Probing Ras function with unnatural amino acids

Hyunho Chung[1] and Peter G Schultz[2]

[1]Lucky Central Research Institute, Dae Duk Sciencetown, Dae Jeon, Choong Nam, South Korea.
[2]Department of Chemistry, University of California, Berkeley, CA 94720 and Lawrence Berkeley Laboratory, Berkeley, CA 94720, USA.

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Mammalian Ras proteins act as molecular switches in the signaling events associated with cell growth and differentiation [1]. The protein cycles between the inactive (off) guanosine diphosphate (GDP) bound state and the active (on) guanosine triphosphate (GTP) bound state (shown in Fig. 1). Point mutations that decrease the intrinsic GTPase activity of Ras, or the GTPase activity stimulated by GTPase activating protein (GAP) [2], are associated with several human cancers. To gain insight into the molecular mechanisms of switch inactivation, we have substituted residues implicated in Ras GTPase activity with a series of unnatural amino acids [3–5]. The ability to substitute amino acids beyond those specified by the genetic code allows us to probe mechanistic and structural issues not addressable by conventional site-directed mutagenesis.

Biochemical and structural studies led to the proposal that Gln61 is important in γ-phosphate binding and GTPase activity. Seventeen natural mutants at position 61 have reduced GTPase activity and are not activated by GAP. It has been proposed that the γ-carboxamide of Gln61 either acts as a general base, polarizing a water molecule for attack on the γ-phosphate of GTP, or stabilizes the incipient transition state during hydrolysis by acting as a hydrogen bond donor [see references in 3]. To test these hypotheses, we replaced Gln6l with the isoelectronic, isosteric nitro analog (NGln), which is a poor base and cannot donate a hydrogen bond [3]. This mutant had normal GTPase activity and was activated by GAP. Gln61 may therefore have a different role in catalysis, perhaps distorting bound GTP toward the transition state geometry.

Mutations at Gly12 of Ras also reduce intrinsic GTPase activity and are often associated with oncogenic activation. Gly12 occurs in a highly conserved type II β-turn [Gly11-X-X-X-Gly-Lys-(Ser or Thr)], a common phosphate-binding loop. Mutation of Gly12 to any natural amino acid other than proline reduces GTPase activity. To examine the role of Gly12 in switch function, we inserted unnatural amino acids, including lactic acid (Lac), pipecolic acid (Pip) and methylglycine (MeGly), at this site. All three mutants had wild-type GTPase activity, although the activity of the Ala12 mutant is reduced. Thus, only mutants that can adopt unusual backbone conformations are active, perhaps because they allow a conformational change in loop L1 that relieves unfavorable interactions between the side chain at position 12 and the transition state. However, although Pro12, Pip12, Lac12, and MeGly12 mutants had normal intrinsic GTPase activity, they were not activated by GAP. Similarly, three mutants at position 13 (Thr13, *allo*Thr13, and Ser13) were not activated by GAP, although their intrinsic GTPase activity was higher than that of wild-type Ras. It may be that loop L1 can adopt two or more conformations in solution and that its position modulates GTPase activity and oncogenic activation. GAP may affect the position of the loop, thus increasing GTPase activity. In this model the effect of the mutations at positions 12 and 13 that alter the backbone structure of the loop may be to prevent switch function by preventing this conformational change.

The most significant difference between the GTP and GDP bound forms of Ras is in loop L2 (residues 32–40). It has been proposed that Pro34, which is conserved in Ras and is close to the active site, controls the conformation of loop L2, perhaps by *cis-trans* isomerization of the Pro34 amide bond [7]. Replacing Pro34 with 2,4-methanoproline, which is strongly biased toward the *trans* configuration by virtue of the C_α substitution, had essentially no effect on the intrinsic and GAP-activated GTPase activity, strongly suggesting that the conformation of Pro34 is not crucial in signal transduction. Similar substitutions may be useful in probing the role of backbone isomerization in protein folding pathways.

In conclusion, the ability to substitute unnatural amino acids into specific sites in proteins such as Ras allows more precise changes in the steric and electronic properties of amino acids than are possible using site-directed mutagenesis and significantly expands the scope of structural perturbations that can be made. This methodology should provide new insights into the nature of biomolecular recognition and catalysis.

