Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5172–5175, June 1993
Cell Biology

Insulin receptor substrate 1 mediates insulin and insulin-like growth factor I-stimulated maturation of *Xenopus* oocytes

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Communicated by William H. Daughaday, February 22, 1993 (received for review December 7, 1992)

ABSTRACT **Insulin and insulin-like growth factor I (IGF-I) initiate cellular functions by activating their homologous tyrosine kinase receptors. In most mammalian cell types, this results in rapid tyrosine phosphorylation of a high-molecular-weight substrate termed insulin receptor substrate 1 (IRS-1). Previous studies suggest that IRS-1 may act as a "docking" protein that noncovalently associates with certain signal-transducing molecules containing src homology 2 domains; however, direct evidence for the role of IRS-1 in the final biological actions of these hormones is still lacking. We have developed a reconstitution system to study the role of IRS-1 in insulin and IGF-I signaling, taking advantage of the fact that *Xenopus* oocytes possess endogenous IGF-I receptors but have little or no IRS-1, as determined by immunoblotting with anti-IRS-1 and antiphosphotyrosine antibodies. After microinjection of IRS-1 protein produced in a baculovirus expression system, tyrosyl phosphorylation of injected IRS-1 is stimulated by both insulin and IGF-I in a concentration-dependent manner, with IGF-I more potent than insulin. Furthermore, after IRS-1 injection, both hormones induce a maturation response that correlates well with the amount of injected IRS-1. By contrast, overexpression of human insulin receptors in the *Xenopus* oocytes does not enhance either IRS-1 phosphorylation or oocyte maturation response upon insulin stimulation. These results demonstrate that IRS-1 serves a critical role in linking IGF-I and insulin to their final cellular responses.**

Insulin and insulin-like growth factor I (IGF-I) induce a wide spectrum of growth and metabolic responses in many cell types. Both peptides initiate their biological effects by activation of receptors containing protein-tyrosine kinase activity (1–4). Several putative substrates for the activated receptors have been identified (5–9), the best characterized of which is insulin receptor substrate 1 (IRS-1), a high-molecular-weight cytoplasmic protein (10–14). IRS-1 in its phosphorylated form has been shown to bind to and activate the enzyme phosphatidylinositol 3-kinase (12, 15); however, as with other growth factor-receptor tyrosine kinases, there has been no direct demonstration that phosphorylation of this or other substrates is linked to the final downstream biological effects of the hormone.

Xenopus oocytes possess endogenous IGF-I receptors, and after gonadotropin priming insulin and IGF-I have been shown to stimulate oocyte maturation as manifested by germinal vesicle breakdown (GVB) and an increase in activity of several cellular enzymes (16–21). These effects can be blocked by microinjection of antiphosphotyrosine antibodies or phosphotyrosine phosphatases, suggesting that tyrosine phosphorylation events play an important role in insulin/IGF-I-stimulated oocyte maturation (22, 23). Although *Xenopus* oocytes from unprimed frogs possess IGF-I receptors, they lack detectable IRS-1 and, thus, represent an ideal system to evaluate the role of IRS-1 in oocyte maturation. We have created a reconstitution system by microinjection of IRS-1 protein produced in a baculovirus expression system into unprimed *Xenopus* oocytes. We find that the microinjected IRS-1 undergoes tyrosyl phosphorylation in response to insulin and IGF-I, and this leads to a hormonal stimulation of the oocyte maturation response.

MATERIALS AND METHODS

Oocyte Preparation and Microinjection. Stage VI oocytes were isolated from unprimed gravid *Xenopus laevis* females (Nasco, Fort Atkinson, WI) by mild collagenase treatment (35). The oocytes were incubated at 19°C in a modified Barth's saline solution (MBS) containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes·NaOH, pH 7.6, with streptomycin (10 μg/ml) and penicillin (10,000 international units per ml) overnight and then microinjected with 50 nl of either buffer (125 mM NaCl/25 mM Tris·HCl, pH 7.6) alone or buffer containing IRS-1 protein.

