A Rho-associated Protein Kinase, ROKα, Binds Insulin Receptor Substrate-1 and Modulates Insulin Signaling*

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Insulin receptor substrate-1 (IRS-1) is phosphorylated on multiple tyrosine residues by ligand-activated insulin receptors. These tyrosine phosphorylation sites serve to dock several Src homology 2-containing signaling proteins. In addition, IRS-1 contains a pleckstrin homology domain and a phosphotyrosine binding domain (PTB) implicated in protein-protein and protein-lipid interactions. In a yeast two-hybrid screening using Xenopus IRS-1 (xIRS-1) pleckstrin homology-PTB domains as bait, we identified a Xenopus homolog of Rho-associated kinase α (xROKα) as a potential xIRS-1-binding protein. The original clone contained the carboxyl terminus of xROKα (xROK-C) including the putative Rho binding domain but lacking the amino-terminal kinase domain. Further analyses in yeast indicated that xROK-C bound to the putative PTB domain of xIRS-1. Binding of xROK-C to xIRS-1 was confirmed in Xenopus oocytes after microinjection of mRNA corresponding to xROK-C. Furthermore, microinjection of xROK-C mRNA inhibited insulin-induced mitogen-activated protein kinase activation with a concomitant inhibition of oocyte maturation. In contrast, microinjection of xROK-C mRNA did not inhibit mitogen-activated protein kinase activation or oocyte maturation induced by progesterone or by microinjection of viral Ras (v-Ras) mRNA. These results suggest that xROKα may play a role in insulin signaling via a direct interaction with xIRS-1.

Phosphorylation of insulin receptor substrate-1 (IRS-1)1 by ligand-activated insulin receptors serves to dock several Src homology 2 domain-containing proteins (1–4). In addition to the multiple tyrosine phosphorylation sites, IRS-1 contains an amino-terminal PH domain (5, 6) and a PTB domain (7, 8) carboxyl-terminal to the PH domain (9, 10). Studies by White and co-workers (11, 12) have shown that the PH domain is required for efficient tyrosine phosphorylation of IRS-1 by the insulin receptor, although the mechanism by which the PH domain regulates this function is not clear. The presence of a PTB domain in IRS-1 explains the earlier observations that mutations in a human insulin receptor autophosphorylation site (NPEY960 (13)) or the equivalent site in insulin-like growth factor I receptor (14) diminished its ability to phosphorylate IRS-1. Biochemical and structural studies indicate that amino acids 161–265 of rat IRS-1 include the required component of the PTB domain which binds the NPEpY motif of the insulin receptor (9, 10). However, using the yeast two-hybrid assay, Gustafson and colleagues have provided evidence that additional amino acids that are carboxyl-terminal to the PTB domain (termed the SAIN domain) are also necessary for binding to the NPEpY sequence of the insulin receptor (15, 16). IRS-1 also interacts with 14-3-3 protein, a process apparently dependent on serine phosphorylation of IRS-1 (17, 18).

We have previously isolated a Xenopus cDNA encoding an IRS-1-like protein (termed xIRS-L) (19). Overall, xIRS-L exhibits 65% amino acid sequence identity to mammalian IRS-1 (1) but only 45% identity to mammalian IRS-2 (20). Sequence identity to the more recently identified IRS-3 (21) or IRS-4 (22) is even less (not shown). Therefore xIRS-L is likely the Xenopus homolog of IRS-1 and hereafter is referred to as xIRS-1. The putative PH and PTB domains of xIRS-1 are highly similar to those of rat IRS-1, with 85 and 90% amino acid sequence identity, respectively. To identify novel proteins that may bind the highly conserved amino-terminal protein modules (PH and PTB in particular) of IRS-1, we conducted a yeast two-hybrid screening of a Xenopus oocyte cDNA library. We report here that the Xenopus homolog of RhoA-associated protein kinase (xROKα) is an xIRS-1-binding protein and that binding of a noncatalytic region of xROKα to endogenous xIRS-1 correlates with inhibition of insulin signaling in Xenopus oocytes.

