Communication

PRK1 Is Targeted to Endosomes by the Small GTPase, RhoB*

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The Rho family of GTPases play a key role in mediating cell motility and adhesion through dynamic regulation of the actin cytoskeleton. In addition, members of this family have other diverse roles, including signaling to stress-activated MAP kinase modules, control of membrane traffic, and regulation of the generation of lipid second messengers (1, 2). The down-stream signaling pathways from Rho GTPases are largely un-}

RhoB has been shown to be an endosomal GTPase both by immunocytochemistry and electron microscopy, however, its role in endocytosis is unknown. Elucidation of the cellular roles of other members of this superfamily of signaling proteins has come with the identification of their downstream partners. We show here that the recently isolated serine/threonine kinase PRK1 is targeted to the endosomal compartment by RhoB. This is established both through immunofluorescence and cell fractionation. PRK1 is shown to interact with activated RhoB in cells and is localized to endosomes through its Rho-binding HR1 domain. Translocation of PRK1 to the endosomal compartment by RhoB is accompanied by a shift in the electrophoretic mobility of the kinase indicative of an accompanying activation.

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EXPERIMENTAL PROCEDURES

Materials

Goat Texas Red-X and BODIPY FL-conjugated secondary antibodies were from Molecular Probes, Inc. Goat polyclonal anti-RhoA (119), goat polyclonal anti-RhoB (119), and rabbit polyclonal anti-HA²-probe (Y-11) antibodies were from Santa Cruz Biotechnology, Inc.

Methods

Plasmid Constructs—Mammalian expression vector constructs were generated in pcDNA3 (Invitrogen) using standard methods. Truncated PRK1 constructs PTS-3 and PTS-6 comprised amino acids 294–943 and 1–307 of PRK1, respectively, and contained a C-terminal HA-epitope tag. A GTPase-deficient RhoB construct, RhoB-QL, was engineered by using oligonucleotide-based site-directed mutagenesis with the Chameleon System (Stratagene), according to the manufacturer’s instructions, to change Gin-63 to Leu. A dominant negative RhoB construct, RhoB-TX, was made by using the same methodology to change Thr-19 to Asn. All constructs were confirmed by sequencing.

Immunofluorescence—Swiss 3T3 cells were microinjected with mammalian expression vectors and prepared for indirect immunofluorescence as described previously (5). 293 cells were transfected using standard calcium phosphate-mediated transfer and were processed for immunofluorescence 36 h later. The cells were fixed for 15 min in 4% fresh paraformaldehyde in PBS and then washed with PBS and permeabilized in 0.2% Triton X-100 in PBS for 5 min. The cells were washed again in PBS and then incubated for 10 min in 0.1% sodium borohydride in PBS. The cells were then washed twice with PBS, once with PBS containing 1% bovine serum albumin (BSA), and then incubated for 1 h with the primary antibody diluted 1:200 in PBS with 1% BSA. The cells were then washed three times with PBS and incubated for 1 h with the secondary antibody diluted 1:200 in PBS. The cells were then washed three times in PBS and mounted under MOWIOL 4–88 (Calbiochem) containing 0.6% 1,4-diazabicyclo[2.2.2]octane as an antiphotobleaching agent. The cells were viewed using a Zeiss Axioskop fluorescence microscope, and the images were captured by a cooled charge-coupled device (Photometrics). The captured images were processed using the Spectrum software package (IP Laboratories).

Immunoprecipitation—293 cells were co-transfected with PRK1 and expression vectors containing various myc epitope-tagged Rho protein constructs. Cells were harvested 36 h after transfection, in cell lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 20 mM NaF, 1% (v/v) Triton X-100, 1 mM dithiothreitol, 100 μM sodium vanadate, 100 mM okadaic acid, 10 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride) and precleared by incubation with 0.1% (v/v) insoluble protein A for 20 min. The extracts were centrifuged at 12,000 × g for 5 min at 4 °C, the supernatants were taken and incubated with 4 μg of 9E10 antibody each for 20 min, tumbling at 4 °C. The extracts were then incubated for a further 1 h with 20-μl packed volume of protein G-Sepharose, tumbling at 4 °C. The Sepharose beads were collected by centrifugation at 12,000 × g for 3 min at 4 °C, washed three times in 1 ml of cell lysis buffer, and then the bound protein was solubilized in SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting.

