Characterization of GFR, a novel guanine nucleotide exchange factor for Rap1

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Abstract Three groups of Rap1-specific guanine nucleotide exchange factors including C3G, CalDAG-GEFI, and Epac/cAMP-GEFI/II have been identified to date. In the present study, we report a new Rap1 guanine nucleotide exchange factor which we have named GFR (guanine nucleotide exchange factor for Rap1). GFR shows close sequence similarity to EPAC/cAMP-GEFI/II although GFR lacks a cAMP binding domain and contains a nuclear localization signal. We demonstrated that GFR can activate Rap1 but not H-Ras in 293T cells and that the cdc25 domain of GFR is required for the activation of Rap1. Northern blot analysis suggested that GFR mRNA is strongly expressed in the brain. In transfected HeLa cells, GFR has been found to be localized in the nuclei.

Key words: Ras family GTPase; Rap1; Guanine nucleotide exchange factor; cdc25 domain; Nuclear localization signal; Human brain

1. Introduction

Ras family GTPases play a crucial role in cell growth, differentiation and malignant transformation [1]. Rap1, a Ras family small GTPase, was originally identified as an anti-oncogenic protein which efficiently reverses the morphologic transformation of the v-Ki-ras-expressing NIH 3T3 cell line, DT [2]. Because the effector domain of Rap1 was virtually identical to that of Ras, it was suggested that Rap1 inhibits the effect of Ras through the formation of an inactivated complex with the effector proteins of Ras [3]. Consistent with this model, the activation of the c-fos promoter from Ras, but not from the activated Raf-1 which is an immediate downstream effector protein of Ras, is antagonized by the expression of Rap1 [4]. In general, overexpression of Rap1 suppresses Ras signaling.

Rap1 is, however, not always activated by the extracellular stimuli which counteract Ras. Rather, a number of stimuli which may induce cell growth or differentiation, such as T cell receptor and B cell receptor activation, thrombin, epidermal growth factor, nerve growth factor, platelet-derived growth factor, cAMP, calcium ionophore, and phorbol 12-myristate 13-acetate (TPA), can activate Rap1 [5]. For example, although Ras is critical for the neuronal differentiation of PC12 cells [6], cAMP [7] and nerve growth factor (NGF) [8] induce neuronal differentiation of PC12 cells through the activation of Rap1. The exact mechanism by which these stimuli activate Rap1 is largely unknown. However, it has become clear that three groups of Rap1-specific guanine nucleotide exhange factors (GEFs), C3G [9,10], CalDAG-GEFI [11], and Epac/cAMP-GEFI/II [12,13] are differently implicated in Rap1 activation. C3G associates with CrkL and Cbl in response to T cell antigen receptor stimulation, resulting in Rap1 activation and T cell anergy [14]. C3G can also bind to Crk and Grb2, which are adapter proteins recruited to receptor tyrosine kinases in response to growth factor stimulation [15]. Thus, it is plausible that the C3G/Rap1 cascade might be coupled to mitogenic signals. CalDAG-GEFI contains calcium or diacylglycerol (DAG) binding motifs and calcium ionophore or TPA increases its catalytic activity for Rap1 in 293T cells [11]. Recent reports demonstrated that direct binding of cAMP to Epac/cAMP-GEFI/II can induce the catalytic activity for Rap1 both in vivo and in vitro [12,13].

Here, we report a new member of Rap1-specific GEF, which we call GFR (guanine nucleotide exchange factor for Rap1). GFR has significant sequence similarity to Epac/cAMP-GEFI/II, although GFR itself lacks a cAMP binding motif and contains a nuclear localization signal (NLS). GFR is strongly expressed in the brain and localizes in nuclei when transfected in HeLa cells.

2. Materials and methods

2.1. Cell culture and transfection

Human 293T embryonic kidney cells and human cervical carcinoma HeLa cells were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo) supplemented with 10% fetal calf serum. 293T cells and HeLa cells were transiently transfected by Superfect transfection reagent (Qiagen).

