Probing the role of loop 2 in Ras function with unnatural amino acids

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ABSTRACT   The YDPT sequence motif (residues 32–35) in loop 2 (residues 32–40) of Ha-Ras p21 protein is conserved in the Ras protein family. X-ray crystal structures have revealed significant conformational differences in this region between the GTP- and GDP-bound forms. Moreover, mutations in this region block neoplastic transformation and prevent interaction with GTPase-activating protein (GAP), suggesting that this region may contribute to the effector function of Ras. To better understand the structural features required for GAP interaction and GTPase activity, the expanded repertoire of unnatural amino acid mutagenesis has been used to investigate the roles of the key residues, Pro-34, Thr-35, and Ile-36. A Pro-34→ methanoproline mutant, in which residue 34 is locked in the trans conformation, was found to retain high levels of intrinsic and GAP-activated GTPase activity, making unlikely conformational isomerization at this position. Deletion of a single methyl group from Ile (Ile-36 → norvaline) abolished GAP activation of Ras, revealing a remarkable specificity in this protein–protein interaction. Finally, replacement of Thr-35 with diastereomeric *allo*-threonine led to inactivation of Ras, demonstrating the importance of the orientation of this critical residue in Ras function.

Mammalian proteins encoded by the *ras* genes function as molecular switches in the signaling events associated with cell growth and differentiation (1–4). The chemical basis for the regulation involves cycling of the protein between the inactive (off) GDP-bound state and the active (on) GTP-bound state. The low intrinsic GTPase activity of cellular Ras protein is increased by interaction with the cytosolic protein GTPase-activating protein (GAP), a possible effector or negative regulator of Ras. In addition, GDP–GTP exchange factors (5–8), which have recently been identified as possible positive regulators, enhance the dissociation of GDP from the protein and facilitate rebinding of GTP. Point mutations that result in a modest decrease in the intrinsic GTPase activity of Ras or GAP (9–12)-stimulated GTPase activity are associated with a large number of human cancers.

The YDPT sequence motif (residues 32–35 in loop 2 of Ras) is conserved throughout the Ras protein family. X-ray crystal structures (13–20) of the GDP-, guanosine 5′-[β,γ-methylene]triphosphate-, and guanosine 5′-[β,γ-imido]triphosphate-bound forms of Ras have shown that the loop 2 region undergoes significant conformational changes upon hydrolysis of GTP. The β-hydroxyl group of Thr-35, which associates with Mg^{2+} in the GTP–Ras complex, moves out toward solvent in the GDP complex. In addition, the side chain of Ile-36, which is solvent-exposed in the GTP complex, is positioned closer to the protein surface in the GDP complex. This flexibility has been proposed to be a functionally important feature of Ras and perhaps in the GTPase family in general. Mutations (21–23) in this region that block neoplastic transformation also prevent interaction of Ras with GAP. We

now report the results of a detailed mutagenesis study in which residues Pro-34, Thr-35, and Ile-36 have been replaced with a series of unnatural amino acid analogues (Fig. 1). The ability to precisely vary the main-chain conformations and steric properties of these residues has allowed us to test structural issues not addressable using the natural 20 amino acids.

MATERIALS AND METHODS

Mutagenesis. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs; reagents and enzymes for DNA mutagenesis and sequencing were obtained from Amersham. Oligonucleotides were synthesized on an Applied Biosystems PCR Mate DNA synthesizer. Amber mutants were constructed by Eckstein mutagenesis (26) using an M13mp19 derivative containing a 576-bp *Cla* I–*Sal* I fragment of the structural gene for Ras. The oligonucleotides 5′-GAGTACGACTAGACTATTGAA, 5′-TACGACCCGTA-GATTGAAGAC, and 5′-GACCCGACTTAGGAAGAC-TCT were used to generate the pRGP34am, pRGT35am, and pRGI36am mutants, respectively. Mutations were verified by dideoxynucleotide chain-termination sequencing (27). The mutant *ras* sequences were then reintroduced into the expression vector pRG in which the *ras* gene is under the control of the *trp* promoter (28).

