

Direct interaction of insulin-like growth factor-1 receptor with leukemia-associated RhoGEF

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Insulin-like growth factor (IGF)-1 plays crucial roles in growth control and rearrangements of the cytoskeleton. IGF-1 binds to the IGF-1 receptor and thereby induces the autophosphorylation of this receptor at its tyrosine residues. The phosphorylation of the IGF-1 receptor is thought to initiate a cascade of events. Although various signaling molecules have been identified, they appear to interact with the tyrosine-phosphorylated IGF-1 receptor. Here, we identified leukemia-associated Rho guanine nucleotide exchange factor (GEF) (LARG), which contains the PSD-95/Dlg/ZO-1 (PDZ), regulator of G protein signaling (RGS), Dbl homology, and pleckstrin homology domains, as a nonphosphorylated IGF-1 receptor-interacting molecule. LARG formed a complex with the IGF-1 receptor *in vivo*,

and the PDZ domain of LARG interacted directly with the COOH-terminal domain of IGF-1 receptor *in vitro*. LARG had an exchange activity for Rho *in vitro* and induced the formation of stress fibers in NIH 3T3 fibroblasts. When MDCKII epithelial cells were treated with IGF-1, Rho and its effector Rho-associated kinase (Rho-kinase) were activated and actin stress fibers were enhanced. Furthermore, the IGF-1-induced Rho-kinase activation and the enhancement of stress fibers were inhibited by ectopic expression of the PDZ and RGS domains of LARG. Taken together, these results indicate that IGF-1 activates the Rho/Rho-kinase pathway via a LARG/IGF-1 receptor complex and thereby regulates cytoskeletal rearrangements.

Introduction

Insulin-like growth factor (IGF)*-1 is a polypeptide hormone that is similar to insulin and plays fundamental roles in mammalian growth processes. Binding of IGF-1 to the extracellular α -subunit of the IGF-1 receptor results in the autophosphorylation of the cytoplasmic β -subunit of the receptor (White and Kahn, 1994). The autophosphorylation of the IGF-1 receptor leads to the activation of various IGF-1-dependent signal transductions (Myers and White,

1996). IGF-1 also plays crucial roles in rearrangements of the cytoskeleton such as those which occur in membrane ruffling (Kim et al., 1998), cell migration (Mañes et al., 1999), and neurite outgrowth (Kim et al., 1997). However, it remains to be clarified how IGF-1 affects such rearrangements. In this study, we identified a cDNA encoding a PSD-95/Dlg/ZO-1 (PDZ) domain as an IGF-1 receptor-interacting molecule. This PDZ domain was almost identical to the PDZ domain of leukemia-associated Rho guanine nucleotide exchange factor (GEF) (LARG) (Kourlas et al., 2000).

The Rho family GTPases play roles in cytoskeletal rearrangements and in cell adhesion in response to extracellular signals. Rho participates in regulation of various cellular functions such as stress fiber and focal adhesion formation (Ridley and Hall, 1992, 1994), smooth muscle contraction (Hirata et al., 1992; Gong et al., 1996), and membrane ruffling (Nishiyama et al., 1994; Takaishi et al., 1995). Rho has GDP-bound inactive and GTP-bound active forms, which are interconvertible by GDP/GTP exchange and GTPase re-

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*Abbreviations used in this paper: AF-6, ALL-1 fusion partner from chromosome 6; DH, Dbl homology; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; HA, hemagglutinin; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; MBP, maltose-binding protein; RGS, regulator of G protein signaling; LARG, leukemia-associated RhoGEF; MBS, myosin-binding subunit; PDZ, PSD-95/Dlg/ZO-1; PH, pleckstrin homology; Rho-kinase, Rho-associated kinase.

Key words: IGF-1; Rho; LARG; PDZ domain; GEF

actions (Nobes and Hall, 1994). The GTPase reaction is regulated by Rho GTPase-activating proteins. The GDP/GTP exchange reaction is regulated by various GEFs and by inhibitory proteins such as Rho GDI (Fukumoto et al., 1990). GTP-bound Rho exerts its biological functions through interaction with specific effectors (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998; Kaibuchi et al., 1999).

GEFs for the Rho family GTPases share a common sequence motif designated as the Dbl homology (DH) domain (Cerione and Zheng, 1996; Whitehead et al., 1997; Stam and Collard, 1999). In addition to the DH domain, GEFs for the Rho family GTPases contain a nearby pleckstrin homology (PH) domain. Both domains are essential for the GEF activity. Although several GEFs have been identified, the molecular mechanisms by which the activation of GEFs is modulated by extracellular signals are largely unknown except in the case of p115 RhoGEF, PDZ-RhoGEF, and Vav. Studies on heterotrimeric G proteins have clarified the mechanism by which p115 RhoGEF is regulated. p115 RhoGEF has the regulator of G protein signaling (RGS) domain, and the RGS domain of p115 RhoGEF specifically stimulates the intrinsic GTPase activity of $G\alpha_{12}$ or $G\alpha_{13}$ (Hart et al., 1998; Kozasa et al., 1998). Activated $G\alpha_{13}$ conversely binds to p115 RhoGEF and stimulates its ability to catalyze nucleotide exchange of Rho. Thus, upon stimulation by extracellular signals the activation of $G\alpha_{13}$ is thought to be linked to the activation of Rho via p115 RhoGEF. Similarly, it has been reported that the RGS domain of PDZ-RhoGEF (KIAA0380) interacts with activated $G\alpha_{12}$ or $G\alpha_{13}$ (Fukuhara et al., 1999). Vav and Vav-2 are other GEFs for the Rho family GTPases (Crespo et al., 1997; Schuebel et al., 1998; Abe et al., 2000). The phosphorylation of Vav and Vav-2 on tyrosine residues leads to the activation of the Rho family GTPases (Crespo et al., 1997; Schuebel et al., 1998). Recently, it has been reported that Vav-2 participates in the hepatocyte growth factor (HGF)-stimulated activation of Rho (Kodama et al., 2000).

It has been suggested that the Rho family GTPases participate in the IGF-1 signaling pathway (Thomson et al., 1997; Cheng et al., 2000); however, the mechanism of regulation of the Rho family GTPases by IGF-1 is unknown. In this study, we found that the IGF-1 receptor formed a complex with LARG and that IGF-1 induced the activation of Rho and its effector Rho-associated kinase (Rho-kinase)/ROK/ROCK (Leung et al., 1995; Ishizaki et al., 1996; Matsui et al., 1996) in MDCKII epithelial cells. The IGF-1-induced activation of Rho-kinase was inhibited by pretreatment of the cells with Rho-kinase inhibitors and by overexpression of the PDZ and RGS domains of LARG.

Results

Identification of LARG as an IGF-1 receptor-interacting molecule

To clarify the mechanism underlying the IGF-1/IGF-1 receptor signaling pathway, we tried to identify IGF-1 receptor-interacting molecules by the yeast two-hybrid screening method. We used the COOH-terminal 20 amino acids of the IGF-1 receptor β -subunit (His-Met-Asn-Gly-Gly-Arg-Lys-Asn-Glu-Arg-Ala-Leu-Pro-Leu-Pro-Gln-Ser-Ser-Thr-

