Genomic Organization of the Human p57KIP2 Gene and Its Analysis in the G401 Wilms’ Tumor Assay

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Abstract

The p57KIP2 gene encodes an inhibitor of cyclin-dependent kinase activity, which negatively regulates cell cycle progression. The human p57 gene is located in 11p15.5, a region of DNA frequently altered in neoplasia. We have isolated a human genomic clone and mapped the p57 gene to a 2.2-kb region between D11S648 and D11S679. Sequence analysis revealed that the coding DNA of the human p57 gene is divided by a 0.5-kb intron. A second intron was detected in the 3' untranslated region, indicating that the human p57 gene contains at least three exons. We now present the genomic structure of the human p57 gene based on restriction mapping and sequence analysis of a cosmid clone. Although the gene mapped within our candidate domain, Northern blot analysis indicated that p57 expression did not correlate with the tumor suppression observed in our functional assay.

Introduction

Cellular proliferation involves an orderly progression through the cell cycle that is controlled by protein complexes of cyclins and CDKs (reviewed in Refs. 1-3). The CDK subunits phosphorylate cell cycle-regulatory proteins, such as the retinoblastoma protein, to release cells from cell cycle arrest. The CDKs are counterbalanced by a family of negative cell cycle regulators. These CDKIs bind with the cyclin-CDK complexes to inactivate their catalytic domains. Mutations in the CDKI proteins result in uncontrolled proliferation typical of neoplasia. Therefore, CDKI proteins are excellent candidates for tumor suppressor genes.

The CDKI proteins are divided into two families based on their sequence homology. One family includes the p16INK4A, p15INK4B, p18INK4B, and p19INK4D proteins, which contain a related series of ankyrin repeats. The second family of CDKI proteins includes p21CIP1/WAF1, p27KIP1, and a recently identified third member, p57KIP2 (4, 5). These proteins have homologous CDK binding domains that are required for the inhibitory activity. The p57 protein inhibits several G₁ and S-phase CDKs, and overexpression of p57 causes a complete cell cycle arrest in G₁ (4, 5).

The human p57 gene is located in 11p15.5 (5). Mutations in this region of DNA are observed in several forms of cancer, including Wilms’ tumor, and in patients with BWS (6-8). In addition, functional studies by our lab (9-11) and others (12) have localized a tumor suppressor gene to a ~500-kb region of 11p15.5 between D11S601 and D11S724. In this report, we present the genomic structure of the human p57 gene based on restriction mapping and sequence analysis of a cosmid clone. Although the gene mapped within our candidate domain, Northern blot analysis indicated that p57 expression did not correlate with the tumor suppression observed in our functional assay.

Materials and Methods

Cell Lines. J1-4a, J1-49, J1-43a, and J1-9 are part of a panel of deletion hybrids derived by mutagenesis of J1, a hamster cell line that stably retains human chromosome 11 (13, 14). MCH 701.8 is a mouse cell line that contains a human t(X;11) (11pter > 11q13::Xq21 > Xqter) chromosome. XMC 708.20 and XMCH 708.25 are mouse cell lines that contain radiation-reduced t(X;11) chromosomes derived from MCH 701.8 (15). G401.6TGTG.6 is a hypoxanthine-guanine phosphoribosyl-transferase-deficient derivative of the G401 Wilms’ tumor cell line (16). MCH 486.1, MCH 486.2G, MCH 486.2J, and MCH 486.2L are G401 hybrid lines that contain the t(X;11) chromosome from XMCH 708.25 and do not form tumors when injected into nude mice (11). MCH 369.18, MCH 485.1, MCH 485.2A, and MCH 485.3 are G401 hybrid lines that contain the t(X;11) chromosomes from XMCH 708.24 or XMCH 708.26 (10, 11). These cell lines do form tumors after mouse inoculation.

