Coamplification of the CDK4 Gene with MDM2 and GLI in Human Sarcomas

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ABSTRACT

The 34-kilodalton cyclin-dependent kinase, p34cdk4, is a major catalytic subunit of mammalian D-type cyclins, which act during the G1 phase of the cell cycle to enforce the decision of cells to enter S phase. A murine complementary DNA clone was used to clone the cognate human CDK4 gene, which was localized to human chromosome 12, band q13, by fluorescence in situ hybridization. Because this chromosomal band contains the GLI and MDM2 genes, which are frequently amplified in human sarcomas, we analyzed CDK4 copy number and expression in a panel of sarcoma cell lines. An osteosarcoma cell line, OsACL, manifested a 25-fold increased copy number of CDK4, amplified concordantly with both GLI and MDM2, whereas a rhabdomyosarcoma cell line, SJRIII, was found to have an amplicon that included CDK4 and GLI but not MDM2. CDK4 mRNA and protein were overexpressed in both cell lines, and nucleotide sequencing analysis indicated that the gene had not sustained mutations. These observations provide the first evidence for amplification of a gene encoding a cell division cycle protein kinase, complement recent data indicating that genes encoding D-type cyclins are targets of chromosomal rearrangement and gene amplification in tumor cells, and suggest that CDK4 amplification might contribute to oncogenesis.

INTRODUCTION

Key cell cycle transitions in all eukaryotes are regulated by cyclins and their catalytic subunits, the CDKs. The prototypic CKD, p34cdk2 (p34cdk1), together with its regulatory subunit, cyclin B, govern mitotic entry and exit (reviewed in Ref. 1), whereas cyclin A associates with both p34cdk2 and a related catalytic partner, p33cdk2, during the S and G2 phases of the cell cycle and may coordinate the events that couple DNA replication to cell division (2-5).

Three recently described classes of mammalian cyclins (designated types C, D, and E) are expressed during G1 phase (6-10), and both cyclins D and E have been inferred to regulate G1 progression and entry into S phase (reviewed in Ref. 11). Active enzyme complexes consisting of cyclin E and p33cdk2 accumulate periodically at the G1-S phase boundary, and their appearance temporally precedes the formation of predominantly S phase-specific complexes between p33cdk2 and cyclin A (12, 13). Both cyclin E and p33cdk2 appear to regulate the G1-S transition because the activity of p33cdk2 is required for entry into S phase (14, 15), and enforced overexpression of cyclin E can shorten the G1 interval (16).

Cyclins D1 and D2 in fibroblasts can shorten their G1 interval and decrease entry into S phase (14, 17). The expression of which is usually manifested earlier during G1 than cyclins A, cyclins D1, D2, and D3 are differentially expressed in various cell lineages in response to growth factor-induced signals (6, 17, 20-24), and they form complexes with a novel catalytic subunit, p34cdk4, to activate its kinase activity (25, 26). Like cyclins A and E, cyclins D2 and D3, but not D1, can productively interact with p33cdk2 (27, 28), whereas cyclins D1 and D3 also form complexes with p31cdk5 in fibroblasts (28). Thus, the interactions of D-type cyclins with catalytic subunits are highly combinatorial and may serve to target the holoenzymes to different substrates, the phosphorylation of which are required for G1 progression (11). For example, the D-type cyclins, but not cyclins E or A, can bind directly to pRB and to the pRB-related protein, p107 (26, 27, 29), and these interactions can direct the p34cdk4 kinase toward pRB, enabling p34cdk4/cyclin D complexes to phosphorylate pRB much more efficiently than other canonical CDK substrates, such as histone H1 (25, 26). Because pRB phosphorylation inactivates its growth suppressive function and accelerates G1 exit (reviewed in Ref. 30), the D-type cyclin-dependent kinases may function in part in this manner. Data implicating D-type cyclins in G1 progression have remained largely inferential, but recent results indicate that overexpression of cyclins D1 and D2 in fibroblasts can shorten their G1 interval and decrease their dependency on serum growth factors (31). It may therefore prove that the D-type cyclins act upstream of cyclin E to commit cells to DNA synthesis.