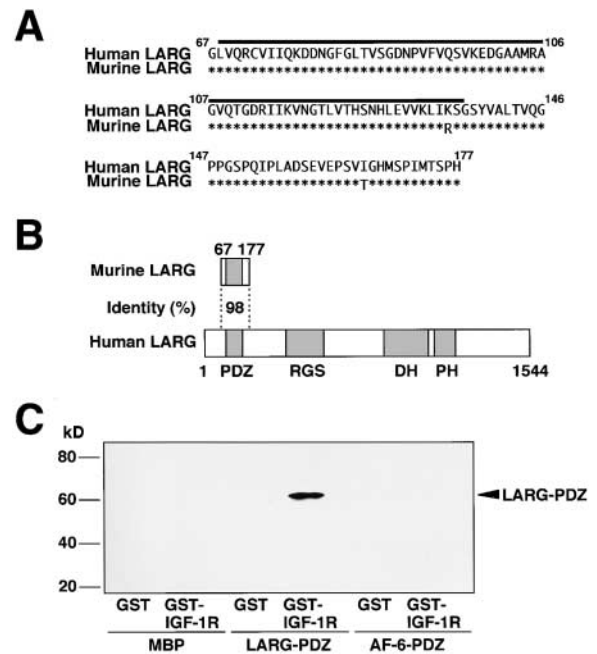


Figure 1. Identification of the IGF-1 receptor-interacting protein. (A) Amino acid sequences of the IGF-1 receptor-interacting peptide and human LARG. The numbers denote amino acid positions. Asterisks denote identical amino acids. The black bars show amino acid positions of PDZ domain. (B) Schematic representation of the IGF-1 receptor-interacting peptide and human LARG. The numbers indicate the amino acid sequence identity in the PDZ domains or amino acid numbers. (C) Interaction of MBP-LARG-PDZ domain with GST-IGF-1 receptor β -subunit in vitro. MBP, MBP-LARG-PDZ domain, or the AF-6-PDZ domain was mixed with GST- or GST-IGF-1 receptor-coated beads. The interacting proteins were coeluted with GST fusion proteins. The eluates were subjected to SDS-PAGE and subjected to immunoblot analysis with anti-MBP or anti-AF-6 antibody. The results shown are representative of three independent experiments.

Cys-COOH) as a bait and identified a cDNA encoding 111 amino acids from the mouse embryo cDNA library. The cDNA product was almost identical to the PDZ domain of human LARG except for Lys-135 and Ile-166 (Fig. 1, A and B). LARG had DH and PH domains (Kourlas et al., 2000) and showed a high degree of sequence similarity to PDZ-RhoGEF (KIAA0380) except for a proline-rich motif COOH-terminally adjacent to the DH/PH domain (Fukuhara et al., 1999; Rümenapp et al., 1999; Togashi et al., 2000). Thus, we regarded the cDNA encoding the 111 amino acids as a part of murine LARG. However, we can not rule out the possibility that the identified fragment by yeast two-hybrid screening is a LARG-like molecule.

To examine whether LARG interacts directly with the IGF-1 receptor, we isolated the human LARG cDNA from the human fetal brain cDNA library and carried out an in vitro binding assay. Affinity beads coated with glutathione *S*-transferase (GST) or GST-IGF-1 receptor β -subunit were mixed with maltose-binding protein (MBP) or MBP-PDZ domain of LARG. The bound MBP-PDZ domain of LARG was then coeluted with the GST fusion proteins by the addition of glutathione, and the eluted MBP-PDZ domain of LARG was detected with anti-MBP antibody. MBP-PDZ domain of LARG was detected in the eluate from GST-

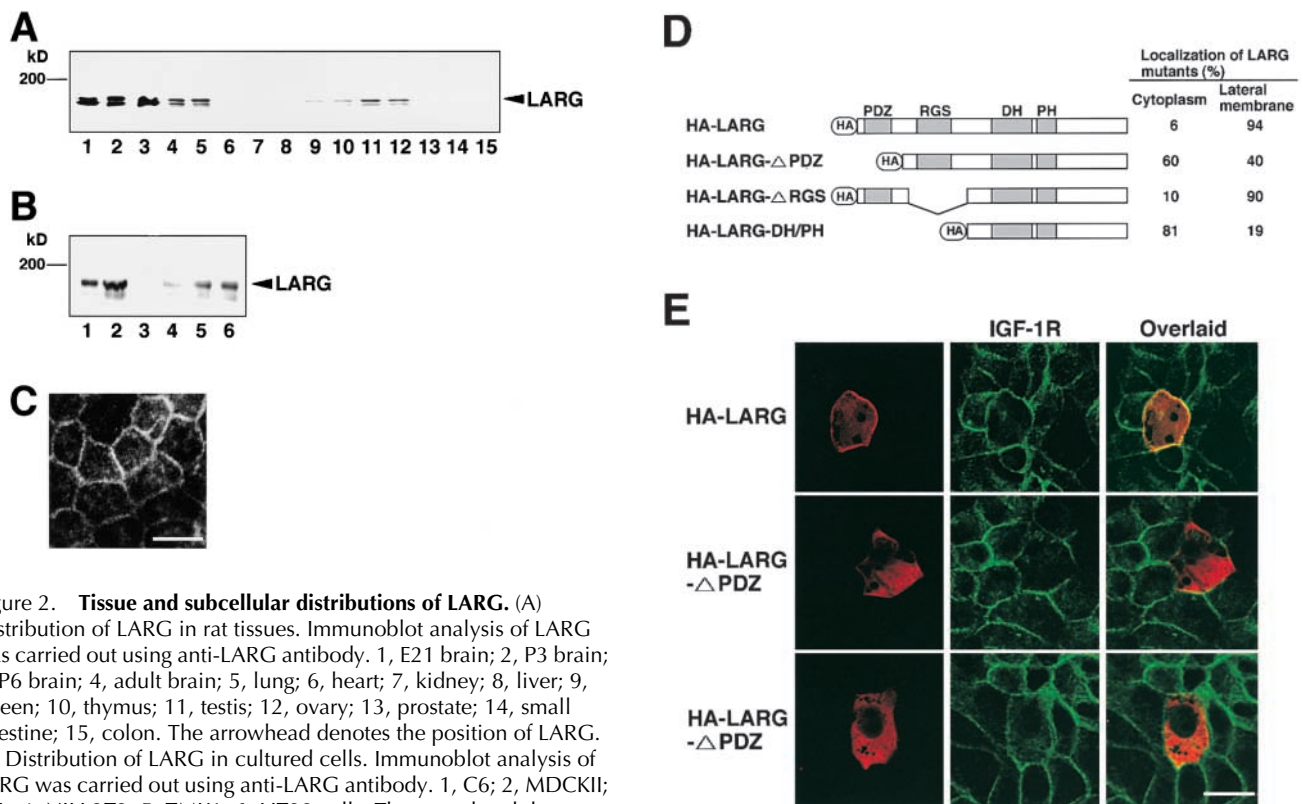


Figure 2. Tissue and subcellular distributions of LARG. (A) Distribution of LARG in rat tissues. Immunoblot analysis of LARG was carried out using anti-LARG antibody. 1, E21 brain; 2, P3 brain; 3, P6 brain; 4, adult brain; 5, lung; 6, heart; 7, kidney; 8, liver; 9, spleen; 10, thymus; 11, testis; 12, ovary; 13, prostate; 14, small intestine; 15, colon. The arrowhead denotes the position of LARG. (B) Distribution of LARG in cultured cells. Immunoblot analysis of LARG was carried out using anti-LARG antibody. 1, C6; 2, MDCKII; 3, L; 4, NIH 3T3; 5, TMK1; 6, HT29 cells. The arrowhead denotes the position of LARG. (C) Subcellular distribution of endogenous LARG in MDCKII cells. Confluent MDCKII cells were stained with anti-LARG antibody. (D) Localization of LARG mutants. MDCKII cells were transfected with various LARG mutants and then stained with anti-HA antibody. The number of cells used for each calculation are >100 , and the values shown are average in three independent experiments. (E) Colocalization of exogenous LARG and endogenous IGF-1 receptor. MDCKII cells were transfected with pEF-BOS-HA-LARG (top) or pEF-BOS-HA-LARG- Δ PDZ (middle and bottom) and then doubly stained with anti-HA and anti-IGF-1 receptor antibodies. The results are representative of three independent experiments. Bars, 10 μ m.

