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Comparison of the Computed Three-Dimensional Structures of Oncogenic Forms (Bound to GDP) of the *ras*-Gene-Encoded p21 Protein with the Structure of the Normal (Non-Transforming) Wild-Type Protein

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The *ras*-oncogene-encoded p21 protein becomes oncogenic if amino acid substitutions occur at critical positions in the polypeptide chain. The most commonly found oncogenic forms contain Val in place of Gly 12 or Leu in place of Gln 61. To determine the effects of these substitutions on the three-dimensional structure of the whole p21 protein, we have performed molecular dynamics calculations on each of these three proteins bound to GDP and magnesium ion to compute the average structures of each of the three forms. Comparisons of the computed average structures shows that both oncogenic forms with Val 12 and Leu 61 differ substantially in structure from that of the wild type (containing Gly 12 and Gln 61) in discrete regions: residues 10–16, 32–47, 55–74, 85–89, 100–110, and 119–134. All of these regions occur in exposed loops, and several of them have already been found to be involved in the cellular functioning of the p21 protein. These regions have also previously been identified as the most flexible domains of the wild-type protein and have been bound to be the same ones that differ in conformation between transforming and nontransforming p21 mutant proteins neither of which binds nucleotide. The two oncogenic forms have similar conformations in their carboxyl-terminal domains, but differ in conformation at residues 32–47 and 55–74. The former region is known to be involved in the interaction with at least three downstream effector target proteins. Thus, differences in structure between the two oncogenic proteins may reflect different relative affinities of each oncogenic protein for each of these effector targets. The latter region, 55–74, is known to be a highly mobile segment of the protein. The results strongly suggest that critical oncogenic amino acid substitutions in the p21 protein cause changes in the structures of vital domains of this protein.

KEY WORDS: p21 protein; oncogenic forms; conformations; molecular dynamics.

1. INTRODUCTION

The *ras*-oncogene-encoded p21 protein is a G-protein, involved in mitogenic signal transduction, which becomes activated by exchanging GDP for GTP (Barbacid, 1987). This event is induced by

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the binding of extracellular growth factors to receptors coupled to activating proteins that ultimately promote the GDP–GTP exchange (Barbacid, 1987). Activated p21 then binds to other proteins in a signal transduction process that results in mitogenesis.

If amino acid substitutions occur at critical positions in the protein, the protein becomes constitutively active and causes malignant transformation of cells (Barbacid, 1987). These substituted proteins have been implicated in causing many human tumors (Almoguerra *et al.,* 1988). Microinjection of oncogenic but not normal p21 proteins into cell lines such as NIH 3T3 cells results in malignant transformation (Stacey and Kung, 1984). When microinjected into oocytes, these proteins induce meiotic division and cell maturation (Birchmeier *et al.,* 1985). The most common oncogenic forms of p21 are those with substitutions at Gly 12 and Gln 61, both of which have been implicated in binding to the β- and γ-phosphates of GDP and GTP, respectively (Pai *et al.,* 1990).

Several proteins are known to interact directly with p21 intracellularly. These include the SOS guanine nucleotide exchange protein (Chardin *et al.,* 1993), GTPase activating protein (GAP) (Trahey and McCormick, 1987; Vogel *et al.,* 1988), the serine/threonine kinase *raf*-p74 protein (Moodie *et al.,* 1993; Leevers *et al.,* 1994), phosphatidylinositol-3-OH-kinase (Rodriguez-Viciana *et al.,* 1994), and a protein of MW 43 kD that stimulates oocyte maturation (Lee *et al.,* 1989; Baskin *et al.,* 1992). GAP is known greatly to enhance an inherent GTPase activity in the normal p21 protein resulting in hydrolysis of bound GTP to GDP, thereby returning the protein to an inactive state (Barbacid, 1987). GAP, *raf*-p74, and phosphatidylinositol-3-OH kinase have been implicated in binding to p21 in an effector domain involving residues 32–47 (Adari *et al.,* 1988; Leevers *et al.,* 1994; Rodriguez-Viciana *et al.,* 1994).

One mechanism by which oncogenic amino acid substitutions are thought to result in the activation of p21 is prolonged binding of oncogenic forms to GTP since a number of mutant forms exhibit markedly diminished GTPase activities (Barbacid, 1987). A linear free energy relationship has been established between the catalytic rate constants for the GTPase activity of wild-type and different mutant p21 proteins and the pK_a values of the terminal phosphate of GTP bound to these different p21 proteins (Schweins *et al.,* 1995). Wild-type and nontransforming proteins were found to have lower pK_a'*s* and higher GTPase activities.