Synthesis of Unnatural Amino Acids. All amino acids were N-protected as nitroveratryloxycarboyl derivatives and converted to the corresponding cyanomethyl esters for attachment to pdCpA. Norvaline, norleucine, *O*-methylserine, *allo*-threonine, and *cis*- and *trans*-4-hydroxyproline are all commercially available. *allo*-Threonine and *cis*- and *trans*-4-hydroxyproline were O-protected as *tert*-butyldimethylsilyl ethers. After attachment of the amino acids to pdCpA, the *tert*-butyldimethylsilyl groups were removed using AcOH/tetrahydrofuran/H_2O, 3:1:1 (vol/vol), at 37°C. 3-Methylproline was synthesized by the method of Belokon *et al.* (24), and methanoproline was prepared by the method of Clardy and coworkers (25).

***In Vitro* Suppression and Synthesis of Ras Proteins.** Reagents and enzymes (pyruvate kinase and inorganic pyrophosphatase) for *in vitro* synthesis reactions were purchased from Sigma. RNasin was obtained from Promega. T4 RNA ligase was prepared as described by Heaphy *et al.* (29). T4 polynucleotide kinase was prepared as described by Cameron and Uhlenbeck (30). T7 RNA polymerase was prepared using *Escherichia coli* BL21/DE3, containing the plasmid pAR1219 (ampicillin resistance) in which the gene for T7 RNA polymerase is under the transcription control of the *lac* promoter (31). All enzymes were stored at −20°C in glycerol solutions. The suppressor tRNA$_{CUA}^{Phe}$(−CA) was prepared using plasmid pYPhe2 as template for runoff transcription according to Ellman *et al.* (31).

Abbreviations: GAP, GTPase-activating protein; WT, wild type; DTT, dithiothreitol.
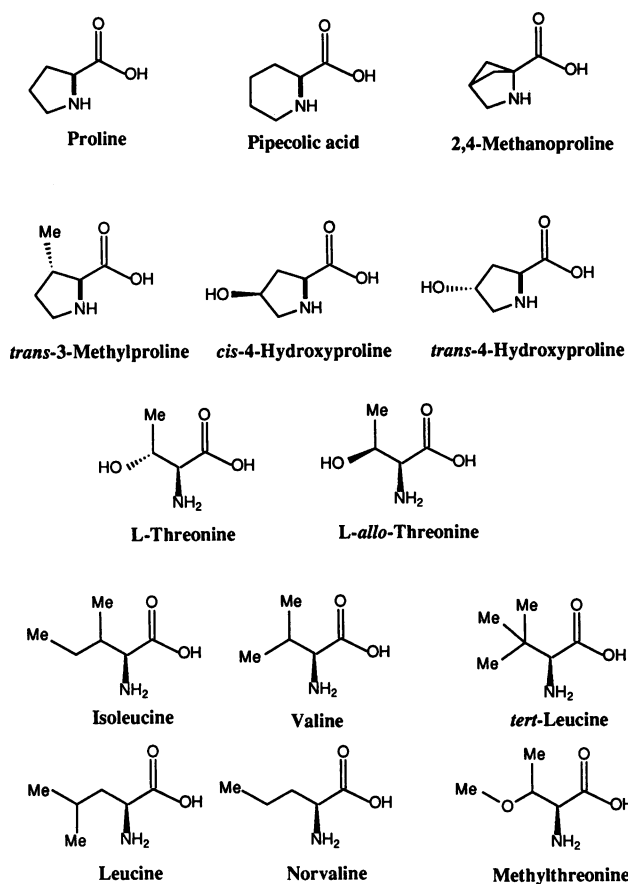*To whom reprint requests should be addressed.

FIG. 1.   Structures of amino acids inserted at positions 34, 35, and 36.

Acylation of suppressor tRNA was carried out as follows: to 700 μM lyophilized acyl-pdCpA was added 55 μM MgCl$_2$, 10% (vol/vol) dimethyl sulfoxide, 20 μg of tRNA$_{CUA}^{Phe(-C^A)}$, 2.5 μM ATP, bovine serum albumin (200 ng/ml), and 200 units of T4 RNA ligase to a total volume of 80 μl (it is important to add and mix the reagents rapidly, since acyl-pdCpA and acyl-tRNA hydrolyze at an appreciable rate). The reaction mixture was incubated at 37°C for 12 min, quenched by addition of 2.5 M sodium acetate (pH 4.5) to 10% (vol/vol), and then precipitated with 3 vol of ethanol (−80°C) after successive extractions with phenol and chloroform. The resulting acyl-tRNA pellet was rinsed with 70% ice-cold ethanol and dried *in vacuo* for 5 min. The acyl-tRNA was then stored at −80°C until immediately prior to the photodeprotection reaction.

