We studied the regulation of three closely related members of Ras family G proteins, R-Ras, TC21 (also known as R-Ras2), and M-Ras (R-Ras3). Guanine nucleotide exchange of R-Ras and TC21 was promoted by RasGRF, C3G, CalDAG-GEF1, CalDAG-GEFII (RasGRF), and CalDAG-GEFIII both in 293T cells and in vitro. By contrast, guanine nucleotide exchange of M-Ras was promoted by the guanine nucleotide exchange factors (GEFs) for the classical Ras (Ha-, K-, and N-), including mSos, RasGRF, CalDAG-GEFII, and CalDAG-GEFIII. GTPase-activating proteins (GAPs) for Ras, Gap1\textsuperscript{\*}, p120 GAP, and NF-1 stimulated all of the R-Ras, TC21, and M-Ras proteins, whereas R-Ras GAP stimulated R-Ras and TC21 but not M-Ras. We did not find any remarkable difference in the subcellular localization of R-Ras, TC21, or M-Ras when these were expressed with a green fluorescent protein tag in 293T cells and MDCK cells. In conclusion, TC21 and R-Ras were regulated by the same GEFs and GAPs, whereas M-Ras was regulated as the classical Ras.

The Ras family of GTP-binding proteins consists of the classical Ras (Ha-, K-, and N-), R-Ras, Rap1, Rap2, Raf, Rheb, Rin, Rit, TC21/R-Ras2 (called TC21 hereafter), and M-Ras/R-Ras3 (M-Ras) (1). Compared with the classical Ras, which plays a pivotal role in cell growth and differentiation, less is known about the other members of this family. It is proposed that three proteins, including R-Ras, TC21, and M-Ras, make up a subfamily (2). It has also been noted, however, that M-Ras differs at the carboxyl terminus from both TC21 and R-Ras, which share a conserved amino acid motif designated the R-Ras box (3). Like the classic Ras proteins (Ha-, K-, and N-Ras), all proteins of the R-Ras subfamily transform NIH3T3 cells, albeit less efficiently than does the classic Ras (3–7). Consistent with its transforming activity, overexpression and mutation of TC21 lead to deregulated kinase/mitogen-activated protein kinase cascade by the R-Ras subfamily proteins. In support of this idea, it is reported that R-Ras subfamily proteins bind to Raf effectors and activate the extracellular signal-regulated kinase/mitogen-activated protein kinase cascade (2, 3, 5, 11, 12). In contrast to these reports, however, it is also reported that the R-Ras subfamily does not bind to Raf (6, 7, 13) and that overexpression of R-Ras did not induce DNA synthesis of Swiss3T3 cells or the differentiation of PC12 cells (14).

Apart from the similarity to Ras, R-Ras seems to have its unique functions. R-Ras inhibits apoptosis of BaF3 cells induced by cytokine deprivation (15) and stimulates cell adhesion by integrin (16). However, so far, no effector molecules for R-Ras have been reported to explain its specific function. Ras family G proteins cycle between GDP-bound inactive and GTP-bound active states. The GDP-bound form is converted to the active form by the guanine nucleotide exchange factor (GEF)\textsuperscript{1} and the GTP-bound form returns to the GDP-bound form by hydrolysis. The intrinsic GTPase activity is stimulated by GTPase-activating proteins (GAPs). The number of known GEFs and GAPs of Ras family G proteins is increasing owing to the progress of genome sequencing projects. A GEF for Rap1, C3G, and GAPs for Ras, CalDAG-GEFII, and RasGRF, also activate R-Ras (17, 18); however, as far as we know, no GAPs specific to R-Ras subfamily G proteins have been reported.

GAPs for Ras family G proteins may be divided into three groups: GAPs for Ras, Rap1, and Raf (19). GAPs for Ras include p120 GAP, GAP1\textsuperscript{\*}, and NF-1. R-Ras GAP shares a high amino acid sequence homology with GAP1\textsuperscript{\*}, and both R-Ras GAP and GAP1\textsuperscript{\*} have been shown to stimulate R-Ras GTPase. GAPs for Rap1 include rap1GAP, SPA-1, tuberin, and GAP\textsuperscript{DPIB}. The activity of GAP for Raf has been demonstrated; however, the genome of this protein has not been isolated (20). Knowledge about GEFs and GAPs of R-Ras subfamily proteins is limited and fragmented in the literature. Sos activates M-Ras (7) but not R-Ras (17); RasGRF promotes guanine nucleotide exchange of R-Ras, TC21, and M-Ras (5, 7, 17); C3G promotes guanine nucleotide exchange of R-Ras (17); R-Ras interacts with p120 GAP and NF-1 (14); p120 GAP activates M-Ras GTPase in vitro (7); and R-Ras GAP and GAP1\textsuperscript{\*} stimulate R-Ras GTPase (21).

