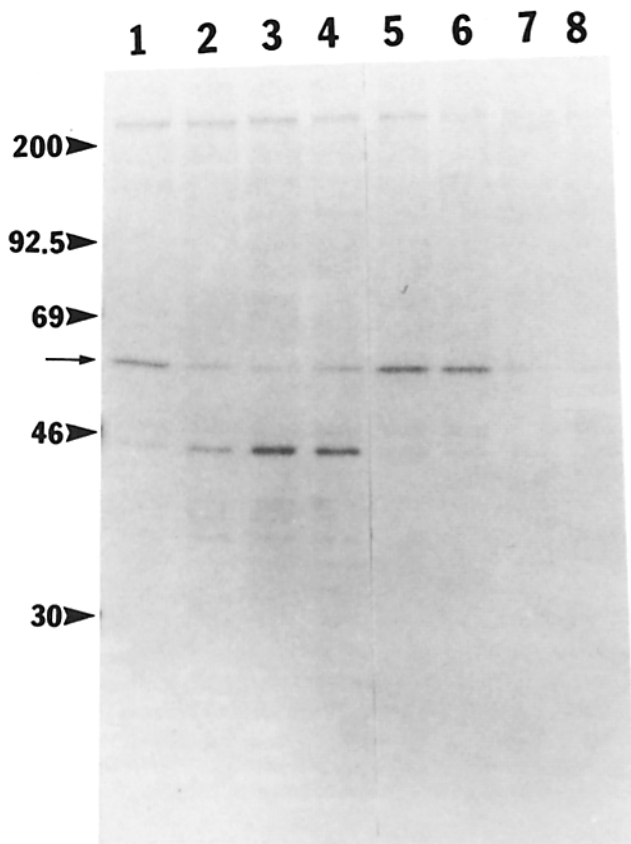
Figure 4. Synthesis of statin in serum-stimulated confluent cultures. Quiescent confluent cultures of young fibroblasts (CPDL = 13) were stimulated with 10% serum for 0 (lanes *1* and *5*), 3 (lanes *2* and *6*), 7 (lanes *3* and *7*), or 14 h (lanes *4* and *8*), and then labeled with [^{35}S]methionine for 2 h in the presence of 10% serum. The Triton X-100–insoluble (lanes *1–4*) and -soluble (lanes *5–8*) fractions obtained from these cells by RIPA buffer extraction were immunoprecipitated by the antistatin antibody. The immunoprecipitates were analyzed by SDS-PAGE. Statin is indicated by an arrow. The band at 43 kD in lanes *1–4* represents actin nonspecifically precipitated.

Table I. DNA Synthesis in Serum-stimulated Cultures of Young Fibroblasts

Hours of serum stimulation before labeling	[methyl-^{3}H]Thymidine incorporation	
	Quiescent confluent cultures (6 × 10^5 cells/cm^2)	Quiescent sparse cultures (9 × 10^3 cells/cm^2)
	cpm	*cpm*
0	10,780	12,778
3	15,242	18,031
7	19,774	24,878
14	37,312	46,341
22	60,210	84,366
26	77,801	104,861

Quiescent sparse and confluent cultures were serum stimulated for designated lengths of time and then labeled with [methyl-^{3}H]thymidine for 1 h. The labeled cells were washed, fixed with TCA, and solubilized in 4 ml of 0.2 N NaOH, followed by neutralization with 0.4 ml of 2 N HCl. To measure the incorporation of radioactive label, 0.5 ml of the neutralized sample was taken out for scintillation counting. The measurement obtained from this scintillation counting was used for calculation of total [methyl-^{3}H]thymidine incorporation into the culture.

10 h before a substantial increase in DNA synthesis was first detected (see Table I). A 9-h serum stimulation caused further decrease (to 67%) in synthesis of the Triton X-100–soluble statin (Fig. 5 *b*, lane *7*). By the time DNA synthesis increased substantially after 16 h of serum stimulation (Table I), synthesis of the Triton X-100–soluble statin had dropped to a very low level (22%) (Fig. 5 *b*, lane *8*), which probably represents a basal level of the protein in the sparse culture serum-stimulated to reenter the S phase of the cell cycle. In comparison to the confluent culture (Fig. 4, lanes *6–8*), synthesis of the Triton X-100–soluble statin in the sparse culture decreased in a more gradual manner and to a lesser extent after 16 h of serum stimulation (Fig. 5 *b*, lanes *6–8*). On the other hand, synthesis of the coprecipitated actin in the sparse culture increased during serum stimulation (Fig. 5 *b*, lanes *1–4*), as is the case for the confluent culture (Fig. 4, lanes *1–4*).

