An Eps Homology (EH) Domain Protein That Binds to the Ral-GTPase Target, RalBP1*

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Ral proteins constitute a family of small GTPases that can be activated by Ras in cells. In the GTP-bound state, Ral proteins bind to RalBP1, a GTPase-activating protein for CDC42 and Rac GTPases. We have used the two-hybrid system in yeast to clone a cDNA for a novel 85-kDa protein that can bind to an additional site on RalBP1. This newly identified protein contains an Eps homology (EH) domain, which was first detected in the epidermal growth factor (EGF) receptor substrate Eps15. Recently, the EH domain of Eps15 has been shown to bind to proteins containing an asparagine-proline-phenylalanine motif. Moreover, EH domains have been found in proteins involved in endocytosis and/or actin cytoskeleton regulation. The RalBP1-associated Eps-homology domain protein, Reps1, is tyrosine-phosphorylated in response to EGF stimulation of cells. In addition, Reps1 has the capacity to form a complex with the SH3 domains of the adapter proteins Crk and Grb2, which may link Reps1 to an EGF-responsive tyrosine kinase. Thus, Reps1 may coordinate the cellular actions of activated EGF receptors and Ral-GTPases.

The Ral-GTPases, RalA and RalB, form a family of Ras-related GTP-binding proteins (1). Like all GTPases, Ral proteins cycle between the active GTP-bound and inactive GDP-bound states. Activation of Ral proteins occurs upon interaction with a Ras-specific guanine nucleotide exchange factor (for review, see Ref. 2). This promotes the release of GDP from Ral, allowing activating GTP to take its place. Deactivation takes place when bound GTP is hydrolyzed back to GDP upon interaction of Ral with a specific GTPase-activating protein,Ral-GAP (3). It is likely that Ral proteins act as molecular switches that become active in response to specific upstream signals and then alter the activities of specific downstream target proteins. A series of recent findings support the idea that Ras proteins are important upstream activators of Ral proteins. In particular, it has been shown that active GTP-bound Ras binds to (4–6) and activates (7) Ras-specific guanine nucleotide exchange factors, which then activate Ral proteins in cells. Thus, activation of Ral-GTPases appears to constitute a distinct downstream signaling pathway from Ras (for review, see Ref. 2). Studies are beginning to reveal how Ras proteins contribute to Ras signaling. Overexpression of Ral-GDS (8) or constitutively active Ral (7) can enhance cellular transformation induced by Ras, and expression of a dominant negative form of Ral can suppress Ras-induced transformation (7). This phenomenon may occur, in part, by a contribution of Ral to fos gene activation (9). Two signaling molecules have been identified that may be affected by Ral protein activity. The first is phospholipase D (PLD), which converts phosphatidylcholine to phosphatidic acid. Phosphatidic acid is itself biologically active, but can also be rapidly converted to diacylglycerol, which can then activate protein kinase C isoforms. It is thought that PLD1 is constitutively associated with the N terminus of Ral proteins (10, 11). Upon Ral activation by Ras, PLD appears to be targeted to a specific signaling complex where its catalytic activity may be elevated by Arf or Rho family GTPases (for review, see Ref. 12).

A second target of Ral may be RalBP1 (also called RLIP or RLP) (13–15). This protein was identified by its ability to bind specifically to active GTP-bound Ral. RalBP1 has been shown to be a GAP for CDC42 and Rac, Ras-related GTPases that influence the actin cytoskeleton and the Jun kinase signal transduction pathway (16). In this paper, we identify another potential binding partner for RalBP1. This protein has an Eps homology (EH) domain and becomes tyrosine-phosphorylated in response to EGF signaling. Thus, RalBP1 associated Eps domain-containing protein (Reps1) may mediate an additional function of RalBP1, and therefore Ral, in cells.

