p21-containing cyclin kinases exist in both active and inactive states

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In normal fibroblasts CDKs exist predominantly in p21/PCNA/cyclin/CDK quaternary complexes, whereas in p53-deficient cells, p21 expression is depressed and the kinases are reduced to a cyclin/CDK binary state. p21 is a universal cyclin kinase inhibitor, but we show here that p21-containing complexes exist in both catalytically active and inactive forms. This finding challenges the current view that active cyclin kinases function only in the binary state and reveals the subtlety with which tumor-suppressor proteins modulate the cell cycle.

[**Key Words:** p21-containing cyclin kinase; cell cycle progression; human fibroblasts; quaternary complex; kinase inhibitor]

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Much of our current understanding of the regulation of the cell division cycle has emerged from studies of a family of protein kinases [cdc2, CDC28, and generically cyclin-dependent kinase (CDK)] and their inhibitors and activators (for review, see Sherr 1993). A critical step in understanding cell cycle control was the discovery that CDKs interact with cyclins, proteins that serve as essential activating subunits and specificity determinants of the kinases (Draetta 1990; Sherr 1993). In human cells, multiple cyclins and CDKs interact in a relatively promiscuous fashion to form a large family of related cyclin kinases, each of which is presumed to play a specific role in cell cycle progression.

The view that mammalian cyclin kinases exist predominantly in a binary (cyclin/CDK) state prevailed until these enzymes were examined in normal human fibroblasts, rather than the many oncogenically transformed cell types that had been investigated hitherto (Xiong et al. 1992). In normal fibroblasts the major population of multiple cyclin kinases exists in quaternary complexes consisting of cyclin, CDK, proliferating cell nuclear antigen (PCNA), and a protein of apparent M_r 21,000, p21 (Zhang et al. 1993). However, in fibroblasts that are transformed with a variety of tumor viral oncoproteins the quaternary complexes essentially reduce to a binary state. Deregulation of cell proliferation is a hallmark of neoplastic transformation. Difference in the composition of cell cycle kinases between normal and transformed cells suggests that fundamental changes in cell cycle regulation contribute to neoplastic transformation.

Recently, it has become apparent that p21 is a universal inhibitor of cyclin/CDK catalytic activity (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993b). Each member of the cyclin kinase family is inhibited by p21, but

their relative affinities vary with each enzyme (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993b). Furthermore, several lines of evidence suggest that p21 expression is regulated by the p53 tumor-suppressor protein (El-Deiry et al. 1993; Xiong et al. 1993b). Thus, cells derived from certain p53-deficient Li-Fraumeni patients lack p21 associated with cyclin kinases (Xiong et al. 1993a). The p21 gene has a p53 transcriptional regulatory motif (El-Deiry et al. 1993), and cells lacking p53 express very low levels of p21 (El-Deiry 1993; Xiong et al. 1993b). These findings have led to a model in which p21 serves as an effector of cell cycle arrest in response to activation of the p53 checkpoint pathway (Xiong et al. 1993a).

The concept that p21 is a universal cell cycle kinase inhibitor creates a paradox, as in normal proliferating fibroblast cells the majority of multiple kinases exist in quaternary complexes (Zhang et al. 1993). Here, we present the unexpected finding that p21 is a component of catalytically active cyclin kinases. It appears that p21-containing enzymes can transition between active and inactive states, probably through changes in the stoichiometry of the p21 subunit. These findings have broad implications for our understanding of the normal cell cycle and the subversion of control pathways in tumor cells.