However, proteins with mutations at position 61 apparently did not fit this linear free energy correlation. Also, one mutant form of p21, which contains Glu in place of Asp 38, binds to GTP and lies on the linear free energy plot, but its GTPase activity is *not* enhanced by binding to GAP, although this mutant protein binds with high affinity for GAP. This protein does not transform cells (Krengel *et al.,* 1990). Furthermore, three different triply substituted mutant p21 proteins have been cloned, one of which transforms cells and two of which are nontransforming (Clanton *et al.,* 1987). None of these three proteins binds nucleotide.

In the original studies that demonstrated that p21 binds to *raf*-p74 (Moodie., 1993), it was shown that the latter protein bound most strongly to Val 12-p21 bound to GTP and hardly at all to wild-type (Gly 12-)p21 bound to GDP. It associated with an enhanced affinity to Val 12-p21 bound to GDP, which, however, was not so strong as its affinity for Val 12-p21 bound to GTP.

All of these findings suggest that oncogenic amino acid substitutions in the p21 protein are sufficient to induce critical conformational changes in these proteins that result in their enhanced activation. In some but not all of these mutant forms, it is possible that the binding of GTP further stabilizes the activated (oncogenic) forms.

In this paper, we study whether oncogenic amino acid substitutions in the p21 protein bound to GDP can cause changes in the structure of the protein that might result in its permanent activation. We then compare these results with those of previous studies on the normal, wild-type protein (Dykes *et al.,* 1993) and on the triply substituted mutants that do not bind nucleotide (Liwo *et al.,* 1994).

2. METHODS

The coordinates for the wild-type p21 protein residues 1–171, obtained for the p21–GDP complex from an energy refinement procedure described elsewhere (Dykes *et al.,* 1993), were used as the starting structure for constructing the conformations of the two mutant transforming proteins, Val 12- and Leu 61-p21. For both mutant proteins, the

substituted amino acid was introduced in the energy-minimized wild-type structure in place of the normally occurring amino acid at that position, i.e., Val for Gly 12 or Leu for Gln 61. The backbone dihedral angles for each substituted residue were the same as for the corresponding amino acid in the wild-type protein. Each side chain was generated in a conformation that was the lowest energy one for the given backbone conformation as determined from the single-residue minima for these amino acids (Vasquez *et al.*, 1983; Zimmerman *et al.*, 1977) from the Empirical Conformational Energies for Peptides Program (ECEPP) (Nemethy *et al.*, 1983).

Each starting conformation was then subjected to energy minimization using the program AMBER (Weiner and Kollman, 1981; Weiner *et al.*, 1984, 1986) and then ECEPP (Nemethy *et al.*, 1983) in four stages. In the first stage, only the substituted amino acid was allowed to move under energy minimization. In the second stage, to determine the lowest energy conformation for the side chain of the substituted amino acid, the side chain was subjected to a 50-psec cycle of molecular dynamics. The lowest energy equilibrated conformation was then used as the starting point for further energy minimizations. In the third stage, the substituted amino acid and five neighboring amino acids on its amino- and carboxyl-terminal ends were allowed to move under energy minimization. Finally, in the fourth stage, energy minimization was performed in which the entire structure was allowed to move.

In these calculations, the united atom approach was used in which explicit hydrogen atoms were present only on polar atoms (Weiner *et al.*, 1984). The resulting energy-minimized structures were then used as starting conformations for molecular dynamics simulations. In all energy minimizations and moelcular dynamics simulations, the effects of solvation were simulated using a distance-dependent dielectric constant of the form $D_0(1 + r_{ij})$, where r_{ij} is the distance between atoms i and j (Weiner and Kollman, 1981; Weiner *et al.*, 1994).

For each energy-minimized structure computed using the above procedures, molecular dynamics simulations were then performed. Initially, the temperature of the system was raised from 0 to 300K in 25-deg increments every 5 psec. After temperature equilibration at 300K, a dynamics simulation run was performed for 100 psec. The energy of the system converged to a minimum value after a maximum of 25 psec. The last 50 isoenergetic structures were used to compute the average structure, the atomic fluctuations for each structure, and residue coordinate fluctuations as described previously (Dykes *et al.*, 1993). The average structures for each of the three proteins, the wildtype and the Val 12 and Leu 61 mutants, were then directly superimposed on one another such that the root-mean-square deviation of the coordinates of the backbone atoms of one average structure (e.g., the Val 12 mutant) from those of the reference structure (e.g., the Gly 12 mutant) was a minimum.

3. RESULTS AND DISCUSSION

3.1. Starting Structures

Energy-minimized structures were obtained for all three proteins which differed in overall rms deviation by < 2 Å. The largest deviation was found for residues 10–16 of the Val 12 protein, in which a reverse turn formed at residues 12 and 13 rather than at positions 11 and 12 as found for the Gly 12- and Leu 61-p21 structures. Molecular dynamics simulations were performed for each of these three structures, and the average structures computed as described in Section 2.