MATERIALS AND METHODS

Animal and Oocyte Manipulation—All procedures involving live oocytes were carried out in a room maintained at 18 °C. Sexually mature, oocyte-positive Xenopus laevis were purchased from NASCO and maintained according to local animal care guidelines. The frogs were injected with pregnant mare serum gonadotropin (Sigma, 50 IU/frog) 3–10 days before oocyte retrieval. A fragment of ovary was removed surgically under hypothermia. Stage VI oocytes (23) were manually defolliculated according to Smith et al. (24). Unless otherwise stated, 10 ng (in 10 nl) of mRNA was injected per oocyte. Microinjection of oocytes was performed in oocyte incubation medium OR2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM Na2HPO4, 5 mM HEPES, pH 7.8) lacking CaCl2. Injected oocytes were incubated in OR2 (containing CaCl2) for 6 h to overnight before the addition of hormones or a second injection.

Progesterone (final concentration 10 μM) stimulation of oocytes was carried out in OR2. To ensure that oocytes respond maximally to insulin, oocytes were incubated with insulin (5 μM) in OR2 lacking K+ ions (25, 26). Elimination of K+ ions from OR2 had no effect on oocyte viability, nor did it induce oocyte maturation by itself (data not shown). To assay for meiotic maturation, oocytes were incubated overnight with

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insulin or progesterone. Oocytes injected with v-Ras mRNA were incubated in OR2 for 4–8 h before being scored for maturation. Oocyte maturation, as indicated by germinal vesicle (GV) breakdown or GVBD, was determined by the appearance of a white spot at the center of the animal hemisphere and confirmed, when in doubt, by bisecting the oocyte and observing for the presence (GVBD-negative) or absence (GVBD-positive) of a GV.

Oocytes were lysed by forcing them through pipette tips in phosphate-buffered saline lysis buffer (10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 μg/ml each of leupeptin and aprotonin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate; 10 μg/ml benzonase). The homogenate was centrifuged in an Eppendorf centrifuge for 15 min at 4°C. Under these conditions, the yolk protein (vitellogenin) was not solubilized and was discarded as a pellet. The cell lysates, which were usually clouded because of the presence of lipids, were suitable for immunoprecipitation and Western blotting.

**Molecular Cloning and Subcloning**—The nucleotide sequence encoding amino acids 3–500 of xIRS-1 (19) was PCR-amplified using the following primers: 5′-TAT GGA TCC GTA GCC CAC AGA C; 5′-TAT GTC GAC TGA GGA GTG AGT CC. The amplified cDNA was digested with BamHI and SalI, restriction sites incorporated into the 5′- and 3′-PCR primers (underlined, respectively). The digested PCR product was then ligated to pAS2 (CLONTECH) which had been digested with the same two enzymes. The resulting plasmid, designated pAS2-xIRS-1 (3–500), was used to express a Gal4 fusion protein containing amino acids 3–500 of xIRS-1 (19) with an extra proline at the junction between Gal4 and xIRS-1 (3–500). The subclones used for mapping binding domains (see Fig. 2) were similarly amplified by PCR using primers containing the same two restriction sites (BamHI and SalI). For the sake of brevity, only the amino acids comprising the domains of xIRS-1 (19) are indicated here: PH, 3–142; PP/PT, 3325; PTB, 137325; SAIN, 306–500; PTB/SAIN, 137–500.

We subsequently transformed a new yeast strain (expressing Gal4-xIRS-1/3-500) with a Xenopus oocyte cDNA library (CLONTECH). Subsequent screening and other yeast procedures were according to protocols provided by CLONTECH. Screening of the agt10 Xenopus oocyte cDNA library (27) was carried out according to standard protocols under conditions of high stringency (28). For PCR amplification of clone PCR-L1, the following primers were used. The two nested primers based on the 5′-end of clone 463 were: 5′-CA GGG AAA TCA AGC ACG TCA ACC AGC and 5′-CTT CGG CTT TCA TTT GAA GCT TGC GG. The anchor primers used on the basis of sequencing of pGAD10 vector were: 5′-CTA GCC TCT ATA GTA TAA CAT ACC TCC ACA CC (forward primer) and 5′-GGT AAC TGG TGG GCT TTA CTA GTA TCT AG AT (reverse primer).

