CalDAG-GEFIII Activation of Ras, R-Ras, and Rap1*

Received for publication, April 21, 2000, and in revised form, June 1, 2000
Published, JBC Papers in Press, June 1, 2000, DOI 10.1074/jbc.M003414200

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We characterized a novel guanine nucleotide exchange factor (GEF) for Ras family G proteins that is highly homologous to CalDAG-GEFII, a GEF for Rap1 and R-Ras, and to RasGRP/CalDAG-GEFII, a GEF for Ras and R-Ras. This novel GEF, referred to as CalDAG-GEFIII, increased the GTP/GDP ratio of Ha-Ras, R-Ras, and Rap1 in 293T cells. CalDAG-GEFIII promoted the guanine nucleotide exchange of Ha-Ras, R-Ras, and Rap1 in vitro also, indicating that CalDAG-GEFIII exhibited the widest substrate specificity among the known GEFs for Ras family G proteins. Expression of CalDAG-GEFIII was detected in the glial cells of the brain and the glomerular mesangial cells of the kidney by in situ hybridization. CalDAG-GEFIII activated ERK/MAPK most efficiently, followed by CalDAG-GEFII and CalDAG-GEFI in 293T cells. JNK activation was most prominent in cells expressing CalDAG-GEFII, followed by CalDAG-GEFIII and CalDAG-GEFI. Expression of CalDAG-GEFIII induced neuronal differentiation of PC12 cells and anchorage-independent growth of Rat1A cells less efficiently than did CalDAG-GEFI. Thus, co-activation of Rap1 by CalDAG-GEFIII apparently attenuated Ras-MAPK-dependent neuronal differentiation and cellular transformation. Altogether, CalDAG-GEFIII activated a broad range of Ras family G proteins and exhibited a biological activity different from that of either CalDAG-GEFI or CalDAG-GEFII.

The Ras family of small GTPases includes Ras, Rap1, Rap2, TC21, R-Ras, M-Ras, Ral, and Rheb (1). By phylogenetic analysis of the amino acid sequences, these Ras family G proteins can be divided further into five subfamilies, the classical Ras (Ha-, K-, and N-), R-Ras, Rap, Ral, and the others. Among them, the classical Ras has been studied most extensively as a critical protein in cell growth and differentiation (2). Like the classic Ras proteins (Ha-, K-, and N-Ras), all proteins of the R-Ras subfamily transform NIH3T3 cells, although less efficiently than does the classic Ras (3–6). In contrast, Rap1 has been reported to be a protein that reverses the cellular transformation induced by K-Ras (7). However, it has been proposed that Rap1 has a function distinct from this anti-Ras effect (8), including the activation of integrin (9, 10).

The Ras family G proteins are activated by external signals via the guanine nucleotide exchange factor (GEF) (1). GEFs promote GDP dissociation and GTP binding of the Ras family G proteins. The GTP-bound Ras family G proteins bind to and activate the effector proteins to transmit signals further downstream. The first mammalian GEF for the Ras family G protein, Ras-GRF, was identified as a protein that shares high sequence homology with the yeast GEF, CDC25 (11). This was followed by the discovery of Sos, RalGDS, and C3G (12), which are GEFs for Ras, Rap, and Rap1, respectively.

Recently, owing to the progress in genome sequencing projects, the number of GEFs for Ras family G proteins has been increasing rapidly. These GEFs may be classified according to their major upstream signaling cascades. Sos and C3G are activated by tyrosine kinases (13, 14), whereas Ras-GRF, CalDAG-GEFI, and RasGRP/CalDAG-GEFII, called CalDAG-GEFII hereafter, are activated by calcium and/or diacylglycerol (15, 16). Epac/cAMP-GEFs are activated by the direct binding of cAMP (17, 18). nRap GEP/PDZ-GEF1/RA-GEF may be activated by direct binding to cell adhesion molecules via the PDZ domain (19–21).

Another classification of GEFs for Ras family G proteins should be based on the substrate specificity. Sos activates only the classical Ras (12), whereas Epac/cAMP-GEF, GFR, and GEF/PDZ-GEF1/RA-GEF are specific to Rap1 (17–21). However, RasGRF and CalDAG-GEFII activate both Ras and R-Ras (16, 22), and C3G activates both Rap1 and R-Ras (23). Of note, even with the recent increase in the number of GEFs, there is no report on a GEF that is specific for the R-Ras subfamily.