Results

p21 associates with PCNA and multiple cyclin kinases

We have demonstrated previously the association of p21 with multiple cyclin kinase complexes. To examine the full spectrum of p21-associated proteins, we raised a specific p21 antiserum. This serum immunoprecipitated from lysates of ^{35}S -labeled normal human diploid fibroblasts (WI38), a prominent ~21-kD protein, and a num-

ber of additional polypeptides (Fig. 1A, anti-p21). Partial V8 protease digestion revealed identity between the ~21-kD band and the p21 protein derived from *in vitro* translation of the p21 cDNA (Fig. 1B). To aid in the identification of the proteins that coimmunoprecipitate with p21, immunoprecipitates of CDC2, CDK2, CDK4, cyclin A, cyclin B1 and cyclin D1 were electrophoresed in parallel. By comparison, the mobilities of most of the p21-associated proteins could be correlated with that of cyclins, CDKs, and PCNA, as described earlier (see Fig. 1A; Xiong et al. 1993a; Zhang et al. 1993). These results suggest that the cyclin kinases and PCNA are the major p21-associated proteins in WI38 cells. To determine whether the majority of cellular p21 exists in complexes with these proteins, we fractionated extracts of ³⁵S-labeled WI38 cells by sedimentation through a glycerol gradient and examined p21 immunoprecipitates from each fraction (Fig. 1C). Little or no monomeric p21 was evident. The majority of p21 cosedimented with cyclin kinase complexes, particularly those that appear to contain cyclin A/CDK2 and cyclin D1/CDK4. The former probably comprise cyclin A/CDK2/p21/PCNA quaternary complexes. We noted that in this gradient, cyclin D1-containing complexes had a higher apparent molec-

ular weight than the cyclin A-containing complexes. This suggests the presence of an additional cyclin D-associated subunit, possibly the ~105-kD protein that cosediments with these complexes (Fig. 1C). Our results are also consistent with the existence of an independent interaction between p21 and PCNA.

*Active and inactive forms of the p21-associated cyclin kinases *in vitro**

p21 is a universal inhibitor of cyclin kinases *in vitro* (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993b). Thus, it was predicted that all p21-containing complexes would lack catalytic activity. However, in previous *in vitro* experiments, progressive addition of p21 to cyclin kinases did not result in a precise, corresponding loss of kinase activity but, instead, caused an abrupt transition from full activity to essentially complete inhibition (Xiong et al. 1993b). Here, we have examined the seeming cooperativity of p21 inhibition of cyclin kinases in greater detail.

Lysates were prepared from metabolically ³⁵S-labeled insect cells infected with baculoviruses directing the ex-

Figure 1. PCNA and cyclin/kinases are the major p21 associated proteins. (A) p21-associated proteins were immunoprecipitated from cell lysates of ³⁵S-labeled normal human fibroblasts (WI38) and compared with the proteins immunoprecipitated by anti-CDC2, CDK2, CDK4, cyclin A, cyclin B, or cyclin D1 antisera as indicated. The positions of protein molecular mass markers (in kD), electrophoresed in parallel, are indicated. (B) p21 produced by *in vitro* translation of the p21 cDNA (left) is compared with p21 purified from anti-p21 immunoprecipitates from ³⁵S-labeled human cell lysates by partial V8 protease digestion (right). (C) Glycerol gradient sedimentation analysis of p21-associated protein complexes. The fractions were taken from top (left) to bottom (right) of the gradient and immunoprecipitated with anti-p21 antibody. The peak of BSA, sedimented in parallel, migrates at fraction 16.

pression of human cyclin A, CDK2, and p21. Progressive addition of the p21-containing lysate to preformed cyclin A/CDK2 complexes resulted in accumulation of p21/cyclin A/CDK2 ternary complexes (Fig. 2A). At a specific point in the titration, the histone H1 kinase activity in cyclin A immunoprecipitates was abruptly lost (Fig. 2A). As noted previously, this transition between active and inactive enzyme was not reflected directly in the binding of p21 to cyclin A/CDK2, which was approximately proportional to the amount of p21 added (Fig. 2A). One explanation for the observed discrepancy between p21 binding and inhibition is that p21 might fail to act as a cyclin kinase inhibitor at subsaturating levels. To test this possibility, we specifically recovered the p21-containing complexes using the p21 antiserum (Fig. 2B). Contrary to previous predictions, complexes formed at subsaturating p21 concentrations possessed substantial histone H1 kinase activity. With the addition of p21, p21-associated kinase activity increased progressively, mirroring the accumulation of ternary complexes, followed by abrupt loss of p21-associated kinase at the same p21 concentration that abolished activity in parallel cyclin A immunoprecipitates. At its maximum, p21-associated kinase was ~70% of that detected in CDK2 immunoprecipitates from the same mixtures (Fig. 2). Similar results were obtained using a histidine-tagged