PCR. Genomic DNA from the somatic cell hybrid panel was amplified using the following primers from the human p57 gene: 5'-CGG GGC TCT TTG GGC TCT-3' and 5'-CCG TGT CCC ATG GCT TCT-3'. They generated a 180-bp amplicon after 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. Each reaction contained 400 ng template, 1 unit Taq polymerase, 10 mm Tris (pH 9), 50 mm KCl, 1.0 mm MgCl₂, and 0.2 µM of each deoxyribonucleoside triphosphate. PCR products were visualized in ethidium bromide-stained 2% agarose gels.

Isolation and Mapping of Cosmids. A human p57 partial cDNA clone was identified in a human cDNA library by hybridization with the mouse p57 cDNA clone. A 0.7-kb fragment from the partial cDNA clone was used to isolate cosmid 14F5 (17). The location of the p57 gene within 14F5 was determined by restriction mapping and hybridizations with fragments from a full-length cDNA clone, hp57. Nonradioactive hybridizations were performed at 65°C, according to the manufacturer’s protocol (Genius system; Boehringer Mannheim).

DNA Sequencing. The 1.2-kb NotI and 1.0-kb HindIII fragments of genomic DNA were isolated from c14F5 and subcloned into pBLUESCRIPT (Stratagene). Plasmid DNA was isolated (Qiagen) and sequenced at the UNC-CH Automated DNA Sequencing Facility on a Model 373A DNA Sequencer (Applied Biosystems). Either external universal primers or internal primers designed from genomic sequence were used.

Northern Analysis. Polyadenylated RNA (2 µg) was isolated using an oligo-dT kit (Collaborative Research), separated on 1% agarose-formaldehyde/MOPS gels, and transferred to nylon membranes (Boehringer Mannheim) in 10× SSC (1× SSC = 0.1 M sodium chloride, 0.01 M sodium). mRNA samples from adult skeletal muscle and kidney tissue (ClonTech) were included as positive controls. RNA blots were hybridized to 32P-labeled DNA probes at 42°C in a 50% formamide solution. The p57 probe was the 1.5-kb EcoRI insert from the human cDNA clone, hp57. The γ-actin probe was the 1.8-kb XhoI insert from the mouse cDNA clone, pHF1.
Results

Localization of the Human p57 Gene and Characterization of Cosmid Clones. Previous analysis mapped the human p57 gene to 11p15.5 using fluorescent in situ hybridization (5). To localize the gene within this chromosomal band, we looked for the presence of p57 in a panel of deletion hybrids that contain fragments of human chromosome 11 in a rodent background (Fig. 1A). DNA from the deletion hybrid panel was amplified by PCR using primers from the human p57 gene. As shown in Fig. 1B, amplified bands were generated in all samples except J1-9. Identical results were observed when Southern blots containing DNA from the hybrid panel were hybridized to human p57 cDNA probes (data not shown). These results indicate that the p57 gene is located in a region of DNA absent in J1-9 but present in XMCH 708.25 and the remaining hybrid lines. The J1-9 cell line contains the D11S724 locus but not the more centromeric D11S1 probe (14, 17). The XMCH 708.25 line contains the D11S648 locus but not the D11S601 probe (11). Therefore, the human p57 gene must be located between the D11S601 and D11S724 loci. This region of 11p15.5 includes cosmids c395 and c469 that contain the D11S648 and D11S679 loci, respectively (17). Neither of these cosmids hybridized to p57 cDNA probes (data not shown).

