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Interactive Roles of Ras, Insulin Receptor Substrate-1, and Proteins with Src Homology-2 Domains in Insulin Signaling in *Xenopus* Oocytes*

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Insulin receptor substrate-1 (IRS-1) serves as the major immediate substrate of insulin/insulin-like growth factor (IGF)-1 receptors and following tyrosine phosphorylation binds to specific Src homology-2 (SH2) domain-containing proteins including the p85 subunit of phosphatidylinositol (PI) 3-kinase and GRB2, a molecule believed to link IRS-1 to the Ras pathway. To investigate how these SH2-containing signaling molecules interact to regulate insulin/IGF-1 action, IRS-1, glutathione S-transferase (GST)-SH2 domain fusion proteins and Ras proteins were microinjected into *Xenopus* oocytes. We found that pleiotropic insulin actions are mediated by IRS-1 through two independent, but convergent, pathways involving PI 3-kinase and GRB2. Thus, microinjection of GST-fusion proteins of either p85 or GRB2 inhibited IRS-1-dependent activation of mitogen-activated protein (MAP) and S6 kinases and oocyte maturation, although only the GST-SH2 of p85 reduced insulin-stimulated PI 3-kinase activation. Co-injection of a dominant negative Ras (S17N) with IRS-1 inhibited insulin-stimulated MAP and S6 kinase activation. Microinjection of activated [Arg^{12},Thr^{59}]Ras increased basal MAP and S6 kinase activities and sensitized the oocytes to insulin-stimulated maturation without altering insulin-stimulated PI 3-kinase. The Ras-enhanced oocyte maturation response, but not the elevated basal level of MAP and S6 kinase, was partially blocked by the SH2-p85, but not SH2-GRB2. These data strongly suggest that IRS-1 can mediate many of insulin's actions on cellular enzyme activation and cell cycle progression requires binding and activation of multiple different SH2-domain proteins.

At the cellular level, insulin and IGF-1[1] produce a wide variety of metabolic effects and stimulate cell growth and differentiation (1). Insulin signaling is initiated by activation of the insulin receptor tyrosine kinase, autophosphorylation of the receptor β-subunit, and stimulation of phosphorylation of an intracellular receptor substrate, termed IRS-1 (2–6). Analysis of the sequences of IRS-1 from rat, mouse, and human has revealed over 20 potential tyrosine phosphorylation sites (6–8). Following insulin-stimulated phosphorylation, these sites serve as a docking sites which bind to the 85-kDa subunit (p85) of PI 3-kinase (6, 9, 10), as well as other proteins possessing Src homology-2 (SH2) domains (11–14). The amino acid residues surrounding the tyrosine determine the high affinity binding to a specific SH2 domain (15–17). Peptides derived from IRS-1 with the p85 recognition motif can also bind to and activate PI 3-kinase *in vitro*, suggesting these non-covalent interactions mediate the insulin signal for activation of this intracellular enzyme (18), although the exact function of this enzyme in cells is still unknown. Phosphorylated IRS-1 also binds to an adaptor protein termed GRB2 which then interacts with a guanine nucleotide exchange factor termed SOS coupling signaling by the insulin receptor to the Ras pathway (19, 20). Thus, binding of multiple molecules to IRS-1 may serve as one point of signal divergence in the insulin action pathway.

We have recently shown that microinjection of IRS-1 enhances oocyte maturation in response to insulin/IGF-1 treatment (21) and that this response can be blocked by microinjection of GST fusion proteins of p85 (22). In the present study, we have further explored the interactions between IRS-1, Ras, and SH2 domain proteins using the *Xenopus* oocyte reconstitution system. We find that both insulin-stimulated PI 3-kinase and GRB2/Ras-mediated activation of MAP and S6 kinases interact in a cooperative and convergent manner during insulin/IGF-1-stimulated oocyte maturation.