p21 that was recovered with Ni⁺-NTA agarose (data not shown). These results demonstrate that p21-containing cyclin kinase complexes can exist in both active and inactive states. Inhibition is achieved only as p21 reaches a saturating concentration.

We note that p21 displays an altered electrophoretic mobility when added to cyclin kinases at noninhibitory levels. Several lines of evidence indicate that altered mobility reflects p21 phosphorylations. These are discussed in detail below.

p21 is a universal inhibitor of cyclin kinases, and non-linear p21 inhibition profiles have been observed with cyclin A/CDK2, cyclin E/CDK2, and cyclin D1/CDK4 enzymes (Xiong et al. 1993b; Fig. 3), suggesting that the association of p21 with active cyclin kinases might be a general phenomenon. Consistent with this prediction, we detected substantial kinase activity in p21/cyclin E/CDK2 and p21/cyclin B/CDC2 complexes formed at subsaturating p21 concentrations (Fig. 3A,B). In the case of cyclin E/CDK2, p21-associated activity reached ~80% of that detected in parallel cyclin E immunoprecipitates.

We have shown previously that the inclusion of PCNA had no gross effect on the inhibition of cyclin kinases by p21. Similarly, PCNA had no effect on the association of p21 with active enzyme (data not shown).

Figure 2. Active and inactive p21/cyclin A/CDK2 kinase complexes. Increasing amounts of ³⁵S-labeled p21-containing Sf9 cell lysates were added to 10 μ l of preformed cyclin A/CDK2 kinase complexes in the presence of 1 mM ATP at 30°C for 30 min. Proteins were recovered from the mixtures by immunoprecipitation with either anti-cyclin A (A) or anti-p21 (B) antibodies. Composition of the immunoprecipitates was monitored by direct visualization of the labeled proteins (*top*). Activity of the complexes was assayed using histone H1 (*middle and bottom*) as the substrate. The relative kinase activities were determined from the band intensities on a Fuji BAS2000 bioimager. The exposure time for anti-p21 is twice that for anti-cyclin A.

Figure 3. Activity of cyclin B/CDC2/p21 and cyclin E/CDK2/p21 kinases. Increasing amounts of p21-containing Sf9 cell lysates were added to 10 μ l of preformed cyclin B/CDC2 (A) or cyclin E/CDK2 (B) kinase complexes in Sf9 cell lysates. After incubation, the p21/cyclin E/CDK2 or p21/cyclin B/CDC2 complexes were recovered by immunoprecipitation with either anti-cyclin B1 (A, top), anti-cyclin E (B, top), or anti-p21 (A, B, bottom) antibodies and assayed for histone H1 kinase activity. In A, the exposure time for anti-p21 is fivefold more than for anti-cyclin B. In B, the exposure time for anti-cyclin E is the same as for anti-p21.

p21 is a component of active cyclin kinases in vivo

We have shown previously that in normal human diploid fibroblasts (e.g., WI38), the majority of cyclin kinases exist in complexes containing p21 (Zhang et al. 1993). Thus, the finding that p21 is an inhibitor of cyclin kinases raised questions concerning the nature of the active kinases in normal cells. The association of p21 with active cyclin kinase in vitro suggested that p21-containing complexes might also possess activity in vivo. Therefore, we immunoprecipitated p21 from several cell lines and assayed for coprecipitation of histone H1 kinase activity. In all cell lines that contain detectable levels of p21 protein, we found catalytically active p21 complexes (Fig. 4, panels WI38, RKO, HeLa, VA13). In WI38 cells that contain functional p53, p21 is relatively abundant

