However, cyclin D/CDK4 binary complexes catalysed substantial Rb phosphorylation (Fig. 4*e*, *f*). Addition of increasing amounts of p21 resulted in the accumulation of cyclin D/CDK4/p21 ternary complexes (Fig. 4*e*), with a corresponding inhibition of Rb phosphorylation (Fig. 4*f*). Again, inclusion of PCNA was essentially without effect (our unpublished results).

The ability of p21 to inhibit such disparate cyclin/CDK kinases suggests that p21 is a universal CDK inhibitor. Thus, overexpression of p21 *in vivo* would be expected to cause cell-cycle arrest. To address this question, we examined the effect of p21 overexpression on cell proliferation using a stable colony formation assay[7]. Transfection of SAOS-2 cells with a pRcCMV vector alone yielded a large number of stable transformants. However, transfection with either of two independent preparations of a plasmid directing the overexpression of p21 failed to produce an appreciable number of colonies (results not shown). The effect of p21 overexpression was virtually identical to the effect of p53 overexpression in a parallel transfection. These results indicate that p21 may be an inhibitor of cell proliferation.

As we have previously found that p21 is absent from cyclin/CDK complexes in cells lacking functional p53 (ref. 1), we isolated the murine p21 cDNA (data not shown) and examined p21 messenger RNA levels in fibroblasts derived from p53-'null' mice. Compared with fibroblasts from normal embryos, p53 'null' fibroblasts showed ~50-fold lower levels of p21 mRNA (data not shown). Furthermore, p21 mRNA is induced ~10-fold by γ-irradiation of a $p53^+$ myeloid leukaemia cell line (mL-7) but is unchanged upon similar treatment of a myeloid leukaemia cell line that lacks p53 (HL-60; data not shown). These results indicate that p21 is regulated by the p53 pathway.

In many transformed cells, cyclins and CDKs associate in binary complexes which form the core of the cell-cycle regulatory machinery. In normal cells, a major fraction of the cyclin kinases acquires two additional subunits and thereby forms quaternary complexes[4,5]. We have isolated a cDNA encoding the one uncharacterized component of these quaternary complexes, p21. Reconstitution of quaternary complexes in insect cells revealed that p21 is a universal inhibitor of cyclin kinases. As such, p21 inhibits cell proliferation upon overexpression in mammalian cells. Taken in conjunction with the previously demonstrated absence of p21 protein in the cell-cycle kinase complexes of cells with deficient p53, our results indicate that p21 could be a transcriptional target of the tumour suppressor protein, p53. One function of p53 is to act in a cell signalling pathway which causes cell-cycle arrest following DNA damage (see, for example ref. 8). We suggest that p21 forms a critical link between p53 and the cell-cycle control machinery. □

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A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4

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THE division cycle of eukaryotic cells is regulated by a family of protein kinases known as the cyclin-dependent kinases (CDKs)[1,2]. The sequential activation of individual members of this family and their consequent phosphorylation of critical substrates promotes orderly progression through the cell cycle[3,4]. The complexes formed by CDK4 and the D-type cyclins have been strongly implicated in the control of cell proliferation during the G1 phase[3–6]. CDK4 exists, in part, as a multi-protein complex with a D-type cyclin, proliferating cell nuclear antigen and a protein, p21 (refs 7–9). CDK4 associates separately with a protein of M_r 16K, particularly in cells lacking a functional retinoblastoma protein[9]. Here we report the isolation of a human p16 complementary DNA and demonstrate that p16 binds to CDK4 and inhibits the catalytic activity of the CDK4/cyclin D enzymes. p16 seems to act in a regulatory feedback circuit with CDK4, D-type cyclins and retinoblastoma protein.

The yeast two-hybrid protein interaction screen[10] was used to search for proteins that can associate with human CDK4. Two-hybrid screening relies on reconstituting a functional GAL4 transcriptional activator from two separate fusion proteins, the activation domain ($GAL4^{ad}$) and the DNA-binding domain ($GAL4^{db}$). A positive cDNA clone was found which contained, in-phase with $GAL4^{ad}$, an open reading frame of 148 amino acids encoding a protein of M_r 15,845 comprising four ankyrin repeats (Fig. 1*a*). We have named this protein $p16^{INK4}$ (inhibitor of CDK4; see below).