Here, by the use of 10 GEFs and 5 GAPs for Ras family G proteins, we report that R-Ras and TC21 are regulated by the same set of GEFs and GAPs and that M-Ras behaves like the classical Ras in terms of its sensitivity to GEFs and GAPs.

\textsuperscript{1} The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; EGFP, enhanced green fluorescent protein; ECFP, enhanced cyan fluorescent protein; GST, glutathione S-transferase; mGDP, 2'-methylanthranol-GDP; ER, endoplasmic reticulum; MDCK, Madin-Darby canine kidney.

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Yusuke Ohba\textsuperscript{\*,\*\*}, Naoki Mochizuki\textsuperscript{\*,\*\*}, Shigeko Yamashita\textsuperscript{\*,}, Andrew M. Chan\textsuperscript{\*,}, Seisuke Hattori\textsuperscript{\*,\*\*}, Kazuo Nagashima\textsuperscript{\*,\*\*}, and Michiyuki Matsuda\textsuperscript{\*,\*\*\*}

From the \& Department of Pathology, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 182-8555, Japan, the \& Laboratory of Molecular and Cellular Pathology, Hokkaido University School of Medicine, N-15 W-7, Kita-ku, Sapporo 060-8638, Japan, the **Division of Biochemistry and Cellular Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan, the \& Donald H. Ruttenberg Cancer Center, Mt. Sinai School of Medicine, New York, New York 10029, and the \& Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.

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\*\* To whom correspondence should be addressed. Tel.: 81-3-3202-7181, ext. 2833; Fax: 81-3-3205-1236; E-mail: mmatsuda@ri.imcj.go.jp

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Regulation of R-Ras, TC21, and M-Ras

EXPERIMENTAL PROCEDURES

Plasmids—cDNAs of R-Ras, c-Ha-Ras, and Rap1A (Krev1) were obtained from A. Hall (University College London, London, United Kingdom), K. Kaibuchi (Nara Institute of Science and Technology, Nara, Japan), and M. Noda (Kyoto University, Kyoto, Japan), respectively. pCEV-TC21 and pBlueScript-M-Ras were described previously (3, 10). Codon sequences of small G proteins were subcloned into pEBG (22), pCAGGS-EGFP, pCAGGS-ECFP, pMSH-HA (Invitrogen, Groningen, the Netherlands), and pGEX-4T3 (Amersham Pharmacia Biotech, Inc.). pCAGGS-EGFP and pCAGGS-ECFP are derivatives of pCAGGS (23) and encode enhanced green fluorescent protein (EGFP) and enhanced cyan fluorescent protein (ECFP) (CLONTECH, Palo Alto, CA), respectively. cDNAs of KIAA0277 (GFR) (24), KIAA0313 (PDZ-GEF1) (25–27), KIAA0286 (CalDAG-GEFI) (28–30). The entire coding region of mouse CalDAG-GEFI and mouse CalDAG-GEFII were subcloned into pGEX-4T3. The entire coding region of rap1GAPII was subcloned into pAcSG2-His (Pharmacia, Utrecht, the Netherlands). pGEM-R-Ras was obtained from K. Kaibuchi. The entire coding regions of mouse CalDAG-GEFII and mouse CalDAG-GEFII were amplified by polymerase chain reaction from a mouse spleen cDNA library. cDNAs of Epac, mouse CalDAG-GEFII, mouse CalDAG-GEFII, and mouse CalDAG-GEFII were subcloned into pCXN2-Flag. pCAGGS-EGFP, pCAGGS-mSos, pCAGGS-C3G, and pCAGGS-Myo-C3G-CD were described previously (17). cDNAs of Ras-GRF, Epac, and C3G-CD were subcloned into pGEX-4T3. The entire coding region of rap1GAPII was subcloned into pAcSG2-His (Pharmingen, San Diego, CA) to generate pAcSG2-His-rap1GAPII. cDNAs of the mCDC5 homology domains of CalDAG-GEFIs were amplified by polymerase chain reaction and subcloned into pGEX-3 to generate pGEX-CalDAG-GEF-CD.

