p57^{KIP2}, a structurally distinct member of the p21^{CIP1} Cdk inhibitor family, is a candidate tumor suppressor gene

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Cyclin-dependent kinases (Cdks) are positive regulators of cell proliferation, whereas Cdk inhibitors (CKIs) inhibit proliferation. We describe a new CKI, p57^{KIP2}, which is related to p21^{CIP1} and p27^{KIP1}. p57^{KIP2} is a potent, tight-binding inhibitor of several G_1 cyclin/Cdk complexes, and its binding is cyclin dependent. Unlike CIP1, KIP2 is not regulated by p53. Overexpression of p57^{KIP2} arrests cells in G_1. p57^{KIP2} proteins have a complex structure. Mouse p57^{KIP2} consists of four structurally distinct domains: an amino-terminal Cdk inhibitory domain, a proline-rich domain, an acidic-repeat region, and a carboxy-terminal domain conserved with p27^{KIP1}. Human p57^{KIP2} appears to have conserved the amino- and carboxy-terminal domains but has replaced the internal regions with sequences containing proline–alanine repeats. In situ hybridization during mouse embryogenesis revealed that KIP2 mRNA displays a striking pattern of expression during development, showing high level expression in skeletal muscle, brain, heart, lungs, and eye. Most of the KIP2-expressing cells are terminally differentiated, suggesting that p57^{KIP2} is involved in decisions to exit the cell cycle during development and differentiation. Human KIP2 is located at 11p15.5, a region implicated in both sporadic cancers and Beckwith–Wiedemann syndrome, a familial cancer syndrome, marking it as a candidate tumor suppressor. The discovery of a new member of the p21^{CIP1} inhibitor family with novel structural features and expression patterns suggests a complex role for these proteins in cell cycle control and development.

[*Key Words*: p57^{KIP2}; p21^{CIP1}; Cdk inhibitor; tumor suppressor; cell cycle]

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The proper development of a multicellular organism is a complex process that requires precise spatial and temporal control of cell proliferation. Cell proliferation in the embryo is controlled via an intricate network of extracellular and intracellular signaling pathways that process growth regulatory signals. This signaling network is superimposed upon the basic cell cycle regulatory machinery that controls particular cell cycle transitions. The ultimate recipient of many of these signals are cyclin-dependent kinases (Cdks), a family of enzymes that catalyze events required for cell cycle transitions.

Cdks require association with cyclins for activation and the timing of Cdk activation is largely dependent on the timing of expression of their cognate cyclin (for review, see Draetta 1993). Several Cdks, including Cdc2, Cdk2, Cdk4, and Cdk6, have been characterized with respect to their temporal activation and their cyclin partners (Matsushime et al. 1992; Meyerson and Harlow 1994; for review, see Pines 1993). Cyclins fall into several classes based on their timing of expression and genetic properties (for review, see Sherr 1994). D-type cyclins associate primarily with Cdk4 and Cdk6, whereas cyclin E associates with Cdk2 and both classes appear to function in the G_1/S phase transition. Cyclin A can associate with Cdk2 or Cdc2 and appears to have roles in S phase and G_2. Cyclin F is expressed in late S and G_2, but its kinase partner and role in the cell cycle are not yet known (Bai et al. 1994). Cyclin B binds Cdc2 and controls entry into mitosis. In addition to cyclin availability, Cdks are regulated both positively and negatively by phosphorylation (for review, see Draetta 1993; Soloman 1993).

Once proliferation and morphogenesis have constructed a particular structure, it is of paramount importance that the proliferative state cease and be replaced with a homeostatic state. Although much attention has been focused on how cells enter the cell cycle, little is known concerning the strategies organisms employ to exit the cycle and maintain the nonproliferative state. This state is of great importance to an organism because the vast majority of its cells exist in a nonproliferative

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state throughout adult life. The inability to appropriately halt growth can lead to malformation during development, and to cancer. Thus, equally important in the execution of developmental programs is the arrest of growth once the program is complete. Although the control of terminal differentiation promises to be complex, cell cycle arrest via inactivation of Cdks is likely to be a central feature. Recently a new class of Cdk regulatory molecules has emerged that are potential mediators of cell cycle exit and maintenance of the nonproliferative state. These are the tight-binding inhibitors of cyclin-dependent kinases, CKIs. Currently two structurally defined classes of CKIs exist in mammals that are exemplified by $p21^{CIP1}$ (El-Diery et al. 1993; Harper et al. 1993; Xiong et al. 1993; Noda et al. 1994) and $p16^{INK4/MTS1}$ (Serrano et al. 1993; for review, see Elledge and Harper 1994).

$p21^{CIP1}$ is a dual specificity inhibitor that can bind and inhibit G_1 cyclin/Cdk complexes and proliferating cell nuclear antigen (PCNA) (Flores-Rozas et al. 1994; Waga et al. 1994), and can arrest the cell cycle in G_1 when overexpressed (Harper et al. 1995). $p21^{CIP1}$ is transcriptionally controlled by the tumor suppressor protein p53 (El-Diery et al. 1993). It is induced by DNA damage in a p53-dependent manner and is found associated with inactive cyclin E/Cdk2 complexes (Dulic et al. 1994; El-Diery et al. 1994). In some fibroblast cell lines $p21^{CIP1}$ basal expression is dependent on p53; however, in embryonic and adult mouse, $p21^{CIP1}$ expression is independent of p53 (Parker et al. 1995), indicating that the regulation of $p21^{CIP1}$ is more complex than originally thought. p53-Independent expression has also been observed under some circumstances in tissue culture cell lines (Jiang et al. 1994; Michieli et al. 1994; Sheiki et al. 1994). $p21^{CIP1}$ is expressed during embryogenesis primarily in a subset of cells that are amitotic, thus potentially contributing to cell cycle exit during differentiation (Parker et al. 1995). $p21^{CIP1}$ induction has also been observed in cell lines undergoing differentiation in vitro (Jiang et al. 1994; Steinman et al. 1994; Halevy et al. 1995; Parker et al. 1995). Thus, organisms utilize $p21^{CIP1}$ in many ways: In proliferating cells in G_1 it can arrest the cell cycle, in S-phase cells it can theoretically block PCNA and slow down DNA synthesis to facilitate repair processes, and in development it may contribute to cell cycle arrest in terminally differentiating cells.

Less is known about the other inhibitors. $p27^{KIP1}$ (Polyak et al. 1994; Toyoshima and Hunter 1994) inhibits both Cdk2 and Cdk4 in vitro. It is not regulated by p53 and does not inhibit PCNA (Flores-Rozas et al. 1994), suggesting specialization for a non-checkpoint function. p27 levels show a modest increase in response to conditions that arrest the cell cycle in some cell types (Kato et al. 1994; Nourse et al. 1994). In contrast, p16, p15, and p18, which form a family of structurally related inhibitors consisting primarily of ankyrin repeats, are specific for Cdk4 and Cdk6 (Serrano et al. 1993; Guan et al. 1994; Hannon and Beach 1994; Jen et al. 1994). p16 and p15 have been found deleted in a number of tumor cells lines and in primary tumors and are likely tumor suppressors (Cairns et al. 1994; Jen et al. 1994; Kamb et al. 1994; Kato et al. 1994; Nobori et al. 1994; Spruck et al. 1994). p15 is induced by TGF-β and is a likely mediator of its growth-arresting properties (Hannon and Beach 1994).

Although considerable information has accumulated concerning the existence of cyclin kinase inhibitors, little is known about the extent of these families, their structural diversity, and potential roles in development. In this paper, we describe the discovery of a new Cdk inhibitor of the $p21^{CIP1}$ family, $p57^{KIP2}$, that has several unique structural features, patterns of expression during development, and is located in a chromosomal region implicated in a number of human cancers.

Results

Isolation of the cDNA encoding KIP2, cyclin kinase inhibitor protein 2

To identify potential regulators of cyclins and cyclin-dependent kinases, we utilized an improved version of the two-hybrid system (Durfee et al. 1993) that we and others have used previously to identify proteins that bind these molecules (Harper et al. 1993; Toyoshima and Hunter 1994). To screen for mouse cDNAs encoding proteins able to interact with cyclin D, Y190/pAS2-cyclin D1 was transformed with a pACT–mouse embryonic cDNA library, and transformants were subjected to selection for histidine prototrophy on SC-his,-trp,-leu plates containing 50 mM 3-amino-triazole as described previously. Transformants (5×10^5) were placed under selection, and rare surviving colonies screened for their ability to produce β-galactosidase. Plasmids recovered from 25 His^+ blue colonies were sequenced from their 5′ ends and their deduced amino acid sequences were compared to GenBank. Three of these clones were related to one another and shared structural homology with $p21^{CIP1}$ and $p27^{KIP1}$. The DNA and deduced amino acid sequence of the protein encoded by the longest cDNA (1.5 kb) is shown in Figure 1A. It encodes a protein of 335 amino acids with a number of unusual structural features. The new inhibitor gene isolated in this study will be referred to as KIP2 because it is most closely related to KIP1, and the protein as $p57^{KIP2}$ based on its size (see below).

