Mutant of Insulin Receptor Substrate-1 Incapable of Activating Phosphatidylinositol 3-Kinase Did Not Mediate Insulin-stimulated Maturation of Xenopus laevis Oocytes*

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Ritsuko Yamamoto-Honda‡, Zen’ichiro Honda§, Kohjiro Ueki‡, Kazuyuki Tobe‡, Yasushi Kaburagi‡, Yoshiko Takahashi‡, Hiroyuki Tamemoto‡, Takeshi Suzuki§, Kohjiro Itoh‡, Yasuo Akanuma‡, Yoshihiko Yazaki‡, and Takashi Kadokawa‡‡

From the ‡Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan, the §Department of Physical Therapy and Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan, and the ¶Institute for Diabetes Care and Research, Asahi Life Foundation, 1-6-1 Marunouchi, Chiyoda-ku, Tokyo 100, Japan

Insulin receptor substrate-1 (IRS-1) is rapidly phosphorylated on multiple tyrosine residues in response to insulin and binds several Src homology 2 domain-containing proteins, thereby initiating downstream signaling. To assess the tyrosine phosphorylation sites that mediate relevant downstream signaling and biological effects, we created site-directed mutants of IRS-1 and overexpressed them in the Xenopus laevis oocyte. In oocytes overexpressing IRS-1 or IRS-1–895F (Tyr-895 replaced with phenylalanine), insulin activated phosphatidylinositol (PI) 3-kinase, p70 S6 kinase, and mitogen-activated protein kinase and induced oocyte maturation. In contrast, in oocytes overexpressing IRS-1–4F (Tyr-460, Tyr-608, Tyr-939, and Tyr-987 of IRS-1 replaced with phenylalanine), insulin did not activate PI 3-kinase, p70 S6 kinase, and mitogen-activated protein kinase and failed to induce oocyte maturation. These observations indicate that in X. laevis oocytes overexpressing IRS-1, the association of PI 3-kinase rather than Grb2 (growth factor-bound protein 2) with IRS-1 plays a major role in insulin-induced oocyte maturation. Activation of PI 3-kinase may lie upstream of mitogen-activated protein kinase activation and p70 S6 kinase activation in response to insulin.

After stimulation with insulin, insulin receptor, a receptor tyrosine kinase, is activated and phosphorylates several signaling molecules including insulin receptor substrate-1 (IRS-1). The tyrosine-phosphorylated proteins become associated with several Src homology 2 domain-containing proteins (SH2 proteins) and thereby transduce downstream signals (1). IRS-1 is a substrate for insulin and IGF-1 receptors and is known to associate with several SH2 proteins, including PI 3-kinase and Ash/Grb2, through distinct phosphorylation sites (2–5). Evidence has been shown to demonstrate the importance of IRS-1 on the pleotropic effects of insulin, such as mitogenesis of 32D cells and maturation of Xenopus laevis oocyte (6, 7). IRS-1-deficient mice exhibit growth retardation and insulin resistance (8, 9). IRS-1 has multiple tyrosine phosphorylation sites, some of which could be redundantly associated with the same SH2 proteins, and it is not clear which phosphorylation sites of IRS-1 mediate relevant downstream signaling and biological effects. Furthermore, ubiquitous expression of IRS-1 makes it difficult to examine the functions of overexpressed IRS-1 and its mutated forms. In the present study, we created site-directed mutants of IRS-1 in which Tyr-895 or a group of four tyrosine residues (Tyr-460, Tyr-608, Tyr-939, and Tyr-987) was replaced with phenylalanine (IRS-1–895F and IRS-1–4F, respectively) and overexpressed them in unprimed the X. laevis oocyte. This system is suitable to study functions of IRS-1 and its mutants, since X. laevis oocytes possess receptors for IGF-1 and signaling molecules such as Ash/Grb2, p85 subunit of PI 3-kinase, Sos, and She-like proteins (10, 11), while the level of endogenous IRS-1 expression is very low (7). Thus, overexpressed IRS-1 is presumed to amplify signals evoked by IGF-1 or by high concentrations of insulin to downstream effectors. We found that IRS-1 overexpression in oocyte actually augmented PI 3-kinase activation in response to insulin, whereas IRS-1–4F expression did not. Overexpressing IRS-1–4F did not augment the activities of downstream signals including MAP kinase, p70 S6 kinase, and maturation of oocytes. These observations suggest that PI 3-kinase activity associated with IRS-1 is necessary for these signaling events. We also noted that oocytes overexpressing IRS-895F, a putative Ash/Grb2 binding site mutant, efficiently transmit signals leading to oocyte maturation, suggesting that the association of overexpressed IRS-1 with Ash/Grb2 may play a minor role in these signals.

