Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5291–5295, June 1994
Cell Biology

A cell cycle-regulated inhibitor of cyclin-dependent kinases

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Communicated by Peter K. Vogt, March 10, 1994

ABSTRACT **Cyclin-dependent kinases (Cdks) previously have been shown to drive the major cell cycle transitions in eukaryotic organisms ranging from yeast to humans. We report here the identification of a 28-kDa protein, p28^{Ick} (inhibitor of cyclin-dependent kinase), that binds to and inhibits the kinase activity of preformed Cdk/cyclin complexes from human cells. p28 inhibitory activity fluctuates during the cell cycle with maximal levels in G_1 and accumulates in G_1- and G_0-arrested cells. These results suggest that control of the G_1/S transition may be influenced by a family of Cdk inhibitors that include p28^{Ick} and the recently described inhibitors p21$^{Cip1/Waf1/Cap20}$ and p16^{Ink4}.**

Cell cycle progression is controlled at several irreversible transition points. Passage through these transitions is based on regulation of the activity of a family of related protein kinases (cyclin-dependent kinases or Cdks). Cyclins have been demonstrated to promote cell cycle transitions by binding and activating specific Cdks (1–3). Progression from G_1 to S phase in mammalian cells involves activation of cyclin E/Cdk2, cyclin D/Cdk4, and cyclin A/Cdk2 (4, 5). Cyclins D1 and E accumulate and activate distinct Cdk proteins in G_1, while the kinetics of cyclin A accumulation and Cdk activation suggest an S-phase role (5). However, cyclin accumulation and Cdk binding do not constitute the only levels of Cdk regulation. Additional modes of regulation of Cdks include positive and negative phosphorylation events and accumulation or activation of inhibitory proteins. Initially demonstrated for the yeast Cdk Cdc28 (6, 7), Cdk inhibitory proteins have recently been implicated in G_1 control of mammalian cells. Whereas p21$^{Cip1/Waf1/Cap20}$ is a p53-induced inhibitor of a broad range of Cdk/cyclin complexes (8–11), p16^{Ink4} specifically inhibits cyclin D/Cdk4 (12). Another inhibitory protein, p27^{Kip1}, has been identified in transforming growth factor β-arrested and in contact-inhibited cells (13, 14).

In the course of investigating the mechanism of lovastatin arrest in HeLa cells, we found high levels of inactive cyclin E/Cdk2 and cyclin A/Cdk2 complexes, suggesting the presence of a Cdk inhibitor. This paper describes the identification of a protein present in lovastatin-arrested and cycling G_1 cells or G_0 cells that inhibits a broad range of cyclin/Cdk complexes.

MATERIALS AND METHODS

Cell Culture and Sychronizations. HeLa cells were grown in suspension culture in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum at a density of 2–6 × 10^5 cells per ml. Asynchronous growing cells were arrested in G_1 by treatment with 66 μM lovastatin for 33 hr as described (15). Lovastatin was kindly provided by A. W. Alberts (Merck Sharp & Dohme).

Cells were synchronized by a thymidine/nocodazole block release protocol using 2 mM thymidine for 19 hr, release for 3 hr, and treatment with nocodazole (75 ng/ml) for 12 hr. Aliquots of the synchronized cells were harvested every hour after release from the nocodazole block and the percentage of cells in different phases of the cell cycle was determined by FACS analysis as described (16). Fibroblasts derived from neonatal foreskin (NHF1) were cultured as described (17) and were made quiescent by contact inhibition (17).

Immunoprecipitation, Immunoblots, and Inhibition Analysis. Immunoblots and immunoprecipitations of cyclin and Cdk complexes from whole cell extracts were performed with polyclonal (anti-cyclins A, B1, E, D1, anti-cdc2, and anti-Cdk2) or monoclonal (anti-cyclin E and anti-PSTAIRE) antibodies as described (18). Antibodies against human Cdk4 were a gift from S. Hanks (Vanderbilt University).

