The small GTPase Rap1 has been implicated in a variety of cellular processes including the control of cell morphology, proliferation, and differentiation. Stimulation of a large variety of cell surface receptors results in the rapid activation of Rap1, *i.e.* an increase in the GTP-bound form. This activation is mediated by second messengers like calcium, cAMP, and diacylglycerol, but additional pathways may exist as well. Here we describe a ubiquitously expressed guanine nucleotide exchange factor of 200 kDa that activates Rap1 both *in vivo* and *in vitro*. This exchange factor has two putative regulatory domains: a domain with an amino acid sequence related to cAMP-binding domains and a PDZ domain. Therefore, we named it PDZ-GEF1. PDZ-GEFs are closely related to Epacs, Rap-specific exchange factors with a genuine cAMP binding site, that are directly regulated by cAMP. The domain related to cAMP-binding domains, like the cAMP binding site in Epac, serves as a negative regulatory domain. However, PDZ-GEF1 does not interact with cAMP or cGMP. Interestingly, PDZ-GEF1 also activates Rap2, a close relative of Rap1. This is the first example of an exchange factor acting on Rap2. We conclude that PDZ-GEF1 is a guanine nucleotide exchange factor, specific for Rap1 and Rap2, that is controlled by a negative regulatory domain.

Rap1 is a Ras-like small GTPase that has been implicated in a variety of cellular processes like T-cell anergy (1), platelet activation (2), and the reversion of oncogenic transformation (3) as well as the induction of oncogenic transformation (4). Because Rap1 has an effector domain that is very similar to Ras, some of these effects may be mediated, by either positive or negative interference in Ras signaling. Indeed it was shown that Rap1 can regulate extracellular signal-regulated kinases specifically and responsive to both of these second messengers. Interestingly, for both DAG and cAMP, Rap1 is activated in various cell types by a variety of stimuli. In fibroblasts, Rap1 is rapidly activated after stimulation with growth factors like platelet-derived growth factor, epidermal growth factor, endothelin, lysophosphatidic acid, and bombesin (10, 11). In blood platelets, Rap1 activation is induced by thrombin and other factors that regulate platelet activation (2); in neutrophils by chemokines and cytokines like f-Met-Leu-Phe, platelet-activating factor, granulocyte macrophage colony-stimulating factor, and tumor necrosis factor-α (15); in T-cells by T-cell receptor cross-linking (16); in B lymphocytes by B-cell receptor cross-linking (17); and in mast cells by cross-linking the FcεRI receptor.

The activation of Rap1 by extracellular stimuli is mediated by different signaling pathways depending on the receptor and the cell type. The best characterized pathways are those that use the second messengers, calcium diacylglycerol and cAMP (2, 10, 18). However, elusive pathways, independent of these second messengers, do exist (15). Three families of guanine nucleotide exchange factors have been identified that control the activation of Rap1. First, C3G, which may control tyrosine kinase-induced activation of Rap1 through the adapter proteins Crk and Cbl (16, 19, 20). Second, the GRPs, which contain calcium and diacylglycerol binding motifs (21). From this family, HCDC25L (22), also called CalDAG-GEF1 (23), is Rap1-specific and responsive to both of these second messengers. Finally, the Epac family, Rap-specific GEFs that are directly regulated by cAMP (24, 25). Interestingly, for both DAG and cAMP, these GEFs represent novel targets that mediate signal transduction independent from the classical targets protein kinase C and protein kinase A.

To further identify GEFs that may be specific for Rap1 we searched data bases and found a conserved family of genes, which we named PDZ-GEFs. The encoded proteins contain the characteristic Ras exchange motif and GEF sequences present in all GEFs for the Ras-like GTPases, as well as a proline-rich motif, a PDZ-domain (26), and a structure that is related to the Ras-effector signaling (10–12). This additional function is still largely elusive. The function of Rap2, a very close relative of Rap1, is also unknown, although Rap2 is able to bind to the same effectors as Rap1 (13). In addition, Rap2 binds to a specific putative effector, RPIPs (14).