The KIP2 protein has a number of unusual structural features

KIP2 has four distinct amino acid sequence domains that will be referred to as domains I–IV for convenience of discussion (Fig. 1C). These are not to be confused with protein structural domains, although they may eventually coincide once structural data are available. Domain I bears significant similarity to $p21^{CIP1}$ and $p27^{KIP1}$ in a region that has been shown to be necessary and sufficient for Cdk inhibition (Polyak et al. 1994; Harper et al. 1995) (Fig. 1D). Domain II is proline-rich region (25% proline) that has a series of alternating prolines interspersed with nonrepetitive sequence. Domain III has an

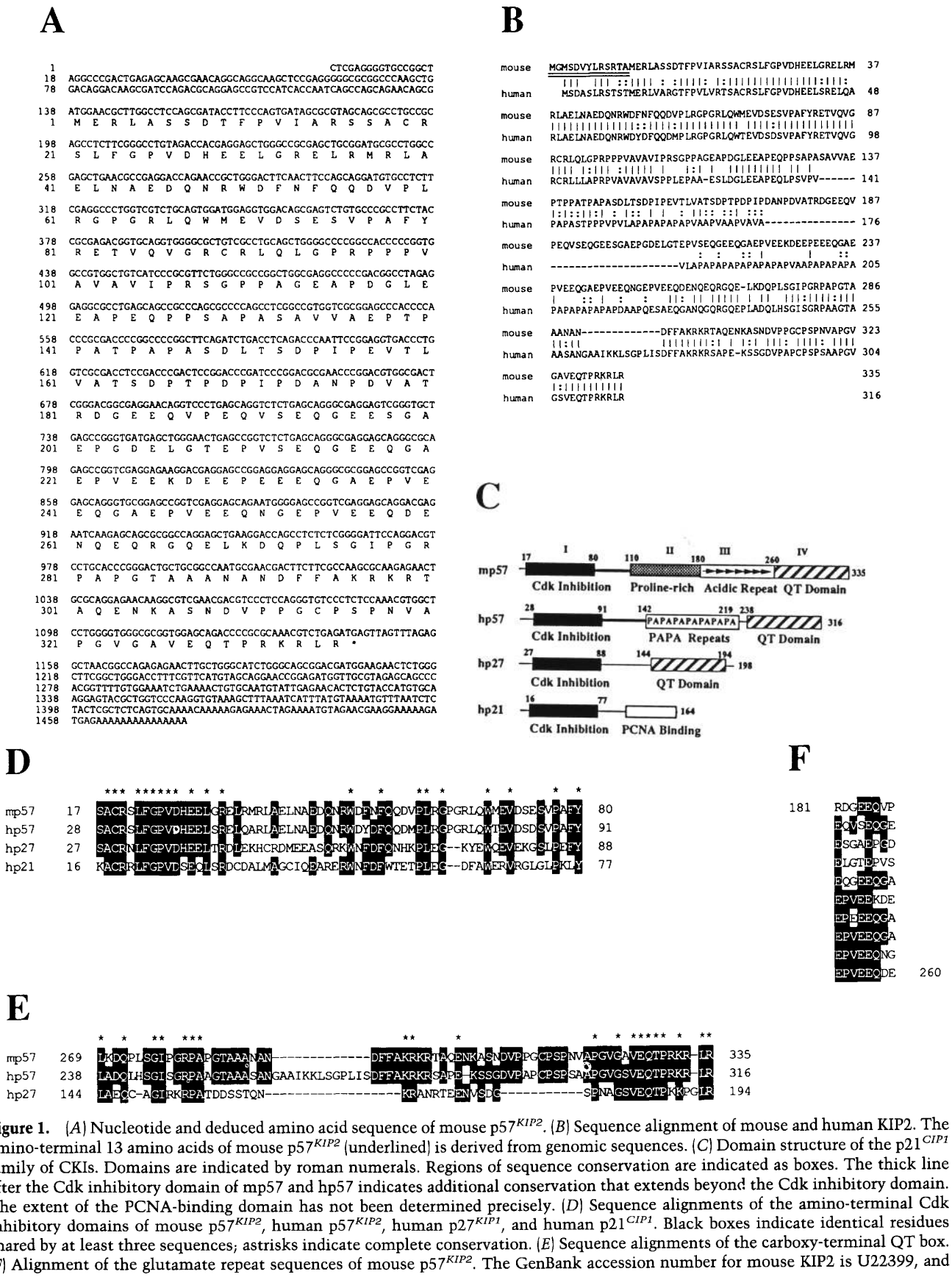

Figure 1. (*A*) Nucleotide and deduced amino acid sequence of mouse p57^{KIP2}. (*B*) Sequence alignment of mouse and human KIP2. The amino-terminal 13 amino acids of mouse p57^{KIP2} (underlined) is derived from genomic sequences. (*C*) Domain structure of the p21^{CIP1} family of CKIs. Domains are indicated by roman numerals. Regions of sequence conservation are indicated as boxes. The thick line after the Cdk inhibitory domain of mp57 and hp57 indicates additional conservation that extends beyond the Cdk inhibitory domain. The extent of the PCNA-binding domain has not been determined precisely. (*D*) Sequence alignments of the amino-terminal Cdk inhibitory domains of mouse p57^{KIP2}, human p57^{KIP2}, human p27^{KIP1}, and human p21^{CIP1}. Black boxes indicate identical residues shared by at least three sequences; astrisks indicate complete conservation. (*E*) Sequence alignments of the carboxy-terminal QT box. (*F*) Alignment of the glutamate repeat sequences of mouse p57^{KIP2}. The GenBank accession number for mouse KIP2 is U22399, and for human KIP2 is U22398.

unusual acidic repeat in that every fourth amino acid is a glutamic or aspartic acid. There is also a slightly degenerate higher order repeat motif of 8 amino acids, EPVEEQXX, shown in Figure 1F. Domain IV has sequence conservation with the carboxyl terminus of $p27^{KIP1}$ (Fig. 1E). The center of the most conserved stretch of sequence identity has the amino acids QT, and we will refer to this conserved motif as the QT box. The carboxy-terminal region of $p27^{KIP1}$ is distinct from the carboxy-terminal region of $p21^{CIP1}$, which is involved in PCNA binding. Sequence conservation with $p27^{KIP1}$ indicates that the QT box is a structural motif that is likely to function in protein–protein interaction.

The human KIP2 homolog, hKIP2, was isolated by low stringency hybridization screening of a human embryonic cDNA library in λgt10. Two cDNAs were recovered and sequenced. The deduced amino acid sequence of one of these revealed a protein of 316 amino acids and is shown in Figure 1B aligned with mKIP2. hKIP2 shares two of the four sequence domains of the mouse gene. It has highly conserved the amino- and carboxy-terminal domains; however, it has replaced the internal domains II and III with a distinct region consisting primarily of an alternating proline–alanine repeat termed the PAPA repeat (Fig. 1B,C). The second hKIP2 cDNA contains amino acids 1–96, deletes the entire PAPA region, and is spliced back in at nucleotide 1076 in the +1 reading frame. Thus, the protein potentially encoded by this smaller cDNA consists primarily of the Cdk inhibitory domain. Whether this cDNA represents a normal splicing variant remains to be determined.

The amino acid sequence alignment derived from the longest mouse and human cDNAs revealed that the human cDNA encodes an extra 13 amino acids at its amino terminus. Limited sequence analysis of the mouse genomic region directly 5′ of the initiating methionine (P. Zhang and S.J. Elledge, unpubl.) revealed further amino acid sequence identity with the amino terminus of the human cDNA. These additional 13 amino acids are underlined in the sequence alignment (Fig. 1). The difference between the human and mouse cDNAs and the correspondence of the human cDNA and mouse genomic sequence suggest that there may be a mouse cDNA with the 5′ end corresponding to the human cDNA. Thus, the difference between the 5′ ends may result from alternative splicing. However, analysis of the genomic DNAs indicates that the difference between the internal domains does not result from alternative splicing (P. Zhang and S.J. Elledge, unpubl.).

$p57^{KIP2}$ can bind to cyclin/Cdk complexes in a cyclin-dependent manner

To examine the association of $p57^{KIP2}$ with cyclin/Cdk complexes, in vitro-translated [^{35}S]methionine-labeled mouse $p57^{KIP2}$ was incubated with [^{35}S]methionine-labeled Cdks or cyclin/Cdk complexes produced in insect sf9 cells, and association was determined by immunoprecipitation with anti-Cdk antibodies. $p57^{KIP2}$ was found to efficiently bind Cdk2, Cdk3, and Cdk4 in a cyclin-dependent manner (Fig. 2A). $p57^{KIP2}$ showed a weaker association with Cdk6/cyclin D2 complexes and no detectable association with Cdk7/cyclin H complexes.

Because $p57^{KIP2}$ was identified in a two-hybrid screen using cyclin D, it is possible that it associates with cyclin/Cdk complexes via direct interaction with cyclins. This is consistent with the fact that association with Cdks is cyclin dependent (Fig. 2A). Furthermore, it has been reported previously that $p27^{KIP1}$ can associate directly with in vitro-translated cyclin D (Toyoshima and Hunter 1994). $p57^{KIP2}$ was tested for its ability to bind cyclins D1, D2, and A in the absence of Cdk subunit using cyclin and $p57^{KIP2}$ proteins produced and [^{35}S]methionine labeled in sf9 cells. Because insect cells contain endogenous Cdks, association of cyclins with $p57^{KIP2}$ could potentially be mediated by endogenous Cdks such as Cdk2 and Cdc2 present in crude sf9 extracts. To reduce the levels of these Cdks and associated cyclins, extracts were precleared with immobilized $p13^{suc1+}$, which is known to bind both monomeric and cyclin-associated Cdks with high affinity. Using such lysates, association between cyclins and $p57^{KIP2}$ was not observed under conditions in which ~5% binding could be detected (Fig. 2B). Similar results have been obtained using $p21^{CIP1}$ and $p27^{KIP1}$ (Harper et al. 1995). Thus, if contacts are made with cyclins, they are very weak. In this situation, the cyclin dependency could result from the integration of weak interactions with surfaces on both cyclins and Cdks. Alternatively, the cyclin dependence could result from conformational changes on the Cdk or cyclin induced upon formation of the cyclin/Cdk complex that favor $p57^{KIP2}$ binding. It is also likely that the endogenous Cdk in yeast, Cdc28, contributed to the interaction between cyclin D and $p57^{KIP2}$ detected in the two-hybrid system.

