THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 43, Issue of October 25, pp. 41070–41078, 2002
Printed in U.S.A.

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

Received for publication, March 5, 2002, and in revised form, August 9, 2002
Published, JBC Papers in Press, August 22, 2002, DOI 10.1074/jbc.M202150200

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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 RalGDS 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-related GTPases.

MATELIALS AND METHODS

Data Base Search and cDNA Cloning of Di-Ras—Data base searches using Entrez (NCBIs search and retrieval system) were performed to find novel Ras-related GTPases. Among the many candidate genes in the retrieval results was a cosmid clone (accession number AC006538) that contains a hypothetical Ras-related gene, which we designated *Di-Ras1*. Sequence data from the open reading frame of the cosmid were used to design specific primers to amplify the cDNA fragment of *Di-Ras1*. Primers used were *Di-Ras1F*, 5′-ATGCCGGAACAGAGTAACGAT-3′, and *Di-Ras1R*, 5′-TCACATGAGGGTGCATTTGCC-3′. PCR amplification was performed using human whole brain cDNA (Clontech) and KOD polymerase for 30 cycles (15 s at 98 °C, 2 s at 65 °C, 30 s at 74 °C). The obtained PCR products were sequenced and used to

* This work was supported in part by research grants from the "Research for the Future" Program of the Japan Society for the Promotion of Science (JSPS-RFTF 96L00505) and the Scientific Research Funds of the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 λ ZAPII cDNA library. Of 8.6×10^6 plaques 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 homologues 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 using the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Biosciences). Samples were electrophoresed on an ABI 373 DNA Sequencer and analyzed by ABI Prism model version 2.1.1 software (PE Applied Biosystems). Multiple alignment of Di-Ras proteins to related protein sequences was carried out using CLUSTALW and viewed in GeneDoc. An unrooted phylogenetic tree was prepared 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 (Clontech). Hybridization was carried out in the ExpressHyb hybridization solution (Clontech) in the presence of the [^{32}P]cDNA probe according 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 (Fuji film) at −80 °C for 2−3 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, respectively, were cloned in pGEX-4T-1 vector to express GST fusion proteins. Overnight culture of *Escherichia coli* BL21-CodonPlus DE3 (Stratagene) containing the expression vector was diluted 1:50 with a 2× YT medium containing 50 μg/ml ampicillin and grown at 37 °C for ~2 h (A_{600} = ~0.5), followed by induction with 0.1 mM 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 consisting of 50 mM Na-Hepes (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT,[1] 2 μg/ml aprotinin, and 0.5 mM Pefabloc SC (Roche Molecular Biochemicals). The suspension was sonicated 10 times for 2 s in ice-cold water and mixed with 0.1 volumes of 10% (w/v) CHAPS. The suspension was rotated 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 consisting of 20 mM Na-Hepes (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 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 $MgCl_2$, 10 mM glutathione, and 0.7% (w/v) CHAPS. The eluted proteins were then applied to a gel filtration column (Sephadex G-25, 9.1-ml bed) that had been equilibrated with 30 ml of buffer D consisting of 50 mM Na-Hepes (pH 7.4), 20 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.7% (w/v) CHAPS, and the fractions containing GST fusion proteins were collected and stocked at −80 °C until use.

Nucleotide Binding and GTPase Assays—[^{35}S]GTPγS binding to the purified proteins was performed as follows. Purified proteins (2 μg) were incubated at 30 °C with different concentrations of the radiolabeled nucleotides (~3,000 cpm/pmol) in a total volume of 40 μl of buffer E consisting of 40 mM Na-Hepes (pH 7.4), 1 mM EDTA, 11 mM $MgCl_2$, 1 mM DTT, and 0.3% (w/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 $MgCl_2$, and 100 mM NaCl) and filtered through a nitrocellulose membrane (0.45-μm pore size, Advantech). The membrane was washed three times with 2 ml of the ice-cold wash buffer 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 unlabeled nucleotides was added, and aliquots (40 μl) of the reaction mixture were withdrawn at the indicated times after the incubation at 30 °C. 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. Purified proteins (4.5 μg) were incubated at 30 °C with 1 μM [γ-^{32}P]GTP (3,000 cpm/pmol) in a total volume of 350 μl of buffer B containing various concentrations of EDTA and $MgCl_2$. The free Mg^{2+} concentration was calculated as described previously (8). After incubation of the indicated times, aliquots (50 μl) of the reaction mixture were withdrawn and mixed with 750 μl of ice-cold 5% (w/v) Norit SX-Plus in 50 mM NaH_2PO_4. The mixture was centrifuged at 15,000 rpm for 5 min at 4 °C, and the supernatant (300 μl) was analyzed for the amounts of $^{32}P_i$ released during the incubation.