IGF-1 receptor affinity beads but not in that from the GST affinity beads (Fig. 1 C). We examined the specificity of the PDZ domain of LARG by using the PDZ domain of ALL-1 fusion partner from chromosome 6 (AF-6), which is a putative Ras effector (Van Aelst et al., 1994; Kuriyama et al., 1996). The PDZ domain of AF-6 was not detected in the eluate from the GST-IGF-1 receptor affinity beads (Fig. 1 C). Taken together, these results indicate that the PDZ domain of LARG binds directly and specifically to the cytoplasmic domain of the IGF-1 receptor *in vitro*.

Distribution of LARG

To understand the functions of LARG, we produced anti-LARG antibody against 249 amino acids of the COOH-terminal site and performed immunoblot analysis of several rat tissues. LARG was expressed in rat adult brain, lung, spleen, thymus, testis, and ovary but not detectable in heart, kidney, liver, prostate, small intestine, and colon (Fig. 2 A). Interestingly, LARG was expressed most abundantly in the developing brains (Fig. 2 A). Moreover, LARG was highly expressed in various cell lines such as MDCKII, C6 glioma, human gastric cancer TMK1, and human intestinal cancer HT29 cells (Fig. 2 B). LARG appeared as a doublet in rat tissues and cells. When we isolated the LARG cDNA from the human fetal brain cDNA library, we obtained the shorter form of the LARG cDNA, which lacked the middle region (235–261 amino acids). The higher band of ~ 172 kD may be an

intact LARG, judging from the molecular mass of intact LARG (173,230 D), and the lower band of ~ 170 kD may be an alternatively spliced form of LARG.

We next examined the localization of LARG in MDCKII epithelial cells. The immunoreactivity of LARG was localized at the lateral membranes and slightly in the cytoplasm (Fig. 2 C). Next, we examined whether LARG is colocalized with the IGF-1 receptor. Because anti-LARG and anti-IGF-1 receptor antibodies were rabbit polyclonal ones, we could not perform double immunofluorescence. Instead, we showed colocalization of endogenous IGF-1 receptor and exogenous LARG (hemagglutinin [HA]-LARG) (Fig. 2 E). The immunoreactivity of HA-LARG was localized at the lateral membranes and slightly in the cytoplasm in 94% of the cells, whereas the immunoreactivity was localized diffusely in the cytoplasm but not at the lateral membranes in the remaining 6% of the cells (Fig. 2, D and E, top). The localization of exogenous HA-LARG was similar to that of endogenous LARG. Thus, it is likely that both LARG and the IGF-1 receptor are colocalized at the lateral membranes. To examine whether LARG is localized at the lateral membranes via its PDZ domain, we examined the localization of LARG using various mutants. The immunoreactivity of HA-LARG- Δ PDZ was localized diffusely in the cytoplasm in 60% of the cells (Fig. 2, D and E, bottom), whereas in the remaining 40% of the cells the immunoreactivity was still localized partially at the lateral membranes but was much weaker than that of HA-LARG (Fig. 2, D and E, mid-

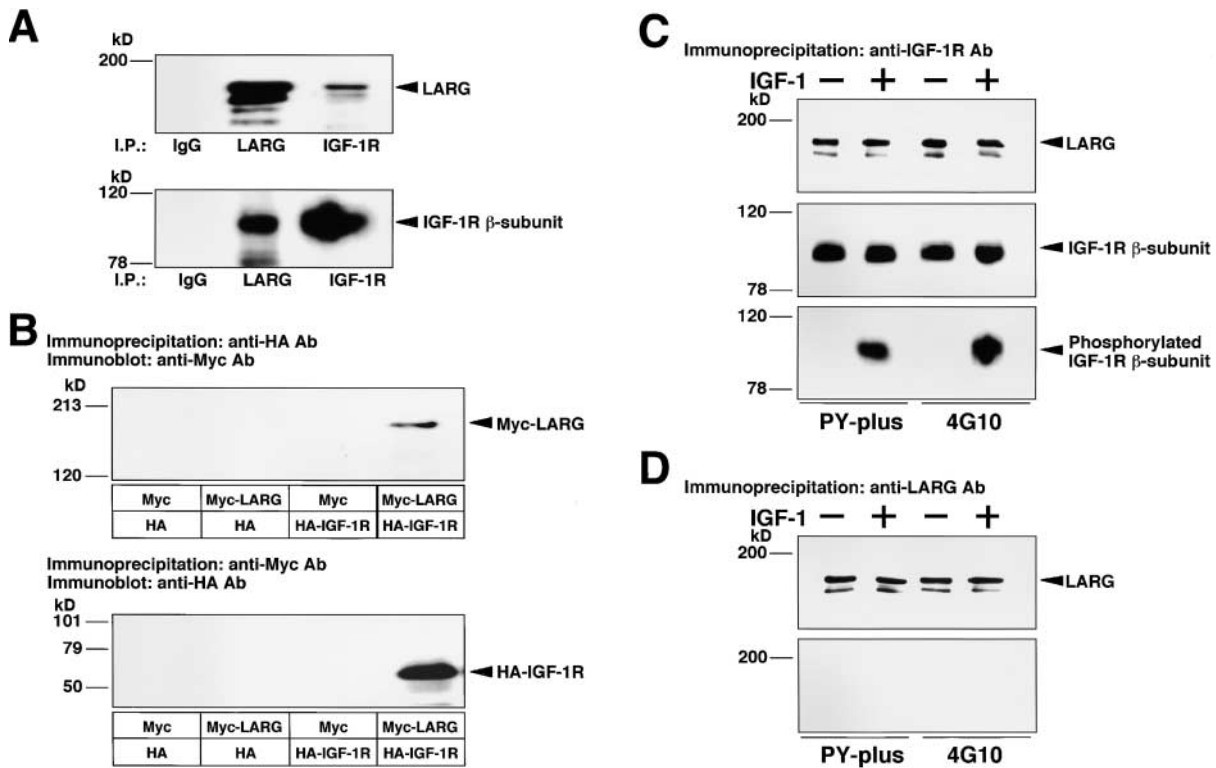


Figure 3. Interaction of LARG with the IGF-1 receptor. (A) Interaction of endogenous LARG with the IGF-1 receptor in vivo. The lysates of MDCKII cells were incubated with rabbit IgG, anti-LARG, or the anti-IGF-1 receptor antibody. The immunocomplexes were then precipitated and subjected to immunoblot analysis with anti-LARG (top) or anti-IGF-1 receptor antibody (bottom). (B) Interaction of the recombinant LARG with the IGF-1 receptor β -subunit. L fibroblasts were cotransfected with pEF-BOS-Myc-LARG and pEF-BOS-HA-IGF-1 receptor β -subunit. These lysates were incubated with anti-HA (top) or with the anti-Myc antibody (bottom). The immunocomplexes were subjected to immunoblot analysis with anti-Myc (top) or anti-HA antibody (bottom). (C) Effect of IGF-1 on the binding state of LARG and the IGF-1 receptor. MDCKII cells were serum starved for 48 h and then stimulated with 10 nM IGF-1 for 20 min. The lysates of MDCKII cells were incubated with anti-IGF-1 receptor antibody. The immunoprecipitates were subjected to immunoblot analysis with anti-LARG antibody (top), anti-IGF-1 receptor antibody (middle), or antiphosphotyrosine antibodies (PY-plus and 4G10) (bottom). (D) Effect of IGF-1 on tyrosine phosphorylation of LARG. Under the same condition as C, the lysates of MDCKII cells were incubated with anti-LARG antibody, and then the precipitated LARG was subjected to immunoblot analysis with anti-LARG antibody (top) or antiphosphotyrosine antibodies (bottom). The results shown are representative of three independent experiments.

dle). The immunoreactivity of HA-LARG-DH/PH was localized diffusely in the cytoplasm in 81% of the cells, whereas the immunoreactivity was localized partially at the lateral membranes in the remaining 19% of the cells (Fig. 2 D). The deletion of the RGS domain alone did not affect the localization of LARG (Fig. 2 D). These results suggest that LARG is translocated to the lateral membranes mainly via its PDZ domain. Although the RGS domain is not essential, it may play a secondary role in the translocation of LARG to the lateral membranes, since the deletion of both PDZ and RGS domains abolished the translocation of LARG to the lateral membranes more efficiently than that of the PDZ domain alone.

