Molecular Switch for Signal Transduction: Structural Differences Between Active and Inactive Forms of Protooncogenic *ras* Proteins

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***Ras* proteins participate as a molecular switch in the early steps of the signal transduction pathway that is associated with cell growth and differentiation. When the protein is in its GTP complexed form it is active in signal transduction, whereas it is inactive in its GDP complexed form. A comparison of eight three-dimensional structures of *ras* proteins in four different crystal lattices, five with a nonhydrolyzable GTP analog and three with GDP, reveals that the "on" and "off" states of the switch are distinguished by conformational differences that span a length of more than 40 Å, and are induced by the γ-phosphate. The most significant differences are localized in two regions: residues 30 to 38 (the switch I region) in the second loop and residues 60 to 76 (the switch II region) consisting of the fourth loop and the short α-helix that follows the loop. Both regions are highly exposed and form a continuous strip on the molecular surface most likely to be the recognition sites for the effector and receptor molecule(or molecules). The conformational differences also provide a structural basis for understanding the biological and biochemical changes of the proteins due to oncogenic mutations, autophosphorylation, and GTP hydrolysis, and for understanding the interactions with other proteins.**

AMONG THE COMMONLY FOUND ONCOGENES IN HUMAN cancer cells are the members of the *ras* oncogene family (*1*). In this family the difference between proto-oncogenes and oncogenes is often a point mutation at or near amino acid residue 12 or 61 of the proteins encoded by the genes (*2*). In analogy to translation elongation factor, EFTu (*3*), and signal transducing G proteins (*4, 5*), the function of *ras* proteins, according to the current view, is that an extracellular signal for cell growth is received by a presumed transmembrane receptor protein (or proteins), which directly or indirectly induces exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) in the *ras* protein inside the cell. The GTP complex is then recognized by an effector protein (or proteins) as an indication for the growth signal. Thus, conformational changes from the GDP to the GTP bound state of *ras* proteins represent the molecular switch from the "off" to the "on" state signaling cell growth. Most of the in vivo transforming *ras* oncoproteins have lost both their intrinsic capacity to hydrolyze GTP (*2*) and their GAP [GTPase (guanosine triphosphatase) activating protein]-mediated GTPase activity (*6*), and, therefore, are thought to be stuck in the signal "on" state. This prolongs the transmission of the growth signal resulting in unregulated cell growth.

The mechanism of the molecular switch is best understood from a comparison of the three-dimensional structures of both GDP and GTP bound forms of the *ras* proteins. Any conformational differences found by the comparison of the crystal structures can be divided into two categories: (i) those resulting from differences in crystal packing, and therefore, the environment around each molecule of the compared structures, and (ii) the functional differences induced by the presence of the γ-phosphate in the GTP complex. To distinguish these two effects, we compared three crystal structures of GDP complexes in two different crystal lattices and two of GTP analog complexes in two different crystal lattices, in one of which there are four independent molecules per asymmetric portion of the unit cell. We now present our findings on the identification of the regions of conformational conservations and differences between GDP bound (inactive) and GTP bound (active) forms of the protooncogeneic *ras* proteins, and on the structural basis for understanding oncogenic mutations, autophosphorylation, and GTP hydrolysis. We can also define the molecular surface that is the best candidate for the recognition site for the effector molecule (or molecules), GAP, and possibly for an upstream regulator such as a receptor. In addition, we identify specific regions of the molecule as potential sites for interactions with other components of the signal transduction pathway.

Crystal structures of GDP complexes and of GTP analog complexes. We have previously determined the crystal structures of a GDP complex of the catalytic domain (residues 1 to 171) of the normal (proto-oncogenic) and of a transforming (oncogenic) form of human c-H-*ras* protein (*7, 8*); a subsequent correction of the structures has been reported (*9*). Both molecules crystallized in the

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Table 1. Crystal parameters and refinement statistics for GDP and GTP analog complexes. GDP · Gly^{12}, GDP · Val^{12}, and GDP · intact are the GDP complexed in normal catalytic domain, transforming catalytic domain (glycine mutated to valine at residue 12), and intact protein of c-H-*ras* proteins, respectively. GDPCP · Gly^{12} and GDPNP · Gly^{12} (*18*) are GTP analog complexes of c-H-*ras* catalytic domains. All crystal structures have one molecule per crystallographic asymetric unit except GDPCP · Gly^{12}, which has four. R factors were calculated with a low resolution cutoff of 6.0Å for GDP · Gly^{12}, GDP · Val^{12}, and GDP · intact; 10.0 Å for GDPCP · Gly^{12}, and GDPNP · Gly^{12}.

Protein complex	Space group	Unit cell (Å)	Total reflections	Resolution (Å)	R factor (%)	rms		Total H_2O	B factor			
						Bond (Å)	Angle (°)		Overall ($Å^2$)	Loop 2 ($Å^2$)	Loop 4 ($Å^2$)	α2 ($Å^2$)
GDP · Gly^{12}	$P6_522$	$a = b = 83.2$, $c = 105.1$	10,837	2.2	19.2	0.026	2.5	38	28.9	38.6	59.7	44.3
GDP · Val^{12}	$P6_522$	$a = b = 83.3$, $c = 105.0$	10,447	2.2	19.6	0.027	2.9	40	29.9	38.1	58.7	46.4
GDP · intact	I4	$a = b = 97.8$, $c = 41.9$	4,951	2.6	19.7	0.024	3.5	0	26.4	27.8	37.2	30.3
GDPCP · Gly^{12}	$P2_1$	$a = 41.5$, $b = 80.1$ $c = 130.5$, $\gamma = 117.5°$	21,061	2.5	20.4	0.029	3.6	0	30.9*	35.4*	42.7*	37.6
GDPNP · Gly^{12}†	$P3_221$	$a = 40.3$, $c = 162.2$	5,378	2.6	22.9							

*Average B factors for all four molecules. †Pai *et al.* (*18*).

same crystal lattice. Both structures are being refined with x-ray diffraction data to 2.2 Å resolution using a combination of the X-PLOR (*10*) and TNT (*11*) refinement programs. The current R factor for both structures is about 19 percent (Table 1). Some of the bound water molecules have been identified, but residues 60 to 68 in one loop are located in weak electron density. The three-dimensional structures are practically identical except at residue 12, the oncogenic mutation site.