Recombinant IRS-1 was produced in a baculovirus expression system using insect cells infected with pBlueBac (Invitrogen, San Diego) containing rat liver IRS-1 cDNA (12). The protein was purified by gel filtration on Sephacryl S-300 HR (Pharmacia). Analysis by SDS/PAGE revealed that the protein was purified to 90% homogeneity (15).

In some experiments human insulin receptors were expressed in *Xenopus* oocytes. The human insulin receptor complementary RNA was produced from the pBluescript plasmid containing human insulin receptor cDNA (HIRc) (25) by a T3 RNA polymerase reaction using *in vitro* transcription and capping system (Stratagene). Expression of the receptors was observed after microinjection of 50 ng of complementary RNA per oocyte.

Oocyte Extract Preparation and Immunoblotting. After microinjection of IRS-1 protein and/or insulin receptor mRNA and preincubation as indicated in the figure legends, groups of oocytes were incubated in the absence or presence of insulin or IGF-I. Oocytes were then homogenized in ice-cold extraction buffer (20 mM NaCl/10 mM Tris·HCl/2 mM EDTA/1% Nonidet P-40/1% deoxycholate/0.1% SDS/aprotinin at 0.1 mg/ml/leupeptin at 10 μg/ml/2 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/1 mM sodium orthovanadate) by pipetting up and down on ice. Soluble protein extract was collected after centrifugation for 5 min at 12,000 × g in a microcentrifuge. Clear extracts were subjected to SDS/PAGE with Laemmli buffer containing 100

Abbreviations: IRS-1, insulin receptor substrate 1; IGF-I, insulin-like growth factor I; GVB, germinal vesicle breakdown; HIRc, human insulin receptor cDNA.

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mM dithiothreitol and transferred to nitrocellulose membrane. Specific protein bands were recognized by ^{125}I-labeled protein A binding and autoradiography (11). The amount of protein was quantitated by laser densitometric analysis using IMAGEQUANT software (Molecular Dynamics).

Antiphosphotyrosine antibody was raised as described in ref. 11. Rabbit anti-C-terminal IRS-1 antibody (raised against the peptide sequence TYASINFQKQPEDRQ from the C terminus of rat liver IRS-1) was protein A affinity-purified and used at 0.3 μg/ml.

Oocyte Maturation and GVB. Stage VI oocytes were isolated and incubated in MBS overnight at 19°C. After microinjection with different amounts of IRS-1 protein, oocytes were incubated for an additional 3 hr and then transferred to medium containing the appropriate concentrations of insulin or IGF-I. After 20-hr incubation, groups of 15–20 oocytes were scored for the presence of GVB by the appearance of a white spot on the animal pole of the oocyte. In some experiments, GVB was confirmed by the microscopic absence of nuclear membrane after fixing oocytes in 5% trichloroacetic acid for 30 min. In experiments with HIRc-injected oocytes, 3 days were allowed for expression of the insulin receptor protein (as confirmed by immunoblotting); then the protocol was continued as above with IRS-1 injected and oocytes stimulated with insulin/IGF-I for GVB.

RESULTS

Endogenous Oocyte IGF-I Receptors Phosphorylate Microinjected IRS-1 Protein on Tyrosine. When oocytes were incubated with 10 μM insulin, there was a rapid stimulation of tyrosine phosphorylation of two protein bands of 105 kDa and 82 kDa, as detected in antiphosphotyrosine immunoblots (Fig. 1, lanes b and d). These two bands represent the β subunit of the endogenous IGF-I receptor and are consistent with the previous reports that in these cells the IGF-I receptor migrates as a doublet (16). A protein band of 170 kDa reactive with antiphosphotyrosine antisera was also observed in the basal state. However, the phosphorylation of this protein was independent of insulin (Fig. 1, lanes a and b) or IGF-I stimulation (data not shown). In addition, this 170-kDa protein was not recognized by either antibodies to the whole baculovirus-produced IRS-1 protein or C- or N-terminal peptide sequences (Fig. 1, lanes e and f). These data suggest that IRS-1 expression in the unprimed *Xenopus* oocytes is very low.