MATERIALS AND METHODS

Oocyte Preparation and Germinal Vesicle Breakdown—Stage VI oocytes were isolated by mild collagenase treatment of ovaries from gravid *Xenopus laevis* frogs (Nasco, Fort Atkinson, WI) which had not received priming with gonadotropin (21). The oocytes were allowed to recover overnight in modified Barth's media and then microinjected with 50 nl of either buffer (125 mM NaCl, 25 mM Tris-HCl, pH 7.6) or combinations of proteins at the indicated concentrations. Recombinant rat liver IRS-1 was produced in a baculovirus expression system and purified to >90% homogeneity as described previously (18). GST fusion proteins were produced from bacterial expression vectors (pGEX) containing the N-terminal SH2 domains of p85 designated GST-SH2-p85) or GRB2 (GST-SH2-GRB2). The GRB2 GST-fusion construct and the SH2-p85 GST fusion construct were kind gifts of J. Schlessinger (New York University, New York) and M. Waterfield (Ludwig Institute of Cancer Research, London, UK). Bacterial lysates were purified on glu-

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[1] The abbreviations used are: IGF, insulin-like growth factor; IRS, insulin receptor substrate; PI, phosphatidylinositol; SH2, Src homology-2; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; GVBD, germinal vesicle breakdown; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein.

tathione-Sepharose columns (Pharmacia Biotech Inc.) as described previously (23). The proteins were then dialyzed extensively against a degased buffer of 88 mM NaCl and 50 mM Tris-HCl, pH 8.0, aliquoted and stored at −80 °C until use. Activated [Arg^{12},Thr^{59}]Ras and the dominant inhibitory Ras (S17N) proteins were produced and purified as described previously (24, 25).

After microinjection with IRS-1, Ras, and/or GST-SH2 fusion proteins, oocytes were incubated for 3 h, then transferred to the media containing insulin at 10 μM. The high concentration of insulin used was due to the fact that the insulin effect in oocytes is mediated by the IGF-1 receptor (21, 22). After a 20-h incubation, groups of 15–20 oocytes were scored for germinal vesicle breakdown (GVBD) by the appearance of a white spot on the animal pole of the oocyte. In some experiments GVBD was confirmed by the microscopic absence of nuclear membrane after fixing oocytes in 5% trichloroacetic acid for 30 min.

PI 3-Kinase Assay—After microinjection with different protein combinations, oocytes were treated with insulin at the indicated concentrations at 19 °C for 5 min. The incubation medium was removed, and oocytes were extracted, and PI 3-kinase activity was measured by *in vitro* phosphorylation of phosphatidylinositol as described by Ruderman *et al.* (26) with minor modifications (22) in immunoprecipitates prepared from the cytosolic extract using either anti-IRS-1 or anti-p85 antibodies collected on protein A-Sepharose (Pharmacia).

Western Blotting—Oocyte lysates were partially purified on phenyl-Sepharose for Western blot analysis of MAP kinase (27). The proteins collected on the phenyl-Sepharose beads were released by boiling in the Laemmli sample buffer, resolved by SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes in Towbin buffer containing 0.02% SDS and blotted with the antibody to phosphotyrosine (5) or antibody to MAP kinase (1B3B9) kindly provided by Michael Weber (University of Virginia). Specific bands were visualized by ^{125}I-protein A (ICN) and autoradiography.

MAP and S6 Kinase Assays—Groups of oocytes were lysed in a buffer containing 50 mM β-glycerophosphate, pH 7.4, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM benzamidine, 0.5 mM Na_3VO_4, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 mM dithiothreitol. Crude cell extracts were aliquoted and stored at −80 °C after centrifugation at 13,000 × *g* for 10 min at 4 °C. Kinase reactions were performed on the cell lysate (equivalent to 1.5 oocytes/tube) by incubating with [γ-^{32}P]ATP and 0.5 mM S6 peptide or 0.5 mM MBP peptide (UBI) in the presence of 10 mM $MgCl_2$ and 2 μM protein kinase A inhibitor (Sigma). Reactions were terminated after 15 min at 30 °C by adding 0.5 volume of 2 M HCl. Samples (25 μl) were spotted onto P81 phosphocellulose paper disks (Life Technologies, Inc.) and washed twice in 1% acetic acid and twice in distilled H_2O. The disks were then dried and counted for radioactivity (Cerenkov method). Nonspecific ^{32}P incorporation was determined in identical assays lacking cell lysates. Each measurement was done in duplicate in at least two independent experiments.

Preparation of GST Fusion Proteins and in Vitro Binding Assay—GST fusion proteins of the SH2 of p85 of PI 3-kinase and GRB2 were prepared as described previously (22). *In vitro* binding of the GST-SH2 fusion protein and the oocyte lysates was carried out at 4 °C for 1 h in the presence of glutathione-agarose beads (Sigma). The beads were then washed four times with phosphate-buffered saline containing 1% Triton X-100 and 0.25 M NaCl. The bound fractions collected on the agarose beads were then solubilized by boiling in the Laemmli's sample buffer containing 100 mM dithiothreitol before gel electrophoresis.