Discussion

In this report we demonstrate by immunoprecipitation analysis of fractionated cell extracts prepared from replicating sparse cultures, quiescent confluent cultures, and quiescent serum-starved cultures of young human fibroblasts that there exist two populations of statin: one that is Triton X-100 soluble and is present in replicating fibroblasts as well as in quiescent ones, and the other that is Triton X-100 insoluble and is present only in quiescent cells. That the Triton X-100–insoluble statin is found only in the quiescent cells is consistent with the previous immunogold labeling data (22) that statin is present in the nuclear envelope of quiescent fibroblasts but absent in that of replicating fibroblasts. Taken together, these results suggest that the Triton X-100–soluble statin, unlike the Triton X-100–insoluble one, is located in cellular compartment(s) other than the nuclear envelope.

Pulse-chase analysis of quiescent confluent cultures of young human fibroblasts shows that statin is synthesized as a 57-kD polypeptide and is not processed from a precursor of different molecular mass. In cells pulse labeled for 30 min, the newly synthesized protein was found in both the Triton X-100–insoluble and -soluble fractions, following a distribution similar to the steady-state one. No precursor–product relationship between the Triton X-100–soluble and -insoluble statins was detected during the pulse and chase periods. However, analysis by two-dimensional gel electrophoresis shows that both populations of statin exhibit an isoelectric point of 5.3 and that only a single isoform is detected by the antistatin mAb. This suggests that there may be only one biochemically distinct species of statin and that the Triton X-100–soluble statin could be converted into the insoluble one by being assembled into the nuclear envelope or associated with other protein complexes there. It is possible that the conversion of the Triton X-100–soluble statin into the Triton X-100–insoluble one is a rapid process and takes less than 30 min, and that a constant level of the Triton X-100–soluble statin is maintained in the cells. If this is the case, the precursor–product relationship between the two populations of statin could not be readily detected during the chase periods of 30 min or longer, and the newly synthesized Triton X-100–insoluble statin detected in the 30-min pulse period could be originated from the Triton X-100–soluble one. More
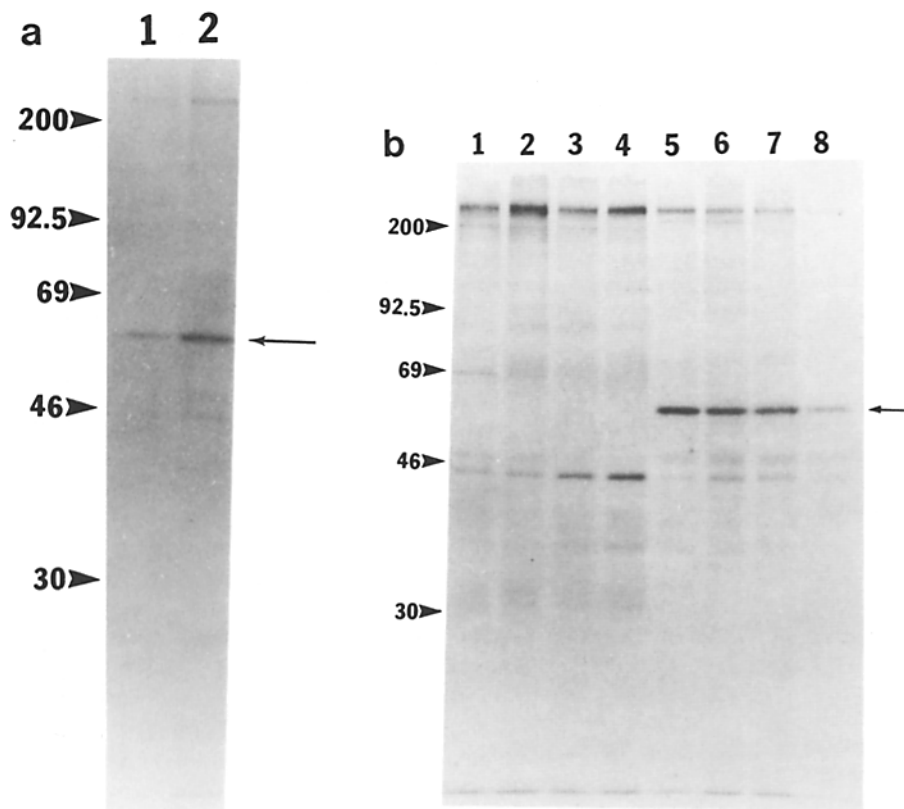
Figure 5. Synthesis of statin in serum-stimulated sparse cultures. (*a*) Quiescent sparse culture of young fibroblasts (CPDL = 13) labeled with [^{35}S]methionine for 2 h in the absence of serum was extracted with RIPA buffer and separated into the Triton X-100–insoluble (lane *1*) and -soluble (lane *2*) fractions. (*b*) Quiescent sparse cultures of young fibroblasts (CPDL = 13) were stimulated with 10% serum for 0 (lanes *1* and *5*), 3 (lanes *2* and *6*), 7 (lanes *3* and *7*), or 14 h (lanes *4* and *8*) and then labeled with [^{35}S]methionine for 2 h in the presence of 10% serum. The labeled cultures were extracted with RIPA buffer and separated into the Triton X-100–insoluble (lanes *1–4*) and -soluble (lanes *5–8*) fractions. All cell fractions were immunoprecipitated by the antistatin antibody and the resulting immunoprecipitates were subjected to SDS-PAGE. Statin is indicated by an arrow. The band at 43-kD represents actin nonspecifically precipitated.