EXPERIMENTAL PROCEDURES

Materials—Type II collagenase and protein kinase A inhibitors were from Sigma. Glutathione-Sepharose beads were from Pharmacia Biotech Inc. All other materials were obtained from the same source as described (12, 13).

Plasmids—To prepare mutant IRS-1s, Tyr-460, Tyr-608, Tyr-895, a group of two tyrosine residues (Tyr-939 and Tyr-987), or a group of four tyrosine residues (Tyr-460, Tyr-608, Tyr-939, and Tyr-987) was substituted for phenylalanine (IRS-1–460F, IRS-1–460F, IRS-1–895F, IRS-1–895F, or IRS-1–4F, respectively). Normal and mutant rat IRS-1 cDNA was subcloned into pCS2+ (kindly provided by Dr. Dave Turner, Fred Hutchinson Cancer Research Center).

Preparation of Oocytes and RNA Injection—Stage VI oocytes were treated with collagenase and manually defolliculated as described pre-
visously (12, 14). Plasmids were linearized with NotI located down-stream of the coding regions, and cRNA was synthesized in vitro as described (12). cRNA was dissolved in distilled water at 50 μg/ml and 50 nl was injected into the cytoplasm of stage VI oocytes. Controls were injected with distilled water. Oocytes were incubated in modified Barth solution (MBS: 0.82 mM MgSO4, 0.41 mM CaCl2, 1 mM KCl, 0.33 mM NaClO4, 88 mM NaCl, 2.4 mM NaHCO3, 10 mM Hepes-NaOH buffer, pH 7.8; 472 h.

Immunoblotting—cIRS-1 (1–6), polyclonal antibodies kindly pro-vided by Dr. Masaki Nishiyama (Jikei University School of Medicine, Tokyo, Japan), were raised against the epitopes corresponding to resi-dues 778-1243 of rat IRS-1 (15). Fifty oocytes microinjected with cRNAs were lysed, the lyses were centrifuged at 15,000 × g for 15 min at 4 °C to remove nuclei and yolk granules, and clear supernatant was ob-tained. Supernatants were incubated with or without 10 μg/ml aprotinin, 20 μg/ml leupeptin, 20 mM MgCl2, 10 μM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 10 μg/ml soybean trypsin inhibitor. After centrifugation, the supernatant was incubated with cIRS-1, and the clear supernatant was obtained by centrifugation. GST fusion protein bound to glutathione-Sepharose beads was incubated with the supernatant of oocytes treated with or without 10 μg insulin for 30 min. The beads were washed, and the bound proteins were eluted by boiling in Laemmli's sample buffer (3). The samples were subjected to SDS-PAGE followed by immunoblotting with a cIRS-1. The primary antibody was probed with anti-rabbit antibody conjugated to horseradish peroxidase antibody, and immunoreactive bands were visualized by a chemiluminescence system (ECL Western blotting analysis system, Amersham Life Science Inc.).

Binding of Ash/Grb2 in Vitro—Glutathione S-transferase (GST) fusion protein of Ash/Grb2 containing amino acids 15–217 of Ash/Grb2 (16) was kindly provided by Dr. Tadaomi Takenawa. Oocytes were lysed with a buffer (20 mM Tris-HCl, pH 7.5, 2 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 10 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 20 μg/ml aprotinin), and the clear supernatant was obtained by centrifugation. GST fusion protein bound to glutathione-Sepharose beads was incubated with the supernatant of oocytes treated with or without 10 μg insulin for 30 min. The beads were washed, and the bound proteins were eluted by boiling in Laemmli's sample buffer (3). The samples were subjected to SDS-PAGE followed by immunoblotting with a cIRS-1 as described in the previous section.

PI 3-Kinase Assay—After microinjection with cRNA, oocytes were treated with or without 10 μg insulin at 19 °C for 10 min. Oocytes were lysed with buffer and the clear supernatant was obtained by centrifugation. Tyrosine-phosphorylated proteins were recovered in α-FY immunoprecipitates, and PI 3-kinase activity in the immunoprecipitates was measured by in vitro phosphorylation of phosphatidylinositol as described (13).