Cell Culture and Transfection—HeLa, HEK293, and HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were transfected with 1 or 3 μg (for 35-mm or 60-mm dish) of plasmid DNA using LipofectAMINE 2000. For ERK kinase assay, the cells were cultured for 24 h after transfection and starved for an additional 12-h incubation in Dulbecco's modified Eagle's medium containing 0.1% fatty acid-free bovine serum albumin (Sigma).

In Vivo Phosphate Labeling and Immunoprecipitation—PCR cloning was used to insert the *Di-Ras* and *Ha-Ras* coding sequences into a modified pCMV5 vector (pMyc-CMV5) that places the Myc tag at its amino terminus. Expression of the Myc-tagged proteins was confirmed by transfection of HeLa or HEK293 cells followed by immunoblotting with an anti-Myc monoclonal antibody. Guanine nucleotides bound to the GTP-binding proteins were analyzed essentially as described previously (9). Briefly, the cells, which had been cultured in 60-mm dishes for ~36 h after transfection, were labeled for 4 h with $^{32}P_i$ (1.85 MBq/dish) in phosphate-free Dulbecco's modified Eagle's medium. The labeled cells (~3×10^6 cells) were lysed with 1 ml of an ice-cold solubilizing buffer (40 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgC_2, 1 mM Na_3VO_4, 1 mM DTT, 1% (w/v) Triton X-100, 2 μg/ml aprotinin, and 0.5 mM Pefabloc SC) and clarified, and the precleared lysates were incubated with the anti-Myc monoclonal antibody (5 μg) and protein G-Sepharose at 4 °C for 2 h. After extensive washing of the immunocomplexes, associated nucleotides were separated by thin-layer chromatography and quantitated with a BAS-1800 image analyzer (Fuji Film).

Yeast Two-hybrid Analysis—A yeast two-hybrid assay was performed according to the method described previously (10). *Di-Ras1, Di-Ras2*, and *Ha-Ras* cDNAs corresponding to the 1–194, 1–195, and 1–180 amino acids, respectively, were cloned in pGBT9 vector. Ras-binding domains (RBD) of Raf-1 and B-Raf corresponding to the 48–131 and 146–226 amino acids, respectively, were cloned in pGAD424 vector. Yeast strain Y190 was double transformed with the pGBT9- and pGAD424-derived vectors. Transformants were grown on a synthetic medium lacking leucine and tryptophan. Colonies were picked 3 days after plating and tested for β-galactosidase activity.

Assay of ERK Activity—For detection of phosphorylated ERK, the cells were washed once with an ice-cold phosphate-buffered saline and lysed in 200 μl of a gel-loading buffer (10% glycerol, 2% SDS, 0.02% (w/v) bromphenol blue, 50 mM Tris-HCl, pH 6.8, and 5% 2-mercaptoethanol). Cell lysates were sonicated briefly and boiled for 5 min. Samples were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. To determine the phosphorylation of endogenous ERK, immunoblot analysis was conducted using an antibody that specifically recognizes Thr-202/Tyr-204-phosphorylated MAP kinase (Cell Signaling Technology). The bands were visualized using Super-Signal West Pico chemiluminescent for the development of immunoblots using a horseradish peroxidase-conjugated secondary antibody according to the manufacturers instructions (Pierce).

Expression of Di-Ras Proteins Fused to EGFP and Fluorescence Microscopy—cDNAs of *Di-Ras1* and *Di-Ras2* were cloned into pEGFP vector (Clontech) for expression of EGFP fusion proteins. HEK293T cells were transfected with 1 μg (for 35-mm dish) of plasmid DNA using LipofectAMINE 2000. After a 2-day culture, cell lysates were prepared and subjected to immunoblot analysis using an anti-EGFP polyclonal antibody (Clontech). For fluorescence microscopy, the cells were grown on poly-L-lysine-coated glass coverslips mounted at the bottom of a 35-mm dish with a 12-mm hole (IWAKI) and transfected with 1 μg of plasmid DNA as described above. After 40–48 h, the cells were viewed with a Carl Zeiss LSM-510 confocal microscope.