3.2. Comparison of Average Structures from Molecular Dynamics

As shown in Fig. 1, the average structures superimposed on one another, the maximal overall rms deviation being 2.5 Å between the Val 12- and the Gly 12-p21 structures. Similar rms deivations of the average structures from their corresponding energy-minimized starting structures were also found. However, in contrast to the superposition of the energy-minimized structures, the relatively low overall rms deviations obscure highly significant deviations in the backbone (and side chain) coordinates for specific segments of the proteins. Figure 2 shows the individual residue backbone deviations for the coordinates of corresponding amino acid residues of the Val 12-p21 (dotted line) and Leu 61-p21 (solid line) average structures from those of the Gly 12 (wild-type) average structure.

Major deviations for both structures are seen to occur at residues 10–16, 32–47, 55–74, 85–89, and 119–134. Lower deviations occur from residues

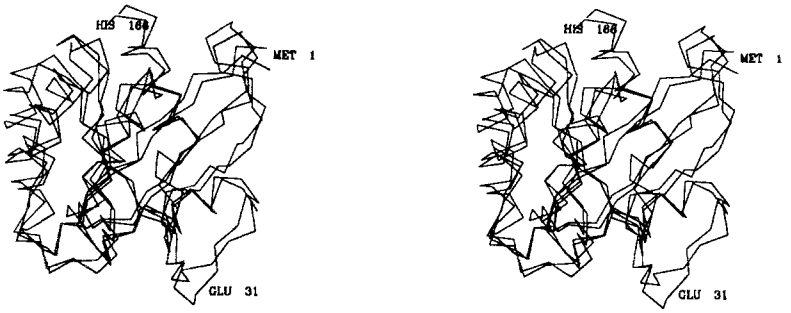

Fig. 1. Stereoview of the superimposed C^{α} traces of the average structures of the Gly 12- and Val 12-p21 proteins (residues 1–166) obtained from molecular dynamics simulations. The numbered residues have been placed on the Gly 12 average structure.

100–108. The largest deviations occur on the amino-terminal half of these two proteins.

3.3. Functional Importance of Corresponding Segments of Differing Structure

As shown in Fig. 3, all of these regions occur on surface loops of the protein, suggesting that they may be involved in interacting with target proteins intracellularly and may constitute effector domains of the protein. Residues 32–40 (and possibly more residues toward the carboxyl end of this sequence) have been implicated in interacting with at least three critical intracellular proteins (see above); residues 58–71 have been implicated as being important in protein activation (Barbacid, 1987) and as constituting part of the epitope to which the inactivating antibody Y13-259 binds (Barbacid, 1987; Furth *et al.*, 1982); and residues 102–103 have been implicated in interacting with guanine nucleotide exchange factors (Willumsen *et al.*, 1991).

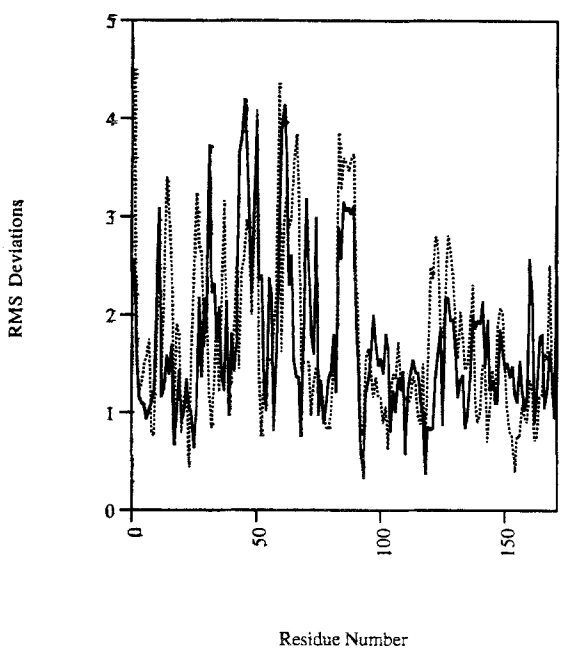

Fig. 2. Plot of the individual residue backbone deivations for the coordinates of corresponding amino acid residues of the Val 12-p21 (dotted line) and Leu 61-p21 (solid line) average structures from those of the Gly 12 (wild-type) average structure.

That these regions may be critical to the functioning of the p21 protein is supported by experimental studies in which peptides containing some of these regions, namely 35–47 (Lee *et al.*, 1990; Chung *et al.*, 1991), 96–110, 115–126 (Haspel *et al.*, 1992), were found strongly to inhibit oocyte maturation promoted by microinjected oncogenic p21 protein. The results found in these calculations indicate that the region from 84 to 89 may also be critical as an effector loop in the protein.