The entire SF7 insert (3.5 kilobases) was excised from the pGAD10 vector by EcoRI digestion, blunted with Klenow, and inserted into pCS2+-MT vector (29) previously treated with Xhol and Klenow, resulting in an in-frame fusion between the N-terminal Myc tags and xROKα (871–1229). The EcoRI fragment was then ligated into the SalI linearized pGAD10 vector to express a Gal4 fusion protein containing amino acids 871–1229 of xROKα (xROKα). Both constructs were confirmed by DNA sequence analyses of the cloning junction. The plasmids were linearized by Sall digestion, and the linear DNA was used as template to synthesize mRNA using Ambion’s Message Machine kit with SP6 RNA polymerase. Concentrations of mRNAs were estimated by gel electrophoresis with RNA standards of known concentrations (Life Technologies, Inc.).

**Yeast Two-Hybrid Assays**—β-Galactosidase assays were performed according to the protocols provided by CLONTECH. The pAS2-based plasmid and pGAD10-based plasmid were cotransformed into Y1090 (CLONTECH) yeast strain. The transformed yeast cells were plated in SD−Trp−Leu and incubated at 30°C. Filter β-galactosidase assays using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as a substrate were carried out (CLONTECH) when the colonies were 1–2 mm in size (2–3 days after transformation). For quantitative β-galactosidase assays, yeast transformants were individually picked and inoculated to 2 ml of SD−Trp−Leu. When the cell density of the cultures was approximately 1×10^6 cells/ml, they were individually diluted to 6 ml of SD−Trp−Leu. The diluted cultures were incubated for a further 6–10 h (until the A600 reached 1). β-Galactosidase assays were carried out using O-nitrophenyl-β-D-galactopyranoside as a substrate, and the results were expressed in micromoles of O-nitrophenyl-β-D-galactopyranoside hydrolyzed/min calculated according to the CLONTECH protocol.

**Results**—An internal BamHI fragment of SF7 was subcloned into the pGEX-KG (30). The resulting plasmid encoded a glutathione S-transferase fusion protein containing amino acids 871–1229 of xROKα. The fusion protein was expressed in and purified from bacteria and used to immunize rabbits. After four injections with 1-month intervals, the rabbits were bled and the antiserum was used without further processing.

**Other Procedures**—Immunoblot assays using myelin basic protein (MBP) as a substrate were carried out essentially as described in Ref. 31 except the immunoprecipitation was carried out in phosphate-buffered saline lysis buffer. Briefly, oocyte lysates (300 μl containing approximately 1 mg of total protein, usually representing the amount from 20 oocytes) were incubated with 5 μl of preimmune or immune serum for 1 h (4°C) before a further 30-min incubation with protein A-Sepharose. Immunoprecipitates were washed three times with phosphate-buffered saline lysis buffer and once with kinase buffer (50 mM HEPES, pH 7.3, 10 mM MgCl2, 2 mM MnCl2, 1 mM dithiothreitol, 0.05% Triton X-100). The kinase reaction was carried out at room temperature for 30 min in 30 μl of kinase buffer containing 10 μl ATP (10 μCi) and 5 μg of MBP. The kinase reaction was terminated by the addition of SDS-sample buffer. 10 μl of the above reaction was resolved by SDS-PAGE (7.5% for autophosphorylation and 15% for phosphorylation of MBP). The 15% gels were directly dried down for autoradiography to reveal phosphorylation of MBP. The 7.5% gels were transferred to nitrocellulose membrane, autoradiographed, and then subjected to Western blotting with anti-xROKα. The presence of 32P-labeled proteins did not interfere with subsequent detection of immunoreactive proteins because the immunobots were typically developed for less than 10 min, whereas the autoradiography took at least overnight.

For Western blotting, xMAP kinase antisera was provided by J. Cooper (32) and used at a 1:20,000 dilution. Antiserum specific to xROKα (see “Materials and Methods”) and xIRS-1 (19) were used at a 1:500 dilution. For reprobing protein blots, the membrane was first incubated with a buffer containing high concentrations of β-mercaptoethanol (according to a protocol provided with the Amersham ECL kit) to strip the first antibody.

