# Communications to the Editor

## Probing the Role of Lysine 16 in Ras p21 Protein with Unnatural Amino Acids

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Mammalian proteins encoded by the ras gene are thought to function as regulators of various signal transduction processes involved in cell growth and differentiation.[1] The chemical basis for the regulation is cycling of the protein between the inactive guanosine diphosphate (GDP)-bound state and the active guanosine triphosphate (GTP)-bound state. Point mutations that result in a modest decrease in the intrinsic guanosine triphosphatase (GTPase) activity of ras or GAP (GTPase activating protein)[2]-stimulated GTPase activity are associated with approximately 30% of human cancers. Loop 1 of ras contains the GXXXXGK(S/T) motif (residues 10–17), which is found in all ras-related proteins, G proteins, and other nucleotide-binding proteins.[3] Structural[4] and biochemical[5] studies have suggested that Lys 16 of loop 1 is critical for substrate binding and catalysis. The $\epsilon$-amino group is involved in ion pair interactions with the $\beta$- and $\gamma$-phosphates of GTP and forms hydrogen bonds with the main-chain oxygens of Gly 10 and Ala 11.[4] In order to better understand the role of this key residue in ras function, we have replaced Lys 16 with a number of unnatural amino acid analogues, including (aminoethyl)cysteine, (hydroxyethyl)cysteine, (aminoethyl)homocysteine, and ornithine (Figure 1).

Incorporation of unnatural amino acids into ras was accomplished by *in vitro* suppression of a Lys 16→TAG amber mutant[6] using a chemically aminoacylated suppressor tRNA.[7] (Aminoethyl)cysteine, (hydroxyethyl)cysteine, and (aminoethyl)homocysteine were incorporated into ras as previously described;[7] ornithine was inserted with the $\epsilon$-amino group protected with an *o*-nitrobenzyl carbamate group (due to the instability of the aminoacylated tRNA). Photolysis of this caged mutant ras led to functional protein. *In vitro* expression of the wild-type (WT) ras gene under control of the T7 promoter (plasmid pRGT7)[8] afforded approximately 100 $\mu$g/mL of active protein.[9] Suppression efficiencies for the mutant proteins varied between 25 and 50% (Table I). WT and mutant proteins were purified to homogeneity from *in vitro* synthesis reactions in approximately 15% overall yield by sequential chromatography on Sephadex-G75 and a CM-Sepharose and DEAE-Sephacel tandem column.[10] The intrinsic GTPase activity, GAP-activated GTPase activity, and GTP dissociation rates for wild-type and the mutant proteins are reported in Table I.[10,11]

The Lys 16→(aminoethyl)cysteine mutant, which has slightly different side-chain bond angles, bond lengths, and p$K_A$ (the p$K_A$

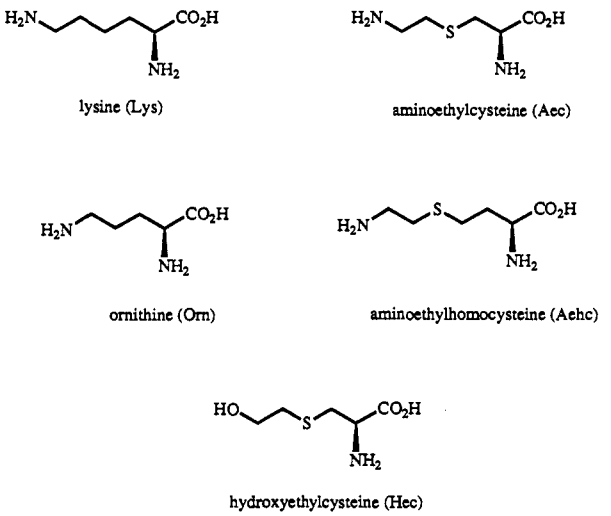


**Figure 1.** Unnatural amino acids substituted for Lys 16 in ras protein.[16]

**Table I.** Suppression Efficiencies, Intrinsic and GAP-Mediated GTPase Activities, and GTP Dissociation Rate Constants

| mutants | suppression efficiencies (%) | intrinsic GTPase activities ($k_{rel}$)[a] | GAP activation[b] | GTP dissociation rate constants ($k_{-1} \times 10^2$ min$^{-1}$)[c] |
|---|---|---|---|---|
| wild-type[d] | | 1.00 | 100 | 1.8 |
| Gly 12/Gly | 35 | 1.00 | 100 | 1.8 |
| Gly 12/Val[d] | | 0.15 | *e* | *e* |
| Lys 16/Aec | 30 | 0.20 | 95 | 1.1 |
| Lys 16/Aehc | 35 | 0.10 | 20 | 1.0 |
| Lys 16/Orn | 25 | 0.50 | 60 | 0.9 |
| Lys 16/Hec | 50 | | | *f* |

[a] The $k_{cat}$ of wild-type ras protein is $2.3 \times 10^{-4}$ s$^{-1}$ (ref 17). [b] Comparison of the rates of conversion of ras(GTP) to ras(GDP) in the presence of recombinant GAP (ref 18). [c] The dissociation rate constants of the ras·GTP complex, $k_{-1}$, were calculated from the slope of the line obtained by plotting ln(Ct/Co) vs $t$, where Ct and Co denote the concentration of ras·[$\alpha$-$^{32}$P]GTP complex at times $t$ and 0, respectively (refs 18 and 19). [d] Protein synthesized *in vivo*; all other proteins were synthesized by suppression of the corresponding nonsense mutations. [e] Not detected. [f] Not determined.

