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Activation of Ha-*ras* p21 by Substitution, Deletion, and Insertion Mutations

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The transforming activity of naturally arising *ras* oncogenes results from point mutations that affect residue 12 or 61 of the encoded 21-kilodalton protein (p21). By use of site-directed mutagenesis, we showed that deletions and insertions of amino acid residues in the region of residue 12 are also effective in conferring oncogenic activity on p21. Common to these various alterations is the disruption that they create in this domain of the protein, which we propose results in the inactivation of a normal function of the protein.

A variety of tumors and tumor cell lines contain mutated *ras* genes, which can be detected by their ability to induce foci of transformed cells upon transfection of monolayer cultures of NIH 3T3 mouse fibroblasts. Several of these activated *ras* genes have been cloned and found to arise by mutation of normal cellular proto-oncogenes. In some cases, the alterations responsible for the activation of these oncogenes are point mutations that affect the protein-encoding portions of these genes and change amino acid 12 or 61 of the 21-kilodalton *ras* protein (p21). The normal cellular Harvey sarcoma virus (Ha), Kirsten sarcoma virus (Ki), and N-*ras* proteins carry glycine as residue 12. Spontaneous mutations causing replacement of Gly 12 by a variety of residues, including valine, aspartic acid, or cysteine, have been shown to convert the human Harvey and Kirsten virus genes (6) into active oncogenes (3, 19, 21, 25, 26, 28, 32–34). Other workers have recently used site-directed mutagenesis to show that almost all point mutations affecting amino acid residue 12 are able to convert the normal gene to an oncogene (27). The *ras* genes transduced by Harvey and Kirsten sarcoma viruses also have replacements at amino acid 12, but the role of these replacements in viral transformation is unclear because other differences distinguish the viral genes from their normal cellular counterparts (10, 35).

One interpretation of these data is that amino acid 12 replacements act by specifically imparting a novel function to the *ras* protein. An alternative hypothesis states that Gly 12 replacements are particularly well suited to derange the local structure (7, 24) and in this way destroy a function normally performed by this domain of the protein. We used site-directed mutagenesis to examine these two possibilities by creating more drastic alterations in this region of the *ras* gene.

MATERIALS AND METHODS

Plasmids. Plasmids pEC6.6 and pEJ6.6 carrying, respectively, the normal and an activated allele of the human Ha-*ras* gene were described previously (pEC and pEJ [32]). The sequences of these *Bam*HI fragments have been reported previously (2). Plasmid pEC5-4 contains a 920-base pair (bp) fragment of pEC6.6 which includes exon 1 of Ha-*ras*1, as well as sequences immediately 5′ and 3′ to it. This fragment, produced by *Xba*I and *Kpn*I cleavage of pEC6.6, was inserted into the *Eco*RI site of pUC13 (36).

Oligonucleotides. Oligonucleotides were synthesized on a solid support by using phosphoamidite chemistry (4). The sequences of the two oligonucleotides used were GGGCGC CGACGGTG and GGGCGCC$\underline{\underline{T}}$GCGGTG, in which the underline indicates the position of mismatch with the normal allele. During the synthesis of the second oligonucleotide, equimolar amounts of the A, C, and T precursors were added at the position of mismatch to obtain a mixture that could encode three different replacements at position 12.

Mutagenesis. Oligonucleotide-directed mutagenesis was done essentially as described by Dalbadie-McFarland et al. (8). Plasmid pEC5-4 (50 μg/ml) was nicked at its unique *Hin*dIII site by treatment with *Hin*dIII (10 U/μg of DNA) in the presence of 60 μg of ethidium bromide per ml for 1 h at 37°C. To expose the site for oligonucleotide hybridization, the nicked DNA was digested at 15°C with exonuclease III as previously described (8), producing a gap of approximately 500 bp. The digested DNA was extracted with phenol-chloroform and then with chloroform and then was ethanol precipitated.