RESULTS

Both p85 Subunit of PI 3-Kinase IRS-1 and GRB2/IRS-1 Interactions Are Required for Insulin-induced Oocyte Maturation—As we have recently shown (21), insulin-induced oocyte maturation, as measured by the appearance of GVBD, is enhanced by the microinjection of recombinant IRS-1 protein (Fig. 1, *left*). This effect is mediated via IGF-1 receptors in the oocyte and is also observed with IGF-1 at 100-fold lower concentrations than insulin (21). Either a GST-SH2 fusion protein from the p85 subunit of PI 3-kinase (GST-SH2-p85) or a GST-SH2-GRB2 in a 4-fold molar excess over IRS-1 inhibited insulin-stimulated IRS-1-dependent oocyte maturation. This correlated with the ability of both GST-SH2 domain fusion proteins to bind to phosphorylated IRS-1 (Fig. 2). Using the GST-fusion proteins, however, at equal concentrations (10 ng/oocyte), SH2-p85 was about 3-fold more effective in precipitating IRS-1 than the SH2 of GRB2. SH2-p85 also precipitated a small amount of

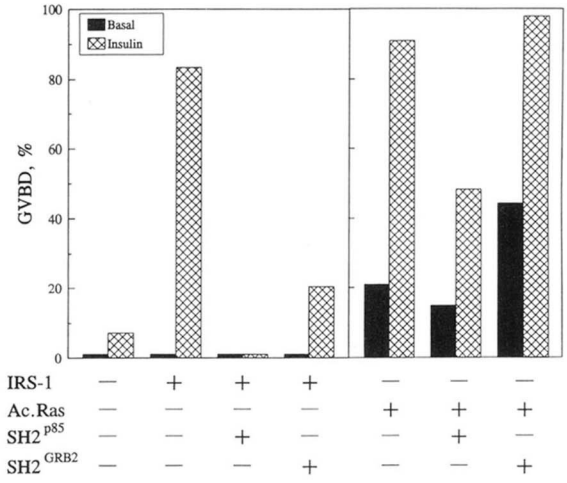

FIG. 1. **Effect of IRS-1 GST-SH2 fusion proteins of p85 and GRB2 and Ras proteins on oocyte maturation.** Oocytes were microinjected with (+) or without (−) recombinant IRS-1 and GST-SH2 fusion proteins (10 ng/oocyte). Oocytes were then incubated with (+) or without (−) insulin for 20 h. Maturation, as indicated by the GVBD, was scored in each group of 20–30 oocytes. The data represent the mean of two separate experiments.

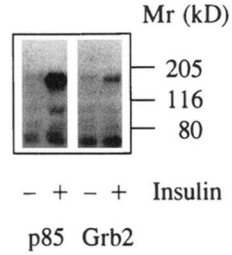

FIG. 2. ***In vitro* binding assay of IRS-1 and GST-SH2 fusion proteins.** Groups of oocytes were treated with or without insulin after microinjection of baculovirus-expressed IRS-1 as described in Fig. 1. After extraction, the oocyte lysates were incubated with GST-SH2 fusion proteins, and the complexes precipitated using glutathione-Sepharose as described under "Materials and Methods." The precipitates solubilized and analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies. The SH2-p85 precipitated both phosphorylated IRS-1 (molecular mass of ~185 kDa) and one β-subunit of the IGF-1 receptor (molecular mass of ~100 kDa). SH2-GRB2 precipitated only phosphorylated IRS-1 GST protein alone did not bind phosphorylated IRS-1.

a phosphorylated protein of molecular mass ~100 kDa which most likely represents the *Xenopus* IGF-1 receptor β-subunit (Fig. 2, *left panel*).

When oocytes were injected with an activated Ras protein, [Arg^{12},Thr^{59}]Ras, the basal level of GVBD was increased and the GVBD response to insulin treatment was further enhanced (Fig. 1, *right*). A similar effect has been recently reported for IGF-1 induced oocyte maturation (28). Injection of SH2-p85 did not reduce the increased basal GVBD produced by injection of activated Ras, but did inhibit the insulin enhancement of oocyte maturation. By contrast, microinjection of SH2-GRB2 further enhanced the basal level of GVBD, but had no effect on the insulin-stimulated response. These data are consistent with the notion that GRB2 serves as an upstream regulator of Ras, and suggests that an additional signal from insulin stimulation via the p85-PI 3-kinase pathway acts in a synergistic manner to cause the insulin bioeffect on oocyte maturation.

