$p27^{Kip1}$, a cyclin–Cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest

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Cell–cell contact and TGF-β can arrest the cell cycle in G_1. Mv1Lu mink epithelial cells arrested by either mechanism are incapable of assembling active complexes containing the G_1 cyclin, cyclin E, and its catalytic subunit, Cdk2. These growth inhibitory signals block Cdk2 activation by raising the threshold level of cyclin E necessary to activate Cdk2. In arrested cells the threshold is set higher than physiological cyclin E levels and is determined by an inhibitor that binds to cyclin E–Cdk2 complexes. A 27-kD protein that binds to and prevents the activation of cyclin E–Cdk2 complexes can be purified from arrested cells but not from proliferating cells, using cyclin E–Cdk2 affinity chromatography. p27 is present in proliferating cells, but it is sequestered and unavailable to interact with cyclin E–Cdk2 complexes. Cyclin D2–Cdk4 complexes bind competitively to and down-regulate the activity of p27 and may thereby act in a pathway that reverses Cdk2 inhibition and enables G_1 progression.

[*Key Words*: $p27^{Kip1}$; Cyclin E–Cdk2; TGF-β; contact inhibition; cell cycle arrest]

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Progression through the cell cycle is marked by a series of irreversible transitions that separate discrete tasks necessary for faithful cell duplication. These transitions are negatively regulated by signals that constrain the cell cycle until specific conditions are fulfilled. Entry into mitosis, for example, is inhibited by incompletely replicated DNA or DNA damage (Weinert and Hartwell 1988). Another feedback pathway delays the transition from M to G_1 if the mitotic spindle is defective (Hoyt et al. 1991; Li and Murray 1991). These restrictions on cell cycle progression are essential for preserving the fidelity of the genetic information during cell division (Hartwell and Weinert 1989). The transition from G_1 to S phase, on the other hand, coordinates cell proliferation with environmental cues, after which the checks on cell cycle progression tend to be cell autonomous (Hartwell et al. 1974; Pardee 1974, 1989). Among the extracellular influences that restrict cell cycle progression during G_1 are proteins that inhibit cell proliferation, growth factor or amino acid depletion, and cell–cell contact. Disruption of these signaling pathways uncouples cellular responses from environmental controls and may lead to unrestrained cell proliferation.

Transitions between phases of the cell cycle are catalyzed by a family of cyclin-dependent kinases (Cdks) (Nurse 1990; Hartwell 1991). In some organisms the physiological signals controlling the G_2 to M transition target a series of steps that activate the mitotic Cdk, Cdc2. Cdc2 activation is positively regulated by phosphorylation on threonine-161 (Gould et al. 1991; Krek and Nigg 1991; Solomon et al. 1992, 1993) and negatively by phosphorylation on tyrosine-15 (Gould and Nurse 1989). Incomplete DNA replication delays dephosphorylation of Tyr-15 (Dasso and Newport 1990; Smythe and Newport 1992), and mutations in Cdc2 that convert Tyr-15 to a nonphosphorylatable residue are lethal and cause premature mitosis (Gould and Nurse 1989). Similarly, either overexpression of the Tyr-15 phosphatase, Cdc25 (Enoch and Nurse 1990; Kumagai and Dunphy 1991), or loss of the Tyr-15 kinases (Lundgren et al. 1991) bypasses the requirement that DNA replication be completed before mitosis begins. Additional levels of control are probably required to fully explain the block to mitosis caused by ongoing DNA replication (Sorger and Murray 1992; Heald et al. 1993; Stueland et al. 1993). There is also evidence that cell cycle arrest induced by DNA damage

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may be related to inactivation of Cdc2 (Rowley et al. 1992; Walworth et al. 1993), but the role of tyrosine phosphorylation in this context has been questioned (Barbet and Carr 1993).

There is some evidence, particularly in yeast, that signals inhibiting the G_1- to S-phase transition block Cdk activation. The mating pheromone α-factor arrests the *Saccharomyces cerevisiae* cell cycle in G_1 (Reid and Hartwell 1977), and this correlates with a decrease in CDC28 kinase activity and a decline in the abundance of active complexes containing G_1 cyclins and CDC28 (Wittenberg et al. 1990). The FAR1 protein binds to G_1 cyclin–CDC28 complexes in cells treated with α-factor, and this is probably necessary for cell cycle arrest (Chang and Herskowitz 1990; Peter et al. 1993). Other inhibitors of CDC28 kinase activity have been identified, but their relationship to physiological signals that control cell cycle progression is not known (Dunphy and Newport 1989; Mendenhall 1993).

Mammalian cells, like yeast, require cyclin-dependent kinases for progression through G_1 and entry into S phase (Blow and Nurse 1990; D'Urso et al. 1990; Furukawa et al. 1990; Fang and Newport 1991; Pagano et al. 1993; Tsai et al. 1993). Both D- and E-type cyclins are rate limiting for the G_1 to S transition, and both reduce, but do not eliminate, the cell's requirement for mitogenic growth factors (Ohtsubo and Roberts 1993; Quelle et al. 1993). There is little information, however, concerning the manner by which these cyclins and Cdks are negatively regulated by extracellular signals that inhibit cell proliferation.

We have studied how two signals that block the cell cycle in G_1, cell–cell contact and transforming growth factor-β (TGF-β), affect the activity of a G_1 cyclin-dependent kinase, Cdk2 (Paris et al. 1991; Elledge and Spottswood 1991; Koff et al. 1991; Tsai et al. 1991; Elledge et al. 1992; Rosenblatt et al. 1992). The cell cycle of Mv1Lu mink epithelial cells can be arrested in G_1 by growth to high density. These contact-inhibited cells express both cyclin E and Cdk2, but cyclin E-associated kinase activity is not present (Koff et al. 1993). Entry into S phase can also be prevented if Mv1Lu cells are released from contact inhibition in the presence of TGF-β, and this correlates with a block to phosphorylation of the retinoblastoma (Rb) protein (Laiho et al. 1990). Both Cdk2 and Cdk4 have been implicated as Rb kinases (Hinds et al. 1992; Matsushime et al. 1992; Dowdy et al. 1993; Ewen et al. 1993a; Kato et al. 1993), suggesting that TGF-β-induced cell cycle arrest may involve inhibition of Cdks during G_1 (Howe et al. 1991). Consistent with this, cells arrested in late G_1 by TGF-β, like contact-inhibited cells, express both cyclin E and Cdk2 but do not contain catalytically active cyclin E–Cdk2 complexes (Koff et al. 1993). Cdk4 synthesis is also repressed by TGF-β (Ewen et al. 1993b). The inactivity of Cdk2, together with the absence of Cdk4, may explain the block to Rb phosphorylation in these cells. We show that contact-inhibited and TGF-β-treated cells, but not proliferating cells, contain a titratable excess of a 27-kD protein that binds to cyclin E–Cdk2 complexes and prevents their activation. The inhibitory activity of p27 can be competed by the cyclin D2–Cdk4 complex suggesting that p27 and cyclin D2–Cdk4 may function within a pathway that transmits growth inhibitory signals to Cdk2.

Results

Nonproliferating cells contain an inhibitor of Cdk2 activation

Cell-free extracts from contact-inhibited, TGF-β-arrested and proliferating cells were used to investigate the mechanism that blocks activation of the cyclin E–Cdk2 complex. We showed previously that addition of physiological amounts of cyclin E to these cell extracts resulted in an increase in the amount of immunoprecipitable cyclin E–Cdk2 complexes; however, only the cyclin E–Cdk2 complexes assembled in extracts from proliferating cells were enzymatically active using histone H1 as a substrate (Koff et al. 1993; see also Fig. 1A). Cell extracts therefore recapitulate the block to Cdk2 activation observed in intact cells.

The block to Cdk2 activation in extracts from nonproliferating cells could be overcome by addition of cyclin E protein to greater than physiological levels (Fig. 1A). Cyclin E was expressed in Sf9 cells using a baculoviral expression vector, and the amount of cyclin E in Sf9 extracts was compared to that in Mv1Lu cell extracts by immunoblotting (not shown). In the experiment illustrated in Figure 1A, 0.05 μl of Sf9 lysate contained as much cyclin E as 50 μg of total cell protein from Mv1Lu cell lysates. Addition of cyclin E (in the form of Sf9 lysate) to an extract from proliferating cells gave a linear increase in cyclin E-associated histone H1 kinase activity (proliferating cells were harvested 15 hr after release from contact inhibition, at which time they were in early S phase). In contrast, titration of up to three times physiological levels of cyclin E into extracts from contact-inhibited or TGF-β-treated cells resulted in no increase in immunoprecipitable cyclin E-associated kinase activity. As more cyclin E was added, cyclin E-associated kinase activity became detectable and increased in proportion. Thus, extracts from nonproliferating cells demonstrated an elevated threshold level of cyclin E necessary to activate Cdk2. Contact-inhibited cells appeared to have a higher threshold than cells arrested in G_1 by exposure to TGF-β, but in both cases the cyclin E requirement was substantially greater than the physiological levels of cyclin E achieved in proliferating cells.