RESULTS

Identification of Novel Members of the Ras GTPase Family—We identified novel members of the Ras GTPase family, termed Di-Ras1 and Di-Ras2. Di-Ras stands for a distinct subgroup of the Ras family. Di-Ras1 was identified in searching the genome data base for new Ras-related GTPases as follows.

[1] The abbreviations used are: DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; GTPγS, guanosine 5′-*O*-(thiotriphosphate); MAPK, mitogen-activated protein kinase; RBD, Ras-binding domain; GST, glutathione *S*-transferase; UTR, untranslated region.

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 8.6×10^6 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 5′-untranslated region (5′-UTR) of 68 bp, a coding sequence of 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. 1*A* 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. 1*B*). 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 C*AAX* 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. 3*A*). 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. 3*B*). 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 glutathione-Sepharose column. Fig. 4*A* shows the time course of GTPγS binding to the purified GST-Di-Ras1 in the presence of 10 mM Mg^{2+}. 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 Mg^{2+}, because the maximum binding reached at 100 nM Mg^{2+} was about 30% of that observed at 10 mM Mg^{2+} (data not shown). The binding properties of Di-Ras1 were also investigated under the various concentrations of GTPγS (Fig. 4*B*). Scatchard analysis indicated that the apparent dissociation constant (K_d) for GTPγS was ~1 μM. Nucleotide specificity was next estimated by competition of various nucleotides with [^{35}S]GTPγS binding (Fig. 4*C*). 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. 5*A* 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 [^{35}S]GTPγS, and dissociation of the bound nucleotide was measured in the presence of 100 μM nonlabeled GTPγS. The radiolabeled GTPγS was rapidly released from GST-Di-Ras1, whereas it was tightly retained on GST-Ha-Ras under the same conditions, indicating that Di-Ras1 exchanges GTPγS extremely faster than Ha-Ras. We next assayed the steady-state rates of GTP hydrolysis catalyzed by GST-Di-Ras1 and GST-Ha-Ras (Fig. 5*B*). GST-Ha-Ras catalyzed GTP hydrolysis at a low concentration of Mg^{2+}. In contrast, GTPase activity of GST-Di-Ras1 was quite low under the conditions of both high and low Mg^{2+}. A thrombin-cleaved form of Di-Ras1 that lacks the GST portion exhibited the same biochemical properties as those of GST-fused Di-Ras1 (data not shown).