3.4. Comparisons with Previous Results

In a previous molecular dynamics study of the Gly 12-p21 protein bound to GDP and surrounded with 494 water molecules using the program DISCOVER (Dauber-Osguthorpe *et al.*, 1988), it was found that the same regions, with the exception of residues 85–89, were the most flexible, i.e., had the highest coordinate fluctuations (Dukes *et al.*, 1993). It was predicted that these regions were the most likely to undergo conformational changes upon the introduction of oncogenic amino acid substitutions and/or the binding of GTP to the protein. The results shown in Fig. 2 confirm this prediction.

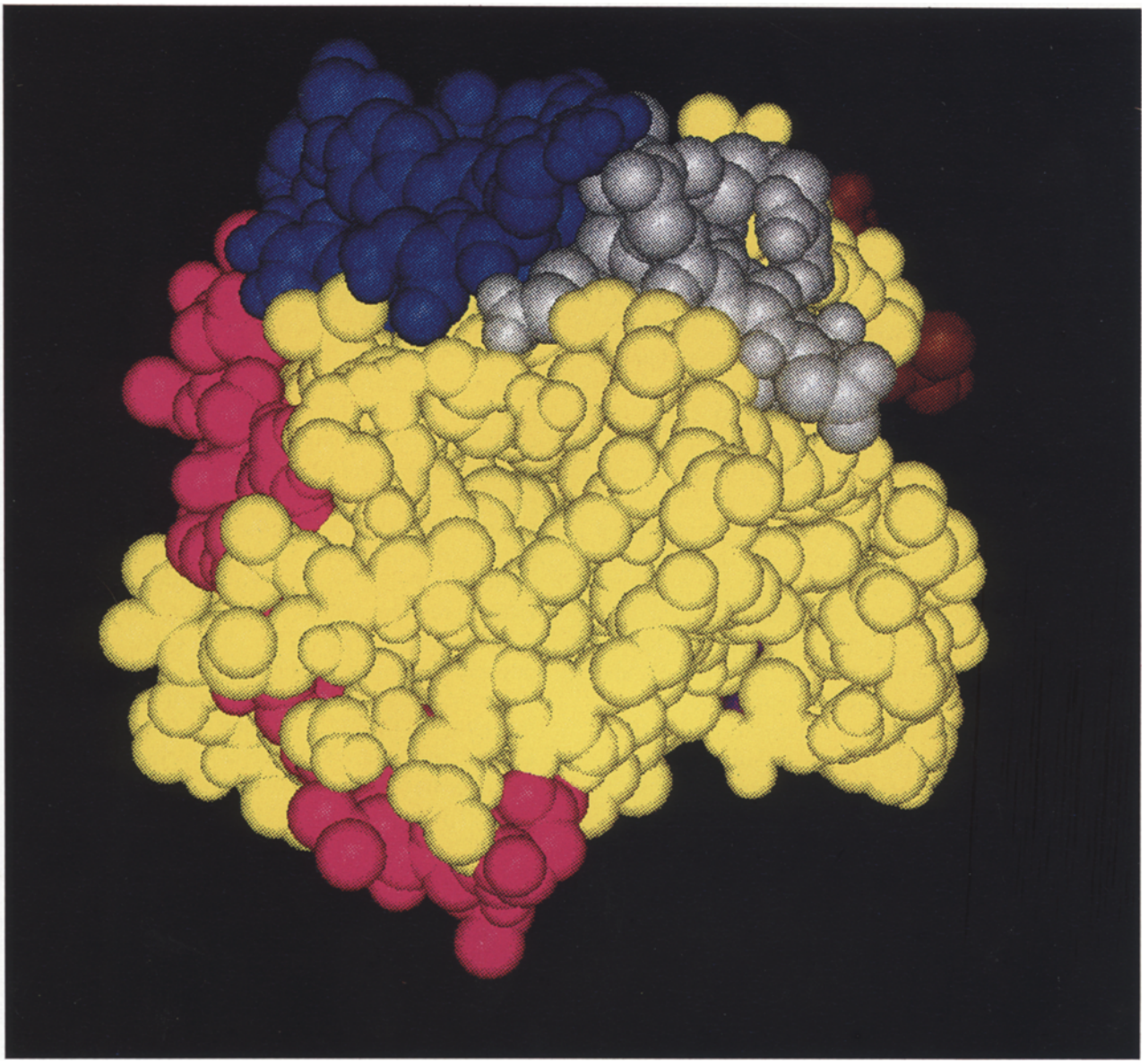

Fig. 3. Color space-filling view of the regions of the two oncogenic proteins that deviate in structure the most from corresponding segments of the Gly 12 protein. The color code is as follows: light purple, residues 32–47; dark blue, 58–73; red, 85–89; gray, 100–108; a small patch of dark purple may be seen on the bottom right of the figure showing residues 10–16 at the back of the molecule in this view. Residues 119–134 occur on the opposite side of the molecule in this view and are therefore not seen in the figure.