Supra-physiological amounts of cyclin E were required to activate Cdk2 in extracts from nonproliferating cells. This could not be explained by lower levels of Cdk2 or cyclin E, nor did these cells appear to lack other factors necessary for Cdk2 activation (Koff et al. 1993; see below). One explanation was that nonproliferating cells contained a titratable inhibitor of Cdk2 activation. Mixing experiments supported this conclusion. Extracts from proliferating cells were mixed with those from either contact-inhibited or TGF-β-treated cells. Physiolog-

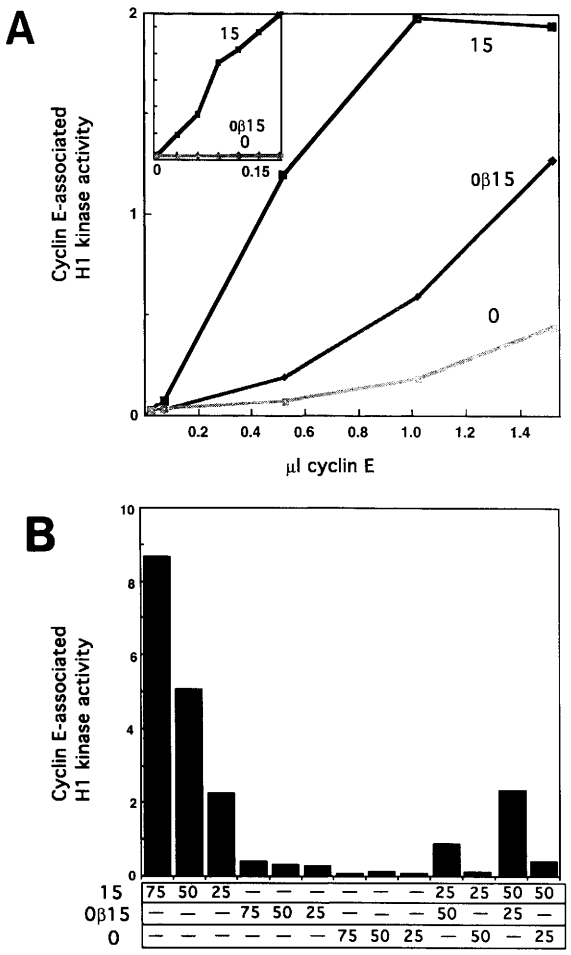

Figure 1. Activation of Cdk2 by cyclin E in extracts from proliferating and growth-arrested cells. (*A*) Cyclin E was added to extracts from contact-inhibited cells (0) and cells released from contact inhibition for 15 hr in the presence (0β15) or absence (15) of TGF-β. The 15-hr cells are referred to in the text as proliferating cells to indicate that they are progressing through the cell cycle and have entered S phase. Cyclin E in the amount of 0.05 μl corresponds to physiological levels of cyclin E in these extracts. (*Inset*) Titrations of up to 3× physiological levels of cyclin E. Cyclin E immunoprecipitates were assayed for histone H1 kinase activity, and the results were quantitated using a PhosphorImager. Background levels of phosphorylation observed in the absence of exogenous cyclin E were subtracted from each sample. (*B*) Extracts were prepared from contact-inhibited cells (0) and cells released from contact inhibition for 15 hr in the presence (0β15) or absence (15) of TGF-β. Physiological amounts of cyclin E were added to various amounts of these extracts and to mixtures of extracts. Extracts from proliferating and arrested cells were mixed in the proportions indicated, and the total amount of protein in each mixture was 75 μg. The amount of each extract used (μg) is indicated. After incubation, cyclin E was immunoprecipitated and assayed for H1 kinase activity. Results were quantitated using a PhosphorImager. Background levels of phosphorylation observed in the absence of exogenous cyclin E were subtracted from each sample.

ical levels of cyclin E were added to the mixed extracts, and cyclin E and any associated kinases were then immunoprecipitated using antibodies to the cyclin. Identical results were obtained using an anti-Cdk2 antiserum (not shown). In mixed extracts cyclin E-associated kinase activity was reduced below that recovered from extracts of proliferating cells alone (Fig. 1B). Thus, extracts from nonproliferating cells contained an excess of an inhibitor of Cdk2 activation. Note that extracts from contact-inhibited cells had both a higher cyclin E activation threshold and a greater inhibitory effect in mixing experiments than extracts from TGF-β-treated cells. However, the abundance of the Cdk2 inhibitory activity depended on the duration of exposure to TGF-β. For instance, we showed previously that an extract from cells exposed to TGF-β for 6 hr beginning in late G_1 did not contain sufficient inhibitory activity to block Cdk2 activation when mixed with an extract from proliferating cells (Koff et al. 1993), and cells exposed to TGF-β for 48 hr had more inhibitory activity that cells exposed for 15 hr (not shown).

A Cdk2 inhibitor binds to cyclin E–Cdk2 complexes

The inhibitor of Cdk2 activation present in extracts from nonproliferating cells could be depleted using a cyclin E–Cdk2 affinity matrix. Cyclin E–Cdk2 complexes were formed by mixing extracts from Sf9 cells infected with baculoviral vectors expressing either Cdk2 tagged with an influenza virus hemagglutinin (HA) epitope or cyclin E. Although neither extract alone contains significant H1 kinase activity, the mixing of the extracts yields high levels of active enzyme (Kato et al. 1993). The cyclin E–Cdk2 (HA) complexes were immunoprecipitated with Sepharose-linked monoclonal antibody directed against the HA tag on Cdk2. Control immunoprecipitations were performed using the monoclonal antibody beads alone. Cell extracts were incubated with either the cyclin E–Cdk2 beads or the control beads, and after pelleting the supernatants were assayed for the ability of exogenously added cyclin E to activate endogenous Cdk2. After depletion of cyclin E–Cdk2-binding proteins, cyclin E was able to activate Cdk2 almost equally in extracts from proliferating and nonproliferating cells (Fig. 2A). Immunoblotting showed that this protocol had no effect on the levels of either cyclin E or Cdk2 in the cell extracts (not shown). In this experiment some stimulatory effect of depleting cyclin E–Cdk2 binding proteins was also observed in extracts from proliferating late G_1 cells, suggesting that they are not completely devoid of the inhibitor (see below). Complexes containing cyclin E and a catalytically inactive mutant of Cdk2 also were able to sequester inhibitory activity when added directly to cell extracts (see Fig. 2C). Thus, reversal of inhibitory activity did not require phosphorylation by the added cyclin E–Cdk2 complexes. These experiments showed that the inhibitor of Cdk2 activation bound to cyclin E–Cdk2 complexes.

In parallel we observed that beads containing just Cdk2 alone were unable to deplete the inhibitory activity from cell extracts (Fig. 2A). This experiment suggested that the inhibitor bound to cyclin E–Cdk2 com-

