Di-Ras, a Distinct Subgroup of Ras Family GTPases with Unique Biochemical Properties*

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The small GTPase Ras family regulates a variety of cell functions including proliferation and differentiation. Here we have identified novel Ras members, human Di-Ras1 and Di-Ras2, belonging to a distinct branch of the GTPase family. Di-Ras1 and Di-Ras2 specifically expressed in heart and brain share 30–40% overall identity with other members of Ras family, however, they have the following characteristic substitutions at highly conserved regions among the Ras family. 1) Thr-63 and Ser-65 in Di-Ras are substituted for Ala-59 and Gln-61 positions in Ha-Ras, respectively, that are known to be critical for GTP hydrolysis. 2) Within the effector domains, Di-Ras has Ile at a position corresponding to Asp-33 in Ha-Ras, which is important for its interaction with the downstream effector Raf. As predicted by these substitutions, Di-Ras has only a quite low level of GTPase activity and exists predominantly as a GTP-bound form upon its expression in living cells. Moreover, Di-Ras fails to interact with the Ras-binding domain of Raf, resulting in no stimulation of mitogen-activated protein kinase. Interestingly, introduction of Di-Ras into HEK293T cells induces large cellular vacuolation. These findings raise the possibility that Di-Ras might regulate cell morphogenesis in a manner distinct from other members of Ras family.

The small GTPase Ras family acts as a molecular switch that regulates a wide range of cell functions including proliferation and differentiation (1–3). Ras is activated in response to a variety of extracellular signals, resulting in stimulation of tyrosine kinases either directly or indirectly. The small GTPase, which cycles between active GTP-bound and inactive GDP-bound states, is activated by guanine nucleotide exchange factors that enhance the exchange of bound GDP for GTP and is deactivated by GTPase-activating proteins that increase the intrinsic rate of hydrolysis of bound GTP. The GTP-bound form of Ras associates with several effector molecules, most notably members of the Raf family, the RafGDS family and phosphoinositide 3-kinase. Amino acid residues 32–40 of Ras are important for its interaction with effector molecules and designated as the “effector domain” (4). The biological consequences of their interactions depend greatly on the cell type and on the context of other signaling events.

The members of Ras family, at least 13 at present, are characterized by extensive similarities in their effector domains (5). Besides three Ras proteins (Ha-Ras, Ki-Ras, and N-Ras), there are four Rap proteins (Rap1A, Rap1B, Rap2A, and Rap2B), two Ral proteins (RalA and RalB), R-Ras, TC21, R-Ras3/M-Ras, and Rheb in the Ras family. Although many Ras members can interact with the same effector molecules as the three Ras proteins, the physiological roles of most Ras-like GTPases are not fully understood.

More recently, some members of the Ras family have begun to be analyzed. For example, Rap1, the closest relative of Ras, has attracted much attention because of the possibility that it functions independently or coordinately with Ras-mediated signaling (6). Rap1 was originally discovered as a suppressor of Ki-Ras-induced transformation, and it has been considered to function as an antagonist of Ras signaling by trapping Ras effectors, particularly the Ser/Thr kinase Raf (7). However, recent studies show that Rap1 is activated by extracellular signals through several regulatory proteins, and raise the possibility that it may function in diverse biological processes, ranging from modulation of cell growth and differentiation to secretion, integrin-mediated cell adhesion, and morphogenesis. It is thus expected that further studies on other members of Ras-like GTPases would also reveal novel signaling pathways involved in a variety of cell functions.

In the present study, we report two novel members, Di-Ras1 and Di-Ras2, belonging to a distinct branch of the Ras GTPase family. Although clearly related in sequence, Di-Ras proteins possess different biochemical and functional properties from other members of the Ras family. It is expected that Di-Ras may be involved in novel cellular functions distinct from other Ras-like GTPases.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY059641 and AY056037 (Di-Ras1) and BC008065 (Di-Ras2).

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screen a human brain c ZAPII cDNA library. Of 8.6 × 10^6 plated
screened, eight positive clones were isolated and sequenced. Four of
the eight positive clones were identified as Di-Ras1 cDNA, and the residual
four positive clones were overlapping cDNA clones homologous to Di-
Ras1 cDNA (thus designated Di-Ras2). The 5′-cDNA fragments of Di-
Ras1 were amplified using human brain Marathon Ready cDNA and
Advantage cDNA PCR Kits (Clontech) according to the manufacturers
instructions.