These findings are further supported by the results of a previous theoretical study (Liwo *et al.*, 1994) on the low-energy conformations of two non-nucleotide-binding p21 proteins, one of which was oncogenic and the other nononcogenic using a completely different search technique, the electrostatically driven Monte Carlo (EDMC) method (Piela and Scheraga, 1987; Ripoll and Scheraga, 1988). In this study, the two proteins were found to differ maximally in the identical regions, although the deviations around positions 100–110 were lower than found in this study (Liwo *et al.*, 1994).

Agreement on the regions of structural differences between oncogenic and nononcogenic forms of the p21 protein has thus now been obtained using three independent methods—

molecular dynamics using DISCOVER and AMBER, and EDMC based on ECEPP—each of which utilizes different models of solvation.

3.5. Similarities and Differences Between the Average Structures of Oncogenic Proteins

Since the two oncogenic proteins were found to differ in structure in surface loop regions from the wild-type protein, we next compared the average structures of the two oncogenic forms with one another. The results are summarized in Fig. 4. While the two structures are superimposable on one another (rms deviation = 2 Å), they differ significantly in three regions: residues 10–16, 32–47, and 55–74. On the other hand, as can be seen in Fig. 4, there is a sharp drop in the residue deviations after residue 76; the two structures lie closer to one another in the carboxyl-terminal domain of the protein. For example, in the region 85–89, the coordinates coincide.

Examples of the comparison of the structures of the loop regions for the three proteins are shown in Fig. 5 for the regions 10–16, 32–47, 55–74, and 83–91. The wild-type protein is shown in red, the Val 12-p21 mutant in blue, and the Leu 61-p21 mutant in purple. In the region 10–16, the Leu 61- and Gly 12-p21 proteins are more similar in structure to one another than to the Val 12 segment, most likely because both the Gly 12 and Leu 61 segments have identical sequences in this region, while the Val 12 protein contains a critical substitution (Val for Gly 12).

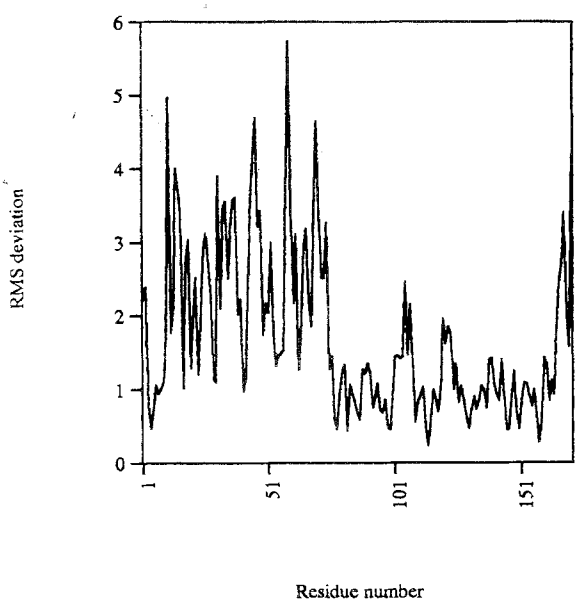

Fig. 4. Plot of the average residue coordinate deviations of the Leu 61-p21 structure from the corresponding coordinates of the Val 12-p21 structure. The two structures compared were the average structures computed for both proteins.

Inspection of the structures for the 10–16 region reveals that one major cause of the difference in conformation between the Val 12 segment and the other two segments is the *absence* of a reverse turn in the Val 12 segment at Ala 11–Gly 12 which is present in the Gly 12 and Leu 61 protein segments. In a previous paper, we predicted that the normal (wild-type) p21 protein with Gly 12 would adopt a bend conformation at positions 11 and 12. Substitution of any noncyclic L-amino acid at position 12 was predicted to eliminate the bend at positions 11 and 12 (Pincus *et al.*, 1983; Pincus and Scheraga, 1985). These results support this conclusion.

As shown in Fig. 5, the structures of the three proteins deviate from one another in the two critical regions, 32–47, implicated in binding to GAP and *raf*-p74 proteins and to phosphatidylinositol-3-OH-kinase, and in the 55–74 segment. The structure of the segment that deviates the most in the 32–47 region is the one from the Leu 61 protein. As discussed in the next section, the coordinate fluctuations for this segment in the Leu 61-p21 protein are the lowest for the three proteins, possibly indicating that this conformation is the activated structure that interacts with downstream target proteins.