Photodeprotection of protected aminoacyl-tRNA was carried out by diluting the tRNA pellet to 1 μg/μl with 1 mM potassium acetate (pH 4.5) and transferring this solution to a transparent Eppendorf tube (Robbins Scientific, Sunnyvale, CA, 1048-00-0). The solution was irradiated at 0°C for 8 min with a 1000-W xenon lamp with either a Pyrex filter or a monochromator set at 350 nm. After photodeprotection, the solution was added directly to the *in vitro* protein synthesis reaction mixture. Because aminoacyl-tRNAs are labile to hydrolysis, the aminoacyl-tRNA solution should be stored at −80°C until immediately before addition to the *in vitro* protein biosynthesis reaction mixture. *E. coli* S-30 extract and low molecular mixture were prepared according to Ellman *et al.* (31). Suppression efficiencies were determined by polyacrylamide gel analysis of [^{35}S]methionine-labeled protein.

Purification of Ras Proteins. *In vitro*-synthesized Ras protein (30–40 μg from a 1.0-ml reaction mixture) was purified

to homogeneity with ≈15% overall yield. A clear supernatant was obtained by centrifugation of the *in vitro* synthesis reaction mixture at 4°C. This supernatant was applied to a column (1.5 × 30 cm) of Sephadex G-75 (Pharmacia–LKB) previously equilibrated with 50 mM Hepes, pH 7.5/1 mM EDTA/1 mM dithiothreitol (DTT) buffer containing 0.01% 1-octyl glucoside, leupeptin (1 μg/ml), antipain (1 μg/ml), and chymostatin (1 μg/ml). The eluted fractions containing Ras protein were then applied to a tandem linear column of DEAE-Sephacel (0.8 × 4 cm; Pharmacia–LKB) and a preadsorption column of CM-Sepharose (0.8 × 4 cm; Pharmacia–LKB) preequilibrated with Hepes buffer. Protein was eluted with a 10-ml linear 0–0.5 M NaCl gradient in Hepes buffer and 100-μl fractions were collected. All chromatographic operations were carried out at 4°C. The fractions were analyzed by SDS/PAGE on a 15% gel followed by silver staining. Wild-type (WT) and mutant Ras proteins were detected by Western blot analysis with the mouse monoclonal antibody 1F6 (32). Complexes were stained with goat anti-mouse IgG conjugated to horseradish peroxidase and visualized after the addition of 4-chloro-1-naphthol and hydrogen peroxide. Protein yields were determined by PAGE with silver staining, immunoprecipitation of [^{35}S]methionine-labeled protein, and GTPase activity assay.

Interactions with Guanine Nucleotides. The GTP exchange reaction was performed as described by Satoh *et al.* (33) with some modifications. To bind labeled nucleotide to Ras, protein samples (10 pmol/ml) were incubated at 25°C for 15 min in exchange buffer {50 mM Hepes/200 mM sucrose/1 mM MgCl$_2$/5 mM EDTA/0.2% NaN$_3$/1 mM DTT, pH 7.4/10 μM [α-^{32}P]GTP (Amersham, 410 Ci/mmol; 1 Ci = 37 GBq)}. The [α-^{32}P]GTP-bound Ras protein was then purified using a PD-10 column (Pharmacia–LKB) and the eluant was collected. An aliquot (200 μl) was immediately removed, added to 0.5 ml of ice-cold wash buffer (50 mM Hepes/10 mM MgCl$_2$, pH 7.5), and filtered through Schleicher & Schuell BA 85 filters. The filters were washed three times with 0.5 ml of ice-cold wash buffer, and the radioactivity on a filter was determined by liquid scintillation counting. GTPase activity was determined by incubating purified Ras (1 μM) with 100 μM [α-^{32}P]GTP (Amersham, 5000 cpm/pmol) in 50 mM Tris·HCl, pH 7.5/1 mM MgCl$_2$/5 mM EDTA/10 mM NH$_4$Cl/5 mM DTT containing bovine serum albumin (20 μg/ml) (100-μl reaction volume). At various times, 5-μl samples were mixed with 5 μl of stop buffer (2 mM EDTA, pH 8.0/0.5% SDS/4 mM GTP/4 mM GDP/4 mM GMP). The conversion of GTP to GDP was monitored by chromatography on polyethyleneimine plates (Sigma) that were developed with 1 M LiCl. After autoradiography of the polyethyleneimine plate, the labeled GDP was excised and quantitated by liquid scintillation counting. The rates were calculated as described (33).