and is associated with substantial histone H1 kinase (Fig. 4, panel WI38). Comparable activity is recovered in CDC2 and CDK2 immunoprecipitates from the same cell lysates. RKO cells also possess functional p53 and active p21-associated cyclin kinases. However, p21-associated activity is exceeded severalfold in these cells by the activity found in CDK2 immunoprecipitates. HeLa and VA13 cells have approximately fivefold reduced levels of p21 because of the presence of p53-inactivating viral oncoproteins (see below), but these cells still contain noticeable p21-associated kinase. In HL60 cells and in the Li-Fraumeni line LCS041, functional p53 is absent and p21 is virtually undetectable. CDC2 and CDK2 immunoprecipitates from these cells contain high levels of histone H1 kinase but, as expected, activity is absent from anti-p21 immunoprecipitates.

Active and inactive states of the p21-associated enzyme

Our results indicate that p21-associated kinases exist in active and inactive states. Transition between these states could be explained by two broadly different classes of models. In the first, modification of one constituent of the kinase complex could regulate its activity. For example, phosphorylation of the cyclin, CDK, or p21 subunit might immunize the complex against inhibition by bound p21. In this regard, kinase-bound p21 displayed an altered electrophoretic mobility under conditions in which cyclin A/CDK2/p21 was active as a histone H1 kinase (Fig. 2A,B, lanes 1–16), and these mobility shifts reflect p21 phosphorylation (data not shown). In an alternative model, changes in subunit stoichiometry might cause the transition from active to inactive p21-associated kinase. In particular, one possible explanation of the nonlinear p21 inhibition profiles (see Figs. 2 and 3) is that multiple p21 subunits are necessary for kinase inhibition.

To distinguish between these alternative mechanisms, we took the approach outlined in Figure 5A. p21/cyclin A/CDK2 complexes were formed at a variety of p21 concentrations yielding both active and inactive p21-associated enzymes as described in Figure 2. These were immunoaffinity purified using the p21 antiserum (see Fig. 5A, step b). Complexes were then challenged with a purified fusion protein consisting of p21 and the maltose-binding protein (MBP-p21, step c). According to the first model (Fig. 5A, top) changes in the phosphorylation state of complex constituents are necessary for changes in activity. Thus, assuming no p21 subunit exchange and assuming that potential modifying enzymes have been removed from immunoprecipitated complexes, incubation with additional MBP-p21 should have no effect (Fig. 5A, top, step d). If, on the other hand, changes in p21 stoichiometry account for changes in activity (Fig. 5A, bottom), MBP-p21 should be incorporated into the complex and inhibit kinase. The experimental results are consistent with the latter scheme (Fig. 5B,C). Addition of saturating amounts of MBP-p21 (see Fig. 5B), but not MBP alone, converted purified, active, p21-containing com-

Figure 4. p21/cyclin/CDK kinases are active in vivo. Proteins were immunoprecipitated from ^{35}S -labeled cell lysates from HL60, LCS041, VA13, HeLa, RKO, or WI38 cells (as indicated) with anti-CDK2, anti-CDC2, or anti-p21 antibodies as indicated. The immunoprecipitates were split and used for direct visualization of immunoprecipitated proteins (*top*) or for histone H1 kinase assays (*bottom*). The positions of protein size markers are indicated.

plexes to an inactive state (Fig. 5C). Examination of labeled proteins present in the immunoprecipitates revealed that inhibition occurred without changes in the phosphorylation state of p21, exchange of p21 subunits, or disruption of the cyclin and CDK subunits of the previously active enzyme. Similar results have been obtained using a glutathione-S-transferase-p21, GST-p21, fusion protein (data not shown).

In parallel experiments, we have identified the phosphorylation sites that cause p21 mobility shifts (see Figs. 2 and 3) as serines 98 and 130. Conversion of either serine to alanine had no obvious effects on either the association of p21 with active kinase or the ability of p21 to inhibit cyclin kinases (data not shown), suggesting that phosphorylation of these sites is irrelevant to the activity of p21-associated enzymes.