Inhibition experiments were performed either by adding inhibitor-containing fractions to immunoprecipitated kinase complexes or by mixing crude extracts or inhibitor-containing fractions before immunoprecipitation. In mixing experiments, extracts containing active kinase complexes were incubated in lysis buffer [50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride (PMSF)/2 μg of leupeptin per ml/2 μg of pepstatin per ml/10 μg of aprotinin per ml] with inhibitor-containing extracts for 30 min at 30°C followed by immunoprecipitation of Cdk/cyclin complexes and analysis of their associated kinase activity. Alternatively, cyclin/Cdk immune complexes were immobilized on protein A-Sepharose beads and incubated with inhibitor-containing extracts or fractions for 30 min at 30°C before they were washed several times in lysis buffer and the associated kinase activity was determined. The assay for cyclin D1-associated kinase activity was performed as described (19).

To release inhibitory activity from complexes, protein extracts were incubated at 100°C for 5 min followed by centrifugation, and the inhibitory activity was recovered in the supernatant containing heat stable proteins. Proteinase K (0.1 mg/ml) digests of gel-purified p28^{Ick} were performed in lysis buffer for 80 min at room temperature. The protease was inactivated by PMSF treatment (2.5 mM PMSF for 15 min at room temperature).

Fractionation of Protein Extracts. *Superose 12 chromatography.* Protein extracts in lysis buffer were spun at 100,000 × *g* for 20 min. Supernatant containing 2 mg of protein was either boiled or directly applied to a Superose 12 column connected to an FPLC system (Pharmacia); 0.5-ml fractions were collected and 50-μl aliquots were assayed for cyclin A/Cdk2 inhibitory activity.

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Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

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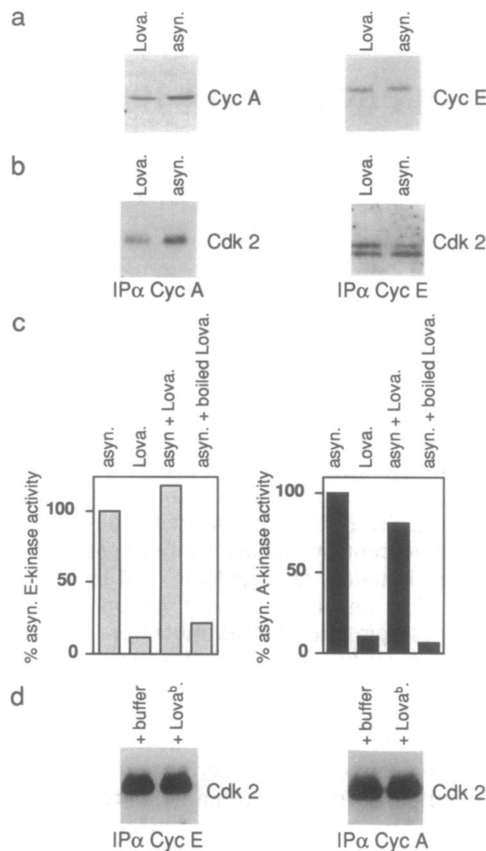

FIG. 1. Lovastatin-arrested HeLa cells contain Cdk2-bound cyclin A and cyclin E proteins with low associated kinase activities and a heat stable cyclin/Cdk inhibitory activity. (*a*) Western blot analysis of protein extracts from lovastatin-arrested (Lova.) and asynchronous (asyn.) HeLa cells. Cyclin A and cyclin E proteins were detected by using polyclonal antibodies (17). (*b*) Cdk2 protein bound to cyclin A and cyclin E was detected after immunoprecipitation from extracts of lovastatin-arrested or asynchronous HeLa cells followed by immunoblotting. Cyclin E-associated Cdk2 was separated into the unphosphorylated inactive form (upper band) and the active faster-migrating phosphorylated form (18, 21). (*c*) Cyclin E- and cyclin A-associated histone H1 kinase activities from lovastatin-arrested or asynchronous HeLa cells were measured after immunoprecipitation (IPα) (18). To detect cyclin/Cdk inhibitory activity, extracts from asynchronous cells were mixed with boiled or nonboiled extracts from lovastatin-arrested cells followed by immunoprecipitation of cyclin A- or E-associated kinases. Kinase activities are shown as percentage of activity obtained from immunoprecipitation of asynchronous cells. (*d*) After mixing protein extracts from asynchronous cells with boiled extracts from lovastatin-arrested cells (Lova^b.), cyclin A or cyclin E bound to Cdk2 was detected as described above.

