Chromosomal Mapping of Members of the cdc2 Family of Protein Kinases, cdk3, cdk6, PISSLRE, and PITALRE, and a cdk Inhibitor, p27^{kip1}, to Regions Involved in Human Cancer

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Abstract

Orderly progression through the cell cycle requires sequential activation and inactivation of cyclin-dependent kinases (cdks). This is achieved in part through the association of cdks with positive regulators called cyclins and inactivation of cyclin-cdk complexes by a rapidly growing number of cyclin-cdk inhibitors. Recently, the role of cell cycle control proteins both as primary effectors and as mediators of tumorigenesis has become a subject of increased interest. Here we report the chromosomal mapping of two cdks, cdk3 and cdk6, two putative cdks, PISSLRE and PITALRE, and one cyclin-dependent kinase inhibitor, p27, to chromosomal regions which may be altered in human tumors and examine their possible involvement in some of these malignancies. In particular, two of the kinases, cdk3 and PISSLRE and PITALRE, the cdc2-related kinases recently cloned by us, map to regions previously shown to exhibit loss of heterozygosity in breast and other tumors.

Introduction

Components of the cell cycle-regulatory machinery have been found to be involved in several human cancers. cdks, their regulatory subunits or cyclins, cdk inhibitors, as well as the associated tumor suppressor proteins p53 and pRb, have been found to be deregulated or mutated in numerous human tumors. The transforming activity of DNA tumor virus proteins EIA, SV40 large T, and E7 is exerted, at least in part, through their binding of the growth suppressor pRb (1) and consequent release of E2F (2, 3). In addition, EIA has been shown to bind to cyclin A/cdk2 complexes, providing the first direct link between the cell cycle and a transformed phenotype (4–8).

Orderly progression through the cell cycle in yeast requires the association of a single cdk, p34/cdc2/CDC28, with cyclins to form active phosphorylating complexes which regulate both the G1-S and G2-M transitions (9, 10). With the discovery and characterization of several murine and human cdc2-related kinases (11), it has become apparent that mammalian cell cycle systems operate under a much higher degree of complexity. Several different cyclin-cdk complexes have been found to be active at different stages in the cell cycle (12–26).

Although termed a cdk, the highly cdc2-related cdk3 does not bind any known cyclin. Nevertheless, it is able to restore growth to cdc28 ts mutants, as well as to arrest cells in G1, in an overexpressed dominant negative mutant form, displaying its key role in G1-S transition (11, 15). The cdk6/cyclin D1 complex is thought to be one of the first cyclin-cdk complexes to appear in the cell cycle as well as being the initial kinase acting on the Rb protein forcing progression into S phase (12, 16). Recurrent abnormalities in chromosome 11q13 were found to involve the Cyclin DI/PRAD1/bcl-1 oncogene in a variety of tumors (reviewed in Ref. 17). In addition, cdk4, which associates with D cyclins, has been mapped to chromosome 12q13 (18) and is part of the MDM2/GLI amplicon in human sarcomas (19, 20).

Recently, two new cdc2-related kinases, PISSLRE and PITALRE, have been cloned in our laboratory (21–23). PISSLRE, sharing 47% identity with cdc2, contains many conserved motifs of cdks. These include the proline in its PSTAIRE-like sequence and target residues for cdk-activating kinase phosphorylation; both are needed for cyclin binding and stabilization (21, 22). Interestingly the PISSLRE gene is predominantly expressed in terminally differentiated tissues, possibly indicating a functional relation to cdk5, which is specifically expressed in nonproliferating neural tissue (21, 22). PITALRE, on the other hand, is a nuclear localized kinase that, although not able to act on histone H1, is able to phosphorylate pRb on serine residues in vitro (23). This would indicate a function not unlike that of cdk4 or cdk6; however, this kinase activity has not been found to be cell cycle regulated.