Since the 32–47 region has been implicated in interacting with three different proteins on the mitogenic signal transduction pathway, namely GAP, *raf*-p74, and phosphatidylinositol-3-OH kinase, another possible reason for the differences in average structure in the 32–47 region of the two oncogenic proteins is that each protein may interact preferentially with a *different* target protein. In this connection, it is known that certain oncogenic proteins such as the Leu 61-p21 protein bind with increased affinity to GAP compared with other oncogenic forms of the protein (Krengel *et al.*, 1990). In fact, the affinity constant of the Leu 61-p21 protein for binding to GAP is on the order of 200 times that of Val 12-p21. The two different average structures for this region for the Val 12- and Leu 61-p21 proteins may therefore reflect differing conformations involved in the interactions of this region with GAP. Possibly, the alternate conformation for the Val 12-p21 protein

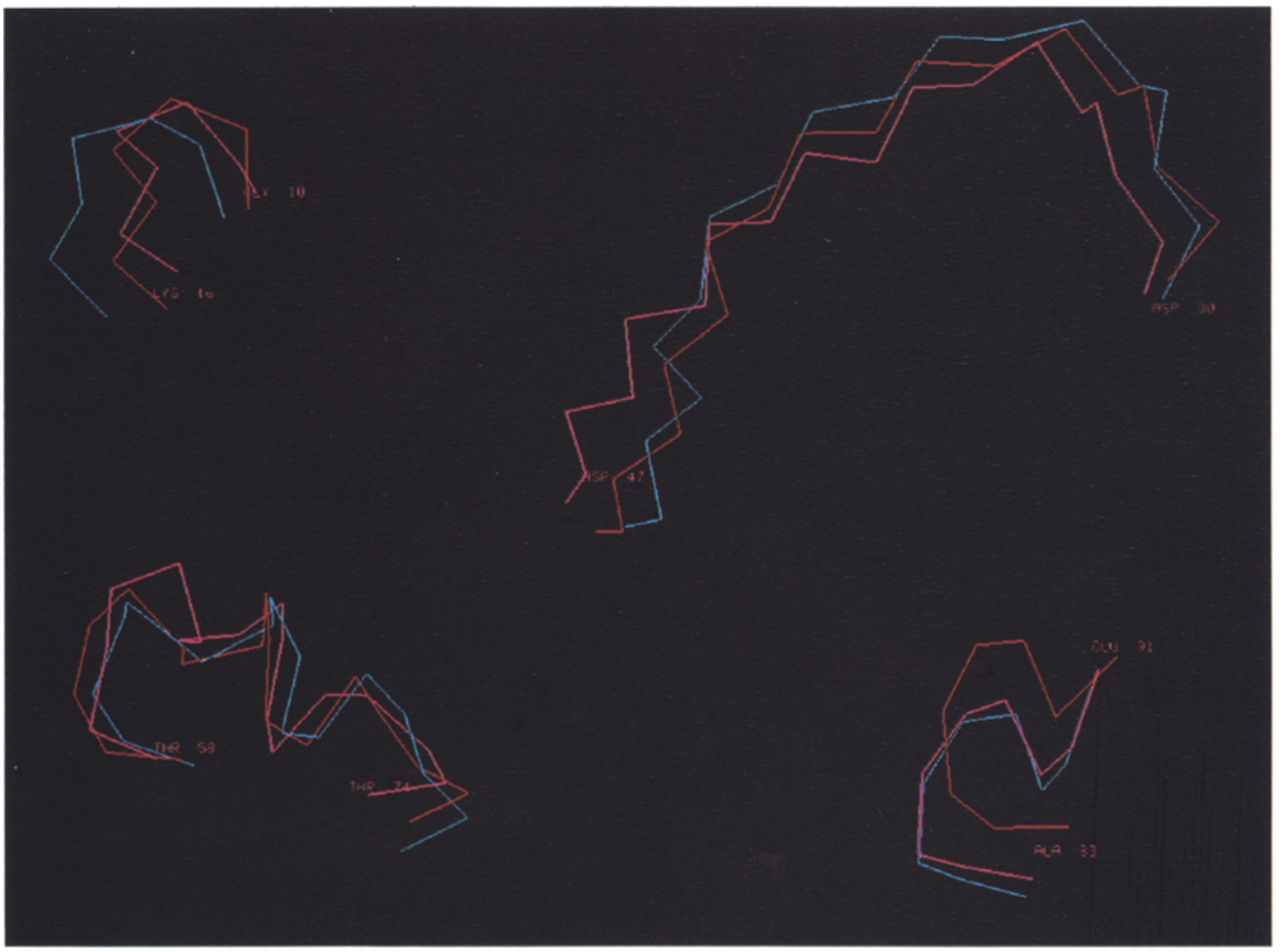

Fig. 5. Comparison of the structures of different regions of the Gly 12-p21 (red), Val 12-p21 (blue), and Leu 61-p21 (purple) proteins for residue 10–16, 32–47, 55–74, and 83–91. These regions were found to differ between the two oncogenic (Val 12 and Leu 61) and normal (Gly 12) proteins.

in this region may favor its preferential binding to one of the other two downstream effector proteins.