$p57^{KIP2}$ can inhibit cyclin/Cdk activity

Having established in vitro association of $p57^{KIP2}$ with cyclin/Cdk complexes, we wished to examine the possible biochemical consequences of this association. Proteins that associate with cyclin-dependent kinases could influence the activity or substrate specificity, modify interactions with other proteins, or alter subcellular localization. We have shown previously that the structurally related protein $p21^{CIP1}$ is an inhibitor of Cdk activity (Harper et al. 1993). To examine whether the $p57^{KIP2}$ alters Cdk function, kinase activities of several cyclin/Cdk complexes were measured in the presence of GST–KIP2 using histone H1 or Rb as substrate (Fig. 2C). $p57^{KIP2}$ is an efficient inhibitor of cyclin E/Cdk2, cyclin A/Cdk2, cyclin E/Cdk3, and cyclin D2/Cdk4 kinase complexes. It shows considerably less activity toward cyclin B/Cdc2 and cyclin D2/Cdk6 complexes. The reduced ability of $p57^{KIP2}$ to inhibit Cdk6 complexes is consistent with the weak association observed in binding assays (Fig. 2B). A potential caveat is that the assays for Cdk6 were performed on cyclin D2/Cdk6 immune complexes, unlike the assays for other kinases, and it is

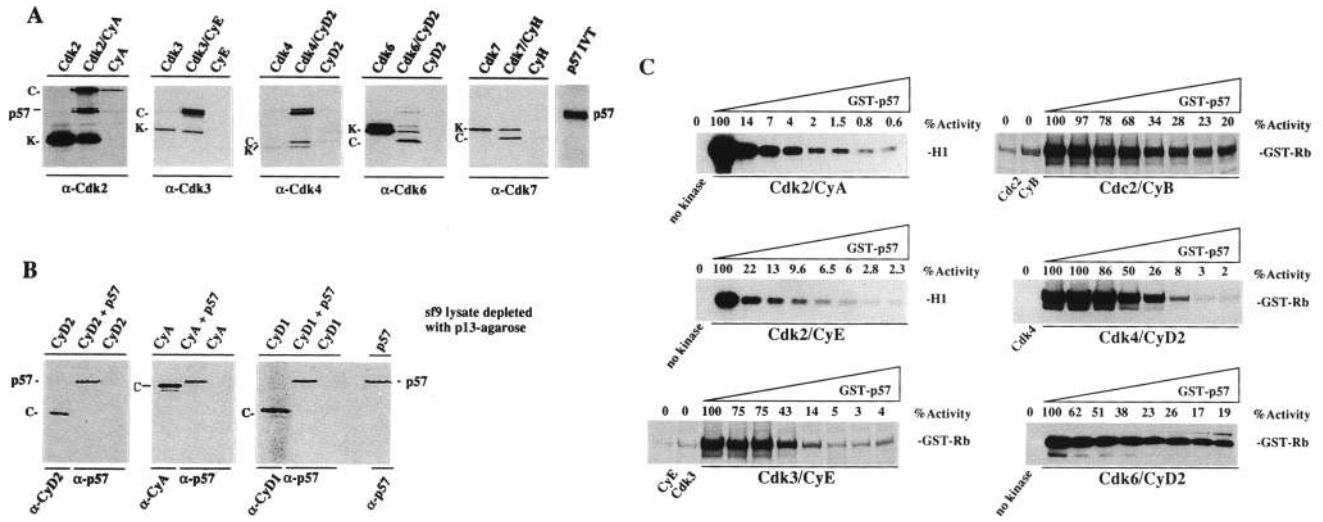

Figure 2. p57^{KIP2} binds and inhibits Cdk family members in vitro. (*A*) Cyclin-dependent association of p57^{KIP2} with Cdks. [^{35}S]Methionine-labeled sf9 extracts prepared from cells infected with baculovirus expressing a cyclin or Cdk alone, or coinfected with the indicated cyclin/Cdk pairs were mixed with [^{35}S]methionine-labeled mouse p57^{KIP2} produced by in vitro translation and Cdk-associated proteins isolated by immunoprecipitation of the Cdk subunit. Immune complexes were separated by SDS-PAGE and analyzed by autoradiography. (C-) The position of the cyclin component; (K-) the kinase component. CyA is a GST fusion protein. Cdk3 is a T7 gene10 fusion protein and is immunoprecipitated with anti-T7 Tag antibodies (Novagen). Cdk7 contains a carboxy-terminal hemagglutinin A (HA) tag and was immunoprecipitated with anti-HA antibodies. (*B*) p57^{KIP2} does not directly associate with CyA, CyD1, or CyD2. [^{35}S]Methionine-labeled sf9 lysates for the indicated cyclins were precleared with p13 beads and then incubated with or without T7 Tag-p57 and immune complexes isolated using either antibodies directed against the cyclin or anti-T7 Tag to immunoprecipitate p57^{KIP2} and any associated proteins. As a control for nonspecific binding of the antibodies, cyclin extracts alone were also immunoprecipitated with anti-T7 Tag antibodies. Immune complexes were separated by SDS-PAGE and analyzed using a Molecular Dynamics PhosphorImager. (*C*) Inhibition of Cdk activity by mouse GST–p57. Inhibition of cyclin A/Cdk2 and cyclin E/Cdk2 (at ~0.4 nM) was examined using kinase complexes purified from insect cells as GST fusion proteins employing histone H1 as substrate. Inhibition of Cdc2/cyclin B, Cdk3/cyclin E, and Cdk4/cyclin D2 was examined using crude insect cell lysates (~1–5 nM in kinase) and 1 μM GST–Rb as substrate (Matsushime et al. 1992; Harper et al. 1993). Cdk6/cyclin D was assayed using anti-Cdk6 immune complexes from insect cells coexpressing Cdk6 and cyclin D2. The concentrations of GST–p57 used were 0, 1.3, 2.7, 5.3, 10.6, 21.3, 42.5, and 85 nM except for Cdc2/cyclin B where the concentrations were 0, 2.7, 5.3, 10.6, 21.3, 42.5, 85, and 170 nM. Counts incorporated into GST–Rb or histone H1 were quantitated using a Molecular Dynamics PhosphorImager. Counts present in the Cdk or cyclin alone lanes were subtracted from total counts in reactions using Cdk/cyclin complexes to calculate percent activities.

possible that the presence of antibodies could interfere with p57^{KIP2} function. Regardless, it is clear that it is a potent inhibitor of cyclin-dependent kinase activity and shows preference for cyclins and Cdks involved in the G_1- to S-phase transitions.

p57^{KIP2} can associate with Cdk2 in vivo

To establish that p57^{KIP2} can associate with cyclin-dependent kinases in vivo, extracts prepared from SAOS-2 cells transfected with either pCMV–p57 or pCMV (negative control) were immunoprecipitated with either anti-p57 or normal rabbit sera and immune complexes subjected to immunoblotting using anti-Cdk2 antibodies. Cdk2 protein was present only in cells transfected with pCMV–p57 and immunoprecipitated with anti-p57^{KIP2} antibodies (Fig. 3C). The presence of Cdk2 in the p57^{KIP2} immunoprecipitates confirms the in vitro binding analysis and demonstrates that p57^{KIP2} can bind to Cdks in vivo.

SAOS-2 cells transfected with mouse pCMV–p57 specifically express KIP2 protein that migrates as a doublet of 57 kD as detected with anti-p57^{KIP2} affinity-purified antibodies (Fig. 3B). As a control, endogenous mouse KIP2 was detected in placental extracts by Western blot. Placental extracts were chosen because placenta express high levels of p57^{KIP2} mRNA (see below). The placental protein also migrates as a doublet but slightly more slowly than p57^{KIP2} from transfected cells. This may be due to the fact that the transfected cells are expressing the mouse KIP2 cDNA whose protein begins at amino acid 14 of the human cDNA (amino acids MERL). The slight difference in mobility could be explained if the placental form of the KIP2 mRNA has an additional 13 amino acids at the amino terminus like the human cDNA (see Fig. 1). Furthermore, human p57^{KIP2} made by in vitro translation of the full-length cDNA produced a protein that comigrated with the faster migrating form of the mouse p57^{KIP2} (data not shown).

Overproduction of p57^{KIP2} leads to G_1 arrest

To investigate whether p57^{KIP2}can arrest the cell cycle, we utilized a transient transfection assay (Zhu et al.