Inhibition of kinase activity and cycle progression is achieved through a growing number of cyclin-cdk inhibitors. Deletions on chromosome 17p13 in a wide variety of human tumors result in the inactivation of the p53 gene (reviewed in Ref. 24) the role of which in cell cycle control (reviewed in Ref. 25) has been strengthened by the demonstration of its ability to directly activate transcription of the p21/Waf1/Cip1 protein (26), a universal inhibitor of cdks (27). Loss of function of a protein such as p21/Waf1 can have catastrophic effects on proliferative regulation, leading to a transformed phenotype. Indeed, the gene coding for p16, an inhibitor of cdk4 activity, which maps within the described region of LOH at 9p21, is deleted or mutated in numerous tumor-derived cell lines (28, 29) and to a lesser extent in primary tumors (30). Recently, a cdk inhibitor, p27^{kip1}, with significant homology to p21 and strong binding affinity for cdk2/cyclin E complexes has been cloned (31, 32). Since cdk2-cyclin E
complexes are crucial for the commitment to S phase, an alteration of p27 inhibitory activity could result in loss of control of the cell cycle.

As a first step in investigating the possible involvement of cdc2-related kinases and cdk inhibitors in human tumors we have screened a rodent-human hybrid panel for the presence of these genes to determine the chromosomal locations of cdk3, cdk6, PISSLRE, PITALRE, and p27.

**Materials and Methods**

**Rodent-Human Hybrids.** Hybrid DNAs were from previously described rodent-human hybrid cell lines (33-35) or from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ).

**PCR Amplification.** PCR amplification was carried out on 100 ng of genomic DNA from human placenta, mouse, hamster, and rodent-human hybrids with 300 ng each of forward and reverse primer. Thirty cycles of amplification were separated as follows: 94°C, 30 s; 60°C, 30 s; 72°C, 30 s. PCR products were separated in 1.5% agarose gels, denatured, neutralized, and transferred to Hybrid-MAT nylon membranes (Amersham). Filters were processed as described below.

For each gene to be mapped, several primer pairs were tested on human, mouse, and hamster DNA in order to obtain a human specific amplification product of the expected size which hybridized with the appropriate oligonucleotide or cDNA probe. Forward primers were given odd numbers and reverse primers were given even numbers. Oligonucleotide primers used for amplification were: 03cdk6, 5'ACCTCCGAGCTGAATACGT3'; 04cdk6, 5'TTCTCGGAGAGAGAGTAAT3'; 01p27, 5'GTGGACCAACCAAGAGTAGT3'; 02p27, 5'TCCTCGCTCAGCTGACTT3'; 01P1T, 5'TTGCCACTAGGGCTCTTG3'; 02P1T, 5'CACTCGTACTT3'; 01P27, 5'CTCTTGCAAGGAGTTA3'; 02p27, 5'GTCAGAG3'; 01p27, 5'GTGGACCACGAAGAGTTA3'; 02p27, 5'CTCTTGCAAGGAGTTA3'. See Fig. 5.

**Southern Blotting.** DNA was isolated from rodent-human hybrids, mouse and hamster cell lines, and CLL tumor samples by standard phenol-chloroform extraction. Restriction enzymes (Boehringer Mannheim) were used in this study were graciously provided by the laboratories using the Prime it II kit (Stratagene). Oligonucleotide probes used in this study were CAGAGAGAGAGAGTAAT3', oligonucleotides internal to the expected amplification products for cdk6 and p27, respectively (see Fig. 5), were used to probe Southern blots of PCR-amplified gene fragments. Oligonucleotides were end-labeled with polyacrylamide gel. Hybridization and washing of PCR Southern blots were carried out as for genomic blots. PCR filters were exposed to X-ray film for 30 min at room temperature.

**Results**

A panel of 20 rodent-human hybrids retaining individual chromosomes and human and rodent control DNAs were tested by Southern blot or PCR amplification for the presence of human cdk3, cdk6, PISSLRE, PITALRE, or p27 gene sequences. Hybrids were scored positive or negative as shown in Fig. 1. Regional localization of each gene within the assigned chromosome was then achieved by testing a small panel of rodent-human hybrids retaining defined subregions of the relevant chromosome. A diagram depicting the results of regional localizations is presented in Fig. 2.