elaborate experiments will be needed to verify this possibility.

Our present studies also demonstrate that synthesis of both the Triton X-100–insoluble and -soluble statin decreases substantially when serum-depleted quiescent cells are serum-stimulated to leave the G0 phase of the cell cycle, and this decrease precedes reinitiation of DNA synthesis. These results suggest that statin is synthesized preferentially and is present in greater abundance during the G0 phase of the cell cycle. In particular, the fact that a large amount of the Triton X-100–insoluble statin is present specifically in the nuclear envelope of quiescent fibroblasts confirms the previous findings (22, 25) that the nuclear statin can be used as a marker for cells arrested at the G0 phase. Thus, cellular expression of statin appears to be associated with the mechanisms controlling the transition between the growing state and the quiescent state.

It should be noted that synthesis of the Triton X-100–insoluble statin is reduced more rapidly by serum stimulation than synthesis of the Triton X-100–soluble statin. In quiescent confluent culture, synthesis of the Triton X-100–insoluble statin dropped drastically to a low basal level after a 5-h serum stimulation. In quiescent sparse culture, synthesis of the Triton X-100–insoluble statin was more responsive to serum and was turned off during the 2-h labeling in the presence of 10% serum. In both kinds of cultures, a substantial increase in DNA synthesis was not detected until ∼14–15 h after serum stimulation. There thus exists a lag of 10–13 h between reinitiation of DNA synthesis and the observed changes in synthesis of the Triton X-100–insoluble statin induced in the sparse cultures and confluent cultures by serum

stimulation. Previous immunofluorescence studies have shown that statin disappears rapidly from the nuclei when serum-depleted cells are promoted to leave the quiescent state upon serum stimulation and that this loss of nuclear statin occurs before DNA synthesis (25). Together with these previous data, the 10–13-h lag suggests that an early inhibition in synthesis of the Triton X-100–insoluble statin induced by serum stimulation may be important to ensure a rapid disappearance of the protein from the nuclear envelope of the serum-stimulated cells, a step which may be necessary before DNA synthesis can be reinitiated. Since it is possible that there may be only one biochemically distinct species of statin and the Triton X-100–soluble statin could be converted into the insoluble form by being assembled into the nuclear envelope (see Discussion above), the observed reduction in the amounts of the newly synthesized Triton X-100–insoluble statin in the serum-stimulated cells may reflect an inhibition in the rate of assembly of the protein into the nuclear envelope rather than synthesis of the protein per se. This inhibition of the assembly rate, accompanied by a decrease in the synthesis of the Triton X-100–soluble statin, could account for the rapid disappearance of the protein from the nuclear envelope of the serum-stimulated cells before reinitiation of DNA synthesis. Presently, it is not known whether the nuclear envelope-localized, Triton X-100–insoluble statin plays any role in maintaining cells in the quiescent state and/or inactivating DNA synthesis, thereby preventing cells from entry into the S phase of the cell cycle.

It is interesting to make a comparison between statin and another nuclear protein, cyclin. Cyclin, also called proliferating cell nuclear antigen (12, 13, 18), is a nuclear protein

initially identified to be present only in replicating or cycling cells and preferentially synthesized during the S phase of the cell cycle (1, 2, 5, 8, 10). Immunofluorescence studies have revealed dramatic changes in its nuclear distribution during the S phase (3, 7). It has recently been shown to be an auxiliary protein for DNA polymerase-δ (6, 17, 19), and required for SV40 DNA replication in vitro (16). At the time this manuscript was prepared, Bravo and Macdonald-Bravo (4) reported existence of two populations of cyclin during the S phase: one that is nucleoplasmic and is extracted by Triton X-100, and another that is associated with the DNA replication sites and is Triton X-100–insoluble. The nucleoplasmic, detergent-soluble cyclin, but not the detergent-insoluble one, is also found in quiescent cells. Although statin and cyclin show different expression patterns and seem to play different roles in the control of cell proliferation, both of them exist in two populations in the cells. Presently, it is not clear what the significance of having two populations of these proteins is, and whether existence of two populations of a protein is a common phenomenon for proteins involved in the mechanisms controlling cell proliferation. It is also not known if there is any interaction between the proteins. Further studies with these and other proteins associated with cell growth are needed to answer the questions.