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

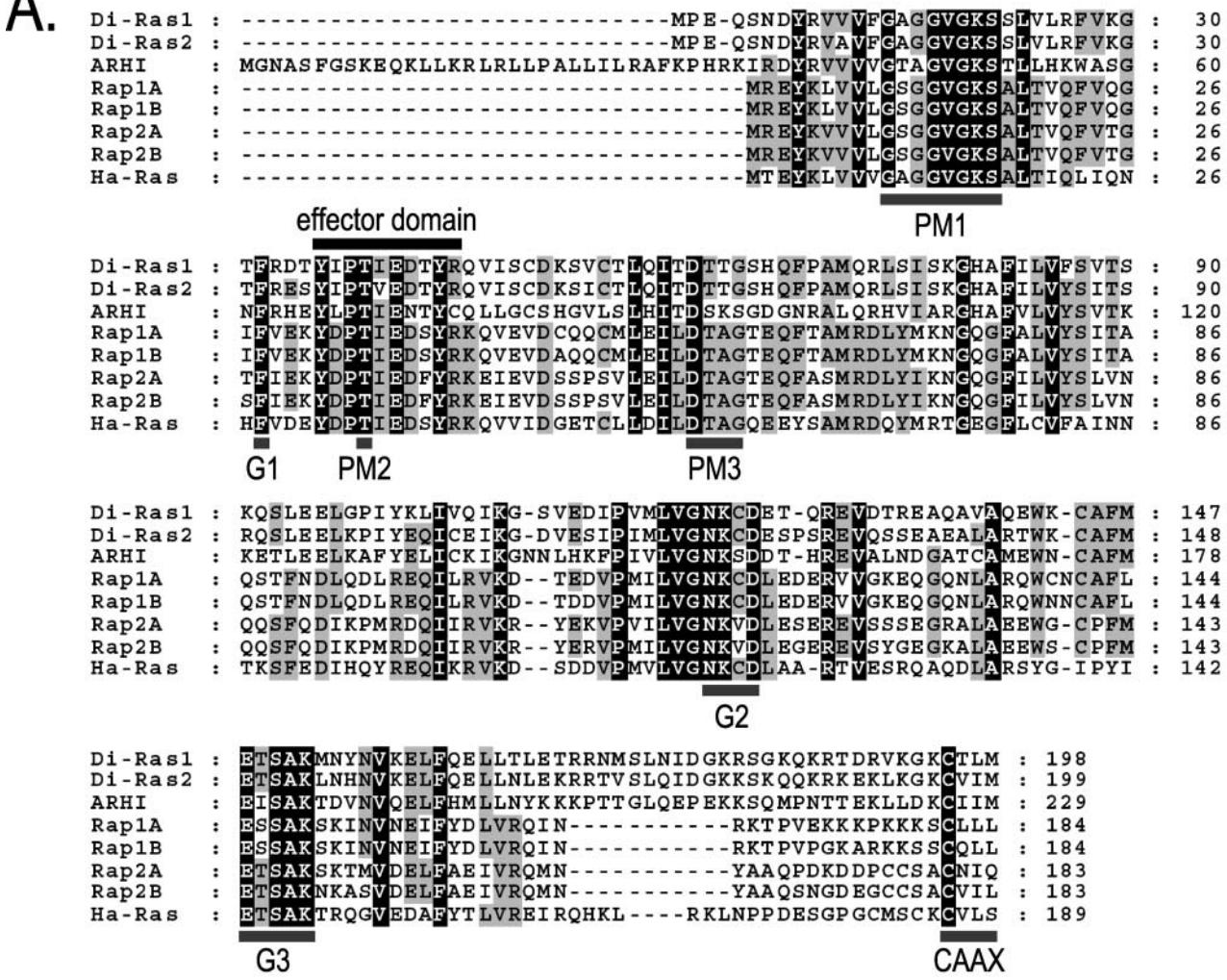

FIG. 1. **Comparison of amino acid sequences of human Di-Ras and other members of Ras family.** *A*, the amino acid sequences were aligned using the CLUSTALW program. Amino acid residues conserved between all proteins are *shaded* in *black*, and those with 60–99% are in *light gray*. PM1–3 and G1–3 show well conserved regions important for binding to phosphate/Mg^{2+} and the guanine base, respectively. The region considered as an effector domain is *overlined*. The COOH-terminal sequence for prenylation (C*AAX* 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.

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Di-Ras1   : ----------------------MPEQSN-DYRVVVFGAGGVGKSSLVLRFVKGTFRDTYIPTIEDTYRQV :  47
Di-Ras2   : ----------------------MPEQSN-DYRVAVFGAGGVGKSSLVLRFVKGTFRESYIPTVEDTYRQV :  47
CG8500    : -----------MTEQKNQVTRAAPEQSN-DYRVVVFGAGGVGKSSLVLRFIKGTFRESYIPTIEDTYRQV :  58
C54A12.4  : MLTFFHNSKKCAKYQLSKLWKQSSEASTSDYRVAVFGAGGVGKSSITQRFVKGTFNENYVPTIEDTYRQV :  70

Di-Ras1   : ISCD-KSVCTLQITDTTGSHQFPAMQRLSISKGHAFILVFSVTSKQSLEELGPIYKLIVQIKG-SVEDIP : 115
Di-Ras2   : ISCD-KSICTLQITDTTGSHQFPAMQRLSISKGHAFILVYSITSRQSLEELKPIYEQICEIKG-DVESIP : 115
CG8500    : ISCN-KNICTLQITDTTGSHQFPAMQRLSISKGHAFILVYSVCSKQSLEELRPIWALIKELKGADIPNIP : 127
C54A12.4  : ISCNQKNVCTLQITDTTGSHQFPAMQRLSISKGNAFILIYSVTNKQSFAELVPIIEMMKEVKGNAIAETP : 140