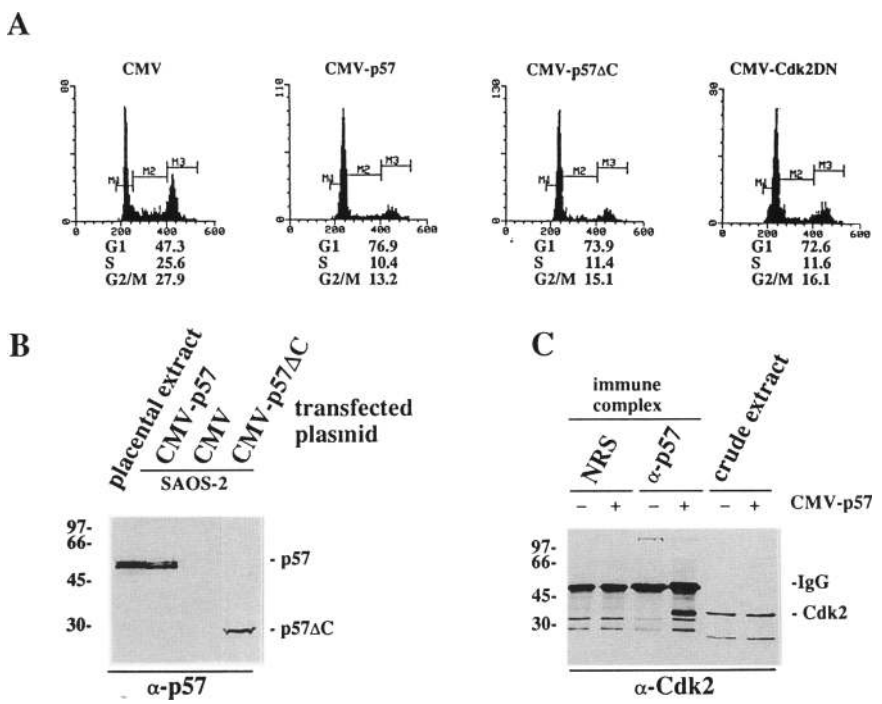

Figure 3. p57^{KIP2} can associate with Cdk2 in vivo and cause G_1 arrest. (*A*) SAOS-2 cells were transiently transfected with 2 μg of pCMVCD20 and 20 μg of either pCMV, pCMV–p57, pCMV–p57ΔC, or pCMV–Cdk2DN using the calcium phosphate method. Forty-eight hours after removal of the precipitate, cells were stained for CD20 and DNA content, and analyzed by flow cytometry. In the histograms shown, DNA content is shown on the abscissa and cell number on the ordinate. The percentages of cells in G_1, S, and G_2/M are given below each histogram. pCMV-p57ΔC expresses an amino-terminal fragment of mouse p57 (residues 1–167). pCMV–Cdk2DN is a dominant-negative Cdk2 mutant (described previously by van der Heuvel and Harlow 1993), which is used here as a positive control for G_1 arrest. (*B*) Expression levels of p57 and p57ΔC in transfected cells as determined by immunoblotting. Whole-cell lysates (10 μg) from SOAS-2 cells transfected with pCMV, pCMV–p57, and pCMV–p57ΔC were separated by SDS-PAGE along with 100 μg of placental extract as a positive control for p57^{KIP2} protein. Proteins were transferred to nitrocellulose and immunoblotted using affinity-purified p57^{KIP2} antibodies, and visualized by ECL (Amersham) detection with an exposure time of 10 sec. The positions of molecular mass markers are shown at *left*. The size of the p57ΔC protein (29 kD) is consistent with the size of the truncated protein observed after in vitro translation (data not shown). (*C*) p57^{KIP2} expressed in SAOS-2 cells associates with Cdk2. Whole-cell extracts from SAOS-2 cells transfected with either pCMV–p57 or pCMV (negative control) were immunoprecipitated with either anti-p57 or normal rabbit sera and immune complexes subjected to immunoblotting using anti-Cdk2 antibodies. As controls, 10 μg of extracts from pCMV–p57 and pCMV-transfected cells were also subjected to immunoblotting with anti-Cdk2 antibodies. The exposure time for ECL detection was 10 sec.

1993). By this approach, it was shown previously that dominant-negative forms of Cdk2 and Cdk3 can block cells in G_1 (van der Heuvel and Harlow 1993) and that overexpression of p21^{CIP1} and p27^{KIP1} cause G_1 arrest (Toyoshima and Hunter 1994; Harper et al. 1995). Expression plasmids for mouse KIP2 expressing full-length p57^{KIP2}, pCMV–p57, or a version expressing the first 167 amino acids, pCMV–p57ΔC, were transfected into SAOS-2 cells along with a plasmid expressing the cell surface marker CD20, and after 48 hr the DNA content of cells expressing high levels of CD20 was measured by flow cytometry (Fig. 3A). SAOS-2 cells are defective for p53 and Rb and have been characterized extensively with respect to cell cycle arrest by both Cdk2-DN (dominant negative) and Rb, both of which lead to predominantly a G_1 arrest (Hinds et al. 1992; van der Heuvel and Harlow 1993). SAOS-2 cells also accumulate in G_1 when transfected with pCMV–p57 (47% vs. 77% in G_1) or pCMV–p57ΔC, indicating that the carboxy-terminal domains are not required for arrest. The overall distribution of cell cycle phases in p57^{KIP2}-arrested cells is similar to cells arrested with Cdk2-DN (Fig. 3A), consistent with the fact that p57^{KIP2} preferentially inhibits Cdks involved in the G_1/S transition. These data indicate that p57^{KIP2} alone can function to arrest the cell cycle in G_1 and does not require p53-regulated proteins or Rb.

KIP2 transcription is not regulated by p53

p21^{CIP1} is transcriptionally regulated by p53. To examine whether KIP2 shares this regulation, we employed Rat A1-5 cells, which contain a temperature-sensitive p53 gene (Martinez et al. 1991). A1–5 cells can grow at 39.5°C when p53 is inactive, but cells arrest in G_1 at 32.5°C when p53 becomes active. We examined whether KIP2 mRNA expression increased when p53 became active. Although CIP1 expression increased upon incubation at 32.5°C, KIP2 mRNA remained constant (Fig. 4B). Thus, p57^{KIP2} is not regulated by p53 under conditions in which p21^{CIP1} transcription is induced in these cells.

p57^{KIP2} shows tissue-specific patterns of expression

In contrast to p21^{CIP1} and p27^{KIP1}, p57^{KIP2} mRNA was detectable in only a subset of adult mouse and human tissues. In general, the mouse expression pattern correlates well with the human distribution. p57^{KIP2} is expressed in heart, brain, lung, skeletal muscle, kidney, pancreas, and testis by Northern analysis (Fig. 4) and by in situ hybridization (see below). It is expressed most highly in placenta and has low levels in liver and is undetectable in spleen (human spleen has not been tested). Furthermore, although the major transcript is ~1.7 kb,

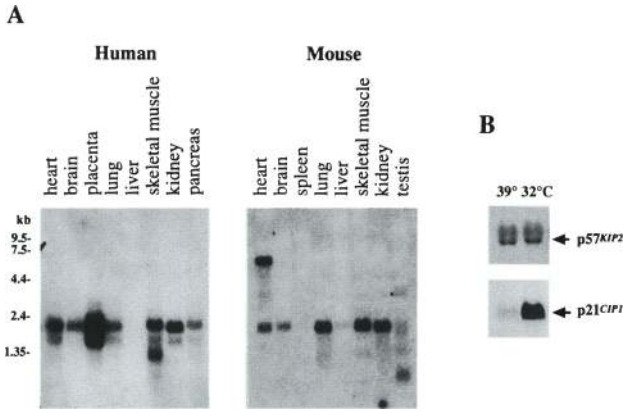

Figure 4. Northern analysis of human and mouse p57^{KIP2}. (*A*) Tissue specificity of KIP2 expression. Blots containing the indicated polyA$^+$ RNA (2 μg per lane) from the indicated tissues were probed with human or mouse p57^{KIP2} cDNAs as described in Materials and methods. The human and mouse probes consisted of the entire cDNA. Exposure times were 15 hr. (*B*) KIP2 is not regulated by p53. Blots containing total RNA prepared from A1–5 cells plated at 39.5°C or 32.5°C for 48 hr were probed with mouse CIP1 or KIP2 probes. The exposure time was 15 hr for CIP1 and 48 hr for KIP2.

there is a minor 1.4-kb species in most human tissues. In addition, there are a number of alternatively sized mRNA species apparent in mouse heart and testis, and human skeletal muscle. The 6-kb mRNA detected in mouse heart is not observed when a probe is used that contains only the kinase-inhibitory domain.

p57^{KIP2} expression during mouse development partially overlaps with p21^{CIP1}

The ability of p57^{KIP2} to function as a cell cycle inhibitor suggests that it might also function to mediate cell cycle arrest during development. Knowledge of the specific timing and location of p57^{KIP2} embryonic expression in vivo could provide evidence that this CKI is involved in terminal differentiation in a developing organism. The in vivo expression pattern of mouse p57^{KIP2} mRNA was examined during embryogenesis and in adult tissues by in situ hybridization. Embryos from day 9.5 postcoitum (pc) through day 15.5 pc were examined. p57^{KIP2} was expressed widely in brain, lens epithelium of the eye, skeletal muscle, and cartilage at all ages examined. A transverse section through the head of a 10-day pc mouse embryo showed expression of p57^{KIP2} in the neural epithelium, Rathke's pouch, and the otocyst (Fig. 5A). In a more caudal region, mRNA expression was observed in the dermamyotome where the first determined myocytes are localized (Fig. 5B), based on coexpression of myogenin in adjacent sections (data not shown). High level expression of p57^{KIP2} was seen later in development in skeletal muscle and cartilage of the cervical region (Fig. 5D) and developing forelimb (Fig. 5E) of 13.5- to 14.5-day pc embryos. Also at this stage in development