To test the specificity of the association between $p16^{INK4}$ and CDK4, yeast cells were co-transformed with a plasmid encoding the $GAL4^{ad}$–$p16^{INK4}$ fusion and with plasmids encoding several different targets (see Fig. 1*b*). Only the $GAL4^{db}$–CDK4 fusion interacted with GAL^{ad}–$p16^{INK4}$ to an extent that allowed growth in the absence of histidine (Fig. 1*b*). The specificity of this interaction was studied in a cell-free system. A fusion protein consisting of glutathione *S*-transferase fused to $p16^{INK4}$ (GST–$p16^{INK4}$) was expressed in bacteria and purified. GST–$p16^{INK4}$ was mixed with different *in vitro*-translated ^{35}S-labelled CDKs (Fig. 1*c*, top), and the GST–$p16^{INK4}$ fusion protein was recovered from the different mixtures on glutathione-Sepharose beads. GST–$p16^{INK4}$ bound much more efficiently to CDK4 (>30-fold) than to the other CDKs tested (Fig. 1*c*, middle). The specificity of the CDK4/$p16^{INK4}$ interaction was also studied in insect cells infected with a recombinant baculovirus encoding $p16^{INK4}$ and with baculoviruses encoding CDK4 or CDK2, respectively (Fig. 1*d*). $p16^{INK4}$ was co-immunoprecipitated with anti-CDK4 (Fig. 1*d*, lane 1), but not with anti-CDK2 (lane 4). Conversely $p16^{INK4}$ antibodies co-immunoprecipitated CDK4 (Fig. 1*d*, lane 3), but not CDK2 (lane 6). These results demonstrate that $p16^{INK4}$ interacts specifically with CDK4. Glycerol gradient centrifugation indicated that CDK4 and $p16^{INK4}$ from insect cell extracts form a binary (1:1) complex (data not shown).