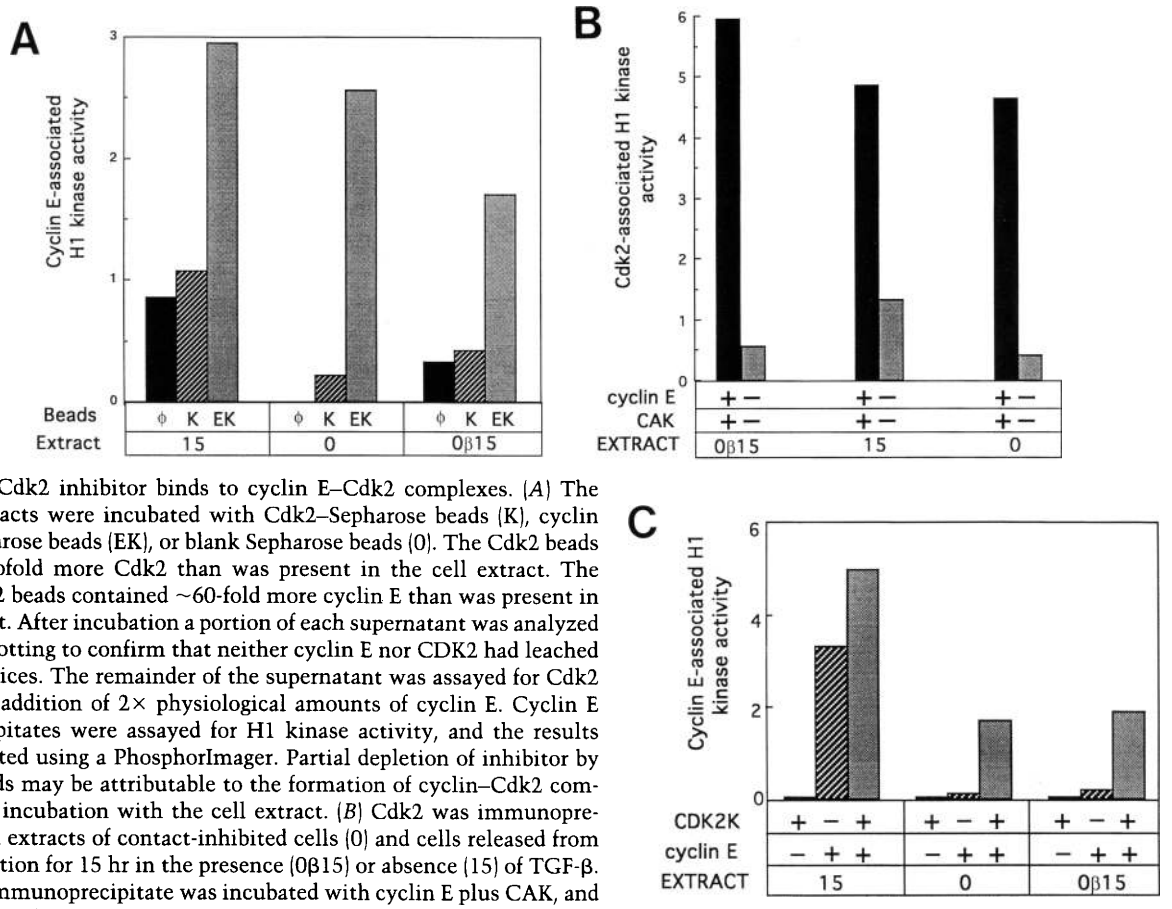

Figure 2. A Cdk2 inhibitor binds to cyclin E–Cdk2 complexes. (*A*) The indicated extracts were incubated with Cdk2–Sepharose beads (K), cyclin E–Cdk2 Sepharose beads (EK), or blank Sepharose beads (0). The Cdk2 beads contained twofold more Cdk2 than was present in the cell extract. The cyclin E–Cdk2 beads contained ~60-fold more cyclin E than was present in the cell extract. After incubation a portion of each supernatant was analyzed by Western blotting to confirm that neither cyclin E nor CDK2 had leached from the matrices. The remainder of the supernatant was assayed for Cdk2 activation by addition of 2× physiological amounts of cyclin E. Cyclin E immunoprecipitates were assayed for H1 kinase activity, and the results were quantitated using a PhosphorImager. Partial depletion of inhibitor by the Cdk2 beads may be attributable to the formation of cyclin–Cdk2 complexes during incubation with the cell extract. (*B*) Cdk2 was immunoprecipitated from extracts of contact-inhibited cells (0) and cells released from contact inhibition for 15 hr in the presence (0β15) or absence (15) of TGF-β. Half of each immunoprecipitate was incubated with cyclin E plus CAK, and the other half underwent mock incubation. Each immunoprecipitate was then assayed for histone H1 kinase activity, and the results were quantitated using a PhosphorImager. In the absence of added CAK, cyclin E had only a very small activating effect on immunoprecipitated Cdk2 (not shown). (*C*) Effect of kinase inactive Cdk2 on cyclin E activity in extracts from growth-arrested cells. Each extract was incubated with 5-fold excess of cyclin E (just at the cyclin E threshold for this lysate), 0.5-fold excess of kinase inactive Cdk2 (Cdk2K), or both. These proportions were chosen based on empirical determinations of the maximum amount of Cdk2K that could be added without sequestering the majority of the added cyclin E. Cyclin E immunoprecipitates were assayed for H1 kinase activity, and the results were quantitated using a PhosophorImager.

plexes but not to Cdk2 alone. To test this idea directly, Cdk2 was immunoprecipated from extracts of proliferating, contact-inhibited, and TGF-β-treated cells. In all cases, the immunoprecipitated Cdk2 protein could be activated by addition of both cyclin E and $p34^{cdc2}$ activating kinase (CAK) (Fig. 2B). Thus, the Cdk2 protein in nonproliferating cells was not intrinsically incapable of activation, nor was it tightly associated with an inhibitor of activation.

Because the Cdk2 inhibitor could bind to cyclin E–Cdk2 complexes, but not to Cdk2, it appeared to recognize either the cyclin–Cdk complex or cyclin. Cyclin E–Cdk2 complexes were more effective at removing the inhibitory activity than was cyclin E, suggesting that the inhibitor interacted preferentially with complexes. Cyclin E was added to a nonproliferating cell extract at a level below the threshold necessary to activate Cdk2 (Fig. 2C). We then induced the assembly of additional cyclin–Cdk2 complexes by supplementing the extracts with an exogenous Cdk2 protein that was rendered catalytically inactive by a mutation of its ATP-binding site (Gu et al. 1992). In the absence of extra Cdk2 no kinase activity was detected in cyclin E immunoprecipitates. When extracts were supplemented with catalytically inactive Cdk2, cyclin E regained H1 kinase activity as a result of activating the endogenous Cdk2. Thus, the cyclin E threshold for Cdk2 activation could be lowered by assembling additional cyclin–Cdk complexes while keeping the total amount of cyclin E constant.

The inhibitor is neither an anti-CAK nor a tyrosine kinase

Previous experiments (Koff et al. 1993) indicated that cyclin E–Cdk2 complexes formed in extracts from nonproliferating cells were not phosphorylated at an essential threonine residue (Gu et al. 1992), possibly accounting for their inactivity. This raised the possibility that CAK was a target of the inhibitor. This initially seemed unlikely because the inhibitor bound directly to the cy-

clin E–Cdk2 complex. We reconsidered this idea in light of recent evidence that CAK is itself a distant member of the Cdk protein family (Fesquet et al. 1993; Poon et al. 1993; Solomon et al. 1993) and therefore might also bind to the inhibitor. Previous work, in another system, indicated that activation of the cyclin B–Cdc2 complex was not blocked by the Cdk2 inhibitor (E. Firpo, A. Koff, and J. Roberts, unpubl.; see below). We therefore used cyclin B and Cdc2 to assay CAK activity, given that CAK is also required to activate the cyclin B–Cdc2 complex (Solomon et al. 1992).

Cdc2 was activated equally when cyclin B was added to extracts from either proliferating cells or TGF-β-arrested cells (Fig. 3A). Therefore, functional CAK was present in extracts from TGF-β-treated cells. CAK was limiting in this experiment because the addition of purified CAK to these extracts catalyzed the activation of additional cyclin B–Cdc2 complexes (Fig. 3B). Moreover, the activity of the added CAK was similar in extracts from TGF-β-treated and proliferating cells (Fig. 3B). Thus, exogenous CAK was not inhibited. Control experiments showed that this CAK was able to activate cyclin E–Cdk2 complexes when they were assembled by mixing Sf9 cell lysates containing cyclin E and Cdk2 expressed from baculoviral vectors (Solomon et al. 1993; data not shown). However, the added CAK did not change the threshold level of cyclin E required to activate Cdk2 (not shown). Thus, the inhibitor did not block CAK and its effects could not be overcome by excess CAK. Inhibition of CAK was not sufficient to explain the block to Cdk2 activation.

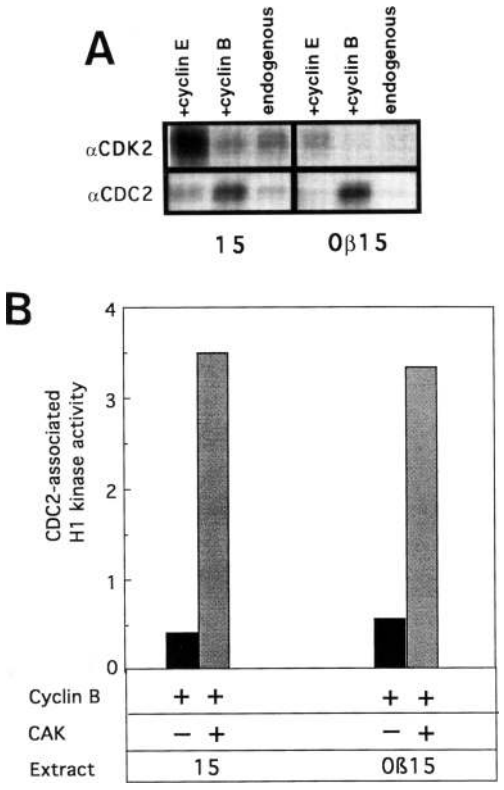

Figure 3. Activation of Cdc2 by cyclin B. (*A*) Cyclin B and cyclin E were added to extracts from cells released from contact inhibition for 15 hr in the presence (0β15) or absence (15) of TGF-β. After addition of cyclins the extracts were divided and immunoprecipitated with either antisera directed to the carboxyl terminus of Cdc2 or the carboxyl terminus of Cdk2. The immunoprecipitates were assayed for H1 kinase activity, and the products were resolved on a 12% polyacrylamide gel. The reactions labeled endogenous contain no added cyclin. (*B*) Cyclin B was added to extracts from cells released from contact inhibition for 15 hr in the presence (0β15) or absence (15) of TGF-β. Half of each reaction was supplemented with purified CAK. Cdc2 was immunoprecipitated with antibody directed toward the carboxyl terminus of Cdc2 and assayed for H1 kinase activity. The results were quantitated using a PhosphorImager.