In this report, we characterize KIAA0846, which was isolated by the KAZUSA human cDNA sequencing project. KIAA0846 shares high sequence homology with CalDAG-GEFI and CalDAG-GEFII. This protein, named CalDAG-GEFIII, shows the widest substrate specificity among the known GEFs.

EXPERIMENTAL PROCEDURES

Plasmids—pBluescript-SKII- (+) -KIAA0846 and pBluescript-SKII- (+) -KIAA0351 were provided by the Kasazu DNA Research Institute. The entire coding region of this human KIAA0846 was amplified by PCR with specific primers (sense oligo, 5′-CTCGAGATGGGATCAAGT-GGCCTTGGGAAA-3′, and antisense oligo, 5′-GCGGCCGCTCAGC-methylanthranolol)-3′). The nucleotide sequence(s) reported in this paper has been submitted to the GenBank (accession numbers AB020653 (for KIAA0846) and AB002349 (for KIAA0351)).

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* This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture, the Health Science Foundation, and the Princess Takamatsu Cancer Research Fund, Japan. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank (accession numbers AB020653 (for KIAA0846) and AB002349 (for KIAA0351)).

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‡ The abbreviations used are: ERK, extracellular signal-regulatory kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun amino-terminal kinase; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; mant-GDP, 2′,3′-bis (N-methylanthranolol)-GDP; PAGE, polyacrylamide gel electrophoresis; oligo, oligonucleotide; PCR, polymerase chain reaction; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; CRD, cysteine-rich domain; RBD, Ras-binding domain.
CATCCTCACATCTGCT-3') and subcloned into pCXN2-FLAG-CalDAG-GEFIII. cDNA of mouse CalDAG-GEFIII was amplified by PCR from a mouse spleen cDNA library, with specific primers (sense oligo, 5'-CTCGAGCTCAATAGATGCCTACCTCCCTCA-3', and antisense oligo, 5'-GCGGCCGTACCAAGCCTCATCTCCAAGTT-3') and subcloned into pCR-BluntII-ToPO-CalDAG-GEFIII. pCXN2-CalDAG-GEFII and pCXN2-CalDAG-GEFIII were similarly constructed by use of specific primer sets for CalDAG-GEFI (sense oligo, 5'-GGTCGACATGGGACAGCATCTGGAGAC-3', and antisense oligo, 5'-AGTCACAGCTTATTTAATGGAATG-3') and CalDAG-GEFIII (sense oligo, 5'-GTCGACATGGGACAGCATCTGGAGAC-3', and antisense oligo, 5'-TAAAGCACTAAGCCTCATCCCTCA-3').

Chimeric cDNAs between CalDAG-GEFII and CalDAG-GEFIII were prepared by a two-step PCR. CalDAG-GEFII/III consisted of the amino-terminal catalytic domain of CalDAG-GEFI (amino acids 1–396) and the carboxyl-terminal regulatory domain of CalDAG-GEFII (amino acid 445–796), including the EF hand and C1 domain. Similarly, CalDAG-GEFII/III consisted of amino acids 1–444 of CalDAG-GEFII and amino acid 496–606 of CalDAG-GEFIII. cDNA of Krev1/Rap1A was isolated by M. Noda (Kyoto University, Kyoto). The CAAX box of the K-ras gene was amplified by PCR and inserted into pCAGGS-His to generate pCAGGS-His-CαAAX as described previously (26).

The coding regions of CalDAG-GEFII and CalDAG-GEFIII were PCR-amplified and subcloned into pCAGGS-His-CAAX so that the CalDAG-GEFs were fused to the CAAX box in frame. pCEV-c-Ha-ras, pCEV-c-Ha-rasV12, pEBHA-RasG12V, and pEXV3-R-Ras were obtained from K. Kaibuchi (Kyoto University, Kyoto), M. Noda (Kyoto University, Kyoto), and A. H. Hall (University of Texas, The Netherlands) (29).

Antibodies—AlexaRap1 polyclonal antibody, anti-Pan-Ras antibody, and anti-MAPFL2 monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Calbiochem, and Sigma. Anti-HA monoclonal antibody was obtained from Roche Molecular Biochemicals.

