Insulin stimulates tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and Shc in Rat1 fibroblasts overexpressing wild type insulin receptors. We investigated the relative role of IRS-1 and Shc in insulin activation of guanine nucleotide releasing factor (GNRF) and p21<sup>ras</sup>-GTP formation. The time course of insulin-stimulated tyrosine phosphorylation of IRS-1 was rapid, whereas Shc phosphorylation was relatively slow. Growth factor receptor bound protein-2 (Grb2) associated with IRS-1 rapidly and gradually dissociated after 5 min, whereas Grb2 association with Shc was slower and reached a maximum at 10 min after insulin stimulation. Thus, the kinetics of Grb2 association with IRS-1 and Shc corresponded closely to the time course of tyrosine phosphorylation of IRS-1 and Shc, respectively. Importantly, 3-13-fold more Grb2 was associated with Shc than with IRS-1. In addition, the kinetics of insulin-stimulated GNRF activity and p21<sup>ras</sup>-GTP formation corresponded more closely to the time course of Shc phosphorylation than to the kinetics of IRS-1 phosphorylation. Furthermore, immunoprecipitation of Shc proteins from cell lysates of insulin-stimulated cells removed 67% of the GNRF activity, whereas precipitation of IRS-1 had a negligible effect on GNRF activity. Thus, although both IRS-1 and Shc associate with Grb2, the current results indicate that Shc plays a more important role than IRS-1 in insulin stimulation of GNRF activity and subsequent p21<sup>ras</sup>-GTP formation.

Insulin binding to the extracellular α-subunits activates the intrinsic tyrosine kinase activity of the cytoplasmic portion of the insulin receptor β-subunit (1). One early molecular event linking the receptor kinase to insulin's biologic actions is tyrosine phosphorylation of IRS-1 (2). Current evidence suggests that IRS-1 acts as a multisite "docking" protein by binding to downstream signal-transducing molecules (3). The physical interaction is mediated by binding of phosphotyrosine IRS-1 motifs with Src homology 2 (SH2) domains of cytoplasmic signaling proteins (4, 5). Recently, Pelicci <i>et al.</i> (6) have characterized a cDNA clone that encodes a protein termed Shc. Shc, which is ubiquitously expressed, contains a single SH2 domain at the C terminus but lacks catalytic activity (6). Shc protein is also tyrosine-phosphorylated after insulin stimulation but does not associate stably with either the insulin receptor or IRS-1 (7, 8).

Current evidence indicates that p21<sup>ras</sup> is a key molecule in the mitogenic pathways initiated by receptor tyrosine kinases, including the insulin receptor (9–12). p21<sup>ras</sup> becomes active as a signaling molecule when it is converted from the GDP- to GTP-bound form, and this process is controlled by Ras GTPase-activating protein (GAP) and GNRF activity (12). Ras GAP promotes the intrinsic GTPase activity of p21<sup>ras</sup> (13), whereas GNRF mediates the dissociation of GDP from p21<sup>ras</sup> (14–16). Recent reports have provided evidence that insulin increases p21<sup>ras</sup>-GTP primarily by stimulation of GNRF activity; rather than by inhibition of GAP activity (17, 18). Other growth factors also appear to increase p21<sup>ras</sup>-GTP levels through GNRF (19, 20). A protein called son of sevenless (Sos) was identified as a GNRF in <i>Drosophila</i> (14, 15), and a mammalian homologue to Sos (16) appears to be the GNRF mediating growth factor actions. The proline rich region of Sos binds to the SH3 domain of Grb2, which is an adapter protein composed of one SH2 domain and two SH3 domains (21), and preformed Grb2-Sos complexes exist within unstimulated cells (16, 22–27). However, activation of the insulin receptor leads to tyrosine phosphorylation of IRS-1 and Shc, both of which can then bind to Grb2-Sos complexes (7, 8, 26, 27). Thus, there are two downstream coupling molecules, IRS-1 and Shc, which can potentially independently connect the activated insulin receptor to Grb2-Sos and stimulation of p21<sup>ras</sup>-GTP formation. We have recently used single cell microinjection studies to inhibit Shc and IRS-1, and we have shown that both molecules are necessary for insulin's overall growth stimulus (28). It is possible that Shc and IRS-1 represent additive complementary pathways allowing insulin to couple more effectively to p21<sup>ras</sup>. On the other hand, one of these pathways could be dominant with the other redundant. Therefore, the purpose of this study was to evaluate the relative roles of Shc and IRS-1 in mediating the insulin-induced increase in cellular p21<sup>ras</sup>-GTP.