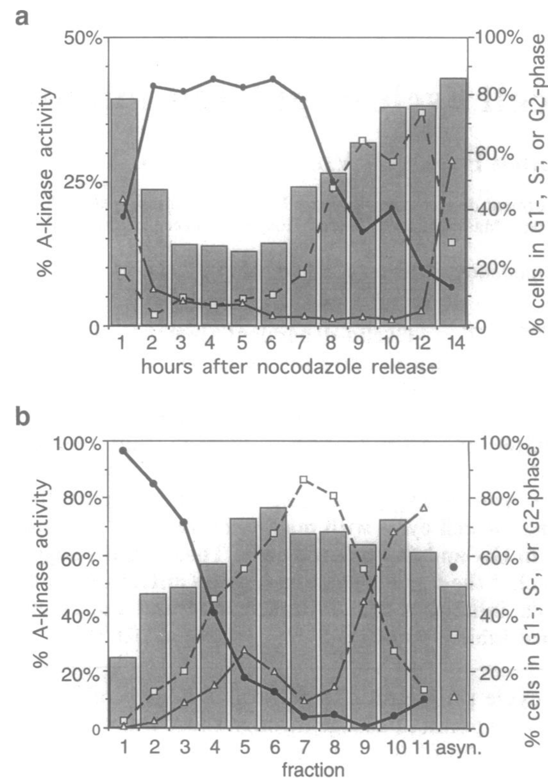

FIG. 2. Cyclin/Cdk inhibitory activity varies during the cell cycle and is maximal in G_1. (*a*) HeLa cells were synchronized by using a thymidine/nocodazole arrest release protocol. (*b*) Human erythroleukemia-derived HL-60 cells were fractionated by elutriation as described (22). Percentages of cells in the different phases of the cell cycle [G_1 (●), S (□), G_2/M (△)] are shown for each synchronized population or the asynchronous cells used for the elutriation experiment. Protein extract (50 μg) from the different synchronized populations was boiled and mixed with 50 μg of protein from asynchronous HeLa cells. Cyclin A-associated kinase activity (bars) was then determined and is shown as percentage of activity obtained from 50 μg of protein from asynchronous cells (asyn.).

SDS/PAGE elution experiments. Protein extracts of double thymidine-blocked cells (30 mg), G_1 synchronized cells (30 mg), or lovastatin-arrested cells (20 mg) were boiled for 5 min, and insoluble proteins were precipitated by centrifugation at 12,000 × *g* for 10 min and at 100,000 × *g* for 20 min. The supernatant (1 mg of protein from S-phase extracts or from G_1-phase extracts) was precipitated with 2 vol of cold (−20°C) acetone, resuspended in 2× sample buffer [100 mM Tris·HCl, pH 6.8/200 mM dithiothreitol/4% SDS/0.2% bromphenol blue/20% (vol/vol) glycerol], and separated by SDS/PAGE on a 12.5% gel. Slices (3 mm) of the stained gel were excised and proteins were extracted, acetone precipitated, and renatured as described (10); 20% of the renatured protein fractions were used in kinase inhibition experiments.

Serial Depletion of Boiled Inhibitor. Protein extracts from lovastatin-arrested cells (50 μg per sample) were boiled and the supernatants were used for depletion experiments. All samples were incubated six times for 15 min at 30°C with protein A-Sepharose beads: x = 0–6 times with protein A-Sepharose beads containing cyclin A immune complexes from 50 μg of protein from asynchronous HeLa cells and $6 - x$ times with protein A-Sepharose beads only. The samples ($0x$–$6x$) were analyzed for inhibitory activity by mixing with additional cyclin A immune complexes from 50 μg of extract from asynchronous cells. After an incubation at 30°C for 30 min, the histone H1 kinase activity was measured.

RESULTS AND DISCUSSION

Inactive Cyclin A/Cdk2 and Cyclin E/Cdk2 Complexes in Lovastatin-Arrested HeLa Cells. The kinase activity of cyclin/Cdk2 complexes varies through the cell cycle in parallel with the abundance of the cyclin E and cyclin A subunits, suggesting that cyclin availability is the primary mode of kinase regulation (5, 18, 20). However, in HeLa cells arrested in G_1 by lovastatin (15), levels of both cyclins A and E were high in the arrested cells (Fig. 1*a*), and these cyclins formed complexes with Cdk2 (Fig. 1*b*), but cyclin/Cdk2 activity was low (Fig. 1*c*). These data suggested that there was likely to be an additional mechanism for inhibiting Cdk2 activity, and we speculated that this might involve binding of inhibitory proteins to the Cdk2/cyclin complexes.