Interaction of LARG with the IGF-1 receptor in vivo

To determine whether LARG interacts with the IGF-1 receptor in vivo, we performed coimmunoprecipitation assay. When the IGF-1 receptor was immunoprecipitated from lysates of MDCKII cells with anti-IGF-1 receptor antibody, the molecule corresponding to LARG was coimmunoprecipitated with the IGF-1 receptor (Fig. 3 A, top). LARG was not coimmunoprecipitated with control rabbit IgG. The IGF-1 receptor was also coimmunoprecipitated with LARG

(Fig. 3 A, bottom) when LARG was immunoprecipitated with anti-LARG antibody. Similar results were obtained when C6 glioma cells were used instead of MDCKII cells (unpublished data). These results suggest that LARG interacts and forms a complex with the IGF-1 receptor in vivo.

We next examined the interaction of the recombinant LARG with the recombinant IGF-1 receptor. Mammalian expression plasmids harboring Myc-LARG or HA-IGF-1 receptor β -subunit were used to cotransfect into L fibroblasts, since the expression levels of LARG were low and the transfection efficiency was high in L cells. When HA-IGF-1 receptor β -subunit was immunoprecipitated with anti-HA antibody, Myc-LARG was coimmunoprecipitated with the HA-IGF-1 receptor β -subunit (Fig. 3 B, top). Myc-LARG was not coimmunoprecipitated with control mouse IgG or the mock plasmid (HA alone). HA-IGF-1 receptor β -subunit was also coimmunoprecipitated with Myc-LARG when anti-Myc antibody was used (Fig. 3 B, bottom). This result indicates that the recombinant LARG can interact with the recombinant IGF-1 receptor β -subunit in L cells.

IGF-1 binds to the IGF-1 receptor and thereby induces the autophosphorylation of this receptor at its tyrosine resi-

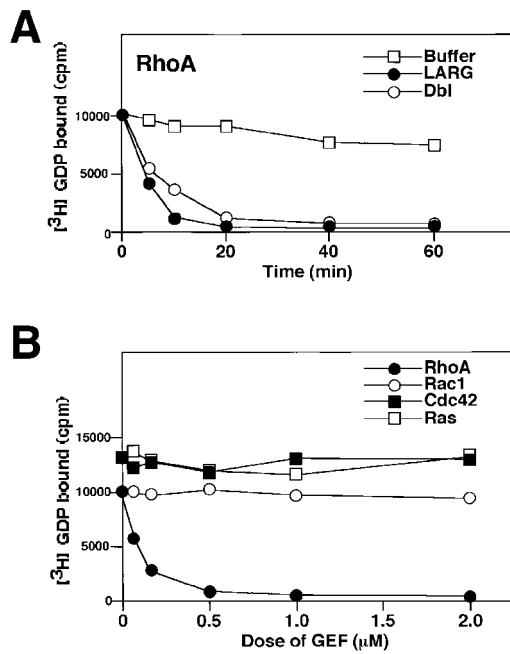


Figure 4. GEF activity of LARG for the Rho family GTPases in vitro. (A) The effect of LARG on the dissociation of GDP from RhoA in vitro. The DH/PH domain of LARG and Dbl enhanced the dissociation of [3 H]-labeled GDP from RhoA in a time-dependent manner. (B) The effect of LARG on other small GTPases in vitro. The DH/PH domain of LARG showed an exchange activity for RhoA but not one for Rac1, Cdc42, or Ras. The results shown are representative of three independent experiments.

dues. The phosphorylation of the IGF-1 receptor is thought to interact with various signaling molecules. We examined whether the treatment of serum-deprived MDCKII cells with IGF-1 affected the binding state of LARG and the IGF-1 receptor. The amounts of LARG that were coimmunoprecipitated with the IGF-1 receptor were not changed under the conditions in which the phosphorylation of IGF-1 receptor was induced (Fig. 3 C). Under the same conditions, the tyrosine phosphorylated LARG was not detected (Fig. 3 D). Taken together, these results suggest that the interaction of LARG and the IGF-1 receptor is constitutive and does not require the phosphorylation of the IGF-1 receptor.

GEF activity of LARG for the Rho family GTPases

We examined the GEF activity for the Rho family GTPases in vitro. In this assay, we used a GST fusion protein carrying the DH/PH domain of LARG for monitoring the exchange activity toward the Rho family GTPases. The DH/PH domain of LARG enhanced the dissociation of [3 H]-labeled GDP from RhoA in a time-dependent manner under the conditions where Dbl stimulated the dissociation of GDP from RhoA (Fig. 4 A). To investigate whether LARG is a specific GEF for Rho, we further examined the effect of the DH/PH domain of LARG on other small GTPases, Rac1, Cdc42, and Ras. The DH/PH domain of LARG showed an exchange activity for RhoA but not for Rac1, Cdc42, or Ras (Fig. 4 B). These results indicate that LARG has the exchange activity for RhoA in vitro but not for Rac1, Cdc42, or Ras.

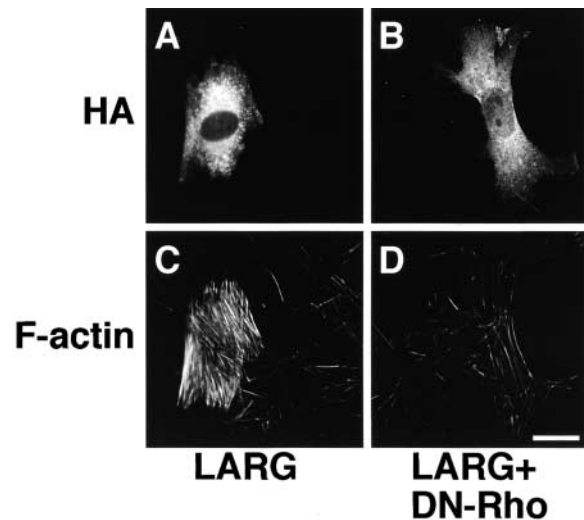


Figure 5. Effect of LARG on the Rho activity in NIH 3T3 fibroblasts. Serum-deprived NIH 3T3 cells were transfected with the pEF-BOS-HA-LARG (A and C) or -Myc dominant negative form of RhoA-HA-LARG (B and D). The transfected cells were doubly stained with anti-HA antibody and TRITC-labeled phalloidin. The results shown are representative of three independent experiments. Bar, 20 μ m.

It has been reported that the constitutive active form of RhoA induced the formation of stress fibers in Swiss 3T3 (Ridley and Hall, 1992, 1994) and NIH 3T3 fibroblasts (Khosravi-Far et al., 1994). Since the expression levels of endogenous LARG and IGF-1 receptor were low in NIH 3T3 cells, we thought that NIH 3T3 cells were appropriate for evaluating the effects of LARG as a RhoGEF. Serum-deprived NIH 3T3 cells were transfected with HA-LARG, and then transfected cells were doubly stained with anti-HA antibody and TRITC-labeled phalloidin. Overexpression of LARG induced the formation of stress fibers (Fig. 5, A and C). The overexpressed LARG was diffusely localized in the cytoplasm of NIH 3T3 cells but not in the plasma membranes (Fig. 5, A and B). This may be due to the high expression levels of HA-LARG and/or the low expression levels of the binding partner of LARG such as the IGF-1 receptor in NIH 3T3 cells. The formation of LARG-induced stress fibers was inhibited by the dominant negative form of RhoA (Fig. 5, B and D). Overexpression of the DH/PH domain of LARG induced the formation of thicker stress fibers than that induced by LARG (unpublished data). This may be due to constitutive activation of Rho.