Autophosphorylation Activity. The autophosphorylation activity of Ras protein was assayed at 37°C in the presence of 10 μM [γ-^{32}P]GTP in 100 μl of reaction buffer containing 50 mM Tris·HCl, pH 7.5/10 mM MgCl$_2$/1 mM DTT/1 mM NaN$_3$. At various times, portions (10 μl) were removed and quenched with 100 μl of ice-cold quenching solution (50 mM Tris·HCl, pH 7.5/5 mM potassium phosphate/5 mM EDTA/1 mM DTT). The samples were filtered through a nitrocellulose membrane filter (Schleicher & Schuell) and washed six times with 1 ml of rinsing buffer (50 mM Tris·HCl, pH 7.5/5 mM MgCl$_2$/10 mM NH$_4$Cl/1 mM 2-mercaptoethanol). The radioactivity retained on the filter was measured by liquid scintillation counting.

GAP-Mediated GTPase Activity. The GAP assay was performed by a modification of a procedure described by Han *et al.* (34). Purified Ras (100 nM) was incubated for 15 min at 25°C with 50 μCi of [α-^{32}P]GTP (410 Ci/mmol; Amersham) in buffer I (50 mM Hepes, pH 7.4/200 mM sucrose/1 mM

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MgCl$_2$/5 mM EDTA/0.2% NaN$_3$/1 mM DTT). The [α-^{32}P]GTP-bound Ras was purified on a PD-10 column (Pharmacia–LKB). More than 95% of Ras bound [α-^{32}P]GTP. The GTPase reaction was initiated by addition of 10 mM MgCl$_2$ in the presence or absence of recombinant GAP (40 nM) (total assay volume, 1 ml). To determine the time required for 50% conversion of Ras and GTP to Ras·GDP, Ras was collected by filter binding at various times. Bound nucleotides were released from the filter by incubation with 20 mM EDTA/1% SDS/4 mM GTP/4 mM GDP/4 mM GMP at 65°C for 5 min and resolved by chromatography on polyethyleneimine plates as described above. The labeled nucleotides were quantitated by scintillation counting. Ras·GTP was 50% converted to Ras and GDP after 1 min. The relative percent conversion of the mutant Ras proteins was determined after 1 min under the same conditions.

RESULTS AND DISCUSSION

The most significant difference between the GTP- and GDP-bound forms of Ras is in the loop 2 region (Fig. 2). Both Thr-35 and Ile-36 undergo considerable changes in orientation upon GTP hydrolysis. Mutagenesis studies have demonstrated that this region is also involved in interactions with GAP (21–23). Within this loop, Pro-34 and Thr-35 are highly conserved in all Ras-related proteins. It has been proposed that Pro-34, which is close to the active site, may play a role in controlling the conformation of loop 2. One possibility could involve a cis–trans isomerization of the Pro-34 amide bond (13). To more precisely examine the structural and mechanistic role of this residue, Pro-34 was replaced with a series of proline analogues including *cis*- and *trans*-4-hydroxyproline, *trans*-3-methylproline, 2,4-methanoproline, and pipecolic acid (Fig. 1).

Incorporation of unnatural amino acids into Ras was accomplished by *in vitro* suppression of TAG nonsense mutations with a chemically aminoacylated suppressor tRNA (31, 35–38). As a control, WT and a Gly-12 → Pro mutant Ras protein were generated *in vivo* and *in vitro* by suppression of the Gly-12 → TAG mutant with glycine and proline, respectively. *In vitro* expression of Ras protein programmed with the plasmid pRG, in which the WT gene is under transcriptional control of the *trp* promoter, afforded Ras at ≈40 μg/ml of reaction mixture. The *in vitro*-synthesized proteins were purified to homogeneity in ≈15% yield by sequential chromatography on Sephadex G-75 and CM-Sepharose and a

