Three-Dimensional Structures of H-ras p21 Mutants: Molecular Basis for Their Inability to Function As Signal Switch Molecules

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Summary

The X-ray structures of the guanine nucleotide binding domains (amino acids 1–166) of five mutants of the H-ras oncogene product p21 were determined. The mutations described are Gly-12→Arg, Gly-12→Val, Gin-61→His, Gin-61→Leu, which are all oncogenic, and the effector region mutant Asp-36→Glu. The resolutions of the crystal structures range from 2.0 to 2.6 Å. Cellular and mutant p21 proteins are almost identical, and the only significant differences are seen in loop L4 and in the vicinity of the γ-phosphate. For the Gly-12 mutants the larger side chains interfere with GTP binding and/or hydrolysis. Gin-61 in cellular p21 adopts a conformation where it is able to catalyze GTP hydrolysis. This conformation has not been found for the mutants of Gin-61. Furthermore, Leu-61 cannot activate the nucleophilic water because of the chemical nature of its side chain. The D38E mutation preserves its ability to bind GAP.

Introduction

The ras proto-oncogene family consists of three members, N-, K-, and H-ras, which code for highly homologous proteins termed p21. By analogy to the hormone receptor–coupled G proteins, the p21 proteins are believed to function as signal switch molecules. In the active GTP conformation, they transmit a signal to an effector molecule that leads to cell proliferation. The p21–effector interaction is switched off by the effector-mediated GTPase reaction, which returns the protein to the inactive GDP bound state. The exact nature of the growth-promoting signal is not yet known.

Point mutations of the ras genes are found in approximately 30% of human tumors and thus constitute the most prevalent oncogene in human carcinogenesis (for reviews see Barbacid, 1987; Spandidos, 1989; Bos, 1989). The mutations are usually found in only two contexts, leading to substitutions in residues Gly-12 or Gin-61 of the p21 protein. Certain mutations are predominantly found in human tumors, but in the case of codon 12 it has been shown that any amino acid except proline renders the protein oncogenic (Seeberg et al., 1984). Also, various substitutions of Gin-61 activate the protein (Bos, 1989; Vogelstein et al., 1988), of which the mutations to Arg, His, or Leu seem to be the most prevalent ones. The mutant proteins can bind to GAP, the GTPase activating protein, which is believed to mediate the signal-transducing effect of p21 on the cell (Trahey and McCormick, 1987; McCormick, 1989; Vogel et al., 1988; Trahey et al., 1988). In contrast to cellular p21, however, oncogenic mutants are not converted to the GDP-bound form during the interaction with GAP and stay in the active GTP conformation. These proteins are thus not able to function as on/off switches and are constantly producing a growth-promoting signal.

We have recently determined the structure of the guanine nucleotide binding domain of H-ras-encoded p21 (amino acids 1–166) in the active conformation complexed to the stable analog guanosine-5′-[(β,γ-imido) triphosphate (GppNp) at 2.5 Å (Pai et al., 1989) and at 1.35 Å (Pai et al., 1990). It has been shown that this structure is identical to that of the "real" p21·GTP complex, the structure of which has been determined using caged GTP and Laue diffraction methods (Schlichting et al., 1990b). The three-dimensional structures of the GppCp and GDP complexes of H-ras p21 have also recently been reported (Tong et al., 1989; Milburn et al., 1990; Schlichting et al., 1990b). Here we describe the three-dimensional structures of five truncated p21 mutants (amino acids 1–166) complexed to the nucleotide analog GppNp. Four of them (G12R, G12V, Q61H, and Q61L) are oncogenic (baccaricin, 1987; Bos, 1989; Table 1); the fifth (D38E) is not transforming, although it is also insensitive to activation by GAP (Calé et al., 1988).

Results

Biochemical Characterization

The p21 mutants, as full-length (amino acids 1–189) or truncated proteins, were prepared as described (Tucker et al., 1986; John et al., 1988). The biochemical properties of these proteins, mostly for full-length proteins, are listed in Table 1. Deletion of amino acids 167–189 does not affect those properties of either mutant or cellular p21 (John et al., 1989; Gross et al., 1985). The crystal structures presented below were determined with the truncated proteins, which for simplicity will also be called p21 henceforth.