Multiple p21 subunits in inactive complexes

The previous experiments suggested that active and inactive p21-containing enzymes might differ in stoichiometry of their p21 subunits and might therefore be physically distinguishable. To test this possibility, complexes containing p21, cyclin A, and CDK2 were formed at subsaturating p21 concentrations, and lysates were fractionated by gel filtration chromatography. p21-containing complexes were recovered from column fractions using p21 antiserum, and immunoprecipitates were tested for both protein composition and histone H1 ki-

nase activity (Fig. 6A). p21-associated complexes distributed into two resolvable peaks. One migrated near the position predicted for a p21/cyclin A/CDK2 ternary complex (fractions 30–36; see legend to Fig. 6), whereas a second migrated at a much higher apparent molecular weight (fractions 16–20). p21-associated histone H1 kinase activity resided almost exclusively in the low-molecular-weight fractions; these complexes possess 20-fold greater activity than the high-molecular-weight fractions, although they contain only about threefold more CDK subunit (see legend to Fig. 6). Identical results were obtained when complexes were formed in the presence or absence of an unfused MBP.

To test the p21 subunit composition of complexes in the low- and high-molecular-weight fractions, we formed p21/cyclin A/CDK2 complexes using a mixture of physically distinct p21 proteins. In this experiment, cyclin A, CDK2, and one of the two types of p21 subunits, native p21, were derived from baculoviral lysates and were ^{35}S -labeled. The second type of p21 was the unlabeled MBP-p21 protein purified from bacteria (see above). If any complexes contain multiple p21 subunits, then native, ^{35}S -labeled p21 should coprecipitate with tagged p21 protein on an MBP-specific amylose resin.

Complexes were formed by incubating the four components at subsaturating p21 concentrations, and low- and high-molecular-weight complexes were separated by gel filtration chromatography. Only cyclin A and CDK2 were recovered in association with MBP-p21 from the

Figure 5. Active p21-associated enzymes are inhibited by additional p21. (A) Experimental design. (a) Formation of active cyclin/CDK/p21 kinase at a subsaturating concentration of p21 in insect cell lysates. p21 is phosphorylated during this reaction. (b) Isolation of the active p21-containing kinase by immunoprecipitation with anti-p21 antibody immobilized on protein A beads. (c) Challenge of the isolated active cyclin/CDK/p21 kinase complex with purified MBP-p21. Outcomes predicted from two possible models are presented. (I) If protein modification is responsible for the maintenance of active cyclin/CDK/p21 kinase, challenge of the kinase with excess MBP-p21 will not affect the activity of the kinase. (II) If increased p21 stoichiometry accounts for kinase inhibition, incorporation of MBP-p21 into the active cyclin/CDK/p21 kinase complex should result in formation of an inactive kinase complex. (B) Formation of ^{35}S -labeled cyclin A/CDK2/p21 kinase complex at various p21 concentration in the insect cell lysates was performed as described in the legend to Fig. 2. The kinase complexes were immunoprecipitated with anti-cyclin A antibody. The purified immunoprecipitates were then challenged with either 2 μg of purified MBP (left) or MBP-p21 fusion protein (right) in the absence of ATP. The protein complexes were reisolated and assayed for labeled protein composition (top) and histone H1 kinase activity (bottom). (C) Mixtures described in B were precipitated with the p21 antiserum before the rechallenge analysis.