Boiled Extracts of Lovastatin-Arrested Cells Contain a Cyclin/Cdk Inhibitory Activity. To assay potential inhibitors,

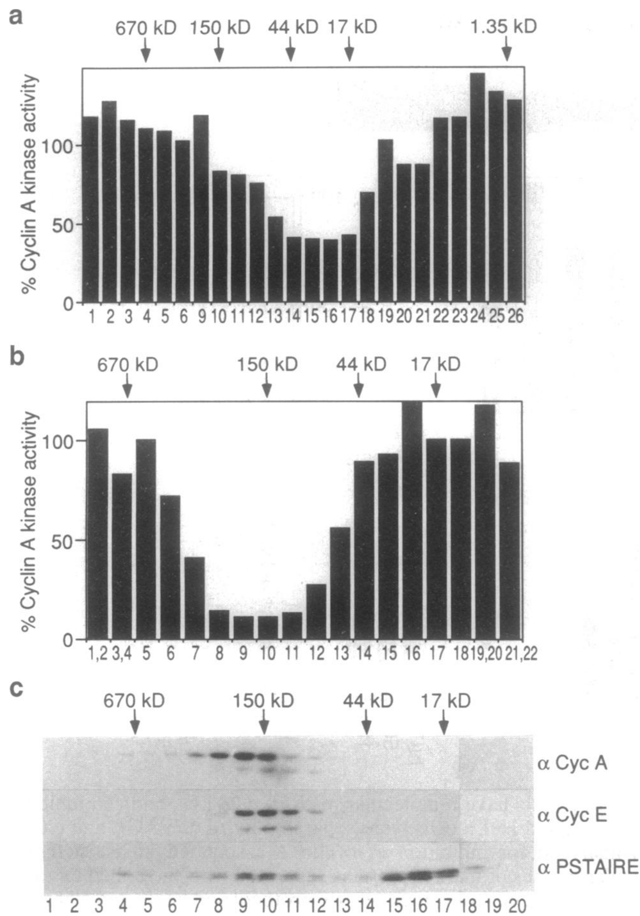

FIG. 3. A small inhibitor is associated with a larger protein complex in crude extracts from which it can be released by boiling. Protein extracts from lovastatin-arrested HeLa cells were either boiled and the supernatant was fractionated on a Superose 12 gel-filtration column (*a*) or they were fractionated without boiling and each fraction was subsequently boiled (*b*). Samples of each fraction were added to asynchronous cell extracts and analyzed for inhibitory activity. Inhibition of histone H1 kinase activity is shown as percentage of uninhibited cyclin A-associated kinase activity obtained from asynchronous cells. (*c*) Fractions obtained in *b* were immunoblotted and analyzed for the presence of cyclin A or E proteins. Cdk protein levels were detected by using a monoclonal antibody against the PSTAIRE motif.

cell lysates of lovastatin-treated cells were added to lysates obtained from cycling cells that contained active cyclin E/Cdk2 or cyclin A/Cdk2 complexes. In the course of numerous experiments, no or very weak inhibition of kinase activities was observed (Fig. 1*c*), suggesting that if inhibitors were present, they must be inaccessible, possibly bound to endogenous cyclin/Cdk complexes or other proteins. We tried a variety of chaotropic treatments to dissociate such ternary complexes and found that simply boiling the lysates for 5 min was sufficient to release a potent inhibitor of cyclin E/Cdk2 and cyclin A/Cdk2 kinases (Fig. 1*c*). Inhibition obtained from boiled extracts was not simply due to degradation of either the cyclin or the Cdk2 subunit of the complex or to cyclin/Cdk2 dissociation (Fig. 1*d*). These results were consistent with the accumulation of a heat stable inhibitory protein that bound to the cyclin E/Cdk2 and cyclin A/Cdk2 complexes in lovastatin-treated cells.