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GEF1. We conclude that PDZ-GEF1 is a novel Rap1- and Rap2-specific GEF, in which the activity is negatively regulated by an intramolecular domain.

EXPERIMENTAL PROCEDURES

Bio-informatics—The presence of domains in amino acid sequences was analyzed using the IBSRC ProfileScan server. Gene sequences in genomic DNA were predicted using programs united in NIX at the Human Genome Mapping Project Resource Center. Alignment of homologous amino acid sequences was conducted using the program ClustalX (27).

Protein Expression and Purification—HA-PDZ-GEF1 contains the full-length PDZ-GEF1 cDNA cloned in the PMT2-SM-HA eukaryotic expression vector. In the same vector, all small GTPases used in overexpression, as well as the deletion constructs of PDZ-GEF1, were cloned. HA-PDZ-GEF1-B5 contains amino acids 1-1002, and HA-PDZ-GEF1-M5 contains amino acids 252-1002. For purification of GST fusion constructs, PDZ-GEF1-M5 and PDZ-GEF1-B5 were cloned in the pGEX4T3 bacterial expression vector. Protein production was induced in BL21 bacteria using 100 μM isopropyl-1-thio-β-D-galactopyranoside for 20 h at room temperature. After protein production, bacteria were pelleted and washed with ice-cold phosphate-buffered saline containing 1% Triton X-100 and protease inhibitors. The lysate was sonicated three times for 10 s and centrifuged at 10,000 × g to remove insoluble material. GST fusion proteins were purified from the cleared lysate by batchwise incubation with glutathione-agarose beads (Sigma), eluted from the beads in buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 10 mM glutathione, and dialyzed for 20 h in the same buffer without glycerol. Small GTPases used for in vitro experiments were as described elsewhere (7, 28, 29).

Northern and Western Blotting—A human multiple tissue Northern blot was purchased from CLONTECH. Western blotting of total lysates and protein samples isolated by activation-specific probe assays were on polyvinylidene difluoride membranes. The antiserum used for detecting endogenous PDZ-GEF1 in total cell lysates was raised against a synthetic peptide containing amino acids 1480-1488 (PYQSQGFSTEED-EDEQVSA) from PDZ-GEF1.

In Vivo Activation of Small GTPases—Cells were transfected with HA-tagged versions of the small GTPases and serum-starved for 20 h prior to all activation experiments. GTP-bound forms of the different GTPases were isolated using activation-specific probes as described (2, 30, 31). R-ras was studied using the Ras-binding domain (RBD) of Rap1. Detection during Western blotting was performed by 12CA5 monoclonal antibodies directed against the HA-tag.

In vivo labeling experiments for Rap1 and Rap2 were performed as described (32). Briefly, serum-starved cells were labeled with [32P]orthophosphate for 5 h. Rap2 was precipitated using 12CA5 antibodies and nucleotides were eluted and separated on PEI-cellulose F thin layer chromatography (TLC) plates (Merck). Labeled nucleotides were visualized using a PhosphorImager, and GTP/GDP ratios were calculated using the program ImageQuant (33).

In Vitro Activation of Small GTPases—Experiments were performed as described (34). Briefly, 100 nM of purified GTPase loaded with fluorescently labeled 2′,3′-bis(O)-N-methylanthranolyl guanosinediphosphate (mant-GDP) was incubated in the presence of excess unlabeled GDP with 50 nM of purified PDZ-GEF1. Release of mant-GDP was measured in real time as a decrease in fluorescence. To calculate reaction rates, single exponential functions were fit using the program Grafit 3.0 (Eritics).