Figure 6. Inactive p21-containing kinases contain multiple p21 subunits. ^{35}S -Labeled p21/cyclin A/CDK2 kinase complexes were formed at subsaturating concentrations of p21 in insect cell lysates as described in the Materials and methods. The lysates were then mixed with subsaturating amounts of either MBP (A) or MBP-p21 (B). The reaction mixtures were loaded onto a Superose 6 gel filtration column and fractionated at 0.5 ml/fraction. Proteins were recovered using anti-p21 (top, middle) antibody, or amylose-agarose (bottom). Protein complexes were assayed for labeled protein composition (top, bottom) or histone H1 kinase activity (middle). Migration of protein molecular mass markers in the Superose 6 column is indicated above the fraction numbers in each panel (kD).

low-molecular-weight fractions, implying that they contained MBP-p21/cyclin A/CDK2 ternary complexes. However, from the high-molecular-weight fractions, native p21, cyclin A, and CDK2 were recovered with MBP-p21 by binding to the amylose resin. This suggests strongly that inactive complexes present in the high-molecular-weight fractions contain multiple p21 subunits and that those in the low-molecular-weight fractions contain a single p21 molecule.

To confirm that partition into low- and high-molecular-weight complexes is attributable to differences in p21 stoichiometry, we isolated low-molecular-weight, p21-containing complexes (fractions 30–36, Fig. 6A) and challenged them by incubation in the presence of either MBP-p21 or MBP alone. Subsequent refractionation by gel filtration showed that challenge with MBP-p21 shifted a significant portion of the low-molecular-weight p21-containing complexes into the high-molecular-weight fraction, and this was accompanied by incorporation of the tagged p21 protein into the complexes (data not shown). Challenge with MBP alone had no effect on

the chromatographic profile of the active complexes. We have also tested the possibility that cyclin/CDK may oligomerize under the conditions we have used. Fusion protein of GST-cyclin A was produced from either bacteria or baculovirus-infected Sf9 cells. The incubation of GST-cyclin A protein with cyclin A/CDK2 kinase only produced GST-cyclin A/CDK2 kinase but not protein complexes that contain GST-cyclin A/cyclin A. Taken together, our results indicate that inhibition of cyclin kinases requires binding of more than one p21 subunit.

Discussion

The most striking finding of this study is that p21, which was thought to function as an inhibitor of cyclin kinases (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993b), can be a component of catalytically active enzymes both in vitro and in vivo. We have shown previously that in normal human diploid fibroblasts, the major fraction of cyclin kinases exists in quaternary complexes containing a cyclin, a CDK, PCNA, and p21 (Zhang et al. 1993).

Thus, the discovery that p21 functions as a kinase inhibitor was paradoxical, as it predicted that normal cells should contain virtually no active cyclin kinase. By demonstrating that p21-containing cyclin kinases exist in both active and inactive states, we have rationalized the inhibitory role of p21 with the ability of normal cells to progress through the cell cycle. It is apparent that some fraction of quaternary complexes are catalytically active *in vivo*. Our results are consistent with the notion that the quaternary complex may represent the normal state of active cyclin kinases and that this form of the complex controls cell cycle progression in normal cells.

The expression of the p21 gene is regulated by the tumor suppressor protein p53 (El-Deiry et al. 1993; Xiong et al. 1993b). Thus, it has been speculated that p21 might be the key effector of cell cycle arrest induced by activation of the p53 checkpoint pathway (El-Deiry et al. 1993; Xiong et al. 1993b; Dulic et al. 1994). Although our results do not affect the basic premise of this model, they do suggest that p53 may also play a more subtle role in the regulation of normal cell growth. For example, the presence of p21 in normal cells may be sufficient to inactivate low levels of cyclin/CDK complexes. Thus, normal cells might need to overcome this p21 threshold before active cyclin kinases promote cell cycle progression. The level of p21 is greatly decreased (>50-fold) in cells lacking functional p53 (see Fig. 4, LCS041, HL60); therefore, p53-deficient cells would not be subject to this type of control. Cyclin kinases exist in different conformations in normal and p53-deficient cells. In normal cells, the active kinases contain PCNA and p21 subunits. In many transformed cells, these two proteins are absent from the binary cyclin/CDK enzymes. Although the presence of PCNA and of a single p21 subunit appear neutral in our *in vitro* assays, it is likely that the quaternary and binary cyclin kinases might have as yet uncharacterized functional differences *in vivo*.