Using a full-length human c-H-*ras* gene product, we recently determined the crystal structure of a GDP complex in a different crystal lattice. The structure was determined by the molecular replacement method (*12, 13*), and has been refined to a current R factor of about 20 percent at 2.6 Å resolution. At present, the carboxyl terminus appears partially disordered (*14*). Crystallographic parameters and refinement statistics for these three GDP complexes are shown in Table 1.

The topological structure of these GDP complexes is the same, and the overall structures are the same except for residues 60 to 68 and the COOH-terminal ends (Fig. 1). Root-mean-square (rms) differences among Cα positions of the three structures, excluding residues 60 to 68, are about 0.5 Å. However, the rms difference for residues 60 to 68 between the two catalytic domains and the intact protein complex are 5.7 and 6.9 Å. We attribute this difference of the GDP complexes to a difference in the environment of two different crystals and to flexibility of this region. There are additional, significant conformational differences in loop 2 and helix α2 when compared to GTP analog complex structures (see below).

We have now determined the crystal structure of a complex between a nonhydrolyzable GTP analog and the catalytic domain of normal human c-H-*ras* protein. The crystals were grown of the protein complexed with guanosine-5′-(β,γ-methylene) triphosphate (GDP-CP), by the vapor phase equilibrium method. Rotation data were collected on x-ray films with synchrotron radiation at the Brookhaven National Laboratory and processed (*15*). The crystals belong to space group $P2_1$ (Table 1) with four molecules in an asymmetric unit.

A series of conventional rotation-translation searches (*12, 13*) failed to yield a unique solution. This is understandable, because the probe structure used in the rotation search was less than one-fourth of the content of an asymmetric unit. The structure was finally determined by means of a new molecular replacement method incorporating Patterson correlation refinement (*16*). The probe used for the rotation-translation search was the crystal structure of the GDP complex of the *ras* catalytic domain (*7, 9*) excluding GDP (*17*). Briefly, the 120 best rotation search peaks were refined by Patterson correlation refinement with three different resolution shells (each from 15 Å to 4, 3.5, and 3 Å, respectively). The combined result revealed four orientations of the probe molecule. A translation search was done to find the location of one probe; then by fixing the probe molecule and searching for the remaining three, all four solutions corresponding to the locations of the four independent molecules were found. The correctness of the structure thus determined was verified when we found good electron density for GDP-CP in all four independent molecules, although the probe did not contain the GDP-CP.

The four crystallographically independent GDP-CP complex molecules have the same backbone conformation except for residues 62 to 65 in the loop 4 region, which are located in weak electron density. This again suggests that the conformation of the latter part of loop 4 is flexible and sensitive to the crystal environment. The crystallographic R factor is currently 20.4 percent at 2.5 Å resolution (Table 1). Among the four molecules, two have better defined electron density in loop 2. We use these two in subsequent comparisons. The crystal structure of a complex between a different GTP analog, guanosine 5′-(β,γ-imido)triphosphate (GDP-NP), and *ras* protein lacking the COOH-terminal 23 residues has been described; the structure was refined at 2.6 Å resolution with an R factor of 22.9 percent. (*18*). A visual comparison between the backbone structure of our GDP-CP complex structure and the GDP-NP complex, which is in a different crystal lattice, shows that the two structures are similar except for residues 61 to 65 in loop 4,

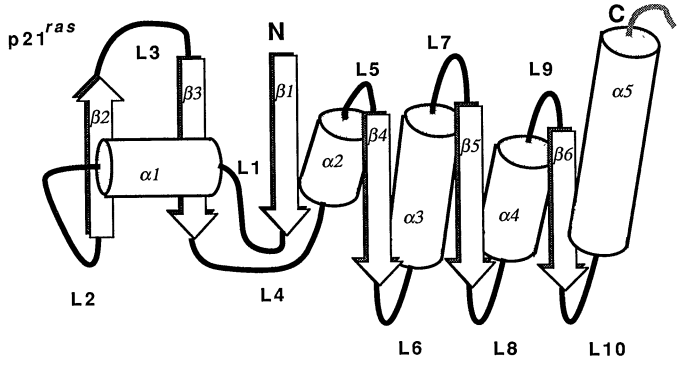

Fig. 1. Topological structure of *ras* proteins. Both GDP and GTP analog complexes of *ras* proteins have the same topological structure. β-strands and α-helices are represented by arrows and cylinders respectively. The current assignments of the beginning and the ending residue numbers for each secondary structural element are; β1 (1–9), β2 (38–46), β3 (50–58), β4 (77–84), β5 (110–117), β6 (140–144), α1 (15–26), α2 (67–75), α3 (87–104), α4 (126–137), α5 (151–171), L1 (10–14), L2 (27–37), L3 (47–49), L4 (59–66), L5 (76), L6 (85–86), L7 (105–109), L8 (118–125), L9 (138–139), and L10 (145–150). The COOH-terminal 18 residues that are lacking or disordered in the crystal structures are represented as a shaded string.

again suggesting that the difference in this region is due to flexibility or differences in crystal environment (or both). When compared to the GDP complex structures, all five GTP analog structures, four with GDP-CP and one with GDP-NP, show additional significant conformational differences in loop 2 and α2 (see below).