As described before, the GTPase and GTP dissociation rates are different for wild-type and mutant p21 proteins (Gibbs et al., 1984; Sweet et al., 1984; John et al., 1988; Der et al., 1986; Feig and Cooper, 1988). The GppNp complexes of p21(G12V) and p21(G12R) bind to GAP with dissociation constants of 20 and 35 μM, which is 3- and 5-fold higher than for wild-type p21. This has been described for p21(G12V) before (Vogel et al., 1988). We find, however, that the absolute values of affinities for both wild-type and mutant proteins are higher with GAP expressed in baculovirus (Frech et al., 1990; Gideon et al., unpublished data) than with bovine GAP (Vogel et al., 1988). The p21(Q61H) and p21(Q61L) mutant proteins form tight complexes with GAP with dissociation constants of 2 and 0.1 μM, respectively, again similar in direction but different in...
Table 1. Biochemical and Biological Properties of p21 Mutants

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>G12V</th>
<th>G12R</th>
<th>Q61H</th>
<th>Q61L</th>
<th>D38E</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTPase, k2 (min⁻¹ x 10⁻³)</td>
<td>28.0⁺</td>
<td>2.0⁺</td>
<td>1.4⁺</td>
<td>1.9⁺</td>
<td>1.3</td>
<td>28.0⁺</td>
</tr>
<tr>
<td>GTP dissociation (min⁻¹ x 10⁻³)</td>
<td>5.0</td>
<td>4.7⁺</td>
<td>1.1⁺</td>
<td>3.7⁺</td>
<td>31.0</td>
<td>5.0⁺</td>
</tr>
<tr>
<td>GAP activation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GAP affinity (µM)</td>
<td>6.6⁺</td>
<td>20⁺</td>
<td>35⁺</td>
<td>2⁺</td>
<td>0.1⁺</td>
<td>7.6⁺</td>
</tr>
<tr>
<td>Transformation activity</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Some of the GTPase and GTP dissociation rate constants have been described before (⁺John et al., 1988, 1989; ᵃCaillis et al., 1988) and are for full-length p21. The values for p21(D38E) have been assumed according to the report that the biochemical properties of this mutant are identical to those of cellular p21 (Caillis et al., 1988). The rate constants have been measured as described (John et al., 1988, 1989). Experiments with GAP were made with human GAP expressed in baculovirus (McCormick et al., unpublished data). The activation of the GTPase activity by GAP was measured with 0.5-1 µM p21-GTP and 10 nM GAP under standard assay conditions (Vogel et al., 1988). Affinities between GAP and p21 were determined by inhibiting the interaction between GAP and wild-type p21-GTP with increasing concentrations of the GppNp complexes of the truncated mutant proteins (⁺Gideon et al., unpublished data; ᵃFrech et al., 1990). The affinity of full-length p21 is 4.8 µM in this assay.

General Comparison of Structures

The overall structural changes between cellular and mutant proteins are very small. Figures 1A and 1B show as examples the Cα plots for two mutants, p21(Q61L) and p21(G12V), in comparison to cellular p21. There are almost no differences in the positions of the Cα atoms between cellular and mutant proteins except for residues 61-65 of loop L4 and—in the case of p21(G12V)—adjacent residues 58-60. This is reflected by the root-mean-square differences, which are 1.60 Å on average for the L4 regions, but only 0.27 Å on average for the rest of the structure (see Table 2). This is in line with proton and phosphorous NMR measurements, where no significant spectral changes between wild-type and mutant proteins were observed (Schlichting et al., 1990a; Poe et al., 1986). Figure 2 shows the differences for the side chains, measured as the divergence of the most distant atom of each residue. These differences are distributed over the whole protein. Most of these can be considered insignificant, such as those of Lys-88 or Asp-108, because these side chains are on the outside of the molecule and are free to move in the crystal. Figure 3 indicates where all of these changes are located in the three-dimensional structure.