A Cell Cycle-Dependent Inhibitor. We wished to determine whether this heat stable inhibitory activity played a regulatory role in cycling cells as well as in arrested cells. We measured the inhibitory activity in boiled lysates prepared from synchronized cells as a function of progression through

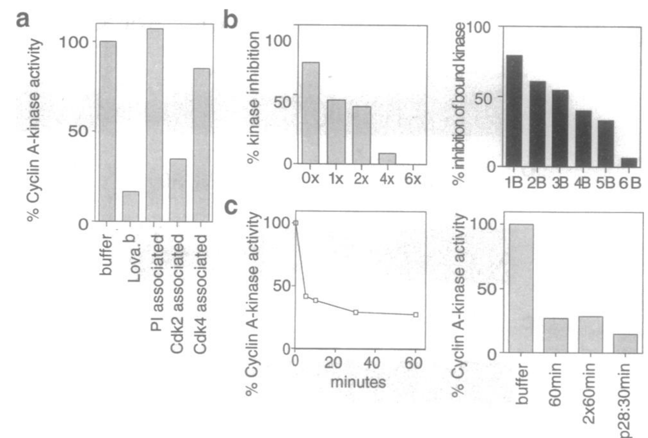

FIG. 4. Inhibitory activity is coprecipitated with Cdk immune complexes and binds *in vitro* to cyclin A/Cdk2 complexes following saturable approximate first-order kinetics. (*a*) Lysates of lovastatin-arrested cells were immunoprecipitated with preimmune (PI), anti-Cdk2, or anti-Cdk4 antiserum. Immunoprecipitates and the non-treated extract (Lova.[b]) were boiled and supernatants were assayed for inhibitory activity as described (see Fig. 1*c*). (*b*) To demonstrate binding of the inhibitor protein to cyclin/Cdk complexes, a depletion experiment was performed. Inhibitor-containing supernatants from boiled extracts of lovastatin-arrested cells were incubated six successive times with or without immobilized cyclin A kinase immune complexes and remaining inhibitor in the supernatant was assayed. 6*x*, Incubation with immunoprecipitated kinase complexes only; 0*x*, incubation without kinase complexes; 1*x*, 2*x*, and 4*x*, intermediate times with or without immunoprecipitated kinase complexes. To prove that the inhibitor was bound to the added kinase complexes, the residual kinase activity of the immune complexes used in successive depletion steps was determined (solid bars). Percentage inhibition was calculated by using the kinase activity of noninhibited cyclin A immune complexes as 0% and no detectable kinase activity as 100% kinase inhibition. 1B, Inhibition of kinase used in the first round of depletion, 2B–6B, successive rounds of depletion. (*c*) Kinetics of inhibitor activity was determined by incubating cyclin A/Cdk2 complexes with a limiting but fixed amount of lovastatin lysate for 5, 10, 30, and 60 min (line graph). The same amount of inhibitor was incubated without cyclin/Cdk complexes for 60 min and then assayed using a 60-min incubation with cyclin A/Cdk2 complexes (2 × 60 min), showing that the inhibitory activity is completely stable under incubation conditions (bar graph). A greater level of inhibition with gel-purified inhibitor (see Fig. 5*a*) is also shown (p28: 30 min).

the cell cycle. In HeLa cells synchronized by a thymidine/nocodazole arrest/release protocol, inhibitory activity was maximal in mid-G_1 and declined as cells approached S phase (Fig. 2*a*). Similarly, in HL-60 erythroleukemia-derived cells fractionated by centrifugal elutriation, inhibitory activity fluctuated through the cell cycle with a peak in the most G_1-enriched fractions (Fig. 2*b*). These results showed that the activity of a heat stable Cdk inhibitor fluctuated during the cell cycle in two unrelated cell types synchronized by disparate methods, with maximal levels occurring in G_1.