As can also be seen in Fig. 5, all three proteins differ in structure in the region 55–74. This region is the least well-defined crystallographically because its thermal motion is high (high *B*-factor) (Krengel *et al.*, 1990). As discussed in the next section, of the three proteins, the Leu 61-p21 protein has the highest fluctuations for the region around Leu 61 (1.5 Å). Both Gly 12- and Val 12-p21 proteins were computed to have lower fluctuations (<1 Å). High fluctuations in this region of the protein may correlate with transmission of conformational changes in the amino-terminal domain of the protein to the carboxyl-terminal domain (Liwo *et al.*, 1994).

As shown in Fig. 5, the two oncogenic proteins, Val 12-p21 (blue) and Leu 61-p21 (purple) adopt identical structures for the region 83–91, while the Gly 12-p21 (red) protein segment adopts a substantially different structure. Very similar results (not shown) were obtained for the segments 100–108 and 119–134. Overall, therefore, the oncogenic proteins exhibit structural differences in the amino-terminal half of the protein, but show strong structural similarities in the carboxyl-terminal half of the protein.

3.6. Coordinate Fluctuations for the Three Proteins

Figure 6 shows the average residue fluctuations for each of the three proteins studied. Despite

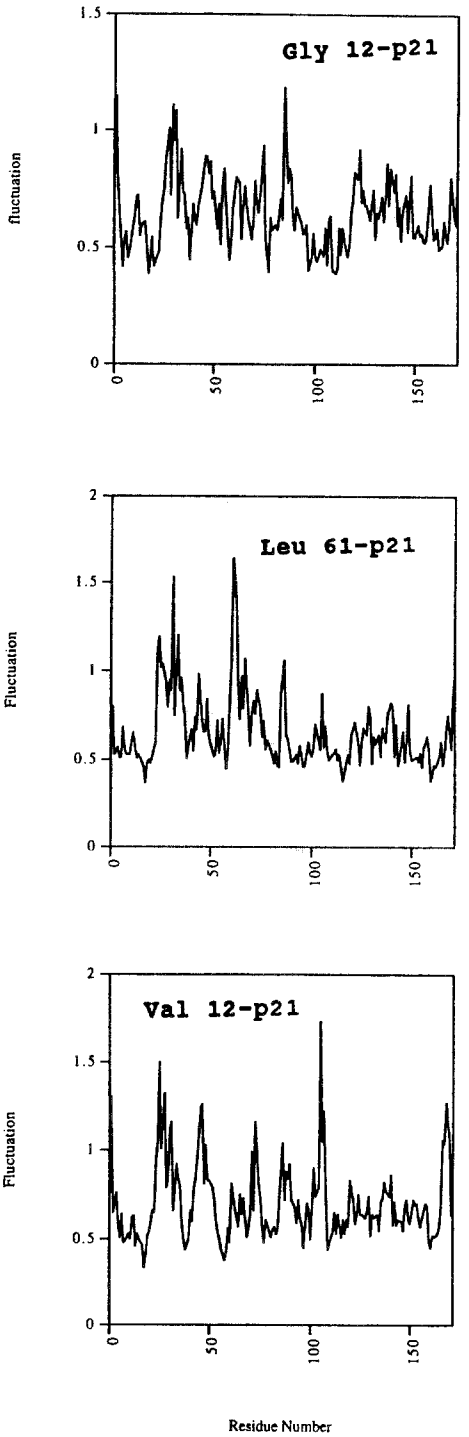

Fig. 6. Plots of average residue deviations for (top) Gly 12-p21, (middle) Val 12-p21, and (bottom) Leu 61-p21.

differences from a previous study (Dykes *et al.*, 1993) with respect to the potential functions (AMBER in this study versus DISCOVER in the previous study) and the specific model of solvation (use of a distance-dependent dielectric constant in this study versus inclusion of explicit water of hydration in the previous study), the fluctuations for the coordinates of the wild-type Gly 12 structure were found to be identical to those obtained in the previous study (Dykes *et al.*, 1993).