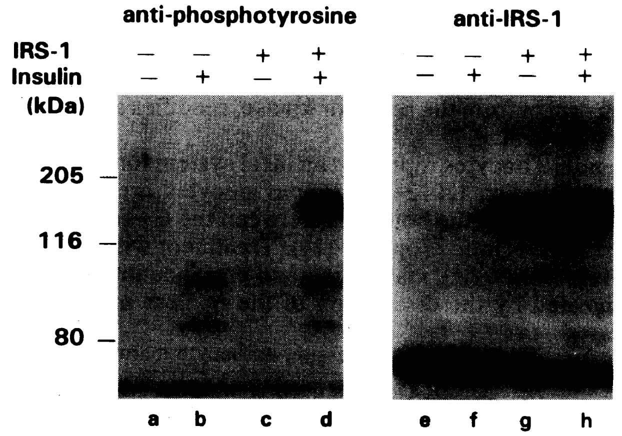

FIG. 1. Effect of endogenous IGF-I receptors on tyrosine phosphorylation of microinjected IRS-1 protein. *Xenopus* oocytes were isolated and microinjected with (+) and without (–) IRS-1 protein at 12.5 ng per oocyte. After stimulation with (+) or without (–) 10 μM insulin for 10 min, oocytes were extracted, and the proteins were resolved by SDS/PAGE under reducing conditions, as described. The position of molecular size markers (kDa) is indicated at left.

After microinjection of baculovirus produced IRS-1, IRS-1 was easily detected by the anti-C-terminal IRS-1 antibody as a doublet band at ≈160–180 kDa (Fig. 1, lanes g and h). The doublet appearance of the baculovirus-expressed IRS-1 may represent some limited amount of proteolysis or heterogeneity of migration due to serine and threonine phosphorylation of the protein in the insect cell system. Insulin stimulation resulted in rapid phosphorylation of the exogenous IRS-1 protein on tyrosine residues as shown in antiphosphotyrosine immunoblots (Fig. 1, lane d). There was also a slight retardation in the mobility of the IRS-1 protein consistent with its increased phosphorylation state (Fig. 1, lane h).

IGF-I and insulin stimulated phosphorylation of injected IRS-1 protein, as well as autophosphorylation of IGF-I receptors, in a dose-dependent way (Fig. 2). When compared with insulin, IGF-I was ≈100-fold more potent in stimulation of receptor phosphorylation, and with both ligands there was a very close correlation between stimulation of receptor autophosphorylation and substrate phosphorylation. These data suggest that insulin-stimulated IRS-1 phosphorylation in *Xenopus* oocytes is mediated through IGF-I receptors, and that IGF-I receptors share the same signaling pathway of insulin receptor in terms of tyrosyl phosphorylation of IRS-1.

Effect of IRS-1 on Insulin-Stimulated GVB Response. The *in vitro* oocyte maturation response varies with different preparations of the frogs and buffer systems. In our hands, using oocytes isolated from the frogs that have not been subject to gonadotropin priming, neither insulin nor IGF-I induces an oocyte maturation response, although the maturation response to progesterone remains intact. When oocytes were microinjected with IRS-1 protein, the maturation response to insulin, as assayed by GVB, increased with the amount of