MAP Kinase Assay—MAP kinase activity was measured according to the method of Itoh et al. (17, 18). After microinjection with cRNA, 10 oocytes were treated with or without 10 μg insulin at 19 °C for 20 h and lysed with a buffer containing 20 mM Tris-HCl, pH 8.0, 10 mM EGTA, 5 mM MgCl2, 1 mM diithiothreitol, 1 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml β-glycerophosphate, 0.1 mM NaF, and 1 mM sodium orthovanadate. The lyses were centrifuged and the clear supernatant was obtained. To measure MAP kinase activity, 5 μl of the extract was mixed with 40 μl of kinase solution (25 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM diithiothreitol, 40 μM [32P]ATP, 2 μM protein kinase A inhibitor, 0.5 mM EGTA) and 5 μl of myelin basic proteins (5 mg/ml). Reactions were performed for 10 min at 19 °C and terminated by addition of 0.6% HCl, 1 mM ATP, and 1% bovine serum albumin. The samples were spotted onto p81 phosphocellulose paper, and the papers were washed with 0.5% phosphoric acid and counted for radioactivity.

Oocyte Maturation and GVBD—After microinjection with cRNA, oocytes were incubated with or without 10 μg insulin for 20 h. Oocytes were scored for the presence of GVBD by the appearance of a white spot on the animal pole of the oocyte.

p70 S6 Kinase Activity in Immune Complex—The epitope of the antibody against p70 S6 kinase was the conserved residues 2–30 of human, rat, and X. laevis p70 S6 kinase (19) (Upstate Biotechnology Inc., Lake Placid, NY.). After microinjection with cRNA, oocytes were treated with or without 10 μg insulin at 19 °C for 1 h and then lysed with a buffer containing 75 mM β-glycerophosphate, pH 7.5, 10 mM MgCl2, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 μg protein kinase inhibitor, and 5 μg/ml leupeptin. After the removal of nuclei and yolk granules by centrifugation, the clear supernatant was incubated with the antibody, and the immune complex was recovered with the aid of protein A-Sepharose. p70 S6 kinase activity was measured in the immunoprecipitate as described previously (20).

RESULTS

Expression and Characterization of Wild-type and Mutant IRS-1—Injected cRNAs for wild-type and mutant rat IRS-1s were efficiently transcribed into IRS-1 proteins (Fig. 1A and 1C, lower panel). We first confirmed decreased binding of IRS-1–895F to Ash/Grb2. As shown in Fig. 1A (upper panel), insulin-dependent binding of wild-type IRS-1 to GST-Ash/Grb2 was demonstrated, but IRS-1–895F failed to bind to GST-Ash/Grb2. Next we examined PI 3-kinase activity in the α-FY immunoprecipitate. PI 3-kinase activity in response to insulin was elevated in oocytes injected with IRS-1 cRNA and in oocytes injected with IRS-1–895F cRNA as compared with oocytes injected with distilled water. PI 3-kinase activity in oocytes injected with IRS-1–4F cRNA was almost comparable in control oocytes injected with distilled water (Fig. 1B). Insulin-induced tyrosine phosphorylation of overexpressed IRS-1 was detectable by immunoblot with α-FY, while insulin-induced tyrosine phosphorylation of IRS-1–4F was not detectable (Fig. 1C, upper panel).

MAP Kinase Activity—Previous studies have demonstrated that almost all of the MBP kinase activity in total cell lysate represented MAP kinase activity (17, 18). Therefore, we measured MAP kinase activity as MBP kinase activity in total cell lysates. As shown in Fig. 2, MBP kinase activity was increased in response to insulin in the oocytes overexpressing IRS-1. In a representative study, insulin treatment increased MBP kinase activity (7.77 fmol/min/oocyte) as compared to basal activity (1.48 fmol/min/oocyte) in oocytes overexpressing IRS-1. Under the same experimental conditions, MBP kinase activity is increased in response to insulin by 1.24-, 8.07-, 9.95-, and 9.23-fold in oocytes injected with distilled water, IRS-1 cRNA, IRS-1–4F cRNA, and IRS-1–895F cRNA, respectively.

Oocyte Maturation in Response to Insulin—In unprimed oo-ocytes injected with water or with IRS-1–4F cRNA, GVBD in response to insulin was observed in only 14.7 or 10.0%, respectively; under the same conditions more than 90% of oocytes matured in response to progesterone (data not shown). In contrast, IRS-1 cRNA and IRS-1–895F cRNA expression augmented insulin-induced GVBD to 83.3 and 85.7%, respectively (Fig. 3). In oocytes microinjected with IRS-1–4F cRNA and IRS-1–895F cRNA, insulin-induced GVBD was observed in 66.7, 53.6, and 60.0%, respectively.