EXPERIMENTAL PROCEDURES

Cloning of Reps1—For two-hybrid screening, RalBP1 cDNA was subcloned as a fusion protein with the DNA binding domain of Gal4, into pAS-CY2 (17). A mouse cDNA library, derived from a differentiated C2C12 muscle cell line and expressed as fusion proteins with the activation domain of Gal4 in pACT, was kindly provided by Dr. Amy Yee (Tufts Medical School) (18). The Saccharomyces cerevisiae Y190 (MATa gal4 gal80 his3 trp1–901 ade2–101 leu2–3, 112 URA3:: GAL-lacZ LYS2::GAL-HIS3, cby1) strain, expressing a GAL4 DNA-binding domain/RalBP1 fusion protein, was transformed with the pACT library, and 2.3 × 106 primary transformants were selected for growth on medium lacking leucine, tryptophan, and histidine and containing 30 mM 3-aminotriazole. The plates were incubated at 30 °C for 3 days. Surviving yeast colonies were transferred to nitrocellulose and laid onto minimal media plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside and screened for expression of β-galactosidase, by incubation of the plates at 30 °C for 1 day. The positive clones were further analyzed by colony hybridization and sequencing. The plasmid insert from the positive clones was subcloned into pGEM (Promega) and sequenced by aENSEQ cycle sequencing kit. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) AFO21929.

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at 30 °C for 1 to 2 days. Blue coloration of a colony was indicative of a positive interaction. His+ LacZ+ colonies were rescued from the plate and grown in selective media. Plasmid DNA was recovered and introduced by electroporation into the leucine deficient Escherichia coli strain, KC8. Transformants were plated on minimal media lacking leucine but only transformants containing the library plasmid were identified.

Positives were tested for target specificity by re-transformation into the reporter strain Y190 alone, in conjunction with the RalBP1-Gal4 DNA-binding domain fusion, or with different Gal4 DNA-binding domain fusions. Only library plasmids that did not activate marker expression on their own or in the presence of a negative control construct,冈SNF1, were analyzed further.

Additional mouse Reps cDNAs were isolated from an oligo(dT) and randomly primed mouse testis library (CLONTECH) by DNA hybridization, using the ~500-base pair Reps1 clone isolated from the two-hybrid system as a probe. The 5’-end of the cDNA (last 5 codons plus 5’-untranslated region) was then cloned by 5’-RACE (rapid amplification of cDNA ends), using a mouse brain cDNA library as template and terminal deoxynucleotidyltransferase to create poly(C) 5’-ends for primer annealing with a poly(G) primer (containing an EcoRI site at the 5’-end). The entire coding sequence for Reps1 was cloned into pBSK. The sequence of Reps1 cDNA was determined on both strands by the dideoxy chain termination technique, using multiple cDNA clones and subclones.

Plasmid Construction—GST-Reps1 (599–743) was generated by subcloning the partial cDNA isolated from the two-hybrid system into pGEX3. Full-length RalBP1 and fragments of it were expressed with N-terminal Myc epitope tags by cloning RalBP1 cDNAs into pJ3M. Full-length RalBP1 was cloned as a BamHI–KpnI fragment. RalBP1 (aa 1–500) was cloned as a BamHI–EcoRI fragment, while RalBP1 (aa 200–647) was cloned as a BglII fragment. Glu-tagged RalBP1 construction was described previously (131). Myc-tagged RalBP1 was generated by cloning full-length Reps from pBSK into pJ3M as an EcoRI fragment.

Cell Culture and Transient Expression—COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% iron-enriched calf serum (HyClone) at 37 °C in 5% CO₂. Cells were plated at a density of ~5 × 10⁵ cells/60-mm plate one day before transfection. pM Reps (19) or pJ3M, containing a Myc epitope tag, were used for the transient expression of the cDNAs in COS-7 cells using the DEAE-dextran method.

Binding Assays—For in vitro binding assays between Reps1 and RalBP1, 1–3 μg of GST-Reps protein bound to 25 μl of glutathione beads were incubated with 500 μl of a COS7 cell lysate derived from one 10-cm dish of semiconfluent cells. The cells had been transiently transfected with Myc-RalBP1 or segments of RalBP1 containing a Myc epitope tag and then lysed in 20 mM Tris-HCl (pH 7.5), containing 10 mM MgCl₂, 20 mM NaCl, 0.5% Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin. After washing beads in binding buffer, the precipitates were run on SDS-PAGE and immunoblotted with anti-Myc antibodies and then incubated with horseradish-conjugated secondary antibodies. Blots were visualized as described above. Cell lysates were also run directly on gels and immunoblotted with either anti-Myc or anti-Glu antibodies to verify Reps1 and RalBP1 expression. Blots were visualized as described above.