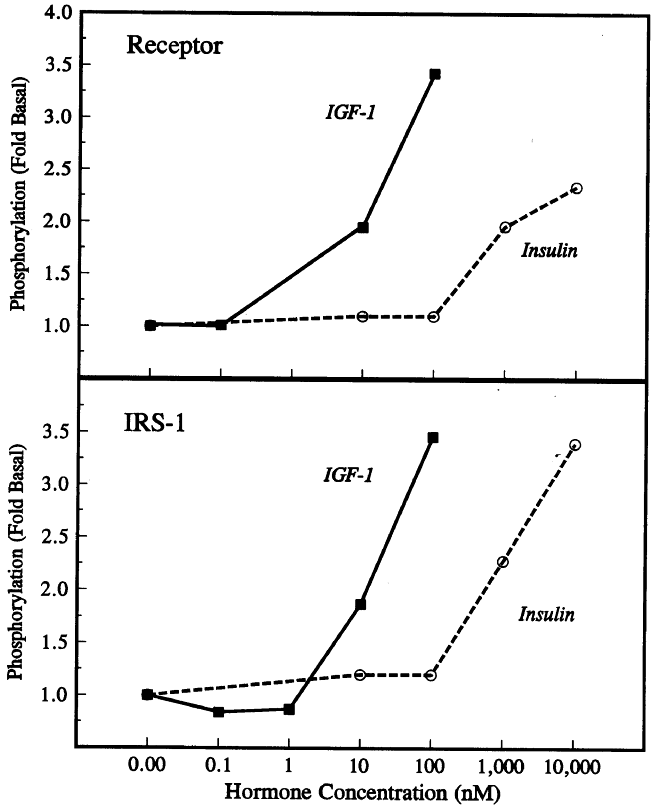

FIG. 2. Concentration-dependence of insulin and IGF-I on tyrosine phosphorylation of β subunit of IGF-I receptors and microinjected IRS-1 protein. Oocytes (each injected with 12.5 ng of IRS-1 protein) were stimulated with IGF-I and insulin at different concentrations for 10 min. The bands corresponding to receptor β subunit and IRS-1 in the antiphosphotyrosine immunoblots were quantitated by a scanning densitometer. ■, Stimulation by IGF-I; ○, stimulation by insulin.

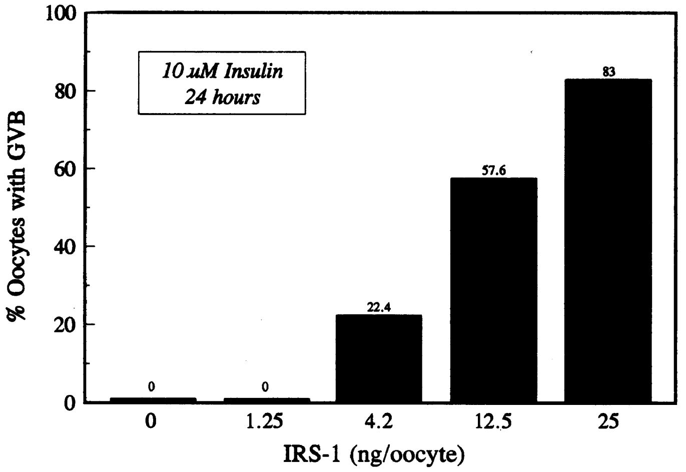

FIG. 3. Effect of IRS-1 on insulin-stimulated GVB response. Insulin (10 µM)-stimulated maturation response was seen in oocytes microinjected with various amounts of IRS-1 protein. GVB was observed in each group of 15–20 oocytes after 20-hr incubation. Data represent the means of two to four separate experiments.

injected IRS-1 protein (Fig. 3). Similarly prepared control extracts from uninfected Sf9 cells or wild-type virus infected-Sf9 cells had no effect (data not shown), indicating that the reconstitution of insulin/IGF-I action was due to the IRS-1 protein rather than some minor contaminant of the preparation. Furthermore, the insulin and IGF-I-induced maturation responses occurred in a concentration-dependent manner in the oocytes that were microinjected with IRS-1 protein (Fig. 4). The ED_{50} of IGF-I for this effect was ≈100-fold more potent as compared with that of insulin (Fig. 4). The dose–response curves of IGF-I and insulin on receptor and substrate phosphorylation coincide with the dose–response curves of IGF-I and insulin-induced oocyte maturation (compare Figs. 2 and 4).