*This work was supported in part by Grant DK33651 from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, by the Veterans Administration Medical Research Service, by the Sankyo Diabetes Research Fund, by a research fellowship grant from the Medical Research Council of Canada, and by an American Diabetes Association Mentor-Based Fellowship Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: IRS-1, insulin receptor substrate 1; SH, Src homology; PI 3-kinase, phosphatidylinositol 3-kinase; GAP, GTPase-activating protein; EGF, epidermal growth factor; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; GNRF, guanine nucleotide releasing factor; Sos, homologue to Sos (16), appears to be the GNRF mediating growth factor actions. The proline rich region of Sos binds to the SH3 domain of Grb2, which is an adapter protein composed of one SH2 domain and two SH3 domains (21), and preformed Grb2-Sos complexes exist within unstimulated cells (16, 22–27). Through the Grb2 SH2 domain, Grb2-Sos complexes can bind to phosphorylated epidermal growth factor (EGF) and platelet-derived growth factor receptors, providing a mechanism whereby these growth factors can stimulate p21<sup>ras</sup>-GTP formation (16, 22–25). However, Grb2-Sos complexes do not associate with the insulin receptor (8, 26). Activation of the insulin receptor leads to tyrosine phosphorylation of IRS-1 and Shc, both of which can then bind to Grb2-Sos complexes (7, 8, 26, 27). Thus, there are two downstream coupling molecules, IRS-1 and Shc, which can potentially independently connect the activated insulin receptor to Grb2-Sos and stimulation of p21<sup>ras</sup>-GTP formation. We have recently used single cell microinjection studies to inhibit Shc and IRS-1, and we have shown that both molecules are necessary for insulin's overall growth stimulus (28). It is possible that Shc and IRS-1 represent additive complementary pathways allowing insulin to couple more effectively to p21<sup>ras</sup>. On the other hand, one of these pathways could be dominant with the other redundant. Therefore, the purpose of this study was to evaluate the relative roles of Shc and IRS-1 in mediating the insulin-induced increase in cellular p21<sup>ras</sup>-GTP.

**EXPERIMENTAL PROCEDURES**

**Materials—**Rat1 cells expressing wild type insulin receptors (HIRc) were maintained as previously described (28). Porcine insulin was kindly provided by Lilly. The p21" probe (v-Ha-Ras) was purchased from Oncor, Inc. (Gaithersburg, MD). [32P]Orthophosphate (0.25 mCi/ml) and [3H]GDP (32 Ci/mmol) were from DuPont NEN. Electrophoresis reagents were from Bio-Rad. Enhanced chemiluminescence reagents were from Amersham Corp. A monoclonal anti-phosphotyrosine antibody (pY20) was from ICN. An anti-p21" monoclonal antibody (Y15–259) was from Oncogene Science, Inc. (Uniondale, NY). A polyclonal and a monoclonal anti-Shc antibody, as well as a monoclonal anti-Grb2 antibody, were from Transduction Laboratories (Lexington, KY). A polyclonal anti-IRS-1 antibody was kindly provided by Dr. Hiroshi Maegawa (Shiga University of Medical Science, Japan). All other routine reagents were purchased from Sigma.

**Western Blotting Studies—**Cells were starved for 24 h in serum-free Dulbecco’s modified Eagle’s medium. The cells were then treated with 17 nM insulin at 37 °C. After the indicated time, cells were lysed in a buffer containing 30 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotonin, 10 μg/ml leupeptin, 1 mM Na3VO4, pH 7.4. The cell lysates were centrifuged, and supernatants were used for immunoprecipitation with the indicated antibodies. Immunoprecipitated or supernatant proteins were separated by SDS-PAGE and transferred onto Immobilon-P by electroblotting. After incubation with the specified antibody, enhanced chemiluminescence detection was performed according to the manufacturer’s instructions (Amersham Corp.).

**GNRF Activity in HIRc Cell Lysates—**Serum-starved cells were stimulated with 17 nM insulin for the indicated time at 37 °C. Cells were then lysed in a buffer containing 50 mM Hepes, 150 mM NaCl, 5 mM MgCl2, 1 mM PMSF, 1 mM Na3VO4, 1% Triton X-100, 0.05% SDS, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 500 μM GTP, and 500 μM GDP, pH 7.5. GNRF activity in the lysates was determined by measuring the dissociation of protein-bound [3H]GDP radioactivity. Purified v-Ha-Ras was incubated with [3H]GDP in a buffer containing 25 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 400 μg/ml bovine serum albumin, pH 7.5, for 15 min at 30 °C. The complex was stabilized by addition of the incubation buffer supplemented with 10.7 mM MgCl2. The Ras-GDP complex was added to the cell lysates and incubated at 23 °C. At the indicated time intervals, aliquots were removed and filtered through 0.45-μm Millipore nitrocellulose filters. The amount of [3H]GDP radioactivity bound to p21" was quantitated by scintillation counting. [3H]GDP binding to p21" was confirmed by immunoprecipitation with anti-Ras antibody (17).