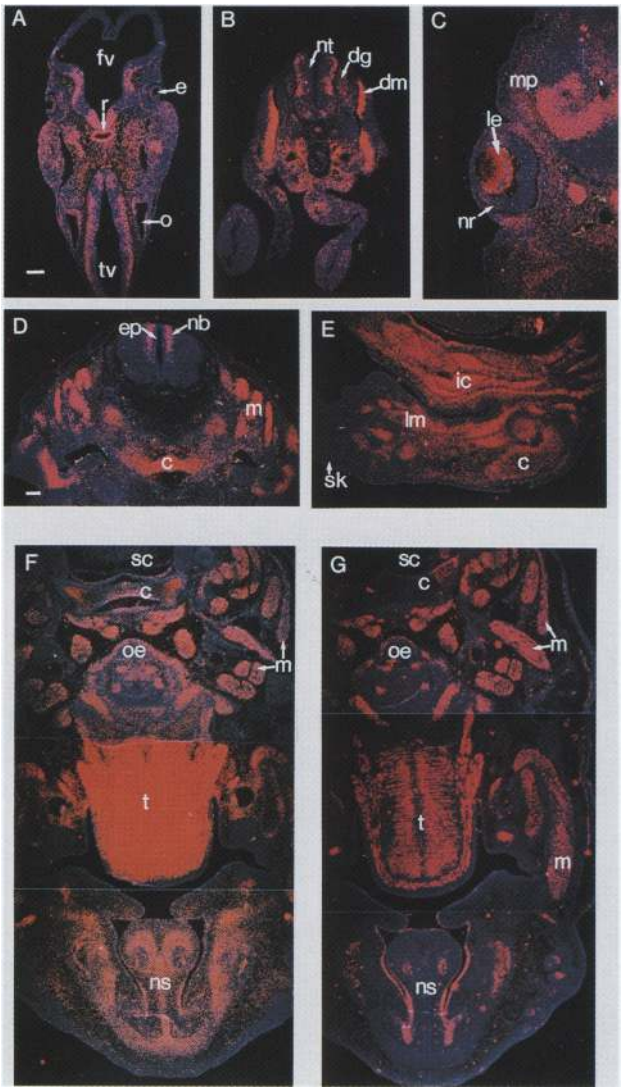

Figure 5. Expression of p57^{KIP2} during mouse embryogenesis. In situ hybridization was performed on specimens as described in Materials and methods. Antisense KIP2 RNA was used as probe for *A–F* whereas p21^{CIP1} was used as probe for *G*. (*A*) Transverse section through the head of a 10-day pc embryo. (*B*) Transverse section through the tail area of a 10-day pc embryo. (*C*) Coronal section through the head of a 13.5-day pc embryo. (*D*) Transverse section through the cervical region of a 14.5-day pc embryo. (*E*) Transverse section through the forelimb of a 14.5-day pc embryo. (*F*) Coronal section through the head and neck of a 14.5-day pc embryo. (*G*) Coronal section through the head and neck of a 14.5-day pc embryo probed for p21^{CIP1} (Parker et al. 1995). Abbreviations are (c) cartilage; (dg) dorsal root ganglion; (dm) dermamyotome; (e) eye; (ep) ependymal layer; (fv) fourth ventricle; (ic) intercostal muscle; (le) lens epithelium; (lm) limb muscle; (m) muscle; (mp) maxillary process; (nb) migrating neuroblasts; (nr) neural retina; (ns) nasal septum; (nt) neural tube; (o) otocyst; (oe) oesophagus; (r) Rathke's pouch; (sk) skin; (sc) spinal cord; (t) tongue; (tv) third ventricle. The scale bar in *A* is 100 μm and represents *A* and *E–G*. The scale bar in *D* is 100 μm and represents *B–D*.

the lens epithelium showed intense expression of $p57^{KIP2}$ (Fig. 5C). In general, $p57^{KIP2}$ expression correlates with cells that have left the cell cycle.

The expression pattern for $p21^{CIP1}$ has been established during embryogenesis and in adult tissues (Parker et al. 1995). It shows specific expression in a number of tissues during development including muscle, epidermis, certain epithelial cells, and cartilage, among others. For comparison, adjacent sections were probed for $p21^{CIP1}$ and $p57^{KIP2}$. As seen in Figure 5, F–G, $p57^{KIP2}$ and $p21^{CIP1}$ coexpress in skeletal muscle, cartilage, and tongue muscle, although $p57^{KIP2}$ seems to be expressed more heavily in these tissues. Differences in expression patterns of $p57^{KIP2}$ and $p21^{CIP1}$ can be observed in the nasal region (Fig. 5F–G) and in the skin of the developing forelimb where $p57^{KIP2}$ is not expressed (Fig. 5E) in contrast to $p21^{CIP1}$ expression in the outer layers of embryonic epidermis (Parker et al. 1995; data not shown). Owing to the abundance of muscle and cartilage tissue in an animal, the coexpression in these lineages gives the incorrect impression that $p57^{KIP2}$ and $p21^{CIP1}$ are generally coexpressed. However, during development, most sites of expression including the brain, epidermis, hair follicles, nasal epithelium, and eyes are nonoverlapping.

A survey of $p57^{KIP2}$ expression by in situ hybridization in adult tissues revealed that it is expressed widely in brain, kidney, heart, lung, liver, and skeletal muscle (Fig. 6A,B,E–H). Expression is limited to the muscularis mucosa layer of the stomach and is completely absent in the small intestine (Fig. 6C,D). In contrast, $p21^{CIP1}$ is expressed heavily in the intestinal villi of the small intestine and in the surface epithelium of the stomach (Parker et al. 1995) but not in the muscularis mucosa layer of the stomach.

KIP2 localizes to chromosome 11p15.5, a locus implicated in several human cancers

The chromosomal localization of the KIP2 gene was identified by fluorescent in situ hybridization (FISH) (Ijdo et al. 1992). It localized to the telomeric end of human chromosome 11 at 11p15.5 (Fig. 7), a position implicated in a number of human cancers (see Discussion). Thus, the CKI $p57^{KIP2}$ is a candidate tumor suppressor gene, consistent with a role in maintenance of the nonproliferative state in adult tissues.

Discussion

We have employed a genetic approach to identify genes encoding proteins that physically associate with cyclins and cyclin-dependent kinases. Using a modified version of the two-hybrid system, we identified KIP2, which en-

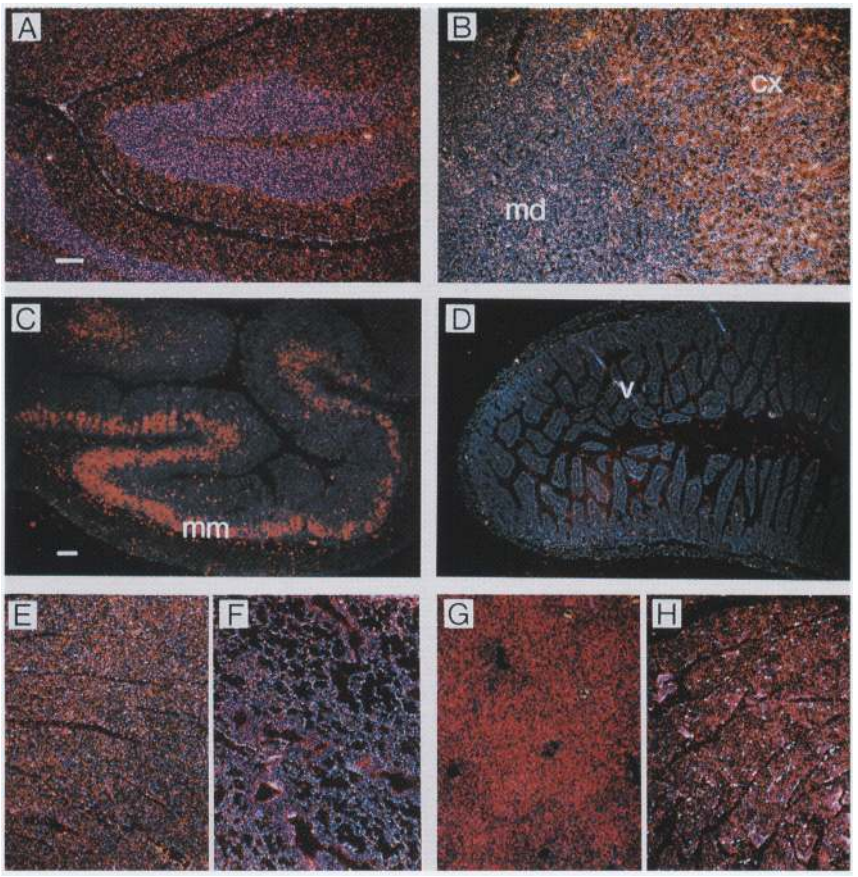

Figure 6. Expression of $p57^{KIP2}$ in adult mouse tissues by in situ hybridization. Sections of brain (*A*); kidney (*B*); stomach (*C*); small intestine (*D*); heart (*E*); lung (*F*); liver (*G*); and skeletal muscle (*H*) from a 4-month-old C57B mouse were probed for KIP2 mRNA as described in Materials and methods. Abbreviations are (cx) renal cortex; (md) renal medulla; (mm) muscularis mucosae; and (v) intestinal villi. The scale bar in *A* is 100 μm and represents *A*, *B*, and *D–H*. The scale bar in *C* is 100 μm.