Effect of Human Insulin Receptor on IRS-1 Phosphorylation and the GVB Response. To study the effect of increased expression of insulin receptors on IRS-1 phosphorylation and the GVB response, *Xenopus* oocytes were microinjected with insulin receptor complementary RNA. Maximal expression of the receptor, as judged by immunoblotting with antireceptor antibody, occurred ≈3 days after microinjection of 50 ng per oocyte. Expression of the insulin receptor could also be detected as an increase in phosphorylation of the 95-kDa β subunit (Fig. 5, lane d). There was also a slight increase in sensitivity of the dose response of phosphorylation of injected IRS-1 (data not shown). In contrast to the effect of microinjecting IRS-1, however, increased insulin receptor expression had little effect on maximal phosphorylation of IRS-1 (Fig. 5, lane d) and did not enhance the ability of insulin to induce the oocyte maturation response (Table 1).

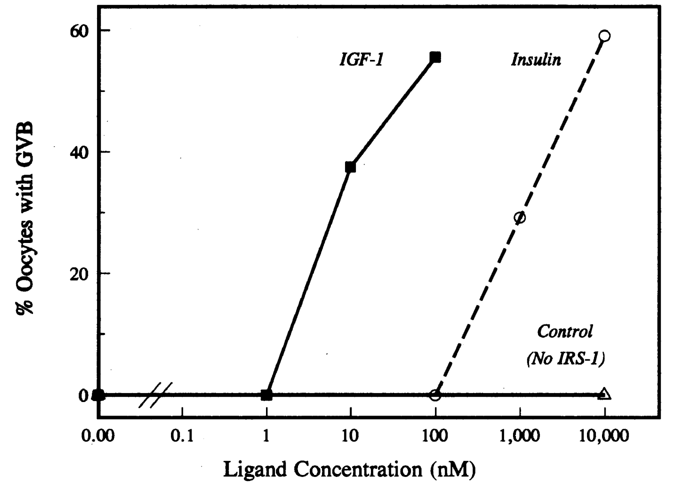

FIG. 4. Concentration dependency of insulin/IGF-I-stimulated GVB response. Oocytes microinjected with 12.5 ng of IRS-1 each were stimulated with various concentrations of IGF-I (■) and insulin (○). Oocytes without microinjection of IRS-1 (△) did not respond to insulin treatment up to 10 µM. GVB response was seen in each group of 15–20 oocytes. Data represent the means of two to four separate experiments.

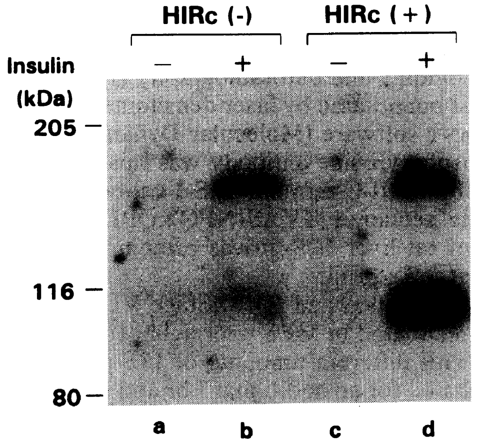

FIG. 5. Effect of expression of human insulin receptor on phosphorylation of injected IRS-1 protein. Oocytes were injected with water [HIRc (−)] or HIRc RNA [HIRc (+)] and incubated for 3 days; they were then injected with IRS-1 protein at 12.5 ng per oocyte. Groups of oocytes were then incubated without (−) or with (+) insulin at 10 µM for 10 min. Insulin stimulation of tyrosine phosphorylation of the receptor β subunit and IRS-1 was visualized in the antiphosphotyrosine immunoblots.

DISCUSSION

As with many hormones and growth factors that act through receptors with tyrosine kinase activity, the molecular basis of insulin and IGF-I postreceptor signaling contains many unknown elements (3). Many proteins that undergo tyrosyl phosphorylation either *in vitro* by partially purified receptors or in intact cells upon ligand stimulation can be regarded as candidates for substrates of the receptor kinase (3, 5–14), but exactly how, or if, these actually link the receptor to its final biological actions remains unclear. The current data indicate that IRS-1 not only acts as a specific substrate of the IGF-I receptor but also enhances IGF-I and insulin-induced maturation of *Xenopus* oocytes. These results clearly show that tyrosyl phosphorylation of the injected IRS-1 protein is under the regulation of the IGF-I receptor kinase and show that the tyrosyl phosphorylation of a substrate protein, one step downstream from the receptor kinase, modifies an important biologic event.