PI 3-Kinase and GRB2 Are on Different Signaling Pathways—To investigate the mechanism of the SH2-p85 and SH2-GRB2 on insulin signaling, we studied their effect on activation of PI 3-kinase and MAP and S6 kinases. As we have previously shown (22), microinjection of IRS-1 enhanced the insulin-

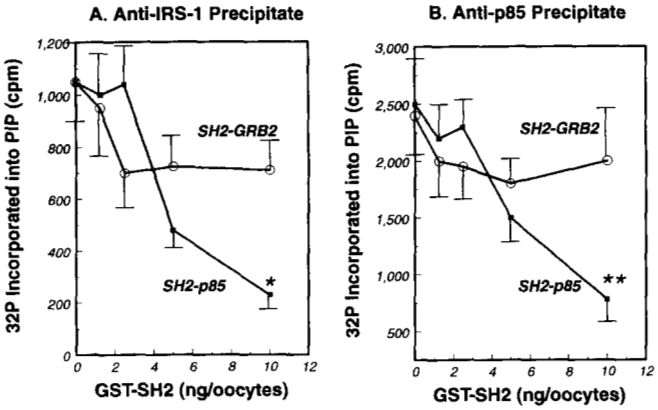

FIG. 3. **Effects of GST-SH2 of p85 and GRB2 and Ras on PI 3-kinase activity.** Insulin-stimulated PI 3-kinase activity was measured in the oocyte with IRS-1 microinjection co-injected with various amounts of GST-SH2 fusion proteins of p85 and GRB2 in anti-IRS-1 precipitates (*Panel A*) and in anti-p85 precipitates. As a measure of total PI 3-kinase activity (*Panel B*). The data shown are the means plus or minus the S.E. By analysis of variance analysis, PI 3-kinase activity was significantly inhibited by the GST-SH2 of p85 both in the anti-IRS-1 ($F = 5.58$, $p < 0.01$) and anti-p85 ($F = 5.63$, $p < 0.01$) immunoprecipitates. The inhibition by GST-SH2 of GRB2 was not significant in either the anti-IRS-1 ($F = 0.34$, $p = 0.846$) immunoprecipitates. The points which showed a significant change as compared to the basal level by the Student-Newman-Keuls test are indicated * for $p < 0.05$ and ** for $p < 0.01$. Data represent the mean of two experiments.

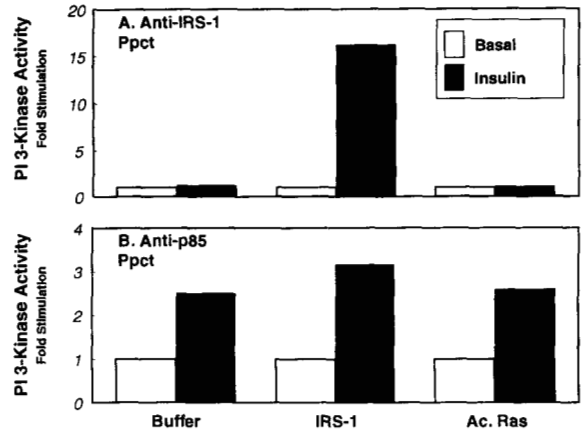

FIG. 4. **Insulin-stimulated PI-kinase was measured in oocytes following microinjection of IRS-1 or the activated [Arg^{12}, Thr^{59}]Ras protein.** Groups of 15 oocytes were incubated with or without (*basal*) insulin for 5 min. PI 3-kinase activity was measured in the immunoprecipitates of anti-IRS-1 or anti-p85 antibodies as described under "Materials and Methods.