Sequence Analysis—DNA-sequencing reactions were performed us-
ing the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Am-
ershram Biosciences). Samples were electrophoresed on an ABI 373 DNA
Sequencer and analyzed by ABI Prism model version 2.1.1 soft-
ware (PE Applied Biosystems). Multiple alignment of Di-Ras proteins to rel-
ated members was carried out using CLUSTALW and viewed in TREEVIEW.

Northern Blot Analysis—Expression patterns of Di-Ras1 and Di-
Ras2 mRNAs were analyzed using human multiple tissue Northern blot
(Clonetech). Hybridization was carried out in the ExpressHyb hybridiza-
tion solution (Clontech) in the presence of the [32P]cDNA probe accord-
ing to the manufacturers instructions. Hybridized blots were washed
twice with 2× SSC (1× = 0.15 M NaCl and 0.015 M sodium citrate) and
0.1% SDS, twice with 0.5× SSC and 0.1% SDS, and once with 0.2× SSC
and 0.1% SDS at 68 °C. Filters were exposed to x-ray film (Fujifilm) at
−80 °C for 2 to 3 days with an intensifying screen.

Expression and Purification of Di-Ras1 Protein—Di-Ras1 and Ha-
Ras cDNAs corresponding to the 1–194 and 1–180 amino acids, respec-
tively, were cloned in pGEX-4T-1 vector to express GST fusion proteins.
Overtight culture of Escherichia coli BL21-CodonPlus DE3 (Strat-
agen) containing the expression vector was diluted 1.5 times with a 2× YT
medium containing 50 μg/ml ampicillin and grown at 37 °C for 2 h (A600 = 0.5), followed by induction with isopropyl-1-thio-
β-D-galactopyranoside at 20 °C for 14 h. The cells (250 ml of culture) were
collected by centrifugation and suspended in 20 ml of buffer A consist-
ing of 50 mM Na-Hepes (pH 7.4), 100 mM NaCl, 5 mM MgCl2, 5 mM DTT,
0.2 μg/ml aprotinin, and 0.5 mM Pefabloc SC (Roche Molecular
Biocchemicals). The suspension was sonicated 10 times for 2 s in ice-
water bath to generate a solution of 10% (w/v) CHAPS. The suspen-
sion was sonicated at 4 °C for 30 min. After centrifugation (100,000 × g, 4 °C,
1 h), the clear supernatant was applied to a glutathione-Sepharose 4B
column (0.8-ml bed) that had been equilibrated with 5 ml of buffer B consist-
ing of 20 mM Na-Hepes (pH 7.4), 100 mM NaCl, 5 mM MgCl2, 1 mM DTT,
and 0.7% (w/v) CHAPS. After washing the column with the same buffer,
proteins were eluted from the column with 5 ml of buffer C consisting of 100 mM Na-Hepes (pH 7.4), 5 mM MgCl2, 10 mM gluta-
thane, and 0.7% (w/v) CHAPS. The eluted proteins were then applied
to an gel filtration column (Sephadex G-25, 9.1-ml bed) that had been equilibrated with 30 ml of buffer D consisting of 20 mM Na-Hepes (pH 7.4),
100 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 0.7% (w/v) CHAPS. Wash-
ing the column with the same buffer, proteins were eluted from the column with 5 ml of buffer E consisting of 40 mM Na-Hepes (pH 7.4), 1 mM EDTA, 11 mM MgCl2, 1 mM DTT, and 0.3% (v/v) CHAPS. After incubation for various periods,
samples were diluted with 400 μl of an ice-cold wash buffer (20 mM Tris-
HCl, pH 7.5, 20 mM MgCl2, and 100 mM NaCl) and filtered through a
nitrocellulose membrane (0.45-μm pore size, Advantec). The membrane
was washed three times with 2× SSC and dried at 40 °C. Radioactivity retained on the membrane was determined by a liquid scintillation counter. For nucleotide dissociation assays, purified proteins were preloaded with the radiolabeled nucleotides in a total volume of 280 μl as described above. A 10-fold excess of unlabelled nucleotides was added, and aliquots (40 μl) of the reaction mixture were left at 30 °C for indicated times after incubation at 30 °C. Then, they were immediately diluted with the wash buffer and subjected to the membrane binding assay as described above.

The steady-state rate of GTP hydrolysis was performed as follows.