RESULTS

Cloning of Reps1 cDNA—To identify new functions for RalBP1, the protein was used as bait in the yeast two-hybrid system. A yeast reporter strain, expressing a RalBP1/GAL4 DNA-binding domain fusion, was transformed with a mouse muscle cell cDNA library expressed as fusions with the GAL4 activation domain (17). From 10⁶ primary transformants, 15 survived the initial histidine selection and only one was also positive for the secondary screen (expression of β-galactosidase) and the tertiary screen (binding to RalBP1 in vitro (see below)). This ~500-base pair clone was used as a probe to clone additional cDNA sequences by hybridization with a λGT10 cDNA library. Finally, the N-terminal end of the cDNA was cloned by 5’-RACE. One continuous open reading frame was detected that encodes a 743 amino acid protein (Fig. 1A). The cDNA clone was used as a probe in a Northern blot of RNA isolated from a variety of rat tissues. One major band, with a size of ~3.5 kilobases, was observed in all tissues examined (data not shown). The highest level of expression was found in kidney and testis.

The amino acid sequence encoded by the cDNA was then compared with known proteins. Although this protein was novel, significant sequence similarity (between 38 and 50%
Connection between Ral and an EH Domain Protein

Fig. 2. Binding of Reps1 to RalBP1 in vitro and in vivo. A. In vitro. Glutathione beads containing a fusion protein of GST (middle column) or a GST fusion with C-terminal amino acids 599–743 of Reps1 (right column) were incubated with lysates of COS7 cells expressing full-length Myc-tagged RalBP1, the first 500 amino acids of RalBP1, containing the GAP and Ral-binding domains, and the final 446 amino acids of RalBP1 containing the Ral binding site and the C-terminal region including a predicted coiled-coil. The beads were then washed, run on SDS-PAGE, and blotted with anti-Myc antibodies. Molecular weight markers are shown on the left of the figure. The various fragments of RalBP1 in the original cell lysates are shown in the left column. B. In vivo. Myc-Reps1 was transfected along with Glu-RalBP1 into COS7 cells. RalBP1 was then immunoprecipitated with anti-Glu antibodies and run on SDS gels. Then, associated RalBP1 was detected in immunoblots using anti-Myc antibodies. The lanes on the left show expression of proteins in the original lysates, and the lanes on the right show anti-Myc immunoprecipitates from duplicate dishes blotted with anti-Myc antibodies. The lower band in the samples is a non-specific band present in all of the immunoprecipitates.

Identification (identity) was found between amino acids 237 and 298 and the EH domains present in a variety of proteins (20). These include the human Eps15 and Eps15-related (Eps15r) proteins, which each contain three EH domains, and S. cerevisiae Pan1 and End3 proteins, which contain two and one EH domains, respectively (Fig. 1, A and B). A putative EF hand resides within many EH domains (see Fig. 1B), suggesting that they are calcium-binding motifs. Recently it has been shown that the EH domains of Eps15 and Eps15R constitute protein-protein interaction modules that bind to segments of proteins containing the amino acid sequence asparagine-proline-phenylalanine (NPF) (21). Thus, one function of Reps may be to interact with a specific protein containing this motif. Based on the possible importance of the EH domain, we have called this RalBP1-binding protein, RalBP1-associated Eps homology protein 1 or Reps1. A linear map containing putative functional domains of Reps1 is shown in Fig. 1C.

Binding of Reps1 to RalBP1 in Vitro—A fusion protein containing glutathione S-transferase (GST) and the coding sequence of the partial Reps1 cDNA clone first isolated from the yeast two-hybrid system (C-terminal amino acids 599–743) was produced in E. coli. After affinity purification on glutathione beads, the immobilized Reps1 protein was incubated with COS cell lysates transfected with various portions of RalBP1, each containing a Myc epitope at its N terminus. After washing the beads, the bound proteins were fractionated by SDS-PAGE and immunoblotted with anti-Myc antibodies (Fig. 2A). Binding in vitro was observed between the C terminus of Reps1 and full-length RalBP1, but not the N-terminal 500 amino acids of RalBP1, which contains a basic α-helix, a CDC42/Rac GAP domain, and a Ral binding site (see Fig. 1C). Binding was also observed between Reps1 and a C-terminal portion (aa 201–647) of RalBP1 containing the Ral binding site and an extended region of predicted α-helix. Thus, Reps1 binds to a region in the C-terminal region of RalBP1 that is distinct from the binding sites for Ral and CDC42/Rac (see Fig. 1C). The C-terminal 43 amino acids of Reps1 (aa 700–743) are predicted to exist as a coiled-coil (Fig. 1, A and C). A GST fusion construct containing these sequences failed to bind to RalBP1 in vitro (data not shown). Thus, although the coiled-coil may participate in binding to RalBP1, it is not sufficient for binding activity.