Cell Fractionation—Endosomal fractions were prepared from 293 cells by sedimentation on discontinuous sucrose gradients as described previously (6). Fractions (500 μl) were removed from the top of the gradient and diluted 5-fold with ice-cold harvesting buffer (20 mM imidazole, pH 7.4, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 100 nM okadaic acid). The membranes were then pelleted by centrifugation at 12,000 × g for 30 min at 4 °C. For gel

* The abbreviations used are: HA, hemagglutinin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.
electrophoresis the membranes were then solubilized in SDS-PAGE loading buffer. The distribution of endosomal membranes on the gradient was determined by incubating cells prior to harvest in medium containing 1 mg/ml horseradish peroxidase for 5 min, followed by a chase in horseradish peroxidase-free media. Chase times were 0 min for early endosomes, 25 min for late endosomes, and 110 min for lysosomes. Sucrose gradient fractions were solubilized in 1 ml of 10 mM Hepes, pH 7.2, 1% (v/v) Triton X-100 and assayed for horseradish peroxidase activity using o-dianisidine as a substrate in a spectrophotometric assay.

RESULTS AND DISCUSSION

PRK1 has been shown to bind activated RhoA and is therefore a candidate RhoA effector. We sought to define a possible role for PRK1 in RhoA signaling through overexpression studies. Overexpression of PRK1 caused no stimulation of two well established RhoA pathways, the formation of actin stress fibers or the activation of the serum response element (data not shown). However, these studies revealed a distinctive subcellular localization pattern of PRK1. When expressed in Swiss 3T3 cells, PRK1 showed a diffuse cytoplasmic staining pattern in most cells (Fig. 1A). However, at later times following microinjection, a subset (approximately 5–10%) of cells showed a punctate staining pattern, suggestive of a vesicular localization (Fig. 1B). RhoA is found largely in the cytoplasm with a small amount of protein present at the plasma membrane (7). In contrast the RhoB GTPase has been shown to be localized exclusively to the endosomal compartment (7). The punctate pattern of PRK1 staining raised the possibility of a functional coupling of PRK1 and RhoB. This was investigated in HEK 293 cells, chosen in part for their low levels of endogenous RhoB in comparison with Swiss 3T3 cells (data not shown). PRK1 overexpressed in 293 cells shows a diffuse cytoplasmic staining pattern with no apparent vesicular localization (Fig. 2A). However, when co-expressed with RhoB, the PRK1 was translocated to the RhoB compartment, indicating that RhoB does indeed target PRK1 to endosomes (Fig. 2, C and D). In agreement with previous studies in other cell types, overexpressed RhoA was largely cytosolic in HEK 293 cells. Co-expression of RhoA with PRK1 did not result in the localization of either protein to endosomes or any other membrane compartment (data not shown).

PRK1 interacts with RhoA in vitro through its N-terminal HR1 domain (8). We examined whether this domain also mediates the targeting of PRK1 to endosomes by RhoB. The PRK1 HR1 domain expressed on its own showed a broad cytoplasmic distribution (Fig. 3A). Its overexpression caused an increase in the number of multinucleate cells in the transfected population. This suggests that this construct had a dominant-negative effect on Rho activity, since inhibition of Rho function has been shown to block cell division (9). Co-expression of the HR1 domain with RhoB caused a translocation of the HR1 polypeptide to the endosomal compartment (Fig. 3, B and C). Moreover, a PRK1 truncation mutant which lacks the HR1 domain failed to localize to endosomes with RhoB (Fig. 3, E and F). Taken together, these data show that the HR1 domain is both necessary and sufficient for the targeting of PRK1 to endosomes by RhoB.