Phylogenetic Analysis of CalDAG-GEFIII—The amino acid sequence of the catalytic domain of CalDAG-GEFIII was aligned with GENE-TYX-Win (SDC Software Development, Tokyo, Japan). The aligned sequence data were then subjected to computation of the pairwise distance by the method of Kimura (30), and the aligned sequence data were then subjected to computation of the pairwise distances by the method of Kimura (30), followed by the construction of a neighbor-joining tree with 100 bootstrap replicates. The reference sequences chosen are C3G (30), RasSos (31), CalDAG-GEFI (16), CalDAG-GEFII (16), nRapGEP/PDGFR-GEFI/RA-GF (19–21), GFR (22), Epac/cAMP-GEFII, and cAMP-GEFII (17, 18).

In situ Hybridization of CalDAG-GEFII to Mouse Brain and Kidney Tissues—Sense and antisense RNA probes were obtained by run-off transcription of pBluescript SKII (+)-CalDAG-GEFII, pBluescript SKII (+)-CalDAG-GEFIII, and PCR-BluntII-ToPO-CalDAG-GEFIII with a digoxigenin-RNA labeling kit and SP6, T3, or T7 polymerase (Roche Molecular Biochemicals). In situ hybridization was performed as described previously (32). Briefly, brain and kidney of B6 mice were embedded in Tissue-Tec OCT compound (Miles) and frozen in liquid nitrogen. 7-μm thickness were prepared with a cryo-microtome, fixed in 4% paraformaldehyde in phosphate-buffered saline, and acetylated in 0.1% triethanolamine with 0.25% acetic anhydride. Sections were prehybridized with hybridization buffer (500 mM formamide, 6× SSPE, 5× Denhardt’s solution, 500 μg/ml tRNA (Roche Molecular Biochemicals)) at room temperature for 16 h. After the addition of hybridization buffer containing a heat-denatured digoxigenin-RNA probe, sections were incubated overnight at 70°C. The hybridization buffer contained anti-digoxigenin antibody and nickel blue tetrazolium/5-bromo-4-chloro-3-indoly phosphate solution containing 0.2 mg/ml levamisole (Roche Molecular Biochemicals).

Cell Culture and Transfection—Human 293T embryonic kidney cells and Rat-LA cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui, Tokyo) supplemented with 10% fetal calf serum. PC12 cells were maintained in DMEM plus 10% fetal calf serum and 5% horse serum. 293T cells were transfected with plasmid DNA by the calcium phosphate precipitation method.

Analysis of Guanine Nucleotide Exchange Activity in 293T Cells—Guanine nucleotide exchange activity of GEFs was analyzed in 293T cells as described previously (26). 293T cells in dishes 3.5 cm in diameter were cotransfected with GEFs and R-GEFIII. Cells were lysed in TLA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl2, 1 ms NaVO4, 1% Triton X-100) and cleared by centrifugation. The supernatant was incubated with glutathione-Sepharose beads at 4°C for 1 h. The beads were then washed twice with TLA buffer solution and then with washing buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl2). Beads were resuspended in 2% SDS and boiled. The supernatant was spotted on polyethyleneimine-coated plates (Macherey & Nagel). The TLC plate was developed with 0.5 μL H2O2-KH2PO4, pH 3.4, and analyzed on a BAS1000 image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Analysis of Guanine Nucleotide Exchange Activity in Vitro—The guanine nucleotide exchange activity of GEFS was analyzed in vitro by use of a fluorescent analogue of GDP, mant-GDP (2',3'-bis-O-(N-methylanthranol))-GDP, as described previously (33). mant-GDP was produced by Doshin Kagaku (Kumamoto, Japan). GST-Ha-Ras, GST-R-Ras, or GST-Rap1A (45 μg) was incubated in exchange buffer (15 mM EDTA, 2.5 mM dithiothreitol, 7.5 mM mant-GDP) for 2 h at 25°C. After the addition of MgCl2 to 20 mM, GST-Ha-Ras, GST-R-Ras, or GST-Rap1A bound to mant-GDP was purified on a Sephadex G-25 column equilibrated with 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, and 5 mM dithiothreitol. The mant-GDP loading efficiency for Ha-Ras or Rap1A was between 80 and 90% and that for R-Ras was between 50 and 60%.