Binding of Reps1 to RalBP1 in Transiently Transfected COS7 Cells—Myc epitope-tagged Reps and Glu epitope-tagged RalBP1 were transfected either alone or together into COS7 cells. Cell lysates were prepared 24 h later, and RalBP1 was immunoprecipitated with anti-Glu antibodies. The immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with anti-Myc antibodies (Fig. 2B). Reps1 protein was clearly detected in RalBP1 immunoprecipitates only when the two proteins were co-expressed. The panels on the left show immunoblots of cell lysates showing that Reps1 and RalBP1 were expressed when their encoded DNAs were transfected. These results document that Reps1 and RalBP1 can form stable complexes in cells.

Tyrosine Phosphorylation of Reps1 Induced by EGF—Eps15 was originally detected because it became tyrosine-phosphorylated in response to EGF. Therefore we tested whether Reps also becomes tyrosine-phosphorylated in response to EGF. COS7 cells were transiently transfected with Myc-Reps1 and then serum starved for 24 h. Duplicate dishes of starved cells were treated with buffer or buffer containing 10 ng/ml EGF for 8 min. Lysates of cells were either run directly on SDS gels and blotted with anti-Myc antibodies or immunoprecipitated with anti-Myc antibodies (Fig. 3A, left) or immunoprecipitated with anti-Myc antibodies and immunoblotted with anti-Tyr(P) antibodies. Fig. 3A (right panel) shows an increase in tyrosine-phosphorylated Reps1 in response to EGF stimulation. This tyrosine phosphorylation may occur just proximal to the EH domain, since a single tyrosine phosphorylation consensus site was detected in Reps1 in sequences surrounding tyrosine 236 (see Fig. 1).

Interaction of Reps1 with SH3 Domain Proteins—Like Eps15, Reps1 also contains polyproline segments, which is suggestive of SH3 binding activity (See Fig. 1A). One motif between amino acids 531 and 540 contains the sequence P-X-P-P-R, which matches the consensus sequence P-X-L/P-X-K found in all proteins known to interact with the SH3 domain of the Crk adapter protein (22). A similar motif is capable of binding Grb2. Thus, SH3 binding activity of Reps1 was tested by incubating lysates of cells containing Myc-Reps1 with Sepharose beads containing fusion proteins of GST and the SH3 domains of Crk, Grb2 (N- and C-terminal domains tested individually), Src, and Abl. The beads were washed, run on
SDS gels, and blotted with anti-Myc antibodies to detect bound Reps1. Fig. 3B shows complexes between Reps1 and GST-Crk(SH3) and GST-Grb2(N-SH3), but not GST-Grb2(C-SH3), GST-Src(SH3), GST-Abl(SH3), or GST alone. These results suggest that Reps1 may form a complex directly with the EGF receptor through the adapter proteins Crk or Grb2. However, we have been unable to detect such a complex in this cell system (data not shown).

DISCUSSION

A growing body of evidence supports the idea that Ras proteins activate a distinct downstream signaling pathway by binding to and activating Ras-specific nucleotide exchange factors. These findings have stimulated interest in defining the function of Ras GTPases. One potential effector of Ras proteins is RalBP1, a 73-kDa protein that binds specifically to the GTP-bound state of Ras proteins (13–15). In addition to a Ras binding site, RalBP1 contains a GAP domain that inactivates CDC42 and Rac GTPases.

Based on its ability to bind to RalBP1, Reps1 is another potential mediator of Ras function. In vitro data presented here argue that Reps binds to a region of RalBP1 that is distinct from the binding sites for Ras and CDC42/Rac. Moreover, transfection studies show that Reps and RalBP1 can interact in vivo, although this remains to be documented for their endogenous cellular counterparts. A clue to the function of Reps1 comes from the presence of an EH domain. EH domains were first detected in Eps15 (23), which contains three tandem EH motifs of ~70 amino acids at its N terminus (see Fig. 1B). Recent experiments using peptide libraries and expression cloning have revealed that the EH domains of Eps15 and Eps15r function as binding motifs for proteins that contain the amino acid sequence asparagine-proline-phenylalanine (NPF) (21). Like other protein-interacting domains, the amino acids neighboring these core sequences were also found to be important and may contribute to binding specificity.