Anti-CDK4 immunoprecipitates from a normal human diploid fibroblast line, W138, revealed that CDK4 associates with several proteins, cyclin D1, proliferating cell nuclear antigen (PCNA), p21 and p16 (Fig. 2*a*, lane 1). Proteins present in this immunoprecipitate probably represent at least two independent

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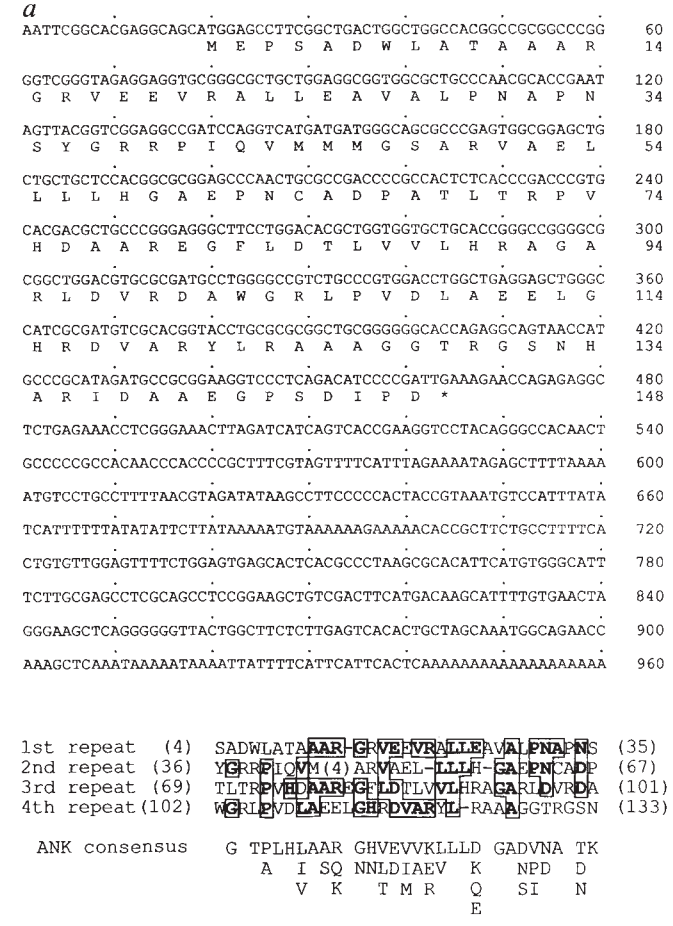
complexes, one comprising the quaternary complex of CDK4, cyclin D1, p21 and PCNA, and a binary complex containing CDK4 and p16 (refs 7–9). In contrast, in VA13 and HeLa cells, CDK4 is predominantly, if not exclusively, associated with p16 (Fig. 2*a*, lanes 5 and 9, respectively[9]). VA13 is a SV40 virus-transformed derivative of W138, and HeLa cells express the papillomavirus E6 and E7 proteins. Anti-p16^{INK4} immunoprecipitates contained a protein of M_r 16K which was readily detectable in the transformed cell lines VA13 and HeLa (Fig. 2*a*, lanes 7 and 11, respectively) and to a much lesser extent in the normal cell line W138 (lane 3). We also found that at least two other proteins, p33 and p38, specifically co-immunoprecipitate with p16^{INK4} (Fig. 2*a*, lanes 3, 7, 11). The *N*-chlorosuccinimide (NCS) partial proteolytic pattern of the CDK4-associated p16 was identical to the pattern generated from immunoprecipitated p16^{INK4} (Fig. 2*b*, compare lanes 1 and 2). In addition, the partial V8 protease patterns of the p16-associated p33 and CDK4 were identical (Fig. 2*b*, compare lanes 3 and 4 with lanes 5 and 6, respectively). These results show unequivocally that our cDNA encodes the CDK4-associated p16. The other apparent p16^{INK4}-associated protein, p38, might correspond to the CDK4-related kinase PSLIRE whose M_r is close to 38K (ref. 11).