RESULTS

A Novel Family of Guanine Nucleotide Exchange Factors—In a data base screen for possible regulators of Rap proteins, a new family of guanine nucleotide exchange factors, which we named PDZ-GEFs, was found. The cDNA encoding the PDZ-GEF1 protein was isolated earlier in a random cloning strategy and was designated KIAA0313 (35). The PDZ-GEF1 gene is conserved in nematode (Z88388 and CA983100) and fly (AC005285), as judged from gene predictions on genomic sequences of these organisms, and has a human homologue (PDZ-GEF2), as predicted from genomic sequences in chromosome 5 (AC004622 and AC004227). The PDZ-GEF2 forms a new family of exchange factors that is most closely related to Epacs, which are Rap1-specific GEFs directly regulated by cAMP (Fig. 1a). PDZ-GEF1s contain a number of conserved domains (Fig. 1b).

First, they have a Ras exchange motif that is present in all CDC25-like exchange factors and is probably needed for protein stabilization (36). Second, they have a catalytic domain that shows considerable homology to previously described Rap1 and Ras GEFs especially in the strongly conserved regions 1, 2, and 3 (Fig. 1c). Third, they have a domain exhibiting the characteristics of a PDZ domain (hence the name PDZ-GEF) (Fig. 1d). This last domain has a hydrophobic amino acid located at position 1 in the second predicted α-helix (Leucine 446, indicated by an arrow in Fig. 1d) and may therefore be a class II PDZ domain (37). Finally, a conserved structure is present in PDZ-GEFs that is related to the cAMP-binding domains in Epac and Pak. However, the RCBD does not contain conserved residues at the positions that interact with cAMP in PKA (38-43), as indicated by arrows in Fig. 1e. This implies that cAMP is probably not the interacting molecule.

PDZ-GEF1 Is Ubiquitously Expressed As a 200-kDa Protein—To investigate whether the PDZ-GEF1 gene is transcribed in human cells we probed a commercial human multiple tissue Northern blot with a radioactively labeled probe spanning the GEF domain of PDZ-GEF1 and found that the gene is expressed in all tissues present on the membrane (Fig. 2a). Furthermore, we raised a polyclonal antibody to a C-terminal peptide of PDZ-GEF1 (in rabbits) that recognized a 200-kDa protein in cell lysates of a number of different cell lines (Fig. 2b). As a control two identical Western blots containing several of these lysates were probed with the antiserum or with antiserum that was preincubated with the peptide used for immunizing the rabbits. In this case the 200-kDa band disappeared (Fig. 2c). From this we conclude that the PDZ-GEF1 gene is ubiquitously expressed and that the cDNA is indeed translated into a protein of the predicted molecular weight.

PDZ-GEF1 Activates Rap1 in Vitro and in Vivo—To investigate the specificity of PDZ-GEF1 for the members of the Ras family of small GTPases we transfected these small GTPases either alone or in combination with PDZ-GEF1 in COS-7 cells (Fig. 3a) and used the appropriate GST-RBD constructs as activation-specific probes to measure activation of the small GTPases (2, 30, 31). As shown in Fig. 3b, cotransfection with PDZ-GEF1 results in a massive increase in the GTP-bound forms of Rap1 (both Rap1A and Rap1B), whereas the amount of GTP-bound Rap1, Ral, Rap2, or R-ras was not affected. From these results we concluded that overexpression of PDZ-GEF1 leads to specific activation of Rap1.