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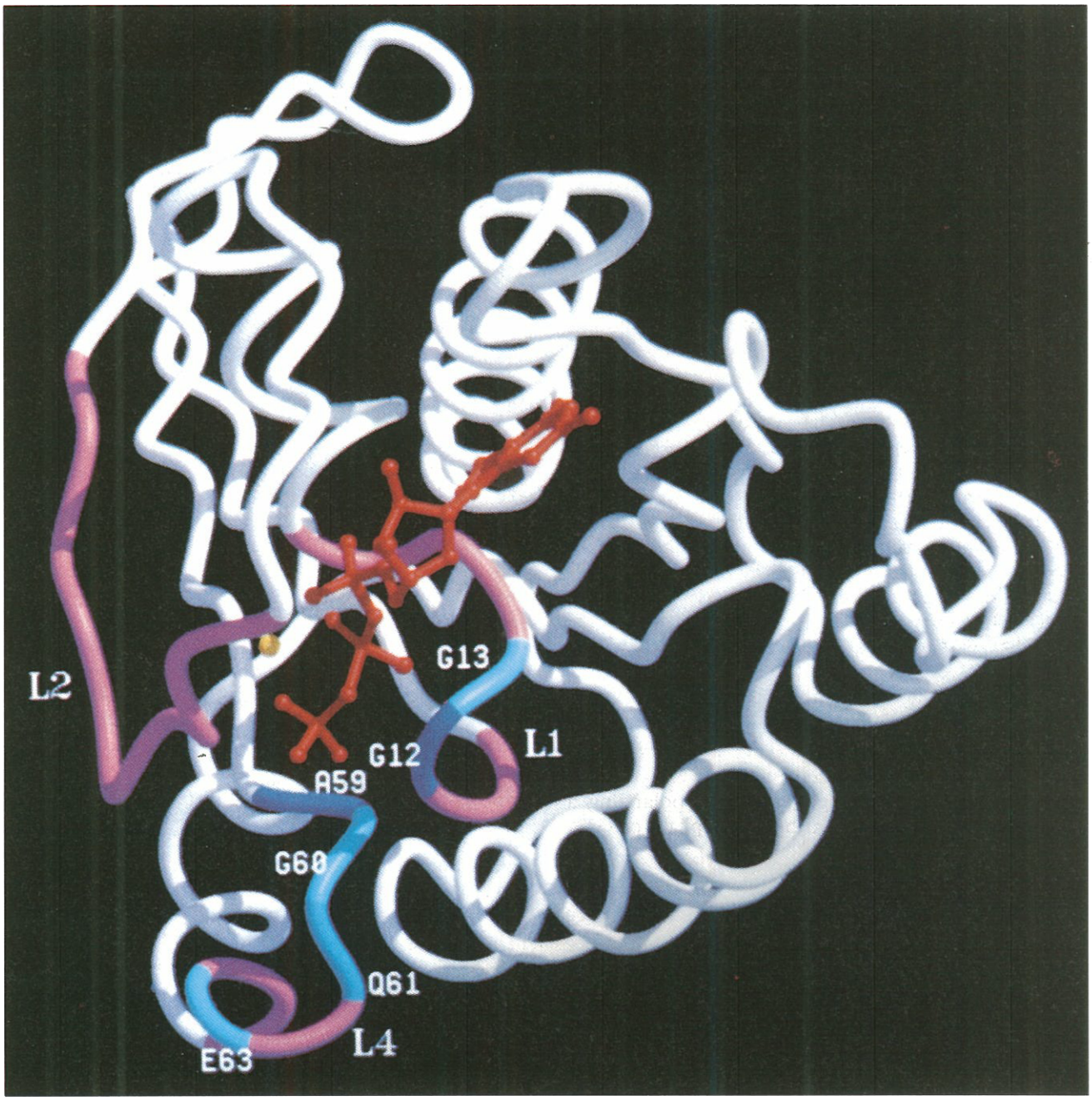

Fig. 1. Model of Ras protein with bound GTP.