2.2. Expression plasmids

Human GFR cDNA, originally reported as KIAA0277 (accession number D87467) [16], was kindly provided by N. Nomura (Kazusa Institute, Japan). The authentic GFR and the cDNA fragment lacking the cdc25 domain of GFR (amino acids 1–315) were subcloned into the pCXN2 eukaryotic expression vector after the addition of a Flag tag sequence to its amino-terminus. The resulting vectors were designated pCXN2-Flag-GFR and pCXN2-Flag-GFRdCD. pEBG-Rap1 and pEBG-H-Ras encoded Rap1 and H-Ras fused to glutathione *S*-transferase (GST) [17], respectively.

2.3. Antibodies

Anti-Flag antibody (M5) was obtained from Sigma. FITC-labeled goat anti-mouse IgG antibody (F-2761) was purchased from Molec-

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Abbreviations: GEF, guanine nucleotide exchange factor; NLS, nuclear localization signal; NGF, nerve growth factor; TPA, phorbol 12-myristate 13-acetate; DAG, diacylglycerol; GST, glutathione *S*-transferase; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride

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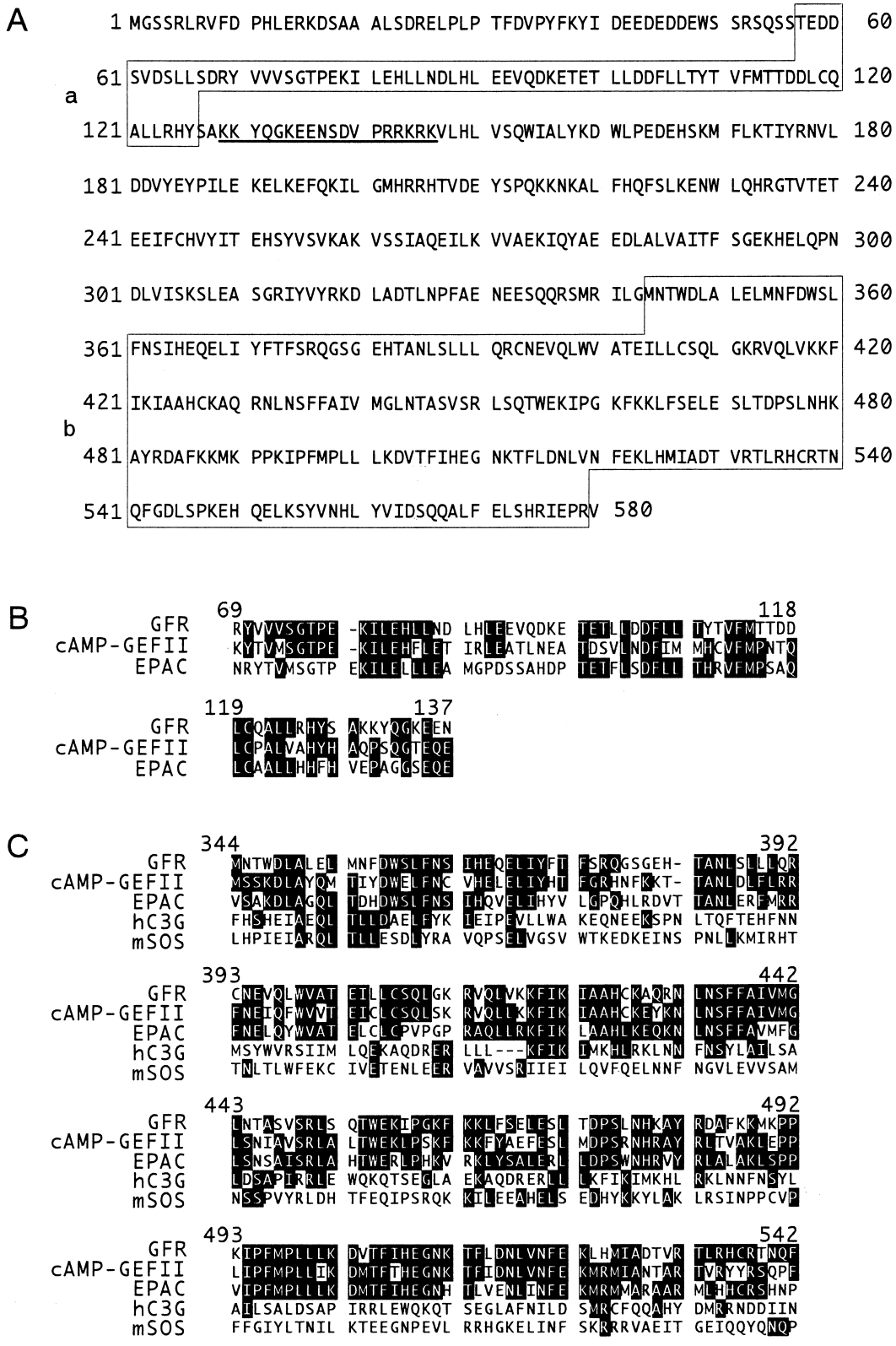