The guanine nucleotide pockets are largely conserved. Except the γ phosphate of the GTP analogs, all the interactions between protein and guanine nucleotide are the same in all eight molecules in four different crystal lattices mentioned above (*7, 8, 9, 17, 18*). Briefly, the guanine base is interacting with the side chains of residues 28, 116, 117, and 119, and the backbone of residue 146; the ribose is interacting with the side chain of residue 117 and the backbone of residue 29; the α-phosphate is interacting with the backbone of residue 18; the β phosphate is interacting with the backbone NH groups of residues 13 or 14 to 17 and the side chain of Lys^{16} (in the GDP-CP complex, the hydrogen bond from residue 13 is lost because of the presence of the methylene group); and the Mg^{2+} ion coordinates to the side chain of Ser^{17} and to one of the oxygens of the β phosphate. In GTP analog complexes, Mg^{2+} makes additional coordination to Thr^{35} and the γ phosphate (Fig. 2). Furthermore, the positively charged side chain of Lys^{16} is surrounded by the carbonyl oxygens of residues 10 and 11 (*18*, and our data) and one of the oxygens of the β phosphate (and γ phosphate in the GDP-CP complex).

At the present stage of refinement, the positions of the β phosphate and the Mg^{2+} ion in the GDP complex (which is coordinated to oxygens of both β and γ phosphates in the GDP-CP complex) appear slightly shifted by about 0.4 Å from those in GDP-CP complex. The ribose conformation in GDP and GTP analog complexes is 2′-endo (*18, 19*), and the orientations and locations of the guanosines are very similar. It remains to be seen whether these small differences are significant.

Confinement of conformational differences between GDP and GTP complexes to two surface regions. A least-squares match of Cα positions of the GDP and GDP-CP complexes of the normal c-H-*ras* protein indicates that the largest differences between the two structures are localized in two regions. The first region, named switch I, spans residues 30 to 38 and corresponds to most of the residues in loop 2, and the second region, switch II, consists of residues 60 to 76 of loop 4 and α-helix 2 (Figs. 3 and 4); these are two regions whose conformation "switches" when GTP replaces GDP in the protein. Therefore, pairwise matches of the structures were recalculated (by a least squares method) for all Cα positions except those two regions. Root-mean-square (rms) differences for all possible pairwise matches are shown in Table 2. For each match, rms differences for the switch I and II regions were calculated separately. The switch II region was further divided into loop 4 (L4) and α-helix 2 (α2) to assess relative differences. The results show that all residues outside the two switch regions match very well (rms of 0.17 to 0.67 Å) among all pairwise matches, including matches between the *ras* proteins complexed with GDP and GDP-CP.

Although the switch I region is well exposed, the temperature factors of the residues in this region in the GDP complex of intact protein and the GDP-CP complex of *ras* protein catalytic domain are at about the same level as the average value for the entire molecules (Table 1), suggesting that this region is fairly well defined in these complexes. In the GDP-CP complex molecules, the side chains of all residues in this region were identifiable even in the electron density map calculated from the structure, but without GDP-CP, loops 2 and 4, and helix α2. The conformation of this region is similar in three GDP complex molecules as manifested by small rms differences (0.6 to 0.7 Å) in Table 2. This is true even though they are in two different crystal forms. Likewise, the conformation of the corresponding region in the GTP bound state is also conserved (rms differences of 0.8 to 0.9 Å) among the four independent molecules

Table 2. Root-mean-squares differences in corresponding Cα positions in angstroms. GTP-B, GTP-C, GDP-F1, GDP-F2, and GDP-I stand for molecule B and C (out of four independent) of human c-H-*ras* protein catalytic domain complexed with GDP-CP, GDP complexes of the normal (Gly^{12}) and transforming (Val^{12}) c-H-*ras* protein catalytic domains, and normal c-H-*ras* intact protein, respectively. Each matrix element has two values. In the lower triangle of the matrix the first values are rms differences for all Cα atoms except those for residues 30 to 38 and residues 60 to 76, and the second values are those for residues 30 to 38. In the upper triangle of the matrix, the rms differences for residues 60 to 76 are further divided into two parts: the first and second values represent rms differences for residues 60 to 68 and for residues 69 to 76, respectively. The small rms values in parentheses reflect that fact that GDP-F1 and GDP-F2 are crystallographically isomorphous (see Table 1).

	GTP-B	GTP-C	GDP-F1	GDP-F2	GDP-I
GTP-B	0	3.57 1.29	7.42 1.62	7.42 1.72	6.98 1.64
GTP-C	0.64 0.92	0	6.49 2.32	7.05 2.43	7.39 2.34
GDP-F1	0.65 2.58	0.64 2.68	0	(2.30 0.29)	6.87 0.77
GDP-F2	0.65 2.59	0.66 2.70	(0.17 0.22)	0	5.67 0.66
GDP-I	0.71 2.56	0.76 2.60	0.51 0.66	0.50 0.59	0