It is clear that p21-containing complexes exist in physically distinct, active and inactive states. Our results are consistent with a model in which these states differ in the relative stoichiometry of the p21 subunit. Active complexes appear to contain a single p21 molecule, whereas inactive complexes possess multiple p21 subunits. Changes in p21 stoichiometry are sufficient to account for the conversion of active to inactive complexes in our *in vitro* experiments. However, association of cyclin kinases with p21 must be intertwined with other modes of regulation *in vivo*. Cyclin kinases are subject to a number of activating and inhibitory phosphorylations. For example, most members of the cyclin kinase family require an activating phosphorylation at a threonine (~161 or its equivalent) residue by CDK-activating kinase (CAK). In this regard, we have found that saturation of CDK complexes with p21 blocks access of CAK to the catalytic subunit. However, inhibition by p21 does not depend on prevention of CAK phosphorylation (H. Zhang, G.J. Hannon, and D. Beach, unpubl.). In addition, CDKs are regulated by inhibitory phosphorylation of threonine and tyrosine residues near their amino termini (for review, see Draetta 1990; Sherr 1993). It is not

known what effect association with noninhibitory levels of p21 might have on the function of these CDK-modifying enzymes *in vivo*. It is also possible that p21 binding might alter the subcellular localization of active kinase complexes. Further study will be required to understand fully how all of these regulatory pathways integrate to affect growth control in normal cells.

The association of p21 with active kinase suggests the possibility that p21 may have roles in cell cycle regulation beyond its action as a CDK inhibitor. Our data suggest that p21 can promote the association of cyclin and CDK subunits (see Fig. 5B). In this manner, p21 might act as a CDK assembly factor and could potentially function as an activator of the kinase. In this regard, we observed a reproducible approximately threefold increase in total kinase activity with the addition of low concentrations of p21 to lysates containing cyclin A and CDK2, and this was accompanied by an increase in cyclin-CDK association (Fig. 5B; H. Zhang, G.J. Hannon, and D. Beach, unpubl.). The existence of a CDK assembly factor was suggested previously (Matsushime et al. 1994) based on the observation that cyclin D and CDK4 did not associate in serum-starved macrophages but did associate with serum stimulation. p21 levels are increased by serum stimulation in p53-deficient mouse embryo fibroblasts (K. Macleod and T. Jacks, pers. comm.), and this, coupled with our results, suggests the possibility that p21 could act as a CDK assembly factor *in vivo*.

Materials and methods

Cell culture

The culture of human normal fibroblast (WI38), Li-Fraumeni cell line (LCS041), SV40 virus-transformed human fibroblast line (VA13), and HeLa cells was described previously (Xiong et al. 1993a). The colorectal carcinoma cell line (RKO) was kindly provided by Dr. Michael Kastan (The Johns Hopkins University, Baltimore, MD) and the myeloid leukemia line (HL60) was obtained from American Type Culture Collection (Rockville, MD). These cells were cultured similarly. Insect Sf9 cells were cultured in Grace's medium supplemented with 10% heat-inactivated fetal bovine serum, lactalbumin hydrolysate, and yeastolate ultrafiltrate at 27°C as described (Xiong et al. 1993b). The expression of baculoviruses encoding p21, PCNA, various CDKs, and cyclins in Sf9 cells was performed as described (Xiong et al. 1993b).

Antibodies and immunoprecipitation

Antibodies against cdc2, CDK2, CDK4, cyclin A, cyclin B1, or cyclin D1 were described earlier (Xiong et al. 1993a). Anti-cyclin E antibody was kindly provided by Dr. Konstantin Galaktionov (Cold Spring Harbor Laboratory). For the production of p21 antiserum, the human p21 gene was cloned into pGEX-KG vector (Guan and Dixon 1991) to create a fusion protein between GST and p21. The fusion protein was expressed in *Escherichia coli* (BL21 strain) and purified on glutathione beads as described previously (Hannon et al. 1993). The purified fusion protein was used to immunize rabbits as described (Hannon et al. 1993). Immunoprecipitation and partial V8 proteolytic mapping were performed as described previously (Xiong et al. 1992; Zhang et al. 1993). In all cases, ~3 µl of crude serum was used for immunoprecipitation of lysates derived from one 10-cm tissue culture dish.