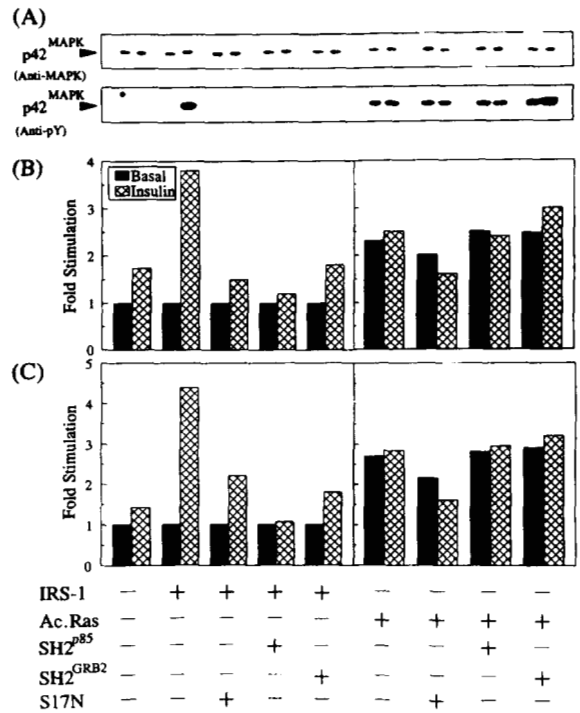

FIG. 5. **Effect of IRS-1, Ras and GST-SH2 of p85 and GRB2 on the insulin-stimulated MAP and S6 kinases activation.** *Panel A*, insulin-regulated tyrosine phosphorylation of MAP kinase. Oocytes were microinjected without (−) or with (+) IRS-1, Ras, or GST-SH2 fusion proteins. After incubation with (+) or without (−) insulin for 20 h, oocytes were then lysed and purified on phenyl-Sepharose beads. Proteins were subjected to SDS-PAGE and Western blotting with anti-MAP kinase and anti-phosphotyrosine (*Anti-pY*) antibodies. The protein band at the position of 42 kDa is shown after visualization by ^{125}I-protein A and autoradiography. Insulin-stimulated MAP kinase (*Panel B*) and S6 kinase (*Panel C*) activities were measured in the extract from the oocytes treated with (+) or without (−) insulin for 20 h. MAP kinase activity was assayed by the phosphorylation of a synthetic fragment of myelin basic protein (UBI); S6 kinase activity was assessed using a synthetic peptide of ribosomal S6 protein (UBI) as a substrate. Data represent the mean fold of stimulation by insulin in two separate experiments as compared to the basal level of the oocytes without injection of activated Ras protein.

stimulated PI 3-kinase activation. Insulin-stimulated PI 3-kinase both associated with IRS-1 and total activity present in anti-p85 precipitates was specifically and significantly inhibited by SH2-p85 ($p < 0.01$) (Fig. 3, *A* and *B*). By contrast, the SH2-GRB2 produced only a slight, non-concentration dependent inhibition which was not statistically significant ($p \geq 0.5$). The lack of the GRB2-GST-SH2 effect on PI 3-kinase was not due to a lack of interaction with IRS-1. Following microinjection of IRS-1 and insulin treatment, there was clear binding of the phosphorylated IRS-1 to the GST-SH2 of GRB-2 (Fig. 2). These data support the notion that the PI 3-kinase and GRB2 are located on different signaling pathways, and the activation of PI 3-kinase in mediated by the SH2-p85.

The regulation of PI 3-kinase activation is independent of Ras. Microinjection of an activated Ras protein, [Arg^{12},Thr^{59}]-Ras, had no effect on either IRS-1-associated or total PI 3-kinase activity (Fig. 4). These data are consistent with the report that insulin-stimulated PI 3-kinase is unaffected by the overexpression of the dominant negative (S17N) Ras in NIH 3T3 cells (29). As previously demonstrated (22), microinjection of IRS-1 significantly increased both IRS-1 associated and total PI 3-kinase activity, although the increase in the latter ranged from only 1.4- to 2-fold.

Activation of MAP and S6 Kinases—It is well recognized that the activity of MAP kinase is regulated by its phosphorylation on the tyrosine and threonine residues (30, 31) and that MAP kinase activation involves a complex network possibly including Ras, raf-1 kinase, and a MAP kinase kinase (32–34). Tyrosine phosphorylation of MAP kinase was observed within 15 min of insulin stimulation in the *Xenopus* oocytes microinjected with IRS-1 protein but did not occur in insulin-stimulated, buffer-injected control oocytes (data not shown). Even after 20 h of incubation, tyrosine phosphorylation of MAP kinase remained markedly stimulated in the oocytes injected with IRS-1 and incubated with insulin (Fig. 5*A*). This increase in the tyrosine phosphorylation correlated well with insulin stimulation of MAP kinase activity using the synthetic 9-amino acid myelin basic protein related peptide as a substrate (Fig. 5*B*). These data indicate that IRS-1 is an upstream element of MAP kinase activation during insulin stimulation. Co-injection of the SH2-GRB2 or the SH2-p85 with IRS-1 completely blocked MAP kinase tyrosine phosphorylation and insulin-stimulated en-