Fig. 1. Features of the GFR gene. A: Predicted amino acid sequence of GFR and domains obtained from the PROSITE search are boxed. a, LTE/rasGRF-associated domain. b, Cdc25 domain. The potential bipartite nuclear localization signal is underlined. B: Multiple sequence alignments of the amino-terminal region of GFR and EPAC/cAMP-GEFI/II (black indicates identity). C: Multiple sequence alignments of the cdc25 domain of GFR, EPAC/cAMP-GEFI/II, human C3G, and mouse SOS (black indicates identity).

ular Probes. Anti-GST rabbit antibody was kindly provided by Dr. S. Matsufuji (Jikei University, Japan).

2.4. Sequence analysis

On-line BLAST searches were performed via the National Center for Biotechnology Information at the National Institute of Health, Bethesda, MD, USA. Protein alignments were carried out with the DNASIS program (Hitachi Corporation). The Profile Scan Output service (PROSITE program) was used to search for protein motifs.

2.5. Northern blot analysis

DNA fragments containing the authentic GFR or β-actin cDNA were labeled with the Multiprime DNA labeling system (Amersham). A multi-tissue Northern blot (Clontech Laboratories) was hybridized with $^{32}P_i$-labeled probes of GFR cDNA or β-actin cDNA and analyzed with a PhosphorImager, BAS2000 (Fuji Film).

2.6. Analysis of guanine nucleotide exchange activity of GFR for Rap1 and H-Ras in human 293T cells

Guanine nucleotides bound to Rap1 were analyzed essentially as described previously [10]. Briefly, 10^5 293T cells plated on 3.8 cm^2 dishes were transfected with 2 μg of expression plasmids. Forty-eight hours after transfection, cells were labeled for 2 h with 0.05 mCi of $^{32}P_i$ in 0.2 ml of phosphate-free medium. GST-fused Rap1 was collected by glutathione-Sepharose 4B (Pharmacia). Guanine nucleotides bound to Rap1 were separated by thin-layer chromatography and quantitated with a PhosphorImager analyzer, FLA2000 (Fuji Film). For the analysis of protein expression, cells were similarly processed without isotopic labeling. The cleared lysates were separated by SDS-PAGE, transferred to a nylon membrane, probed with anti-Flag or anti-GST antibodies, and detected using the ECL chemiluminescence system (Amersham).

2.7. Immunofluorescence

HeLa cells transfected with pCXN2-Flag-GFR were fixed with 4% paraformaldehyde in phosphate-buffered saline supplemented with 30% sucrose for 15 min at 37°C and permeabilized with 0.3% Triton X-100 for 3 min. After blocking with 1% bovine serum albumin, the cells were incubated with the anti-Flag antibody at a dilution of 1:100. The FITC-conjugated goat anti-mouse IgG at a dilution of 1:200 was used for the detection of Flag-tagged GFR, followed by DNA staining by 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim). The cells were viewed using a Carl Zeiss fluorescence microscope.