DEAE-Sephacel tandem column. The purified proteins had the same chromatographic properties, intrinsic GTPase activity, and GAP-stimulated GTPase activity as the corresponding Ras proteins synthesized *in vivo* (Table 1). A Western blot of *in vitro*-synthesized Ras mutants also showed bands having mobilities identical to *in vivo*-synthesized protein. Importantly, *in vitro* synthesis reactions containing the TAG mutants in the absence of suppressor tRNA or in the presence of full-length unacylated suppressor tRNA$_{CUA}$ afforded <0.5% of Ras protein. These controls demonstrate that the *in vitro* system does not contain endogenous suppressor tRNAs capable of reading through the amber stop codon and that the aminoacyl tRNA synthetases present in the *E. coli* extract S-30 do not aminoacylate the suppressor tRNA$_{CUA}$ with any of the other 20 natural amino acids.

Suppressor tRNAs were then aminoacylated with the proline analogues and added to *in vitro* protein synthesis reaction mixtures containing the Pro-34 → TAG mutant Ras gene. Suppression efficiencies were typically between 20 and 40% for these amino acids (Table 1 and Fig. 3). The Pro-34 mutants were examined for both intrinsic and GAP-mediated GTPase activity. Both the *trans*-3-methylproline and 2,4-methanoproline mutants have intrinsic GTPase activity ≈2 times that of WT Ras. The high intrinsic GTPase activity of the methanoproline mutant in which the amide bond of residue 34 is strongly biased toward the trans configuration (by virtue of Cα substitution) makes unlikely a conformational isomerization in the GTP hydrolysis reaction. This result is consistent with the absence of *cis*-proline in the x-ray crystal structures of Ras–GTP analogue and –GDP complexes. The Pro-34 → pipecolic acid mutant, which is expected to have a more negative Φ value (39) than proline, also retains WT intrinsic GTPase activity. Thus these results suggest that the conformation of Pro-34 does not play a critical role in controlling the intrinsic GTPase activity of Ras.

All of the Pro-34 mutants retain a high degree (≥68% WT Ras) of their ability to be activated by GAP. In addition, the *cis*- and *trans*-4-hydroxyproline mutants (in which the hydroxyl group is pointed toward solvent) retain WT intrinsic GTPase activity and are GAP-activated. Therefore, it is also unlikely that the unique structure of Pro-34 plays a key role in the interaction of Ras with GAP. The double mutation Asp-33 → His,Pro-34→Ser in Ras leads to a decrease in GAP activation (40). However, this double mutation may lead to significant structural changes at other sites in the Ras protein.
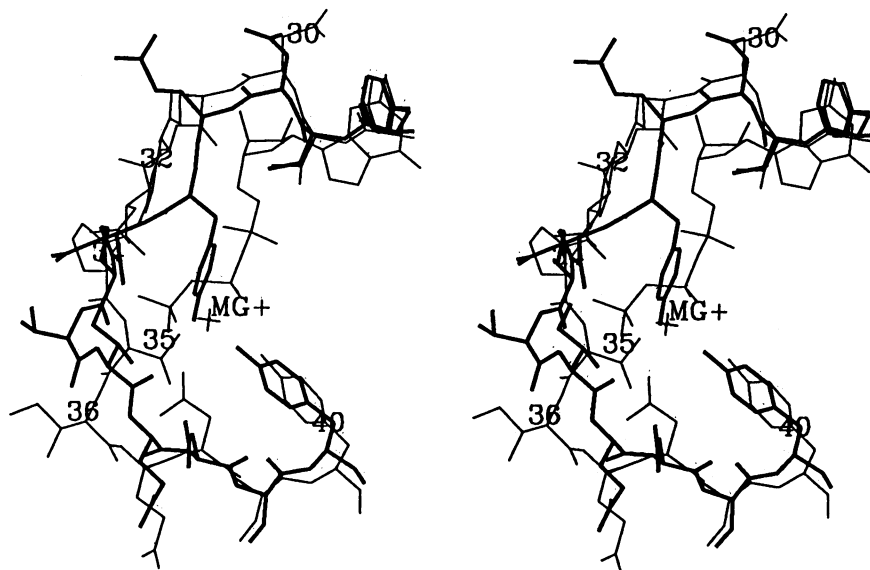

FIG. 2. Comparison of the backbone structures of loop L2 in GDP-bound state (heavy line) and GTP-bound state (light line) (19).