To determine whether tyrosine phosphorylation contributed to the inhibition of Cdk2 activity, cyclin E was added to nonproliferating cell extracts at sub-threshold levels and the cyclin E–Cdk2 complexes were immunoprecipitated using anti-cyclin E antibodies. No tyrosine phosphorylation of Cdk2 in the inactive cyclin E–Cdk2 complexes was detected by immunoblotting with antiphosphotyrosine antibodies (not shown). As a positive control, phosphotyrosine was readily detected in Cdc2 immunoprecipitated from human cells.

Cyclin D2–Cdk4 complexes facilitate Cdk2 activation

As cells traverse G_1, complexes between Cdk4 and the D-type cyclins appear prior to the formation of active complexes containing cyclin E and Cdk2 (for review, see Sherr 1993). Contact-inhibited Mv1Lu cells do not express significant levels of cyclin D1 or D2 (not shown), and Cdk4 synthesis is repressed in cells arrested in G_1 by exposure to TGF-β (Ewen et al. 1993b; K. Polyak and J. Massague, unpubl.; see also Fig. 5D, below). Thus, accumulation of cyclin D–Cdk4 complexes is limiting in G_1-arrested cells. These observations suggested that cyclin D–Cdk4 complexes could potentially have a role in removing the Cdk2 inhibitor during cell cycle progression. Ewen et al. (1993b) recently showed that constitutive ectopic expression of Cdk4 can override the TGF-β block to Cdk2 activation and cell cycle progression. We tested this in our system by asking whether the restoration of cyclin D–Cdk4 complexes to extracts from nonproliferating cells might overcome the block to Cdk2 activation.

Cdk4 is a partner of the D-type cyclins and does not form active complexes with cyclins E, A, or B. It interacts equally well with each of the D-type cyclins when they are coexpressed in insect cells. Cyclin D–Cdk4 complexes phosphorylate histone H1 poorly but show strong activity using the Rb protein as substrate (Matsushime et al. 1992; Kato et al. 1993). Complexes between Cdk4 and either cyclin D1, D2, or D3 were assembled by coinfection of Sf9 cells with baculoviral vectors, and Sf9 lysates were added to extracts from proliferating and nonproliferating Mv1Lu cells. Subthreshold amounts of cyclin E were then added, and activation of Cdk2 was tested after immunoprecipitation

of cyclin E–Cdk2 complexes with antibodies to cyclin E. Addition of cyclin D2–Cdk4 complexes, but neither subunit alone, to extracts from contact-inhibited and TGF-β-arrested cells allowed cyclin E to activate Cdk2 to a level equivalent to that observed in extracts from proliferating cells (Fig. 4A). Titrations demonstrated that the amount of cyclin D2–Cdk4 necessary to block the Cdk2 inhibitor was less than that present in an equivalent amount of extract from proliferating cells (not shown). In contrast, the activity of cyclin E was not increased when cyclin D2–Cdk4 complexes were added to extracts from proliferating cells. Moreover, the cyclin D2–Cdk4 complex did not have CAK activity, as it was unable to substitute for CAK in promoting the activation of cyclin E–Cdk2 complexes assembled from proteins expressed in Sf9 cells (not shown). Thus, the cyclin D2–Cdk4 complex reversed the inhibition of Cdk2 activation. Equal amounts of cyclin D1–Cdk4 and cyclin D3–Cdk4 complexes, as estimated by immunoblotting of Sf9 lysates, were much less effective in lowering the cyclin E threshold for Cdk2 activation (Fig. 4B). The inability of cyclin D1– or cyclin D3–Cdk4 complexes to sequester the Cdk2 inhibitor was not because those complexes were unstable in cell lysates (not shown).

Quite surprisingly, the ability of cyclin D2–Cdk4 to reverse Cdk2 inhibition did not require Cdk4 catalytic activity. Complexes formed between cyclin D2 and a catalytically inactive mutant Cdk4 subunit were as effective as enzymatically active cyclin D2–Cdk4 complexes in removing the Cdk2 inhibitor (Fig. 4A). Titrations with different amounts of cyclin D2 complexes containing either catalytically active or inactive Cdk4 revealed that their specific activities in reversing Cdk2 inhibition were very similar (not shown). This ruled out the possibility that cyclin D2–Cdk4 must phosphorylate the inhibitor to inactivate it and excluded any model in which cyclin D2–Cdk4 bypassed the inhibitor by functioning as a CAK. It therefore seemed likely that cyclin D2–Cdk4 removed the Cdk2 inhibitor by binding to it directly and sequestering it from Cdk2 (see below).

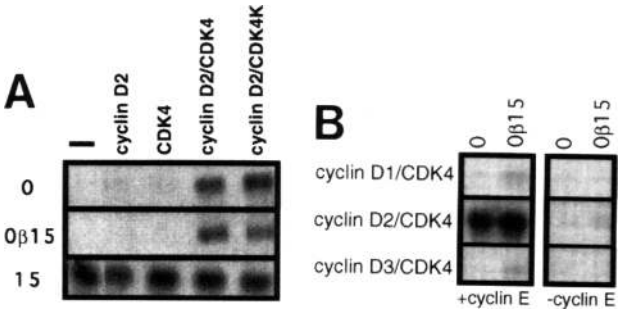

Figure 4. Effect of cyclin D–Cdk4 complexes on cyclin E activity. (*A*) Extracts were prepared from contact-inhibited cells (0) and cells released from contact inhibition for 15 hr in the presence (0β15) or absence (15) of TGF-β. Sf9 cell lystates (0.05 μl) containing cyclin D2, Cdk4, cyclin D2–Cdk4 complexes, or complexes containing cyclin D2 bound to catalytically inactive Cdk4 (Cdk4K) were added to these extracts together with physiological amounts of cyclin E. These amounts of cyclin D2 and Cdk4 closely correspond to physiological amounts of these proteins. Cyclin E was immunoprecipitated and assayed for associated histone H1 kinase activity. (*B*) Extracts were prepared from cells released from contact inhibition for 15 hr in the presence (0β15) or absence (15) of TGF-β. Sf9 cell lysates (0.05 μl) containing the indicated cyclin D–Cdk4 complexes were added to these extracts in the presence or absence of cyclin E. Cyclin E was immunoprecipitated and assayed for associated histone H1 kinase activity.

The Cdk2 inhibitor is a 27-kd protein

The above observations indicated that (1) a functional cyclin E–Cdk2 inhibitor was present in extracts from contact-inhibited cells or cells released from contact inhibition in the presence of TGF-β, but not in extracts from proliferating cells; (2) that this molecule preferentially associated with cyclin E–Cdk2 complexes as opposed to either subunit alone; and (3) it could be depleted by preincubation of cell extracts with catalytically active or inactive cyclin D2–Cdk4 complexes. To identify a factor that might display these properties, Mv1Lu cells were metabolically labeled with [^{35}S]methionine, and lysates were incubated with Sepharose beads that contained immunoadsorbed recombinant Cdk2, either alone or in complexes with recombinant cyclin E. Denatured ^{35}S-labeled proteins, eluted by heating the beads with buffer containing 1% SDS, were visualized by gel electrophoresis and fluorography (Fig. 5A). All cell lysates yielded a similar pattern of cyclin E–Cdk2-binding proteins with the exception of a 27-kD protein that was recovered from extracts of contact-inhibited or TGF-β-inhibited cells but not late G_1-phase cells (Fig. 5A). This protein, referred to as p27, was isolated using cyclin E–Cdk2 complexes but not Cdk2 alone (Fig. 5A). The recovery of p27 increased in proportion to the amount of cyclin E–Cdk2 complex used until it reached a maximum (Fig. 5B), indicating that binding of p27 to cyclin E–Cdk2 complexes was saturable. This was consistent with the observation that Cdk2 inhibitor activity could be depleted by cyclin E–Cdk2 complexes. As expected, stoichiometric amounts of p27 were also observed in cyclin E immunoprecipitates from growth-arrested cells (not shown).