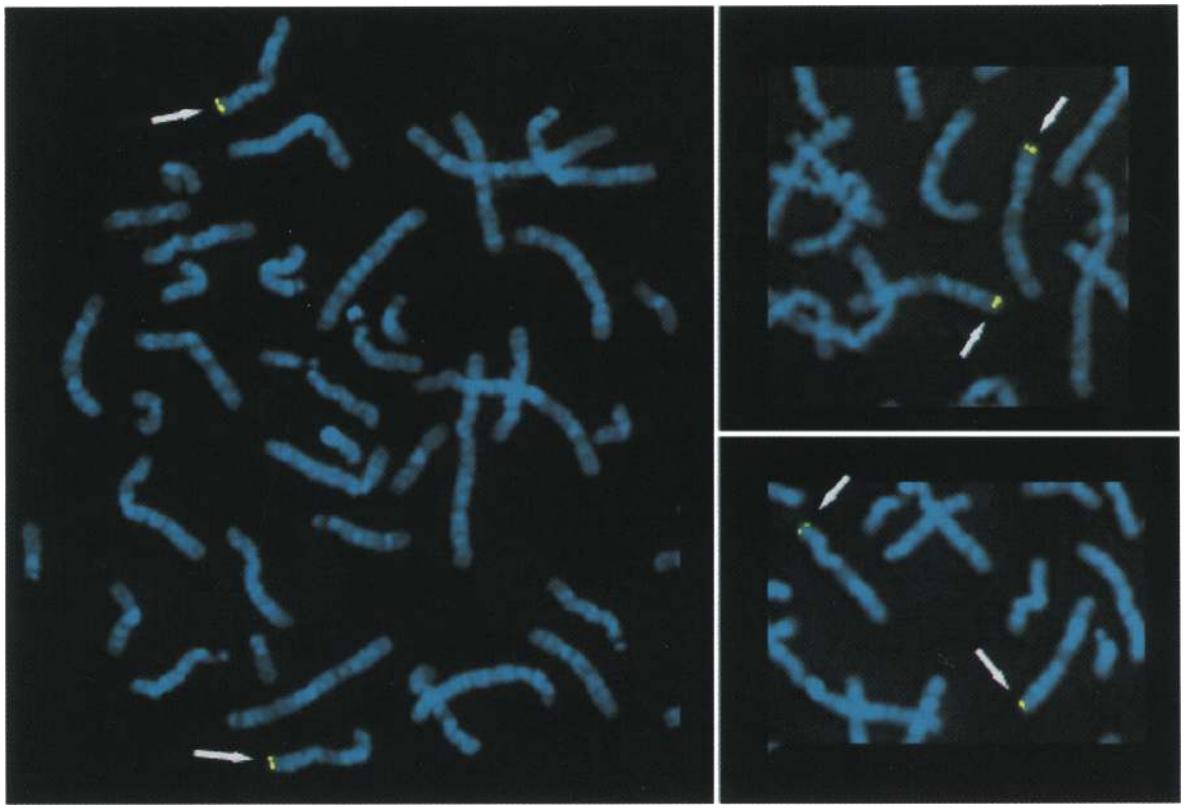

Figure 7. The human KIP2 gene is located on chromosome 11p15.5. In situ hybridization was performed on mitotic chromosomes using fluorescently labeled KIP2 DNA. Three independent spreads of metaphase chromosomes are shown. Arrows indicate the localization of KIP2 on the telomeric end of chromosome 11 at 11p15.5.

codes a novel inhibitor of cyclin-dependent kinases. KIP2 encodes a 335 amino acid protein in mouse and a 316 amino acid protein in humans, both of which migrate as 57 kD by SDS-PAGE, very close to cyclin A at 58 kD. Comigration with cyclin A may have prevented earlier biochemical detection. KIP2 shows specificity for G_1 Cdks. It can bind several Cdks in a cyclin-dependent manner, including cyclin A/Cdk2, cyclin E/Cdk2, cyclin E/Cdk3, cyclin D2/Cdk4, and, to a lesser extent, cyclin D2/Cdk6. Furthermore, $p57^{KIP2}$ can inhibit the kinase activity of the G_1 cyclin Cdk2, Cdk3, and Cdk4 complexes. Thus, KIP2 is capable of inhibiting several cyclin-dependent kinases with demonstrated roles in the G_1/S-phase transition. This places KIP2 in a position to play an important regulatory role in control of cell proliferation.

The cyclin dependence of binding and inhibition of $p57^{KIP2}$ is similar to that observed for $p21^{CIP1}$ and $p27^{KIP1}$ (Harper et al. 1995) and suggests that $p57^{KIP2}$ can also act as a buffer to titrate cyclin/Cdk complexes as they are formed. One notable difference is the reduced activity toward cyclin D2/Cdk6 complexes that are efficiently inhibited by $p21^{CIP1}$ (Harper et al. 1995). This difference may indicate a potential mechanism of $p57^{KIP2}$ resistance, that is, dependence on Cdk6 instead of Cdk4 for cyclin D kinase activity. Overproduction of $p57^{KIP2}$ can efficiently arrest SAOS2 cells in the G_1 phase of the cell cycle, consistent with its specificity for inhibition of G_1 cyclin/Cdk complexes. Furthermore, it can be found associated with Cdk2 in these transfected cells indicating that $p57^{KIP2}$ is likely to mediate cell cycle arrest through its known biochemical activity of binding and inhibiting Cdks in vivo.

$p57^{KIP2}$ is the most structurally diverse member of the $p21^{CIP1}$ family of CKIs, consisting of four distinct regions. It is more closely related to $p27^{KIP1}$ in the kinase inhibitory domain. In addition, it shares strong similarity with $p27^{KIP1}$ in the carboxy-terminal region termed the QT box. The relatedness suggests that the QT box has a defined function, most likely in protein–protein interaction. Because $p21^{CIP1}$ appears to have a dual inhibitory function in vitro, it is tempting to speculate that the other members of this class will also have dual functions. However, a cytomegalovirus (CMV) expression construct expressing domains II–IV of $p57^{KIP2}$ or the carboxy-terminal domain of $p21^{CIP1}$ had no effect on cell cycle distribution of SAOS2 cells (C. Bai, J.W. Harper, and S.J. Elledge, unpubl.). Thus, a secondary inhibitory function for either of these CKIs has not been demonstrated in vivo.

The most striking features of the primary structure of KIP2 are the internal repeat domains. Mouse $p57^{KIP2}$ has

a proline-rich region and an acidic repeat region. The human cDNA that we have isolated has a proline–alanine repeat region termed the PAPA repeat. A number of issues concerning these motifs remain unanswered. For example, it is not known definitively whether the mouse and human genes are true homologs or members of a related family. The sequence conservation in the Cdk inhibitory and QT box domains suggests that they are closely related. The absence of sequence conservation in the internal region could be attributable to the lack of functional conservation. It is possible that the internal domains are merely spacer regions needed to appropriately separate the amino- and carboxy-terminal domains, and their length, not their composition, is the critically conserved feature. Alternatively these sequences may serve as important sites of protein–protein interaction. One possible function for domains II–IV is to recruit substrates to the Cdk/cyclin complexes. It has been shown recently that multiple molecules of $p21^{CIP1}$ are necessary to inhibit Cdks (Zhang et al. 1994; Harper et al. 1995). Thus, these inhibitors can be part of active complexes and any additional proteins recruited into these complexes are potential substrates of these Cdks. These multidomain inhibitors could serve as adapters that allow signals to be generated from Cdks in a cell during the process of terminal differentiation.

$p57^{KIP2}$ is expressed in several embryonic and adult tissues derived from the ectoderm, mesoderm, and endoderm. Overall there is a strong correlation between arrest of cell proliferation and $p57^{KIP2}$ expression, strongly suggesting that expression of $p57^{KIP2}$ and other cyclin kinase inhibitors will be coupled to activation of cell differentiation in multiple cell lineages. In muscle it has been shown that $p21^{CIP1}$ is also induced during terminal differentiation indicating a potentially redundant role with $p57^{KIP2}$ in this tissue. It is likely that a number of redundant mechanisms will cooperate to ensure permanent exit from the cell cycle in terminally differentiated tissues. Although there may be some redundancy with $p21^{CIP1}$, there are also complementary patterns of expression suggesting unique functions for each protein. In adults, $p57^{KIP2}$ mRNA is found in a wide range of tissues, indicating a role in maintenance of the nonproliferative state throughout life.