To prove that the activation of Rap1 by PDZ-GEF1 results from a direct interaction, we measured Rap1 activation in vitro. Because full-length PDZ-GEF1 could not be purified, we constructed GST fusions of truncated versions of PDZ-GEF1 (Fig. 3a). GST-PDZ-GEF1-B5 contains amino acids 1-1002, thus lacking the 497-amino acid C-terminal region with no recognizable domains. GST-PDZ-GEF1-M5 contains amino acids 252-1002, thus lacking both the 497-amino acid C-terminal region and the RCBD. Both constructs were expressed in Escherichia coli and purified by glutathione-agarose beads (Fig. 3a, right panel). Purified Rap1 loaded with fluorescent mant-GDP (34) was incubated with the different GST-PDZ-GEF1 proteins in the presence of excess unlabeled GDP, and exchange of guanine nucleotide bound to Rap1 was measured in real time as a decrease in fluorescence (Fig. 3c). Exchange of the nucleotide bound to Rap1 was slightly stimulated by GST-PDZ-GEF1-B5 but was stimulated more dramatically by an equal amount of GST-PDZ-GEF1-M5, which lacks the RCBD. First of all, these results confirm the in vivo data that proves that PDZ-GEF1 can function as a Rap1-GEF. In addition, these results indicate that the RCBD is an inhibitory domain in...
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PDZ-GEF. This latter conclusion was supported by experiments in which increasing amounts of HA-tagged versions of PDZ-GEF1-B5 and PDZ-GEF1-M5 (Fig. 3a) were expressed together with Rap1 in COS-7 cells. At low levels of expression, PDZ-GEF1-M5 induced a much stronger activation of Rap1 than PDZ-GEF1-B5 (Fig. 3d). In this respect RCBD acts in a way that is similar to the cAMP domain of Epac (24).

To investigate whether cAMP or a related molecule could, analogous to Epac, relieve PDZ-GEF1-B5 from the inhibitory effect of the RCBD, we measured PDZ-GEF1-B5 activity in the presence of cAMP. However, as expected from the sequence of RCBD, cAMP did not induce the activation of PDZ-GEF1-B5 in vitro or in cotransfection in COS-7 cells and in A14 fibroblasts (data not shown). Also, cGMP had no effect on PDZ-GEF1-B5 (Fig. 3e). Recently, the NAD derivatives cADPr and B-NAADP were shown to act as second messengers in T-cell

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signaling (44, 45). Also, these molecules did not modulate the activity of PDZ-GEF1-B5 in vitro (data not shown).

PDZ-GEF1 Is a Dual Specific GEF for Rap1 and Rap2—To confirm the specificity of PDZ-GEF1 for Rap1, the GST fusion constructs were incubated in the same assay with mant-GDP-loaded Ras, Ral, R-ras, and Rap2. As expected from the cotransfection experiments, no stimulation by PDZ-GEF1 of the guanine nucleotide exchange on Ras, Ral, or R-ras was observed (data not shown). However, Rap2 was slightly activated by GST-PDZ-GEF1-B5 and more strongly by GST-PDZ-GEF1-M5 in this in vitro setup (Fig. 4a). To compare the activity of PDZ-GEF1 toward Rap1 versus Rap2, the reaction rates of the catalyzed exchange reaction were calculated (see “Experimental Procedures”), compared with the non-catalyzed (intrinsic) exchange rates, and scored as -fold stimulations of guanine nucleotide exchange (Fig. 4, b and c). We observed that PDZ-GEF1 can activate Rap2A more efficiently than Rap1A. Deletion of the RCBD results in a 10-fold higher stimulation of exchange activity on both Rap1 and Rap2. From these experiments we concluded that PDZ-GEF1 is a dual specific GEF that can catalyze the activation of both Rap1 and Rap2.