Boiling Releases an ≈30-kDa Inhibitor from 150- to 250-kDa Cdk/Cyclin Complexes. To determine the approximate size of the inhibitor, boiled lysates were fractionated on a Superose 12 column, and inhibitory activity was recovered in a single peak centered around 30 kDa (Fig. 3*a*). Lysates that had not been boiled contained no detectable inhibitor, but the inhibitor could be assayed after fractionation by boiling the individual fractions. Inhibitor in unboiled lysates was recovered in fractions corresponding to an apparent molecular mass of 150–250 kDa (Fig. 3*b*), the same fractions containing cyclin A/Cdk2 and cyclin E/Cdk2 complexes (Fig. 3*c*). In contrast, no inhibitor was recovered in the fractions that contained monomeric Cdk proteins. These data indicate that

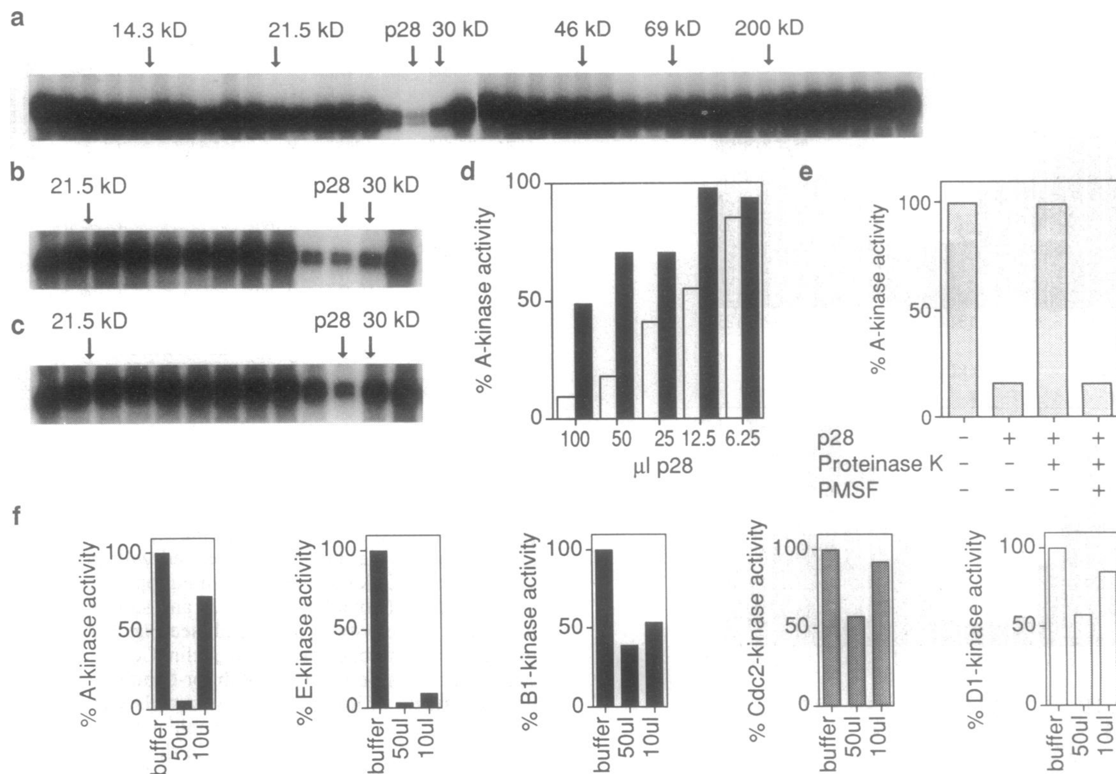

FIG. 5. Inhibitory proteins found in lovastatin-arrested cells and cycling HeLa cells have a molecular mass of ≈28 kDa and can inhibit a broad spectrum of cyclin/Cdk complexes. (*a*) Protein extracts from lovastatin-arrested HeLa cells were separated by SDS/PAGE and eluted proteins of fractions obtained from 3-mm gel slices were renatured and analyzed for inhibition of cyclin A-associated kinase activity. Autoradiograph of histone H1 kinase assay is shown with the apparent molecular masses of marker proteins separated with the boiled extracts above the autoradiograph. (*b*) HeLa cells were synchronized by using the thymidine/nocodazole block release protocol and harvested 5 hr after the release from nocodazole to obtain G_1 cells. Heat stable extracts were fractionated and analyzed as in *a*. (*c*) Double thymidine-blocked HeLa cells arrested in S phase were analyzed as in *a* and *b*. Experiments in *b* and *c* were performed in parallel and both autoradiographs were obtained after identical exposure time (5 min). (*d*) Between 100 and 6.25 µl of the eluted peak fractions containing p28 of mid-G_1 (open bars) or S-phase-arrested (solid bars) HeLa cells obtained in *b* and *c* were used to inhibit cyclin A-associated kinase activity. Histone H1 kinase activity is shown as percentage of uninhibited cyclin A-associated kinase activity. (*e*) Gel-purified p28 obtained from the experiment shown in *a* was incubated with proteinase K or proteinase K that had been inactivated with PMSF. After incubation for 80 min at room temperature, the protease was inactivated by PMSF treatment and inhibition of cyclin A-associated kinase activity was assayed. (*f*) p28 isolated from lovastatin-arrested cells as described in *a* was used to determine the specificity of $p28^{Ick}$. Cyclin/Cdk complexes immunoprecipitated from 100 µg of double thymidine-blocked cells (cyclins A and E), 100 µg of nocodazole-arrested cells (cyclin B1 and Cdc2), or 500 µg of asynchronous fibroblast extracts (cyclin D1) were prebound to protein A-Sepharose and 50 or 10 µl of the eluted p28 was added. After incubation for 30 min at 30°C, the remaining kinase activity was determined with histone H1 (cyclins A, E, and B1, Cdc2) or a glutathione *S*-transferase–pRb fusion protein (23) (cyclin D1) as substrates. Since different amounts of kinases of different origins were used for the inhibition experiment, results cannot be taken as quantitative. In particular, low efficiency of the cyclin D-associated kinase assay required immunoprecipitation of large amounts of Cdk4/cyclin D1 complexes.