**RESULTS**

**Identification of xROKα as an xIRS-1-binding Protein**—We initially identified 10 positive clones in a yeast two-hybrid screening of about 700,000 colonies using xIRS-1(3–500) as bait. After isolation of the plasmid and retransformation into yeast, four clones still demonstrated bait-dependent activation of the Gal4 promoter. Two of these clones (SF7 and SF107) contained similar 3.5-kilobase inserts. Upon DNA sequence analysis, the two clones were determined to be identical and contained an open reading frame of 499 amino acids, in-frame with the amino-terminal Gal4 sequence. A search of data bases revealed high amino acid sequence identity (90%) with the carboxyl terminus of mammalian ROKα (31, 33, 34) (Fig. 1). SF7 contained the putative Rho binding domain and the carboxyl-terminal pleckstrin/cysteine-rich domain (PH/CRD) (31, 35) (Fig. 1A). Indeed, SF7 interacted strongly with an activated mutant of RhoA (V14-RhoA) (36) in the yeast two-hybrid assay (Fig. 2A). Based upon these criteria, we termed the partial cDNA xROK-C.

To clone the remaining coding region of xROKα, we screened a Xenopus oocyte cDNA library (27) with a 1.1-kilobase probe derived from the 5′-end of SF7. Multiple clones were isolated, and the longest one (clone λ45) was sequenced. This clone contained an additional 1-kilobase coding sequence 5′ to SF7 (Fig. 1A). A subsequent screening of the same phage library using a 500-base pair probe derived from the 5′-end of clone λ45 resulted in the isolation of clone λ63 which, upon sequence analysis, was found to contain the entire kinase domain of xROKα but was still missing about 40 amino acids compared with rat ROKα. The remaining 5′-coding region of xROKα was obtained by PCR amplification from the original CLONTECH pGAD10 library using two nested primers specific to the 5′-end of clone λ63 and the pGAD10 anchor primers (see “Materials and Methods”). xROKα (Fig. 1B) exhibited 82% overall amino acid identity with rat ROKα.
acid sequence identity to ROK\(^a\) (31, 33, 34) and 64% to ROK\(^b\) (31, 37). The amino-terminal kinase domain and the carboxyl-terminal PH/CRD of xROK\(^a\) are particularly similar to those of ROK\(^a\), having 93 and 91% identity, respectively. In contrast, the Rho binding domain exhibited only 76% identity to that of ROK\(^a\) (Fig. 1A).

To delineate the xIRS-1 sequence responsible for binding xROK-C, we subcloned the various regions of xIRS-1 into the FIG. 1. Cloning of xROK\(^a\). Panel A, the various regions of ROK\(^a\) (31) are presented schematically. SF7/SF107 were isolated in a yeast two-hybrid screening using xIRS-1(3–500) as bait. Clones \(l\) and \(l\) were isolated from a \(l\)gt10 Xenopus oocyte cDNA library by hybridization screening. Clone PCR-L1 was isolated from the pGAD10 yeast library (CLONTECH) by PCR using pGAD10 reverse primer and two nested antisense primers based on the 5' sequence of clone \(l\).

\(R\) kinase, serine/threonine kinase domain; \(R\)ho binding domain; \(P\)leckstrin homology/cysteine-rich domain.

Panel B, coding sequence of xROK\(^a\) with the corresponding amino acid sequence (in one-letter code). The **boldface** regions (from amino terminus to carboxyl terminus) correspond to the kinase domain, Rho binding domain, and PH/CRD, respectively.

The binding domain of xROK\(^a\) is composed of the corresponding amino acid sequence (in one-letter code). The **boldface** regions (from amino terminus to carboxyl terminus) correspond to the kinase domain, Rho binding domain, and PH/CRD, respectively.
Fig. 2. xIRS-1 PTB domain binds xROK-C. Panel A, pAS2, pAS2/V14-RhoA, or pAS2 containing the various regions of xIRS-1 was cotransformed with pGAD10/xROK-C. Six colonies (except for V14-RhoA wherein one colony was picked) were picked individually from each cotransformation and assayed separately for β-galactosidase activity. Shown are means of six determinants with standard errors. ONPG, O-nitrophenyl-β-D-galactopyranoside. Panel B, the six colonies used in panel A were pooled and analyzed by Western blotting using anti-hemagglutinin antibodies. Lane 1 was hemagglutinin-tagged xIRS-1(204–1089), which was expressed in insect cells (19). Lanes 2–7 were hemagglutinin-tagged Gal4 DNA binding domain fusion proteins containing the indicated regions of xIRS-1.