We have reconstituted active CDK4/cyclin D complexes that can phosphorylate a fusion protein consisting of gluthathione *S*-transferase and a fragment of the retinoblastoma protein (Rb) termed the large pocket[12,13] (Fig. 3*a*, lanes 1, 2). Addition of extracts containing p16^{INK4} abolished phosphorylation of GST–Rb by cyclin D2/CDK4 (Fig. 3*a*, lanes 3–5) whereas extracts



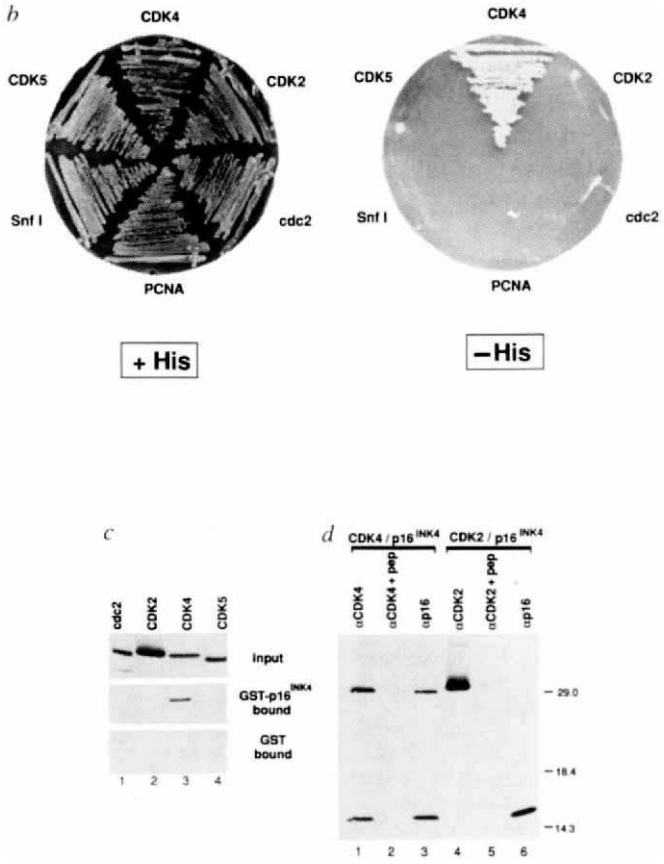


FIG. 1 Sequence of the human p16^{INK4} cDNA and specific interaction between p16^{INK4} and CDK4. *a*, Top, sequence of p16^{INK4}. We have noted a motif at the amino terminus of p16^{INK4} ($MX_3ADWLATAX_4RVEEVX_2LL$) which shows homology to the amino-terminus of the cyclin box[19]. This is the same region, termed the P-box, which in B-type cyclins contributes to the activation of the cdc25 phosphatase[20]. However, mutation of this motif affected neither CDK4 binding nor inhibition of CDK4 kinase by p16^{INK4} (our unpublished results). Bottom, p16^{INK4} is formed by four ankyrin repeats[21]. *b*, Yeast cells were simultaneously transformed with a plasmid expressing a GAL4^{db}–INK4 fusion and with plasmids expressing the GAL4^{ad} fused to the indicated CDK, PCNA or the budding yeast kinase, Snfl. Cells containing both plasmids were streaked on plates with or without histidine. The ability to grow in the absence of histidine depends on the expression of the *HIS3* gene that is under the control of a GAL4-responsive promoter. *c*, Purified, bacterially produced GST–p16^{INK4} fusion protein was mixed with ^{35}S-labelled *in vitro*-translated CDKs, as indicated. Top, analysis of an aliquot of the *in vitro*-translation products. Centre, ^{35}S-labelled proteins recovered on glutathione–Sepharose beads following incubation with GST–p16^{INK4}; 1% of the input CDK4 was recovered in lane 3. Bottom, ^{35}S-labelled proteins recovered following incubation with GST. Purification of GST–p16^{INK4} and binding assays were done as described[15]. *d*, ^{35}S-labelled insect cells lysates expressing CDK4 and p16^{INK4} (lanes 1–3) or CDK2 and p16^{INK4} (lanes 4–6) were immunoprecipitated with the indicated antibodies.

METHODS. *a*, For two-hybrid screening, human CDK4 (ref. 6) was fused to the GAL4 DNA-binding domain in the pGBT9 vector (P. Bartel and S. Fields, unpublished data). The HeLa cDNA library was constructed as described[15] except that a low-expression derivative (pGAD-GL) of pGAD-GH was used which lacks a 900-base pair (bp) *Sph*I fragment from the alcohol dehydrogenase promoter. Screening was done as described[15]. *d*, The recombinant baculovirus expressing p16^{INK4} was constructed using the vector pVL1393 and the Baculo-Gold kit (Pharmingen). Baculoviruses expressing CDK2 and CDK4 were obtained from D. Morgan and H. Zang, respectively. Preparation of cell lysates and immunoprecipitation were as described[15]. Antisera against GST–p16^{INK4} were generated in rabbits by Pocono Rabbit Farm and Laboratory, Inc. Antibodies were immunoaffinity purified using a CNBr-activated Sepharose 4B column (Pharmacia) as described[22]. The anti-CDK4 peptide antibody has been described previously[9]. The anti-CDK2 antibody was raised against a synthetic peptide corresponding to the carboxy-terminal region of human CDK2 (provided by K. Galaktionov).