RESULTS

Identification of Novel Members of the Ras GTPase Fam-
ily—We identified novel members of the Ras GTPase family, termed Di-Ras1 and Di-Ras2. Di-Ras stands for a distinct sub-
group of the Ras family. Di-Ras1 was identified in searching the genome data base for new Ras-related GTPases as follows.
We found that a cosmid clone derived from the chromosome 19.q13.1 locus contains an open reading frame, which encodes a putative Ras-related protein. To confirm the actual transcription of the gene, PCR analysis was performed using human brain cDNA library. We obtained a PCR product with the expected molecular weight, of which the nucleotide sequence matched the coding region within the cosmid DNA. The resulting PCR product was then used to screen a human brain λ ZAPII cDNA library (Stratagene). Eight positive colonies were isolated from 8.6 x 10⁶ clones, and their inserts were sequenced. Comparison with genomic sequence revealed that four of the eight colonies were derived from the Di-Ras1 gene. The combined nucleotide sequence of the four clones revealed a four of the eight colonies were derived from the gene. Comparison with genomic sequence revealed that matched the coding region within the cosmid DNA. The result expected molecular weight, of which the nucleotide sequence

Di-Ras1

594 bp, and 3'-UTR of 1.1 kilobase pairs. 5'-Rapid amplification of cDNA ends experiments identified further a 5'-UTR sequence, which included a termination codon upstream of the predicted ATG translation initiation site. The predicted protein was designated as Di-Ras1. The remaining four of the eight colonies were overlapping clones similar to Di-Ras1 cDNA. The combined nucleotide sequence revealed a 5'-UTR region of 32 bp with a termination codon upstream of the predicted ATG translation initiation site, a coding sequence of 597 bp, and 3'-UTR of 1.8 kilobase pairs. The predicted sequence encoded a protein highly related to Di-Ras1 (79% identity, 88% similarity), thus we designated it as Di-Ras2. There are some unpublished DNA sequences in the EMBL data base, of which coding sequences are identical to Di-Ras1 and Di-Ras2.

Di-Ras As a Distinct Subgroup of the Ras GTPase Family—

Fig. 1A shows amino acid alignment of human Di-Ras and other members of the Ras family. Di-Ras1 (Di-Ras2) shares 40% (41%) amino acid identity with Rap2A, 36% (39%) with Rap1A, and 32% (32%) with Ha-Ras. Besides these Ras members, Di-Ras proteins share 40% identities with ARHI, a recently identified tumor suppressor gene product (11). An unrooted phylogenetic tree of Di-Ras and other members of the Ras family indicates that Di-Ras1, Di-Ras2, and ARHI can be classified into a distinct subgroup of the Ras family (Fig. 1B). We found several overlapping clones of mouse- and rat-expressed sequence tags whose sequences are highly homologous to human Di-Ras1 (data not shown). In addition, BLAST search of the protein data base revealed that Di-Ras proteins share significant homologies with two predicted gene products of Caenorhabditis elegans C54A12.4 and Drosophila melanogaster CG8500 (Fig. 2). However, we could not find any homologue of human Di-Ras in budding yeast, fission yeast, Arabidopsis, and Dictyostelium.

Motif searches of the predicted Di-Ras sequences revealed that they contain a highly conserved GTP-binding domain, a putative effector domain (corresponding to the amino acid sequence 32–40 of Ha-Ras), and the membrane localizing CAAX motif (where C is Cys, A is an aliphatic amino acid, and X is any amino acid) at the carboxyl terminus. However, Thr-63 and Ser-65 in Di-Ras are substituted for Ala-59 and Gln-61, positions that are known to be critical for GTP hydrolysis in the Ras family. These positions in Ras, when substituted with a variety of amino acids, confer oncogenicity by rendering Ras defective for GTP hydrolysis and resistance to the stimulation by GTPase-activation proteins, thereby causing Ras to be maintained as an activated state (12, 13). Moreover, within the core effector domain, Di-Ras differs from most Ras members in that Ile-37 (both Di-Ras1 and Di-Ras2) and Val-40 (Di-Ras2) are substituted for Asp-33 and Ile-36 in Ha-Ras.

