A Human *Ki-ras* Oncogene Encodes Two Transforming p21 Proteins

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The human *Ki-ras* gene was previously reported to contain two alternative fourth exons which encode two distinct p21 proteins differing only at their carboxy termini. The present study shows that either p21 protein is able on its own to transform NIH 3T3 cells to a tumorigenic state.

The human *Ki-ras* gene has been found to be activated in a number of human tumors and tumor cell lines (for reviews, see references 2 and 10). The activation of this gene appears to occur via somatic point mutations that most frequently affect the amino acid residues present at either position 12 or 61 of the encoded p21 protein (1, 4, 5, 7, 8, 12). This gene has also been shown to contain two alternative fourth exons, termed 4A and 4B (1, 4, 8). The presence of alternative fourth exons allows the *Ki-ras* gene to express two distinct p21 proteins, termed p21_{4A} and p21_{4B}, which differ only in their carboxy termini. Transcripts derived from the human *Ki-ras* gene contain either exons 1, 2, 3, 4A, and 4B or exons 1, 2, 3, and 4B (1), serving as templates for the synthesis of p21_{4A} and p21_{4B}, respectively. The presence of a translation termination codon at the end of exon 4A results in the p21_{4A} protein being produced from the RNA containing both exons 4A and 4B (1). The genome of Kirsten sarcoma virus contains both fourth exons and in this way resembles the cellular mRNA encoding p21_{4A} (8). In the cell lines examined to date, 90 to 95% of the *Ki-ras* mRNA specifies the synthesis of p21_{4B} (1).

The carboxy termini of the two p21 proteins are quite distinct. p21_{4B} has a basic carboxy terminus which is not closely related to the carboxy terminus of the Kirsten sarcoma virus-encoded p21 protein. In contrast, the p21_{4A} carboxy terminus is virtually identical to the carboxy terminus encoded by Kirsten sarcoma virus (1, 4, 8).

Initial studies of the transforming potentials of the two *Ki-ras*-encoded p21 proteins left unresolved the question of whether either p21 protein, acting alone, is able to transform cells in a focus assay (1). This initial work showed that a plasmid specifying both *Ki-ras*-encoded p21 proteins displays detectable biological activity (1). Such data could be interpreted to suggest that both types of p21, acting in concert, are required to achieve transformation (1).

To further investigate the transforming abilities of p21_{4A} and p21_{4B}, we constructed the plasmids (Fig. 1) that carry cDNA copies of the *Ki-ras2* oncogene present in the human SW480 colon carcinoma. This oncogene carries a point mutation that causes amino acid residue 12 of the proto-oncogene-encoded p21 protein to be replaced by a valine (4). All of these plasmids were transfected into NIH 3T3 cells (4). As can be deduced from Fig. 1, Pst⁻pCD11-1 and pCD1234B both direct the synthesis of only p21_{4B}. Both of these plasmids induced foci of NIH 3T3 cells with a frequency of approximately 400 foci per μ g of cloned

transfected DNA. Foci carrying the Pst⁻pCD11-1 plasmid were picked and expanded into cell lines. These cell lines were analyzed for p21 production and tumorigenicity in mice (4). Cells from a focus carrying the Pst⁻pCD11-1 plasmid produce p21 protein (Fig. 2, lane H). These cells were found to be highly tumorigenic in mice (six of six mice injected with these cells developed tumors). These data show that expression of the *Ki-ras* p21_{4B} protein alone is sufficient to induce foci on monolayers of NIH 3T3 cells and to form tumors in mice.

Two other plasmids, pCD4A and pCD1234A (Fig. 1), should direct the synthesis of only p21_{4A} proteins. We observed that pCD1234A failed to induce foci of NIH 3T3 cells. Cells carrying pCD1234A were isolated by cotransfection with pSV2 Neo (9) as previously described (4). Analysis of p21 production in neomycin-resistant cells carrying intact pCD1234A DNA indicated that these cells contain little or no p21 protein beyond that of the recipient NIH 3T3 cells. We believe that the low level of p21 expression associated with pCD1234A may be due to the presence of a poorly functioning splice acceptor preceding exon 4A. This notion is suggested by the observation that only 5% of the cellular *Ki-ras* mRNA derives from use of the 4A splice acceptor sequence during mRNA formation.

The pCD4A plasmid also encodes a p21_{4A}. Because this plasmid carries a cDNA version of the *Ki-ras* gene, production of its mRNA does not depend on utilization of the exon 4A splice acceptor sequence. This DNA construction induced foci of NIH 3T3 cells at a frequency of approximately 500 foci per μ g of cloned transfected DNA. Immunoprecipitation of cells derived from pCD4A-induced foci by using p21 monoclonal antibodies demonstrated that these cells produced p21 protein (Fig. 2, lane E). Cells derived from these foci were also found to be highly tumorigenic in mice (10 of 12 mice developed tumors). These data indicate that expression of p21_{4A} alone is also sufficient to induce foci on monolayers of NIH 3T3 cells and to form tumors in mice. Such results represent the first unambiguous identification of a human *Ki-ras*-encoded p21_{4A}. These data also demonstrate (Fig. 2) that the p21_{4B} protein migrates more quickly on sodium dodecyl sulfate-polyacrylamide gels than the closely related p21_{4A} protein.