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(8) A 576bp *NdeI*/*XhoI* fragment of the ras gene[18] was subcloned into pSW1,[21] placing the *ras* gene under the transcription control of T7 promoter in the resulting vector, pRGT7.

(9) The yield of ras was determined by polyacrylamide gel electrophoresis, immunoprecipitation of [$^{35}$S]-methionine-labeled protein, and GTPase activity assay.

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is reduced by 1 unit)[12] relative to lysine, has a reduced level (20% of WT) of intrinsic GTPase activity. This level is similar to that of the transforming Gly 12→Val mutant (15% of WT). Similarly, the one methylene homologue, (aminoethyl)homocysteine, has diminished intrinsic GTPase activity (10% of WT), while the shorter homologue of lysine, ornithine, has 50% the intrinsic GTPase activity of WT ras. However, in contrast to other ras mutants with low intrinsic GTPase activity, including the dominant negative Gly 12→Val mutant, the GTPase activity of all three Lys 16 mutants was enhanced in the presence of GAP (GAP increases the rate of GTP hydrolysis in WT ras by a factor of more than $10^4$).[13] In fact, the GAP-dependent GTPase activity of the (aminoethyl)cysteine mutant is 95% that of WT. Moreover, the dissociation rate constants of GTP for all three mutants are comparable to that of WT ras.

Previously, it has been shown that mutation of Lys 16 to Asn leads to a mutant protein with decreased intrinsic GTPase activity and reduced affinity for GTP.[5] In addition, point mutations of the equivalent lysine residue, Lys 13, in adenylate kinase (an ATP binding protein which has a similar structural arrangement of Lys)[14] have large effects on the kinetics and structure of the enzyme.[15] Based on these studies it has been argued that Lys 16 of ras plays a key role in stabilizing the transition state for GTP hydrolysis. We were therefore surprised to find that the "unnatural" mutant ras proteins retained high levels of GAP-stimulated GTPase activity and GTP dissociation rates comparable to that of WT ras. These results may indicate that, in the GAP-activated form, Lys 16 is not involved in transition-state stabilization or GTP binding. However, replacement of Lys 16 with the isosteric uncharged Lys analogue, (hydroxyethyl)-cysteine, led to a complete loss of GAP-stimulated GTPase activity, demonstrating the importance of the charged ammonium side chain. The high GAP-dependent activities of the mutants more likely reflect some conformational flexibility in loop 1, consistent with earlier mutagenesis studies of Gly 12 and Gly 13 in ras[10] and Lys 13 in adenylate kinase.[15] The ability of loop 1 of ras to undergo conformational change could provide a mechanism for regulating the rate of GTP hydrolysis or GTP/GDP exchange. The absence of conformational changes in loop 1 would require that the salt bridge between the ammonium ion of the side chain and the transition state/ground state be relatively insensitive to changes in distance. In order to distinguish the latter two alternatives, we are attempting to structurally characterize one or more of the Lys 16 mutants. In either case, these rather conservative lysine mutations, which are only possible through an expanded genetic code, are surprisingly well tolerated in the ras·GTP complex.

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(11) *In vitro* synthesis reactions containing the nonsense mutants in the absence of suppressor tRNA or in the presence of full-length unacylated suppressor $tRNA_{CUA}$ afforded less than 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 *Escherichia coli* S-30 extract do not aminoacylate the suppressor $tRNA_{CUA}$ with the 20 natural amino acids.

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(16) Aminoethylcysteine was purchased from Sigma. Nitroveratryloxycarbonyl (NVOC)-protected aminoethylhomocysteine was obtained from the reaction of *N*-NVOC-2-bromoethylamine with the sodium salt of *N*-NVOC-L-homocysteine. Protected ornithine[22] and hydroxyethylcysteine[10] were synthesized as previously described.

(17) 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 $NH_4Cl$, and 5 mM dithiothreitol (DTT) containing BSA (20 μg/mL) (100 μL reaction volume). At various times, 5-μL samples were mixed with 5 μL of stop mix [2 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate (SDS), and 4 mM of GTP, GDP, and GMP]. Conversion of GTP to GDP was monitored by chromatography on polyethyleneimine (PEI) plates (Sigma), the labeled GDP was excised and quantitated by liquid scintillation counting. The rates were calculated as described by: Satoh, T.; Nakamura, S.; Nakafuku, M.; Kasiro, Y. *Biochem. Biophys. Acta* **1988**, *949*, 97–109.

(18) The GAP assay was performed by a modification of a procedure described by: Han, J. W.; McCormick, F.; Macara, I. G. *Science* **1991**, *252*, 567. Purified ras (100 nM) was incubated for 15 min at 25 °C with 50 μCi [α-$^{32}$P]GTP (410 Ci/mmol; Amersham) in buffer I [50 mM Hepes (pH 7.4), 200 mM sucrose, 1 mM $MgCl_2$, 5 mM EDTA, 0.2% $NaN_3$, and 1 mM DTT]. The [α-$^{32}$P]GTP bound ras was purified on a PD10 column (Pharmacia-LKB). More than 95% of ras was loaded with [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 was 1 mL). In order to determine the time required for 50% conversion of ras·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, GDP, and GMP at 65 °C for 5 min and resolved by chromatography on PEI plates as described previously. The labeled nucleotides were quantitated by scintillation counting. Ras·GTP was 50% converted to ras·GDP after 1 min in the presence of GAP. The relative percent conversions of the mutant ras proteins were determined after 1 min under the same conditions.

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