Expression of Di-Ras mRNAs in Human Tissues—Di-Ras cDNAs could be detected in the oligo(dT)-primed cDNA library, indicating that Di-Ras mRNA can be essentially classified into the poly(A)+ class. To determine the expression pattern of Di-Ras mRNAs, Northern blotting analysis was performed using a human multitissue Northern blot. Di-Ras1 mRNA (4.0 kb) was detected in brain and heart, whereas Di-Ras2 mRNA (4.4 kb) was expressed specifically in brain (Fig. 3A). Other tissues expressed quite low or undetectable levels of both transcripts. Closer examination of the expressions in human brain revealed that the Di-Ras1 mRNA was expressed at a high level in the cerebral cortex, the occipital pole, and the frontal and temporal lobes (Fig. 3B). The expression level was quite low in both medulla and spinal cord. The expression pattern of Di-Ras2 was similar to that of Di-Ras1 except that its expression was high in the cerebellum.

Biochemical Properties of Di-Ras—To examine the biochemical properties of Di-Ras1, we expressed it in bacteria as a GST fusion protein and purified it using a gluthathione-Sepharose column. Fig. 4A shows the time course of GTPγS binding to the purified GST-Di-Ras1 in the presence of 10 mM Mg²⁺. More than 15 pmol of GTPγS bound to 40 pmol of GST-Di-Ras1, indicating that about 40% of the purified protein is properly folded and binds the nucleotide. The nucleotide binding to Di-Ras1 required millimolar order concentrations of Mg²⁺, because the maximum binding reached at 100 mM Mg²⁺ was about 30% of that observed at 10 mM Mg²⁺ (data not shown). The binding properties of Di-Ras1 were also investigated under the various concentrations of GTPγS (Fig. 4B). Scatchard analysis indicated that the apparent dissociation constant (Kd) for GTPγS was ~1 μM. Nucleotide specificity was next estimated by competition of various nucleotides with [35S]GTPγS binding (Fig. 4C). GTP and GDP competed for GTPγS (1 μM) binding with half-maximal inhibition being observed at 0.5 and 2 μM, respectively, indicating that the affinity of Di-Ras1 for GTP is higher than that for GDP. In contrast, ATP did not compete for the binding even at a concentration as high as 100 μM. Thus, Di-Ras1 appears to specifically bind guanine nucleotides.

Fig. 5A shows the dissociation of GTPγS from Di-Ras1 in a comparison with that from Ha-Ras. GST-Di-Ras1 and GST-Ha-Ras were first loaded with [35S]GTP and then competition was measured for various concentrations of GTP (Fig. 5B). Scatchard analysis indicated that the apparent dissociation constant (Kd) for GTPγS was ~1 μM. Nucleotide specificity was next estimated by competition of various nucleotides with [35S]GTPγS binding (Fig. 4C). GTP and GDP competed for GTPγS (1 μM) binding with half-maximal inhibition being observed at 0.5 and 2 μM, respectively, indicating that the affinity of Di-Ras1 for GTP is higher than that for GDP. In contrast, ATP did not compete for the binding even at a concentration as high as 100 μM. Thus, Di-Ras1 appears to specifically bind guanine nucleotides.

Identification of Nucleotide Form Associated with Di-Ras in Living Cells—We next examined the nucleotide form associated with Di-Ras1 and Di-Ras2 in living cells. For the analysis, epitope-tagged proteins were expressed in HeLa and HEK293 cells, and the proteins were purified by means of immunoprecipitation. Expression of the transfected constructs was confirmed by immunoblotting (data not shown). The guanine nucleotides associated with the immunocomplex were analyzed by thin-layer chromatography. Fig. 6 shows that wild-type Ha-Ras existed predominantly as a GDP-bound form, whereas about 80% of Ha-Ras/G12V was a GTP-bound form in HeLa cells. In contrast, 78 and 54% of wild-type Di-Ras1 and Di-Ras2, respectively, existed as GTP-bound forms in the cells. A high ratio of the Di-Ras GTP-bound form was also observed in HEK293...
FIG. 1. Comparison of amino acid sequences of human Di-Ras and other members of Ras family. A, the amino acid sequences were aligned using the CLUSTALW program. Amino acid residues conserved between all proteins are shaded in black, and those with 60–99% are in light gray. PM1–3 and G1–3 show well conserved regions important for binding to phosphate/Mg and the guanine base, respectively. The region considered as an effector domain is overlined. The COOH-terminal sequence for prenylation (CAAX box: A, aliphatic; X, any amino acid) is underlined. The sequence data are available from GenBank under accession numbers AB076888 (Di-Ras1) and AB076889 (Di-Ras2). B, the unrooted tree was constructed using the CLUSTALW with standard parameters and viewed using TREEVIEW.
cells. These results indicate that Di-Ras proteins are predominantly GTP-bound forms in living cells.