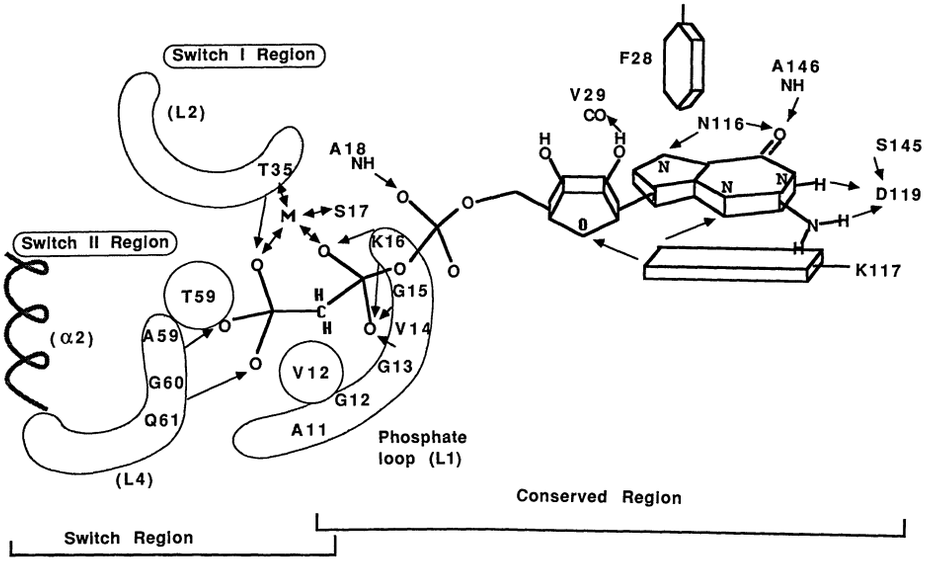

Fig. 2. Schematic drawing of the environment of the guanine nucleotide found in the crystal structures of GDP and GDP-CP complexes of normal and oncogenic human c-H-*ras* proteins. The nucleotide is contained in a pocket composed primarily of loops L1, L2, L4, L8, and L10. Interaction between the proteins and the GDP portion of the nucleotide is conserved. The switch region, which changes its conformation on GDP-GTP exchange, is composed of L2, L4, and α2, and the former two interact with the γ phosphate of GDP-CP. Loop L1 surrounds the β phosphate of the guanine nucleotide, and the backbone NH groups of the loop form hydrogen bonds to the phosphate. Other hydrogen bonds and coordination bonds are indicated by single and double-headed arrows respectively. The observed side chain positions of Val^{12} and Thr^{59} (*9*) are also shown schematically. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M. Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

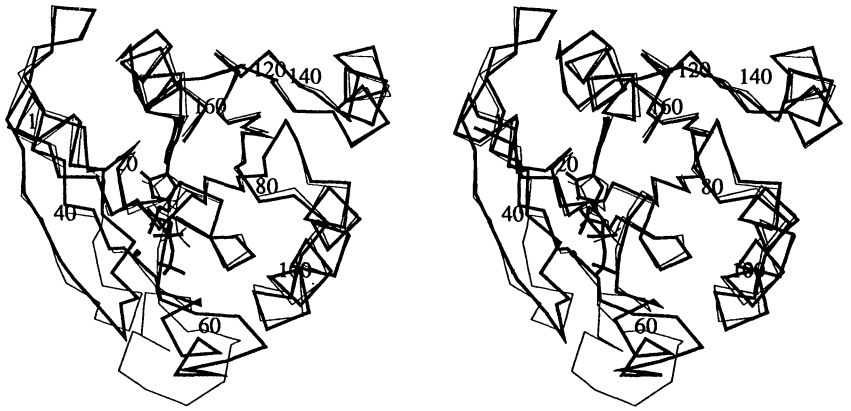

Fig. 3. A stereo view showing the superposition of Cα positions (residues 1 to 163) of GDP complex (thin lines) and GDP-CP complex (thick lines) of the catalytic domain of normal human c-H-*ras* protein. Looking down the guanine nucleotide binding pocket, two regions of major structural differences are localized in residues 30 to 38 and 60 to 76.

of the GDP-CP complex, and, on visual comparision of Cα positions, also between the GDP-CP (our data) and GDP-NP (*18*) complexes.

In contrast, the conformational difference in the switch I region between the GDP complex and the GTP analog complexes is quite large as indicated by relatively large rms differences in Cα position (2.6 to 2.7 Å) (Table 2). This is seen in a stereo view of the match between the GDP and GDP-CP complexes (Fig. 3). The detail of the conformational difference in the switch I region is shown in Fig. 5. The most prominent differences are (i) a hydrogen bond between the side chains of Tyr^{32} and Tyr^{40} in the GDP complex is broken in the GDP-CP complex, and the side chain of Tyr^{32} now swings out, covering a part of the phosphate pocket of GDP-CP and partially blocking the entrance of the guanine nucleotide pocket; (ii) the side chain of Thr^{35} of loop 2 in the GTP analog complex is coordinated to the Mg^{2+} ion (*18*, and our data) and hydrogen bonded to the γ phosphate, while the same residue is pointing out toward the solvent in the GDP complexes; and (iii) the side chain orientations of residues 36 and 38 in the GDP-CP complex are substantially different from those in the GDP complexes. Such extensive conformational differences can certainly represent two different states of the molecular switch that can be distinguished by the effector protein (or proteins). This notion is consistent with the earlier observation that mutations in this loop alter transforming activity of oncogenic mutants, thus suggesting this region as "putative effector recognition region" (*20*).