For the measurement of GEF activity, 400 μl labeled Ha-Ras, R-Ras, or Rap1A was incubated with or without 100 mM GEFS in reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 2 mM dithiothreitol) at 20°C. The reaction was started by the addition of GTP to 200 μM, and the decrease in fluorescence was monitored in a luminescence spectrometer (Fujifilm Venus, Tokyo, Japan). The GTPase activity of each GEF was measured as described previously (34).

In Vitro ERK and JNK Assay—The activity of ERK and JNK was examined as described previously (34). 293T cells in dishes 3.5 cm in diameter were cotransfected with pEBG-ERK or pEBG-JNK and with expression vectors for GEFS. Twenty four hours post-transfection, cells were lysed in kinase lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 10% glycerol). Glutathione-Sepharose beads were added to the lysates and rotated at 4°C for 1 h. The beads were washed with KLB and kinase buffer (500 mM HEPES-NaOH, pH 7.4, 10 mM MgCl2) and incubated with 15 μl of kinase reaction mixture at 30°C for 10 min. Kinase reaction mixture is kinase buffer containing 100 μM ATP, 5 μM of [γ-32P]ATP, and 0.2 mg/ml myelin basic protein (Sigma) for ERK or Rap1A was incubated with or without 100 mM GEFS in reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 2 mM dithiothreitol) at 20°C. The reaction was terminated by the addition of 5 μl of 5× Laemmli sample buffer, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The radioactivity of the substrates was quantitated with a BAS1000 image analyzer.

Neurite Outgrowth of PC12 Cells—PC12 cells were transfected with expression plasmids by the use of LipofectAMINE 2000 (Life Technologies, Inc.) and 48 h later observed under a microscope (34). PC12 cells that extended neurites longer than 2-fold of the diameter of the cell body were counted as differentiated cells.

Analysis of Rat-IA Cell Lines Expressing CalDAG-GEFII—Rat-IA cells were transfected with pCXN2-FLAG-derived CalDAG-GEF II expression vectors by use of LipofectAMINE (Life Technologies, Inc.). Cells were selected with 1 mg/ml G418 (Life Technologies, Inc.) and well isolated colonies were maintained in medium containing 400 μg/ml G418. A colony formation assay was performed as described previously (35). Briefly, 103 cells before and after cloning were cultured in DMEM containing 0.8% agarose with 2 or 10% fetal bovine serum at 37°C in 5% CO2. Two weeks later, colonies larger than 0.5 mm in diameter were counted.

Analysis of GTP-bound Ras Family G Proteins in Rat-IA Cells—Analysis of GTP-bound Ras family G proteins was performed by the method of Bos and co-workers (29). Rat-IA cells were serum-starved for 36 h, lysed in pull-down lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 0.5 mM dithiothreitol, 0.1% SDS, 1 mM NaVO4), and clarified by centrifugation. The supernatant was incubated with GST-tagged Rap1DSBD or GST-tagged Rap1B +...
expression of CalDAG-GEFs did not overlap each other. CalDAG-GEFII was expressed in the interstitial cells, CalDAG-GEFIII in the epithelium of the distal convoluted tubules and collecting tubules, and CalDAG-GEFII in the mesangial cells of the glomeruli. Thus, this observation showed that the three CalDAG-GEFs play a role in the cell type-specific regulation of Ras family G proteins by calcium and diacylglycerol.

Guanine Nucleotide Exchange Activity of CalDAG-GEFII in Vivo—The substrate specificity of CalDAG-GEFII was examined first in 293T cells (Fig. 3A). The expression of CalDAG-GEFII significantly increased the GTP-bound form of Ha-Ras, R-Ras, Rap1A, and Rap2A. CalDAG-GEFII did not activate RalA; therefore, the activity of CalDAG-GEFII was restricted to the Ras, R-Ras, and Rap subfamilies. We used KIAA0351 as a positive control for the Ral guanine nucleotide exchange factor. As reported previously (15, 16), CalDAG-GEFII activated Rap1 and, to a lesser extent, R-Ras, and CalDAG-GEFII activated both Ha-Ras and R-Ras.