Effect of IGF-1 on the Rho signaling pathway

We have shown that LARG binds directly to and is colocalized with the IGF-1 receptor. This result led us to examine whether IGF-1 controls the Rho activity via LARG. It has been reported that the IGF-1 receptor plays roles when stimulated by IGF-1 in MDCK cells (Sukegawa et al., 1987; Hauguel-de Mouzon and Kahn, 1991), and we detected the high expression levels of LARG and the IGF-1 receptor. Therefore, we thought that MDCKII cells are suitable cells to monitor the activation of Rho upon the IGF-1 stimulation. To understand the role of IGF-1 in Rho signaling, we

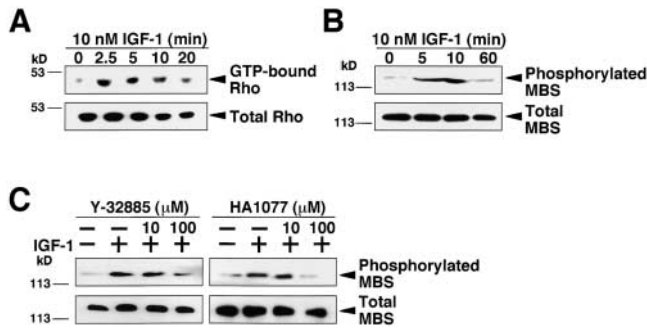


Figure 6. Effect of IGF-1 on the Rho/Rho-kinase signaling pathway. (A) Activation of exogenous RhoA by IGF-1. MDCKII cells stably expressing EGFP-RhoA were serum starved and then stimulated with 10 nM IGF-1 for the indicated minutes. The lysates were incubated with GST-Rho-binding domain of rhotekin. The bound EGFP-RhoA was subjected to immunoblot analysis with anti-RhoA antibody. Arrowheads indicate the positions of GTP-bound EGFP-RhoA and total EGFP-RhoA. (B) IGF-induced phosphorylation of MBS at Ser-854. Serum-deprived MDCKII cells were stimulated with 10 nM IGF-1 for the indicated minutes, and the whole cell lysates were resolved by SDS-PAGE followed by immunoblot analysis with anti-pS854 (top) or anti-MBS antibody (bottom). Arrowheads indicate the positions of the phosphorylated MBS and total MBS. (C) Inhibition of the IGF-1-induced MBS phosphorylation by Rho-kinase inhibitors. HA1077 (right)- or Y-32885 (left)-pretreated serum-deprived MDCKII cells were stimulated with 10 nM IGF-1 for 10 min, and then the lysates were resolved by SDS-PAGE followed by immunoblot analysis with anti-pS854 (top) or anti-MBS antibody (bottom). Arrowheads indicate the positions of phosphorylated MBS and total MBS. The results shown are representative of three independent experiments.

examined Rho activity in IGF-1-stimulated MDCKII cells by a pull-down assay using the GST-Rho-binding domain of rhotekin (Zondag et al., 2000). However, we could not detect any increase in GTP-bound Rho in the IGF-1-stimulated cells. The reason for this result may be low expression levels of endogenous Rho. Therefore, we next performed a pull-down assay using MDCKII cells stably expressing EGFP-RhoA. The expression level of EGFP-RhoA was more than 10-fold greater than that of endogenous Rho. MDCKII cells stably expressing EGFP-RhoA were deprived of serum and then incubated in medium containing 10 nM IGF-1. The IGF-1-induced Rho activation peaked at ~2.5–5 min after stimulation and then returned to basal levels at 20–30 min (Fig. 6 A).

Next, we used another method in order to examine the effect of LARG on Rho activity under physiological conditions. We reported previously that Rho activates Rho-kinase and thereby Rho-kinase phosphorylates substrates including myosin light chain, ezrin/radixin/moesin family proteins, adducin, and myosin-binding subunit (MBS) of myosin phosphatase (Kaibuchi et al., 1999). Using anti-pS854 antibody, which specifically recognizes the phosphorylated Ser of MBS at residue 854, we have shown that MBS is phosphorylated specifically at Ser-854 via the Rho/Rho-kinase pathway during the action of HGF in MDCK cells (Kawano et al., 1999). To examine whether IGF-1 affects the activation of Rho/Rho-kinase, we measured the amount of MBS phosphorylated at Ser-854 in IGF-1-stimulated MDCKII cells. MDCKII cells were deprived of serum and then incubated in medium

containing 10 nM IGF-1. When total cell lysates of nonstimulated MDCKII cells were immunoblotted with anti-pS854 antibody, phosphorylated MBS was detected weakly. The addition of IGF-1 enhanced the phosphorylation of MBS within 5–10 min (Fig. 6 B). Similar results were obtained when the cells were stimulated with HGF (Kawano et al., 1999). The maximal phosphorylation level of MBS was ~5–10-fold over the basal level, and the phosphorylation level of MBS returned to basal levels at ~60 min (Fig. 6 B). Furthermore, we examined the effects of Rho-kinase inhibitors, such as Y-32885 and HA1077 (Uehata et al., 1997), on IGF-1-stimulated phosphorylation of MBS. The IGF-1-induced phosphorylation of MBS was inhibited by the pretreatment of the cells with Y-32885 or HA1077 in a dose-dependent manner (Fig. 6 C). Similar result was obtained when the cells were treated with Y-27632, which is also an inhibitor of Rho-kinase (unpublished data). Taken together, these results indicate that IGF-1 activates the Rho/Rho-kinase pathway.

Roles of LARG in the IGF-1/Rho signaling pathway

It has been reported that the RGS domain of PDZ-RhoGEF (KIAA0380) inhibits stress fibers formation induced by the heterotrimeric G protein-coupled lysophosphatidic acid receptor (Rümenapp et al., 1999). The RGS domain is thought to be a functional interface for interaction with heterotrimeric G proteins. Here, we showed that the PDZ domain of LARG interacted with the IGF-1 receptor and that IGF-1 activated the Rho signaling pathway. We speculated that the PDZ domain of LARG serves as the dominant negative mutant for the IGF-1-induced Rho/Rho-kinase activation. We examined whether the PDZ domain of LARG inhibits the complex formation of LARG with the IGF-1 receptor. The beads, which were coated with GST-IGF-1 receptor β -subunit, were mixed with endogenous LARG from lysates of MDCKII cells and MBP alone, MBP-LARG-PDZ domain, or AF-6-PDZ domain, and then the amounts of endogenous LARG bound to GST-IGF-1 receptor β -subunit were measured. Endogenous LARG interacted with GST-IGF-1 receptor β -subunit but not with GST alone (Fig. 7 A). The amounts of endogenous LARG bound to GST-IGF-1 receptor β -subunit were reduced by the addition of MBP-LARG-PDZ domain but not by the addition of MBP alone or the PDZ domain of AF-6 (Fig. 7 A). Thus, it is probable that the PDZ domain of LARG can dissociate endogenous LARG from the IGF-1 receptor and serve as the dominant negative form.

We examined the effect of the PDZ domain of LARG on the IGF-1/Rho signaling pathway to determine whether IGF-1 activates Rho/Rho-kinase via LARG. Serum-deprived MDCKII cells were transfected with HA alone, HA-LARG-PDZ domain, HA-LARG-RGS domain, or HA-AF-6-PDZ domain. The transfected cells were stimulated with IGF-1 for 10 min. In the cells expressing HA-LARG-PDZ and HA-LARG-RGS domains, the levels of phosphorylated MBS were lower than that in the cells expressing HA alone or HA-AF-6-PDZ domain. The expression levels of HA-LARG-PDZ, HA-LARG-RGS, and HA-AF-6-PDZ domains were almost the same (Fig. 7 B).