The filters carrying putative mutants were screened by differential hybridization with end-labeled mutagenic oligonucleotide as the probe, essentially as described by Wallace (R. B. Wallace, *in* S. Woo [ed.], *DNA Recombinant Technology: Methods*, in press). Colonies whose DNA retained strong hybridization to the probe even at wash temperatures close to the dissociation temperature were further screened by *Nae*I digestion. Almost all the colonies that were positive by differential hybridization had also lost the exon 1 *Nae*I site which is present in the normal human Ha-*ras*1 gene, suggesting that they were genuine mutants. Selected colonies were further screened by DNA sequencing across the mutation site.

Deletion and insertion mutations. The construction of the amino acid 12 deletion mutant pEC5-4 D12 is described below. Mutations encoding the insertion of amino acids between amino acids 11 and 12 of Ha-*ras*1 were made by insertion of linkers (Collaborative Research, Inc.) into the unique *Nae*I site of pEC5-4 by standard methods. In one

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case an *Hpa*I linker (GTTAAC) encoding Val-Asn was inserted. In another case, a *Bam*HI linker (CCCGGATCCGGG) encoding Pro-Gly-Ser-Gly was inserted. To verify that only one copy of each linker was inserted, candidate insertions and wild-type pEC5-4 were digested with *Nar*I, end-labeled with [γ-^{32}P]ATP by using the exchange reaction (1), recut with *Pvu*II, and resolved in adjacent channels on denaturing sequencing gels. A DNA sequencing ladder was used as reference marker. This procedure, when done on wild-type pEC5-4, generates a labeled band of 37 bases which spans the *Nae*I site. The corresponding band in the *Hpa*I insertion was 6 bp longer than this and 12 bp longer in the *Bam*HI insertion, indicating single insertions.

Nucleotide sequencing. Nucleotide sequencing was performed as described by Maxam and Gilbert (18).

Reconstruction of complete *ras* genes. All the mutations described were introduced into pEC5-4. To reconstruct complete *ras* genes that contain the above-described mutations, we isolated the 620-bp *Bst*EII-*Xba*I fragment from each mutant. This fragment was then ligated with the 10-kilobase *Xba*I-*Xho*I fragment from pEC6.6 and the 1,100-bp *Xho*I-*Bst*EII fragment from pEC6.6. Correct ligation of these three fragments places the mutations in a pEC6.6 background and was verified by restriction analysis of candidates.

All other procedures were performed as previously described (15, 16, 19, 23, 29).

RESULTS

Analysis of position 12 amino acid replacements. We used oligonucleotide-directed mutagenesis to make replacements of amino acid 12 of p21 (see above). Two different 14-base synthetic oligonucleotides were used in these experiments. The first encodes a substitution of aspartic acid for glycine at position 12. The second was synthesized as a mixture of all the possible replacements of the first nucleotide of codon 12 of the Ha-*ras* gene. Use of this oligonucleotide mixture allowed recovery of mutated genes encoding three different replacements of amino acid 12: serine, arginine, and cysteine. Candidate clones which reacted positively in a differential hybridization test and which lacked the diagnostic *Nae*I restriction site (see above) were further evaluated by sequencing across the mutation site.

These mutations were introduced into a subclone of the gene that included only exon 1 of the normal human Ha-*ras*1 gene. The altered subclones were then used in conjunction with subclones of the remaining portions of the gene to reconstruct complete Ha-*ras* genes, and these were then tested for biological activity by transfection of NIH 3T3 cultures. The three mutated Ha-*ras* genes were all strongly focus inducing, having biological activities (foci per microgram of DNA transfected) that were comparable to that of the human EJ/T24 bladder carcinoma oncogene (Table 1). These data suggest that all of these replacements at position 12—Asp, Arg, and Cys—impart oncogenicity to the cHa-*ras*1 protein. This was verified by characterization of cells transformed by the mutant protein (see below). These data are in accord with results which have been reported since we began these studies (11, 26, 27).