Di-Ras1   : VMLVGNKCDET--QREVDTREAQAVAQEWKCAFMETSAKMNYNVKELFQELLTLETRRNMSLNIDGKRSG : 183
Di-Ras2   : IMLVGNKCDESP-SREVQSSEAEALARTWKCAFMETSAKLNHNVKELFQELLNLEKRRTVSLQIDGKKSK : 184
CG8500    : VMLVGNKCDETAELREVSQAEGQAQATTWSISFMETSAKTNHNVTELFQELLNMEKTRTVSLQLDTKKQK : 197
C54A12.4  : IMLVGNKKDEES-KREVSSNSGQKVATNMECGFIETSAKNNENITELFQQLLALEKKRQLALTMDDPDGK : 209

Di-Ras1   : KQKRTDR----------------------VKGKCTLM : 198
Di-Ras2   : QQKRKEK----------------------LKGKCVIM : 199
CG8500    : KQKKEKKSKDTNGSIPENGDAGASASGGAKEKCRVM : 233
C54A12.4  : NGK--------------------------KKGCHIM : 219
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FIG. 2. **Alignment of human, *D. melanogaster* and *C. elegans* Di-Ras proteins.** Deduced amino acid sequences from the indicated genes were aligned using CLUSTALW. Amino acid residues conserved among all proteins are shaded in *black*, and those with 50–99% are in *light gray*. Accession numbers of *D. melanogaster* CG8500 and *C. elegans* C54A12.4 are AAF54453 and AAA68305, respectively, in the NCBI Entrez Protein data base.

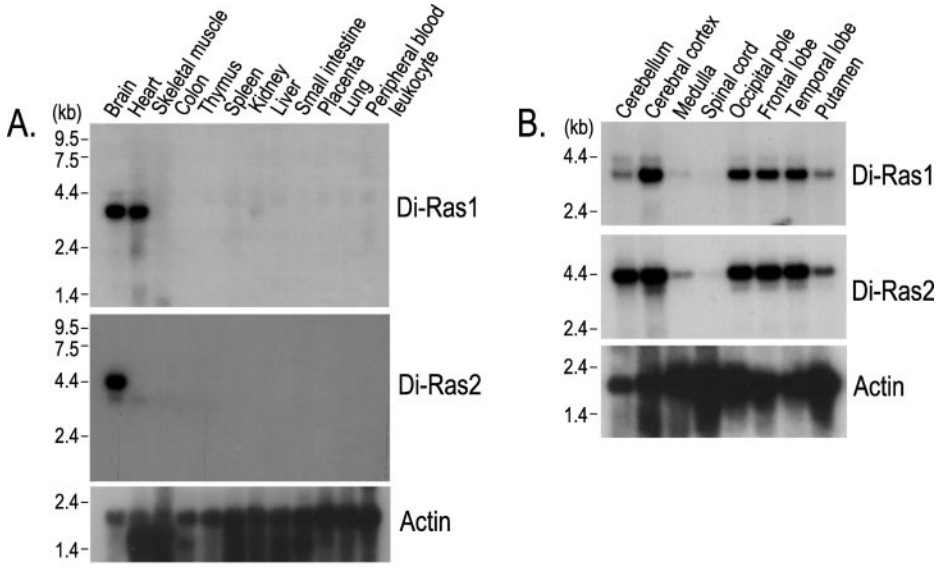

FIG. 3. **Expression of *Di-Ras1* and *Di-Ras2* mRNAs in human tissues.** *A*, Northern blots of human poly(A)$^+$ RNAs from various human tissues (Clontech, 7780-1) were hybridized with ^{32}P-labeled *Di-Ras* probes. *B*, Northern blots of human poly(A)$^+$ RNAs from eight different regions of the human brain (Clontech, 7755-1) were hybridized with ^{32}P-labeled *Di-Ras* probes. Hybridization and washing were performed as described under "Experimental Procedures."