By intensive mutational analysis (Barbacid, 1987; McCormick, 1989; Adari et al., 1988; Sigal et al., 1988; Gibbs et al., 1988), a number of residues have been found to be especially important for the function of p21: the nucleotide
Table 2. Details of the Crystallographic Analysis

<table>
<thead>
<tr>
<th></th>
<th>D38E</th>
<th>G12R</th>
<th>G12V</th>
<th>Q61H</th>
<th>Q61L</th>
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<tbody>
<tr>
<td>Space group</td>
<td>$P 3_{21}$</td>
<td>$P 3_{21}$</td>
<td>$P 3_{21}$</td>
<td>$P 3_{21}$</td>
<td>$P 3_{21}$</td>
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<tr>
<td>Axes (Å)</td>
<td>$a = b = 40.3$</td>
<td>$a = b = 40.4$</td>
<td>$a = b = 40.5$</td>
<td>$a = b = 40.4$</td>
<td>$a = b = 40.3$</td>
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<tr>
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<td>2.2</td>
<td>2.6</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Observed reflections (no.)</td>
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<td>27,137</td>
<td>11,868</td>
<td>38,198</td>
<td>48,526</td>
</tr>
<tr>
<td>Unique reflections (no.)</td>
<td>7,633</td>
<td>8,628</td>
<td>5,411</td>
<td>7,875</td>
<td>13,288</td>
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<tr>
<td>$R_{sym}$ (%)$^a$</td>
<td>4.9</td>
<td>6.2</td>
<td>4.7</td>
<td>4.9</td>
<td>6.8</td>
</tr>
<tr>
<td>Refining cycles (no.)</td>
<td>5</td>
<td>7</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>$R_F$ (%)$^b$ without water molecules</td>
<td>22.2</td>
<td>22.8</td>
<td>21.4</td>
<td>22.5</td>
<td>22.4</td>
</tr>
<tr>
<td>with water molecules</td>
<td>18.3</td>
<td>19.2</td>
<td>0</td>
<td>18.0</td>
<td>19.2</td>
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<tr>
<td>Fitted water molecules (no.)</td>
<td>211</td>
<td>203</td>
<td>0</td>
<td>225</td>
<td>192</td>
</tr>
<tr>
<td>rm differences (Å)</td>
<td>amino acids 1-60, 66-166</td>
<td>0.24</td>
<td>0.24</td>
<td>0.41 (0.34)</td>
<td>0.26</td>
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<tr>
<td></td>
<td>amino acids 61-65</td>
<td>1.68</td>
<td>1.44</td>
<td>1.53 (1.50)</td>
<td>1.75</td>
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</tbody>
</table>

For each of the five mutants analyzed, unit cell parameters and statistics of data collection are listed. Resolution ranges from 2.0 to 2.6 Å with R-factors lower than 20% after the final round of refinement. For comparing wild-type and mutant structures, root-mean-square (rms) differences of equivalent C$_\alpha$ positions are given. In the upper line, C$_\alpha$ of the flexible region in loop L4 are left out, the bottom line gives root-mean-square differences only for this part. Values in brackets for p21(G12V) are for amino acids 1-57/66-166 and 57-65, respectively.

$^a$ $R_{sym} = \frac{\sum_{h} \sum_{k}}{\sum_{h} \sum_{k}|I_{hk} - \langle I_{hk}\rangle|}{\sum_{h} \sum_{k} \langle I_{hk}\rangle}$, where $I_{hk}$ are symmetry related intensity observations and $\langle I_{hk}\rangle$ is the mean intensity of reflections with unique indices $h$.

$^b$ $R_F = \frac{\sum |F_o| - |F_c|}{\sum |F_o|}$.  

binding site, comprising various regions of the protein (see Pai et al., 1990, Figure 5), and the effector region, residues 32-40, which has been shown to be involved in GAP binding. For the mutants analyzed here, no significant deviations from the cellular structure can be detected in the phosphate binding loop (except for G12V), for the Mg$^{2+}$ ion coordination (Figure 4A) and in the other regions involved in nucleotide binding, such as amino acids 28, 35, 116-119, and 144-146. For Lys-147, the structural changes observed do not affect the aliphatic interaction between Phe-28 and part of the side chain (C$_\alpha$ to C$_\gamma$). Some changes are seen in the effector region around Tyr-32, but they are due to the Gly-12 mutations of the symmetry-related molecules in the crystals and are thus not relevant to the protein structure in solution.

