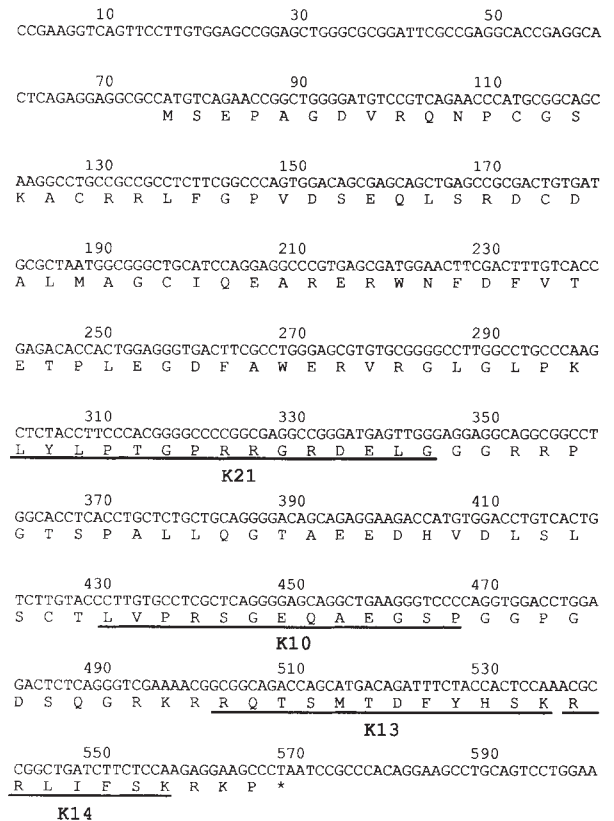


FIG. 1 Sequence of the p21 cDNA. The sequence of the p21 cDNA and the deduced amino-acid sequence of the p21 protein are shown. The positions of the peptides obtained from microsequencing are underlined (see text). Asterisk indicates stop codon.

METHODS. For the purification of p21, WI38 cells (passage 24) were grown to late log phase on 400 15-cm tissue culture dishes in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum. All subsequent steps were carried out at 4 °C. Cells were rinsed in phosphate-buffered saline (PBS), collected and lysed in NP40 lysis buffer¹. The lysate was clarified by centrifugation at 5,000g for 10 min, and the supernatant was added to 2.5 mg of affinity-purified cyclin D1 antibodies, covalently crosslinked to protein A-Sepharose beads with dimethyl palmitate (Pierce). Immunoprecipitation was carried out for 4 h, after which the beads were washed four times with lysis buffer. The supernatant was subjected to a second round of affinity chromatography by the addition of fresh anti-cyclin D1-Sepharose. Proteins were released from the beads by boiling in SDS sample buffer and loaded onto a 12% SDS-polyacrylamide gel (acrylamide:bis-acrylamide 37.5:1). The gel was stained with 0.05% Coomassie brilliant Blue G (Sigma) for 15 min, destained and soaked in water for 1 h. The p21 protein (~2 µg) was excised from the gel and subjected to in-gel digestion with *Achromobacter* endoprotease I for 24 h at 30 °C as described previously⁹. Digests were cleared by centrifugation and the supernatant was filtered through a 0.22-µm membrane (Ultrafree-MC, Millipore). Peptides were separated by reverse-phase HPLC (Hewlett Packard 1090) using a Vydac C18 column (2.1 × 250 mm, 5 µm, 300 Å) with an anion-exchange pre-column (Brownlee GAX-013, 3.2 × 15 mm). Peptides were eluted with an acetonitrile gradient and their absorbance was monitored at 214, 280, 295 and 550 nm. Amino-acid sequencing was performed on an automated microsequencer (ABI model 470) with on-line HPLC (ABI model 120A) analysis of phenylthiohydantoin-tagged amino acids. Four peptide sequences were obtained as follows: K10, tSLVP?SGEQAEGSPsk; K13, ?RQTSMTDFYHSK; K14, RRLIFS; K21, LYLPTGPPRSRDEDLGIS. Lower-case letters indicate likely identification and question marks indicate uncertainty. Degenerate oligonucleotide primers were designed based on the three longest peptides, K10, K13 and K21. Primers were synthesized in both directions and codons for serine were split to reduce degeneracy. One or two amino acids were reserved at each end for the verification of any PCR product. Template DNA was prepared from several human cDNA libraries (WI38 and HeLa cell, Stratagene; U118 cell, a gift from M. Wigler) as described¹⁰. Thirty-five cycles of amplification were performed (50 or 55 °C annealing and 72 °C extension) after which products were resolved on a 1.5% agarose gel. All DNA fragments smaller than 500 bases were excised and inserted into pUC118 for sequence analysis. Amplification with one pair of primers derived from the K10 and K13 peptides (K10 primer