**Failure of Di-Ras to Stimulate Mitogen-activated Protein Kinase (MAPK) Signaling Pathway**—Growing evidence has suggested that members of the Ras family exert their biological effects through the activation of several divergent downstream pathways (4, 14). One of the major pathways activated by Ras is a Raf-dependent MAPK signaling cascade, which involves the sequential phosphorylation of protein kinases. As an initial attempt to unravel the biological function of Di-Ras, we examined the ability of Di-Ras to stimulate the MAPK pathway.

HEK293 cells transfected with Myc-tagged Ras or Di-Ras were serum-starved, and the total cell lysates were collected. The activation of ERK in these cells was determined by immunoblotting of the cell lysates using an antibody that specifically recognizes Thr-202/Tyr-204-phosphorylated MAP kinase. As shown in Fig. 7A, overexpression of oncogenic Ras/G12V-stimulated MAPK kinase activity in HEK293 cells, however, neither wild-type Di-Ras1 nor its mutant (Di-Ras1/G16V) activated the MAPK pathway. Overexpression of Di-Ras2 also had no effect on the kinase activity.

We thus addressed whether the observed inability of Di-Ras to activate the MAPK pathway is correlated with its inefficiency to bind Raf. The interaction between Di-Ras1 (or Di-Ras2) and Raf was investigated using a yeast two-hybrid system. We constructed a pGBT9 vector containing the cDNA fragment of Di-Ras1 that lacks the COOH terminus CAAX motif to optimize targeting to the yeast nuclear compartment. As a positive control, a similar construct was generated with a pHa-Ras/G12V cDNA that has been shown to bind strongly to RBD of the Raf family (21). RBDs of Raf-1 and B-Raf corresponding to the 48–131 and 146–226 amino acids, respectively, were cloned in pGAD424 vector. Fig. 7B shows that binding of Ha-Ras/G12V to each Raf-RBD was clearly demonstrated by the stimulation of transcriptional activity of the lacZ reporter gene. In contrast, neither Di-Ras1 nor Di-Ras2 binds Raf. We conclude from these results that the inability of Di-Ras to activate the MAPK pathway is because of its inefficiency to bind Raf.

We also investigated whether Di-Ras activates other Ras-effector molecules, such as phosphoinositide 3-kinase. However, expression of Di-Ras in HEK293 cells did not induce Akt
activation, which is a downstream target of the lipid kinase (data not shown). It thus appears that Di-Ras does not interact with the Ras-binding domain (RA domain) of phosphoinositide 3-kinase unlike Ha-Ras.

**Large Vacuolar Formation Induced by Overexpression of Di-Ras in HEK293T Cells** — To examine the subcellular localization of Di-Ras proteins and their functions in living cells, HEK293T cells were transfected with pEGFP vectors containing various forms of Di-Ras at the COOH terminus of EGFP. When lysates of the transfected cells were subjected to immunoblot analysis with an anti-EGFP polyclonal antibody, only a single band of ~48 kDa was observed (Fig. 8), showing that the EGFP signal detected in the transfected cells represented the intact EGFP-Di-Ras fusion proteins. Interestingly, high level expression of EGFP-Di-Ras1 or EGFP-Di-Ras2 induced large intracellular vacuolation, and the proteins were localized to the vacuole membranes as well as the plasma membrane, whereas the proteins were mainly associated with plasma membranes in the cells where their expression levels were low (Fig. 9, D).
and F). The large vacuolar formation was also observed when Myc-tagged Di-Ras proteins were expressed in HEK293T or HEK293 cells (data not shown). In contrast, expression of a control empty vector or the mutant EGFP-Di-Ras1, which lacked the COOH-terminal membrane-anchoring signal sequence, CAAAX, caused diffuse, widespread signals and did not induce cellular vacuolation even at high expression levels (Fig. 9, B and H). As the presence of the CAAAX motif is crucial for normal plasma membrane localization and biological functions of Ras family proteins, the importance of this motif is also likely to be true of Di-Ras proteins. We compared the Di-Ras-induced vacuolar formation with action of Ha-Ras, because oncogenic Ha-Ras/G12V has also been reported to induce cellular vacuolation in some cell lines (15). However, the vacuolation induced by Ha-Ras/G12V was modest and morphologically different from the Di-Ras-induced effect at least in HEK293T cells (data not shown). The expression of EGFP-Di-Ras1 also induced a significant change in the morphology of HeLa cells (data not shown), indicating that the effect of Di-Ras is not specific to HEK293 cells.