Plasmids and DNA recombination techniques

For the production of MBP-p21 fusion protein, a DNA fragment encoding the human p21 gene was cloned in-frame to the carboxyl terminus of MBP in the pMAL-c2 vector between *Sall* and *HindIII* sites. The fusion protein was produced in *E. coli* (BL21) and purified on amylose resin according to the instructions provided by the manufacturer (New England Biolabs). The MBP was similarly purified. The purified proteins were dialyzed into kinase buffer [50 mM Tris (pH 7.5), 10 mM MgCl₂, 0.5 mM DTT] and stored at -80°C. MBP and MBP-p21 proteins were >95% pure. For the generation of p21 mutants, the oligonucleotide-directed mutagenesis method was used (Kunkel 1985). For the change of serine 98 to alanine, an oligonucleotide CCTGGCACCGCACCTGCTCTG was synthesized with the underlined G changed from T. For the change of serine 130 to alanine, the oligonucleotide GCTGAAGGGGCCCCAGGTGGA was synthesized with the underlined G changed from T. The mutated p21 genes were cloned into baculovirus expression vector pVL1393 and expressed in Sf9 cells as described previously (Xiong et al. 1993b). The change of threonine 57 to alanine was also made, but no effect on the p21 phosphorylation or p21 activity was observed (unpublished data).

Assembly of active and inactive quaternary complexes

Typically, insect Sf9 cells were individually infected with baculoviruses encoding cyclin, CDK, or p21. At 48–72 hr postinfection, cells were labeled with 100 µCi/ml of [³⁵S]methionine for 3 hr. Infections and preparation of cell lysates were exactly as described previously (Xiong et al. 1993b). The cell lysates containing cyclin or CDK2 (5 µl each) were mixed in the presence of 1 mM ATP, incubated for 30 min at 30°C, and cooled on ice for 5 min to form cyclin/CDK kinase complexes. Increasing amounts of p21-containing lysates were then added to each 10 µl of kinase lysate. After incubation on ice for 15 min to allow complex formation, the reaction was further incubated at 30°C for 15 min. The reaction was stopped by addition of 20 mM EDTA. Complexes were recovered by immunoprecipitation. The kinase assay using histone H1 as the substrate was conducted as described previously (Xiong et al. 1993b). The relative kinase activities were determined from the band intensities on a Fuji BAS2000 bioimager.

Glycerol gradient sedimentation and gel filtration chromatography

For sedimentation analysis, labeled cell lysates were loaded onto 9 ml of 15–30% glycerol gradients and sedimented at 40,000 rpm for 38 hr in an SW41.Ti rotor (Beckman) as described (Zhang et al. 1993). The fractions (300 µl each) were collected from the top of the gradient, and proteins were recovered by immunoprecipitation with the anti-p21 antiserum. For gel filtration analysis, 40 µl of ³⁵S-labeled p21-containing lysates was mixed with 30 µl of cyclin A/CDK2 lysate to form p21/cyclin A/CDK2 kinase complexes at 30°C for 30 min. Reactions were then incubated in the presence of either 2 µg of purified MBP or MBP-p21 for 20 min at 30°C. The reaction mixtures were then loaded onto a Superose 6 gel filtration column (Pharmacia) equilibrated in 50 mM Tris (pH 7.5) and 250 mM NaCl. The fractions (0.5 ml) were split and immunoprecipitated with anti-cyclin A (not shown) or anti-p21 antibodies or mixed with amylose beads. The protein complexes on the beads were assayed for labeled protein composition or histone H1 kinase activity. The protein molecular mass markers fractionated in parallel are bovine gamma globulin (158 kD), bovine serum albumin (67 kD), chicken ovalbumin (44 kD), and equine myoglobin (17 kD).

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