It has been reported that IGF-1 affects actin organization in mammalian cells (Kadowaki et al., 1986; Lev-

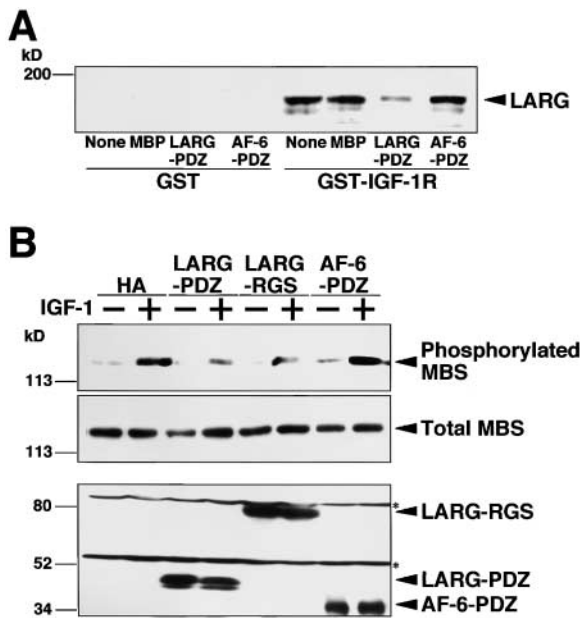


Figure 7. Effect of the PDZ domain of LARG. (A) Inhibition of the complex formation of IGF-1 receptor and LARG by the PDZ domain of LARG. Endogenous LARG from MDCKII lysate and MBP, MBP-LARG-PDZ domain, or the AF-6-PDZ domain (500 pmol) were mixed with GST or GST-IGF-1 receptor-coated beads (100 pmol). The bound LARG was subjected to immunoblot analysis with anti-LARG antibody. (B) Effect of the PDZ and RGS domains of LARG on the IGF-1-induced MBS phosphorylation. MDCKII cells were transfected with pEF-BOS-HA, or -LARG-PDZ, -LARG-RGS, or -AF-6-PDZ domain. These transfected cells were serum starved for 48 h and then stimulated or not with 10 nM IGF-1 for 10 min. The lysates were resolved by SDS-PAGE followed by immunoblot analysis with anti-pS854 (top), anti-MBS antibody (middle), or anti-HA antibody (bottom). Arrowheads indicate the positions of phosphorylated MBS, total MBS, or expressed proteins. Asterisks indicate nonspecific bands. The results shown are representative of five independent experiments.

enthal et al., 1997). These observations raise the possibility that the Rho/Rho-kinase pathway participates in the IGF-1-induced rearrangements of the cytoskeleton. To examine whether IGF-1 affects actin organization in MDCKII cells, MDCKII cells were serum starved and then stimulated with 10 nM IGF-1 for various minutes. Under the serum-starved conditions, ~10% of the cells showed thick stress fibers, and the rest of the cells showed thin and weak stress fibers (Fig. 8 A). The stimulation by IGF-1 induced thick stress fibers in ~50% of cells within 20–60 min (Fig. 8, A and C). The IGF-1-induced enhancement of stress fibers appeared 20–60 min after stimulation and then returned to basal levels later than ~120 min. The PDZ domain of LARG partially inhibited the IGF-1-induced enhancement of stress fibers, whereas GST (negative control) or the PDZ domain of AF-6 had no effects (Fig. 8, B and C). The RGS domain of LARG partially inhibited the IGF-1-induced enhancement of stress fibers, though the inhibitory effect was weaker than that of the PDZ domain of LARG. Taken together, these results suggest that IGF-1 activates the Rho/Rho-kinase pathway via LARG.

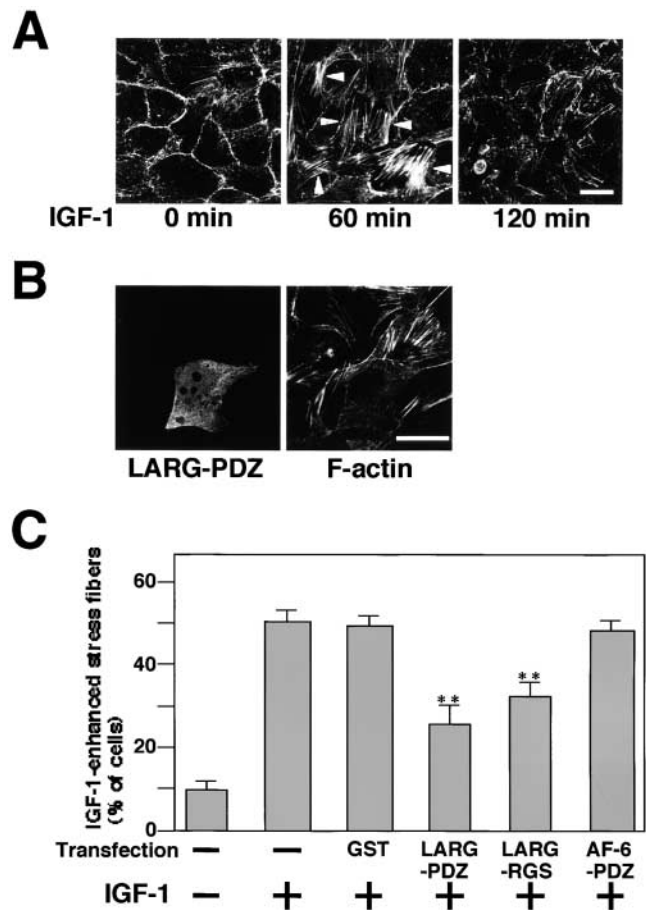


Figure 8. Effect of LARG on the IGF-1-induced enhancement of stress fibers. (A) IGF-1-induced enhancement of stress fiber formation. MDCKII cells were serum starved for 48 h and then stimulated with 10 nM IGF-1 for the indicated minutes. The stimulated cells were stained with TRITC-labeled phalloidin. Arrowheads indicate the cells showing IGF-1-induced enhancement of stress fibers. (B and C) Effect of the PDZ and RGS domains of LARG on the IGF-1-induced enhancement of stress fibers. MDCKII cells were transfected with the pEF-BOS-HA-LARG-PDZ domain. The transfected cells were serum starved for 48 h and then stimulated with 10 nM IGF-1 for 60 min. The transfected cells were doubly stained with anti-HA antibody and TRITC-labeled phalloidin (B). The cells showing the IGF-1-induced enhancement of stress fibers, which were transfected with pEF-BOS-GST, -HA-LARG-PDZ, -HA-LARG-RGS, or -HA-AF-6-PDZ, were calculated (C). The number of cells used for each calculation is >300, and the values shown are means \pm SE of triplicates. ** $p < 0.01$, significance of difference from the cells of IGF-1-induced stress fibers analyzed by Student's *t* test.

Discussion

LARG as an IGF-1 receptor-interacting molecule

IGF-1 binds to the IGF-1 receptor and thereby induces the autophosphorylation of the β -subunit of this receptor at tyrosine residues. The autophosphorylation of the IGF-1 receptor is thought to initiate a cascade of actions mediating IGF-1-triggered cellular signal transduction (Myers and White, 1996). For example, insulin receptor substrate-1 binds to phosphotyrosine residues on the IGF-1 receptor and thereby initiates IGF-1-mediated cellular signal transduction (White and Kahn, 1994). Here, we showed that LARG interacted with the nonphosphorylated IGF-1 re-

ceptor through the PDZ domain. The IGF-1 receptor contains Ser-Thr-Cys, which is not a typical consensus motif for the PDZ-binding domain, in the COOH-terminal end (His-Met-Asn-Gly-Gly-Arg-Lys-Asn-Glu-Arg-Ala-Leu-Pro-Leu-Pro-Gln-Ser-Ser-Thr-Cys-COOH), and this motif might be responsible for the binding of the IGF-1 receptor to LARG. This also raises the possibility that receptors other than the IGF-1 receptor can interact with LARG through its PDZ domain.