the inhibitor in lovastatin-arrested HeLa cells is a small protein of ≈30 kDa that is bound in large complexes.

To determine whether the inhibitor was bound to cyclin/Cdk complexes *in vivo*, accounting for their inactive state, Cdk2 and Cdk4 immunoprecipitates were boiled and supernatants were analyzed for inhibitory activity on cyclin A/Cdk2 complexes (Fig. 4*a*). Significant amounts of inhibitor activity were recovered from Cdk2 but not from Cdk4 immune complexes. The inability to recover inhibitory activity from Cdk4 immune complexes may be due to the low abundance of cyclin D/Cdk4 complexes in HeLa cells (unpublished results).

If inhibition of cyclin/Cdk activity *in vivo* and *in vitro* is due to stoichiometric binding of the inhibitor to kinase complexes, then incubation of inhibitor-containing lysates with immobilized cyclin/Cdk complexes should deplete the inhibitor from the lysate. Conversely, if the inhibitor acts in a catalytic fashion that does not involve tight binding to kinase complexes, it should remain in the lysate upon removal of the complexes. To distinguish between these possibilities, boiled inhibitor-containing lysates from lovastatin-treated cells were incubated sequentially with beads carrying bound cyclin A/Cdk2 kinase complexes or control beads (to control for the effects of the sequential incubation procedure). Cyclin A/Cdk2 beads clearly depleted inhibitor from the lysate, whereas control beads did not (Fig. 4*b*), consistent with a mechanism based on binding. Binding of the inhibitor to cyclin/Cdk complexes does not prove a stoichiometric mechanism of action. We showed, however, that inhibition of cyclin/Cdk complexes follows saturable approximate first-order kinetics, strongly suggestive of a stoichiometric rather than a catalytic mechanism (Fig. 4*c*).

The Inhibitor from Cycling or Lovastatin-Arrested HeLa Cells or Contact-Inhibited Fibroblasts Is a Protein of ≈28 kDa That Can Inhibit the Kinase Activity of a Broad Spectrum of Cdk/Kinase Complexes. The biochemical properties of this inhibitor (a small heat stable protein that inhibits cyclin/Cdks through direct binding) were similar to those of a recently identified 21-kDa Cdk inhibitor, Cip1/Waf1/Cap20 (8–11). To determine whether the inhibitor in lovastatin-treated cells was identical to $p21^{Cip1/Waf1/Cap20}$, we obtained a more accurate estimate of its molecular mass by SDS/PAGE (Fig. 5*a*). A single major peak of Cdk2/cyclin A inhibitory activity was eluted from gels at ≈28 kDa (Fig. 5*a*). This molecular mass