Rap2 Activity Is Increased by PDZ-GEF1 in Vivo—Although guanine nucleotide exchange on Rap2 is strongly stimulated by PDZ-GEF1 in vitro, no clear increase in GTP-bound Rap2 was found when coexpressed with PDZ-GEF1 in COS-7 cells (Fig. 3a). However, this discrepancy caused us to question the validity of the use of Rap1 GDS-RBD as an activation-specific probe for Rap2. Therefore, we performed a classic GTP/GDP loading experiment with [32P]orthophosphate-labeled cells. HA-tagged Rap2a was coexpressed with the different PDZ-GEF1 constructs (Fig. 3a) in A14 or COS-7 cells. Cells were labeled with...
orthophosphate, Rap2 was immunoprecipitated, and the GTP/GDP content was analyzed by TLC as described (32). As shown in Fig. 5a, over 60% of Rap2 was bound to GTP in resting A14 and COS-7 cells. Rap2-GTP levels were increased to 95, 94, and 85% by PDZ-GEF1-M5, PDZ-GEF1-B5, and full-length PDZ-GEF1, respectively. The high doses of PDZ-GEF1 DNA used in these experiments explains why no difference is observed in the PDZ-GEF1-M5 and PDZ-GEF1-B5 cotransfections. Differences are only observed at low levels of expression, as shown in Fig. 3d. To test whether the high basal Rap2 activity is due to abnormal signaling activity in the cells used, Rap1 activation was measured in COS-7 cells using the same method. As shown in Fig. 3b, only 6% of Rap1 was bound to GTP in resting COS-7, and Rap1 GTP levels were increased to 44, 46, and 35% by PDZ-GEF1-M5, PDZ-GEF1-B5, and full-length PDZ-GEF1, respectively. From these experiments we conclude that Rap2 can be activated by PDZ-GEF1 both in vitro and in vivo. The high basal level of Rap2-GTP in resting fibroblasts and COS-7 cells allows a maximal activation of less than 2-fold. This explains why no clear activation of Rap2 was found in the initial experiments using the activation-specific probe approach. However, using the PDZ-GEF1-M5 and PDZ-GEF1-B5 constructs in cotransfection of COS-7 cells, a small increase in Rap2-GTP was observed using Ral GDS-RBD as an activation-specific probe (Fig. 5c).

**DISCUSSION**

Here we describe the identification of a novel family of guanine nucleotide exchange factors named PDZ-GEFs. The human PDZ-GEF1 gene encodes a 200-kDa protein that is expressed ubiquitously. PDZ-GEF1 is a dual specific GEF that activates both Rap1 and Rap2 but not Ras, Ral, or R-ras. Apart from the catalytic domain that mediates activation of the small GTPases and the Ras exchange motif that is needed for stability of the protein (36), PDZ-GEFs contain two conserved structures: a domain similar to PDZ-domains and a domain related to cAMP binding sites. PDZ-domains are found in a large variety of signaling proteins, where they mediate protein-protein interactions via the binding of specific C-terminal peptides (26, 37, 46, 47). Thus far, attempts to identify the binding partners of PDZ-GEF1 using two-hybrid experiments have failed.

The primary sequence of the RCBD resembles the cAMP-binding domains of Epac, PKA, and the cyclic nucleotide-gated channels, although clear differences exist at the positions that interact with cAMP in PKA (38–43). cAMP-binding domains function as inhibitory domains in the absence of the second messenger. When cAMP binds to the domain or when it is deleted, this inhibitory effect is abolished. Similarly, RCBD functions as an inhibitory domain, and deletion of the domain results in a much more active protein. As expected, we could not detect any interaction of cAMP or cGMP with the RCBD in vitro (data not shown). Also, two other small second messen-
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gers, the NAD derivatives, cyclic ADP-ribose and B-NADP (44, 45), did not affect the activity of PDZ-GEF. We also failed thus far to find an extracellular stimulus that can augment the activity of PDZ-GEF. We predict that PDZ-GEF is regulated by a ligand, either a small second messenger or a protein or lipid, that binds to the RBKD. In this model the PDZ domain may serve as a membrane anchor to get the GEF close to Rap1. A similar function is predicted for the DEP domain in Epac (24).

PDZ-GEF1 differs from C3G and CalDAG-GEFI in that it activates Rap2 as well (Ref. 48 and data not shown). It is currently unclear what determines this difference in specificity. Comparing the sequences of the catalytic domains of these GEFs, an insert appears to be present in PDZ-GEF1. As judged from the structure of the complex between Ras and Sos, how-