Several lines of evidence implicate overexpression of the cyclin D1 gene, located on human chromosome 11, band q13 (8, 18, 19), in oncogenesis. Cyclin D1 was independently isolated (as PRADI) at the breakpoint of a chromosome 11 inversion in human parathyroid adenomas, where its intact coding sequences are fused to the parathyroid hormone promoter, resulting in its deregulated overexpression (8, 32). Cyclin D1 (alias BCLI) is overexpressed as a result of a chromosomal translocation, t(11;14)(q13;q32), that is commonly found in centrocytic B-cell lymphomas (33-35) and pursuant to gene amplification in 15-20% of human breast cancers and squamous cell tumors of the head and neck (36-38). Expression of cyclins D1 and D2 can also be inappropriately induced by retroviral insertions in rodent lymphoid malignancies (39, 40). A reasonable hypothesis is that deregulated expression of D-type cyclins can contribute to tumor formation by interacting with CKDs, the kinase activities of which are rate limiting for G1 progression.

We therefore reasoned that CDK4, a unique D-type cyclin partner, might be a target for genetic alterations leading to aberrant cell proliferation in some human cancers. As a first step in investigating its potential role in tumorigenesis, we determined the chromosomal location of CDK4 and assigned the gene to band q13 of human chromosome 12. This region, which also contains the GLI and MDM2 protooncogenes, is rearranged or amplified in a variety of human sarcomas, such as malignant fibrous histiocytoma, rhabdomyosarcoma, osteosarcoma, and liposarcoma (41-45). Our results now demonstrate that CDK4 is included in the 12q13 amplicon and is overexpressed in sarcoma cell lines with gene amplification.

MATERIALS AND METHODS

Genomic DNA Cloning. Bacteriophage and cosmids human genomic DNA libraries were screened with a 32P-labeled 1.3-kilobase EcoRI fragment of the murine CDK4 cDNA (25). Hybridization was performed overnight at 42°C in buffer containing 5× standard saline citrate (1× standard saline citrate is 0.15 NaCl-0.015 sodium citrate, pH 7.0), 50% formamide, 1.0% sodium dodecyl...
of the hybrid cell lines are described in "Materials and Methods." Discordant is defined as the sum of the hybrids containing the chromosome but lacking human CDK4 and those lacking the chromosome but containing human CDK4 divided by the total number of hybrid cell lines examined and multiplied by 100.

**Protein Analysis and Antisera.** Subconfluent adherent cells in T-75 flasks (Falcon) were starved for 30 min in 3 ml of methionine-free medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and glutamine) and metabolically labeled for 2 h with 200 µCi/ml [35S] methionine (120 Ci/mmol; NEN/Dupont, Wilmington, DE). Cells were rinsed with phosphate-buffered saline and lysed with 1% Nonidet P-40 detergent, and the cleared lysates were precipitated with rabbit antisera. Radiolabeled proteins separated on denaturing polyacrylamide gels containing sodium dodecyl sulfate were detected by autoradiography of the dried gels (51). Immunoprecipitates prepared with antisera to CDK4 or to the D-type cyclins were separated on gels, transferred to nitrocellulose, and immunoblotted with the same antisera at 1:250 dilution followed by 125I-labeled *Staphylococcus aureus* protein A (Amersham) (52).

Rabbit antisera to mouse D-type cyclins and p34<sup>cdc2</sup> were raised to bacterially expressed, full length polypeptides (6, 25). The antisera to cyclins D1 and D2 readily precipitate the human proteins and cross-react with one another but do not immunoprecipitate cyclin D3, whereas the antisera to cyclin D3 reacts only with the cognate mouse and human cyclins. Although the antisera to D-type cyclins coprecipitate p34<sup>cdc2</sup>, they react preferentially with the "free" regulatory subunits. None of these antisera react with cyclins A, B, C, or E. The antisera to p34<sup>cdc2</sup> does not significantly cross-react with p34<sup>cdk4</sup>, p33<sup>cdk2</sup>, p35<sup>cdk4</sup>, and p31<sup>cdk4</sup>, but preferentially precipitates p34<sup>cdk4</sup> unbound to cyclins (25).