To isolate genomic clones containing the p57 gene, we screened a human chromosome 11 cosmid library with a p57 partial cDNA probe. This analysis identified one cosmid c14F5 that contained the p57 gene. NotI restriction analysis and hybridization to genomic fragments mapped c14F5 between c395 and c469 (Fig. 2A). This analysis also indicated that c14F5 and c395 had substantial overlap in genomic sequences. Blots containing EcoRI, HindIII, and NotI digests of c14F5 were hybridized to a p57 full-length cDNA probe. This analysis detected an 11.4-kb EcoRI band and a 10.0-kb HindIII band in c14F5. Similarly-sized EcoRI and HindIII fragments were observed in Southern blots of genomic DNA (data not shown), suggesting that the c14F5 cosmid contained an intact p57 gene. Double digests with these enzymes indicated that the gene was located within a 4.8-kb EcoRI-HindIII fragment. This fragment contained two NotI sites that divided it into three smaller sections: a 2.6-kb EcoRI-NotI fragment; a 1.2-kb NotI fragment; and a 1.0-kb NotI-HindIII fragment (Fig. 2B). p57 cDNA probes hybridized to the 1.2-kb NotI and 1.0-kb NotI-HindIII fragments (data not shown) but not to flanking DNA, suggesting that the p57 coding DNA is contained within this 2.2-kb region.

Genomic Organization of the Human p57 Gene. The 1.2-kb NotI and 1.0-kb NotI-HindIII fragments from c14F5 were subcloned and sequenced (Fig. 3). To determine the genomic organization of the human p57 gene, the sequences derived from the two genomic fragments were compared to published cDNA sequence (5). This analysis revealed that the coding region of the human p57 gene is divided at codon 275 into two exons that are separated by a 0.5-kb intron. A second, 83-bp intron was detected in the 3' untranslated region. Some single base pair differences in the untranslated regions of the p57 gene were observed between the genomic and cDNA sequences. No differences were detected in the coding DNA.

Human p57 Expression in G401 Hybrid Cell Lines. We have used somatic cell hybrids in a functional assay to identify regions of chromosome 11 that suppress tumorigenicity in the G401 Wilms' tumor cell line (9–11). The G401 cells form tumors when injected s.c. into nude mice. They have a pseudo-diploid karyotype with two cytogenetically normal copies of chromosome 11 but presumably lack expression of a tumor suppressor gene. In our assay, we introduced t(X;11) chromosomes into G401 cells and analyzed the tumorigenic potential of the hybrid cell lines in nude mice. In some cases, the introduced t(X;11) chromosome complemented the G401 defect so that the hybrid lines did not form tumors after inoculation. In other cases, the introduced chromosome had no influence on the tumorigenicity of the G401 cells. This functional assay has enabled us to localize a tumor suppressor gene to a region of 11p15.5 telomeric to the D11S601 locus (11). Since p57 maps to this same region, we investigated whether p57 expression contributed to the tumor suppression observed in our assay.

Previous experiments demonstrated that four G401 hybrid lines,
These results indicate that p57 expression does not correlate with detected in G401 or in the hybrid lines. However, a 0.8-kb transcript containing polyadenylated RNA isolated from the G401, MCH 369, expression in G401 and the microcell hybrid lines. Northern blots G401 cell line (data not shown). To determine the level of p57 lowercase tellers. The translation initiation and termination sites are underlined. Nucleotides at positions 151-168. 263. and 2200 differ from the cDNA sequence published previously served in adult skeletal muscle and adult kidney mRNA samples. was detected in all of the tumorigenic lines and some of the nontu-
tions were detected by Southern blot analysis in the p57 alíeles in the tumorigenic (10, 11). Each of these G401 hybrid lines contains an MCH 369.18, MCH 485.1, MCH 485.2A, and MCH 485.3, were (5). The Genhank accession number is U4SS69.

We have mapped the p57 gene to a 2.2-kb region in llpl5.5 Fig. 4. p57 expression in the G401 hybrid lines. Northern blot of polyadenylated RNA isolated from the G401.6TG.c6 parental cell line, nonlumorigenic CUOI hybrid lines hybridized with a p57 cDNA probe. The translation initiation and termination sites are underlined. Nucleotides at positions 151-168. 263. and 2200 differ from the cDNA sequence published previously (5). The Genbank accession number is U48869.