Creation of insertion and deletion mutants. Aspartic acid, arginine, cysteine, and valine (which is found at position 12 in the p21 encoded by the EJ/T24 oncogene) have distinct chemical properties. The fact that replacement of Gly 12 by all of these amino acids leads to oncogenic activation suggests that most or all of the possible replacements at position 12 would give a similar result (see reference 27). This left undetermined whether amino acid 12 replacements act by imparting a specific new function to the protein or by destroying a function. To address this question, we created three additional mutations, each of which changes p21 in a more drastic fashion than the point mutations described above.

The first mutant, borne on plasmid pD12, carried an exact deletion of amino acid 12. It was made by using a one-nucleotide-deletion mutant that was fortuitously obtained in screening for mutants arising after use of the previously

TABLE 1. Induction of focus formation and soft agar growth by mutant Ha-*ras*1 constructs

Plasmid[a]	Mutation in p21 encoded by plasmid, relative to normal p21 (human Ha-*ras*1)	Potency in focus induction relative to pEJ6.6[b]	G418-resistant cell lines derived by cotransfection with pSV2neo	Growth of cell lines in soft agar (visible colonies/10^3 cells)
pEC6.6	None (Gly 12)	0.02	EC-3	0
			EC-6	36[c]
pEJ6.6	Val 12	1.0	EJ-4	175
pArg12a[d]	Arg 12	0.4		
pArg12b	Arg 12	0.5	Arg-5	162
pAsp12a[d]	Asp 12	0.5	Asp-1	289
pAsp12b	Asp 12	0.5		
pCys12a[d]	Cys 12	0.9		
pCys12b	Cys 12	0.8	Cys-5	212
pD12a[d]	Deletion of Gly 12	0.3	D12-4	196
pD12b		0.3		
pNiH	Insertion of two amino acids (-Val-Asn-) between Ala 11 and Gly 12	0.06	NiH-2[e]	246
			NiH-3	240
			NiH-4	147
pNiB	Insertion of four amino acids (-Pro-Gly-Ser-Gly-) between Ala11 and Gly 12	0.25	NiB-5	103
			N-3[f]	

[a] pEC6.6 and pEJ6.6 have been described previously (32). pEC6.6 encodes the normal human Ha-*ras* gene. pEJ6.6 encodes an oncogenic allele of the Ha-*ras* gene which was isolated from a human bladder carcinoma. All of the other plasmids are identical to pEC6.6 except for the indicated mutations. Their construction is described in the text.

[b] Plasmids were transfected into NIH 3T3 cells as described in the text. In these assays, transfection of pEJ6.6 DNA yielded 4 to 5 $\times$ 10^2 foci per μg of DNA.

[c] These colonies were much smaller than those formed by the other cell lines listed here.

[d] Plasmids termed a and b were constructed with independently derived clones of mutated pEC5-4 obtained from in vitro mutagenesis, each of which had the correct nucleotide sequence across the mutation site.

[e] Unlike the other cell lines described here, this line was not established from a G418-resistant colony, but from a focus. This focus resulted from a culture which was cotransfected as usual with the pSV2neo and pNiH plasmids but not subjected to subsequent G418 selection. Cells of this focus proved to be resistant to and were subsequently carried in G418. They contain the same slowly migrating p21 protein as NiH-3 and NiH-4 cells (see Fig. 2).

[f] N-3 is a G418-resistant cell line derived by transfection of only pSV2neo DNA.