**Fluorescence in Situ Hybridization.** Bromodeoxyuridine-synchronized, phytohemagglutinin-stimulated peripheral blood lymphocytes from a normal donor were used as a source of metaphase chromosomes. Genomic DNA subcloned into phage or cosmid vectors was nick-translated with digoxigenin-11-UTP and hybridized overnight at 37°C to fixed metaphase chromosomes according to the method of Pinkel et al. (53), except for the inclusion of 0.33 µg/µl of highly reiterated human DNA self-aneled to C<sub>2</sub>1 (Bethesda Research Laboratories, Gaithersburg, MD). Signals were detected by incubating the slides with fluorescein-conjugated sheep antidigoxigenin antibodies (Boehringer Mannheim, Indianapolis, IN) followed by counter staining in propidium iodide solution containing antifade [1,4-diazabicyclo(2.2.2)octane; Sigma Chemical, St. Louis, MO]. Fluorescence microscopy was performed with a Zeiss microscope equipped with fluorescein epifluorescence filters.

**Somatic Cell Hybrids.** Hybrid cell lines with the prefix "GM" in Table 1 were obtained from the National Institute of General Medical Sciences' Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ); the characterization and human chromosome content of these hybrids are described in the Repository catalogue. The preparation of the human X

| Hybrid | CDK4 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X | Y |
|-------|-----|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| GM 06317 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 07297 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 07299 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 07300 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 07301 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 09927 | - | - | - | + | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 09934 | + | - | - | + | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 10027 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 10114 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 10253 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 10324 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 10449 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 10479 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 10498 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| GM 10611 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| A3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| A4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| A5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| A6 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| C1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| C2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| % Discordant | 43 | 24 | 24 | 33 | 29 | 19 | 19 | 29 | 33 | 29 | 24 | 0 | 33 | 43 | 33 | 24 | 33 | 24 | 33 | 29 | 14 | 24 | 38 | 33 |

* + and – indicate the presence or absence, respectively, of the CDK4 gene, as determined by Southern blotting, and each human chromosome in the hybrid cell lines. The origins of the hybrid cell lines are described in "Materials and Methods." % Discordant is defined as the sum of the hybrids containing the chromosome but lacking human CDK4 and those lacking the chromosome but containing human CDK4 divided by the total number of hybrid cell lines examined and multiplied by 100.
CDK4 Amplification in Human Sarcomas

Chromosomal Assignment of the Human CDK4 Gene. Human genomic libraries were screened with a murine CDK4 cDNA probe (25), and one recombinant phage (λ-cdk4) and two cosmids clones (cos-10-3 and cos-14-3) were obtained that contained overlapping hybridizing sequences. Human sequences hybridizing to the probe were contained within a 6.7-kilobase BamH1 fragment, and a 4.0-kilobase MboI-BamH1 portion of this fragment was subcloned (Fig. 1). Specific oligonucleotide primers were prepared based on the previously published human CDK4 cDNA sequence (46) and used to amplify a 534-base pair product by PCR, using the subcloned DNA as a template. The pK4-PCR product contained one complete and two partial CDK4 exons (denoted by capital letters in Fig. 1) with nucleotide sequences identical to previously reported CDK4 coding sequences (44), as well as two small introns, confirming that the genomic clones were derived from the human CDK4 locus.

The chromosomal location of human CDK4 was determined by fluorescence in situ hybridization, using probes prepared from each of the genomic clones (λ-cdk4, cos-10-3, and cos-14-3) which were hybridized to normal metaphase human chromosomes. The fluorescence signals obtained with each probe were localized to the long arm of chromosome 12, as indicated by cobyridization with the chromosome 12-specific centromeric probe, D12Z1 (Fig. 2A). The CDK4 locus was sublocalized to band q13 based on the distance of the hybridization signal from the chromosome 12 centromere.

The chromosomal assignment was confirmed by hybridizing the pK4-PCR probe to Southern blots containing DNAs extracted from a panel of rodent × human hybrid cell lines containing different combinations of human chromosomes. A 12-kilobase EcoRI restriction fragment contained within the authentic human CDK4 genomic locus was detected concordantly with human chromosome 12 (Table 1), consistent with results obtained by fluorescence in situ hybridization.