Table 1. Suppression efficiencies and intrinsic and GAP-mediated GTPase activities of Pro-34 and Ile-36 mutants

Mutant	Suppression efficiencies, %	Intrinsic GTPase activities, k_{rel}*	GAP activation[†]
WT[‡]	NA	1.00	100
Gly-12/Gly	35	1.00	99
Gly-12/Pro	30	1.10	—[§]
Pro-34/*trans*-4Hyp	30	1.04	91
Pro-34/*cis*-4Hyp	25	0.95	93
Pro-34/*trans*-3-MePro	35	2.07	75
Pro-34/2,4-methanoPro	20	2.11	68
Pro-34/pipecolic acid	33	1.23	74
WT[‡]	NA	1.00	100
Ile-36/Ala	34	1.04	—
Ile-36/Val	50	1.02	—
Ile-36/*tert*-Leu	45	1.00	—
Ile-36/norvaline	45	0.98	—
Ile-36/MeThr	40	1.00	—

—, No detectable activity was observed; NA, not applicable; Hyp, hydroxyproline.
*The k_{cat} of WT Ras protein is 2.3×10^{-4} sec^{-1}.
[†]Comparison of the rates for the conversion of Ras(GTP) to Ras-(GDP) in the presence of 40 nM recombinant GAP.
[‡]Protein was synthesized *in vivo*; all other proteins were synthesized by suppression of the corresponding nonsense mutations.

The x-ray crystal structures of Ras complexed with GTP, GTP analogues, and GDP indicate that upon GTP hydrolysis, the hydrophobic side chain of Ile-36 leaves an energetically unfavorable position in which it is completely exposed to the solvent and moves toward the protein surface. The structures adopted by the loop 2 region in GDP- and GTP-bound forms are thus different enough to be easily distinguished by GAP. Previously, it has been shown (41) that retroviral Ras proteins
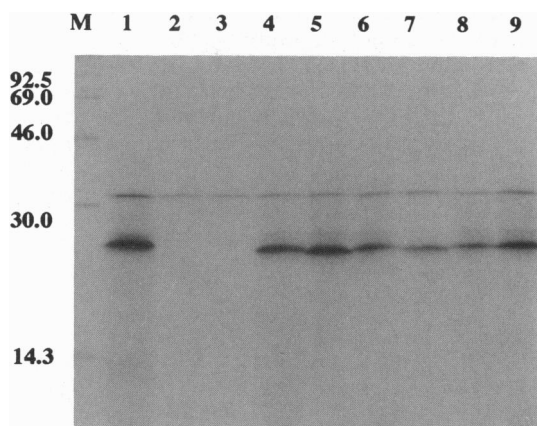

FIG. 3. Autoradiograms of *in vitro* suppression reaction products (30 μl) labeled with L-[^{35}S]methionine and containing the following plasmids and tRNAs. Lanes: M, standards; 1, pRG (WT); 2, pRG34am (G34am refers to an amber mutation at position 34 in the WT gene) without tRNA; 3, pRG34am with 5 μg of full-length unacylated tRNA$_{CUA}$; 4, pRG34am and 5 μg of prolyl-tRNA$_{CUA}$; 5, pRG34am and 5 μg of pipecolyl-tRNA$_{CUA}$; 6, pRG34am and 5 μg of 4-*trans*-hydroxyprolyl-tRNA$_{CUA}$; 7, pRG34am with 5 μg of 4-*cis*-hydroxyprolyl-tRNA$_{CUA}$; 8, pRG34am with 5 μg of 2,4-methanoprolyl-tRNA$_{CUA}$; 9, pRG34am with 5 μg of 3-*trans*-methylprolyl-tRNA$_{CUA}$. Cleared supernatants from terminated *in vitro* reaction mixtures were incubated with RNase A and analyzed by SDS/PAGE (15% gel). *In vitro* suppression experiments were performed. The band at 30 kDa is β-lactamase.

(Gly-12 → Arg,Ala-59 → Thr) carrying the three most conservative substitutions at position 36 (leucine, valine, and methionine) were still able to transform cells but with an efficiency that was significantly lower than that of WT retroviral Ras. The less-conservative Ala-36 mutant was not active.