Xenopus oocytes represent an ideal system for the study of hormone action (20–24). *Xenopus* oocytes are physiologically arrested at the boundary between the G_2 and M phases of the first meiotic prophase (24). Treatment with progesterone initiates oocyte maturation and entrance into meiosis I, as signaled by the dissolution of the nuclear membrane or

Table 1. Effect of HIRc expression on oocyte maturation

	Oocytes undergoing GVB with insulin, %			
Group	0 µM	0.1 µM	1 µM	10 µM
$HIRc^-$, $IRS\text{-}1^-$	0	0	0	0
$HIRc^-$, $IRS\text{-}1^+$	0	0	29.2	55.7
$HIRc^+$, $IRS\text{-}1^-$	0	0	0	0
$HIRc^+$, $IRS\text{-}1^+$	0	3.7	37.2	50.6

Data present the means of three separate experiments; µM indicates insulin concentration.

GVB (21, 22). Previous studies have shown that insulin and IGF-I can also induce oocyte maturation with various response rates, depending on the nature of the incubation medium and the condition of the frog at the time of oocyte harvest (22, 26–28). In the absence of gonadotropin priming or *in vitro* treatment with progesterone, however, insulin and IGF-I have a minimal effect on oocyte maturation and, thus, provide an ideal system in which to study the role of IRS-1, the major insulin/IGF-I receptor substrate in most cells. When oocytes are microinjected with IRS-1 protein, the maturation response to insulin and IGF-I, as assayed by the appearance of GVB, increases in proportion to the amount of injected protein. Control extracts prepared from uninfected cells or cells infected with the virus only have no effect. Furthermore, insulin and IGF-I induce GVB responses in a concentration-dependent manner that coincides with the dose–response curves for insulin and IGF-I stimulation of receptor and substrate phosphorylation. This effect is mediated via the endogenous IGF-I receptor and, thus, IGF-I is ≈100-fold more potent than insulin in producing these effects. By contrast, increased expression of the insulin receptor by microinjection of receptor cRNA has no effect on ligand-stimulated oocyte maturation. This result is consistent with the finding in mammalian cells that has shown that overexpression of IRS-1 alone increases maximal insulin-stimulated DNA synthesis, whereas overexpression of insulin receptor increases primarily insulin sensitivity, and overexpression of both actually results in a decrease in maximal response possibly due to competition of the phosphorylated IRS-1 in src homology 2-containing proteins (29).

The identification of a role of IRS-1 in oocyte maturation represents an important opportunity to further our understanding of the relationship of this substrate protein and other downstream signal-transducing molecules (31) and phosphorylation-cascade intermediates (32–34) involved in insulin/IGF-I actions. Oocytes possess many of the serine and threonine kinases implicated in insulin/IGF-I action including the ribosomal protein S6 kinases, microtubule-associated protein kinase, and cdc2 kinase (20–24, 28, 30–34). Interestingly, oocytes from gonadotropin-primed frogs increase their insulin responsiveness without an apparent induction of IRS-1, suggesting that there may be converging pathways that act on these downstream intermediates of signaling (data not shown). In preliminary experiments we have found that oocytes possess a phosphatidylinositol 3-kinase activity that is activated during association with tyrosyl-phosphorylated IRS-1 in a manner similar to that seen in mammalian cells (12, 15). In addition, some insulin-responsive proteins, such as the GLUT4 glucose transporter, can be expressed in oocytes by injection of mRNA (19, 20, 35). Thus, reconstitution of insulin/IGF-I action in *Xenopus* oocytes may provide a system for dissecting the complex pathways of action of these hormone/growth factors.

This work was supported, in part, by grants from the National Institutes of Health DK33201 (C.R.K.), DK43808 (M.F.W.), and DK39519 (M.J.B.) and the Juvenile Diabetes Foundation (M.J.B.), as well as National Institutes of Health-sponsored Joslin Diabetes amd Endocrinology Research Center Grant (DK36836.).

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