CDK4 Amplification in Sarcomas. We and others have previously identified an amplicion at chromosome 12q13 involving the GLI1 and MDM2 genes in soft tissue sarcomas (44, 45). To determine whether CDK4 is included in this amplicion, we performed Southern blot analysis of EcoRI-digested DNAs extracted from 14 human sarcoma cell lines, including 2 osteosarcomas, 6 rhabdomyosarcomas, 5 Ewings’ sarcomas, and 1 undifferentiated sarcoma, as well as a control lymphoblastoid cell line (CJTW) (Fig. 3). The Southern blot was sequentially hybridized with probes from the pK4-PCR CDK4 fragment (Fig. 3A), the human MDM2 cDNA (Fig. 3B), and the human GLI cDNA (Fig. 3C). As a control for DNA loading, we performed parallel experiments using a hematopoietic cell phosphatase cDNA probe, which detects a gene located on the short arm of human chromosome 12 (Ref. 55; Fig. 3D). We detected approximately 25-fold amplified levels of the 12-kilobase CDK4 EcoRI fragment in two cell lines, OsA-CL and SJRH30 (Fig. 3A), which have previously been shown to have amplification of the GLI locus (Ref. 44; Fig. 3C). By contrast, no amplification of CDK4 was observed in 12 other sarcoma cell lines or in control CJTW cells (Fig. 3A). Our results also confirmed those obtained previously with MDM2 in OsA-CL cells (Ref. 45; Fig. 3B). Thus, all three genes were coamplified in OsA-CL, but only CDK4 and GLI (not MDM2) were coamplified in SJRH30.

To confirm these findings, we analyzed both metaphase and interphase cells from the two cell lines by fluorescence in situ hybridization with a CDK4 cosmide probe (cos-10-3; Fig. 2, B-D). In metaphase cells from OsA-CL (Fig. 2B), we found increased levels of hybridization in a coalesced pattern, indicating amplification of this locus as an intrachromosomal homogeneously staining region located on an abnormal marker chromosome, which also hybridizes to GLI sequences (44). In interphase nuclei from OsACL cells (Fig. 2C) and SJRH30 (Fig. 2D), the CDK4 cosmide probe also demonstrated localized areas of increased hybridization, consistent with amplification as components of homogeneously staining regions in each of the cell lines.

CDK4 Expression in Sarcoma Cell Lines. Northern blot analysis was performed with total cellular RNAs extracted from OsA-CL and SJRH30 cells, along with those from control rhabdomyosarcoma (SRJH28) and Ewings sarcoma (SJS8B) cell lines (Fig. 4). The two cell lines containing the CDK4 amplicion each had 8- to 10-fold increased levels of CDK4 mRNA expression (Fig. 4, Lanes 1 and 2) when compared to controls (Fig. 4, Lanes 3 and 4), indicating that the increased CDK4 copy number resulted in augmented levels of gene expression.

Metabolically labeled lysates from the same four sarcoma cell lines were immunoprecipitated with anti-p34cdk4 antibodies to determine the levels of p34cdk4 expression (Fig. 5, Lanes 2, 7, 12, and 17). The levels of synthesis p34cdk4 were approximately five-fold higher in OsA-CL and SJRH30 cells (Fig. 5, Lanes 2 and 7) than those in the control cell lines (Fig. 5, Lanes 12 and 17) but were somewhat lower than that predicted from the augmented CDK4 mRNA levels detected by Northern blotting. The same lysates were precipitated with antisera raised against cyclins D1, D2, and D3. Although the antisera to cyclins D1 and D2 are cross-reactive, they preferentially precipitate the cognate sequences (44). In interphase nuclei from OsACL cells (Fig. 2C) and SJRH30 (Fig. 2D), the CDK4 cosmide probe also demonstrated localized areas of increased hybridization, consistent with amplification as components of homogeneously staining regions in each of the cell lines.

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Fig. 2. Fluorescence in situ hybridization analysis of CDK4. Fluorescence signals were localized to human chromosome 12, band q13, in normal metaphase cells using a digoxigenin-labeled CDK4 genomic probe (cos-10–3) (4). Arrows, the CDK4 signals; arrowheads, the chromosome 12 centromere detected using a chromosome 12-specific centromeric probe, D12Z1. Fluorescence in situ hybridization analysis of the OsA-CL (B, metaphase; C, interphase) and SJRH30 (D, interphase) cell lines showed amplified copies of CDK4 with patterns consistent with homogeneously staining regions (indicated by * in B).

Gest that the levels of cyclin D1/p34<sup>cdc28</sup> complexes detected by coimmunoprecipitation were paradoxically decreased in the OsA-CL cell line, despite CDK4 gene amplification and overexpression.

