PTPL1 is an intracellular protein-tyrosine phosphatase that contains five PDZ domains. Here, we present the cloning of a novel 150-kDa protein, the four most C-terminal amino acid residues of which specifically interact with the fourth PDZ domain of PTPL1. The molecule contains a GTPase-activating protein (GAP) domain, a cysteine-rich, putative Zn\(^{2+}\)- and diacylglycerol-binding domain, and a region of sequence homology to the product of the Caenorhabditis elegans gene ZK669.1a. The GAP domain is active on Rho, Rac, and Cdc42 in vitro but with a clear preference for Rho; we refer to the molecule as PTPL1-associated RhoGAP 1, PARG1. Rho is inactivated by GAPs, and protein-tyrosine phosphorylation has been implicated in Rho signaling. Therefore, a complex between PTPL1 and PARG1 may function as a powerful negative regulator of Rho signaling, acting both on Rho itself and on tyrosine phosphorylated components in the Rho signal transduction pathway.

The Rho family of Ras-like GTPases, which includes Rho, Rac, and Cdc42, control actin-based cytoskeletal rearrangements (reviewed in Refs. 1 and 2). Rho regulates receptor-mediated assembly of focal adhesions and stress fibers (3), whereas Rac regulates the formation of membrane ruffles (4) and Cdc42 controls the formation of filopodia (5). Rho proteins have also been shown to be important in the regulation of cell proliferation (reviewed in Ref. 6). As members of the Ras superfamily, Rho proteins function as molecular switches, having an active, GTP-bound form and an inactive, GDP-bound form. The active, GTP-bound form is negatively regulated by GTPase-activating proteins (GAPs)\(^1\), which enhances the intrinsic GTPase activity of Rho proteins. A number of GAPs that are active on proteins of the Rho family have been identified (reviewed in Ref. 7).

Intracellular protein-tyrosine phosphatases (PTPs) are a diverse group of proteins involved in signal transduction (reviewed in Ref. 8). They contain a conserved PTP domain that specifically dephosphorylates tyrosine residues and, in addition, domains that regulate their subcellular localization and activity (reviewed in Ref. 9). For example, the SH2 domains of SHP-1 and SHP-2 enables these PTPs to localize to and interact with activated growth factor receptors (9). Correct localization of PTPs is of importance, because the PTP domains usually have broad substrate specificity.

PTPL1 (10), also called PTP-BAS (11), hPTP1E (12), and FAP-1 (13), is a 250-kDa protein expressed in many tissues and cell lines. It contains an N-terminal leucine zipper motif followed by a domain with homology to the Band 4.1 superfamily. Band 4.1-like domains are found in proteins involved in the linkage of actin filaments to the plasma membrane (14). Five PDZ domains (PDZ is derived from PSD-95 (15), Dlg-A (16), and ZO-1 (17), each of which contains three such domains) are present between the Band 4.1-like domain and the C-terminal PTP domain. The PDZ domain, which also has been called LglGFP repeat or DHR, is a domain of about 90 amino acid residues and has been identified in a variety of proteins (18). Recently, PDZ domains have been shown to interact with C-terminal tails of target proteins (reviewed in Ref. 19). The second PDZ domain of PTPL1 has been shown to bind to the C-terminal tail of the membrane receptor Fas (13).

To identify proteins that interact with PTPL1, we searched for potential binding proteins in cell lysates using the PDZ domains of PTPL1 as baits. Here, we present the cDNA cloning of PTPL1-associated RhoGAP 1, PARG1, a 150-kDa protein that contains a GAP domain that displays strong activity toward Rho. Furthermore, the C-terminal tail of PARG1 specifically interacts with PDZ 4 of PTPL1.

**MATERIALS AND METHODS**

**Construction and Production of GST Fusion Proteins—**DNA fragments coding for PDZ domains of PTPL1 were produced by polymerase chain reaction and subcloned into the glutathione S-transferase (GST) fusion protein expression vector pGEX1T (Pharmacia Biotech Inc.).

**Cell Culture, Metabolic Labeling, Binding Assay, and SDS Gel Electrophoresis—**PC-3 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured as described (10). Recombinant Rho, Rac, and Cdc42 proteins were subjected to thrombin cleavage (11). After labeling, the cells were solubilized in buffer containing 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate, 1 mM dithiothreitol, 1.5% Trasylol (Bayer), and 1 mM phenylmethylsulfonyl fluoride (Sigma). After 15 min on ice, cell debris was removed by centrifugation. Samples (1 ml) were then incu-
bated for 1.5 h at 4 °C with 10 μg of GST-PDZ fusion proteins bound to glutathione-Sepharose 4B beads (Pharmacia). The beads were pelleted and washed four times with solubilization buffer. The protein complexes were eluted by boiling for 5 min in SDS sample buffer (100 mM Tris-HCl, pH 6.8, 0.01% bromphenol blue, 36% glycerol, 4% SDS, 10 mM dithiothreitol) and analyzed by SDS gel electrophoresis using 5–12% polyacrylamide gels (20). The gel was fixed, incubated with Amplify (Amersham Corp.) for 20 min, dried, and subjected to fluorography.