GGNGA[A/G]CA[A/G]GCNGGNTCCNC, encoding GEQAEGSP; K13 primer TTNCA[A/G]TG[A/G]TA[A/G]AA[A/G]TCNGTCATNCANGT[C/T]TG, encoding QTSMTDFYHSK) gave rise to a specific 96-bp PCR fragment that encoded 13 amino acids in addition to those encoded by the PCR primers. This sequence encoded KRR preceding the K13 primer. This was consistent with the sequence K?R which was predicted to precede the sequence encoded by the K13 primer. The PCR fragment was excised from pUC118 and used as a probe for screening a human U118 cDNA library. Several positive clones were obtained, and 10 were chosen for subsequent analysis. One of these contained an ~600-base insert which was shown to carry the entire p21-coding region.

and CDKs. We cannot, however, formally exclude the possible presence of insect cell PCNA in the p21/CDK/cyclin complexes.

We used the same baculovirus reconstitution system to assess the effect of p21 on the enzymatic activity of the cyclin kinases. Anti-CDK2 immunoprecipitates were prepared from lysates of ³⁵S-labelled insect cells co-infected with viruses encoding CDK2 and cyclin A. The precipitates contained cyclin A/CDK2 and displayed substantial histone H1 kinase activity (Fig. 4a-d). Addition of increasing amounts of a lysate containing p21 resulted in progressive formation of cyclin A/CDK2/p21 ternary complexes (Fig. 4a). As p21 neared stoichiometry, CDK2 kinase activity was abruptly inhibited (Fig. 4b). Addition of PCNA, in an otherwise identical experiment, resulted in the formation of quaternary complexes (Fig. 4c) but had no effect on either histone kinase activity or its inhibition by p21 (Fig. 4d). Similar inhibition by p21 was observed with cyclin B/CDC2 and cyclin E/CDK2 kinases (our unpublished results).

Cyclin/CDK4 kinase differs from the other cyclin kinases in its inability to utilize histone H1 as a substrate. To date, the only substrates known for cyclin D/CDK4 kinases are the members of the retinoblastoma (Rb) family of 'pocket' proteins⁶. We therefore tested the effect of p21 on the ability of cyclin D/CDK4 to phosphorylate Rb. As previously described, insect cell lysates containing cyclin D or CDK4 alone showed little activity towards glutathione S-transferase (GST) fused to Rb.

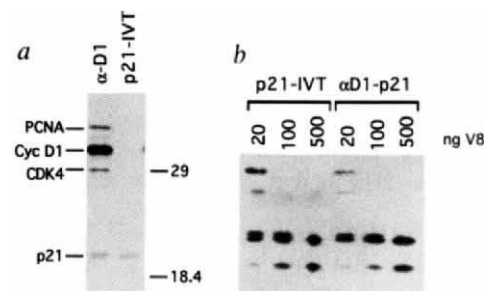


FIG. 2 *a*, Cyclin D1-associated proteins were immunoprecipitated from ³⁵S-methionine-labelled WI38 cells using an anti-cyclin D1 antiserum (α-D1, left lane) and electrophoresed in parallel with *in vitro*-translated, ³⁵S-methionine-labelled putative p21 protein (p21-IVT, right lane). For reference, the positions of labelled protein markers are shown. *b*, *In vitro*-translated putative p21 and cyclin D1-associated p21 were electrophoretically purified from a gel similar to that shown in *a*. These polypeptides were then subjected to partial V8 protease digestion using the indicated amounts of enzyme. Resulting peptide fragments were fractionated on a 17.5% polyacrylamide gel. Cell culture, immunoprecipitation and partial V8 protease digestion were performed exactly as described previously⁴.

FIG. 3 Reconstitution of p21/PCNA/cyclin/CDK quaternary complexes in baculovirus-infected cells. Exponentially growing Sf9 cells were co-infected with baculoviruses encoding p21 and the indicated cyclins and CDKs in the presence (+) or absence (-) of a baculovirus directing the expression of PCNA. At 40 h post-infection, cells were labelled with ^{35}S -methionine for 3 h and lysed in NP40 lysis buffer. The protein complexes were immunoprecipitated with anti-CDK4 (panel 1), anti-CDK2 (panels 2, 3) or anti-CDC2 (panel 4) antibodies and analysed by gel electrophoresis. The positions of protein *M*, standards are indicated.