zyme activation (Fig. 5, *A* and *B*). Similar results were observed using S6 kinase activity as an end point (Fig. 5*C*). IRS-1-mediated MAP and S6 kinase activation was also blocked by co-injection of the dominant negative (S17N) Ras.

Microinjection of activated [Arg^{12},Thr^{59}]Ras resulted in an increase in tyrosine phosphorylation of MAP kinase and increased basal activity of MAP and S6 kinases to a level about 65% that observed following IRS-1 injection and insulin stimulation. Insulin stimulation produced no further increase in these parameters, despite its effect to further augment germinal vesicle breakdown (compare Figs. 5*B* and 1*B*). These effects of activated Ras were not blocked by SH2-p85, SH2-GRB2, or by the dominant negative (S17N) Ras at the concentration which inhibited the IRS-1 mediated effects.

DISCUSSION

Although considerable progress has been made over the past decade, the exact molecular mechanism of insulin action at the cellular level remains obscure. The discovery of the insulin receptor tyrosine kinase and its intracellular substrate IRS-1 have pointed to a tyrosine phosphorylation cascade as the initiating event in insulin's pleiotropic downstream effects. IRS-1 itself contains multiple tyrosine phosphorylation sites which in their phosphorylated form can bind to different signaling molecules which contain SH2 and SH3 domains, including the 85 kDa subunit of PI 3-kinase, GRB2 and the protein tyrosine phosphatase Syp/SHPTP2 (9, 14, 18, 20, 35, 36). These interactions result in activation of these proteins and result in further downstream signaling. This is analogous to the interaction of SH2 domain proteins with different phosphorylated residues in the platelet-derived growth factor and epidermal growth factor receptors which is critical for signaling by these receptors (16). Recently, we demonstrated that IRS-1 and these SH2 interactions play an essential role in mediating insulin's effects on PI 3-kinase and progression of the cell cycle from G_2 to M phase in *X. laevis* oocytes (21, 22). To further understand the role of IRS-1 in mediating insulin's multiple functions, in this report we have studied the effects of IRS-1 and SH2 domain proteins on phosphorylation and activation of MAP and S6 kinases, as well as PI 3-kinase and oocyte maturation, and attempted to determine their relationship to the Ras pathway of action.

Our data indicate that IRS-1 utilizes at least two convergent pathways in exerting its role in insulin action in oocytes: one mediated via the SH2 domain of p85 of PI 3-kinase and the other mediated via the SH2 domain of GRB2. Thus, injection of the GST fusion protein of either SH2 domain significantly inhibits IRS-1-mediated oocyte maturation, MAP kinase phosphorylation and MAP and S6 kinase enzyme activation, but only the SH2 of p85 blocks PI 3-kinase activation. Interestingly, in this oocyte system the effect of the SH2-GRB2 blockade, but not the SH2-p85 blockade, is overcome by co-injection of activated Ras. On the other hand, activated Ras can almost completely or completely mimic the effect of insulin on MAP and S6 kinases, but only partially mimics the effect on oocyte maturation. Taken together, these data suggest that two convergent pathways, one mediated by the PI 3-kinase and the other by GRB2 and Ras, are required for oocyte maturation.

From genetic studies, GRB2, and the related molecules sem-5 and drk, have been implicated in linking receptor tyrosine kinase to Ras signaling (37–40). In some cases, an additional element, the protein Shc, is involved in linking the receptor tyrosine kinase. Tyrosine phosphorylation of Shc is stimulated by activated platelet-derived growth factor, epidermal growth factor, and, in some cells, insulin receptors, as well as by the non-receptor tyrosine kinases v-*src* and v-*fps* (41–43). We cannot assess the role of Shc in this system from the present data, however, we find that SH2-GRB2 can block insulin/IRS-1 stimulated MAP and S6 kinases activation, suggesting that IRS-1, GRB2 and the MAP/S6 kinases are on the same pathway. Recently, a GST-SH2 fusion protein of GRB2 has also been shown to block an epidermal growth factor receptor signaling to the nucleus in an *in vitro* system (44). In addition, SH2-GRB2 interfered the insulin-stimulation of certain small membrane-associated GTP binding proteins (data not shown). Since the microinjection of an activated Ras protein can also activate MAP and S6 kinases bypassing the blockade of SH2-GRB2, this indicates that GRB2 or a GRB2-like molecule is an upstream regulator of Ras in *Xenopus* oocytes.