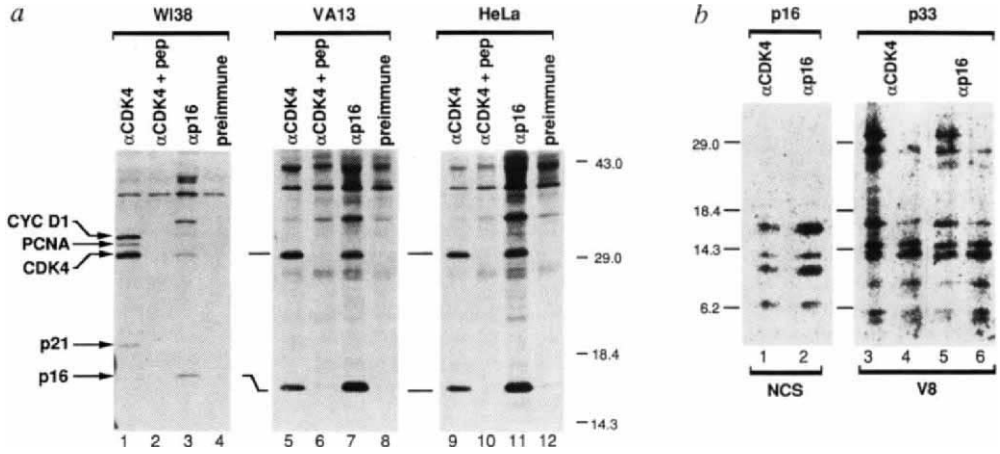


FIG. 2 p16^{INK4} associates with CDK4 in human cells. *a*, Proteins were immunoprecipitated from ^{35}S-labelled lysates of W138 (lanes 1–4), VA13 (lanes 5–8) or HeLa (lanes 9–12) cells with the indicated antibody. The identity of the proteins marked with arrows was determined previously for the anti-CDK4 immunoprecipitates[7,9], and in this report for the anti-p16^{INK4} immunoprecipitates (see *b*). *b*, p16 co-immunoprecipitated from HeLa cell lysates with anti-CDK4 (lane 1) and p16^{INK4} immunoprecipitated from HeLa cell lysates with anti-p16^{INK4} (lane 2) were digested with 15 mM NCS and digestion products were analysed by SDS–PAGE. CDK4 immunoprecipitated from HeLa cell lysates (lanes 3, 4) was compared with the p16^{INK4}-associated p33 (lanes 5, 6) by partial digestion with V8 protease, 100 ng (lanes 3, 5) or 500 ng (lanes 4, 6).

METHODS. *a*, Immunoprecipitations were done as described[7]. The immunoprecipitates were separated in a 12% polyacrylamide gel (30:1 acrylamide:bis-acrylamide). In this gel system, the mobility of PCNA with respect to cyclin D1 is reversed (data not shown) in comparison with the gel system used in other reports (12.5% polyacrylamide, 125:1 acrylamide:bis-acrylamide[7–9,18]). *b*, In-gel partial digestion with V8 protease was performed as described[22]. For NCS partial digestion, gel slices were rehydrated by soaking in water and equilibrated in NCS buffer (8.3 M urea; 50% acetic acid). NCS was added and gel slices were incubated for 30 min at room temperature. Digestion was stopped by soaking in water. Before electrophoresis, gel slices were equilibrated in gel buffer (62.5 mM Tris–HCl pH.7.0, 10% glycerol, 3% SDS, 15% β-mercaptoethanol). Electrophoresis was done in gels containing 17.5% polyacrylamide (250:1 acrylamide:bis-acrylamide).

containing p53 (lanes 6–8) had no effect. Identical results were obtained using extracts containing CDK4/cyclin D1 and CDK4/cyclin D3 (data not shown). We have also found that two other members of the Rb protein family, p107 and p130 (refs 14–16), are phosphorylated by cyclin D/CDK4 kinase *in vitro*, and that p16^{INK4} inhibits phosphorylation of these substrates (data not shown). The CDK2/cyclin D2 kinase also phosphorylates GST–Rb *in vitro*[13]. Neither p16^{INK4} (Fig. 3*a*, lanes 11–13) nor p53 (lanes 14–16) inhibited the CDK2/cyclin D2 kinase, suggesting that the inhibition of CDK4 might be related to the interaction between p16^{INK4} and CDK4. To confirm that this inhibition was due solely to the presence of p16^{INK4} in the insect cell lysates, we purified a histidine-tagged p16^{INK4} protein produced in insect cells (Fig. 3*b*). The purified His–p16^{INK4} protein inhibited the activity of the CDK4/cyclin D2 complex (Fig. 3*c*, lanes 3–6) whereas a mock purification from