METHODS. Insect Sf9 cells were maintained in Grace's medium supplemented with 10% heat-inactivated fetal bovine serum, lactalbumin hydrolysate and yeastolate ultrafiltrate at 27 °C. Recombinant baculoviruses were constructed using the Baculo-Gold transfection system (Pharmingen) according to the manufacturer's instructions. CDK2, CDC2, cyclin A, cyclin D1 and cyclin E baculoviruses were gifts from D. Morgan, C. Sherr or S. Grunwald. For the production of recombinant proteins, Sf9 cells were infected with multiplicities of infection above 10. After 36–48 h, the cells were labelled with 100 $\mu\text{Ci ml}^{-1}$ of ^{35}S -methionine (NEN) for 3 h. Cell labelling, cell lysis and immunoprecipitation were carried out as described previously¹¹.

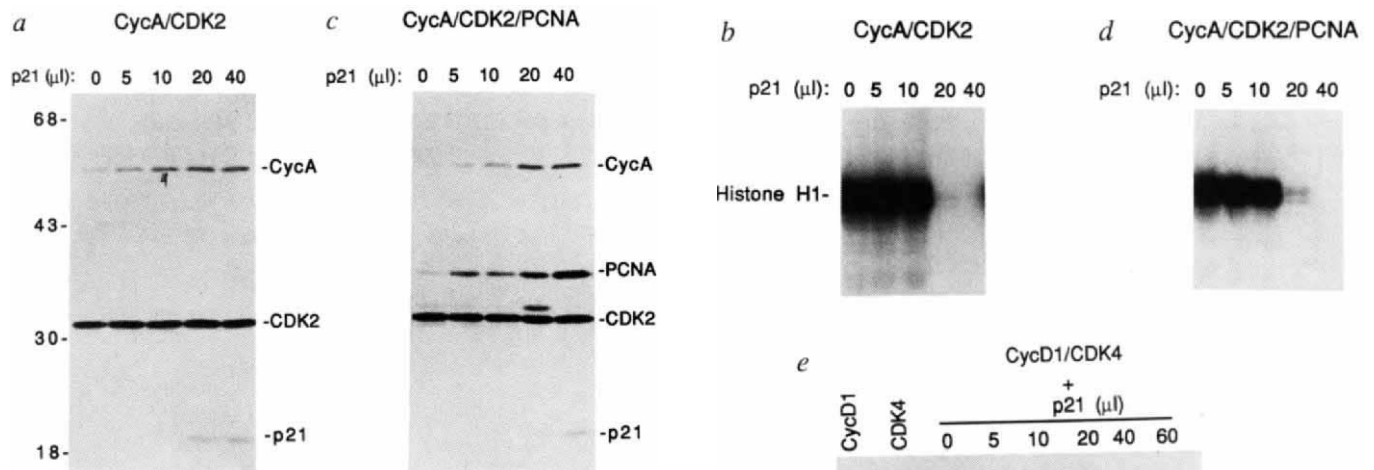
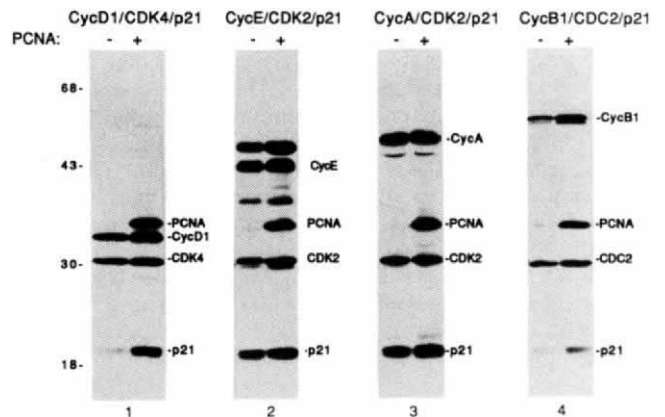