3. Results

3.1. Identification and features of GFR cDNA

To identify a potential Rap1 GEF, we performed a BLAST homology search using the cdc25 domain of C3G. The cdc25 domain is common to Ras family GEFs and mediates guanine nucleotide exchange activity [18]. We found that clone KIAA0277 has high amino acid similarity and selected it for biochemical analysis. Since KIAA0277 showed catalytic activity for Rap1 as described below, we renamed it GFR (guanine nucleotide exchange factor for Rap1). The predicted molecular weight of GFR is 67 730 Da with a theoretical p*I* of 5.87. The Profile Scan Output shows that GFR contains an LTE/rasGRF-associated domain, potential bipartite nuclear localization signal [19,20], and a cdc25 domain (Fig. 1A).

From the BLAST sequence search results, we found that GFR shares significant sequence similarity to EPAC/cAMP-GEFI/II [12,13], which are Rap1 GEFs activated by cAMP, although the open reading frame of GFR lacks a cAMP binding motif. The cdc25 domain of GFR also shows sequence similarity to other members of Ras family GEFs including C3G [9], and SOS [21] (Fig. 1C). Remarkably, in addition to the cdc25 domain, EPAC/cAMP-GEFI/II displays close similarity to the amino-terminal region of GFR which contains the LTE1/rasGRF-associated domain (Fig. 1B).

3.2. Activation of Rap1 by GFR in 293T cells

293T cells were transfected with expression vectors encoding Flag-tagged GFR, C3G, GST-tagged Rap1, and H-Ras. After 48 h, guanine nucleotides bound to GST-Rap1 were analyzed by thin-layer chromatography. As shown in Fig. 2, the expression of GFR significantly increased the ratio of GTP/(GDP+GTP) on Rap1. The extent of Rap1 activation by GFR was comparable to that by C3G. Although mSOS activated GST-H-Ras (data not shown), GFR did not increase the ratio of GTP to GDP on GST-tagged H-Ras, suggesting that it has a strict exchange activity for Rap1. The same expression levels of GFR, Rap1, and H-Ras protein were confirmed in each experiment (data not shown). To confirm that Rap1 activation was mediated by the catalytic activity of GFR, we constructed a plasmid which lacks the cdc25 domain of GFR and designated it GFRdCD. As expected, the expression of GFRdCD did not affect the ratio of guanine nucleotides bound to Rap1 (Fig. 3). From these results, we concluded that GFR is a novel guanine nucleotide exchange factor for Rap1.

3.3. Northern blot analysis of GFR mRNA

Next, we investigated the tissue distribution of GFR mRNA (Fig. 4). Consistent with the fact that GFR was originally identified from a brain cDNA library [16], GFR was predominantly expressed in the brain, although to some extent, it was also expressed in the heart, spleen, kidney, liver, placenta, and lung.