Overall comparison of the coordinate fluctuations for all three proteins reveals that the two oncogenic proteins show higher fluctuations in the loop regions discussed above. For the Gly 12-p21 structure, only two regions were computed to have fluctuations greater than 1 Å, namely 32–47 and 100–108. In computations on the flexibility of Pro 12-p21, which is nononcogenic (Seeburg *et al.*, 1984), the coordinate fluctuations for this protein were found to be even lower than for the Gly 12 protein (results not shown).

Compared with the Gly 12 protein, the Val 12-p21 protein was found to have fluctuations that were greater than 1.5 Å for residues 32–47 and 100–108 and, in addition, around residues 70–75 and 85–89. The Leu 61-p21 structure showed relatively high flcutautions in the 32–47 region (>1.5 Å), in the Leu 61 region (>1.5 Å), and 85–89 (Fig. 6).

These increased fluctuations of the oncogenic proteins may reflect the presence of activated structures that contribute to the average structure. The oncogenic substitutions may lower the energy of the activated conformations relative to the conformational energy of the "resting" or inactive conformations. These activated structures would be of sufficiently higher energy for the Gly 12-p21 protein that they would make a significantly lower contribution to the average structure and hence a lower contribution to the coordinate fluctuations.

In a previous study on the low-energy structures for two triply substituted non-nucleotide-binding p21 proteins, one of which was oncogenic and the other nononcogenic, we found that the oncogenic protein exhibited high fluctuations in the region around Gln 61 while the nononcogenic protein showed much lower fluctuations (Liwo *et al.*, 1994). We further found that, for the oncogenic protein, changes in structure in the carboxyl-terminal domains such as the 100–108 and 119–134 domains correlated uniquely with changes in structure in the 55–74 region. We concluded that the 55–74 segment is a "conformation switch" region that may be involved in propagating conformational changes in the amino-terminal domains to those in the carboxyl-terminal regions of the protein.

If these conclusions are valid, from the results obtained in this study, there may be several different ways in which the 55–74 region can propagate the activating conformational changes from one part of the protein to the other. Increased motion of any part of the 55–74 segment may increase the likelihood of protein activation.

4. CONCLUSIONS

Substitution of amino acids (Val for Gly 12 or Leu for Gln 61) for the normally occurring amino acids at critical positions in the polypeptide chain in the p21 protein causes changes in the conformation of specific regions of the protein that are the ones that were found to be the most flexible for the normal (Gly 12, Glm 61)-p21 protein. Both oncogenic proteins show increased flexibility relative to that of the wild-type protein, most likely because the activated form(s) of the protein contribute significantly to the average structure.

There are significant differences in structure between the two oncogenic proteins, especially in the 32–47 and 55–74 segments. Since the former segment has been implicated in interacting with at least three target intracellular proteins, our results suggest that each of these proteins may show different affinities for each of the target proteins.

Both oncogenic proteins differ in structure from one another, but exhibit high flexibility at different residues in this region: the Val 12 protein shows high flexibility around residue 74, while the Leu 61 protein shows high flexibility around residue 61. Since increased flexibility of this region seems to correlate with activation of the protein, we conclude that amino acid substitutions that decrease mobility of this region may cause the protein to become nononcogenic even with substitutions at position 12 or 61. Prior results on a p21 protein that contains Pro 61 (Der *et al.*, 1986) but does not transform cells support this conclusion. Because of its limited conformational flexibility due to the pyrrolidine ring, this residue limits the motion of the entire segment (Chen *et al.*, 1989).

We have also identified another region of the p21 protein that may be involved in the activation process, viz residues 85–89. These residues adopt the same structure in both oncogenic proteins that differs significantly from that of the same segment in the wild-type protein. Peptides containing these residues may inhibit (or possibly enhance) the effects of microinjected oncogenic p21 protein in cells.

The above conclusions are based on the assumption that regional differences between the average structures of the wild-type and oncogenic proteins computed from molecular dynamics are the ones that result in activation of the oncogenic proteins. It is possible, however, that activated structures are ones of significantly higher energy that may be kinetically more accessible to the mutant proteins than to the wild-type protein or which may be of lower energy compared with that of the mutant proteins. Further, it is possible that the activated structure may be only one of several structures on the dynamics trajectory that does (do) not contribute significantly to the average structure. The differences between mutant and wild-type proteins observed would then be due to differences in nonactivated structures.

The results obtained from these and preceding calculations, however, indicate that the regions where structural differences occur are the same regardless of the oncogenic substitutions and regardless of whether the p21 protein binds nucleotide (Liwo *et al.*, 1994). These regions are the same ones that are the most flexible in the wild-type protein. Furthermore, peptides containing these structurally different regions strongly inhibit the mitogenic effects of oncogenic p21 (Chung *et al.*, 1991; Haspel *et al.*, 1992). These considerations support our conclusion that the above regions are the ones involved with protein activation, and the average structures of the oncogenic proteins are those of the activated proteins.

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