Cell extracts that received recombinant cyclin D2–Cdk4 complex no longer yielded p27 when the mixture was adsorbed to cyclin E–Cdk2–Sepharose (Fig. 5C). After removal of the cyclin E–Cdk2–Sepharose beads from samples that received cyclin D2–Cdk4, the precleared supernatants were incubated with Cdk4 antibody to recover Cdk4 and its associated proteins. This yielded p34Cdk4 itself, the levels of which were highest in extracts from cells in late G_1 and lowest in TGF-β-treated cells (Fig. 5D) (Matsushime et al. 1992; Ewen et al. 1993b). With the same antiserum, Ewen et al. (1993b) used partial proteolytic digestion to confirm the authenticity of Mv1Lu Cdk4. In addition, these immunoprecipitates contained a 27-kD protein in samples from contact-inhibited and TGF-β-treated cells (Fig. 5D). Lesser

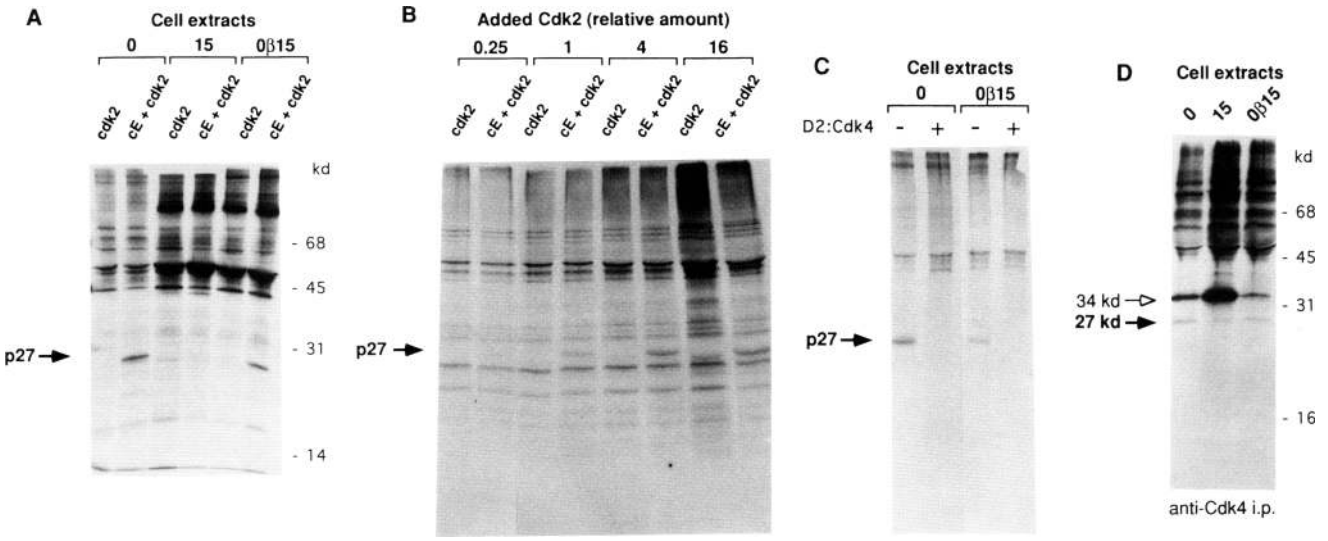

Figure 5. A 27-kD cyclin E–Cdk2-binding protein. Contact-inhibited Mv1Lu cells were released from quiescence by replating at lower density; and extracts were prepared from [^{35}S]methionine-labeled cells at 0 and 15 hr. Some cultures were incubated in the presence of 100 pM TGF-β for 15 hr (0β15). These metabolically labeled extracts were treated as described, and bound proteins were eluted in sample buffer and analyzed by SDS-PAGE followed by fluorography. Migration of molecular mass markers are shown (hD). (*A*) [^{35}S]methionine-labeled cell extracts were incubated with Cdk2 or cyclin E–Cdk2 complexes, and bound proteins were eluted in sample buffer. The arrow indicates the migration of a 27-kD protein (p27) specifically associated with cyclin E–Cdk2 complexes in extracts from contact-inhibited and TGF-β-treated cells. (*B*) Extracts from metabolically labeled contact-inhibited Mv1Lu cells were incubated with varying amounts of Cdk2 or cyclin E–Cdk2 relative to standard conditions and bound proteins were analyzed as described above. The presence of p27 is indicated (arrow). (*C*) Cyclin D2–Cdk4 complexes prevent binding of p27 to cyclin E–Cdk2. Extracts from contact-inhibited cells were preincubated with 4 μl of baculovirus-produced cyclin D2–Cdk4 complex for 30 min at 4°C prior to addition of the cyclin E–Cdk2 complex. (*D*) Recovery of p27 in Cdk4 immunoprecipitates. Supernatants from *C* were immunoprecipitated with an anti-Cdk4 antiserum, and immunoprecipitates were analyzed on 12% SDS-PAGE. The open arrow at 34 kD shows the endogenous mink Cdk4 protein; the solid arrow indicates p27, associated with the cyclin D2–Cdk4 complexes.

amounts of p27 were also recovered in Cdk4 immunoprecipitates from late G_1 cell samples, even though p27 could not be recovered from those same extracts by cyclin E–Cdk2 affinity chromatography. This suggested that p27 was present in proliferating cells but in a form unavailable to interact with exogenously added cyclin E–Cdk2 complexes (see below). Side-by-side comparison showed that p27 purified on cyclin E–Cdk2 beads or by coprecipitation with Cdk4 had the same apparent molecular weight (not shown).

Experiments done to characterize the stability of this factor showed that heating cell extracts to 100°C for a brief period preserved both the ability of p27 to bind to cyclin E–Cdk2 (Fig. 6A) and the inhibitory activity as well (Fig. 6C). Furthermore, when applied to extracts from cells in late G_1 phase, heat treatment unexpectedly induced the appearance of p27 (Fig. 6A) and concomitantly increased the level of Cdk2 inhibitory activity (Fig. 6B). These results indicated that p27 and the inhibitory activity were both heat stable and that they could be reactivated in late G_1 extracts by a heat-sensitive mechanism. As expected, cyclin D2–Cdk4 complexes were also able to sequester p27 from heat-treated lysates (not shown).

Extracts from metabolically labeled TGF-β-treated cells were subjected to chromatography over cyclin E–Cdk2–Sepharose or, as a control, Cdk2–Sepharose. After washing, the beads were eluted with an acidic buffer and one portion of the eluate was analyzed by SDS-PAGE. This showed that p27 was the predominant labeled species recovered and was present only in the eluate from cyclin E–Cdk2 beads (Fig. 7A). Samples from the same eluates were assayed for the presence of the Cdk2 inhibitor, and this activity was present in the eluate from cyclin E–Cdk2 beads but not Cdk2 beads (Fig. 7B). The remainder of the eluate was concentrated by acetone precipitation, denatured in 6 M guanidium hydrochloride, renatured by dialysis against isotonic buffer, and subjected to a second round of binding to cyclin E–Cdk2–Sepharose. Elution from these beads by boiling in buffer containing 1% SDS yielded p27 as the only labeled band (Fig. 7C). These results strongly supported the possibility that p27 and the inhibitory activity are one and the same. This conclusion was directly confirmed by fractionating the cyclin E–Cdk2 eluate by PAGE and extracting the fractionated proteins from gel slices. Renatured proteins were tested for their ability to inhibit activation of Cdk2 by cyclin E (Fig. 7D). The protein recovered from the gel slice containing p27 completely inhibited Cdk2 activation, and no additional inhibitory activity was recovered from any other gel slice.