**DISCUSSION**

We have herein described the identification and characterization of novel members of the Ras family, human Di-Ras1 and Di-Ras2, which form a distinct branch of the Ras family. The Ras GTPase family normally exists as an inactive GDP-bound state and is converted to an active GTP-bound state in response to a variety of extracellular signals. Di-Ras has a G domain that is highly conserved in the GTPase family, and indeed we have shown that recombinant Di-Ras1 efficiently binds GTP. It is noteworthy that Di-Ras exchanges GTP faster than Ha-Ras and exhibits quite low levels of intrinsic GTPase activity. Di-Ras proteins expressed in HeLa and HEK293 cells were found mostly to be in GTP-bound states. Although we cannot totally rule out the possibility that the high GTP/GDP ratio on Di-Ras in living cells might result from overexpression of the proteins, the biochemical properties of Di-Ras1 support the idea that Di-Ras proteins are largely in GTP-bound states under physiological conditions.

Di-Ras has the following substitutions at two positions when Myc-tagged Di-Ras proteins were expressed in HEK293T or HEK293 cells (data not shown). In contrast, expression of a control empty vector or the mutant EGFP-Di-Ras1, which lacked the COOH-terminal membrane-anchoring signal sequence, CAAAX, caused diffuse, widespread signals and did not induce cellular vacuolation even at high expression levels (Fig. 9, B and H). As the presence of the CAAAX motif is crucial for normal plasma membrane localization and biological functions of Ras family proteins, the importance of this motif is also likely to be true of Di-Ras proteins. We compared the Di-Ras-induced vacuolar formation with action of Ha-Ras, because oncogenic Ha-Ras/G12V has also been reported to induce cellular vacuolation in some cell lines (15). However, the vacuolation induced by Ha-Ras/G12V was modest and morphologically different from the Di-Ras-induced effect at least in HEK293T cells (data not shown). The expression of EGFP-Di-Ras1 also induced a significant change in the morphology of HeLa cells (data not shown), indicating that the effect of Di-Ras is not specific to HEK293 cells.

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Di-Ras has the following substitutions at two positions.
known to be involved in GTPase activity and conserved in most of other small GTPases: amino acids 63 (Thr) and 65 (Ser), equivalent to 59 (Ala) and 61 (Gln) in Ha-Ras, respectively. Substitution of these amino acids in Ha-Ras decreases its intrinsic GTPase activity, which resulted in constitutive activation of Ha-Ras with transforming property. Such substitutions have been reported in other members of the Ras family. For example, Rap2 has a Thr instead of highly conserved Gln at position 61 (16), and the GTP-bound form exceeded 50% of the total protein in HEK293T and NIH3T3 cells (17). Recently, some members of Rho family have also been shown to have substitutions at three positions know to be important for GTP hydrolysis (18–20). RhoE/Rnd3 has a Ser at each position (17, 64, and 66), equivalent to amino acids 12 (Gly), 59 (Ala), and 61 (Gln) in Ras, respectively. RhoE/Rnd3 has no detectable level of GTPase activity and appears to be a constitutively GTP-bound state. It is considered that RhoE/Rnd3 has an antagonistic effect on Rho/Rac-regulated signaling pathways, but its physiological function remains to be defined.

To determine whether the amino acid differences in Di-Ras1 that correspond to oncogenic Ras mutations account for the lack of its GTPase activity, we performed site-directed mutagenesis to replace amino acids 63 (Thr) and 65 (Ser) with their normal counterparts (Ala-59 and Gln-61, respectively) seen in other members of the Ras family. The double substitution (T63A,S65Q) increased the intrinsic GTPase activity of Di-Ras1, but did not completely restore the GTPase activity to the level of that seen with Ha-Ras (data not shown). These results indicate that besides these amino acids, other amino acid residue(s) of Di-Ras1 may also be responsible for its reduced GTPase activity.