We examined the cellular interaction of RhoB and PRK1 further using mutant Rho proteins. The Rho proteins were co-expressed in 293 cells with PRK1 and then isolated by immunoprecipitation. The presence of any associated PRK1 was detected by Western blotting. The activated mutants of RhoA and RhoB were seen to form an association with PRK1 in cells that was stable to extraction (Fig. 4). Although there was some variation in the level of expression of the various Rho proteins in cells, the interaction with RhoA appeared to be qualitatively similar to that with RhoB. PRK1 fulfilled the criteria required for a RhoB effector in that it formed a stable association with the activated RhoB-QL mutant but not with the wild-type protein or with the dominant-negative RhoB-TN mutant (Fig. 4).

The failure of PRK1 to co-immunoprecipitate with wild-type RhoB would seem to be at variance with translocation of PRK1 to endosomes by wild-type RhoB observed by immunofluorescence. We have shown recently that PRK1 makes two interactions with the RhoA GTPase: a weak interaction through the PRK1 HR1b motif, which is not dependent on GTP-loading of RhoA, and a strong interaction through the PRK1 HR1a motif, which requires activated (GTP-loaded) RhoA (10). It would seem likely that a similar situation occurs with RhoB and that a weak interaction with wild-type RhoB is sufficient to target PRK1 to endosomes. Indeed, the activated RhoB-QL mutant is no better than the wild-type protein at translocating PRK1 to endosomes as judged by immunofluorescence (data not shown). The weak interaction would then survive the procedures in-

![Image 1](https://example.com/image1.jpg)

![Image 2](https://example.com/image2.jpg)

**FIG. 1.** Cellular distribution of PRK1 in Swiss 3T3 cells. PRK1 expression vector was introduced into Swiss 3T3 cells by nuclear microinjection. Cells were fixed approximately 2 h (A) or 3 h (B) after injection and processed for immunofluorescence using a rabbit polyclonal anti-PRK1 antibody.

**FIG. 2.** Co-localization of PRK1 and RhoB by fluorescence microscopy. 293 cells were transfected with PRK1 (A), RhoB (B), PRK1 and RhoB (C and D). C and D show the same field of cells double-stained for PRK1 (anti-PRK1 polyclonal, red) and RhoB (9E10 anti-Myc epitope monoclonal, green). Bar represents 10 μm.
Endosomal Targeting of PRK1

Fig. 3. Localization of PRK1 mutants. PRK1 truncation mutants and RhoB were expressed in 293 cells. A–C, cells were transfected with the HR1 domain construct, PTS-6. D–F, cells were transfected with the HR1-deleted construct, PTS-3. A, PTS-6 alone; B, PTS-6 with RhoB stained for PTS-6; C, B stained for RhoB; D, PTS-3 alone; E, PTS-3 with RhoB stained for PTS-3; F, E stained for RhoB. PRK1 truncation mutants were detected with polyclonal anti-HA epitope antibody. RhoB was detected with monoclonal anti-Myc epitope antibody 9E10. Bar represents 10 μm.

Fig. 4. Co-immunoprecipitation of PRK1 by Rho proteins. Myc-epitope-tagged Rho protein constructs and PRK1 were co-expressed in 293 cells. Cells were harvested 36 h after transfection, and the Rho proteins were immunoprecipitated with 9E10 antibody as described under “Experimental Procedures.” The immunoprecipitated proteins were solubilized in SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting for PRK1 (A) and Rho (B). PRK1 was co-expressed with: RhoA-QL (lane 1), RhoB (lane 2), RhoB-TN (lane 3), and RhoB-QL (lane 4). In B there is a band derived from the 9E10 IgG, which reacts with the second antibody and migrates close to the position of RhoA-QL. This is indicated to the right of the figure. C shows a Western blot for PRK1 of samples taken from the cell extracts prior to immunoprecipitation as a control for equivalent PRK1 expression in the samples.

Figs. 5A and 5B. Membrane fractions from the gradient were pelleted, washed, and analyzed by SDS-PAGE and Western blotting for PRK1 (A, C) and RhoB (B, D). A and C show equivalent exposures (15 min). As a qualitative indication of the level of RhoB overexpression, B shows a 15-min exposure, whereas D was exposed for approximately 1 s.

