Biochemical Characterization of a Novel KRAS Insertion Mutation from a Human Leukemia*

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A novel alteration in exon 1 of KRAS was detected by single strand conformational polymorphism analysis of DNA amplified from the bone marrow of a 4-year-old child with myeloid leukemia. Sequencing of this mutant allele revealed an insertion of three nucleotides between codons 10 and 11 resulting in an in-frame insertion of glycine. Expression of the mutant protein in NIH 3T3 cells caused cellular transformation, and expression in COS cells activated the Ras-mitogen-activated protein kinase signaling pathway. Surprisingly, Ras-GTP levels measured in COS cells established that this novel mutant accumulates to 90% in the GTP state, considerably higher than a residue 12 mutant. Biochemical analysis confirmed that the higher Ras-GTP levels correspond to a dramatic decrease in intrinsic GTP hydrolysis as well as resistance to GTPase-activating proteins. This mutation is the first dominant Ras mutation found in human cancer that does not involve residues 12, 13, or 61, and its biochemical properties should help elucidate the mechanism of oncogenic activation.

Mutations of RAS proto-oncogenes are among the most common alterations detected in human cancer cells (1, 2). Activated RAS oncogenes function as dominant alleles and encode proteins with aberrant biochemical activity. Wild-type Ras proteins regulate cellular growth and differentiation by cycling between inactive GDP-bound and active GTP-bound forms (reviewed in Refs. 3 and 4). Oncogenic mutant proteins show defective intrinsic GTP hydrolysis and therefore accumulate elevated levels of Ras-GTP. This, in turn, leads to increased signaling through effector proteins. The defect in intrinsic GTP hydrolysis is augmented by an insensitivity to the Ras GTPase-activating proteins p120-GAP and neurofibromin, which normally bind to Ras-GTP and accelerate the rate of hydrolysis to Ras-GDP (reviewed in Ref. 5).

Ras activation leads to increased flux through a number of effector pathways, including the Raf/mitogen-activated protein (MAP) kinase cascade (6). In normal cells, this is primarily achieved by activation of nucleotide exchange on Ras. In contrast, tumor cells with activated RAS oncogenes accumulate high levels of Ras-GTP because of a defect in the rate of GTP hydrolysis. A wide variety of mutations have been described which mimic oncogenic RAS by transforming the phenotype of cultured cell lines, yet only residues glycine 12, glycine 13, and glutamine 61 have been found mutated in actual human tumors. This observation suggests that such point mutations are uniquely poised to alter the biochemical properties of Ras such that a constitutive signal is induced in the cell.

In analyzing the DNA of a patient with myeloid leukemia, we found a novel activating mutation in the KRAS gene that involves the insertion of three nucleotides between codons 10 and 11, such that an anomalous glycine residue is introduced. We denote this mutant Gly11 to signify this genetic alteration. This mutant Ras protein shares with the codon 12, 13, and 61 mutations the ability to activate the Ras pathway and morphologically transform cell lines. Analysis of the nucleotide dissociation and hydrolysis activities reveals similarities, but also differences, with other activating mutations.

EXPERIMENTAL PROCEDURES

Materials—The Gly11 mutation was introduced by polymerase chain reaction (PCR) into a plasmid encoding the Gau-epitope tagged K-Ras(4B) protein for baculovirus expression (7). An NcoI-EcoRI fragment from this plasmid was introduced into the Neurotrophin-3 (8) and generated pTRc99A (9) for bacterial expression. An NcoI-XbaI fragment from the baculovirus plasmids for K-Ras(wild-type), K-Ras(Gly11), and K-Ras(Gly11) was ligated into the SacI-XhoI sites of pcDNA (9) using SacI and XhoI linker in order to generate the corresponding pcDNA-Ras plasmids. Ras and GAP proteins were purified as described previously (7, 10). The pEXV3-ERK2-tag plasmid has also been previously described (11).

Mutation Detection Techniques—Genomic DNA was extracted from bone marrow cells, amplified with RAS-specific oligonucleotide primers by PCR, and screened for mutations by single strand conformational polymorphism analysis as exactly as described elsewhere (12). Mutations were confirmed by DNA sequence analysis of cloned PCR products using Sequenase, version 2.0.