Discussion

We have mapped the p57 gene to a 2.2-kb region in 11p15.5 between D11S648 and D11S679. Molecular analyses have detected frequent loss of heterozygosity in 11p15.5 in a variety of human tumors (6), suggesting that a common tumor suppressor gene may reside in this area. Moreover, many of the chromosome alterations observed in BWS patients map to the same region of this 11p15.5. For example, the breakpoint sites of balanced chromosome rearrangements in five BWS patients and one rhabdoid tumor map to a ~320-kb region that includes p57 (8). These alterations probably do
not disrupt the small p57 gene but may alter the activity of nearby regulatory elements.

Several studies have suggested that the 11p15.5 tumor suppressor gene may be imprinted. Loss of heterozygosity analyses have detected a preferential loss of the maternal allele in BWS, sporadic Wilms’ tumor, and rhabdomyosarcoma patients (18–21). In addition, balanced translocations in BWS patients are generally transmitted through the maternal germline (22). The human 11p15.5 band contains two imprinted genes, H19 and insulin-like growth factor II (23–27). These genes have reciprocal expression and methylation patterns. That is, H19 is expressed from the maternally-derived chromosome, which has a hypomethylated H19 promoter, whereas insulin-like growth factor II is expressed from the paternally-derived chromosome, which has a hypermethylated H19 promoter (28, 29). Alterations in these expression and methylation patterns have been observed in BWS patients, Wilms’ tumors, and rhabdomyosarcomas (26, 27, 30–32). These loss-of-imprinting mutations result in a “paternal” appearance on both copies of chromosome 11, suggesting that the 11p15.5 tumor suppressor gene is imprinted so that only the maternal allele is normally expressed. The mouse p57 gene displays this type of monoallelic expression (33). If the human p57 gene is also imprinted, then loss of imprinting may be one method used to inactivate the p57 gene.

Many CDKI proteins map to chromosomal regions that are altered frequently in cancer cells (1–3). These locations, along with their role in negative growth control, suggest that CDKI proteins may be inactivated during tumor development. Deletions, mutations, and methylation changes that inactivate p15 and p16 are detected frequently in a variety of tumors (reviewed in Ref. 34). In contrast, mutations in the family of CDKI proteins including p21, p27, and p57 are extremely rare. Few mutations have been reported in p21 (35, 36), and no structural alterations have been detected after extensive analysis of p27 (37–39). Similar analyses have not yet revealed mutations in the p57 gene after examining 51 Wilms’ tumors and 75 soft tissue sarcomas (40). These results suggest that alternative methods of inactivation are required if the loss of these CDKI proteins contributes to tumor development.

We have used a somatic cell hybrid assay to identify a genetic element in 11p15.5, which suppresses tumor formation in the G401 Wilms’ tumor line (9–11). These experiments, in combination with similar functional analyses by Koi et al. (12), localized the suppressive element to a ~500-kb region between D11S601 and D11S724 (11). We have now mapped the p57 gene within this region of 11p15.5. If p57 expression modulated the tumorigenic potential of the G401 hybrid lines, we would expect to observe p57 mRNA in all of the nontumorigenic lines but not in the tumorigenic lines. Northern analysis, however, detected a 0.8-kb p57 transcript in both tumorigenic and nontumorigenic samples. Therefore, the p57 gene does not appear to be responsible for the tumor suppression observed in our functional assay.

Interestingly, the 0.8-kb transcript observed in the G401 hybrid panel is too small to encode the intact p57 gene. This reduced message has been detected previously in normal human tissues (5) and is most likely generated by alternative splicing. Differential use of three splice acceptor sites and an exon containing 5′ untranslated sequences was detected in the mouse p57 gene (4). Our sequence analysis identified an 83-bp intron downstream of the translation termination signal, indicating an exon containing 3′ untranslated sequences. Further investigation may reveal additional untranslated exons in the human p57 gene. Our sequence results also demonstrated that the coding region in the human p57 gene is divided by a 0.5-kb intron. The splice


\*W. S. El-Deiry and J. Pietenpol, personal communication.


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