The exact relationship between Ras and the PI 3-kinase pathways is still not settled. Although there is one report showing that PI 3-kinase activity can be precipitated with an anti-Ras monoclonal antibody in cells transformed with Ha-Ras (45), this has not yet been demonstrated in insulin-stimulated systems. Furthermore, biochemical evidence for a direct association of these proteins is lacking. In our study microinjection of an activated Ras did not affect the basal and the insulin-stimulated PI 3-kinase activity, but did sensitize the oocyte to insulin stimulation, suggesting some ability to bypass the IRS-1 mediated response. Likewise, overexpression of a dominant negative (S17N) Ras did not block insulin stimulation of PI 3-kinase, although MAP kinase activation is blocked by this protein (29). However, the MAP kinase activation induced by insulin in IRS-1 injected cells is blocked by SH2-p85. Taken together, these data suggest there may be alternative pathways of insulin signaling, one involving IRS-1 and another not involving IRS-1, and that PI 3-kinase may also play a role upstream of Ras. A similar conclusion has been reached from studies using *in vitro* mutagenesis of the platelet-derived growth factor receptor (46) and in studies of insulin action on gene expression in cells transiently overexpressing the p86 subunit of PI 3-kinase (47). Furthermore, the blockade of PI 3-kinase by the SH2-p85 was specific, and no interaction between the SH2-GRB2 and IRS-1's ability to interact with PI 3-kinase can be demonstrated *in vitro* (data not shown). Of course, it is possible that the SH2-p85 GST-fusion protein inhibits some other SH2 domain interaction with IRS-1 which was not measured. If so, however, this must be a high affinity, specific interaction, since the GST fusion protein was only used at a 4-fold molar excess and *in vitro* studies have shown that different SH2 domain proteins bind to specific sites on IRS-1 with 60–100-fold differences in affinity (48). Thus, there appears to be some cross-talk between the two pathways mediated by p85 and GRB2.

The site and nature of the convergent signaling by the p85 and GRB2 pathways is not clear. This convergence appears to occur downstream of SH2-IRS-1 interactions, since the binding of different SH2 molecules to IRS-1 is specific (22, 48). Furthermore, injection of activated Ras-sensitized oocytes to insulin in the absence of exogenous IRS-1, suggesting that some small amount of PI 3-kinase activation occurs in the absence of exogenous IRS-1 which is sufficient to synergize with Ras to augment insulin stimulation of GVBD. Indeed, the SH2 domains of both p85 and GRB2 may participate in signaling by binding to upstream molecules other than IRS-1, such as Shc and a recently described 60 kDa PI 3-kinase associated protein (49). Whatever the mechanisms, IRS-1 can play an important role in mediating the pleiotropic insulin actions, including the activation of many different cytoplasmic enzymes, changes in the GTP binding of certain membrane associated proteins, and stimulation of cell cycle progression from G_2 to M phase. This response appears to require two pathways, one linking IRS-1, p85 of PI 3-kinase, and possibly MAP and S6 kinases to ger-

minal vesicle breakdown, and another linking IRS-1, GRB2, Ras, or Ras-related GTP-binding proteins, MAP/S6 kinases, and germinal vesicle breakdown. The latter pathway alone is not enough for the full biologic response, since insulin stimulation further enhances GVBD even in oocytes injected with activated Ras possessing high basal MAP and S6 kinase activities. Similar phenomena have also been observed in the case of platlet-derived growth factor, where a mutant receptor could activate Ras but not stimulate DNA synthesis (46), and in the case of pp60v-Src where both tyrosine kinase activity and Ras are required for the c-*raf*-1 kinase activation (50). These data strongly suggest that a network of converging Ras-dependent and Ras-independent pathways is required to mediate insulin/IRS-1 signaling on complex biologic responses such as those observed in oocyte maturation.

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