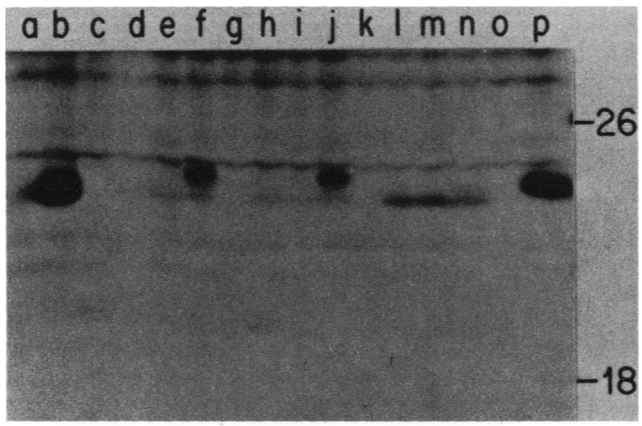

FIG. 1. Immunoprecipitation of p21 from cell lines containing pNiH (which encodes a p21 molecule that has a two-amino-acid insertion) and pAsp12a (which encodes a p21 molecule that has aspartate as residue 12). [^{35}S]methionine-labeled cell lysates were prepared as described in the text and precipitated with either normal rat serum (lanes a, c, g, k, and o) or one of three different anti-p21 monoclonal antibodies (13): YA6-172 (lanes b, d, l, and p), Y13-238 (lanes e, i, and m), and Y13-259 (lanes f, j, and n). Immunoprecipitations were done with lysates containing equal levels of incorporated ^{35}S radioactivity. Electrophoresis through sodium dodecyl sulfate-polyacrylamide gels and subsequent fluorography were performed as described in the text. Molecular size is shown in kilodaltons. Lysates were from EC-6 cells (lanes a and b), NiH-3 cells (lanes c to f), NiH-4 cells (lanes g to j), Asp-1 cells (lanes k to n), and EJ-4 cells (lanes o and p). Table 1 shows the derivation of these cell lines.

described mixed oligonucleotide. The single-nucleotide deletion created a new *Sac*II endonuclease site which was cleaved by treatment with this enzyme. The resulting ends were blunted by treatment with T4 DNA polymerase. The rejoining of these ends by T4 DNA ligase resulted in the deletion of the three nucleotides constituting codon 12. Two independent plasmids obtained by this procedure were sequenced and found to have the desired deletion.

The other mutants studied here encoded insertions of amino acid sequences between residues 11 and 12 of normal p21 protein. Construction of these mutated genes was facilitated by the presence of an *Nae*I endonuclease site that is located precisely between codons 11 and 12 of the human Ha-*ras*1 gene. This endonuclease leaves blunt ends after cleavage, making possible the insertion of commercially available oligonucleotide linkers. In one case, we inserted a 12-nucleotide linker that carries a *Bam*HI cleavage site and encodes the amino acid sequence Pro-Gly-Ser-Gly. This created the mutant plasmid pNiB. In the second case, we inserted a linker that carries an *Hpa*I cleavage site and encodes the sequence Val-Asn, resulting in mutant plasmid pNiH. The mutations described were introduced, as before, into a subclone that contains only exon 1 of the normal Ha-*ras*1 gene. The resulting subclones were then used in conjunction with subclones of the remaining portions of the normal gene to reconstruct complete mutated alleles of the Ha-*ras* gene.

The deleted and expanded genes were tested by transfection for ability to induce foci on monolayers of NIH 3T3 cells. Table 1 shows the results of the focus assays of these mutants. DNAs of the amino acid 12 deletion mutant and the four-amino-acid insertion mutant clearly induced foci at rates comparable to that of pEJ6.6, which carries the EJ/T24 bladder carcinoma oncogene. Plasmid pEC6.6 which carries the normal Ha-*ras*1 gene, had the expected very low rate of focus induction. The plasmid encoding the two-amino-acid insertion exhibited a rate of focus induction which was higher than that of pEC6.6 but reproducibly much lower than that of plasmid pEJ6.6. Two of the foci induced by pNiH, which encodes a p21 molecule bearing a two-amino-acid insertion, were established as cell lines and shown to contain the p21 protein encoded by this plasmid (see below), thus ruling out the possibility that these lines arose spontaneously. We conclude that a variety of mutations altering this N-terminal domain of p21 can cause oncogenic activation.

Characterization of stable cell lines. To further characterize the mutant genes, it was desirable to have clonally derived cell lines carrying these genes. These were obtained by cotransfection of the mutant plasmid DNAs with pSV2neo DNA (30) followed by selection of colonies resistant to the drug G418. For each constructed mutation, as well as for pEJ6.6 and pEC6.6, several G418-resistant colonies were picked and established as cell lines. There was no reproducible qualitative difference in the morphologies of cells transformed by the various mutant Ha-*ras* genes (data not shown).