To determine the chromosome location of the cdk3 gene, DNAs from the hybrid panel shown in Fig. 1 were digested with EcoRI, electrophoresed, transferred, and hybridized to the cdk3 cDNA. As summarized in Fig. 1, the cdk3 gene was present only in hybrids retaining human chromosome 17. Hybrids used to further localize the cdk3 gene to 17q22-qter are depicted on Fig. 2A. Southern blotting results of a sublocalization experiment for this gene are
depicted in Fig. 3A. The cdk3 locus is telomeric to the recently identified BRCA1 gene (38). Two distinct regions of LOH telomeric to the BRCA1 locus have been described in breast cancer (39). In order to investigate the possible involvement of the cdk3 gene in breast cancer; we looked for alterations of the gene by Southern blotting on several breast cancer cell lines known to carry LOH at 17q. No evidence of cdk3 deletion was found. However, preliminary data indicate the presence of a cdk3 rearrangement or polymorphism in the MDA-MB-543 breast cancer cell line (Fig. 4). Abnormal bands in MDA-MB-453 DNA were detected with two of eight enzymes tested (Fig. 4). The band of highest molecular weight in the EcoRV digest might represent hybridization to undigested DNA in the lane.

The chromosomal location of the loci for cdk6, PISSLRE, PITALRE, and p27 was determined by screening individual hybrid DNAs from the hybrid mapping panel (summarized in Fig. 1) for the presence of a specific PCR-amplified product of the expected size, using oligonucleotide pairs specific for each gene sequence. In order to increase specificity, primers were sometimes selected within 3' or 5' untranslated regions of available cDNA sequences. When untranslated regions were not suitable for primer design, primers were chosen within the least conserved regions of the cDNAs. Positions of oligonucleotide pairs within specific genes used for amplification or hybridization are depicted in Fig. 5. The same oligonucleotide pairs were then used to sublocalize these genes to specific regions within assigned chromosomes. The specificity of amplified PCR products was confirmed by hybridization to cDNA (PISSLRE and PITALRE) or oligonucleotide probes.

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5 M. L. Veronese, unpublished observation.
6 Unpublished results.
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Fig. 3. Southern blot (A, and bottom panels, B–E) and PCR amplification (top panels, B–E) for regional localization of the cdk3 (A), cdk6 (B), PSSILRE (C), PITALRE (D), and p27 (E). A: Lane 1, human placental DNA; Lane 2, 275S hybrid DNA; Lane 3, parental rodent control. In B, C, D, and E, Lanes 1–4 are control lanes and contain no template, human placental DNA, mouse DNA, and hamster DNA, respectively; other lanes contain hybrid DNA. B: Lane 5, Nu9 hybrid DNA; Lane 6, 11753 hybrid DNA; Lane 7, CI366 hybrid DNA; Lane 8, CI21 hybrid DNA. C: Lane 5, 10567 hybrid DNA; Lane 6, ALN3 hybrid DNA; Lane 7, 11102 hybrid DNA; Lane 8, 11100 hybrid DNA. D: Lane 5, 10611 hybrid DNA; Lane 6, 11102 hybrid DNA; Lane 7, 10095 hybrid DNA; Lane 8, B4–2 hybrid DNA. E: Lane 5, 7301 hybrid DNA; Lane 6, GL3 hybrid DNA; Lane 7, G5 hybrid DNA; Lane 8, M44 hybrid DNA. kbp, kilobase pairs; bp, base pairs.

Fig. 4. Southern blot of MDA-MB-453 (MDA) cell line DNA digested with BamH1 and EcoRV restriction enzymes and probed with the cdk3 cDNA probe. HP, human placenta DNA. kbp, kilobase pairs.

(cdk6 and p27). Diagrams depicting the regional localization of each of the genes are presented in Fig. 2 with cdk6 at 7p13–cen (Fig. 2B), PSSILRE at 16q24 (Fig. 2C), PITALRE at 9q34.1 (Fig. 2D), and p27 at 12p12–pter (Fig. 2E). Corresponding Southern blots of PCR sublocalization experiments are shown in Fig. 3.