Another large difference between the GDP and GTP analog complex structures appears in the switch II region of residues 60 to 76, corresponding to loop 4 and the following helix, α2. The difference in helix α2 is primarily manifested in a difference in orientation of this helix with respect to the rest of the molecule in the two structures (Fig. 3). By far the largest difference is in the region of residues 60 to 68 (rms range of 3.6 to 7.4 Å). Weak electron density is associated with a portion of this region in both the GDP and GTP analog complexes (residues 60 to 65 in the GDP complex of the intact protein, and residues 62 to 65 in four independent molecules of the GDP-CP complex), suggesting high flexibility of this portion. The magnitude of the difference, however, is comparable to that of the same region between GDP complexes in different crystal lattices. Therefore, it is difficult to distinguish differences in a part of loop 4 due to crystal packing and flexibility of the region from those due to the γ phosphate of GDP-CP (if any). We conclude that helix α2 (and possibly some part of loop 4) is the second region of the molecular switch which distinguishes GTP bound states from GDP bound states of *ras* proteins.

Two specific interactions are noteworthy. The backbone NH groups of residues 60 and 61 are in positions to form hydrogen bonds to the γ phosphate. This portion of the sequence is highly conserved among all *ras* and *ras* related proteins, and similar backbone-phosphate interactions are observed in another conserved region, namely, residues 12 to 18 (Fig. 2). The functional importance of the switch II region is indicated by the observation that the second most commonly found oncogenic mutation site, residue 61, is located at the beginning of this region, and that the monoclonal antibody Y13-259, which binds to this region (*20*), neutralizes the transforming activity of oncogenic mutants.

Although only one residue, Thr^{35}, of loop 2 interacts with the γ phosphate in the GTP analog complexes (Fig. 2), the switch I region covering almost the entire loop 2 has a different conformation (not induced by different crystal lattices) from that in the GDP complexes. A similar extended conformational change is seen for the switch II region; the backbone of residues 60 and 61 interacts with the γ phosphate, inducing conformational changes in the entire loop 4 and reorientation of helix α2. Thus, the presence of the γ phosphate induces concerted changes in two separate regions in the amino acid sequence of the molecule propagating the conformational changes over a span of 40 Å. This "conformational domino effect" suggests that the entire length is conformationally linked; if one end (for example, helix α2) of this span changes its conformation, the change may propagate, like a row of dominos, to the guanine nucleotide pocket and beyond to the other end (for example, the effector site). Both regions are on the surface of the molecule.

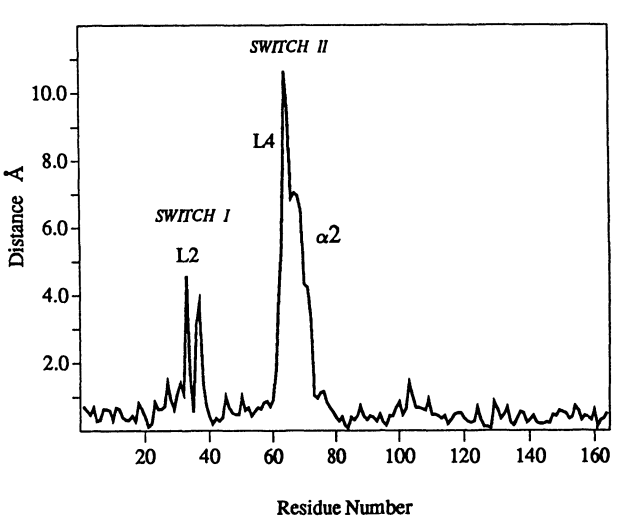

Fig. 4. Distances between corresponding Cα atoms in the crystal structures of GDP complex of the intact protein and GDP-CP complex of normal human c-H-*ras* proteins are plotted as a function of residue numbers. Two major differences are localized to switch I region in L2 and switch II region (L4 and a2).

Structural correlations to biological and biochemical functions. Examination of the crystal structures of the seven normal (Gly^{12}) proteins and one transforming (Val^{12}) catalytic domain supports reasonable correlations between the observed structural features and the known biochemical and biological functions of the *ras* proteins, such as oncogenic mutations, autophosphorylation of viral *ras* protein, GAP interaction, antibody induced neutralization of *ras* function, and GTP hydrolysis. Comparison of all the guanine nucleotide complexes of *ras* protein whose structures have so far been determined reveals that the conformation of the phosphate binding loop, L1, is identical within experimental error. [Our earlier result (*8*) that L1 conformation of normal (Gly^{12}) and transforming (Val^{12}) *ras* protein is different was due to an error in the tracing of the loop (*9*)]. As pointed out earlier, the backbone NH groups of residues 13 or 14 to 17 of this loop and the side chain of Lys^{16} are involved in hydrogen-bonding to the β phosphate. However, a superposition of loop 1 of the GDP-CP complex of normal (Gly^{12}) protein and the GDP complex of oncogenic (Val^{12}) *ras* protein reveals that the hydrophobic side chain of Val^{12} would be in contact with the highly hydrophilic and charged γ phosphate of GDP-CP, creating an energetically unfavorable situation (Fig. 6A). This may result in changes in the catalytically favorable position and orientation of the γ phosphate. Furthermore, the side chain of Val^{12} partially blocks the entrance of the guanine nucleotide pocket, thus possibly preventing the entry of a nucleophilic attacking group or departure of the γ phosphate after hydrolysis (or both). This observation suggests that other substitutions of Gly^{12} would create a similar situation, thus decreasing GTPase activity of the mutants. On the other hand, the observed oncogenic activation by substitution of Gly^{13} by valine or aspartate (*2*) may arise from distortion of the loop 1, which binds β phosphate. This model provides a qualitative structural explanation for the earlier observation that the substitution of Gly^{12} or Gly^{13} by almost any amino acid except proline endows the *ras* protein with transforming activity (*21*). Preliminary model building with proline at these positions show little steric blocking of the entrance or distortion of the loop.