Growth in soft agar provided yet another measure of transformation and serves as a good in vitro correlate of tumorigenicity. For each mutant construct, two or more G418-resistant cell lines transfected with the construct were tested for growth in soft agar. Table 1 shows the results for the best-characterized cell lines. These data show that all of these mutant Ha-*ras* genes can confer growth in soft agar.

Transformation by a *ras* gene can occur by mechanisms that affect either p21 structure or the level of its expression (5). Thus, low levels of mutant *ras* protein or extremely high expression of normal Ha-*ras*1 p21 can result in transformation. We wished to confirm that the transformed lines described here were transformed by low levels of altered p21 rather than by over expression of a functionally normal protein. To this end, cell cultures were grown for 16 to 20 h in a [^{35}S]methionine-containing medium, and metabolically labeled p21 protein was quantitatively immunoprecipitated by using the anti-p21 monoclonal antibody Y13-259 (13). For standards, we analyzed in parallel lysates of cells transformed by the EJ/T24 bladder carcinoma oncogene (the EJ-4 cell line), as well as cells partially transformed by the presence of very high levels of normal p21 (the EC-6 cell line).

For each of the six mutant genes, at least two cell lines were obtained that had levels of p21 comparable to the small amounts found in EJ-4 cells. Moreover, these cell lines grew much better in soft agar than did EC-6 cells, which contain much higher (50×) levels of a p21 protein that has a normal amino acid sequence (data not shown). Figure 1 shows representative data for cells expressing the p21 that has aspartate as residue 12 (lanes l to n) and the p21 that bears the two-amino-acid insertion (lanes f and j). We concluded that each of the six mutant constructs carrying point mutations, deletions, or insertions encodes a p21 molecule that has transforming activity comparable in potency to that of the EJ oncogene-encoded p21 and much greater than the weak transforming activity exhibited by the normal p21 protein.

Effects of mutations on protein conformation. The replacement of Gly 12 by valine decreases the electrophoretic mobility of the protein (9, 32). This property of aberrant migration has also been observed for several other *ras* proteins that carry activating point mutations (11). The mutations described here also resulted in unexpected shifts in the electrophoretic mobility of p21. For example, im-

munoprecipitations of lysates from the cells line carrying p21 with a four-amino-acid insertion (Fig. 2, lanes g and h) show that this protein migrates more rapidly than p21 with a simple valine-glycine substitution (lane b). Other data, not shown here, indicate that p21 containing a two-amino-acid insertion migrates more slowly than does the valine-substituted protein. Other data show that the protein encoded by pAsp12 comigrates with the normal Ha-*ras* p21 (Fig. 1), while the proteins encoded by pArg12 and pCys12 have mobilities intermediate between those of normal p21 and EJ p21, which has valine at position 12 (data not shown). The range of mobilities of p21 molecules with activating lesions suggests that many of these proteins have undergone strong shifts in conformation.

Additional evidence for conformational changes in some of the mutant proteins was obtained by immunoprecipitation with two anti-p21 monoclonal antibodies, Y13-238 and YA6-172 (13), whose specificities differ from those of the often-used Y13-259 antibody (22). Both of these antibodies precipitated the normal p21 as well as p21 molecules carrying residue 12 replacements of Asp, Arg, Cys, and Val (Fig. 1, lanes l and m; data not shown). The p21 bearing the two-amino-acid insertion was not precipitated by Y13-238 or by Y6-172 (Fig. 1, lanes e, h, and i). The mutant p21 containing the four-amino-acid insertion was quite well precipitated by Y13-238 (Fig. 2, lanes g and h). The protein encoded by the amino acid residue 12 deletion mutant was either poorly or not at all precipitated by Y13-238 and YA6-172 (Fig. 2, lanes d, e, and f). Such data suggest, once again, that conformational changes have rendered the protein encoded by this mutant at best poorly recognizable. We conclude that changes in the N-terminal domain of p21 have complex effects on its conformation, as manifested by changes in electrophoretic mobility and antibody reactivity.