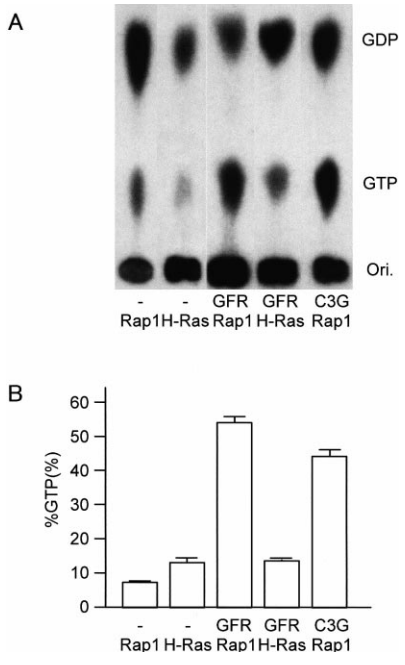

Fig. 2. GFR activates Rap1 in 293T cells. A: 293T cells were transfected with the expression vectors encoding the bottom. After 48 h, the transfected cells were labeled with $^{32}P_i$ for 2 h. GST-tagged GTPases were collected by glutathione-Sepharose from the cell lysates. Guanine nucleotides bound to GTPases were analyzed by thin-layer chromatography. B: The radioactivity of each spot was measured by a PhosphorImager, and the ratio of GTP to GTP+GDP was calculated. Mean values obtained from three samples are shown with the S.D.

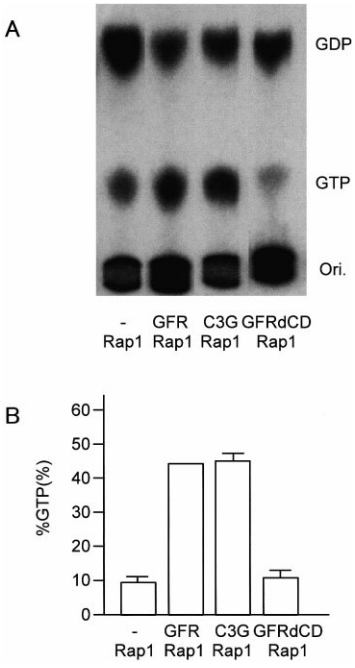

Fig. 3. Cdc25 domain of GFR is required for the activation of Rap1 in 293T cells. A: 293T cells were transfected with pEBG-Rap1 and the expression vectors encoding the bottom. After 48 h, the transfected cells were labeled with $^{32}P_i$ for 2 h. GST-tagged Rap1 was collected by glutathione-Sepharose from the cell lysates. Guanine nucleotides bound to Rap1 were analyzed by thin-layer chromatography. B: The radioactivity of each spot was measured by a PhosphorImager, and the ratio of GTP to GTP+GDP was calculated. Mean values obtained from three samples are shown with the S.D.

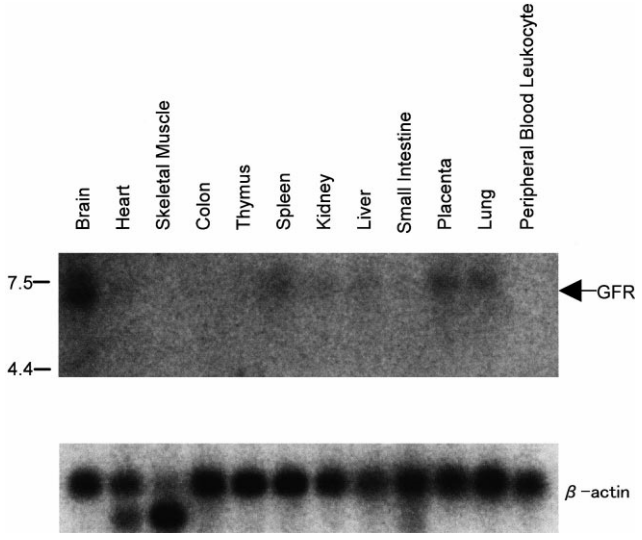

Fig. 4. Expression of GFR mRNA in different tissues. A human multiple-tissue Northern blot (Clontech) was hybridized in separate experiments with $^{32}P_i$-labeled GFR (upper panel) and β-actin (lower panel) probes as described. Lanes contain 2 μg of poly(A)$^+$ RNA from the indicated tissues with loading normalized to β-actin. RNA size markers are shown in the left margin (upper panel), the GFR transcript is indicated by an arrow (upper panel), and the β-actin transcripts are shown in the lower panel (note the alternative β-actin transcripts in the heart and skeletal muscle).