As seen in Figures 1A, 1B, 2, and 3, consistent changes for all the mutants are seen in loop L4 (residues 59-65). The high resolution analysis of cellular p21 (p21C) has shown that several residues in loop L4, such as Gin-61, Glu-62, Glu-63, and Tyr-64, are not well defined in the electron density map (Figure 4D). The crystallographic temperature factors for residues 61 to 67 are very high (54 Å$^2$ on average) for the cellular protein, much higher than those for the rest of the chain (16 Å$^2$ on average) (Pai et al., 1990, Figure 5).
Figure 4. Comparison of the Three-Dimensional Structures of Wild-Type and Mutant p21 Molecules

(A) The octahedral Mo$^{VI}$ ion coordination of the p21(Q61L) mutant in comparison to wild-type p21. The multicolored model is p21(Q61L); wild-type p21 is shown in red.

(B) Part of the effector region of p21(D38E) around Glu-38 in electron density at 15% of its maximum. The multicolored model is the mutant; the red model is wild type p21.

(C) Comparison of the phosphate binding region of p21, (multicolored), p21(G12V) (red), and p21(G12R) (blue). Loop L1 is at top left, loop L4 at top right, and the effector region at the bottom.

(D–F) Equivalent views of loop L4 (Gly-60, Gin-His-61, Glu-62, Glu-63, and Tyr-64) and the electron density at 10% of the maximum level for p21 (D), p21(Q61H) (E), and p21(G12V) (F). γ-phosphate and amino acid 12 are in the top right of the figure.

al., 1990), which means that this region is highly flexible. How many of the observed changes in L4 are due to the inherent flexibility in this region of the crystal structure (Pai et al., 1990) is unclear at the moment and will be clarified in future work using data collection at subzero temperatures. In p21, all of these residues occupy at least two different conformations. In the case of Gln-61, we have defined a conformation where it can activate a water mole-

Figure 3. Three-Dimensional Structures of p21 Mutants with Side Chain Movements

Grey shading illustrates the 1.5 Å changes; black the changes >1.5 Å (see Figure 2). For the cellular protein (p21c), the position of the nucleotide is indicated.
cule that is in a position to attack the γ-phosphate (Pai et al., 1990). Therefore, Gln-61 probably plays an important role in catalysis. We believe that, in addition to amino acids 32-40 (L2), the residues of loop L4 are involved in the interaction with GAP for the following reasons: this region is highly conserved, and it is situated in the vicinity of the effector region. It is located on the outside of the protein and points in the same direction as the effector region. Antibody Y13-259, which inhibits the biological activity of p21, has been shown to bind to this site (Furth et al., 1982; Lacal and Aaronson, 1986; Sigal et al., 1986). We could show that the effector region is not sufficient for a productive p21-GAP interaction, because the protein of the Krev-1 gene, which has the same sequence as p21 in the effector region but not in loop L4, is not activated by GAP, but binds strongly to it (Frech et al., 1990).

Below, we describe the significant changes around the phosphate binding loop, the effector region, and loop L4 separately for each group of mutants. These changes are located in the vicinity of the γ-phosphate, where they interfere with GTP hydrolysis.

Effector Region Mutant D38E

The region spanning amino acids 32 to 40 is commonly called "effector loop," although a better name would be effector region, because amino acids 37-40 are not in a loop but form part of the second β strand. This region is almost unchanged in p21(D38E) (Figures 2 and 3). Figure 4B shows residues 35-40 of the effector region in comparison with cellular p21. No differences can be seen, except for Ser-39 and the Asp-98+Glu mutation itself. Since p21(D38E) is unaltered in the nucleotide and Mg²⁺ ion binding site, it is not surprising that the nucleotide binding properties of the D38E mutant are identical to p21c (Cales et al., 1988).