To exclude the possibility that the regulatory domain modifies the substrate specificity of CalDAG-GEFs, we constructed chimeric proteins between CalDAG-GEFII and CalDAG-GEFIII. CalDAG-GEFII/II consisted of the catalytic domain of CalDAG-GEFII and the regulatory domain of CalDAG-GEFII, whereas CalDAG-GEFII/I consisted of the catalytic domain of CalDAG-GEFI and the regulatory domain of CalDAG-GEFI. As shown in Fig. 3B, CalDAG-GEFII/II and CalDAG-GEFII/I showed substrate specificity identical to that of CalDAG-GEFII and CalDAG-GEFII, respectively, indicating that the regulatory domains did not alter the substrate specificity of CalDAG-GEFII or CalDAG-GEFIII.

Guanine Nucleotide Exchange Activity of CalDAG-GEFII in Vitro—We confirmed the guanine nucleotide exchange activity of CalDAG-GEFII in vitro. For this purpose, we expressed and purified the catalytic domains of CalDAG-GEFs from Escherichia coli. Rap1A, R-Ras, and Ha-Ras were loaded with mant-GDP, and its dissociation in the presence of CalDAG-GEFs was monitored with a fluorospectrometer (Fig. 4). Rate constants were calculated as described previously (Table I) (33). Release of the mant-GDP from R-Ras was promoted by CalDAG-GEFII and, to a lesser extent, CalDAG-GEFII, whereas the release of mant-GDP from Rap1A was promoted by CalDAG-GEFI and, less efficiently, by CalDAG-GEFIII. Dissociation of mant-GDP from R-Ras was promoted by CalDAG-GEFII, followed by CalDAG-GEFII and CalDAG-GEFI. Thus, the
CalDAG-GEFIII promoted guanine nucleotide exchange of Ha-Ras, R-Ras, and Rap1 both in vivo and in vitro.

**Activation of ERK/ MAPK and JNK by CalDAG-GEFIII**—To understand the role of the broad substrate specificity of CalDAG-GEFIII, we measured the activity of ERK and JNK in 293T cells expressing CalDAG-GEFs (Fig. 5). Activation of ERK/MAPK was most prominent by CalDAG-GEFIII, followed by CalDAG-GEFII. In contrast, JNK was activated most strongly by CalDAG-GEFII, followed by CalDAG-GEFIII. Because we did not find any significant difference in the expression levels of CalDAG-GEFs, the difference in the activation of ERK/MAPK and JNK seemed to reflect the difference in the substrate specificity.

**Neurite Outgrowth of PC12 Cells by CalDAG-GEFIII**—It is known that constitutive activation of ERK/MAPK induces neuronal differentiation of PC12 cells (36). To gain further insight into the biological activity of CalDAG-GEFIII, we introduced CAX box-containing active CalDAG-GEFs into PC12 cells (Fig. 6). Neuronal differentiation of PC12 cells, which was evaluated by the number and the length of the neurites, was most prominent when we expressed CalDAG-GEFII-CAX, followed by CalDAG-GEFIII-CAX. CalDAG-GEFII-CAX did not induce neurite outgrowth. Interestingly, whereas CalDAG-GEFII-expressing cells exhibited typical neurite extension, cells expressing CalDAG-GEFIII showed spreading of the cytoplasm with wide neurites. The transfection efficiency of the expression vectors in PC12 cells exceeded 50% in all cases when the cells were immunostained with anti-FLAG antibody (data not shown). The amount of each protein was also within a comparable level.
equivalent expression levels (Fig. 7C). Again, we found that the colony formation efficiency of cells expressing CalDAG-GEFIII was significantly lower than that of cells expressing CalDAG-GEFI (Fig. 7B). We observed similar levels of increase in GTP-Ras in cells expressing CalDAG-GEFII and CalDAG-GEFIII (Fig. 7D). The level of GTP-Rap1 was highest in cells expressing CalDAG-GEFII, followed by CalDAG-GEFIII-expressing cells. This result suggests that the activation of Rap1 by CalDAG-GEFIII attenuated the Ras-dependent growth of Rat-1A cells in soft agar.