Cell Culture and Transfection—The cell lines used in this study, 293T (obtained from B. J. Mayer, Harvard Medical School) and MDCK (ATCC CCL34), were cultured in Dulbecco’s modified Eagle’s medium (Nissui, Tokyo) supplemented with 10% fetal calf serum. Expression plasmids were introduced into 293T cells by the calcium-phosphate precipitation method and into MDCK cells with FuGENE6 (Roche Molecular Biochemicals) according to the manufacturer’s protocol.

Preparation of Glutathione S-Transferase (GST)-tagged, Maltose-binding Protein-tagged, and His-tagged Proteins—Recombinant proteins fused to GST were expressed in E. coli from pGEX-derived vectors and purified as described previously (31). His-tagged and maltose-binding protein-tagged proteins were purified from bacterial lysates according to the manufacturers’ protocols. Purification of His-tagged rap1GAPII from baculovirus-infected cells will be described elsewhere.

Analysis of Guanine Nucleotides Bound to G Proteins—Guanine nucleotides bound to R-Ras, TC21, and M-Ras were analyzed essentially as described previously (17). Briefly, 293T cells were transfected with pEBG-R-Ras, TC21, and M-Ras in the presence or absence of the expression plasmids of GEFs or GAPs. Twenty-four hours after transfection, cells were labeled for 2–4 h with 32P, in phosphate-free modified Eagle’s medium (Life Technologies, Inc.). For activation of Epac, Sp-cAMPS triethylamine (Research Biochemical International, Natick, MA) was added at 100 μM for 10 min before cell harvest. GST-tagged R-Ras, TC21, and M-Ras were collected on glutathione-Sepharose beads, and guanine nucleotides bound to the G proteins were separated by TLC and quantitated with a BAS-1000 image analyzer (Fuji Film, Tokyo, Japan).

Guanine Nucleotide Exchange Reaction in Vitro—A fluorescent analogue of GDP, 2′,3′-bis-(O-[(N-methylanthranolyl)]-GDP (mGDP), was purchased from Dojin Kagaku (Kumamoto, Japan). His-R-Ras, His-TC21, and His-M-Ras were loaded with mGDP as described previously (32). The mGDP loading efficiency for R-Ras was between 50 and 60%; for TC21 and M-Ras, it was between 80 and 90%. For the measurement of GDP, 400 μM of labeled small G protein was incubated with or without 100 nM GEFs in reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 2 mM dithiothreitol) at 20 °C for R-Ras and TC21) or at 30 °C (for M-Ras). The reaction was started by the addition of GTP at 200 μM. The decrease in fluorescence was monitored with a JASCO FP-750 fluorescence spectrometer, with excitation and emission wavelengths of 366 and 450 nm, respectively. The value showing significant activation compared with the control is marked with an asterisk at the top of the each column.

Regulation of R-Ras, TC21, and M-Ras

A

FIG. 1. Promotion of guanine nucleotide exchange reaction of R-Ras, TC21, and M-Ras in vivo. A and B, 293T cells were transfected with expression vectors encoding proteins listed at the top and labeled with 32P. C3G-WT and C3G-CD are the wild type and the activated mutant, consisting mostly of the catalytic domain, respectively. For the activation of Epac, cells were incubated with 100 μM cAMP analogue, Sp-cAMPS triethylamine (cAMP), before analysis. Guanine nucleotides bound to the GST-tagged G proteins were separated by TLC (upper panels). Mean values obtained from three experiments are shown with S.D. (lower panels). The value showing significant activation compared with the control is marked with an asterisk at the top of the each column.

In Vitro GAP Assay—GAP activity was measured in vitro as described previously (28). His-R-Ras, His-TC21, His-M-Ras, GST-Ha-Ras, or GST-Rap1, 5 μM each, was loaded with γ-[32P]GTP in loading buffer (20 mM Tris, pH 8, 1.5 mM GTP, 10 mM 2-mercaptoethanol, 5 mM MgCl2, 20 mM EDTA, 10% glycerol, 0.5 mg/ml bovine serum albumin) at 30 °C for 15 min, followed by the addition of MgCl2 to 20 mM. Purified GAP proteins were added to 250 mM of each small G protein in reaction buffer (20 mM Tris, 5 mM MgCl2, 0.5 mg/ml bovine serum albumin) at 30 °C for 10 min. The reaction was terminated by the addition of ice-cold washing buffer (20 mM Tris-HCl, pH 8, 5 mM MgCl2, 100 mM NaCl). Samples were adsorbed to nitrocellulose filters (Schleicher & Schuell). The filters were washed three times with washing buffer, dried, and analyzed with a BAS-1000 image analyzer.