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. 7*A*, overexpression of oncogenic Ras/G12V-stimulated MAP 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 of COOH terminus C*AAX* motif to optimize targeting to the yeast nuclear compartment. As a positive control, a similar construct was generated with a *Ha-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. 7*B* 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 RBD of each 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

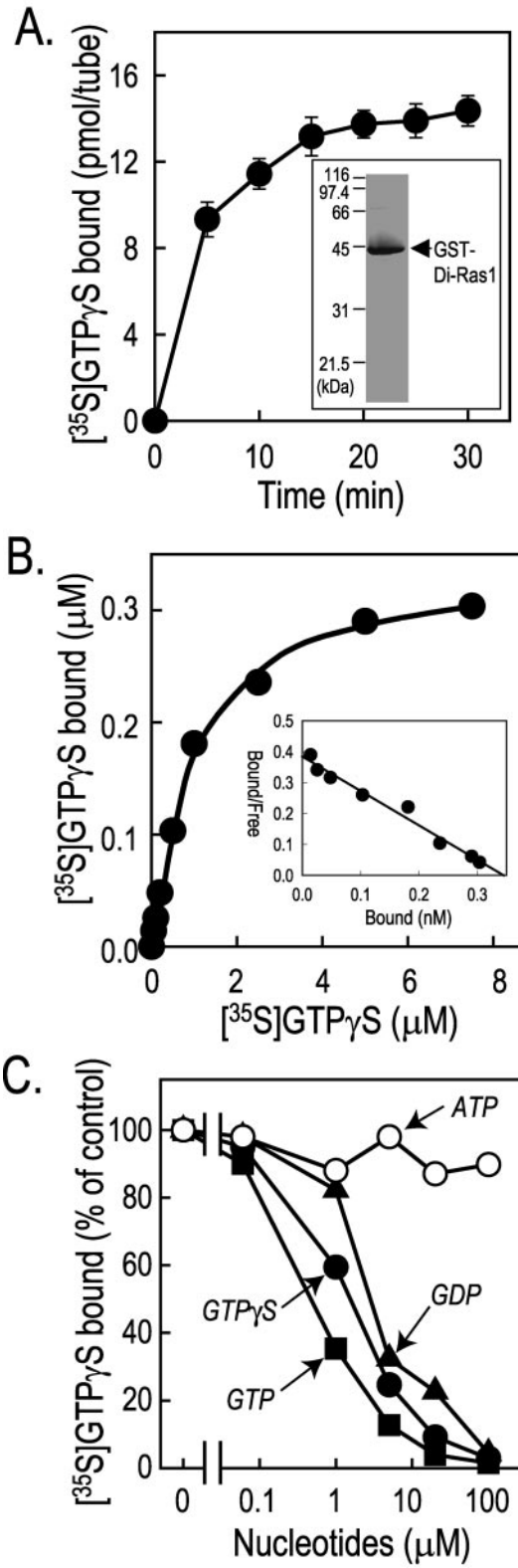

FIG. 4. **Nucleotide binding properties of Di-Ras1 protein.** *A*, the purified GST-Di-Ras1 (50 μg/ml) was incubated with 10 μM [^{35}S]GTPγS for the indicated times, and [^{35}S]GTPγS binding was determined as described under "Experimental Procedures." The *inset* shows SDS-PAGE analysis of the purified GST-Di-Ras1 (Coomassie Blue stain). *B*, the purified GST-Di-Ras1 protein (50 μg/ml) was incubated for 30 min with the indicated concentrations of GTPγS. The *inset* shows the Scatchard plot obtained with the [^{35}S]GTPγS binding data. *C*, the purified GST-Di-Ras1 (50 μg/ml) was incubated for 30 min with 1 μM [^{35}S]GTPγS and the indicated concentrations of unlabeled GTPγS, GTP, GDP, or ATP. The [^{35}S]GTPγS binding data are expressed as percentages of the bound GTPγS obtained with [^{35}S]GTPγS alone.