**Purification of PARG1, In-gel Digestion, Separation, and Sequencing of Peptides**—Confluent cultures of PC-3 cells from a total of 1750 cm² area were used. Cultures were solubilized as described above. Samples (20 ml) were incubated for 1.5 h at 4 °C with 200 μg of GST-PDZ fusion protein bound to glutathione-Sepharose 4B beads. The beads were washed, and protein complexes were subjected to SDS gel electrophoresis as described above. The gel was stained with Coomassie Brilliant Blue, and the band containing PARG1 was excised, transferred to Eppendorf tubes, and subjected to in-gel digestion (21). In brief, the gel piece was washed with 0.2 M ammonium bicarbonate (for digestion with trypsin) or 0.5 M Tris-HCl, pH 9.2 (for digestion with EndoLysC protease), and 50% acetonitrile and then dried completely. During rehydration, 0.5 μg of modified trypsin, sequence grade (Promega), or 0.5 μg of EndoLysC (WAKO Chemicals) was added, and 0.2 M ammonium bicarbonate (for trypsin) or 0.1 M Tris-HCl, pH 9.2 (for EndoLysC) was added in aliquots until the gel piece was immersed. After overnight incubation at 30 °C, the supernatant was saved and combined with two further extractions from the gel piece. Generated peptides were isolated by reversed phase liquid chromatography using the SMART System (Pharmacia). Peptides were sequenced on an Applied Biosystems model 470A or 476A following the manufacturer’s instructions.

**Cloning and Characterization of PARG1 cDNA**—Complementary and overlapping oligonucleotides corresponding to nucleotides 2–41 and 68–29 (noncoding strand) of an Expressed Sequence Tag with the GenBank accession number Z28520 was made using a DNA synthesizer and labeled by a fill-in method (22) using the Klenow fragment of DNA polymerase I (Amersham Corp.) and [α-32P]dATP (300 Ci/mmol, Amersham Corp.). A λgt11 human skeletal muscle cDNA library (HL5002b, Clontec) was screened as described (10) using the 32P-labeled oligonucleotides as a probe. A positive clone was isolated, subcloned into pBluescript SK (Stratagene), and thereafter sequenced. A Northern blot Hybridization—A Northern blot filter with mRNA from different human tissues was purchased from CLONTECH. The filter was hybridized with the 32P-labeled oligonucleotide probe described above at 42 °C overnight in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 2 × Denhardt’s solution, 0.5% SDS, 50 μM sodium phosphate, pH 6.9, and 0.1 μg/ml salmon sperm DNA. The filter was washed twice in 0.5 × SSC, 0.1% SDS at 55 °C for 15 min. After washing, the filter was exposed to Amersham Corp. Hyperfilm MP.

**GAP Assay**—The GAP assay was performed as described (23). Reconstituted Rho, Rac, and Cdc42 (500 nM) were incubated at 30 °C with either 125 nM GST-PDZ fusion proteins bound to glutathione-Sepharose 4B beads or 125 nM GST-PTPL1. The GAP assay was performed as described (23). The GAP assay was performed as described (23). The GAP assay was performed as described (23). The GAP assay was performed as described (23).

**RESULTS**

**Production of PDZ Fusion Proteins**—To identify proteins that bind to the PDZ domains of PTPL1, regions of PTPL1 cDNA corresponding to the various PDZ domains were cloned into the bacterial expression vector pGEX1 AT (Fig. 1A). The different GST fusion proteins were produced in E. coli and then subjected to SDS gel electrophoresis. Fig. 1B shows that pure preparations of fusion proteins with expected sizes were obtained.

A Novel 150-kDa Protein Binds to PDZ 4—The GST PDZ fusion proteins were immobilized on glutathione-Sepharose 4B and incubated with [35S]methionine- and [35S]cysteine-labeled cell lysates from the human carcinoma cell line PC-3. After washing, the bound material was analyzed by SDS gel electrophoresis. A component of 150 kDa that bound to the fusion proteins GST-PDZ 4 and GST-PDZ 4–5 could be observed (Fig. 2); this component did not bind to GST fusion proteins containing PDZ domains 1, 2, 3, or 5 only, indicating that the 150-kDa component interacts specifically with PDZ 4 of PTPL1.