The chromosomal location of human KIP2, 11p15.5, marks it as a candidate tumor suppressor gene. This region has been investigated intensively because of frequent loss of heterozygosity at this locus in a number of human cancers including breast cancer, bladder, lung, ovarian, kidney, and testicular carcinomas (for review, see Seizinger et al. 1991). Several types of childhood tumors, including Wilms' tumor, adrenocortical carcinoma, rhabdomyosarcoma, and hepatocellular carcinoma, display a specific loss of maternal 11p15 alleles, suggesting a role for genomic imprinting. Chromosome transfer experiments have provided evidence that a tumor suppressor gene resides at this locus, the WT2 gene involved in Wilms' tumor and possibly rhabdomyosarcoma (for review, see Hastie 1994), either of which could be attributable to loss of a Cdk inhibitor. In addition, rearrangements in the 11p15 region have been linked to Beckwith–Wiedemann Syndrome (BWS), which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, visceromegely (enlarged organs), exomphalos (umbilical protrusion), and an increased risk (7.5%) of childhood tumors (Weidemann 1983). The BWS occurs with an incidence of 1 in 13,700 births, 85% of which are sporadic and 15% familial (Pettenatti et al. 1986). Genetic analysis indicates maternal carriers, also suggesting a role for genomic imprinting (for review, see Junien 1992). Several features of the KIP2 gene make it a reasonable candidate for a mediator of some of the phenotypes associated with BWS. First, a Cdk inhibitor could potentially explain both overgrowth and tumorigenesis phenotypes. Furthermore, the expression pattern of KIP2 in mouse correlates with areas known to be affected in BWS, including the tongue, kidney, and muscle. Finally, the presence of the PAPA repeat region might provide a high frequency mechanism for expansion mutation akin to the triplet repeat mechanism known to operate in fragile X syndrome and other diseases. Genetic analysis of alterations in the KIP2 gene in tumors or affected individuals will be necessary to determine which, if any, of these 11p15-associated diseases are attributable to $p57^{KIP2}$.

Materials and methods

Isolation of $p57^{KIP2}$ cDNAs

Two-hybrid screens were performed using pAS1-cyclin D1 in yeast strain Y190 (Durfee et al. 1993; Harper et al. 1993) and a mouse day-10.5 pc embryonic cDNA library fused with the GAL4 activation domain. Plasmids were recovered into *Escherichia coli* strain JA226 and reintroduced into Y190 strains containing either pAS1–cyclin D1 or other pAS2 plasmids expressing various GAL4 DNA-binding domain fusion proteins to test for specificity of the interaction with cyclin D1. Clones selective for interaction with cyclin D1 were subjected to DNA sequence analysis (Sequenase, U.S. Biochemical) using either library plasmids or pBluescript subclones.

A human homolog of mouse $p57^{KIP2}$ was isolated by low stringency hybridization (Elledge and Davis 1990) using a embryonic library in λgt10 and a probe containing nucleotides 1–581 of the mouse cDNA. Phage inserts were subcloned into pBluescript for sequence analysis.

Protein expression and purification

For expression of mouse $p57^{KIP2}$ as a glutathione *S*-transferase (GST) fusion protein, the open reading frame was engineered to contain an *NcoI* site at the initiation codon (MERL), cloned into a modified version of pGEX–2TK, and purification was accomplished as described previously for GST–p21 (Harper et al. 1993). The concentration of GST–p57 was determined by Bradford analysis. $p57^{KIP2}$ in vitro translation was accomplished using TnT reticulocyte system (Promega) in conjunction with pBluescript–p57. For expression of $p57^{KIP2}$ in sf9 cells, the KIP2 open reading frame was cloned into pBlueBacHis (Invitrogen) and recombinant baculovirus generated using Baculogold (Pharmingen) as described by the manufacturer. This virus produces a p57 protein with a 41 amino acid amino-terminal extension containing a polyhistidine tag and sequences from T7 gene 10,

which are recognized by anti-T7 Tag antibodies (Novagen). [^{35}S]Methionine-labeled sf9 extracts and purified cyclin/Cdk complexes were generated 44 hr postinfection as described previously (Matsushime et al. 1992; Harper et al. 1993, 1995). GST–Rb and histone H1 kinase assays were performed as described (Harper et al. 1993).

Antibody production

Polyclonal antibodies against mouse p57^{KIP2} were generated in rabbits using GST–p57ΔC (residues 1–167) purified by preparative SDS-PAGE. Antibodies for immunoprecipitation were depleted of GST antibodies using GST protein covalently linked to glutathione–Sepharose followed by affinity purification using GST–p57 linked to glutathione–Sepharose. Proteins were coupled to Sepharose using dimethyl palmidate (Harlow and Lane 1988). Antibodies for immunoblotting were purified by first depleting antisera of GST reactive antibodies and then affinity purifying p57-specific antibodies using GST–p57 immobilized on nitrocellulose. Antibody concentrations were determined by Bradford analysis.

In vitro binding

To examine the interaction of p57^{KIP2} with Cdks, 10 μl of [^{35}S]methionine-labeled sf9 cell lysate containing the indicated proteins were mixed with 4 μl of in vitro translated p57. After 10 min on ice, extracts were diluted with 150 μl of binding buffer (40 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 10 mM NaF, 2 mM EDTA, 5 μg/ml of antipain, leupeptin, and aprotinin) and Cdks then were immunoprecipitated using 10 μl of protein A–Sepharose (Pharmacia) and 0.5–2 μg of the appropriate antibody. Immune complexes were washed three times with 1 ml of binding buffer prior to SDS-PAGE and autoradiography. To examine the interaction of p57 with cyclins, [^{35}S]methionine-labeled sf9 lysates for cyclins A, D1, and D2, and T7Tag–p57 were first depleted of Cdk-associated cyclins using 25 μl of p13–agarose (Oncogene Science). The indicated extracts were mixed and immunoprecipitated with either antibodies directed against the cyclin or anti-T7 Tag prior to SDS-PAGE and autoradiography. Cyclin A antibodies were from a previous study (Elledge et al. 1992), whereas anti-cyclin D1 and D2 were from Santa Cruz Biochemicals.

To examine the association of p57^{KIP2} with Cdk2 in transfected SAOS-2 cells, extracts were generated from cells transfected with either pCMV–p57 or pCMV, a negative control, as described below. Cells from two 10 cm dishes were lysed in binding buffer. Extracts (0.4 mg of protein) were immunprecipitated with 0.3 μg of either affinity purified anti-p57 or normal rabbit sera. Immune complexes washed three times with binding buffer and immune complexes separated by SDS-PAGE prior to immunblotted with anti-Cdk2 (Santa Cruz). Detection was accomplished using ECL (Amersham).

Transfections

SAOS-2 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37°C in 5% CO_2. Transient transfections for flow cytometry were performed using the calcium phosphate method (van der Heuvel and Harlow 1993; Zhu et al. 1993). Briefly, the indicated plasmids (20 μg) were cotransfected with pCMV–CD20 (2 μg) into SAOS-2 cells (20% confluence). Forty-eight hours post-transfection, cells were harvested with phosphate-buffered saline (PBS) containing 0.1% EDTA prior to staining with fluoroscein isothiocyanate-conjugated anti-CD20 antibodies (Becton-Dickinson). Cells were washed with PBS, then fixed with 80% ethanol, and the DNA stained with propidium iodide (10 μg/ml) containing 250 μg/ml of ribonuclease A prior to flow cytometry using a Becton-Dickinson FACScan. DNA content in 4000–6000 CD20-positive cells is presented in the DNA histograms. For immunoblot analysis of transfected cells, 10 μg of whole-cell lysates was separated by SDS-PAGE, transferred to nitrocellulose, and probed with affinity-purified anti-p57.

p57^{KIP2} expression

Human and mouse tissue Northern blots (Clonetech) were probed at high stringency with full-length human or mouse cDNAs labeled with [α-^{32}P]dCTP. For in situ hybridization, embryos or tissues were collected at the indicated times, fixed in 3% paraformaldehyde, embedded in paraffin, and sectioned on a Microm microtome at 6 μm. Specimens were hybridized with riboprobes labeled with α-^{35}S-labeled UTP essentially as described previously (Lutz et al. 1994). pBluescript p57 was linearized with *Kpn*I or *Bam*HI, and sense and antisense transcripts generated using either T7 or T3 polymerase, respectively. Specimens were photographed by double exposure using dark-field illumination with a red filter and Hoechst epifluorescence optics.

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References

Bai, C., R. Richman, and S.J. Elledge. 1994. Human cyclin F. *EMBO J.* **13:** 6087–6098.

Cairns, P., L. Mao, A. Merlo, D. Lee, D. Schwab, Y. Eby, K. Tokino, P. Van Der Riet, J. Blaugrund, D. Sidransky, A. Kamb, Q. Liu, K. Harshman, S. Tavtigian, C. Cordon-Cardo, and M. Skolnick. 1994. Rates of p16[MTS1] mutations in primary tumors with p9 loss. *Science* **265:** 415–417.

Draetta, G. 1993. cdc2 activation: The interplay of cyclin binding and Thr161 phosphorylation. *Trends Cell Biol.* **3:** 287–289.

Dulic, V., W.K. Kaufman, S. Wilson, T.D. Tlsty, E. Lees, J.W. Harper, S.J. Elledge, and S.I. Reed. 1994. p53-dependent inhibition of cyclin dependent kinase activitites in human fibroblasts during radiation-induced G1 arrest. *Cell* **76:** 1013–1023.

Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, Y. Yang, A. Kilburn, W.-H. Lee, and S.J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes & Dev.* **7:** 555–569.

El-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75:** 817–825.

El-Deiry, W.S., J.W. Harper, P.M. O'Connor, V. Velculescu, C.E. Canman, J. Jackman, J. Pietenpol, M. Burrell, D.E. Hill, K.G. Wiman, W.E. Mercer, M.B. Kastan, K.W. Kohn, S.J. Elledge, K.W. Kinzler, and B. Vogelstein. 1994. WAF1/CIP1 is induced in p53 mediated G1 arrest and apoptosis. *Cancer Res.* **54:** 1169–1174.