As in the cellular protein, there are several conformations for residues 61-65 in loop L4. The side chain of Arg-68 in the mutant contacts residues 71, 59, and 60 in such a way as to fix this loop, whereas in wild-type p21 Arg-68 only contacts Gin-61.

In vivo, this mutant remains complexed to GTP, because its GTPase activity is not stimulated by GAP (Cales et al., 1988). We find, however, that p21(D38E) binds to GAP with almost wild-type affinity (Table 1; Gideon et al., unpublished data). It is generally assumed that any p21 protein that stays in the active conformation during the interaction with GAP is transforming, because the growth-promoting signal is not switched off. However, since p21(D38E) is not oncogenic (Cales et al., 1988), we have to assume that an interaction between p21-GTP and GAP per se is not sufficient to mediate the effect of p21 on cellular growth. Since p21(D38E) binds to GAP with wild-type affinity, but is not activated, we have to assume that Asp-38 is more important for p21 activation than for GAP binding.

Gly-12 Mutants G12R and G12V

An important part of the nucleotide binding site is the phosphate binding loop that connects the first β strand with the first α helix. This loop is highly conserved as the GXXXXGKSf motif in many nucleotide binding proteins (Gay and Walker, 1983; Wierenga and Hol, 1983; Dreusicke and Schulz, 1986; Fry et al., 1986). Conformational energy calculations on an N-terminal peptide of p21 indicate that a well-defined structural change takes place at Ala-11-Gly-12 on substituting Gly-12 by any other amino acid except proline (Pincus et al., 1983; Pincus and Scheraga, 1985). It has further been proposed that Gly-12 and Gly-13 have unusual dihedral angles that are forbidden for other amino acids (McCormick et al., 1995). However, we find that only Gly-10, Gly-13, and Gly-15 have such unusual dihedral angles, whereas Gly-12 has normal values (Pai et al., 1990). Thus it is not unreasonable to assume that Gly-12 can be substituted by other amino acids without destroying the geometry of the phosphate binding loop L1. Our results for the Gly-12 mutants confirm this assumption: for p21(G12R) the chain tracing is identical to that of cellular p21, and for p21(G12V) there are only slight shifts of the Cα positions of amino acids 12 (0.2 Å) and 13 (0.8 Å) (see Figure 4C). Similar findings also exist for the GDP complex of p21(G12V) (Milburn et al., 1990). In the triphosphate structure presented here, the large side chains of valine and arginine are the main differences in the phosphate binding loop between the mutants and p21c.

In p21(G12R), the guanidinium group is placed in front of the γ-phosphate and is involved in hydrogen bonding with one of its oxygens (Figures 4C and 5A). It takes the place of the water molecule WAT-175 in cellular p21, which is proposed to act as a nucleophile in GTP hydrolysis. The side chain of Arg-12 is also close to the carbonyl oxygen of Thr-35 and thus blocks the exit for GppNp release from the nucleotide binding pocket. This explains why the nucleotide dissociation is slower for this mutant (see Table 1). Due to the large side chain of Arg-12, Gln-61 cannot occupy either of the two conformations found in p21c and is pushed further away into the solvent. The new orientation of Gln-61 causes a complete reorientation of residues 62 to 65 in loop L4.

The structure of p21(G12V) was determined after photolysis of the complex with caged GTP and thus shows the structure of the "real" GTP complex. It has been shown that in the crystal, the p21(G12V)-GTP complex catalyzes the GTPase reaction with a rate constant similar to that found in solution (1.6 × 10⁻³ per min at room temperature compared with 3.0 × 10⁻³ per min at 37°C) (John et al., 1988; Schlüchting et al., 1989).

The structure of p21(G12V) is determined in roughly the same direction as that of Arg-12 (Figures 4C and 5B). Because of the branched nature of its side chain, it comes close to the oxygen atoms of the γ-phosphate and is thus pushed away. The distance between the main chain amide group of Gly-13 and the γ-bridging oxygen is now 3.7 Å instead of 3.0 Å in cellular p21 (Figure 4C). The mutation also clearly affects loop L4: the valine side chain would lead to steric hindrance with atoms C60, N60, and N61. This is relieved by shifting Ala-59, Gly-60, and Gin-61. Gly-60 can no longer form a hydrogen bond between its main chain amide and one γ-phosphoryl oxygen. Instead, it forms a hydrogen bond from its carbonyl oxygen to the side chain hydroxyl group of Tyr-96. Figures 2 and 3 show that the
Structures of H-ras p21 Mutants

G12V mutation also affects the other residues of loop L4. Furthermore, the electron density is much better defined for residues 61–65, which have only one major conformation. This seems to be caused by additional hydrogen bonds between Glu-62 and Tyr-64 and from the Arg-68 side chain to Tyr-64, Ala-59, and Tyr-71.