**DISCUSSION**

We have characterized CalDAG-GEFIII, which shows the broadest substrate specificity among GEFs for Ras family G proteins, i.e. this is the first GEF that activates G proteins of the Ras, R-Ras, and Rap subfamilies. The phylogenetic analysis of the catalytic domain of GEFs of Ras family G proteins showed that CalDAG-GEFII, CalDAG-GEFIII, and CalDAG-GEFIII made up a subfamily. All three CalDAG-GEFs promoted the guanine nucleotide exchange of R-Ras, although the levels of activation differed significantly for each CalDAG-GEF. CalDAG-GEFII and CalDAG-GEFIII differed in that the former stimulated Rap1, whereas the latter stimulated Ras. Identification of CalDAG-GEFIII, which activated Ras, R-Ras, and Rap1, strongly suggests that the prototype CalDAG-GEF catalyzed the guanine nucleotide exchange of all of the Ras, R-Ras, and Rap subfamilies and that CalDAG-GEFII and CalDAG-GEFIII lost the capability to catalyze Ras and Rap1, respectively, during evolution. Interestingly, there is only one CalDAG-GEF in Caenorhabditis elegans. Determination of the substrate specificity of C. elegans CalDAG-GEF will provide insights into the evolution CalDAG-GEFs.

GEFs that activate both R-Ras and Rap1 or R-Ras and Ras have already been reported (23); however, the finding that CalDAG-GEFIII promoted the guanine nucleotide exchange of these three subfamilies of G proteins questioned about the specificity in the signaling from GEFs to Ras family G proteins. The Ras, R-Ras, and Rap subfamilies of G proteins are expressed ubiquitously (37), suggesting that CalDAG-GEFIII-expressing cells also express all of these three Ras subfamily G proteins. Although we showed that CalDAG-GEFIII activates Ras, R-Ras, and Rap1 in vitro and in cultured cells, this does not necessarily confirm that CalDAG-GEFIII promotes the guanine nucleotide exchange reaction of these G proteins in vivo, particularly in highly differentiated cells.

There are two possibilities to explain the redundancy in the substrate specificity. CalDAG-GEFIII may be used to activate simultaneously all of the Ras, R-Ras, and Rap1 subfamilies. Alternatively, there may be a mechanism that restricts the substrate specificity of CalDAG-GEFIII in vivo. For example,
Rap1 is believed to be localized in the Golgi apparatus, whereas Ras is localized mostly at the plasma membrane (38). Thus, CalDAG-GEFIII may be used to activate Ras at the plasma membrane and Rap1 at the Golgi apparatus. To solve these problems, we must develop methods to stimulate GEFs without overexpression and to measure the level of GTP-binding form without disrupting the structure of the cell.

CalDAG-GEFIII, like CalDAG-GEFII, retained biological phenotypes attributable to the activation of Ras, including the activation of ERK/MAPK, the induction of neuronal differentiation of PC12 cells, and the transformation of Rat-1A cells (36). However, there were apparent differences between CalDAG-GEFII and CalDAG-GEFIII in the level of these biological effects. CalDAG-GEFIII consistently activated Ras and ERK/MAPK more strongly than did CalDAG-GEFII in 293T cells or in vitro. Nevertheless, CalDAG-GEFIII induced neuronal differentiation of PC12 cells and transformation of Rat-1A cells less efficiently than did CalDAG-GEFII. Therefore, the attenuated phenotype of CalDAG-GEFIII appeared to result from its less efficient activation of PC12 cells used in our experiments, negating the positive contribution of Rap1 in CalDAG-GEFIII-induced neuronal differentiation of PC12 cells used in our laboratory.2 Rather, our observation suggested that co-stimulation of Rap1 by CalDAG-GEFIII attenuated the effect of Ras. It has been reported that Rap1 inhibits Ras transformation by the competitive binding to c-Raf (40, 41). However, more recently, it has also been reported that Rap1 may enhance cell attachment by activating integrins, suggesting that Rap1 exerts its anti-Ras effect apart from c-Raf inhibition (9, 19, 24, 42).

In conclusion, we characterized CalDAG-GEFIII, which showed the broadest substrate specificity among the known GEFs of Ras family G proteins. Mutually exclusive expression patterns of CalDAG-GEFs in the brain suggest that each CalDAG-GEF plays an important role in the regulation of higher order brain functions.

Acknowledgments—We thank J. L. Bos, A. Wittinghofer, B. J. Mayer, S. Hattori, K. Kaibuchi, J. Miyazaki, Y. Nakabeppehu, K. Kitayama, and M. Noda for providing materials used in this study.

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