Fig. 3. xROK-C binds xIRS-1 in Xenopus oocytes. Panel A, un.injected oocytes (lane 6) or oocytes injected with Myc-tagged xROK-C (lanes 1–5) were either left unstimulated (lanes 1, 2, 4, and 6) or stimulated with insulin for 10 min (lanes 3 and 5). Oocyte lysates (100 μg of total protein, lanes 4–6), or immunoprecipitates (IP) using anti-glutathione S-transferase (GST, lane 1) or anti-xIRS-1 (lanes 2 and 3) were analyzed by SDS-PAGE followed by Western blotting (upper panel). Anti-Myc antibodies were stripped off, and the membrane was reprobed with antibodies against xIRS-1 (lower panel). Panel B, oocytes injected with xROK-C mRNA were lysed. Total lysate (lane 2) or immunoprecipitates using anti-Myc (lane 1) or anti-hemagglutinin (HA, lane 3) were analyzed by SDS-PAGE followed by Western blotting using anti-xIRS-1 antibodies (upper panel). Anti-xIRS-1 antibodies were stripped off, and the membrane was reprobed with anti-Myc antibodies (lower panel).

pAS2 yeast Gal4 DNA binding domain vector and analyzed their ability to bind xROK-C (in pGAD10 Gal4 activating domain vector) in the yeast two-hybrid assay. We employed a β-galactosidase assay in solution (Fig. 2A) for better quantification. Yeast lysates from pooled colonies were also analyzed by anti-hemagglutinin Western blotting (Fig. 2B) to ensure expression of the respective fusion proteins. An activated RhoA (V14-RhoA) (36) interacted strongly with xROK-C, an anticipated result given that xROK-C contained the putative Rho binding domain (Fig. 1A). Among the xIRS-1 subclones, the strongest binding to xROK-C was observed with the original bait (xIRS-1(3–500)), xIRS-1 PTB, and xIRS-1 PTB/SAIN. The control plasmid, pAS2, or pAS2 containing the xIRS-1 PH domain, conferred no detectable binding to xROK-C. Interestingly, xIRS-1 PH/PTB conferred only weak binding (compared with (xIRS-1 PTB) despite a comparable expression of both fusion proteins (Fig. 2B, lanes 4 and 5). The SAIN domain also exhibited weak binding, although the significance of this is less certain because of the large amount of xIRS-1 SAIN expressed in the transformed yeast (Fig. 2B, lane 7). Similarly, the stronger binding of PTB/SAIN compared with that of PTB may be caused by the higher amount of PTB/SAIN expressed (comparing lanes 5 and 6).

xROK-C Binds Endogenous xIRS-1 and Inhibits Insulin Signaling in Xenopus Oocytes—We used mRNA injection in Xenopus oocytes to confirm the xIRS-1-xROKα interaction and to characterize the function of xROKα in insulin signaling. Injection of Myc-tagged xROK-C mRNA (Fig. 3A, lanes 1–5) but not a control RNA (lane 6) into Xenopus oocytes resulted in the production of a protein of approximately 80 kDa (slightly higher than the predicted molecular mass of 70 kDa) which was recognizable by anti-Myc antibodies (Fig. 3A, lanes 4 and 5). Furthermore, immunoprecipitation using an anti-xIRS-1 antibody (lanes 2 and 3) but not a control antibody (anti-glutathione S-transferase lane 1) coprecipitated the Myc-tagged xROK-C. Binding of xROK-C to endogenous xIRS-1 did not appear to be influenced by insulin stimulation (comparing lanes 2 and 3). Reciprocal experiments were also carried out wherein anti-Myc immunoprecipitation of xROK-C coprecipitated endogenous xIRS-1 (Fig. 3B, lane 1). A control antibody...
Rho-kinase Binds IRS-1