Glu-61 Mutants Q61H and Q61L

Of the five mutants analyzed, p21(Q61H) shows the smallest differences in structure compared with the cellular protein. As for the other mutants, the nucleotide binding and the effector region are similar to p21. Gln-61 in the cellular protein adopts at least two different conformations. In one of these, it can activate the attacking water molecule. Similarly, for His-61 we find three conformations, none of which is identical with the "catalytically active" conformation of Gln-61 in the cellular structure. Glu-62, Glu-63, and Tyr-64 also have conformations different from cellular p21.

The guanidinium group of Arg-68 interacts with the main chain carbonyls of Ala-59, Gly-60, Tyr-64, and Ser-65 (Figure 5C) and thereby stabilizes this conformation. These additional hydrogen bonds seem to reduce the flexibility in the L4 region (Figure 4E).

The other Glu-61 mutant, p21(Q61L), also has an identical main chain structure (Figure 1A), and only a few scattered changes in its side chains (Figures 2 and 3), which are again concentrated in loop L4. Stabilization of loop L4 occurs again through Arg-68, which makes additional hydrogen bonds to the main chain carbonyl oxygens of Tyr-64 and Ser-65. The nucleotide binding region is structurally well conserved.

Leu-61 in loop L4 has two conformations. One corresponds to the inactive position of Gln-61 in cellular p21, and in the other the side chain is even further away from the γ-phosphate. The Leu-61 side chain is hydrophobic and does not occupy a conformation where it would be very close to the γ-phosphate. This could be the reason why the nucleotide dissociation rates are much faster for this mutant (Table 1). Glu-61 is postulated to activate the nucleophilic attacking water molecule via the carbonyl group of its carbamoyl side chain (Pai et al., 1990), and the aliphatic side chain of Leu-61 would obviously not be able to perform the same function.

Discussion

The mutations described do not change the overall structure of the molecule. They only result in minor changes of some amino acid side chains. More significant differ-
We propose that the two mutants of position 61 analyzed here are oncogenic because the side chains of Leu-61 and His-61 cannot perform the same function as glutamine in cellular p21. Leu-61 is not able to activate the nucleophile, because of the chemical nature of its side chain, while His-61 probably cannot be oriented in such a position as to activate the water molecule. In the conformation nearest to that corresponding to Gln-61(2) in the model of p21(G12R) with threonine and find that the side chain oxygen of Thr-59 is within 1 Å of the nucleophilic water molecule. In the conformation involving the γ-phosphate, Arg-12 physically blocks access to the γ-phosphate and makes a hydrogen bond to one of the γ-phosphate oxygens, but it does not change the geometry of the phosphate binding loop. Val-12 with its hydrophobic branched side chain comes too close to the negatively charged γ-phosphate group. To relieve this unfavorable interaction, the geometry of the phosphate binding loop around Val-12 and Gly-13 is slightly perturbed. The main effect of the Val-12 mutation, however, is to push Gly-60 away, both of which are involved in γ-phosphate binding and the enzymatic reaction (Pai et al., 1990). These mutants have a reduced ability to undergo the conformational change, as shown for N-ras p21(G12D) by Neal et al. (1990) and for H-ras p21(G12V) by J. John (unpublished data). Thus the Gly-12 mutants are oncogenic because the larger side chains interfere with the "catalytically active" conformation of p21, although by different mechanisms.