References

1. Bravo, R. 1986. Synthesis of the nuclear protein cyclin (PCNA) and its relationship with DNA replication. *Exp. Cell Res.* 163:287–293.
2. Bravo, R., and J. E. Celis. 1980. A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. *J. Cell Biol.* 84:795–802.
3. Bravo, R., and H. Macdonald-Bravo. 1985. Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:655–661.
4. Bravo, R., and H. Macdonald-Bravo. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J. Cell Biol.* 105:1549–1554.
5. Bravo, R., S. J. Fey, J. Bellatin, P. Mose Larsen, J. Arevalo, and J. E. Celis. 1981. Identification of a nuclear and of a cytoplasmic polypeptide whose relative proportions are sensitive to changes in the rate of cell proliferation. *Exp. Cell Res.* 136:311–319.
6. Bravo, R., R. Frank, P. A. Blundell, and H. Macdonald-Bravo. 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. *Nature (Lond.).* 326:515–517.
7. Celis, J. E., and A. Celis. 1985. Cell cycle-dependent variations in the distribution of the nuclear protein cyclin in cultured cells. *Proc. Natl. Acad. Sci. USA.* 82:3262–3266.
8. Celis, J. E., P. Madsen, S. Nielsen, and A. Celis. 1986. Nuclear patterns of cyclin (PCNA) antigen distribution subdivide S-phase in cultured cells. *Leuk. Res.* 10:237–249.
9. Hollenberg, M. D., and P. Cuatrecasas. 1975. Insulin and epidermal growth factor: human fibroblast receptors related to deoxyribonucleic acid synthesis and amino acid uptake. *J. Biol. Chem.* 250:3845–3853.
10. Kurki, P., M. Vanderlaan, F. Dolbeare, J. Gray, and E. M. Tan. 1986. Expression of proliferating cell nuclear antigen (PCNA)/cyclin during the cell cycle. *Exp. Cell Res.* 166:209–219.
11. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
12. Mathews, M. B., R. M. Bernstein, B. R. Franza, and J. I. Garrels. 1984. The identity of the proliferating cell nuclear antigen and cyclin. *Nature (Lond.).* 309:374–376.
13. Miyashi, K., M. J. Fritzler, and E. M. Tan. 1978. An autoantibody to a nuclear antigen in proliferating cells. *J. Immunol.* 121:2228–2234.
14. O'Farrell, P. H. 1977. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
15. Ottaviano, Y., and L. Gerace. 1985. Phosphorylation of the nuclear lamins during interphase and mitosis. *J. Biol. Chem.* 260:624–632.
16. Prelich, G., M. Kostura, D. R. Marshak, M. B. Mathews, and B. Stillman. 1987. The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication *in vitro*. *Nature (Lond.).* 326:471–475.
17. Prelich, G., C. K. Tan, M. Kostura, M. B. Mathews, A. G. So, K. M. Downey, and B. Stillman. 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. *Nature (Lond.).* 326:517–520.
18. Takasaki, J., D. Fischwild, and E. M. Tan. 1984. Characterization of proliferating cell nuclear antigen recognized by autoantibodies in lupus sera. *J. Exp. Med.* 159:981–992.
19. Tan, C. K., C. Castillo, A. G. So, and K. M. Downey. 1986. An auxiliary protein for DNA polymerase-δ from fetal calf thymus. *J. Biol. Chem.* 261:12310–12316.
20. Wang, E. 1985. A 57,000-mol-wt protein uniquely present in nonproliferating cells and senescent human fibroblasts. *J. Cell Biol.* 100:545–551.
21. Wang, E. 1985. Are cross-bridging structures involved in the bundle formation of intermediate filaments and the decrease in locomotion that accompany cell aging? *J. Cell Biol.* 100:1466–1473.
22. Wang, E. 1985. Rapid disappearance of statin, a nonproliferating and senescent cell-specific protein, upon reentering the process of cell cycling. *J. Cell Biol.* 101:1695–1701.
23. Wang, E., and D. Gundersen. 1984. Increased organization of cytoskeleton accompanying the aging of human fibroblasts *in vitro*. *Exp. Cell Res.* 154:191–202.
24. Wang, E., and J. G. Krueger. 1985. Application of a unique monoclonal antibody as a marker for nonproliferating subpopulations of cells of some tissues. *J. Histochem. Cytochem.* 33:587–595.
25. Wang, E., and S. L. Lin. 1986. Disappearance of statin, a protein marker for nonproliferating and senescent cells, following serum stimulated cell cycle entry. *Exp. Cell Res.* 167:135–143.