DISCUSSION

In the present study, we attempted to evaluate the relative importance of IRS-1 phosphorylation sites that could bind PI 3-kinase or Ash/Grb2 for downstream signals by comparing the signaling activities of wild-type IRS-1, mutated IRS-1 unable to activate PI 3-kinase, and that unable to bind to Ash/Grb2. This strategy enabled us to specify signals derived from IRS-1 and to assess the roles of each phosphorylation site of IRS-1 on downstream signaling.

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Mutant IRS-1 were treated with or without 10^{-5} M insulin for 20 h. The lysates were centrifuged to obtain clear lysate. MAP kinase activities were assayed as described under "Experimental Procedures." The results were converted to the ratio against the unstimulated kinase activity of oocyte injected with water and were the means ± S.D. of three independent assays in duplicate.

In the X. laevis oocyte, insulin-induced maturation was achieved either by pretreatment with gonadotropin (priming) or by overexpressing IRS-1 to unprimed oocytes (7). The present study was undertaken to define mechanisms of overexpressed IRS-1 that could induce maturation in unprimed X. laevis oocytes. Previous studies have shown the importance of p21^ras^-MAP kinase cascade in insulin-induced oocyte maturation (21). Activation of p21^ras^ is necessary (22, 23) and sufficient (24) for insulin-induced germinal vesicle breakdown. MAP kinase cascade lies downstream of p21^ras^.

Recent experiments exhibited that MAP kinase activation is also necessary (25, 26) and sufficient (27–29) to induce oocyte maturation. Ash/Grb2 is one of the SH2 proteins associated with IRS-1 or Shc that mediates p21^ras^ activation through Sos in mammalian cells (2, 3, 30). Previous studies showed that microinjection of the SH2 domain of Ash/Grb2 inhibited insulin-induced X. laevis oocyte maturation (36). Here we show that overexpressed IRS-1–895F, a mutant that could not bind Ash/Grb2 (4), was able to transmit signals to MAP kinase activation and oocyte maturation as well as overexpressed IRS-1. It could be that in oocytes overexpressing IRS-1–895F insulin-induced signals might well be compensated by interaction between Ash/Grb2 and phosphoproteins other than IRS-1, such as Shc (31). Although at present reports on X. laevis Shc-like protein are limited (11) and cDNA encoding this protein has not been reported, the role of Shc in insulin-induced maturation of X. laevis oocytes should be investigated in future experiments. In 32D cells, overexpressed IRS-1–895F was reported to affect insulin-induced mitogenesis and MAP kinase activation (4). The discrepancy between our observations and the results in 32D cells could be that the sets of phosphoproteins that bound to Ash/Grb2 might be different in these cells.