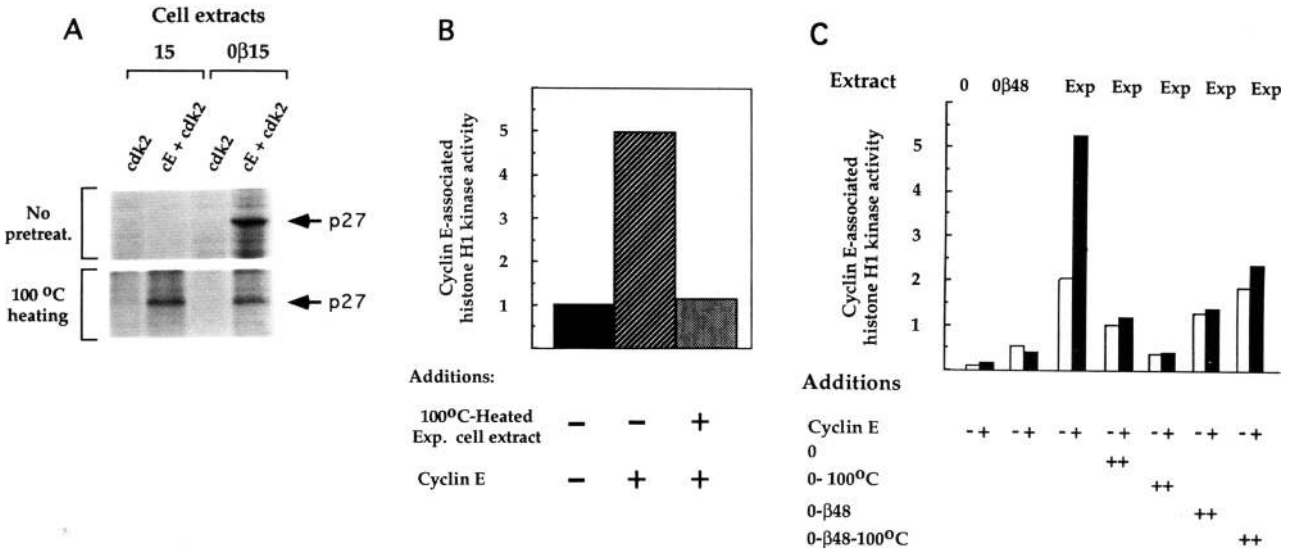

Figure 6. Heat stability of p27 and the Cdk2 inhibitor. (*A*) p27 binding is heat stable, and p27 can be recovered from proliferating cell extracts by heat treatment. Mv1Lu cells were released from contact inhibition for 15 hr with (0β15) or without (15) TGF-β. Cells were metabolically labeled using [^{35}S]methionine. Prior to incubation with Cdk2 or cyclin E–Cdk2 complexes, cell extracts either received no pretreatment or were heated to 100°C for 3 min. Note the appearance of p27 (arrow) in the heat-treated 15-hr cell extract. (*B*) Cdk2 inhibitory activity can be recovered from proliferating cell extracts by heat treatment. Cyclin E-associated kinase activity was measured in extracts from asynchronous proliferating cells by immunoprecipitation with antibodies against human cyclin E. Histone H1 was the substrate, and results were quantitated using a PhosphorImager. (Lane *1*) No additions; (lane *2*) the extract was supplemented with 3× physiological amounts of cyclin E; (lane *3*) as in lane *2* except that heat-treated extract from proliferating cells (see Materials and methods) was also added to the cell extract. (*C*) Cdk2 inhibitory activity was heat stable. Extracts were prepared from contact-inhibited cells (0), cells released from contact inhibition for 48 hr in the presence of TGF-β (0β48), or asynchronous proliferating cells (Exp). Cyclin E-associated kinase activity was measured with or without the addition of exogenous cyclin E. In the indicated lanes proliferating cell extracts were mixed with equal amounts of extract from nonproliferating cells that had either been untreated or heated to 100°C for 5 min.

Discussion

We have identified an inhibitor in nonproliferating cells that prevents activation of complexes containing the G_1 cyclin, cyclin E (Koff et al. 1991; Lew et al. 1991; Ohtsubo and Roberts 1993), and its catalytic subunit, Cdk2 (Dulic et al. 1992; Koff et al. 1992). This inhibitory activity is, at least in part, attributable to a 27-kD polypeptide, which we have named $p27^{Kip1}$ (Cdk inhibitory protein 1). The inhibitor and $p27^{Kip1}$ share the following characteristics: They bind to cyclin E–Cdk2 complexes but not to Cdk2 alone; they are only detected in extracts from growth-arrested cells; they can be sequestered by cyclin D2–Cdk4 complexes but not by either component alone; they are heat stable; and they are latent in extracts of proliferating cells but can be unmasked by brief heat treatment. In addition, purified $p27^{Kip1}$ inhibits Cdk2 activation by cyclin E when added to an extract from proliferating cells. Although these data strongly suggest that $p27^{Kip1}$ is at least a component of the Cdk2 inhibitor, we have not determined whether inhibition is attributable to $p27^{Kip1}$ alone or whether $p27^{Kip1}$ recruits additional proteins to the cyclin E–Cdk2 complex.

The mechanism of $p27^{Kip1}$ inhibition has features that distinguish it from pathways that control activation of the mitotic cyclin–Cdc2 complexes. First, $p27^{Kip1}$ appears to act stoichiometrically rather than catalytically. Second, tyrosine phosphorylation of Cdk2 was not detected in inactive cyclin E–Cdk2 complexes containing $p27^{Kip1}$, suggesting that $p27^{Kip1}$ does not have tyrosine kinase activity or inhibit a tyrosine phosphatase. Complexes containing $p27^{Kip1}$ were not phosphorylated efficiently by the $p34^{Cdc2}$ activating kinase, CAK, and this might be sufficient to explain their inactivity. It is possible that $p27^{Kip1}$ dephosphorylates Thr-160, although it would be surprising if the enzymatic activity of a phosphatase were stable to heating to 100°C. It is more likely that binding of $p27^{Kip1}$ to the cyclin E–Cdk2 complex prevents Thr-160 phosphorylation by altering the conformation of the T160 domain, or by sterically obstructing CAK. It would not be surprising if $p27^{Kip1}$ functioned similarly to the negative regulatory subunits or domains of other protein kinases, perhaps even interacting with the kinase active site as a pseudosubstrate.

It is intriguing that in addition to $p27^{Kip1}$ other potential regulators of Cdk activity during G_1 also bind directly to cyclin–Cdk complexes, including FAR1 (Peter et al. 1993), p40 (Mendenhall 1993), p16 and p21 (Xiong et al. 1992, 1993), and Rb (Dowdy et al. 1993; Ewen et al. 1993a; Kato et al. 1993). Thus far, however, only p40 has been shown to directly inhibit Cdk activity, but it seems likely that at least some of the others will also be Cdk

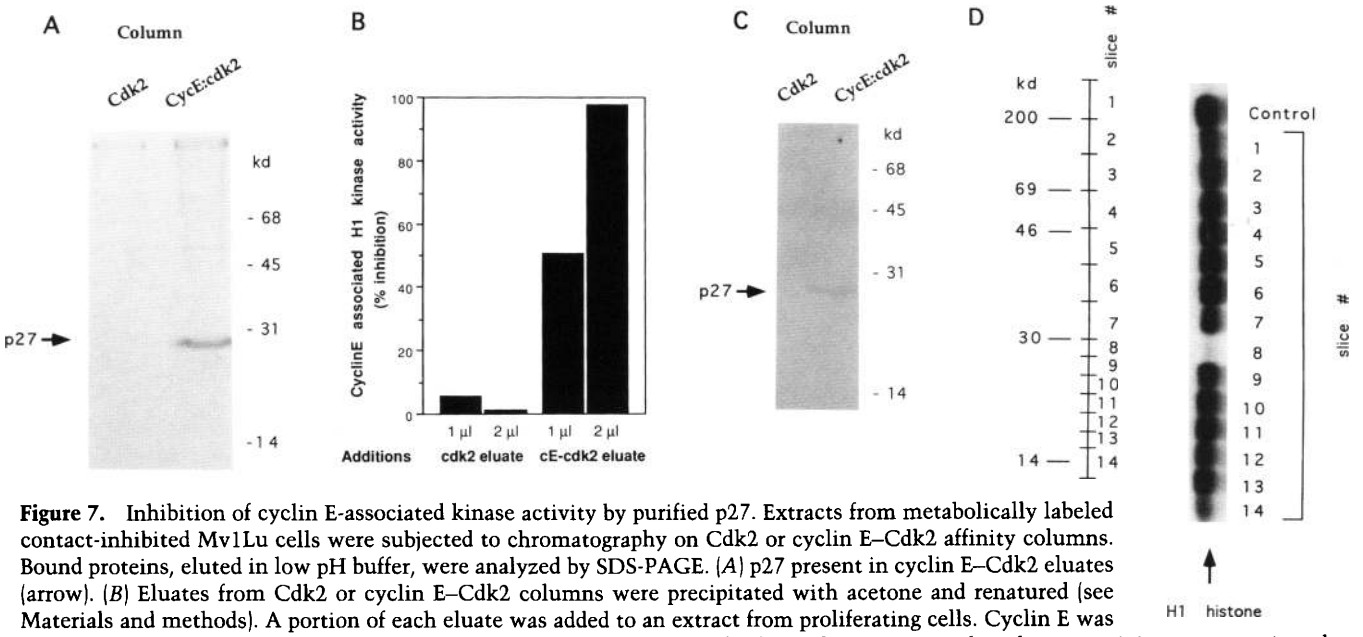

Figure 7. Inhibition of cyclin E-associated kinase activity by purified p27. Extracts from metabolically labeled contact-inhibited Mv1Lu cells were subjected to chromatography on Cdk2 or cyclin E–Cdk2 affinity columns. Bound proteins, eluted in low pH buffer, were analyzed by SDS-PAGE. (*A*) p27 present in cyclin E–Cdk2 eluates (arrow). (*B*) Eluates from Cdk2 or cyclin E–Cdk2 columns were precipitated with acetone and renatured (see Materials and methods). A portion of each eluate was added to an extract from proliferating cells. Cyclin E was added, and cyclin E-associated histone H1 kinase activity was measured. The cyclin E-associated H1 kinase activity was quantitated and plotted as percent inhibition relative to extracts receiving no additions. (*C*) Renatured eluates were incubated with Cdk2 or cyclin E–Cdk2 complexes. p27 (arrow) bound to cyclin E–Cdk2 after renaturation. (*D*) Eluates from cyclin E–Cdk2 columns were fractionated on 12% acrylamide gels. The gels were sliced as shown, and proteins were eluted and renatured. A portion of the protein recovered from each gel slice was added together with cyclin E to extracts prepared from proliferating Mv1Lu cells. Cyclin E immunoprecipitates were assayed for histone H1 kinase activity.

inhibitors. Direct protein–protein interactions may be a way to focus inhibitory signals on specific cyclin–Cdk complexes in a cellular environment containing other more promiscuous *trans*-acting regulators of Cdk activity.

p27Kip1 links growth inhibitory signals to cell cycle arrest

We discovered $p27^{Kip1}$ in cells arrested in G_1 by either contact inhibition or TGF-β. A similar activity has also been found to block Cdk2 activation in various cell types deprived of specific growth factors, including serum-starved fibroblasts and interleukin-2 (IL-2)-deprived lymphocytes (E. Firpo, M. Ohtsubo, A. Koff, and J. Roberts, unpubl.). Inhibition of Cdk2 activation by $p27^{Kip1}$, or functionally similar proteins, may be a general mechanism through which diverse extracellular and intracellular signals exert control on cell proliferation.