By analogy to previously characterized GTP-binding proteins, it is expected that the GTP-bound Di-Ras is the active state. If this is true, how is the cellular activity of Di-Ras regulated? One possibility is that Di-Ras function is attenuated by inhibitory binding proteins or by sequestration at specific intracellular locations. An alternative possibility is that the Di-Ras activity is regulated at an expression level. To date, we could not detect Di-Ras1 by immunoblotting using an anti-Di-Ras1 antibody in some human cell lines, where Di-Ras1 mRNA could be detected (data not shown). The inability to detect the Di-Ras1 protein may be because of low titer of the antibody and/or the low level of protein expression, however, it is possible that the protein level of Di-Ras1 is regulated at the translation level.

The members of Ras family, at least 13 at present, are characterized by extensive similarities in their effector domains, and many Ras members can interact with the same effector proteins including Raf (4, 5). As for Di-Ras1 and Di-Ras2, their putative effector domains are the same as other Ras members, except for amino acid 37 (Ile), corresponding to position 33 (Asp) in Ha-Ras. This position is important in the Ras family for interaction with the downstream effector Raf, because substitution of Asp-33 to Asn in Ha-Ras impairs both its interaction with Raf-1 and its transforming activity (21). Indeed, we have found that Di-Ras neither binds RBD of both Raf-1 and B-Raf nor activates the MAPK pathway. We also investigated whether Di-Ras can bind to the other known Ras effector molecule. RIN1 (Ras interaction/interference) is known to bind to the GTP-bound Ha-Ras and stimulate guanine nucleotide exchange on Rab5 (22, 23). Unlike Ha-Ras, we could not detect the interaction of Di-Ras with RIN1 in a yeast two-hybrid assay (data not shown). Although we cannot totally rule out the possibility that Di-Ras activates other effector molecule(s) commonly activated by Ras family members, the novel GTPase might selectively stimulate unidentified downstream effector(s).

Northern blot analysis of mRNA isolated from various human tissues shows that Di-Ras transcripts are highly restricted to the brain and heart. This intriguing expression pattern is similar to that of a recently identified Ras-related GTPase, R-Ras3/M-Ras, of which the transcript is also abundant in brain and heart (24, 25). R-Ras3/M-Ras has been shown to have transforming activity in NIH 3T3 cells (26, 27), but Di-Ras lacked the activity in the cells (data not shown). R-Ras3/M-Ras and Di-Ras1 may have different biological functions in brain and heart, although the expression pattern is similar.

We have demonstrated that high expression of Di-Ras proteins caused cellular vacuolation, although the nature of the large vacuoles induced by the expression has not been determined. Overexpression of activated Ras has been known to stimulate fluid-phase endocytosis (28), and a recent report shows that the stimulation seems to occur upstream of the action of Rab5 (23). Our preliminary data, however, showed that a large fraction of the vacuoles induced by Di-Ras did not incorporate a fluid-phase tracer (Texas Red-conjugated dextran) or pH-sensitive probes (neutral red and LysoTracker). Although we cannot formally rule out the possibility that the observed localization and vacuolation are because of overexpression of the proteins, Di-Ras might be involved in regulation of membrane transport, and their lipid-anchoring motifs could be important for their localization and functions. The eventual identification of this vacuolar structure will likely provide an important clue to the biological function of Di-Ras.

Di-Ras1 and Di-Ras2 have significant homology to ARHI (NOEY2), which has been recently identified as a tumor suppressor (11). ARHI is expressed consistently in normal ovarian and breast epithelial cells but is down-regulated in ovarian and breast cancers (11, 29). Re-expression of ARHI through transfection suppressed clonogenic growth of ovarian and breast cancer cells (11). Thus, ARHI may function as a molecular switch that regulates cell growth, although neither the signaling mechanism(s) regulated by the molecule nor its biochemical characterization remain to be defined. Significant sequence homology between Di-Ras and ARHI indicates that these proteins can be classified into a subgroup of the Ras family, and they may have similar roles in cell function. It would be thus worthwhile to investigate the possibility that Di-Ras might function as growth suppressors in neuronal and cardiac muscle cells, because these cells are highly differentiated and cannot divide to proliferate in adult tissues.

In general, the cellular effects of small GTPases are mediated through their GTP-dependent interaction with intracellular targets or effectors, and numerous candidates have already been identified using the yeast two-hybrid system and affinity chromatography techniques. Identification of effector molecule(s) or binding partner(s) to Di-Ras would provide important clues to understanding of their physiological functions.

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Addendum—During revision of this paper, the characterization of a novel Ras-related protein, Rig, which is identical to the present Di-Ras, was reported by Ellis et al. (30).

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