To characterize the 150-kDa component further, it was purified from PC-3 cells. Briefly, immobilized fusion protein GST-PDZ 4 was incubated with cell lysate from 1750 cm² of confluent PC-3 cells. The beads were washed, and the bound proteins were eluted and subjected to SDS gel electrophoresis. After staining of the gel with Coomassie Brilliant Blue, the band that contained the 150-kDa component was excised and subjected to in-gel digestion using modified trypsin or EndoLysC protease. The recovered peptides were separated by reversed phase chromatography, and selected peptides were sequenced. Sequences were obtained from 10 peptides, and searches in different data bases showed that none of these sequences were found in any known gene or protein, but the human expressed sequence tags with GenBank accession numbers T32345, Z28837, and Z28520 contained cDNA sequences corresponding to three of the obtained peptides. Oligonucleotides corresponding to the nucleotide sequences of the expressed sequence tags were designed and used as probes for Northern blots and screening of cDNA libraries.

**cDNA Cloning of PARG1**—The expressed sequence tag-derived oligonucleotides were used to screen different human cDNA libraries. A clone of 5238 base pairs was found in a cDNA library from skeletal muscle. Nucleotide sequencing revealed an open reading frame of 3783 base pairs, coding for a protein of 1261 amino acid residues. The open reading frame is flanked by a 5′-untranslated sequence of 183 base pairs that contains an inframe stop codon at positions 166–168 and a 3′-untranslated sequence of 1270 base pairs that has a poly(A) tail. The calculated molecular mass of the translated product is 142 kDa, and the protein was, for reasons described below, denoted PARG1. The amino acid sequence of PARG1 is shown in Fig. 3A; the nucleotide sequence can be obtained at GenBank accession number U59020.

**Structure of the PARG1 Protein**—The amino acid sequence of PARG1 contained all peptide sequences obtained (Fig. 3A). In the deduced amino acid sequence of PARG1, the transmembrane domains and signal sequence for secretion were found, indicating that PARG1 is an intracellular protein. Three regions with homologies to other proteins could be identified: a GAP domain with similarity (23–33% amino acid sequence identity) to proteins of the RhoGAP Family (7) is found at amino acid residues 668–855; a cysteine-rich region at amino acid residues 613–657 has homology to a regulatory, phorbol ester-, diacylglycerol-, and Zn²⁺-binding domain of members of the protein.
kinase C (PKC) family (24); and a region at amino acid residues 193–509 has homology (27% identity) to the gene product of the C. elegans gene ZK669.1a (EMBL accession number Z37093).

Fig. 3B shows an alignment of the latter homology region, denoted ZPH region (for ZK667.1a-PARG homology), the function of which is unknown. Like PARG1, the gene product of ZK669.1a contains in addition to the ZPH region a cysteine-rich domain and a GAP domain (Fig. 3C).

Expression of PARG1 mRNA—Northern blot analysis of mRNA from various human tissues shows that a single PARG1 transcript of 5.5 kilobases is found in all screened tissues (Fig. 4). The expression is high in skeletal muscle and heart and moderate in placenta, liver, and pancreas. Low expression is seen in brain, lung, and kidney. The size of the transcript suggests that the obtained cDNA clone was close to full-length.

GAP Activity of PARG1—To determine the GAP activity of PARG1 on proteins of the Rho family, the GAP domain of PARG1 was produced as a GST fusion protein in E. coli (Fig. 5A). Rho, Rac, and Cdc42 were preloaded with [γ-32P]GTP and incubated for various time periods in the presence of the GST-GAP fusion protein or the presence of GST protein as a control. Thereafter, the radioactivity bound to the GTPase was determined as a measurement of the GTP hydrolysis activity. The results show that the GAP domain of PARG1 at the concentration of 1 nM had a strong GAP activity on Rho (Fig. 5B). At this concentration, no GAP activity on Rac or Cdc42 could be detected (Fig. 5, C and D). However, at a concentration of 20 nM, the GST-GAP fusion protein was also active on Rac and Cdc42 (Fig. 5, C and D). Thus, this indicates that PARG1 has a functional GAP domain that, in vitro, is active on Rho, Rac, and Cdc42 but with a clear preference for Rho. It is therefore likely that Rho is the physiological target of PARG1. The name PARG1 is consequently derived from PTPL1-associated RhoGAP.