MAP Kinase Assays—COS cell plates were transfected with 2 μg of pEXV3-ERK2-tag plasmid and 5 μg of pcDNA-Ras plasmid by electroporation (13). In order to measure MAP kinase activity, the ERK2-tag protein was selectively immunopurified using antibodies directed to the epitope tag. Cells were lysed in 20 mM Tris, pH 8, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 50 mM NaF, 1.5 mM MgCl2, 1 mM EGTA, 1 mM vanadate, 1 mM Pefabloc, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and cell debris was removed by centrifugation. 25 μg of ERK2-tag antibodies and 10 μl of protein A-Sepharose FF (Pharmacia Biotech Inc.) were added and incubated for 1 h at 4 °C with constant rotation. The Sepharose beads were pelleted and washed three times with lysis buffer and then once with kinase buffer (30 mM Tris, pH 8, 20 mM MgCl2, 2 mM MnCl2). Kinase activity was then measured by adding 30 μl of kinase buffer containing 1 μM ATP (300 Ci/mmol) and 7 μg of myelin basic protein (Upstate Biotechnology, Inc.) and incubating for 30 min at 30 °C with constant agitation. Assays were stopped by addition of SDS sample buffer and analyzed by 14% polyacrylamide gels. Incorporation of 32P into the myelin basic protein was judged by autoradiography and quantitation.

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Novel KRAS Insertion Mutation

FIG. 1. Detection of an insertion in exon 1 of KRAS in the bone marrow of a child with acute myelogenous leukemia. A, the samples in the first and third lanes show a normal migration pattern (Normal). These bands are also seen in the patient sample (Patient) (second lane) along with two abnormal fragments (indicated by the arrows) that are visible above the normal products. B, an in-frame insertion of a GGA triplet between codons 10 (GGA) and 11 (GCT) was detected in multiple independent clones.

Expression vector pcDB, expressed in NIH 3T3 cells and scored for transformed foci after 10–14 days. All results are the average of duplicate dishes.

| KRAS allele | DNA | Foci per dish |
|-------------|-----|--------------|
| Wild-type   | 5   | 0            |
| Gly11       | 5   | 19 ± 1       |
| Asp12       | 5   | 10 ± 1       |
| Experiment 2 | Wild-type | 5 | 0           |
| Gly11       | 5   | 10 ± 2       |
| Asp12       | 5   | 12 ± 1       |
| Experiment 3 | Gly11 | 20  | 27 ± 1       |
| Asp12       | 20  | 5 ± 1        |

As a further test for activation of Ras signaling, the ability to activate the Rac-MEK-ERK pathway was examined. As shown in Fig. 2, K-Ras(Gly11) induced an activation of recombinant ERK2 activity when cotransfected into COS cells. This activation was considerably higher than that caused by K-Ras(wild-type) and similar to that caused by K-Ras(Asp12).

Since the ability of oncogenic Ras to activate downstream effectors derives from an increased percentage of bound GTP, the nucleotides bound to the various K-Ras proteins were determined in transfected COS cells. The percentage of Ras-GTP was determined after labeling the unstimulated, transfected cells with inorganic [32P]phosphate (Fig. 3A). The Rac-GTP levels of K-Ras(Gly11) (93.5%) were considerably higher than those of K-Ras(Asp12) (45.7%). This suggested that the K-Ras(Gly11) intrinsic GDP dissociation rate may be considerably higher, the GTP hydrolysis rate may be considerably lower, or that an endogenous cellular factor may selectively down-regulate K-Ras(Asp12).

In order to test this latter possibility, purified K-Ras proteins were incubated with excess GTP and the nucleotide dissociation and hydrolysis activities allowed to equilibrate for 5 h. Bound nucleotides were then analyzed using methods similar to those for the COS cell labelings (Fig. 3B). The Rac-GTP levels on the mutant proteins were comparable in COS cells and in solution, while the Ras-GTP levels on the wild-type protein was considerably lower in the CO2 cells (7.0%) than in the cell-free reaction (31.1%). This observation suggests that neither K-Ras(Gly11) nor K-Ras(Asp12) is down-regulated in the CO2 cells. In contrast K-Ras(wild-type) is markedly down-regulated in the CO2 cells, and this is consistent with the hypothesis that the cellular GAPs are active on K-Ras(wild-type) but not on either mutant protein.