It has been reported that RhoGEFs interact with transmembrane proteins or receptor-associated proteins. "Trio" was identified originally as the binding protein of the intracellular domain of leukocyte antigen-related protein, which is a transmembrane protein tyrosine phosphatase (Debant et al., 1996). The NH₂-terminal GEF domain of Trio has exchange activity for Rac1 and RhoG, and the COOH-terminal GEF domain of Trio has exchange activity for RhoA *in vitro* (Debant et al., 1996; Blangy et al., 2000). To our knowledge, the present results are the first demonstration, indicating the direct binding of RhoGEF to a growth factor receptor.

Roles of LARG in the Rho signaling pathway

LARG has the PDZ, RGS, DH, and PH domains and shows a high degree of sequence similarity to PDZ-RhoGEF (KIAA0380). LARG had the exchange activity for RhoA but not for Rac1, Cdc42, or Ras. The RGS domain of PDZ-RhoGEF interacts with activated G α_{12} or G α_{13} of heterotrimeric G proteins, and extracellular signals activate Rho via this interaction (Fukuhara et al., 1999). A recent report suggests that the RGS domain of LARG interacts with G α_{12} or G α_{13} (Fukuhara et al., 2000). Thus, PDZ-RhoGEF and LARG may be structurally and functionally similar to each other. It is possible that PDZ-RhoGEF also interacts with the IGF-1 receptor, since the PDZ domain of PDZ-RhoGEF shows a high sequence similarity (75% identical) with that of LARG (Kourlas et al., 2000). Indeed, our preliminary experiments suggest that recombinant PDZ-RhoGEF interacts with the IGF-1 receptor in L fibroblasts (unpublished data). Further studies are required for evaluating the involvement of PDZ-RhoGEF in the IGF-1 signaling pathway. It has been reported that PDZ-RhoGEF (KIAA0380) translocates to the plasma membrane via its proline-rich motif COOH-terminally adjacent to its DH/PH domain and induces cortical actin reorganization in Swiss 3T3 fibroblasts (Togashi et al., 2000). The proline-rich motif truncated PDZ-RhoGEF induced the formation of stress fibers but not the cortical actin reorganization. We showed that LARG, which did not contain a proline-rich motif, induced the formation of stress fibers in NIH 3T3 fibroblasts. Our results together with the previous observations suggest that LARG is partly different from PDZ-RhoGEF in terms of the localization site on the membrane and as a result shows a different phenotype of actin reorganization.

Activating mechanism of LARG in the IGF-1 signaling pathway

In this study, we showed that the IGF-1 receptor forms a complex with LARG, IGF-1 induced the activation of Rho and Rho-kinase, and overexpression of the PDZ or RGS domain of LARG partially inhibited the IGF-1-induced acti-

vation of Rho-kinase and the enhancement of stress fibers in a dominant negative fashion. The IGF-1-induced Rho activation peaked at \sim 2.5–5 min after stimulation and then returned to basal levels at 20–30 min. The IGF-1-induced MBS phosphorylation peaked at \sim 10 min and then returned to basal levels at \sim 60 min. The IGF-1-induced enhancement of stress fibers appeared 20–60 min after stimulation and then returned to basal levels later than \sim 120 min. We think that the time courses of IGF-1/Rho/Rho-kinase signaling pathways are reasonable. These results suggest that IGF-1 activates the Rho/Rho-kinase pathway via a complex of the IGF-1 receptor and LARG. As to the molecular mechanism in which the activation of GEFs can be modulated by extracellular signals, Vav and Vav-2 are thought to be phosphorylated on tyrosine residues, and then this leads to activation of the Rho family GTPases (Crespo et al., 1997; Schuebel et al., 1998). Thus, we speculated that the phosphorylation of LARG on tyrosine residues might be necessary for the activation of LARG. We examined whether IGF-1 induced the tyrosine phosphorylation of LARG in MDCKII cells and found that the tyrosine phosphorylation of LARG was not detected under our conditions in which the IGF-1 receptor was coimmunoprecipitated with LARG and tyrosine phosphorylated (Fig. 3, C and D). These results suggest that the interaction of the IGF-1 receptor with LARG is constitutive and the tyrosine phosphorylation of LARG is not necessary for its activation. Since Rho/Rho-kinase is activated by the addition of IGF-1, the phosphorylation of the IGF-1 receptor and/or some other unidentified mechanism may account for the activation of LARG. In this context, molecules, which interact with the RGS domain such as heterotrimeric G proteins, may participate in the activation of LARG. Alternatively, the binding of IGF-1 to the IGF-1 receptor may induce the conformational change of the receptor, leading to the activation of LARG. Further studies are necessary for understanding the mode of activation of LARG by IGF-1.

Materials and methods

Materials and chemicals

The cDNA encoding KIAA0382 was provided by Drs. T. Nagase and N. Nomura (Kazusa DNA Research Institute, Kisarazu, Japan). The plasmid of GST-rhotekin was provided by Dr. M.A. Schwartz (Scripps Research Institute, La Jolla, CA). FITC-conjugated anti-rabbit and anti-mouse IgG antibodies and Texas red-conjugated anti-mouse IgG antibody were purchased from Amersham Pharmacia Biotech. TRITC-phalloidin, anti-IGF-1 receptor, and anti-myc (9E10) antibodies were purchased from Sigma-Aldrich. Anti-HA monoclonal antibody (12CA5) was purchased from Boehringer. Anti-MBP polyclonal antibody was purchased from New England Biolabs, Inc. Anti-RhoA antibody was purchased from Cytoskeleton, Inc. Antiphosphotyrosine antibody (PY-plus) was purchased from Zymed Laboratories, and 4G10 was purchased from Upstate Biotechnology. Anti-MBS and anti-pS854 antibodies were generated previously (Kawano et al., 1999). Rabbit anti-LARG antibody was prepared and purified by use of GST-LARG (1296–1544 amino acids) as an antigen. Human recombinant IGF-1 was purchased from GIBCO BRL. HA1077 was provided by Asahi Chemical Industry. Y-32885 was synthesized as described (Uehata et al., 1997). All materials used in the nucleic acid study were purchased from Takara Shuzo Corp.

Plasmid constructions

The human IGF-1 receptor cDNA was amplified by PCR from the human fetal liver library. The pLexA- or pBTM116-IGF-1 receptor plasmid, the fragments of the IGF-1 receptor (1348–1367 amino acids) inserted into pLexA or pBTM116, were provided by Dr. Hollenberg (Fred Hutchinson

Cancer Research Center, Seattle, WA), respectively. The 9.5- and 10.5-d mouse embryo cDNA-VP16 fusion libraries and pLexA-Lam were also provided by Dr. Hollenberg (Fred Hutchinson Cancer Research Center). The pLexA-Lam plasmid expressed human lamin C (66–230 amino acids) as a fusion product between it and the DNA-binding domain of LexA. For the mammalian expression plasmid pEF-BOS-HA-IGF-1 receptor β -subunit or the *Escherichia coli* expression plasmid pGEX-IGF-1 receptor β -subunit, the fragment of the IGF-1 receptor (899–1367 amino acids) was inserted into pEF-BOS-HA or pGEX-4T-1 (Amersham Pharmacia Biotech), respectively. The human LARG cDNA was amplified by PCR from the human fetal brain MATCHMAKER cDNA library (CLONTECH Laboratories, Inc.) and then cloned into pGEM-T-Easy (Promega). The fragment of LARG (32–1544 amino acids), LARG- Δ PDZ (153–1544 amino acids), LARG- Δ RGS (the deletion of 262–685 amino acids), LARG-DH/PH (686–1544 amino acids), LARG-PDZ domain (32–295 amino acids), or LARG-RGS domain (153–670 amino acids) was cloned into pEF-BOS-Myc or -HA, respectively. The fragment of the LARG-PDZ domain (32–190 amino acids) or LARG-DH/PH (686–1544 amino acids) was inserted into pMAL-c2 (New England Biolabs, Inc.) or pGEX-4T-2, respectively.