Biochemical analysis strongly suggests that Eps15 is involved in receptor-mediated endocytosis in mammalian cells. Eps15 has been detected in the clathrin-coated vesicles, where it is constitutively associated with α-adaptin (24–26). Eps15 is also complexed with the light chain of clathrin (24). Finally, Eps15 can bind to the SH3 domain of the Crk adapter molecule, which may promote its association with activated EGF receptors (22, 27). These findings have led to the hypothesis that Eps15 promotes the association of EGF receptor with newly forming coated vesicles, an early step in the internalization and down-regulation of the receptor.

Support for the idea that EH domains, in particular, participate in endocytosis comes from studies on the EH domain yeast proteins End3 and Pan1. S. cerevisiae expressing mutant End3 or Pan1 proteins display defects in both receptor-mediated and fluid phase endocytosis (28, 29). In addition, evidence points to a role of these EH domain proteins in cortical actin cytoskeleton-related processes (28, 29). For example, altering the activity of Pan1 results in an abnormal distribution of the actin cytoskeleton.

The presence of an EH domain in Reps1 suggests this protein may also influence endocytosis and/or the actin cytoskeleton. Interestingly, Eps15 and Reps1 have additional features in common. Like Eps15, Reps1 becomes tyrosine-phosphorylated in response to EGF stimulation. Although the site of tyrosine phosphorylation has not been mapped for either protein, both have a single consensus tyrosine phosphorylation immediately N-terminal to an EH domain. Both proteins also have the capacity to bind to the SH3 domain of the adapter protein Crk. This may allow Reps1 to form a complex with the EGF receptor. However, we have not been able to detect such a complex in the cell system we have used. For Eps15, a complex with the EGF receptor has been detected only in cells overexpressing the EGF receptor (24). Finally, both Reps and Eps15 have a coiled-coil region. In Eps15 this region is in the middle of the protein, and appears to promote homo-oligomerization (30). In Reps1, the coiled-coil is at the extreme C terminus. It cannot bind RalBP1 on its own, raising the possibility that Reps1 may also homodimerize.

A major distinction between Reps1 and Eps15 is that the C terminus of the latter contains repeats of aspartate, proline, and phenylalanine, which are responsible for the constitutive association of Eps15 with clathrin-coated vesicles (31). In contrast, Reps1 contains a RalBP1 binding site at a comparable region of the protein. Presumably, this leads to the regulated localization of Reps1 to a site in the cell where Ral exists. Ral proteins have been detected in plasma membrane fractions, where they are thought to become activated by Ras-induced activation of Ral-specific nucleotide exchange factors. However, a majority of Ral proteins are found in intracellular vesicles, including clathrin coated vesicles as well as exocytic vesicles (32, 33). Thus, Reps1 may function at the cell surface or in vesicles.

What does this imply about the function of Reps? Based on the information gained from experiments reported here, and previous work on Ral GTPases, a model of potential protein-protein interactions involving Reps1 is shown in Fig. 4. EGF receptor may influence Reps1 in two ways. First, EGF receptor activation leads to the tyrosine phosphorylation of Reps1. Like Eps15, this tyrosine phosphorylation may be promoted by an adapter protein that connects the kinase to its substrate. Moreover, the site of tyrosine phosphorylation on both proteins may be directly proximal to the EH domain. Thus, both proteins tyrosine phosphorylation may influence EH domain function directly by altering its activity or indirectly by producing a docking site for an SH2 domain protein that can interact with the EH domain.

EGF receptor stimulation also leads to the activation of Ras, which can activate Ral and lead to the association of Ral with RalBP1. In this way, a signaling complex connecting EGF receptor, Ral, RalBP1 and tyrosine-phosphorylated Reps1 may be formed (see Fig. 4). Since Ral has been shown to be

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constitutively associated with PLD1 (10, 11), this enzyme may also be part of this signaling complex. Interestingly, the known biochemical activities of these signaling molecules are consistent with their participation, along with the EH domain of Reps1, in the regulation of endocytosis and/or the cytoskeleton.

For example, RalBP1 is known to be a GAP for CDC42 and Rac. Thus, it has the capacity to inhibit these two actin-regulating GTPases. Since Rac has also been shown to negatively influence on this process. Furthermore, a growing body of evidence supports a role for phospholipase D in the promotion of vesicle budding, possibly through its ability to enhance the production of phosphatidic acid (35–37).

Future studies will attempt to identify proteins that interact with the Reps1 EH domain. Based on studies on the EH domain of Eps15, these proteins will contain NPF motifs. Their biochemical activities may yield insight into the function of both EGF receptor and Ral GTPase signaling mechanisms.

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