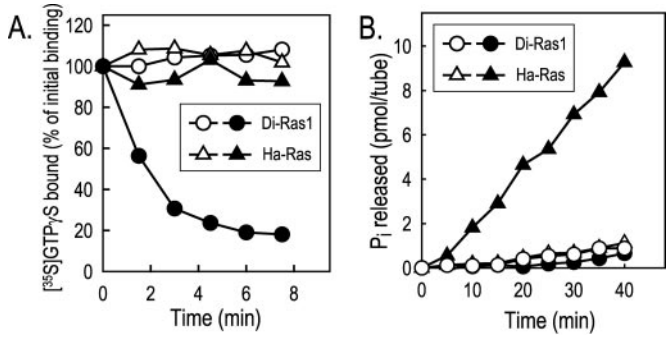

FIG. 5. **Different properties of nucleotide exchange and GTP hydrolysis reactions between Di-Ras1 and Ha-Ras.** *A*, the GST-fused protein (50 μg/ml) of Di-Ras1 (*circles*) or Ha-Ras (*triangles*) was incubated with 10 μM [^{35}S]GTPγS for 30 min, and unlabeled GTPγS (*closed symbols*, 100 μM at the final concentration) or control buffer (*open symbols*) were added to the reaction mixture. At the indicated times, aliquots (40 μl) were withdrawn and analyzed for [^{35}S]GTPγS binding as described under "Experimental Procedures." *B*, the GST-fused protein (13 μg/ml) of Di-Ras1 (*circles*) or Ha-Ras (*triangles*) was incubated with 1 μM [γ-^{32}P]GTP in the presence of 0.1 μM (*closed symbols*) or 10 mM (*open symbols*) Mg^{2+}. At the indicated time, aliquots (50 μl) of the reaction mixture were withdrawn and analyzed for the amounts of $^{32}P_i$ released as described under "Experimental Procedures."

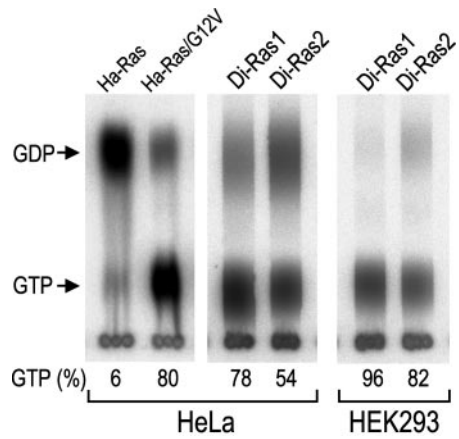

FIG. 6. **Analysis of the nucleotide form associated with Di-Ras1 and Di-Ras2.** HeLa or HEK293 cells were transfected with expression vectors encoding the proteins listed at the *top* and metabolically labeled with $^{32}P_i$. The expressed proteins were immunoprecipitated with an anti-Myc monoclonal antibody. Guanine nucleotides associated with the expressed proteins were separated by thin layer chromatography. The radioactivity of GTP and GDP was quantitated, and the percentages of GTP/[GTP + GDP] are shown at the *bottom*.