Chromosome 12 numerical abnormalities are associated with a variety of human tumors. In particular, trisomy 12 has been associated with a high percentage of CLL (reviewed in Ref. 40) and ovarian tumors (41). Trisomy 11 in ALL has been shown to represent a masked rearrangement of the ALL-1 gene involved in translocations of chromosome 11 at band q23 characteristic of acute leukemias (42). In order to determine whether a similar phenomenon resulting in activating rearrangements of putative oncogenes (cdk2, cdk4, and MDM2) located on chromosome 12 or in the production of dominant negative forms of the cyclin-cdk inhibitor p27 might be occurring in CLL, we have examined more than 20 DNAs from randomly selected CLL patients by Southern blotting with cDNA probes for cdk4, cdk2, MDM2, and p27. We found no evidence of rearrangement for p27, cdk4, or MDM2 in Southern blots of CLL tumor DNA digested with EcoRI, BamH1, and HindIII restriction enzymes (Fig. 6).

Discussion

Recurrent chromosomal abnormalities associated with human solid and hematopoietic tumors are known to result in the activation of oncogenes or inactivation of tumor suppressor genes which contribute to the malignant phenotype. Oncogene activation by chromosomal translocation in leukemias, lymphomas, and soft tissue sarcomas can occur either by enhancement of transcription or by gene fusion (43–45). In addition, loss of function through mutation or deletion of tumor suppressor genes such as p53 and pRb contribute to both solid and hematopoietic tumor development (24, 46).

The previously described involvement of a cyclin-dependent kinase (cdk4), a cyclin (cyclin D1), and an inhibitor of cyclin-cdk complexes (p16) in human neoplasia and the present localization of new members of
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A

1051 TCCCACTGTC CGCGCCGCGCA GACACCTCAG GCTGTGAATA CAGCCTGTGG
1101 CTCAGCCACTG GCCGACGCGCC GCTGTGACGG GCCGGCGCGA
1151 ATGGGTGATCC ACCCACTGAC CGAGCCTGGC TGGGTCCTGG
1201 AGGGAGTGGG GAGGAGGAGG GAGGAGGAGG GAGGGAGGAG
B

1001 ATGGTCTGTT CTAGGCGGTC GACGGCGGCG GAGGGCGCG
1051 TCCAGTCTCT CCACTGACCG GCTGTGACGG GCCGGCGCGA
1101 CTGCGGGCGG AAGCGGGCGG GAGGAGGAGG GAGGGAGGAGG
1151 GGGCTGCGGG GAGGAGGAGG GAGGGAGGAGG GAGGGAGGAGG
1201 GAGGGGGTGG ACCGGGGGGG ACCGGGGGGG GACGGGCGCG
1251 ACCACTGGTT CCTGGGAGGA GCCGACGGGC GAGGGCAGG
C

1151 ACCAACGACG CGGCGTACGG GACGGCGCGC GAGGGCGCG
1201 TACTGGGAGG TGGGAGGAGG GAGGAGGAGG GAGGGAGGAG
1251 CAGGGGATCC GAGGGGATCC GAGGGGATCC GAGGGGATCC
D

61 GCGGACGACG CCAACGGCCTC GACGAGCTGG GACGGGGTGG
66 GCGGATCC GCAACGGCCTC GACGAGCTGG GACGGGGTGG
111 CCGACAGAGG TAGCCCGGGG ATCTGGGAGG GACGAGCTGG
1161 GCGGGAGCG GCGGGAGCG GCGGGAGCG GCGGGAGCG
1211 GGGAGGGAGA AGAGG TGGGAGA AGAGG TGGGAGA AGAG