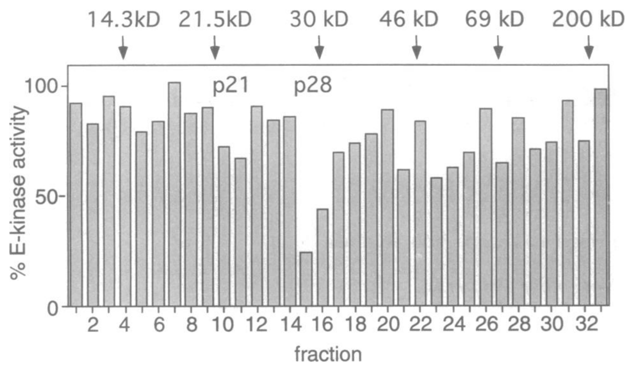

FIG. 6. A fibroblast strain derived from neonatal foreskin (NHF1) was made quiescent by contact inhibition. Extracts were boiled, separated by SDS/PAGE, eluted, and analyzed for inhibition of cyclin E-associated kinase activity as described in Fig. 5.

distinguishes the inhibitor in lovastatin-treated cells (henceforth $p28^{Ick}$) from $p21^{Cip1/Waf1/Cap20}$. Furthermore, reconstruction experiments in which recombinant $p21^{Cip1/Waf1}$ was added to p28-containing lysates prior to electrophoresis confirmed that the 28-kDa inhibitory activity was clearly distinct from the $p21^{Cip1/Waf1}$ inhibitory activity (data not shown). The molecular mass of $p28^{Ick}$ suggests that it may be the human homologue of the recently described Cdk inhibitor from mink epithelial cells $p27^{Kip1}$ (13).

To determine whether the inhibitor identified in cycling cells corresponded to p21 or p28 (or another protein), mid-G_1 HeLa cell lysates (high inhibitory activity) and thymidine-blocked S-phase cell lysates (low inhibitory activity) were separated by SDS/PAGE and analyzed as described above (Fig. 5 *b* and *c*). A nearly 10-fold higher level of 28-kDa inhibitory activity was detected in the G_1 sample (Fig. 5*d*).

Strong inhibitory activity has also been detected in lysates of human fibroblasts rendered quiescent by contact inhibition. The predominant inhibitory activity from growth-arrested fibroblasts corresponded to a 28-kDa protein based on the SDS/PAGE elution procedure and was therefore likely to be $p28^{Ick}$ (Fig. 6), suggesting that p28 also accumulates in fibroblasts subjected to density-mediated growth arrest.

We used the gel-purified $p28^{Ick}$ from lovastatin-arrested HeLa cells to prove that it is a protein (Fig. 5*e*) and to determine its specificity against a panel of immunoprecipitated cyclin/Cdk complexes. Inhibitory activity could be detected for cyclin A/Cdk2, cyclin E/Cdk2, cyclin B1/Cdc2, and total Cdc2-associated kinase immunoprecipitated from HeLa cells, as well as for cyclin D1-associated kinase immunoprecipitated from human fibroblasts (Fig. 5*f*). Although this analysis was not quantitative, it indicated that p28 was capable of inhibiting a broad spectrum of cyclin-dependent kinases.

The finding that p28 activity is cell cycle regulated and able to inhibit various cyclin/Cdk complexes suggests that it may play a role in the timing of cyclin/Cdk activation in proliferating cells, perhaps by preventing premature activation of those complexes in early and mid-G_1. The relationship between human $p28^{Ick1}$ and the similar mink $p27^{Kip1}$ (13) cannot be established at present, since the sequence of neither protein is available. Definitive assignment of the roles played by p28 and/or p27 as well as p21 in cell cycle control and in exit from the cell cycle awaits further characterization of these inhibitors.

We thank A. W. Alberts for lovastatin, E. Harlow and A. Fattaey for GST-pRB protein, M. Yamashita and Y. Nagahama for antibodies against the PSTAIRE motif, C. McGowan and J. Westendorf for antibodies against cyclin B1 and Cdc2, S. Hanks for antibodies against Cdk4, and D. Morgan for sharing unpublished data and the gel elution method. We also thank D. J. Lew, D. Resnitzky, and F. Melchior for many helpful discussions, ideas, and critical reading of the manuscript. L.H. was supported by a research fellowship of the Deutsche Forschungsgemeinschaft. This work was supported by National Institutes of Health Grant GM4006 and Grant 3165 from the Council for Tobacco Research to S.I.R.

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