**Sequence Analysis of CDK4 mRNA.** In view of the apparently lower levels of cyclin D–p34<sup>cdc28</sup> complexes in cell lines overexpressing p34<sup>cdc28</sup>, it seemed possible that CDK4 mutations might have resulted in the overexpression of a defective p34<sup>cdc28</sup> protein that was unable to interact with the D-type cyclins. Cellular mRNA extracted from OsA-CL and SJRH30 cells was therefore used as a template to prepare CDK4 cDNAs by reverse PCR. DNA sequencing analysis of the complete CDK4 cDNA coding sequences from two independent PCR reactions performed with each cell line did not reveal any mutations. Thus, the failure of the overexpressed p34<sup>cdc28</sup> proteins to form increased numbers of complexes with D-type cyclins must have another mechanistic basis.

**DISCUSSION**

Gene amplification is an important mechanism leading to the overexpression of specific protooncogenes in human solid tumors. Well studied examples involve the N-MYC gene in childhood neuroblastoma (56–58) and the HER-2/NEU gene in breast carcinoma (59, 60), which are amplified in subsets of patients who are more likely to have metastatic disease at diagnosis and a poor prognosis. Amplicons can be quite large, spanning more than a megabase (59), and it has frequently proven difficult to pinpoint target genes whose overexpression acts to maintain the amplicon and functionally contribute to the neoplastic phenotype. In human breast carcinomas and squamous cell carcinomas of the head and neck, the amplicon on chromosome 11, band q13, includes two genes (INT2 and HST1) encoding fibroblast growth factors (62), the former being first identified as a target of retroviral insertion in murine breast carcinomas (63). Despite their potential to contribute to oncogenesis, these genes are not always

nate isoforms; note also that the three D-type cyclins can be clearly distinguished by differences in their electrophoretic mobilities on denaturing gels (25, 26) (Fig. 5), as predicted from their molecular masses (17). OsA-CL cells synthesized mostly cyclin D1 (Fig. 5, Lane 3), whereas SJRH30 and SJRH28 predominately expressed cyclin D2 (Fig. 5, Lanes 9 and 14). The control cell line, SJES8, expressed detectable levels of all three proteins, with cyclin D1 observed at high, D2 at intermediate, and D3 at comparatively low levels (Fig. 5, Lanes 18–20). Given that cells of different lineages express varying amounts of the three D-type cyclin proteins, it is not surprising that the cell lines examined here had unique patterns of cyclin D expression.

In samples immunoprecipitated with anti-p34<sup>cdc28</sup>, some complex formation could be demonstrated between p34<sup>cdc28</sup> and the major D-type cyclin found in each cell line, as indicated by the open arrowheads in Fig. 5 (Lanes 2, 7, 12, and 17). In reciprocal experiments, it was much more difficult to detect proteins with the mobility of p34<sup>cdc28</sup> in immunoprecipitates prepared with antisera to the cyclins. At least in part, these results may reflect the propensity of the antibodies to react with the free subunits (25) (see “Materials and Methods”). However, despite the preponderance of p34<sup>cdc28</sup> in the two cell lines exhibiting gene amplification, the levels of coimmunoprecipitated cyclin D proteins were not increased compared to those in control cell lines exhibiting much lower levels of p34<sup>cdc28</sup> expression.

Western blot analysis of lysates from OsA-CL or SJES8 cells immunoprecipitated with antisera to cyclin D1 and probed with the same antibodies revealed that the steady state levels of cyclin D1 in SJES8 cells were somewhat higher (Fig. 6, Lanes 2 and 5) in general agreement with the results obtained by metabolic labeling (Fig. 5, Lanes 3 and 18). Paradoxically, when immunoprecipitated proteins prepared with antisera to p34<sup>cdc28</sup> were probed with antibodies to the cyclin, approximately 5-fold lower levels of cyclin D1 were complexed with p34<sup>cdc28</sup> in OsA-CL cells compared to control SJES8 cells (Fig. 6, Lanes 3 and 6, open arrowheads). Thus, the available evidence suggests that the levels of cyclin D1/p34<sup>cdc28</sup> complexes detected by coimmunoprecipitation were paradoxically decreased in the OsA-CL cell line, despite CDK4 gene amplification and overexpression.