Fluorescence Microscopy—293T and MDCK cells were grown on poly-L-lysine-coated glass plates, and transfected with pCAGGS-EGFP-R-Ras, pCAGGS-EGFP-TC21, and pCAGGS-EGFP-M-Ras. After 24 h, the

* Y. Ohba, N. Mochizuki, and M. Matsuda, unpublished results.
cells were observed with an LSM-510 confocal microscope (Carl Zeiss, Jena, Germany). In another experiment, MDCK cells maintained on collagen-coated glass dishes were transfected with expression vectors encoding ECFP-R-Ras, ECFP-TC21, or ECFP-M-Ras and YFP-ER or YFP-Golgi (CLONTECH) for 24 h and observed with the confocal microscope.

RESULTS

Effect of GEFs on R-Ras, TC21, and M-Ras in 293T Cells—We first investigated whether GEFs for Ras and Rap1 promote guanine nucleotide exchange of R-Ras, TC21, and M-Ras in vivo. 293T cells expressing GST-tagged G proteins and GEFs were labeled with $^{32}$P$_i$, and guanine nucleotides bound to G proteins were analyzed by TLC. As shown in Fig. 1A, the basal level of GTP-bound R-Ras was 22%. The amount of GTP-R-Ras was increased significantly by the expression of RasGRF, C3G, CalDAG-GEFII, CalDAG-GEFI, and CalDAG-GEFIII. Very similarly to R-Ras, the basal level of GTP-bound TC21 was 25%, and the amount of GTP-bound TC21 was increased by the expression of RasGRF, C3G, CalDAG-GEFII, CalDAG-GEFI, and CalDAG-GEFIII. By contrast, GTP-bound M-Ras was 6% without GEFs and increased by the expression of mSos, RasGRF, CalDAG-GEFII, and CalDAG-GEFIII, all of which have been shown to activate Ha-Ras. Because the effect of C3G expression was marginal, we utilized C3G-CD, which is the activated form of C3G (Fig. 1B). Expression of C3G-CD increased the amount of GTP-R-Ras and TC21 more efficiently than did the full-length C3G, whereas M-Ras was not activated by either the full-length C3G or C3G-CD. Other GEFs for Rap1, including GFR (24), PDZ-GEF1 (25–27), and Epac (33, 34), and KIAA 0351, a GEF for Ral, did not activate R-Ras, TC21, or M-Ras (Fig. 1A).

Guanine Nucleotide Exchange Reaction in Vitro—We examined the guanine nucleotide exchange reaction of R-Ras, TC21, and M-Ras in vitro. A fluorescent analogue of guanine nucleotide, mGDP, was loaded on G proteins, and its replacement with GTP was monitored by a fluorescence spectrometer in the presence of GEFs as described previously (35). The intrinsic exchanging rate under our experimental conditions was examined, because TC21 is reported to show a high intrinsic exchange activity (5). As shown in Table I, the intrinsic exchange rate of R-Ras was the highest, followed by those of TC21 and M-Ras at the examined temperatures. These intrinsic guanine nucleotide exchange rates were significantly higher than those of Ha-Ras and Rap1. In order to normalize the intrinsic exchange rate, the effect of GEFs was monitored at 20 °C for R-Ras and TC21 and at 30 °C for M-Ras. As shown in Fig. 2 and Table II, RasGRF, CalDAG-GEFII, and CalDAG-GEFIII efficiently promoted dissociation of mGDP from all three G proteins. C3G and CalDAG-GEFI promoted guanine nucleotide exchange of R-Ras and TC21, albeit less efficiently than did the other three GEFs. Epac did not promote the guanine nucleotide exchange of any of the tested G proteins, irrespective of the presence or absence of the cAMP analogue. Under the same conditions, guanine nucleotide exchange of Ha-Ras was promoted by CalDAG-GEFII and CalDAG-GEFIII. Similarly, under these conditions, we confirmed that nucleotide exchange of

| G proteins | Reaction temperature (°C) | 20  | 30  | 37  |
|------------|--------------------------|-----|-----|-----|
| R-Ras      |                          | 6.8 | 15  | 45  |
| TC21       |                          | 4.8 | 11  | 25  |
| M-Ras      |                          | 3.6 | 7.0 | 8.6 |
| Ha-Ras     |                          | 1.8 | ND* | ND  |
| Rap1A      |                          | 2.3 | ND  | ND  |

* ND, not determined.
Rap1 was promoted by C3G, CalDAG-GEF1, CalDAG-GEFII, and Epac3. Thus, the same set of GEFs regulates R-Ras and TC21, whereas M-Ras was stimulated by the GEFs for Ha-Ras.