Insulin-induced activation of PI 3-kinase also contributes to MAP kinase activation in various degrees in both p21^ras^-dependent and -independent pathways (31–33). Recently, the importance of PI 3-kinase in p21^ras^-dependent oocyte maturation has also been proposed. Constitutive active mutant of p85-p110 fusion subunit of PI 3-kinase was able to induce oocyte maturation, and the oocyte maturation was inhibited by dominant negative p21^ras^ (34), indicating that activation of PI
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3-kinase seems to be sufficient for oocyte maturation and that PI 3-kinase may act upstream of p21ras. Activation of PI 3-kinase was also necessary for insulin-induced germinal vesicle breakdown, since pretreatment with wortmannin (35) or microinjected fusion protein for SH2 domains of p85α subunit of PI 3-kinase (36, 37) abolished this process. However, these results should be interpreted with caution. Wortmannin could also inhibit the other isoforms that did not bind to tyrosine-phosphorylated Tyr-X-X-Met motifs. Microinjection of SH2 domains of PI 3-kinase might inhibit interaction of p85 subunit of PI 3-kinase with molecules other than IRS-1, namely insulin receptor, p21ras, pp60, or other unidentified molecules (11–14, 38). Therefore, it was difficult to conclude that PI 3-kinase activity associated with overexpressed IRS-1 was crucial in insulin-induced oocyte maturation. To circumvent these issues and to examine more directly the contribution of PI 3-kinase associated with IRS-1, we overexpressed wild-type IRS-1 and IRS-1 mutated at possible binding sites for PI 3-kinase (IRS-1–4F) in the X. laevis oocyte. We found that IRS-1–4F failed to stimulate PI 3-kinase, MAP kinase, and oocyte maturation in response to insulin. The following conclusions can be drawn from the results presented in this paper. First, as predicted by cell-free experiments (39, 40), we proved that Tyr-X-X-Met motifs of some of Tyr-460, -608, -939, and -987 of IRS-1 might be necessary for association and full activation of PI 3-kinase in intact cell systems. Similar observations that insulin-induced activation of PI 3-kinase was impaired in 293 cells overexpressed with IRS-1 mutated at Tyr-608 were recently reported by Rocci et al. (41). Under our present experimental conditions, in which a high concentration of insulin is required to induce effects, IRS-1–4F failed to activate PI 3-kinase in response to insulin. However, a significant amount of PI 3-kinase activity in response to insulin was observed when IRS-1–4F was overexpressed to primary hepatocytes. Thus in insulin-sensitive tissues, Tyr-X-X-Met motifs other than Tyr-460, -608, -939, and -987 of IRS-1 might bind and activate PI 3-kinase in response to insulin. As the present study does not address whether Tyr-X-X-Met motifs of Tyr-460, -608, -939, and -987 of IRS-1 are sufficient for activating PI 3-kinase, PI 3-kinase activity of added-back IRS-1 mutant possessing only Tyr-X-X-Met motifs should next be examined. Second, the interaction between some of Tyr-X-X-Met motifs of Tyr-460, -608, -939, and -987 of IRS-1 and the SH2 proteins bound to these sites was required for insulin-induced activation of MAP kinase and oocyte maturation. Molecules that bound to tyrosine-phosphorylated IRS-1 reported to date include p85 subunit of PI 3-kinase (4, 5), Ash/Grb2 (2, 3), SHPTP2 (42), p53(PIK) (40), and Nck (43). Although Nck was reported to bind to Tyr-751 of platelet-derived growth factor receptor (44), one of the binding sites of p85 subunit of PI 3-kinase, only p85 subunit of PI 3-kinase and p53(PIK), a variant form of the regulatory subunit of PI 3-kinase, are able to bind to phosphopeptides containing Tyr-460, -608, -939, or -987 of IRS-1 (39, 40). Thus the present observation suggested that activation of IRS-1-associated PI 3-kinase was the upstream mechanism for activation of p21ras-MAP kinase cascade and induction of oocyte maturation. In the present system, however, the possible interaction of any one of the four Tyr-X-X-Met motifs with some unidentified SH2 domain-containing molecules cannot be completely ruled out. To examine the possibility that phosphorylation of any one of the Tyr-X-X-Met motifs could be required for oocyte maturation, we carried out experiments for insulin-induced maturation of oocytes overexpressing IRS-1–460F, IRS-1–608F, or IRS-939F/987F. The result indicated that not any one of the four Tyr-X-X-Met motifs played a predominant role in oocyte maturation. Finally, Yamauchi and Pessin reported that overexpressed IRS-1 resulted in a concomitant reduction of Ash/Grb2 associated with Shc and inhibited insulin signaling (45). These observations hence raised the possibility that overexpression of mutant IRS-1 could somehow affect the insulin signaling of endogenous IRS-1-independent pathway. However, differences in IRS-1-induced and mutated IRS-1-induced signaling are most likely caused by the mutation itself, since overexpression of wild-type IRS-1 might also affect this putative IRS-1-independent pathway in a similar fashion.

p70 S6 kinase is known to be involved in G1 progression (46) and protein synthesis by phosphorylating ribosomal protein S6 and translation initiation factors in mammalian cells (47–49). In 32D cells, overexpression of IRS-1 stimulated p70 S6 kinase activity in response to insulin, suggesting that IRS-1-mediated signals are required for p70 S6 kinase activation (6). p70 S6 kinase seems to lie downstream of one or some of the PI 3-kinase family molecules, because it is activated by autoactive fusion subunit of p85-p110 PI 3-kinase (50) and inhibited by wortmannin and LY294002, inhibitors of PI 3-kinase family molecules (51, 52). To examine more directly the contribution of Tyr-X-X-Met motifs of IRS-1 on insulin-induced p70 S6 kinase activation, we analyzed p70 S6 kinase activity in oocytes overexpressing normal and mutant IRS-1s. We observed that insu-
lin-induced p70 S6 kinase activity was increased by overexpression of IRS-1 but not by that of IRS-1–4F (Fig. 4). These results indicated that interaction between some of the Tyr-460, -608, -939, and -987 of IRS-1 and the SH2 proteins bound to these sites, most likely PI 3-kinase, was necessary for insulin-induced activation of p70 S6 kinase in this system. Recently overexpression of truncated mutant of p85 subunit of PI 3-kinase induced activation of p70 S6 kinase in this system. Recently overexpression of truncated mutant of p85 subunit of PI 3-kinase was associated with IRS-1.

In conclusion, MAP kinase activation, p70 S6 kinase activation, and oocyte maturation in response to insulin appears to link with PI 3-kinase, which was associated with IRS-1.

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