TABLE I

Uninjected oocytes or oocytes injected with control or xROK-C mRNA were incubated in OR2 (no hormone) or OR2 containing insulin or progesterone. In the case of v-Ras, the previously injected oocytes were injected further with v-Ras mRNA. After overnight incubation, GVBD was scored. The numbers represent sum of GVBD-positive oocytes/sum of total test oocytes in several independent experiments (numbers indicated within parentheses).

| Treatment          | n   | %     | Control RNA | n   | %     | xROK-C |
|--------------------|-----|-------|-------------|-----|-------|--------|
| No hormone         | 0/115 | 0 (5) | 1/126       | 1 (6) | 0/106 | 0 (5)  |
| Insulin            | 118/127 | 93 (4) | 146/159     | 92 (5)* | 99/197 | 50 (5)* |
| Progesterone       | 43/46  | 93 (2) | 77/82       | 94 (2) | 68/75  | 91 (3) |
| v-Ras              | 54/58  | 93 (2) | 60/63       | 95 (2) | 56/59  | 95 (2) |

* These two were compared by χ² test (p < 0.0001).

FIG. 4. xROK-C blocks insulin-induced Xp42 MAP kinase activation. Following GVBD scoring as presented in Table I, MAP kinase (MAPK) activation was assayed by Western blotting using polyclonal anti-Xp42 MAP kinase antibodies. Both experiments (panels A and B) were repeated several times with similar results. C, no hormone; I, insulin; P, progesterone; R, v-Ras mRNA injection. Each of the three treatments (I, P, and R) induced GVBD (Table I).

(anti-hemagglutinin, lane 3) did not precipitate xROK-C or xIRS-1.

To explore the possible biological effect of xROK-C, we incubated xROK-C mRNA-injected oocytes with insulin overnight and scored for GVBD. Injection of xROK-C, but not of the control mRNA, partially blocked insulin-induced GVBD (Table I). In contrast, neither xROK-C nor the control mRNA affected progesterone-induced GVBD, suggesting that xROK-C specifically blocked insulin signaling. Consistent with previous reports (38), injection of v-Ras mRNA caused GVBD (Table I). Interestingly, v-Ras-induced GVBD was not affected by injection of xROK-C mRNA. These results suggested that xROK-C was acting upstream of Ras and therefore is consistent with the notion that xROK-C interferes with insulin signaling via its interaction with endogenous xIRS-1.

Both insulin and progesterone activate MAP kinase, which precedes GVBD during Xenopus oocyte maturation (39–41). Microinjection of a neutralizing antibody against MAP kinase blocks progesterone-induced MAP kinase activation with a concomitant blockage of progesterone-induced activation of maturation promoting factor and GVBD, suggesting that the MAP kinase cascade lies upstream of and is necessary for, maturation promoting factor and GVBD (42). To determine if insulin-induced MAP kinase activation would be blocked by xROK-C, oocyte extracts (after GVBD scoring) were analyzed for xMAP kinase activation using an anti-xMAP kinase Western blot (32, 40). Microinjection of xROK-C mRNA, but not the control RNA, partially blocked insulin-induced MAP kinase activation (Fig. 4). As expected, MAP kinase activation induced by progesterone (Fig. 4A) or v-Ras (Fig. 4B) was not affected by the xROK-C mRNA injection.

Characterization of Endogenous xROKa—An immunoprecipitation-coupled in vitro kinase assay was performed using either preimmune or xROKa-specific immune serum. Anti-xROKa specifically brought down a protein of >200 kDa, detected both by in vitro kinase assay (Fig. 5A) and by Western blotting (Fig. 5B). In addition, the immunoprecipitated kinase was capable of phosphorylating MBP (Fig. 5C). We did not observe any consistent stimulation of xROKa kinase activity, either by xROKa autophosphorylation or phosphorylation of MBP, in oocytes stimulated with insulin (comparing lanes 2 and 3). The size of the putative xROKa was considerably larger than either the predicted molecular mass of the cloned xROKa (159 kDa) or the reported size of mammalian ROKα (160 kDa) (31). We have recently pasted together all of the subclones (Fig. 1A) for in vitro transcription/translation experiments. The full-length xROKa produced in these experiments was indeed >200 kDa on SDS-PAGE and was recognizable by anti-xROKa serum.²