Elledge, S.J. and R.W. Davis. 1990. Two gene differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits in ribonucleotide reductase. *Genes & Dev.* **4:** 740–751.

Elledge, S.J. and J.W. Harper. 1994. Cdk Inhibitors: On the threshold of checkpoints and development. *Curr. Opin. Cell Biol.* **6:** 874–878.

Elledge, S.J., R. Richman, F.L. Hall, R.T. Williams, N. Logsdon, and J.W. Harper. 1992. CDK2 encodes a 33 kd cyclin A-associated protein kinase and is expressed before CDC2 in the cell cycle. *Proc. Natl. Acad. Sci.* **89:** 2907–2911.

Flores-Rozas, H., Z. Kelman, F. Dean, Z.-Q. Pan, J.W. Harper, S.J. Elledge, M. O'Donnell, and J. Hurwitz. 1994. Cdk-interacting protein-1 (Cip1, Waf1) directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase δ holoenzyme. *Proc. Natl. Acad. Sci.* **91:** 8655–8659.

Guan, K., C.W. Jenkins, Y. Li, M.A. Nichols, X. Wu, C.L. O'keefe, A.G. Matera, and Y. Xiong. 1994. Growth suppression by p18, a $p16^{INK4/MTS1}$ and $p14^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes & Dev.* **8:** 2939–2952.

Halevy, O., B.G. Novitch, D.B. Spicer, S.X. Skapek, J. Rhee, G.J. Hannon, D. Beach, and A. Lassar. 1995. Terminal cell cycle arrest of skeletal muscle correslates with induction of p21 by MyoD. *Science* (in press).

Hannon, G.J. and D. Beach. 1994. $p15^{INK4B}$ is a potential effector of TGF-β induced cell cycle arrest. *Nature* **371:** 257–261.

Harlow, E. and D. Lane. 1988. *Antibodies: A laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Harper, J.W., G. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The 21 kd Cdk interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75:** 805–816.

Harper, J.W., S.J. Elledge, K. Keyomarsi, B. Dynlacht, L.-H. Bai, P. Zhang, S. Dobrowoski, C. Bai, L. Connell-Growley, E. Swindell, M.P. Fox, and N. Wei. 1995. Inhibition of cyclin-dependent kinases by p21. *Mol. Biol. Cell* (in press).

Hastie, N.D. 1994. The Genetics of Wilm's tumor. A case of disrupted development. *Annu. Rev. Genet.* **28:** 523–558.

Hinds, P.W., S. Mittnacht, V. Dulic, A. Arnold, S.I. Reed, and R.A. Weinberg. 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70:** 993–1006.

Ijdo, J.W., E.A. Lindsay, R.A. Wells, and A. Baldini. 1992. Multiple variants in subtelomeric regions of normal karyotypes. *Genomics* **14:** 1019–1025.

Jen, J., J.W. Harper, S.H. Bigner, D.D. Bigner, N. Papadopoulos, S. Markowitz, J.K.V. Willson, K.W. Kinzler, and B. Vogelstein. 1994. Deletion of p16 and p15 genes in brain tumors. *Cancer Res.* **54:** 6353-6358.

Jiang, H., J. Lin, Z.-Z. Su, F.R. Collart, E. Huberman, and P.B. Fisher. 1994. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21 WAF/CIP1, expression in the absence of p53. *Oncogene* **9:** 3397–3407.

Junien, C. 1992. Beckwith-Wiedemann Syndrome, tumourigenesis and imprinting. *Curr. Opin. Genet. Dev.* **2:** 431–438.

Kamb, A., N.A.Gruis, J. Weaver-Feldhaus, Q. Liu, K. Harshman, S.V. Tavtigan, E. Stockert, R.S.I. Day, R.E. Johnson, and M.H. Skolnick. 1994. A cell cycle regulator potentially involved in the genesis of many tumor types. *Science* **264:** 436–440.

Kato, J., M. Matsuoka, K. Polyyak, J. Massague, and C.J. Sherr. 1994. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor ($p27^{KIP1}$) of cyclin-dependent kinase 4 activation. *Cell* **79:** 487–496.

Martinez, J., I. Georgoff, J. Martinez, and A.J. Levine. 1991. Cellular localization and cell cycle regulation by a temperature-sensitive p53-protein. *Genes & Dev.* **5**:152–159.

Matsushime, H., M.E. Ewen, D.K. Strom, J.-Y. Kato, S.K. Hanks, M.F. Roussel, and C.J. Sherr. 1992. Identification and properties of an atypical catalytic subunit ($p34^{PSK\text{-}J3}$/cdk4) for mammalian D-type G1 cyclins. *Cell* **71:** 323–334.

Meyerson, M. and E. Harlow. 1994. Identification of G1 kinase activity for Cdk6, a novel cyclin D partner. *Mol. Cell Biol.* **14:** 2077–2086.

Michieli, P., M. Chedid, D. Lin, J.H. Pierce, W.E. Mercer, and D. Givol. 1994. Induction of WAF1/CIP1/ by a p53-independent pathway. *Cancer Res.* **54:** 3391–3395.

Nobori, T., K. Miura, D.J. Wu, A. Lois, K. Takabayashi, and D.A. Carson. 1994. Deletion of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* **368:** 753–756.

Noda, A., Y. Ning, S.F. Venable, O.M. Pereira-Smith, and J.R. Smith. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression cloning screen. *Exp. Cell Res.* **211:** 90–98.

Nourse, J., E. Firpo, W.M. Flanagan, S. Coats, K. Polyak, M. Lee, J. Massague, G.R. Crabtree, and J.M. Roberts. 1994. Interlukin-2-mediated elimination of the $p27^{KIP1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* **372:** 570–573.

Parker, S.B., G. Eichele, P. Zhang, A. Rawls, A.T. Sands, A. Bradley, E.N. Olson, J.W. Harper, and S.J. Elledge. 1995. p53-Independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells. *Science* **267:** 1024–1027.

Pettenati, M.J., J.L. Haines, R.R. Higgins, R.S. Wappner, C.G. Palmer, and D. Weaver. 1986. Beckwith-Wiedemann syndrome: Presentation of clinical and cytogenetic data on 22 new cases and review of the literature. *Hum. Genet.* **74:** 143–154.

Pines, J. 1993. Cyclins and cyclin-dependent kinases: Take your partners. *Trends Biochem. Sci.* **18:** 195–197.

Polyak, K., M.H. Lee, H. Erdjument-Bromage, P. Tempst, and J. Massague. 1994. Cloning of $p27^{KIP1}$, a cyclin-dependent kinase inhibitor and potential mediator of extracellular antimitogenic signals. *Cell* **78:** 59–66.

Seizinger, B., H.P. Klinger, C. Junien, Y. Nakamura, M. Le Beau, W. Cavenee, B. Amanuel, B. Ponder, S. Naylor, F. Mittelman, D. Louis, A. Menon, I. Newsham, J. Decker, M. Kaelbling, I. Henry, and A.V. Deimling. 1991. Report of the committee on chromosome and gene loss in human neoplasia. *Cytogenet. Cell. Genet.* **58:** 1080–1096.

Serrano, M., G.J. Hannon, and D. Beach. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366:** 704–707.

Sheikh, M.S., X. Li, J. Chen, Z. Shao, J.V. Ordonez, and J.A. Fontana. 1994. Mechanisms of regulation of WAF1/Cip1 gene expression in human breast carcinoma: Role of p53-dependent and independent signal transduction pathways. *Oncogene* **9:** 3407–3415.

Sherr, C.J. 1994. G1 phase progression: Cycling on cue. *Cell* **79:** 551–555.

Solomon, M.J. 1993. Activation of the various cyclin/cdc2 protein kinases. *Curr. Opin. Cell Biol.* **5:** 180–186.

Spruck, C.H., M. Gonzalez-Zulueta, A. Shibata, A.R. Simoneau, M.F. Lin, F. Gonzales, Y.C. Tsai, and P. Jones. 1994. p16 gene in uncultured tumours. *Nature* **370:** 183–184.

Steinman, R.A., B. Hoffman, A. Iro, C. Guillouf, D.A. Liebermann, and M.E. El-Houseini. 1994. Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* **9:** 3389–3396.

Toyoshima, H. and T. Hunter. 1994. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78:** 67–74.

van der Heuvel, S. and E. Harlow. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **26:** 2050–2054.

Waga, S., G.J. Hannon, D. Beach, and B. Stillman. 1994. The p21 inhibitor of cyclin- dependent kinases controls DNA replication by interacting with PCNA. *Nature* **369:** 574–578.

Wiedemann, H.R. 1983. Tumours and hemihypertrophy associated with Wiedemann-Beckwith syndrome. *Eur. J. Pediatr.* **141:** 129.

Xiong, Y., G. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* **366:** 701–704.

Zhang, H., G. Hannon, and D. Beach. 1994. p21-containing cyclin kinases exist in both active and inactive states. *Genes & Dev.* **8:** 1750–1758.

Zhu, L., S. Heuvel, K. Helin, A. Fattaey, M. Ewen, D. Livingston, N. Dyson, and E. Harlow. 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes & Dev.* **7:** 1111–1125.