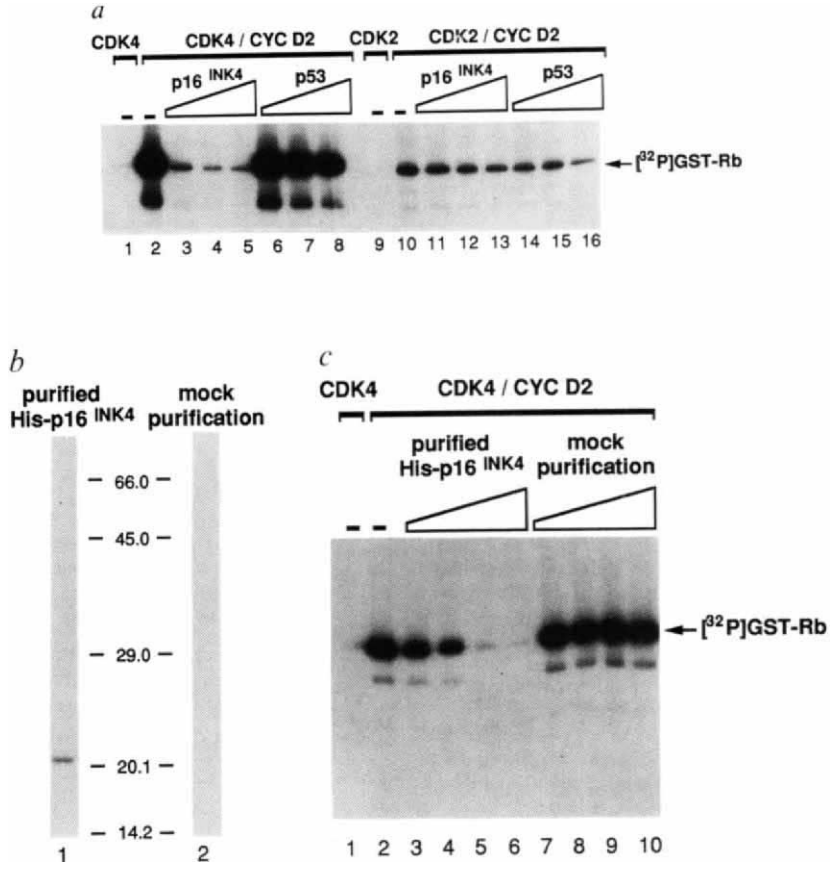


FIG. 3 p16^{INK4} inhibits CDK4/cyclin D2 kinase. *a*, Extracts (2 μl) from baculovirus-infected insect cells expressing CDK4 (lane 1), CDK4 plus cyclin D2 (lanes 2–8), CDK2 (lane 9) or CDK2 plus cyclin D2 (lanes 10–16) were mixed with different amounts (1 μl, lanes 3, 6, 11, 14; 2 μl, lanes 4, 7, 12, 15; and 4 μl, lanes 5, 8, 13, 16) of similar extracts containing p16^{INK4} (lanes 3–5, 11–13) or human p53 (lanes 6–8, 14–16). Mixtures were then assayed for their ability to phosphorylate GST–Rb. *b*, Coomassie-blue-stained gel with purified His-p16^{INK4} (lane 1) and an equivalent volume of the mock purification (lane 2). *c*, Extracts (2 μl) containing CDK4 (lane 1) or CDK4 plus cyclin D2 (lanes 2–10) were mixed with increasing amounts of purified His-p16^{INK4} (final concentrations: 2.5 ng μl^{-1}, lane 3; 5 ng μl^{-1}, lane 4; 10 ng μl^{-1}, lane 5; 20 ng μl^{-1}, lane 6) or with the equivalent volume of the mock purification, as indicated in the figure (lanes 7–10), and tested for their ability to phosphorylate GST–Rb.

METHODS. *a*, The recombinant baculovirus directing the expression of cyclin D2 was obtained from S. Gruenwald. Extracts were prepared as described[12]. For kinase assays, extracts were mixed in kinase buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol) up to a final volume of 10 μl, and incubated for 10 min at 30 °C. Reactions were started by addition of 2 μl containing 125 ng of purified GST–Rb large-pocket protein[12,13] (the plasmid was provided by M. Ewen), 25 μM ATP and 5 μCi[^{32}P-γ]ATP (3,000 Ci mmol; NEN). Reactions proceeded for 1 min at 30 °C, and were stopped by addition of 50 μl of a solution 0.5% NP40 and glutathione–Sepharose beads. Beads were loaded onto a 10% polyacrylamide gel. *b*, A recombinant baculovirus expressing His-tagged p16^{INK4} was constructed using vector pAcSG-His-NT-C and the Baculo-Gold kit (Pharmingen). Purification was done as described[23].

insect cells infected with a non-recombinant baculovirus (expressing the polyhedrin protein) produced no detectable inhibition (Fig. 3*c*, lanes 7–10).