$p27^{Kip1}$ constrains cell proliferation by setting the threshold level of cyclin E necessary to activate Cdk2. If $p27^{Kip1}$ acts stoichiometrically, as our data suggest, then the Cdk2 activation threshold is reached soon after the amount of cyclin E in the cell exceeds the amount of active $p27^{Kip1}$. In arrested cells this threshold is set higher than physiological cyclin E levels; and consequently, only inactive cyclin E–Cdk2 complexes assemble. The cyclin A–Cdk2 complex may be subject to similar control (Koff et al. 1993; E. Firpo, A. Koff, and J. Roberts, in prep.), and an inability to activate this complex should also contribute to cell cycle arrest (Girard et al. 1991; Pagano et al. 1992, 1993; Tsai et al. 1993).

How might growth inhibitory signals be linked to the activity of $p27^{Kip1}$? The simplest idea would be that growing cells do not contain much $p27^{Kip1}$ and that signals that inhibit cell proliferation induce $p27^{Kip1}$ synthesis or stabilization and thereby increase its amount above a critical basal level. This model cannot be strictly correct because greatly increased quantities of $p27^{Kip1}$ can be recovered from a latent pool once extracts from proliferating cells are subject to heat treatment. A substantial pool of $p27^{Kip1}$ must be present in these extracts and must be sequestered by other molecules. This implies that $p27^{Kip1}$ plays a normal role during the proliferative cell cycle and is not simply a response element for signals that induce growth arrest. The abundance of "free" $p27^{Kip1}$ that is able to interact with the cyclin E–Cdk2 complex might therefore be modulated by an upstream regulator, such as the cyclin D2–Cdk4 complex, which also binds to $p27^{Kip1}$ directly. This prevents association with cyclin E–Cdk2 and enables its functional activation, at least in vitro. The idea that p27 activity is governed by an upstream regulator does not exclude the possibility that the total cellular level of p27 may increase in arrested cells, and our experiments have not directly compared the total amounts of p27 in proliferating and arrested cells.

D-type cyclins are themselves targets of growth inhibitory signals (for review, see Sherr 1993). Their synthesis is rapidly reduced in growth factor-deprived cells (Mat-

sushime et al. 1991; Won et al. 1992; Kato and Sherr 1993) and in contact-inhibited cells (N. Polyak, J.-Y. Kato, C. Sherr, and J. Massagne, unpubl.) leading to a reduction in cyclin D–Cdk4 levels (Matsushime et al. 1992). Although D-type cyclin levels are not greatly affected by TGF-β blockade, TGF-β does reduce synthesis of Cdk4 so that a net reduction in cyclin D–Cdk4 complexes is achieved nevertheless (Ewen et al. 1993b). In TGF-β-inhibited cells, where Cdk4 is limiting, expression of excess Cdk4 should lead to the formation of additional cyclin D–Cdk4 complexes and sequester $p27^{Kip1}$. Enforced expression of Cdk4 in vivo reverses the block to Cdk2 activation in cells exposed to TGF-β (Ewen et al. 1993b). However, the addition of Cdk4 alone to extracts from TGF-β-treated cells in vitro does not reverse the interaction of $p27^{Kip1}$ with cyclin E–Cdk2. Unlike complexes with cyclin E and Cdk2, which can be formed in vitro by mixing the recombinant proteins produced in insect cells, D-type cyclins and Cdk4 do not assemble efficiently unless Sf9 cells are coinfected with baculoviruses encoding both components (Kato et al. 1993). Although we have not defined the reasons underlying these differences in complex formation, all results are internally consistent and support the idea that cyclin D–Cdk4 complexes act upstream of cyclin E–Cdk2 by interacting with $p27^{Kip1}$. Although these ideas are based on many observations made in intact cells, the proposed pathway containing cyclin D–Cdk4, $p27^{Kip1}$, and cyclin E–Cdk2 has been tested directly only in vitro. One might expect that Cdk2 will be regulated by additional mechanisms and that other proteins, including Cdk complexes other than cyclin D2–Cdk4, could contribute to the titration of $p27^{Kip1}$.

We do not believe that the only role of cyclin D2–Cdk4 is to titrate $p27^{Kip1}$ but, rather, suggest that complex accumulation is also likely to trigger the Cdk4-mediated phosphorylation of particular substrates necessary for G_1 progression. Thus, cyclin D complexed with catalytically inactive Cdk4 is sufficient to sequester $p27^{Kip1}$ but is unlikely to fully substitute for all essential Cdk4 functions in vivo.

One feature of $p27^{Kip1}$-induced cell cycle arrest is that cells can accumulate inactive cyclin E–Cdk2 complexes. Recovery from cell cycle arrest, therefore, might not require synthesis of new cyclin E and assembly of new cyclin E–Cdk2 complexes. Rather, the cell may make use of this latent pool of inactive complexes when cell proliferation resumes. This might be essential under circumstances where the signals that promoted cyclin synthesis were transient and absent when the growth inhibitory signals ceased. Thus far, however, conditions have not been defined that allow reactivation of inactive cyclin E–Cdk2–$p27^{Kip1}$ complexes. In vitro, only cyclin E–Cdk2 complexes that assemble after titration of $p27^{Kip1}$ are active, and the same may be true in vivo as well.

The presence of $p27^{Kip1}$ in proliferating cells suggests that its role may not be restricted to inducing cell cycle arrest in response to extracellular signals. It may also set the cyclin E threshold for execution of the G_1 to S transition during each mitotic cycle. Cell fusion experiments have indicated that entry into S phase in mammalian fibroblasts is controlled by an activator that accumulates continuously during G_1 (Fournier and Pardee 1975; Rao et al. 1977). By comparing the rate of S-phase entry in mono- bi- and trinucleate cells it was concluded that the amount of this activator, rather than its concentration, was critical in determining the start of S phase. These observations are consistent with a model in which the limiting step in Cdk2 activation is not assembly of the cyclin–Cdk2 complex, which should be concentration dependent but, instead, involves the assembly of a sufficient number of complexes to overcome a threshold level of a stoichiometric inhibitor, such as $p27^{Kip1}$. We also point out that spontaneous decay of $p27^{Kip1}$-inhibited complexes to free $p27^{Kip1}$ and active cyclin–Cdk2 might occur with first-order (exponential) kinetics and could underlie the first-order rate constants reported frequently for S-phase entry in mammalian cells (Smith and Martin 1973; Brooks et al. 1980).

$p27^{Kip1}$ may enforce order during G_1 progression

Cyclin–Cdk complexes appear in a specific order as cells transit G_1 (Sherr 1993). If we assume that this temporal order is essential for normal G_1 progression, then cells must solve the problem of restoring order during recovery from cell cycle arrest. For example, contact inhibition and TGF-β interfere with the accumulation of cyclin D–Cdk4 complexes but do not affect synthesis of cyclin E– and cyclin A–Cdk2 complexes, which act later in the cell cycle. If the cyclin E–Cdk2 and cyclin A–Cdk2 complexes were active during cell cycle arrest, then the normal order of Cdk action would be lost. $p27^{Kip1}$ might ensure that this does not happen by preventing activation of these pre-existing complexes during cell cycle arrest. In addition, if the activity of $p27^{Kip1}$ is itself controlled by cyclin D2–Cdk4, then this would provide an efficient mechanism for maintaining Cdk2 inactive until cyclin D–Cdk4 complexes assemble and execute their functions.