Oncogenic *ras* proteins encoded by retroviral *ras* genes have one additional mutation at residue 59, substituting threonine for alanine, and this threonine is autophosphorylated (*22*). The location of this residue in both GDP complexes and GTP analog complexes is approximately the same, and is near the γ phosphate in the structure of the GDP-CP complex (Fig. 6B). This close proximity immediately suggests that the hydroxyl group of threonine at this position would be a good acceptor of the γ phosphate resulting in a covalently bound phosphate on the threonine. It can even be possible, in case of the virally encoded protein, that the side chain of Thr^{59}, polarized by other side chains or water nearby, may act as an attacking group in the hydrolysis reaction.

GAP binds to GTP complexes of normal and transforming mutants, but catalyzes GTPase activity of normal *ras* proteins only (*6, 23, 24*). Whether GAP is an upstream negative regulator or downstream effector of *ras* proteins is still uncertain. GAP sensitivity of various mutants (*25–28*) suggests that a large portion of switch I and a part of switch II regions are involved with GAP interaction.

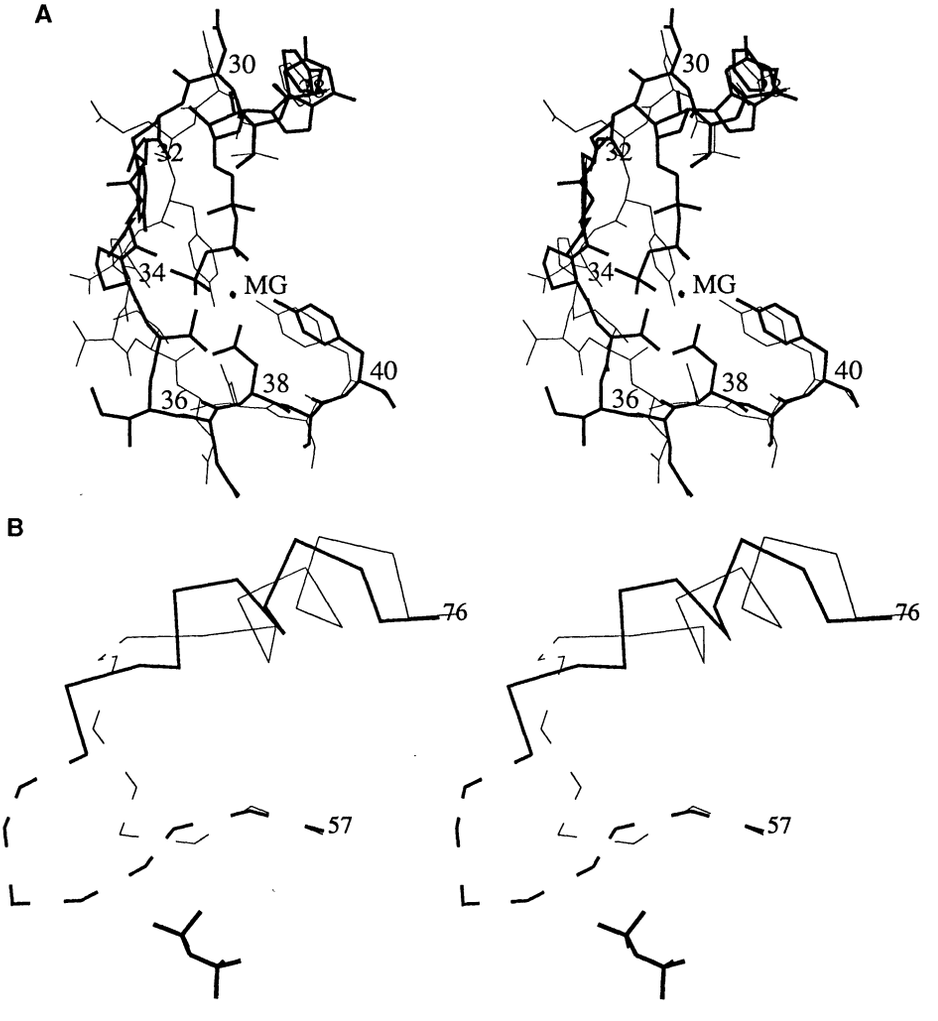

Fig. 5. Stereo views showing the details of conformational differences in switch I region (**A**) and switch II region (**B**) of GDP complex (thin lines) and that of GDP-CP complex (thick lines) structures. In (B) only Cα positions are shown for clarity. Flexible regions are shown in broken lines.