DISCUSSION

Replacement of Gly 12 of Ha-*ras* p21 by Asp, Arg, or Cys led to oncogenic activation of the protein, in accord with the results of others (3, 11, 26, 28). Because this is a diverse group of amino acids, such data suggest that most or all of the possible replacements can cause oncogenic activation, a prediction borne out by the recent work of Seeberg et al. (27).

One implication of the amino acid 12 replacement data is that these mutations may act by destroying some function exhibited by normal p21. To further test this possibility, we constructed three mutants which encode proteins with more drastic lesions in the N-terminal domain. One protein lacked amino acid 12, and the other two had two- and four-amino-acid insertions between amino acids 11 and 12. Our data show that each of those p21 proteins can transform cells, even when they are present in the cell in low amounts. This means that there are mutations in the N-terminal domain other than replacements of residue 12 which can activate the transforming potential of p21. Yet another example has recently been reported: replacement of glycine 13 by aspartate was shown to activate the Ha-*ras* gene (11). The deletion and insertion mutations that we have constructed are likely to induce alterations in protein structure which are different from and more severe than the frequently observed amino acid substitutions. It seems that the only property common to all of these various activating events is the ability to disrupt local structure. Thus, the activating mutations in the amino acid 12 region appear to act by destroying a configuration in the Ha-*ras*1 protein that is essential for normal function.

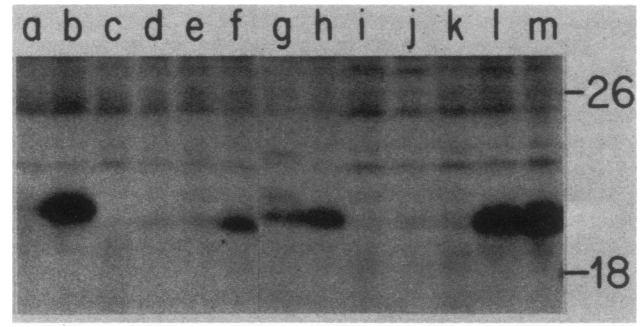

FIG. 2. Immunoprecipitation of p21 from cell lines containing pD12 (which encodes the deletion of glycine 12 of p21) and pNiB (which encodes the insertion of four amino acids between residues 11 and 12 of p21). The preparation of lysates, immunoprecipitation, and electrophoresis were as described in the legend to Fig. 1. The immunoglobulins used were normal rat serum (lanes a, c, i, and k) or one of three anti-p21 monoclonal antibodies: YA6-172 (lanes b, d, and j), Y13-238 (lanes e and g), and Y13-259 (lanes f, h, and m). The lysates were from EJ-4 cells (lanes a and b), D12-4 cells (lanes c to f), NiB-5 cells (lanes g and h), N-3 cells (a G418-resistant cell line derived from transfection of only pSV2neo DNA; lanes i and j), and EC-6 cells (lanes k to m). Molecular sizes are in kilodaltons. See Table 1 for the derivation of these cell lines.

We suggest that these activating mutations in the amino acid 12 region destroy a negative regulatory function of normal p21, resulting in constitutive expression of some other, positive activity of the protein. Other workers have suggested that the amino acid 12 region is part of a nucleotide binding site on the basis of its sequence homology with binding site sequences of known nucleotide-binding proteins (14, 37). However, normal and oncogenic p21 appear to bind GTP equally well (12, 19). More recently, it has been shown that the normal Ha-*ras* p21 can hydrolyze GTP and that this activity is greatly reduced in oncogenic EJ/T24 p21 (17, 19, 31). This suggests that the negative regulatory function we propose may be related to GTP hydrolysis. It remains to be seen whether reduced GTPase activity is common to all of the different transforming p21 molecules. Most puzzling is the repeated observation of spontaneously arising point mutations at residue 12 and nowhere else in this region of the protein. The present work and that of others (27) suggests that mutations affecting a variety of residues in this region of the protein should be equally effective in creating potent oncogenes. This paradox may be resolved only by crystallographic analysis of the *ras*-encoded p21 proteins.

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