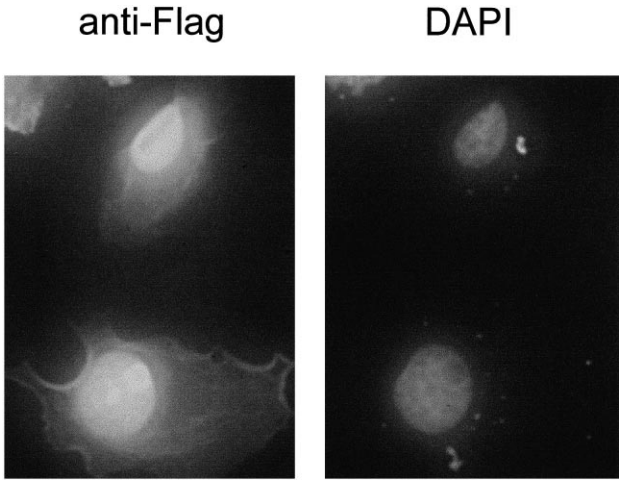

Fig. 5. Nuclear localization of GFR in HeLa cells. HeLa cells were transfected with expression vector (pCXN2-Flag-GFR). After 48 h, fixed cells were incubated with anti-Flag antibody, followed with FITC-conjugated anti-mouse IgG. Then, cells were probed with DNA staining by DAPI and visualized by a fluorescence microscope.

3.4. Nuclear localization of GFR in HeLa cells

HeLa cells were transfected with expression vector encoding Flag-tagged GFR, and its cellular localization was probed with FITC-labeled goat anti-mouse IgG and DNA staining by DAPI. As shown in Fig. 5, GFR has been co-localized with DAPI staining, suggesting its existence in the nucleus.

4. Discussion

We have characterized a novel GEF for Rap1 which we have named GFR. GFR shares significant sequence similarity to EPAC/cAMP-GEFI/II [12,13]. GFR is abundantly expressed in the brain, and it is also detected in the heart, spleen, kidney, liver, placenta, and lung. In addition to a cdc25 domain, GFR contains a LTE1/rasGRF-associated domain and a bipartite nuclear localization signal [19,20]. Consistent with the existence of NLS, GFR localizes in nuclei when transfected in HeLa cells.

The LTE1/rasGRF-associated domain, also called the Ras exchange motif region, is common to Ras family GEFs and may be critical for the stabilization of GEF structure [22]. Endogenous Rap1 is mainly localized at endocytic and lysosomal vesicles in the cytoplasm [23]. Probably, GFR might have to migrate from the nucleus to the cytoplasm in response to stimuli that activate the GFR-Rap1 pathway. Alternatively, GFR might stimulate nuclear GTPases other than Rap1.

The amino-terminal portion of GFR lacks a cAMP binding domain which controls the catalytic activity of EPAC/cAMP-GEFI/II. However, there is a possibility that this region may control its catalytic activity. In the case of C3G, truncation or tyrosine phosphorylation of the amino-terminal region can enhance its catalytic activity [24].

Since GFR mRNA is strongly expressed in the brain, the GFR/Rap1 cascade might be implicated in neuronal proliferation or differentiation. PC12 rat adrenal medullary cells differentiate into sympathetic neurons upon stimulation with NGF or cAMP. Rap1 is activated immediately after this stimulation and triggers B-Raf/ERK activation. This cascade

seemed to be critical for neuronal differentiation [7,8]. However, a recent report suggests that endogenous Rap1 displays no activation on treatment with NGF [5]. To clarify the potential role of GFR in neuronal differentiation, the expression of GFRdCD may be useful because it will block GFR-dependent pathway(s) like the C3G mutant lacking a catalytic domain [25].

Some GEFs couple with growth factors by binding to their receptors either directly or indirectly upon stimulation [15]. Thus, isolation of GFR binding proteins may be useful to determine GFR-specific cascade(s). Furthermore, in analogy with C3G, whose catalytic activity is enhanced by the expression of C3G binding proteins [17], it is possible that proteins which associate with GFR may control its activity in vivo.

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