Stimulation of GTPase Activity of R-Ras, TC21, and M-Ras—We next examined the specificity of GAPs to R-Ras and TC21, whereas M-Ras was stimulated by the GEFs for Ha-Ras.

GTPase activity of R-Ras, TC21, and M-Ras—We next examined the specificity of GAPs to R-Ras and TC21, whereas M-Ras was stimulated by the GEFs for Ha-Ras.

Regulation of R-Ras, TC21, and M-Ras

Rap1 was promoted by C3G, CalDAG-GEF1, CalDAG-GEFII, and Epac3. Thus, the same set of GEFs regulates R-Ras and TC21, whereas M-Ras was stimulated by the GEFs for Ha-Ras.

Table II
Summary of the interaction of GEFs with R-Ras, TC21, and M-Ras, as measured by fluorescence assay

| Rate constant (units/s) | RasGRF | C3G | CalDAG-GEF1 | CalDAG-GEFII | CalDAG-GEFIII | Epac with cAMP |
|-------------------------|--------|-----|-------------|--------------|---------------|----------------|
| R-Ras                   | 5.2    | 20  | 8.0         | 8.8          | 18            | 43             | 4.5            |
| TC21                    | 3.2    | 14  | 7.9         | 9.6          | 56            | 79             | 3.4            |
| M-Ras                   | 3.3    | 13  | 3.3         | 3.7          | 9.5           | 7.5            | 3.3            |

FIG. 3. GAP activity in vitro. The recombinant G proteins indicated at the bottom were loaded with [γ-32P]GTP and incubated in the absence (open columns) or presence of GAPs (filled columns) for 10 min. Radioactivity remaining on G proteins was quantitated and plotted with S.D.

FIG. 4. GAP activity in vivo. 293T cells expressing GAPs and G proteins indicated at the bottom with (filled columns) or without (open columns) RasGRF were labeled with 32P. Guanine nucleotides bound to the G proteins were quantitated as shown in Fig. 1, and mean values obtained from three experiments were plotted. Bars indicate S.D.

Subcellular localization of R-Ras, TC21, and M-Ras—By using green fluorescent protein-tagged proteins, Choy et al. have shown that the choice of exon at the carboxyl terminus of...
K-Ras determines whether it reaches the plasma membrane from Golgi or ER (48). Thus, we compared the subcellular localization of R-Ras, TC21, and M-Ras using green fluorescent protein-tagged proteins. As shown in Fig. 5A, green fluorescent protein-R-Ras, TC21, and M-Ras were localized mainly at the plasma membrane in 293T cells. When we used MDCK cells, they were more enriched in the cytoplasm. To investigate further whether these G proteins were localized at the Golgi apparatus or endoplasmic reticulum in MDCK cells, we co-expressed enhanced yellow fluorescent protein-tagged markers of ER and Golgi. After 24 h, cells were observed with a confocal microscope.

**DISCUSSION**

In this study, we have shown that R-Ras and TC21 are regulated by the same set of GEFs (Table III) and GAPs. In addition, R-Ras and TC21 have another feature in common; the basal level in the GTP-bound form of R-Ras and TC21 exceeds 20% without stimulation, whereas those of M-Ras, Ras, Rap1, and Ral are less than 15% in 293T cells. By contrast, M-Ras is very similar to Ha-Ras in the regulation by GEFs and GAPs. This observation is particularly important because the amount of M-Ras exceeds that of Ha-Ras in many tissues and cell lines (3). In these cells, signals that have been postulated to be transmitted by Ras seem also to be transmitted by M-Ras. However, it should be pointed out that Raf activation by M-Ras is significantly weaker than that by the classical Ras. In agree-
ment with this difference, the transforming activity of M-Ras is significantly weaker than that of the classical Ras (3). Thus, M-Ras does not necessarily function as the fourth member of the classical Ras.