PRK1 was distributed in two peaks on the gradient (Fig. 5A), one of which was in the endosomal fraction coinciding with the distribution of endogenous RhoB. This suggests that the targeting of PRK1 to endosomes by RhoB is not simply a consequence of overexpression of these proteins. PRK1 protein was also seen in the heavier (40.6% sucrose) fractions. These fractions were of particular interest as the PRK1 showed the presence of additional, slower migrating forms on SDS-PAGE. PRK1 has been shown to become heavily phosphorylated upon activation (11), and these slower migrating species appear to correspond to hyperphosphorylated forms of the enzyme. The slowest migrating form of PRK1 was present in Fraction 10 from the gradient, which contains insoluble cytoskeletal proteins. PRK1 has been shown to interact with the intermediate filament protein, vimentin (12). We therefore determined the distribution of vimentin in the sucrose gradient fractions. Interestingly, vimentin was seen to be concentrated in Fraction 10 (data not shown), suggesting the possibility that the PRK1 associated with this intermediate filament component in vivo may be hyperphosphorylated.

Finally, we determined what effect overexpression of RhoB involved in preparing cells for immunofluorescence (which involves a rapid fixation step), but not the more stringent immunoprecipitation protocol where only the strong, GTP-dependent interaction is observed.

Overexpression studies can sometimes yield anomalous results. As the RhoB and PRK1 antibodies are not good enough to detect endogenous proteins by immunofluorescence, we determined the subcellular localization of endogenous PRK1 and RhoB in 293 cells by cell fractionation. Crude cell membranes were purified by centrifugation on a discontinuous sucrose gradient, optimized for the isolation of endosomes (6). Endosomes were isolated as a single peak on these gradients (Fraction 5) as determined by quantifying the distribution of endocytosed horseradish peroxidase marker (data not shown). By following a time course of horseradish peroxidase uptake, it was seen that Fraction 5 contained both early and late endosomes (data not shown). The gradient fractions were analyzed for distribution of endogenous RhoB and PRK1. Although endogenous RhoB could not be detected by Western blotting in crude 293 cell lysates, it could be readily detected after enrichment by sucrose gradient fractionation, and it was seen to be present solely in the endosomal fraction (Fig. 5B). Endogenous RhoB was detected by sucrose gradient fractionation, and it was seen to be present solely in the endosomal fraction (Fig. 5B). Endogenous RhoB

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would have on the localization of endogenous PRK1. Overexpression of RhoB caused a highly reproducible increase in the amount of endogenous PRK1 associated with the endosome fraction (Fig. 5C). Interestingly, the endogenous PRK1 that was recruited to this compartment appeared to be composed of a single, slower migrating form of the kinase on SDS-PAGE. This suggests that activation of PRK1 occurs on translocation. Similar results were observed with the overexpression of the constitutively active RhoB-QL mutant (data not shown). The efficiency of transfection of the 293 cells in these experiments was estimated to be approximately 20%. Although the level of RhoB expression in these cells is very high, the experiment samples the whole cell population, so the translocation of endogenous PRK1 is only about 20% of that which would be observed if 100% of the cells were transfected.

There is increasing evidence for an involvement of Rho GTPases in endocytosis (2). Although RhoB has been shown to be restricted in its localization to endosomes, both by immunofluorescence (7) and electron microscopy (13), its function in cell trafficking in unknown. The elucidation of the cellular function of other Rho family members has come with the identification of downstream effectors. Presently, the signaling pathways downstream from RhoB are totally obscure. In this study we have shown that PRK1 interacts with activated RhoB through its HR1 domain and is targeted to the endosomes. This translocation leads to a decreased mobility of PRK1 on SDS-PAGE, indicating phosphorylation and activation of the PRK1 kinase. Taken together, these data suggest that PRK1 is the downstream effector of RhoB and executes Rho-mediated control of endocytosis.

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