² R. Booth and X. J. Liu, unpublished observations.
Rho-kinase Binds IRS-1

Members of the Rho subfamily of small monomeric GTP/GDP-binding proteins (including Rho, Rac, and Cdc42) function in mediating growth factor/cytokine-induced actin cytoskeleton reorganization (43). Specifically, Rho (RhoA, B, and C) is involved in growth factor/cytokine-induced focal adhesion/stress fiber formation, Rac is involved in membrane ruffling, and Cdc42 promotes filopodia (44). In addition, these GTPases have been implicated in mediating v-Ras-induced cell transformation (45), transcriptional activation (46, 47), and in mediating integrin-MAP kinase activation (48). ROKα was first identified as a serine/threonine protein kinase capable of binding to GTP-bound RhoA (33). The function of ROKα as an effector of Rho in promoting actin polymerization and focal adhesion formation has been well established (31, 35). It is unclear, however, whether ROKα may play a role in the other functions of Rho.

ROKα is a relatively large protein (160 kDa) consisting of an amino-terminal kinase domain, a central coiled coil domain that also includes the Rho binding domain, and a carboxy-terminal putative PH domain that is split by the insertion of a cysteine-rich motif (31, 37) (Fig. 1A). In the present study, we have identified a novel interaction between the carboxyl terminus of ROKα and the IRS-1 PTB domain. We have also demonstrated that this binding correlated with the inhibition of insulin signaling in Xenopus oocytes. Two important lines of evidence support the specificity of xROK-C-induced inhibition of insulin signaling. First, injection of xROK-C mRNA did not affect progesterone-induced MAP kinase activation or GVBD, suggesting that xROK-C acted in an insulin-specific pathway. Second, injection of xROK-C mRNA did not affect MAP kinase activation or GVBD induced by injection of v-Ras mRNA, suggesting that xROK-C acted upstream of cellular Ras in the insulin signaling pathway. Although it is possible that the PH/CRD domain found at the carboxyl terminus of ROKα may nonspecifically interfere with the function of other PH-containing proteins, notably IRS-1, such a possibility is quite doubtful. Whereas there is limited sequence homology between the PH/CRD of ROKα and the IRS-1 PH domain, the carboxyl-terminal domain has been implicated as one of the binding sites for the PTB domain of IRS-1 (or xIRS-1) (not shown).

The xROK-C binding site was mapped to the PH/CRD domain of IRS-1. This raises the possibility that xROKα-xIRS-1 interaction may interfere with the presumed interaction of the PH domain of IRS-1 with the Xenus protein and insulin-like growth factor I receptor. This provides a potential explanation for the xROKα-mediated inhibition of insulin signaling. Interestingly, a potential serine phosphorylation site (Ser-270) within the PH domain of IRS-1 (or IRS-1) has been implicated in negative regulation of IRS-1 tyrosine phosphorylation and insulin signaling (51, 52). Alternatively, the PTB domain may serve a dual, but sequential, function in linking both Xenus insulin-like growth factor I receptor and xROKα. For example, the PTB domain may mediate a transient interaction between xIRS-1 and the receptor (NPEP γ autophosphorylation site) which results in tyrosine phosphorylation of xIRS-1. Tyrosine-phosphorylated xIRS-1 may interfere with the presumed interaction of the PTB domain with the receptor and xROK-C-induced inhibition of insulin signaling. One of the effects of ROKα is the phosphorylation of the serine/threonine protein kinase ROKα (33, 37) or xROKα. In this regard, xROKα may be a negative regulator of insulin signaling because serine/threonine phosphorylation of IRS-1 has often been implicated in negative regulation of IRS-1 tyrosine phosphorylation and insulin signaling (51, 52). Alternatively, the PTB domain may mediate a transient interaction between xIRS-1 and the receptor (NPEP γ autophosphorylation site) which results in tyrosine phosphorylation of xIRS-1. Tyrosine-phosphorylated xIRS-1 may turn bind xROKα via another PTB-mediated interaction resulting in modulation of xROKα kinase activity and/or subcellular localization. Studies are under way to examine the role of full-length xROKα in insulin signaling.

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