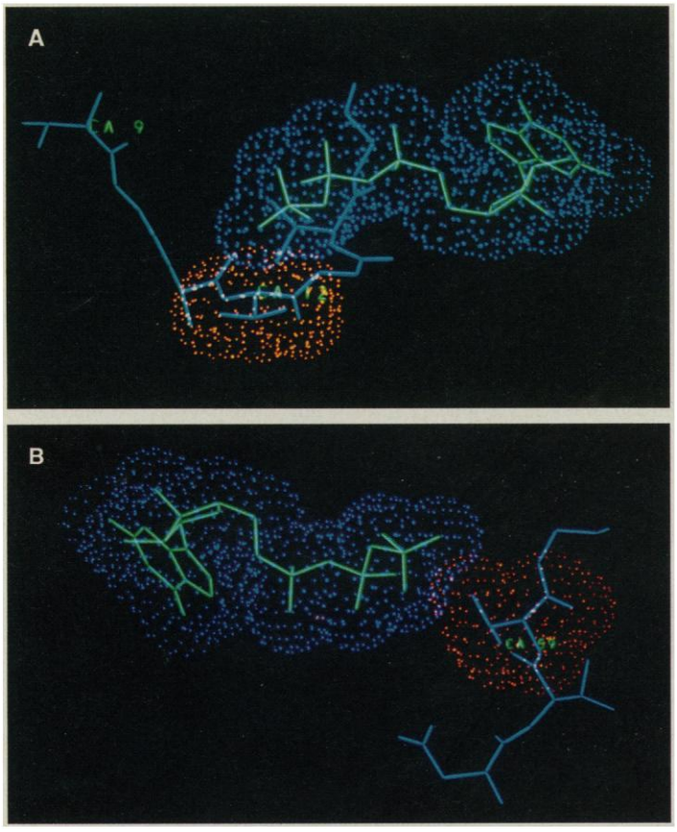

Fig. 6. Molecular models showing proximity between the γ phosphate of GDP-CP and the side chains of Val^{12} and Thr^{59}. (**A**) Loop 1 of a GDP complex crystal structure of a transforming (Val^{12}) *ras* protein is superposed to that of GDP-CP complex of normal (Gly^{12}) protein to show that the van der Waal's surface (in orange) of Val^{12} side chain contacts that of the γ phosphate (in blue). (**B**) A side chain of threonine was attached to Cβ of Ala^{59} in the crystal structure of GDP-CP complex to show the proposed proximity of Thr^{59} (in orange) of viral *ras* protein to the γ phosphate (in blue).

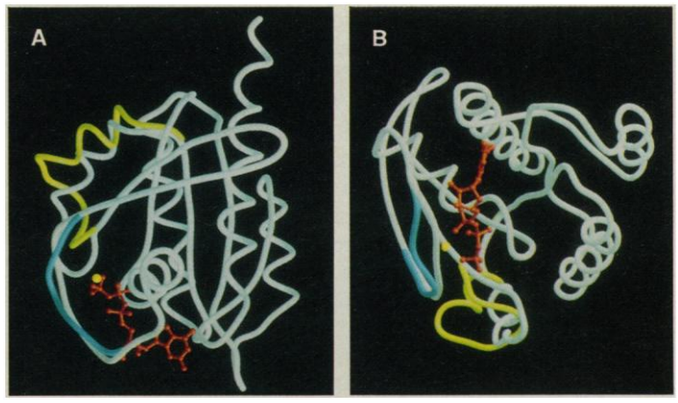

Fig. 7. Two views of the backbone structure of normal human c-H-*ras* protein showing two regions of conformational differences. The backbone of the GDP-CP complex is in white and that of switch I and II of the GDP complex are in blue and green, respectively. Guanine nucleotide is in red, and Mg^{2+} ion is shown as a yellow sphere.

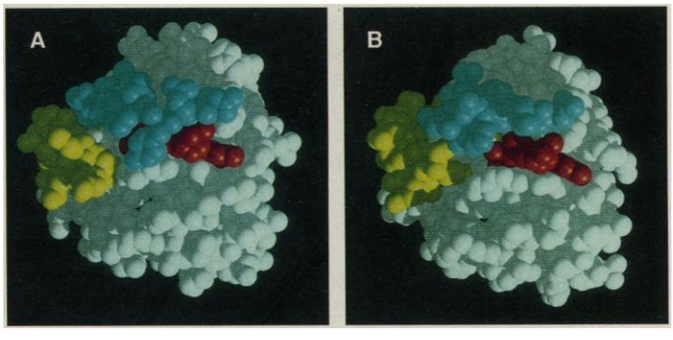

Fig. 8. Switch I (blue) and II (green) regions form a continuous patch on the molecular surface of normal human c-H-*ras* proteins. These are two regions whose conformation changes on GDP-GTP exchange. Guanine nucleotide is in red. (**A**) GDP-CP complex and (**B**) GDP complex of catalytic domain of human c-H-*ras* protein.

When Thr^{35} was mutated to alanine, the mutant was no longer sensitive to GAP. This can be explained by the fact that, unlike Thr^{35}, alanine cannot interact with Mg^{2+} or the γ phosphate of the GTP to induce the conformational changes of the switch I region that are required for GAP recognition. However, substitution by serine partially preserves GAP sensitivity, presumably because serine may still be able to interact with Mg^{2+} and the γ phosphate. Furthermore, mutations at residue 36 and 38 are known to eliminate GAP sensitivity, but not for residues 39 and 40, consistent with our finding that the switch I region does not extend beyond residue 38.

The epitope for the neutralizing antibody, Y13-259, has been identified as the region of residues 63 to 73 (*20*). Our GDP-CP complex structure shows that this region covers most of the switch II region, and is not a part of the guanine nucleotide binding pocket. Furthermore, the structure shows that the residues 63 to 70 (except 68), and 73 are well exposed. Of these, residues 63 to 65 are in weak electron density, but the remaining residues are on the exposed side of helix α2. These observations are in agreement with earlier studies showing that mutations at residues 63, 65, 66, 67, 70, and 73 inhibit binding of the antibody to *ras* proteins (*20*), and that the antibody does not affect guanine nucleotide binding or GTP hydrolysis. This epitope is a part of the switch II region, which has a different conformation in the GDP and the GDP-CP complexes. We propose that conformational changes of this region are necessary for GDP-GTP exchange (that occurs either by enhancing dissociation of bound nucleotide and subsequent GTP binding because of higher intracellular concentration of GTP, or by catalyzing the exchange process directly). This interpretation is consistent with the observation that the antibody binding inhibits GDP-GTP exchange (*29*). A simple explanation for the neutralizing effect of the antibody would be that the antibody binding to *ras* proteins would freeze the conformation of this region and thus prevent the release of the bound nucleotide or exchange of GDP for GTP with concomitant activation of *ras* proteins.