FIG. 4 *a–d*, p21 is an inhibitor of cyclin A/CDK2 kinase. Sf9 cell lysates containing cyclin A and CDK2 were mixed with the indicated amounts of a p21-containing lysate. In *c* and *d*, an additional 40 μl of a lysate containing PCNA was added. After incubation of 30 °C for 30 min in the presence of 1 mM ATP, proteins were immunoprecipitated from cell lysates with an anti-CDK2 antiserum. Immunoprecipitates were split for direct analysis of co-immunoprecipitated proteins (*a*, *c*) or for kinase assays using histone H1 as a substrate (*b*, *d*). *e*, *f*, p21 inhibits cyclin D1/CDK4 kinase. Lysates from Sf9 cells co-infected with baculoviruses encoding cyclin D1 and CDK4 were incubated with increasing amounts of a p21-containing lysate (as indicated) at 30 °C for 30 min. The first two lanes show lysates from cells infected with baculoviruses encoding either cyclin D1 or CDK4. The lysates were split for analysis of proteins immunoprecipitated with an anti-cyclin D1 antiserum, except for lane 2 which was immunoprecipitated with anti-CDK4 (*e*), or for kinase assays using recombinant retinoblastoma protein (GST-Rb) as the substrate (*f*).

METHODS. Cell culture, baculovirus infection and metabolic labelling were as described in Fig. 3 legend. For preparation of kinase extracts, cells were rinsed once with PBS and once with kinase buffer (50 mM Tris pH 7.4, 10 mM MgCl_2 , 5 mM ethylene glycol tetra-acetate and 2 mM dithiothreitol). Cells were then lysed in kinase buffer by four passages through a 25-gauge needle. The cell lysates were cleared of insoluble material by two centrifugations at 12,000g and stored at -80 °C until use. For cyclin D1/CDK4 complexes, the lysates were from cells co-infected with baculoviruses encoding cyclin D1 and CDK4. For *a–d*, lysates containing cyclin A, CDK2, p21 or PCNA were mixed in the presence of 1 mM ATP for 30 min at 30 °C. In all experiments, differences in reaction volumes were compensated for with lysates from Sf9 cells infected with wild-type baculovirus. After incubation, the reactions were stopped by adding 20 mM EDTA and the cyclin/CDK protein com-

plexes were immunoprecipitated as described¹¹. After immunoprecipitation, the samples were split in half; half was used for the analysis of the protein composition and half for kinase assay. Histone H1 kinase assays were performed as described¹². For the analysis of cyclin D1/CDK4 kinase activity, lysates were incubated at 30 °C for 30 min without ATP and kinase activity was assayed using bacterially produced GST-Rb as described¹³.

However, cyclin D/CDK4 binary complexes catalysed substantial Rb phosphorylation (Fig. 4e, f). Addition of increasing amounts of p21 resulted in the accumulation of cyclin D/CDK4/p21 ternary complexes (Fig. 4e), with a corresponding inhibition of Rb phosphorylation (Fig. 4f). 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 assay⁷. 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. □

Received 12 November; accepted 1 December 1993.

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ACKNOWLEDGEMENTS. We thank J. Smith and P. Burfiend for assistance with preparation of the manuscript and flow cytometry, respectively, M. Ockler, P. Renna and J. Duffy for help with photography and graphics, M. Nichols, S. Matsumoto and C. Gawel for technical assistance with cloning and sequencing the p21 cDNA, S. Allan for help with the colony formation suppression assays, and M. Serrano for discussion and for help in compiling figures. Y.X. thanks B. Marzluff for his help and encouragement. We also acknowledge the gift of baculovirus reagents from D. Morgan (UCSF) and S. Gruenwald (Pharmingen). Y.X. is supported by the program in molecular biology of the University of North Carolina, Chapel Hill. D.B. is an investigator of the Howard Hughes Medical Institute. H.Z. is a Research Associate of the Howard Hughes Medical Institute. G.J.H. is a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Research Fund. This work was supported by the NIH. Y.X. and G.H. contributed equally to this work.

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 phase³⁻⁶. 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*, 16K, particularly in cells lacking a functional retinoblastoma protein⁹. 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 screen¹⁰ 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*, 15,845 comprising four ankyrin repeats (Fig. 1a). 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. 1b). Only the GAL4^{db}-CDK4 fusion interacted with GAL4^{ad}-p16^{INK4} to an extent that allowed growth in the absence of histidine (Fig. 1b). 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 ³⁵S-labelled CDKs (Fig. 1c, 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. 1c, 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. 1d). p16^{INK4} was co-immunoprecipitated with anti-CDK4 (Fig. 1d, lane 1), but not with anti-CDK2 (lane 4). Conversely p16^{INK4} antibodies co-immunoprecipitated CDK4 (Fig. 1d, 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. 2a, lane 1). Proteins present in this immunoprecipitate probably represent at least two independent

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