To gain increased insight into the critical steric features of the side chain at position 36 required for GAP activity, we replaced Ile-36 in WT Ras with a series of aliphatic amino acid analogues, including *tert*-leucine, norvaline, and O-methyl-threonine, as well as the natural amino acid valine. Norvaline is an analogue of isoleucine that lacks the methyl group on the β-carbon and *tert*-leucine is a structural isomer of isoleucine that contains a tertiary β-carbon. O-Methylthreonine is an isostere of isoleucine in which the γ-carbon is substituted with oxygen. The biochemical activities of WT and mutant proteins are reported in Table 1. None of the mutants, including the highly conservative substitutions with O-methylthreonine, norvaline, and valine, was activated by GAP even though the mutant Ras proteins retain WT levels of intrinsic GTPase activity. This represents at least 100-fold loss in catalytic activity in the ras–GAP complex or ≈3 kcal/mol. The fact that deletion of a single methyl group from the isoleucine side chain (Ile → norvaline and Ile → Val) has such a dramatic effect on the ability of GAP to stimulate the GTPase activity underlies a remarkable dependence in this protein–protein interaction on hydrophobic packing interactions.

X-ray structures (13–20) of Ras bound to GDP, guanosine 5'-[β,γ-imido]triphosphate, and guanosine 5'-[β,γ-methylene]triphosphate show that a Mg^{2+} is coordinated to the β- and γ-phosphates of GTP and to the β-hydroxyl groups of Thr-35 and Ser-17. In the GDP-bound state, the Thr-35 side chain points away from the nucleotide and toward the solvent. This significant change in the orientation of Thr-35 induced by GTP binding has been postulated to be crucial for the action of Ras protein as a molecular switch (19). To better understand the catalytic role of this residue, Thr-35 was replaced with L-*allo*-threonine, which differs only in the stereochemical configuration of the secondary alcohol. Replacement of L-Thr-35 by L-*allo*-threonine led to an ≈10-fold decrease in intrinsic GTPase activity (Table 2). This decrease is similar to that found for the transforming Gly-12 → Val mutant. In addition, a double mutant, *allo*-Thr-35,Thr-59 (in which Eckstein mutagenesis was used to mutate Ala-59 → Thr) did not show any significant autophosphorylation of Thr-59, in contrast to the behavior of the Thr-35,Thr-59 mutant. Moreover, neither *allo*-threonine mutant was activated by GAP. Previous studies have also demonstrated that the Thr-35 → Ser mutant is not activated by GAP (42). These results suggest that the orientation of the β-hydroxyl group of Thr-35, which is controlled by the β-methyl substituent, is critical to the intrinsic and GAP-dependent GTPase activity of Ras. In general, the presence of threonine in the genetic code, in addition to the less sterically hindered and more nucleophilic serine residue, may reflect the importance of restricting side chain conformational entropy in proteins.

CONCLUSION

The ability to make precise changes in the structures of Ile-36 and Thr-35 using unnatural amino acids has revealed a remarkable sensitivity in the intrinsic GTPase activity and the GAP-stimulated GTPase activity of Ras to the presence and/or chirality of a single side chain methyl group. These studies underscore the degree to which protein–protein recognition and catalytic activity are controlled by hydrophobic packing interactions and side chain conformation (43). In addition, the ability to substitute methanoproline site-specifically into proteins should prove a useful tool for

Table 2. Suppression efficiencies, intrinsic and GAP-mediated GTPase activity, and autophosphorylation activity of Thr-35 mutants

Mutant	Suppression efficiency, %	Intrinsic GTPase activity, k_{rel}*	Autophosphorylation activity (k), sec^{-1} ($\times 10^6$)	GAP activation[†]
WT	NA	1.00	NA	100
Gly-12,*allo*-Thr-35	36	0.10	NA	—
Gly-12,Thr-59[‡]	NA	0.18	6.53	—
Gly-12,Thr-59,*allo*-Thr-35	35	0.12	—	—

—, No detectable activity was observed; NA, not applicable.
*The k_{cat} of WT Ras protein is 2.3×10^{-4} sec^{-1}.
[†]Comparison of the rates for the conversion of ras(GTP) to ras(GDP) in the presence of 40 nM recombinant GAP.
[‡]Protein was synthesized *in vivo*; all other proteins were synthesized by suppression of the corresponding nonsense mutations.

analyzing the effects of proline conformation on protein structure and folding.

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