Recent analyses have revealed that the switch II region (amino acids 57–75) and residues 40, 61, and 70 of Ha-Ras are critical in the recognition by GEFs (36, 37). The amino acid sequence of the M-Ras switch II region differs from that of classical Ras and TC21 containing serine at this position, whereas R-Ras and TC21 contain glycine. To examine whether the amino acid of this position determined the interaction to GEFs, we made two mutants, TC21Ser-75 and M-Ras Gly-75. Against our expectation, neither substitution changed the interaction to GEFs, compared with each wild type. Hence, GEFs discriminate between TC21 and M-Ras by a region(s) other than the switch II region.

Surprisingly, although five GEFs have been shown to activate R-Ras and TC21, none of them is specific to this R-Ras subfamily. RasGRF, CalDAG-GEF II, and CalDAG-GEFIII also activate G proteins of the classical Ras subfamily. C3G, CalDAG-GEF, and CalDAG-GEFII also activate the Rap1 subfamily. Officially, it is possible that an unidentified GEF is highly specific to the R-Ras subfamily; however, it should be noted that, in many circumstances, activation of the R-Ras subfamily is accompanied by activation of either the Ras or the Rap1 subfamily. In this regard, the substrate specificity of R-Ras GAP seems to be more specific (21), and thus, R-Ras GAP may play a critical role in the specific regulation of the R-Ras subfamily.

Apart from the substrate specificity, the tissue distribution of G protein, GEFs, and GAPs is critical in the understanding of the regulation of the G proteins in a physiologic milieu. R-Ras, TC21, and M-Ras are all expressed ubiquitously, although their expression levels vary among tissues; for example, M-Ras is enriched in the brain (2, 3, 6, 7, 17, 38, 39). The expression levels of TC21 and M-Ras also vary depending upon the stimulation of growth factors and cytokines (38, 40). Some GEFs, including mSos and C3G, are expressed ubiquitously (17, 41). Expression of other GEFs is limited to some tissues; for example, RasGRF is expressed mostly in brain and testis (42), and CalDAG-GEF I and CalDAG-GEFII are concentrated in brain and hematopoietic organs (18, 43, 44). Some GAPs are also expressed in a tissue-specific manner. Of note, p120 GAP, NF-1, Gap1α, and R-Ras GAP were expressed abundantly in the brain, suggesting a specific role of G proteins in this organ (21, 28, 45). Extensive examination of localization of G proteins and their regulatory proteins will be required to decipher the cell type-specific signaling cascades.

Subcellular localization of the G proteins and the corresponding regulatory proteins should also be important for understanding the signaling cascade of Ras family G proteins. Sos and C3G are activated by translocation to the plasma membrane by the adaptor proteins (46, 47). However, GEFs such as Epac and CalDAG-GEFs may be activated at the intracellular membrane compartments (19). In our preliminary experiments, we noticed that fixatives used for the indirect immunofluorescent method significantly altered the staining pattern, hindering correct estimation of the subcellular localization of G proteins. Thus, we utilized green fluorescent protein-tagged G proteins in this study. Against our expectation, there was no remarkable difference in the subcellular distribution of R-Ras, TC21, and M-Ras. All three G proteins were localized both at the plasma membrane and also within the cells, although the ratio varied significantly between 293T cells and MDCK cells. It may be important to examine the localization of G proteins together with that of GEFs and GAPs.

In conclusion, we showed that TC21 and R-Ras form a subgroup and that M-Ras belongs to the classic Ras subfamily when we classified these G proteins according to their specificity to GEFs and GAPs.

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REFERENCES

1. Bos, J. L. (1997) Biochim. Biophys. Acta 1333, M19–M31
2. Kimmelman, A., Tolkacheva, T., Lorenzi, M. V., Osada, M., and Chan, A. M. (1999) Oncogene 18, 2675–2683
3. Ehrhardt, G. R., Leslie, K. B., Lee, F., Wieler, J. S., and Schrader, J. W. (1999) Blood 94, 2433–2444
4. Cox, A. D., Brotva, T. R., Lowe, D. G., and Der, C. J. (1994) Oncogene 9, 3281–3288
5. Movilla, N., Crespo, P., and Bustelo, X. R. (1999) Oncogene 18, 5860–5869
6. Graham, S. M., Vojtek, A. B., Huff, S. Y., Cox, A. D., Clark, G. J., Cooper, J. A., and Der, C. J. (1996) Mol. Cell. Biol. 16, 6132–6140
7. Quilliam, L. A., Castro, A. F., Rogers-Graham, K. S., Martin, C. B., Der, C. J., and Bi, C. (1999) J. Biol. Chem. 274, 23850–23857