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CDK4 amplification in human sarcomas

A. cogene, MDM2, included in the same amplicon (45, 69), was originally identified in a spontaneously transformed subclone of BALB/c mouse fibroblasts (70). The MDM2 product binds directly to p53 and can interfere with the ability of wild-type p53 to transactivate reporter gene expression (71, 72) and to suppress ras-induced transformation of rat embryo fibroblasts (73). Consistent with this hypothesis, MDM2 has been found to be amplified in over one-third of human sarcomas tested (45), and tumors with MDM2 amplification generally do not have inactivating p53 mutations, which are frequently found in sarcomas with normal MDM2 gene dosage (69). At least one other locus (SAS, for sarcoma amplified sequence) is included in the chromosome 12q13 amplicon (41, 74), but its nucleotide sequence remains unavailable and its physiological role is unknown. The amplicon in the SJRH30 cell line does not include MDM2, suggesting that other genes, such as CDK4 or GLI, may be the relevant targets of gene amplification in some tumor subsets. An analysis of fresh tumor samples and further investigations of the biochemical properties of the CDK4 gene product will be needed to determine whether it contributes directly to oncogenesis or whether it is simply coamplified with a more relevant target locus.

When complexes of different mammalian cyclins and CDKs were reconstituted in vitro or in insect cells, only the three D-type cyclins were found to interact with p34cdk4 to activate its kinase activity (25, 26). Cyclin D proteins have a very short half-life in mammalian cells (half-life, <20 min), but p34cdk4 is much more stable (half-life, 4–6 h) (6, 11, 25). These results imply that cyclin, rather than p34cdk4, synthesis is both necessary and rate limiting for G1 progression, and this included in the 11q13 amplicon and are not invariably expressed in tumor cells, even when amplified (36, 64, 65). Indeed, the cyclin D1 gene now appears to be a more relevant candidate because it is both amplified and overexpressed in many such tumors (36–38).

A similar problem is presented by the amplicon in human sarcomas involving sequences at chromosome 12, band q13. This region was initially shown to be amplified in a human glioma, and positional cloning techniques were used to identify the GLI gene as a potentially relevant target within the amplicon (66). GLI encodes a potential transcriptional regulatory protein with five zinc finger DNA binding motifs and sequence similarity to the Krüppel drosophila segmentation gene (67, 68). GLI is both amplified and overexpressed in the two sarcoma cell lines, OsACL and SJRH30 (44), which also show amplification and overexpression of CDK4. Another potential protoonco-
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REFERENCES


Fig. 5. Immunoprecipitation of p34(PSK)/cdk4 and the D-type cyclins. Open arrowheads. Right, the positions of the D-type cyclins and of p34(PSK)/cdk4.

Fig. 6. Detection of p34(PSK)/cyclin D1 complexes. Immunoprecipitates prepared with anti-sera to cyclin D1 or to p34(PSK)/cdk4 (top) were separated on a denaturing gel and immunoblotted with antiserum to cyclin D1. Approximately equal amounts of cyclin D1 were present in the cell lines OsA-CL and SJES8, as indicated by the solid arrowheads in Lanes 2 and 5. The levels of p34(PSK)/cyclin D1 complex detected by immunoblotting of the p34(PSK)/immunoprecipitates with anti-cyclin D1 are shown in Lanes 3 and 6 (open arrowheads). The exposure time was 3 days.

has now proven to be the case, at least in fibroblasts (31, 75). However, in cells sensitive to the growth inhibitory action of transforming growth factor β, overexpression of p34(PSK) overcomes the transforming growth factor β block in late G1 phase, resulting in activation of the cyclin E/cdk2 histone H1 kinase and entry into S phase (76). Based on immunoprecipitation and Western blotting analyses, the levels of cyclin D/p34(PSK) complexes were paradoxically reduced in cell lines exhibiting CDK4 amplification, although the amplified gene had not sustained detectable mutations. Similar results have recently been observed in other transformed cells, in which cyclin D/p34(PSK) complexes undergo dissociation, and the catalytic subunits become bound to a series of low molecular weight proteins (28, 77). One possibility, then, is that the latter proteins act as stoichiometric inhibitors of p34(PSK) kinase activity (i.e., as anticyclins) and compete with D-type cyclins for binding to catalytic subunits. In principle, the overexpression of inactive, monomeric p34(PSK) subunits might titrate out such negative regulators, resulting in the increased availability of D-type cyclin subunits and their reassembly into complexes containing other catalytic partners, such as cdk2, or their increased binding to pRb or pRb-related proteins (27, 29). Whatever the mechanism, our results provide the first example of amplification of a gene encoding a known cell division cycle kinase in human tumor cells.

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Ziad A. Khatib, Hitoshi Matsushime, Marcus Valentine, et al.


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