Although either p21 protein appeared able to induce transformation when present alone, it remained possible that the two, when present together, might act synergistically to induce transformation even more efficiently. Consequently, we examined the transforming abilities of pCD1234A4B and pCD1234B4A, both of which have the ability to direct

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FIG. 1. Structure of plasmids characterized in analysis of the transforming potentials of the *Ki-ras*-encoded p21 proteins. Each plasmid has the eucaryotic expression vector pCD (6) as its backbone. The promoter, indicated by P_E , is the early simian virus 40 promoter. A 5' splice site (denoted 5'ss) and a 3' splice site (denoted 3'ss) allow the mRNAs transcribed from each plasmid to be spliced even if a cDNA is inserted into the vector. The polyadenylation site is indicated by poly(A) site. The construction of Pst^- pCD11-1 was described in detail by McCoy et al. in 1984 (4). This plasmid has a valine at residue 12 of the encoded p21 protein. Cleavage of Pst^- pCD11-1 with *EcoRI* and *XbaI* excises a 600-base-pair (bp) fragment which contains the most 3' 50 bp of exon 3 and all of exon 4B. In the construction of the intermediate plasmid pCD123, this 600-bp *XbaI-EcoRI* fragment was replaced with a 300-bp *EcoRI* fragment which contains the most 3' 50 bp of exon 3 and 250 bp of the intron between exons 3 and 4A. pCD1234A was constructed by inserting a 3.0-kilobase (kb) *EcoRI-XbaI* fragment carrying exon 4A at the 3' end of the pCD123 insert. pCD1234B4A was constructed by inserting a 1.8-kb *EcoRI-XbaI* fragment carrying exon 4B into pCD1234A in a position immediately upstream of the 3.0-kb fragment containing exon 4A. pCD1234B carries the 1.8-kb exon 4B-containing fragment 3' to the pCD123 insert. Cleavage of pCD1234B and insertion of the 3.0-kb *EcoRI-XbaI* fragment carrying exon 4A into a position immediately upstream of the 1.8-kb exon 4B-containing fragment led to the isolation of pCD1234A4B. pCD4A was constructed by excising the 600-bp *EcoRI-XbaI* fragment present in Pst^- pCD11-1 and replacing it with a 1,160-bp *EcoRI-SalI* fragment which contains the most 3' 50 bp of exon 3 as well as all of exons 4A and 4B. This 1,160-bp *EcoRI-SalI* fragment was obtained from pSVESW4A (plasmid IV in reference 1).

synthesis of both *Ki-ras*-encoded p21 proteins. Both of these plasmids induced foci at a frequency of approximately 400 foci per μ g of cloned DNA transfected. In addition, cotransfection of Pst^- pCD11-1 and pCD4A induced foci at a rate of approximately 500 foci per μ g of cloned DNA transfected. Thus, it appears that the presence of both types of p21 protein neither stimulates nor inhibits focus formation.

To further support the present conclusions, we transfected a plasmid termed pSVESW4A (plasmid IV in reference 1) and generously provided by Arthur Levinson. This plasmid is very similar in structure to the pCD4A plasmid which contains a fragment derived from pSVESW4A. pSVESW4A contains exons 1, 2, 3, 4A, and 4B in a cDNA form and therefore can direct the synthesis of p21_{4A}. We found that pSVESW4A induces foci of NIH 3T3 cells at a frequency of approximately 200 foci per μ g of cloned DNA transfected. Cell lines derived from these foci produce p21 protein (Fig. 2, lane I) and are tumorigenic in mice (six of six mice injected with these cells developed tumors).

Thus, it appears that mammalian cells may express as many as four p21 proteins (*Ki-ras* p21_{4A} and p21_{4B}, *Ha-ras*

FIG. 2. Immunoprecipitation of p21 from transfected cell lines. ^{35}S -labeled lysates were prepared as previously described (4) and immunoprecipitated with either normal rat serum (lanes A, C, F, G, and J) or p21 monoclonal Y13-259 (3) (lanes B, D, E, H, and I). The immunoprecipitated lysates were subjected to electrophoresis through a 15% sodium dodecyl sulfate-polyacrylamide gel, and the gel was fluorographed as described by McCoy et al. in 1984 (4). Lysates were from NIH 3T3 cells (lanes A and B), transfectants containing Pst^- pCD11-1, which contains exon 4B (lanes C, D, G, and H), transfectants containing pCD4A, which contains exon 4A (lanes E and F), and transfectants containing pSVESW4A, which contains exon 4A (lanes I and J). Molecular weight markers appear on the left.

p21, and N-*ras* p21), any one of which, if appropriately altered, can transform cells. The work of others (11) recently showed that alteration of some regions near the carboxy terminus of a Ha-*ras* oncogene-encoded p21 protein has little influence on transforming activity. Taken together with the present data, these results suggest that the effector functions important for transformation reside in the more N-terminal portions of the p21 protein.

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