PDZ 4 Binds to the C-terminal of PARG1—PDZ domains has recently been shown to specifically interact with the C-terminal ends of proteins containing an aliphatic residue (valine, leucine, or isoleucine) at the absolute C-terminal end (reviewed in Ref. 19). In some cases a C-terminal valine residue is critical for binding (25, 26, 36). Because PARG1 was identified through a specific interaction with PDZ 4 of PTPL1 and because it has a valine residue at the C-terminal end, we found it likely that the interaction is mediated via PDZ 4 and the C-terminal tail of PARG1. To verify this possibility, peptides corresponding to the last four, five, or six C-terminal amino acid residues of PARG1 (PQFV, IPQFV, and EIPQFV) and, as a control of specificity, a peptide with an alanine residue instead of the C-terminal va-
line residue (PQFA) were synthesized and coupled to Affi-Gel beads via their N-terminal ends. The beads were incubated with the different GST-PDZ fusion proteins and washed, and the bound material was then eluted and analyzed by immuno-blotting with an antiserum raised against GST. As shown in Fig. 6, the fusion proteins GST-PDZ 4 and GST-PDZ 4–5 but not GST fusion proteins containing PDZ 1, PDZ 2, PDZ 3, or PDZ 5 only bound to the peptide corresponding to the last four amino acid residues of PARG1. Similar results were obtained using the longer peptides (results not shown), indicating that a maximum of four amino acid residues at the C-terminal end of PARG1 is enough for a strong and specific interaction with PDZ 4 of PTPL1. Furthermore, no affinity could be detected between a peptide in which the valine residue was replaced with an alanine residue (PQFA) and PDZ 4 or any other PDZ domain of PTPL1 (results not shown), strengthening the notion that the interaction between the C-terminal end of PARG1 and PDZ 4 of PTPL1 is specific.

DISCUSSION

We have cloned a cDNA coding for PARG1 that contains a GAP domain with a strong activity on Rho in vitro. GAP activities were also detected on Rac and Cdc42; however, because these were observed only at high concentrations, it is likely that the in vitro target of PARG1 is Rho. A number of proteins have been shown to have GAP activity for Rho, including p50rhoGAP (27), Myr5 (28), and p190 (29), which are active also on Rac and Cdc42, whereas p122-RhoGAP seems to be
A cysteine-rich domain is located directly N-terminal of the GAP domain of PARG1. This domain has been identified in various proteins including most PKC isoforms (which have two copies each of the domain), the protooncogene products Vav and Raf, diacylglycerol kinase, and chimaerins (reviewed in Ref. 24). The cysteine-rich domain has been shown to bind Zn$^{2+}$ (31), and the domains found in PKCs and in chimaerins also bind phorbol esters and diacylglycerol (31, 32). Generation of diacylglycerol or addition of phorbol ester increases the affinity of PKC molecules for membranes, and the resulting translocation of PKC from the cytosol to the plasma membrane is likely to involve interactions between the cysteine-rich domains and the lipid second messenger (24, 33). The role of the cysteine-rich domain of PARG1 remains to be determined. It is possible that it mediates regulatable binding to the membrane and could possibly also be involved in regulation of the GAP activity. Interestingly, $n(a1)$-chimaerin, a Rac-specific GAP, contains a copy of the cysteine-rich domain, and it has been shown that phospholipids and phorbol esters regulate the GAP activity of $n(a1)$-chimaerin (34).

In the N-terminal part of PARG1, a region of about 300 amino acid residues with similarity (27% identity) to the gene product of the C. elegans gene ZK669.1a was identified and denoted the ZPH region. Currently, no information regarding the function of the ZK669.1a gene product is available, and the function of the ZPH region is unknown. The overall domain structure of the ZK669.1a gene product is similar to PARG1, and it is possible that PARG1 is the human homolog of the C. elegans ZK669.1a gene product. However, the RhoGAP domain and the cysteine-rich domain of the ZK669.1a gene product is not significantly more similar to PARG1 (29% identity within the RhoGAP domains and 24% identity within the cysteine-rich domains) compared with other human proteins containing these domains (24–31% identity within the RhoGAP domains and 16–27% identity within the cysteine-rich domains).

PDZ domains have been identified in a diverse set of proteins (18). These proteins seem to be involved in signal transduction, and many of them, if not all, are found in structures at the plasma membrane. The size of the PDZ domain of about 90 amino acid residues, and its appearance in signal transduction specific for Rho (30).

![FIG. 4. Expression of PARG1 mRNA in different human tissues. A multiple human tissue Northern blot (CLONTECH) was hybridized with a PARG1 cDNA-specific probe. Each lane contained 2 μg of polyadenylated RNA from the indicated tissues.](image-url)
proteins suggested that it, like SH2 and SH3 domains, might mediate direct interactions with other molecules. We have shown that PARG1 binds specifically to PDZ 4 of PTPL1 in vitro and that a binding site for PDZ 4 resides in the four most C-terminal amino acid residues of PARG1. When the intracellular part of Fas was used as a bait in a screening for interacting proteins using a two-hybrid system in yeast, it was shown that PDZ 2 of PTPL1 interacted with the 15 most C-terminal amino acid residues of Fas (Ref. 13; PDZ 2 is in this publication that PDZ 2 of PTPL1 interacted with the 15 most C-terminal ing proteins using a two-hybrid system in yeast, it was shown that a binding site for PDZ 4 resides in the four most C-terminal amino acid residues of PARG1. When the intracellular part of Fas was used as a bait in a screening for interact-

This is the first example of an interaction between a PTP and PARG1 in Rho signal transduction are highly warranted.

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