Since the difference in Rac-GTP levels between K-Ras(Gly11) and K-Ras(Asp12) is apparently not due to cellular factors, it appeared that intrinsic properties should be accountable. Measurement of intrinsic GTPase activities confirmed this hypothesis (Fig. 4A). While K-Ras(Asp12) has only a 2-fold lower GTPase rate than K-Ras(wild-type), that of K-Ras(Gly11) is over 10-fold lower. In addition, both mutant proteins are insensitive to both p120-GAP (Fig. 4B) and neurofibromin (Fig. 4C), consistent with the observed insensitivity of these Ras proteins to endogenous COS cell down-regulators.

Ras-GTP levels are determined by a counterbalance between...
the rates of GTP hydrolysis and nucleotide dissociation. Therefore, it was of interest to compare the nucleotide dissociation rates of the mutant proteins. As shown in Table II, while the GTP dissociation rates are similar, the GDP dissociation rate off K-Ras(10Gly11) is faster than that off K-Ras(Asp12). This property further accentuates the biochemical differences between the mutant proteins and contributes to the dramatic differences seen in Fig. 3. As discussed below, these findings support the idea that intrinsic biochemical properties are important determinants of the transforming potency of mutant Ras proteins. The altered nucleotide dissociation rates collaborate with reduced GDPase activity and insensitivity to the Ras GAPs to raise the Ras-GTP levels.

**DISCUSSION**

The discovery of a novel activating RAS mutation in the bone marrow of a child with myeloid leukemia has implications for the role of Ras proteins in human cancer. Deregulated signaling through Ras appears to play a central role in myeloid leukemogenesis and may occur by at least three different genetic mechanisms (16). First, 20–40% of leukemic bone marrow of a child with myeloid leukemia has implications for Ras deregulation and progression, the biochemical properties of the Ras proteins are subjected to rigorous selection criteria. Previously, mutations of only three residues, namely Gly12, Gly13, and Gly11, as well as the role of Ras proteins in human cancer. Deregulated signaling through Ras appears to play a central role in myeloid leukemia.
K-Ras(10Gly11) is insensitive to GAPs (Fig. 4). Genetic experiments in yeast and mammalian cells also implicate GAPs as a key mechanism of GTP hydrolysis. The biochemical properties of K-Ras[10Gly11] underscore the importance of GAP proteins in limiting cellular growth by down-regulating Ras. In this context, the ability to interact with effectors is preserved, as judged by the ability to activate the MAP kinase pathway and induce cellular transformation.

The insertion between Gly10 and Ala11 falls within the phosphate binding loop of Ras (22). From the three-dimensional structure of Ras, it appears that the side chain of Lys16 makes contacts with the carbonyl of Gly10 and Ala11, and it is possible that the glycine insertion in K-Ras[10Gly11] disrupts this interaction. This disruption could account for the altered biochemical properties of K-Ras[10Gly11], since Lys16 also makes critical contacts with the γ-phosphate of GTP. The structural and mechanistic consequences of phosphate binding loop mutations are still not understood (23). It will be of interest to determine the structure of K-Ras[10Gly11] in more detail, since this may shed light on the mechanism of GTP hydrolysis.

The biochemical properties of K-Ras[10Gly11] underscore the importance of GAP proteins in limiting cellular growth by down-regulating Ras. In this context, it is striking that a difference in Ras-GTP levels between K-Ras(wild-type) and K-Ras[Asp12] proteins is only evident in transfected COS cells (compare Fig. 3, A and B). This clearly implicates a cellular factor that down-regulates Ras as an important determinant of resistance to transformation. Like other mutant Ras proteins, K-Ras[10Gly11] is insensitive to GAPs (Fig. 4). Genetic experiments in yeast and mammalian cells also implicate GAPs as playing a central role in controlling growth. Yeast strains that are homozygous for targeted disruptions of either the p120-GAP or NF1 genes are nonviable (25–27). Finally, human tumors with NF1 are predisposed to myeloid leukemia and other types of cancer, and these tumor cells frequently show loss of the normal NF1 allele (5, 18). While these biochemical and genetic data emphasize that GAP function is essential to properly regulate Ras signaling in vivo, the intrinsic GTPase activities of Ras proteins strongly influence their transforming potential. Thus, a Ras mutant with Gly12 replaced by Pro or Ala is not transforming, despite insensitivity to GAPs, since intrinsic GTPase and GDP dissociation rates are not sufficiently altered (28). Analysis of K-Ras[10Gly11] provides additional insights into the specific requirements for determining the transforming potency of Ras mutants. A better understanding of the biochemical defects associated with oncogenic Ras may lead to improved treatments for a number of human cancers.

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