Normal *ras* proteins have intrinsic GTPase activity in the absence of GAP. Direct attack of the γ phosphate by a water molecule has been shown to be the mechanism for the GTP hydrolysis in elongation factors Tu and G as well as *ras* proteins (*30*). The environment around the γ phosphate suggests that there are three or four amino acids that may be involved, individually or in combination, in the hydrolysis of the γ phosphate. First, the highly conserved amino acid residue, Lys^{16} has its amino group in a position to polarize the γ phosphate for nucleophilic attack. Second, two other highly conserved amino acids, Asp^{57} and Gln^{61}, could serve to activate a water molecule for a nucleophilic attack of the γ phosphate in a general base type mechanism. Third, the hydroxyl group of Thr^{35} is coordinating to the Mg^{2+} in the GTP structures and may be the amino acid responsible for liberating the attacking water molecule from the previously Mg^{2+}-coordinated state. GAP

may achieve enhancement of the intrinsic hydrolysis rate by providing a better attacking group or an improved catalytic environment.

Gln^{61} is one of the most commonly found in vivo activating positions in *ras* proteins. However, Glu^{61}, Pro^{61}, and to a lesser extent Gly^{61} are not activating. It is possible for Glu^{61}, like Gln^{61}, to act as a polarizing group for the attacking water molecule, but probably not for Pro^{61}. This suggests that Pro^{61} may be behaving differently for deactivation of this critical location—possibly preventing the conformational changes required for GDP-GTP exchange needed in this particular loop, that is, hydrogen-bonding of the backbone NH groups of residues 60 and 61 to the γ phosphate.

Switch II region: Recognition site for upstream regulator or regulators? Comparison of GDP and GDP-CP complex structures clearly shows that the conformation of the switch region is significantly different (especially in the orientation of helix α2) between the two complexes (Fig. 2), and the difference is not due to the difference in crystal lattice, implying the importance of this region. The monoclonal antibody Y13-259, which binds to the same region (*20*), can neutralize the transforming activity of oncogenic *ras* proteins (*2*), yet a linker insertion-deletion experiment (*31*) showed that residues 64 to 76 in this region are dispensable for transforming activity. These observations can be explained if we hypothesize that the switch II region is the recognition site for a putative upstream regulator, such as a receptor, while the switch I region is the effector recognition site, and that the two regions are conformationally "coupled" or "linked" by the γ phosphate. One scenario for transmitting signals through *ras* protein could be that the upstream regulator induces the conformational change of the switch II region, allowing the release of the bound nucleotide followed by the binding of GTP or the direct exchange of GDP for GTP. This in turn would enable the conformational changes in the switch I region, and finally the effector to recognize the new conformation in the switch I region.

Under this hypothesis the results of the linker insertion-deletion experiment can be understood because transformation activity of mutants was monitored in the experiment independent of the upstream regulators, thus, the presence of the switch II region was not necessary. Likewise, Y13-259 binding would freeze the conformation of the switch II region and prevent GDP-GTP exchange (which requires a conformational change of the region), thus neutralizing the transforming activity of oncogenic *ras* proteins.

Molecular surface. Both switch I and switch II regions are located on the molecular surface, and occupy a contiguous stretch of the surface as shown in Fig. 8. This contiguous surface region is the best candidate as the recognition sites for upstream and downstream regulator molecule (or molecules). This region spans over 40 Å from the binding site of putative upstream molecule (the switch II region) to the guanine nucleotide pocket, then to the putative effector binding site (the switch I region). Examination of the surface near the γ phosphate shows that in three out of four molecules of the GDP-CP complex, the guanine nucleotide pocket is partially covered by Tyr^{32}, leaving the γ phosphate exposed (Fig. 8B) through a small opening. In a transforming mutant (Val^{12}), this opening would be covered by the side chain of the Val^{12}.

The NH_2- and COOH-terminii of the molecules are near to each other in space (see Fig. 3), and are located at one side of the molecule (top side of Figs. 1 and 7A), and the guanine nucleotide binding site is on the opposite side. Since the COOH-terminii of *ras* proteins are modified with an isoprenyl, most likely farnesyl, group (*32–34*), by which the molecules are anchored directly to the cytoplasmic membrane, or indirectly through a "farnesyl receptor," the crystal structures suggest that loops 3, 5, 7, and 9 and helices α2 and α5 (at the "top" side of the molecule as represented in Fig. 7A) are near the membrane and that some of them may be potential sites for the interaction with membrane-bound upstream or downstream (or both) regulators such as GDP-GTP exchange proteins, receptors, and effectors. Exposed residues on the membrane side are residues 47 to 50 of loop L3; 76 of loop L5; 102 to 108 of loop L7; 137 of loop L9; 66, 67, 69, 70, 73, and 74 of helix α2; and 164 to 171 (and possibly beyond) of helix zα5. Mutation of some of these residues may disrupt the interaction between *ras* proteins and these putative membrane-bound proteins.

Our comparative studies on the structures of the GDP and GTP analog couplèxes underscore the compactness of the information content on the NH_2-terminal half in this small protein and the complexity of conformation changes associated with signal transduction. The role of the COOH-terminal half, except membrane anchorage, is not evident from the structural comparison described here. However, further structural studies on molecular complexes of oncogenic *ras* proteins with GTP analogs and other interacting proteins are needed to understand the detailed mechanisms of biological functions of the proteins (*35*).

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35. Coordinates of the Cα positions of the GDP-CP complex will be submitted for deposit in the Brookhaven Protein Data Bank. Those for the GDP complex have already been deposited.

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