Fig. 5. Position of specific oligonucleotide primers in cdk6 (A), PISSLRE (B), PITALRE (C), and p27 (D). 5' to 3' sequences of forward (F) and reverse (R) oligonucleotide primers used for amplification are underlined. Sequences of oligonucleotides used for hybridization are boxed. Stop codons for ctlko, PISSLRE, and PITALRE as well as base differences between human (top sequence in D) and mouse (bottom sequence in D) p27 cDNAs are in boldface.

these gene families near chromosomal regions known to be involved in alterations in several human malignancies suggests that one or more of these genes could similarly be candidates for contribution to oncogenic transformation. The gene for cdk3 was mapped to chromosome 17q22–qter telomeric to the BRCA1 locus. We investigated the possible involvement of cdk3 within reported regions of LOH telomeric to BRCA1 in breast cancer and a possible rearrangement of the gene was found in the MDA-MB-543 cell line (Fig. 4). It is possible that this rearrangement results in deregulation of cdk3 gene expression or in the production of an abnormal transcript. Alternatively, the rearrangement in the MDA-MB-543 cell line could result in a deletion encompassing a target gene in the region. Such a rearrangement with concurrent amplification of the ERBB2 gene was described in the BT474 breast cancer cell line. A deletion, possibly targeting the BRCA1 gene in this cell line, was found to result in the expression of THRA1-BTR fusion transcripts (47).

cdk6 and PITALRE map to chromosomes 7p13–cen and 9q34.1, respectively. Both regions are involved in nonrandom chromosomal alterations. Deletions involving chromosome 7 at band p13 have been reported to be associated with non-Hodgkin’s lymphoma where they probably represent a secondary chromosomal abnormality (48). T-cell non-Hodgkin’s lymphomas, on the other hand, have been reported to carry abnormalities involving chromosome 9 at band q34 (49). Also, a t(8;9) translocation involving 9q34 has been observed in myeloproliferative disorders (50). Allelic losses at 9q34 have been reported in several malignancies. In particular, more than 40% of bladder tumors demonstrate LOH at 9q34.1–2 (51).

MDM2 expression, like that of the cyclin-cdk inhibitor, p21, at 6p21 (26), is regulated by the p53 tumor suppressor and was found to be one of the amplification targets at band 12q13 in human sarcomas (19). As mentioned previously, cdk4 and GLI are also included in the 12q13 amplicon (20). The p27 gene was mapped to chromosome 12 at band p12.3–pter. Chromosome 12 abnormalities have been reported in a significant number of CLLs (40). We have tested more than 20 patient DNAs by Southern blotting for abnormalities in the MDM2, cdk2, cdk4, and p27 genes (Fig. 6). We found no evidence of rearrangement at the genomic level with any of the cDNA probes. A similar location and absence of mutations for p27 in solid and hematopoietic malignancies is reported by Pietenpol et al. (52) and Ponce-Castañeda et al. (53). Overexpres-
sion of MDM2 without evidence of DNA rearrangements was recently reported in CCL suggesting a role for this gene as a secondary mediator of transformation or tumor progression (54). Indeed, our results do not exclude the possibility of involvement of these genes in CCL through point mutations or rearrangements occurring outside the regions we have examined, which could include regulatory sequences. However, since gross alterations of the type known to result in oncogene activation do not appear to occur in a significant number of cases, it seems unlikely that any of these genes play a role as primary effectors of tumorigenesis in CCL. Additional chromosomal abnormalities which could be the involve the p27 gene at 12p12–pter include isochromosomes of the short arm of chromosome 12 which constitute a characteristic abnormality in male germ cell tumors (55). More relevant to the possible involvement of a putative tumor suppressor, deletions of 12p13 have been observed in chronic lymphoproliferative disorders and deletions of 12p11–p13 have been observed in acute lymphoblastic and acute myeloblastic leukemia (50).

Finally, the gene for PISSLRE which maps to 16q24 is located in a region of reported LOH in breast (56), prostate (57), and other lymphoblastic and acute myeloblastic leukemia (50). Additional chromosomal abnormalities which could the in the type known to result in oncogene activation do not appear to occur in a significant number of cases, it seems unlikely that any of these genes play a role as primary effectors of tumorigenesis in CCL.

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