Yeast strains and media

The genotype of the *S. cerevisiae* reporter strain used, L40, was MATA trp1 leu2 his3 ade2 LYS2::lexA-HIS3 URA3::lexA-lacZ and that of NA87-11A was MAT α leu2 his3 trp1 pho3 pho5. Yeast strains were grown at 30°C in rich medium (1% yeast extract, 2% bacto-peptone, 2% glucose) or in Burkholder's minimal medium with appropriate supplements (Tohe et al., 1973).

Library screening

S. cerevisiae L40, which contained the pLexA-IGF-1 receptor, was transformed with the mouse embryo cDNA library constructed in pVP16. Plasmid DNA transformations were performed by the lithium acetate method (Schieftel and Gietz, 1989). His⁺ colonies were grown in synthetic medium at 30°C for 3 d. Approximately 1.8×10^7 transformants were screened, and then 1,683 colonies were picked up as His⁺. Of these colonies, 99 were His⁺ and LacZ⁺ as confirmed by replicate plating. The 99 His⁺ and LacZ⁺ clones were cured of pLexA-IGF-1 receptor by growing the cells in Trp-containing medium and then mating them to yeast strain NA87-11A that had been transformed with pLexA-IGF-1 receptor or pLexA-Lam. Mated cells were selected for growth in medium that lacked Trp (pBTM116) and Leu (pVP16) and were tested for their ability to transactivate HIS3 and a lacZ reporter gene by a β -galactosidase colorimetric filter assay. 60 diploids transactivated the reporter constructs. Of these 60 diploids, 10 did not transactivate the reporter construct in the presence of the LexA-lamin fusion protein (Vojtek et al., 1993). The library plasmid was recovered from the 10 clones and sequenced by an ABI 377 DNA sequencer (Perkin-Elmer Applied Biosystems).

In vitro binding assay

GST or MBP fusion proteins were expressed in *E. coli* BL21(DE3) and purified according to the manufacturer's instructions. MBP or MBP-LARG PDZ domain (600 pmol) was mixed with glutathione-Sepharose 4B beads coated with 100 pmol of either GST or GST-IGF-1 receptor β -subunit in buffer A (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 5 mM MgCl₂). The bound MBP-LARG PDZ domain was coeluted with GST fusion proteins by the addition of buffer A containing 10 mM glutathione. Portions of the eluates were subjected to SDS-PAGE followed by immunoblot analysis with anti-MBP antibody.

Cell culture

NIH 3T3 and MDCKII cells were grown in DME containing 10% calf serum, penicillin, and streptomycin. L cells were grown in DME containing 10% FBS. Although IGF-1 induced the enhancement of actin stress fibers in parental MDCKII cells, the background of stress fibers was relatively high. To monitor the enhancement of actin stress fibers, the cell line, which showed low background of actin stress fibers and high response to IGF-1, was subcloned and employed for whole studies. The cells showed the same characteristics as typical MDCK cells, which show characteristics of polarized epithelial cells. MDCKII cells stably expressing EGFP-RhoA were grown in DME containing 10% calf serum and 300 μ g/ml of G418.

Immunofluorescence and laser scanning confocal microscopy

MDCKII cells plated on 13-mm round glass coverslips were fixed in 3.7% formaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 10 min. The fixed cells were incubated with primary antibodies for 12 h at 4°C and then were incubated for 1 h with secondary antibodies. The distributions of LARG and the IGF-1 receptor were examined with a laser scanning confocal microscope LSM510 (ZEISS).

Coimmunoprecipitation assay

For coimmunoprecipitation assay of the endogenous proteins, MDCKII cells were lysed with buffer B (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 0.5% [wt/vol] Triton X-100, 10 μ M *p*-APMSF, and 10 μ g/ml leupeptin). The lysate was sonicated and then clarified by centrifugation at 12,000 *g* for 30 min at 4°C. The soluble supernatant was incubated with anti-LARG antibody, anti-IGF-1 receptor antibody, or rabbit IgG. For coimmunoprecipitation assay of the recombinant proteins, L cells were transfected with pEF-BOS-Myc-LARG and pEF-BOS-HA-IGF-1 receptor β -subunit by lipofectamine (GIBCO BRL) and cultured for 16 h. The soluble supernatant was incubated with anti-HA or anti-Myc antibody. The immunocomplexes were then precipitated with protein A-Sepharose (Amersham Pharmacia Biotech) eluted by boiling in sample buffer for SDS-PAGE, and subjected to immunoblot analyses with the appropriate antibodies.

In vitro GEF assays

Effects of LARG on the dissociation of [³H]GDP from the Rho family proteins were assayed as described previously (Hoshino et al., 1999). The [³H]GDP-bound form of small G proteins was obtained by incubating 10 pmol of each small G protein with 1 μ M [³H]GDP (1,000–2,000 cpm/pmol) for 20 min at 30°C in reaction mixture I (20 mM Tris/HCl, pH 7.5, 10 mM EDTA, 1 mM DTT, 5 mM MgCl₂). To prevent the dissociation of [³H]GDP from the G proteins, we added MgCl₂ to a final concentration of 20 mM and then cooled the mixtures immediately to 4°C. The dissociation of [³H]GDP was performed at 25°C by adding a 200-fold excess of unlabeled GTP and the indicated amounts of GST-DH/PH domain of LARG or GST-Dbl to reaction mixture II (50 mM Tris/HCl, pH 8.0, 2.9 mM EDTA, 1 mM DTT, 10 mM MgCl₂). The diluted mixtures were filtered through nitrocellulose filters, and the radioactivity trapped on the filters was counted.

Transfection of NIH 3T3 or MDCKII cells with the expression plasmid of LARG

NIH 3T3 cells were transfected with pEF-BOS-HA-LARG or -LARG-Myc dominant negative form of RhoA by using lipofectamine and cultured for 24 h. The transfected cells were serum starved for 24 h in DME. MDCKII cells were transfected with pEF-BOS-GST, or the -HA-LARG-PDZ, -HA-LARG-RGS, or -HA-AF-6-PDZ domain by using lipofectamine 2000 (GIBCO BRL) and cultured for 24 h. The transfected cells were serum starved for 48 h in DME and then stimulated with 10 nM IGF-1 for 1 h. The fixed cells were incubated with primary antibody and then incubated with secondary antibody and TRITC phalloidin. Fluorescent images were taken with a laser scanning confocal microscope LSM510.

Pull-down assay (Rho activity assay)

To obtain MDCKII cells stably expressing EGFP-RhoA, MDCKII cells were transfected with pEGFP-RhoA along with pSVIISRA α vector containing the neomycin resistance gene using lipofectamine 2000, and neomycin-resistant clones were selected. MDCKII cells stably expressing EGFP-RhoA were incubated for 24 h and then were deprived of serum for 48 h. The cells were next incubated in DME containing 10 nM IGF-1 at 37°C for various minutes and then lysed with buffer C (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 1% NP-40, 10 μ M *p*-APMSF, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin). The soluble supernatant was incubated with 2 nmol of GST-rhotekin Rho-binding domain (Zondag et al., 2000). The bound EGFP-RhoA was eluted by boiling in sample buffer for SDS-PAGE and subjected to immunoblot analysis with anti-RhoA antibody.

Detection of phosphorylated MBS by immunoblot analysis

MDCKII cells were incubated for 24 h, and the cells were deprived of serum for 48 h. For inhibitor assays, serum-deprived cells were treated with various doses of HA1077 or Y-32885 for 30 min. In the experiments involving the PDZ domain of LARG, serum-deprived cells were transfected with pEF-BOS-HA, or the -LARG-PDZ, -LARG-RGS, or -AF-6-PDZ domain by using lipofectamine 2000 and then were cultured. The cells were thereafter incubated in DME containing 10 nM IGF-1 at 37°C for various minutes. The IGF-1-stimulated cells were treated with 10% (wt/vol) TCA. The resulting precipitates were subjected to immunoblotting with anti-MBS or anti-pS854 antibody.

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