The biochemical properties of $p16^{INK4}$ suggest that it could act as a negative regulator of the proliferation of normal cells. The relative abundance of D-type cyclins and $p16^{INK4}$ could determine the activity of the CDK4 kinase and thus regulate cell-cycle progression. The exclusive presence of the p16/CDK4 complex in cells transformed by a variety of DNA tumour viruses poses a paradox. This can, however, be resolved assuming that Rb and related 'pocket proteins' are the exclusive critical substrates of cyclin D/CDK4. If this is the case, in those cells lacking functional Rb the activity of CDK4 would be unnecessary and thus the inhibitory role of $p16^{INK4}$ would be without effect. Consistent with this, it has been found that microinjection of cyclin D1 antibodies into SV40-transformed fibroblasts or Rb-defective SAOS-2 cells fails to cause cell-cycle arrest[17]. $p16^{INK4}$ may act in normal cells in a negative feedback loop whose role is to downregulate CDK4 once Rb has been inactivated by phosphorylation. In cells in which Rb is constitutively inactive, $p16^{INK4}$ expression is increased, with consequent inhibition of CDK4. However, in this situation, the negative feedback loop is futile as CDK4 kinase is not required for cell-cycle progression.

We have identified two different negative regulators of cyclin kinases. Our data suggest that p21 affects cell-cycle arrest induced by p53 (ref. 18). $p16^{INK4}$ is proposed to act both upstream and downstream of Rb to form a negative feedback loop which regulates the ability of Rb to prevent cell proliferation. □

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Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit

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THE major events of the cell division cycle are triggered by periodic changes in the activity of cyclin-dependent protein kinases (CDKs). In mammals, the members of the CDK family include CDK2 and CDC2, which are thought to be involved in the control of DNA replication and mitosis, respectively[1–3]. The protein kinase activity of these enzymes is controlled by a complex array of mechanisms[4–6]. Activation of the CDK catalytic subunit requires association with a positive regulatory subunit (cyclin) and phosphorylation (at Thr 160 in CDK2). This activated complex can be inhibited by additional phosphorylation at Thr 14 and Tyr 15. Here we report the identification of a new mechanism for the regulation of CDK2 activity. We find that CDK2/cyclin complexes in mouse fibroblasts associate tightly with a 20K protein (CAP20). Complexes containing CAP20 were isolated from cell lysates and found to have negligible kinase activity, indicating that CAP20 association *in vivo* may inhibit CDK2 activity. We purified CAP20 from 3T3 cells and found that low concentrations of the protein completely inhibit the kinase activity of CDK2 *in vitro*. Thus CAP20 represents a new negative regulatory subunit that inhibits the activity of CDK2/cyclin complexes in mammalian cells.

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Our search for new regulators of CDK2 began with analyses of proteins that associate with CDK2 in mouse BALB/c 3T3 fibroblasts. When CDK2 is immunoprecipitated from metabolically labelled lysates of these cells, two other proteins are also specifically immunoprecipitated (Fig. 1*a*, lanes 1–4). The larger protein ($M_r \sim 55{,}000$ (55K)) is recognized by antibodies directed against cyclin A (results not shown) and probably represents cyclin A, the major cyclin partner for CDK2. The nature of the smaller protein ($M_r \sim 20$K) is not known; we refer to this protein as CDK2-associated protein-20 (CAP20). CAP20 is not detectable in immunoprecipitates of CDC2 (Fig. 1*a*, lanes 5 and 6).

We found that cation-exchange chromatography can be used to isolate a subpopulation of CDK2 that is associated with CAP20. Metabolically labelled cell lysates were passed over an S-Sepharose column, which was washed and then eluted with low salt (100 mM NaCl) and then with high salt (500 mM NaCl). Immunoprecipitation analysis revealed that CDK2 in the high-salt eluate, but not in other fractions, is associated with cyclin A and CAP20 (Fig. 1*b*). Immunoprecipitates of cyclin A from the high-salt eluate also contained CAP20 (Fig. 1*c*). Thus CAP20 is present in immunoprecipitates of both CDK2 and cyclin A from this fraction, suggesting that CAP20 is associated with CDK2/cyclin A complexes.

We next used a similar procedure to separate CDK2/cyclin complexes containing CAP20 from those lacking the protein. Crude cell lysates were injected onto a Mono-S column, which was washed with 100 mM NaCl and then eluted with a linear salt gradient up to 500 mM NaCl. CDK2 eluted at two distinct positions: peak I eluted at ~250 mM NaCl and Peak II eluted at ~450 mM NaCl (Fig. 2*a*, *b*). Gel filtration analysis showed that CDK2 in both peaks migrated at the large size characteristic of CDK/cyclin complexes (Fig. 2*c*). Interestingly, measurement of CDK2 activity in Mono-S fractions revealed that CDK2 in peak II was essentially inactive (Fig. 2*b*). We estimate that the