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*

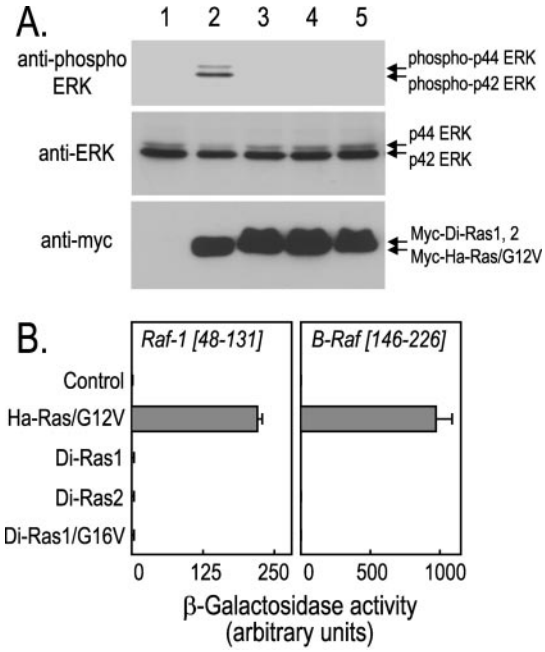

FIG. 7. **Effects of Di-Ras and Ha-Ras on the MAPK signaling pathway.** *A*, HEK293 cells were transfected with pMyc-CMV5 expression vectors encoding various proteins. *Lane 1*, control; *lane 2*, Ha-Ras/G12V; *lane 3*, Di-Ras1; *lane 4*, Di-Ras2; *lane 5*, Di-Ras1/G16V. The cells were cultured for 24 h and starved for an additional 12 h as described under "Experimental Procedures." The cell lysates were separated by SDS-PAGE and subjected to immunoblotting using anti-phospho-ERK (*upper panel*), anti-ERK (*middle panel*), and anti-Myc (*lower panel*) antibodies. *B*, Ha-Ras/G12V, Di-Ras1, Di-Ras2, and Di-Ras1/G16V in pGBT9 vector were transformed in the yeast Y190 along with pGAD424 vector containing human Raf-1 RBD (*left panel*) or B-Raf RBD (*right panel*) cDNA corresponding to 48–131 and 146–226 amino acid sequences, respectively. Interactions were measured by assay of the β-galactosidase activity.

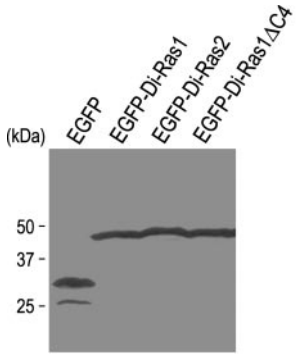

FIG. 8. **Immunoblot analysis of the EGFP-Di-Ras fusion proteins expressed in HEK293T cells.** HEK293T cells were transfected with pEGFP alone or pEGFP containing cDNAs of *Di-Ras1*, *Di-Ras2*, and *Di-Ras1*ΔC4 that lacks the COOH terminus C*AAX* motif. After a 2-day culture, the cell lysates were separated by SDS-PAGE and subjected to immunoblotting using anti-EGFP polyclonal antibody.

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, C*AAX*, 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 C*AAX* 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

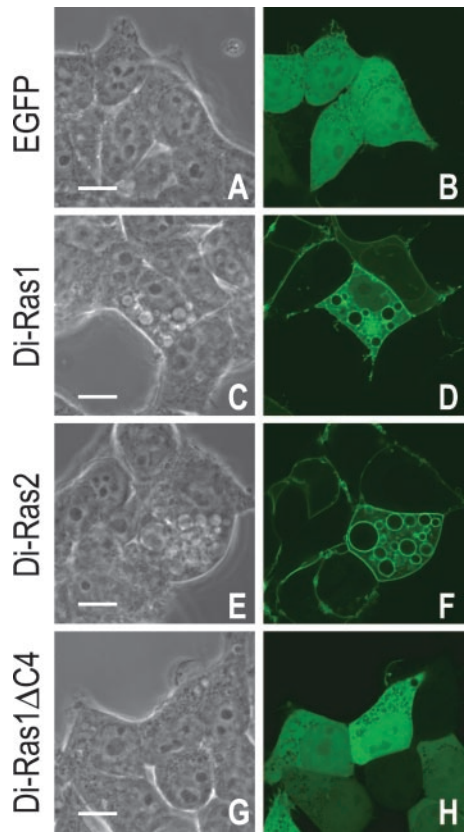

FIG. 9. **Detection by confocal microscopy of the EGFP-Di-Ras fusion proteins in HEK293T cells.** HEK293T cells were grown on poly-L-lysine-coated glass coverslips and transfected with pEGFP vectors as described under "Experimental Procedures." After 40–48 h, the cells were viewed with a Carl Zeiss LSM-510 confocal microscope. *B*, *D*, *F,* and *H*, confocal microscopic analysis of HEK293T cells expressing EGFP (*B*), EGFP-Di-Ras1 (*D*), EGFP-Di-Ras2 (*F*), and EGFP-Di-Ras1ΔC4 (*H*). Note that high expression levels of Di-Ras1 and Di-Ras2 lead to the formation of large vacuolar structure. *A*, *C*, *E*, and *G*, corresponding phase-contrast photographs. *Bars* indicate 10 μm.

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.

DISSCUSSION

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-Ras1 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

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.

Acknowledgments—We thank Dr. John Colicelli for providing *RIN1* cDNA and Dr. David W. Russell for the gifts of pCMV5 vector.

Addendum—During revision of this paper, the characterization of a novel Ras-related protein, Rig, which is identical to the present Di-Ras1, was reported by Ellis *et al.* (30).

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

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J. Biol. Chem. 2002, 277:41070-41078.
doi: 10.1074/jbc.M202150200 originally published online August 22, 2002