Viral p21, which has an Ala-59→Thr mutation in addition to Arg 12, can be phosphorylated at Thr 60 (see Barbaoid, 1987). This is an autophosphorylation reaction, where the side chain oxygen is presumed to attack the γ-phosphate (John et al., 1988). We have substituted Ala-59 in the model of p21(G12R) with threonine and find that the side chain oxygen of Thr-59 is within 1 Å of the nucleophilic water molecule. This would explain why Thr-59 autophosphorylates, but at a reduced rate as compared with the GTP hydrolysis reaction (John et al., 1988).

Since oncogenic mutants of p21 are found in approximately 30% of human tumors, and since p21 is the most frequently activated oncogene associated with human malignancies, it may be worthwhile to develop drugs that would specifically inhibit the oncogenic activity of p21 ras proteins. Considering the high degree of homology between cellular and mutant structures, where the GTP binding site in the interior of the protein is almost unperturbed, it is unlikely that rational antitumor drug design directed toward modifying the guanine nucleotide would achieve such a goal. From the structure, it appears that only sub-

![Figure 6. Schematic View of the Active Site of p21](image)
otients of the nucleotide that reach into the active center around Gln-61 could be potential lead compounds for such an approach. It may be more appropriate to search for drugs that modify the p21–GAP interaction. This interaction is suggested to involve loop L2 and L4 of p21 (Frech et al., 1990; Pai et al., 1990), which are both located on the outside of the molecule. Alternatively, if the conformational change undergone by loop L4 is also the rate-limiting step of the GTPase reaction for the p21 mutants, and if their actual GTPase reaction is in fact much faster than measured in vitro, it would be advantageous to look for chemical compounds that enhance the GTPase reaction by inducing this structural rearrangement and thus switching off the growth-promoting signal.

Our studies on D38E have shown that an interaction between p21 and GAP is not alone sufficient for cellular transformation. This could mean that GAP is not the effector molecule, but just a negative regulator that keeps the concentration of p21-GTP low. It could also mean that GAP alone is not the effector molecule, but that other compounds like p190 or p62 (Molloy et al., 1989; Ellis et al., 1990) are involved in such an interaction, which would be modified by the D38E mutation. Another explanation could be that GAP catalyzes the conformational change in p21 that is important for GTP hydrolysis. In turn, GAP itself would undergo a conformational change, and it would be this structural change that is disturbed by the effector loop mutant p21(D38E).

**Experimental Procedures**

**Purification and Crystallization**

The p21 mutants were prepared as described (Tucker et al., 1990; John et al., 1988). They contain a tightly bound nucleotide, mostly GDP (Poe et al., 1985; Feuerstein et al., 1987). This nucleotide was exchanged for the slowly hydrolyzing GTP analog GppNp as described (John et al., 1990) and crystallized in general according to Scherer et al. (1989), using standard buffer with 10 mM MgCl₂. For the mutants Gl2V, Gl2Y, G61H, and G61L, PEG 1450 was replaced by 30% (w/v) PEG 400 (Serva). To obtain single crystals of G61L, it was necessary to use seeding techniques.

Crystals of the G12V mutant complexed to GppNp grew to large sizes but did not diffract to better than 3 Å resolution. The structure of this mutant was determined in a different way: p21(G12V) was crystallized as a complex with caged GTP, which is a photolabile precursor of GTP (Schlichting et al., 1989). A Xenon flash lamp was used to remove the protecting group in the crystal by photolysis (Schlichting et al., 1989). A complete data set of the resulting p21(G12V),GTP complex was taken at 4°C. At this low temperature, GTP hydrolysis is extremely slow (half-life at 25°C is 433 min). After data collection the nucleotide content of the crystal was verified by high pressure liquid chromatography to be 80% GTP and 20% GDP (Serva). To obtain single crystals of Q61L, it was necessary to use seeding techniques.

**Data Collection and Crystallographic Refinement**

All X-ray diffraction data from cooled (4°C) crystals were recorded on the program X-PLOR (Brünger et al., 1987). After one or two cycles of minor rebuilding using FRODO (Jones, 1978) adapted for an IRIS 4GT work station by C. M. Cambillau and further refinement, water molecules were fitted to the electron density, which resulted in a drastic improvement of the R-factor. The water molecules occupy similar sites in the different mutants.

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**References**