Materials and methods

Cell culture

Exponentially growing Mv1Lu cells were growth arrested by culturing them to confluence in the presence of 10% fetal bovine serum (FBS). Cells were released from contact inhibition by trypsinization and reseeding in sparse conditions. TGF-β1 (100 pM) was added to the cells at the indicated times. Cell entry into S phase was confirmed routinely by measuring ^{125}I-labeled deoxyuridine incorporation into DNA (Laiho et al. 1990).

Preparation of recombinant proteins.

Cyclin E, Cdk2, Cdk2–HA, and Cdk2K were prepared by the method of Desai et al. (1992). Briefly, 100-mm plates of confluent Sf9 cells were infected with the appropriate baculovirus at an m.o.i. of 5–20 PFU/cell. After 48 hr of infection the cells were collected and lysed by Dounce homogenization or cup–

horn sonication in hypotonic buffer. The extract is clarified by ultracentrifugation and stored at −70°C. The baculoviral vectors containing cyclins D1, D2, D3, Cdk4, and catalytically inactive Cdk4 have been described previously (Matsushime et al. 1992; Kato et al. 1993).

CAK

CAK was purified from *Xenopus* egg extracts through the Mono Q step exactly as described (Solomon et al. 1993) and was used at a final concentration of 1–2 U/ml.

Metabolic labeling

Mv1Lu cultures in 150-mm dishes were incubated for 30 min in methionine-free medium supplemented with 10% dialyzed FBS, followed by incubation for 2 hr in the same medium with 200 μCi/ml of [^{35}S]methionine (Trans ^{35}S-label, ICN). Cells were collected by trypsinization and centrifuged at 2000*g* for 5 min. Cell pellets were lysed by gentle agitation for 30 min at 4°C in 10 volumes of NP-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2 mM EDTA, 0.5% NP-40, 0.3 mM Na orthovanadate, 50 mM NaF, 80 μM β-glycerophosphate, 20 mM Na pyrophosphate, 0.5 mM DTT, and protease inhibitors), and lysates were clarified by centrifugation (10,000*g* for 15 min at 4°C). Prior to binding reactions the supernatants were precleared twice with Sepharose and once with protein A–Sepharose.

Cdk activation assays

Indicated amounts of baculovirus-expressed recombinant cyclin, Cdk, or cyclin–Cdk complex were added to 50 μg of extracts prepared by sonication in a hypotonic buffer as described previously (Koff et al. 1993). In all cases, the exogenous cyclins and Cdks were added in the form of an unfractionated Sf9 cell lysate. Cyclins and Cdks typically comprise at least 1–3% of total cell protein. Uninfected Sf9 cell lysates have been tested in all assays and have no activity. After 30 min at 37°C the reaction was adjusted to 0.5% NP-40, 250 mM NaCl, and immunoprecipitated with the indicated antibody. Immunoprecipitates were subsequently assayed for histone H1 kinase activity as described (Koff et al. 1993). For experiments in which the effect of the D cyclins and Cdk4 on cyclin E activity were tested, all cyclins and Cdks were added to the cell extract together. Heat treatment of extracts was performed by incubating extracts to 100°C for 5 min. Coagulated protein was then pelleted by microcentrifugation. For experiments in which Cdk2 immunoprecipitates were tested for activation by cyclin E, 20 μl of antiserum to the carboxyl terminus of CDK2 (Koff et al. 1993) was adsorbed to protein A–Sepharose and washed into NP-40 RIPA buffer. Extract (300 μg) was subsequently incubated with the anti-CDK2–Sepharose for 90 min at 4°C. The precipitate was washed twice with NP-40 RIPA buffer and four times with buffer A containing 10 mM ATP. Cyclin E and CAK were added as described above, and reactions were incubated for 30 min at 37°C and subsequently assayed for H1 kinase activity.

Inhibitor depletion

Cyclin E–Cdk2–Sepharose was prepared by mixing 1.2 μl of Sf9 cell lysate containing HA-tagged Cdk2 (Cdk2–HA) with 12 μl of lysate containing cyclin E in buffer A (30 mM HEPES–KOH at pH 7.5, 7.5 mM $MgCl_2$, 1 mM DTT) containing 10 mM ATP and incubated at room temperature for 30 min to allow complex formation. The assembly reaction was then adjusted to 250 mM NaCl and 0.5% NP-40. The Cdk2–HA-containing complexes were immunoprecipitated with the 12CA5 monoclonal antibody (BabCo) and collected on protein A–Sepharose. Cdk2–Sepharose was prepared in an identical manner except cyclin E was omitted. Immunoprecipitates were washed twice with NP-40 RIPA buffer (0.5% NP-40, 250 mM NaCl, 10 mM EDTA, 20 mM Tris-HCl at pH 7.4) and four times with buffer A. The matrix was divided into four aliquots and incubated with 100 μg of cell extract in buffer A containing 3 mM ATP, 20 μg/ml of creatine phosphokinase, and 40 mM phosphocreatine for 45 min at 37°C. After incubation, the supernatant was collected and assayed for Cdk2 activation by addition of recombinant cyclin E as described above. A critical parameter in the execution of this experiment is to ensure that no cyclin, Cdk, or complex leaches from the beads into the cell extract. In our hands, this is unpredictable and must be checked by immunoblotting each time the experiment is performed.

Cyclin E–Cdk2-binding assays

Complexes of baculoviral cyclin E with baculoviral Cdk2 containing the influenza virus HA epitope HA1 were formed as described below. The complexes were immunoprecipitated in NP-40–RIPA buffer (50 mM Tris-HCl at pH 7.4, 250 mM NaCl, 0.5% NP-40, 50 mM NaF, 0.3 mM Na-orthovanadate, 5 mM EDTA, and protease inhibitors) with anti-HA monoclonal antibody (12CA5, BabCo) and bound to protein A–Sepharose. Cdk2 or cyclin E–Cdk2 adsorbed to protein A–Sepharose was incubated with metabolically labeled cell lysates from 10^7 cells for 2 hr at 4°C. Unless indicated otherwise, the beads were washed several times with SDS–RIPA buffer, and the proteins were eluted by heating in SDS-PAGE sample buffer and analyzed on 12% polyacrylamide gels followed by fluorography. For heat treatment, metabolically labeled cell lysates were heated for 3 min at 100°C, the precipitated proteins were removed by microcentrifugation, and the clarified lysates were incubated with protein A–Sepharose-bound Cdk2 or cyclin E–Cdk2 complexes. In binding assays using cyclin D2–Cdk4 complexes, metabolically labeled cell extracts were preincubated with 4 μl of cyclin D2–Cdk4 complex for 30 min at 4°C before the addition of protein A–Sepharose-bound Cdk2 or cyclin E–Cdk2. After removing the Sepharose beads, the cell extracts were immunoprecipitated with Cdk4 antiserum and the immunoprecipitates were analyzed on 12% SDS-PAGE.

Affinity purification of p27 and denaturation–renaturation experiments

HA-tagged Cdk2, alone or in complex with cyclin E, was bound to HA antibody immobilized on protein A–Sepharose beads (ImmunoPure Orientation Kit, Pierce) and used to isolate proteins from metabolically labeled cell lysates. Bound proteins were eluted from the column in 0.1 M glycine (pH 2.8) and precipitated with 4 volumes of ice-cold acetone and kept at −20°C for 20 min. The precipitates collected by microcentrifugation for 30 min were washed several times with cold acetone and dissolved in 6 M guanidium chloride in 1× HBB buffer (25 mM HEPES–KOH at pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 0.05% NP-40, 1 mM DTT). For renaturation (Kaelin et al. 1992), samples were dialyzed overnight against 1× HBB buffer and used either in kinase inhibition assays or for binding to cyclin E–Cdk2 Sepharose. For cyclin E-associated H1 kinase inhibition assays, aliquots (37.5 μg of protein) from 100,000*g* supernatants of lysates prepared from exponentially growing Mv1Lu cells were incubated for 30 min at 37°C with physiological amounts of baculoviral cyclin E either alone or in the presence of the indicated volumes of rena-

tured eluates. After incubation, samples were precipitated with cyclin E antiserum and assayed for histone H1 kinase activity. The relative cyclin E-associated H1 kinase activity was quantitated using a Molecular Dynamics PhosphorImager ImageQuant software.

To assay the activity of protein eluted from gel slices, cyclin E–Cdk2–HA affinity column eluates were run on 12% polyacrylamide gels along with molecular weight markers (Amersham). Part of the sample was run on the same gel, stained with Commassie, destained, and detected by fluorography. The gel was cut as indicated (between 0.5 and 1 cm per slice), and the proteins were isolated from the gel as described (Boyle et al. 1991). The isolated proteins were renatured and used for kinase inhibition assays as described above.

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Note added in proof

Microsequence data obtained from purified $p27^{Kip1}$ do not show significant similarity to the recently described p21 protein (Harper et al. 1993. *Cell* **75** 805–816; El-Diery et al. 1993. *Cell* **75** 817–825), or to any other protein in available databases.

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