**Measures of GTP- and GDP-bound p21"—**Serum-starved cells were incubated for 1 h in phosphate-free serum-free Dulbecco’s modified Eagle’s medium and labeled with [32P]Orthophosphate for 4 h. Insulin (17 nM) was added at 37 °C. After the indicated time, cells were lysed in a buffer containing 50 mM Hepes, 150 mM NaCl, 5 mM MgCl2, 1 mM PMSF, 1 mM Na3VO4, 1% Triton X-100, 0.05% SDS, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 500 μM GTP, 500 μM GDP, pH 7.5. The lysates were centrifuged, and GTP- and GDP-bound p21" was immunoprecipitated with the monoclonal antibody Y13–259. Nucleotides were eluted by suspending immunoprecipitates, and heating at 85 °C for 3 min. Separation of GTP from GDP was then performed by thin layer chromatography (17).

**RESULTS**

To investigate the kinetics of tyrosine phosphorylation of Shc, the insulin receptor β-subunit, and IRS-1, we studied the time course of tyrosine phosphorylation after insulin stimulation in Rat1 fibroblasts overexpressing insulin receptors. As shown in Fig. 1A, phosphorylation of the receptor β-subunit and IRS-1 was observed after 30 s of insulin stimulation, peaked by ~1 min, and declined after 10 min. The bands corresponding to the insulin receptor β-subunit and IRS-1 were confirmed by using specific antibodies (data not shown). To assess Shc phosphorylation, cell lysates were immunoprecipitated with a polyclonal anti-Shc antibody, and the immunoprecipitates were immunoblotted with a monoclonal anti-phosphotyrosine antibody. The time course of insulin-induced Shc phosphorylation was slower compared with the insulin receptor β-subunit and IRS-1. Phosphorylation was first observed at 1 min, reached a maximum at 10 min, and then declined (Fig. 1B). These results are summarized in Fig. 1C. It is clear that tyrosine phosphorylation of the insulin receptor β-subunit and IRS-1 proceeds more rapidly compared with Shc.

It has been shown that both IRS-1 and Shc associate with Grb2 following insulin stimulation (8, 26, 27), and we assessed the time course of Grb2 association with these two molecules. After insulin treatment, cell lysates were immunoprecipitated with antibodies against IRS-1 or Shc, and the precipitates were immunoblotted with a Grb2 antibody. The IRS-1-Grb2 association was rapid, with complexes detected after 30 s of insulin treatment, peaking at 1 min, and declining thereafter (Fig. 2A). On the other hand, complex formation between Shc and Grb2 proceeded more slowly. Association was first apparent at 1 min, reached a maximum by 10 min, and declined thereafter (Fig. 2B). These results are summarized in Fig. 2C, and the pattern of these time courses were comparable to the kinetics of Shc versus IRS-1 phosphorylation, as seen in Fig. 1.

To assess Grb2 association with IRS-1 or Shc more directly, we performed sequential immunoprecipitation studies using anti-IRS-1 and anti-Shc antibodies. After insulin stimulation, cell lysates were first immunoprecipitated with the anti-Shc antibody, and the remaining supernatants were re-immunoprecipitated with anti-IRS-1 antibody. The anti-Shc and anti-
IRS-1 immunoprecipitates and the final supernatants were then immunoblotted with anti-Grb2 antibody. As can be seen in Fig. 3A, there was minimal association of Grb2 with IRS-I or Shc in the basal state. After 1 min of insulin stimulation, a small amount of Grb2 associated with both IRS-1 and Shc, but even at this early time, the absolute amount of Shc-Grb2 complexes far exceeded the amount of IRS-1-Grb2. By 10 min, a large amount of Grb2 was associated with Shc, whereas Grb2 association with IRS-1 had already declined (Fig. 3A). Three separate experiments are summarized in Fig. 3B. Of total Grb2, 5.1 ± 1.3 and 14.1 ± 0.8% associated with IRS-1 or Shc, respectively, after 1 min of insulin treatment. At 10 min, 2.4 ± 1.3 and 31.7 ± 1.1% of the Grb2 was complexed with IRS-1 versus Shc, respectively. Reversing the order of the immunoprecipitation (first with anti-IRS-1 and second with anti-Shc) produced the same results (data not shown), thus, demonstrating that Grb2 does not bind to IRS-1 and Shc simultaneously.

Preformed Grb2-Sos complexes exist within the cell (16, 22–27), and insulin stimulation leads to the association of these complexes with either IRS-1 or Shc (8, 26, 27). To further explore which association is more important for insulin-stimulated GNRF activity, we measured GNRF activity in cell lysates before and after precipitation of the lysates with anti-IRS-1 or anti-Shc antibody. As can be seen in Table I, precipitation of Shc from insulin-stimulated cells reduced 67% of the total GNRF activity, whereas precipitation of IRS-1 had a negligible effect on GNRF activity.

To confirm that our results were not related to the particular antibodies used in these studies, comparable experiments were conducted with a different set of antibodies, with identical results. Thus, a monoclonal anti-Shc antibody was used to perform the Grb2 association and GNRF immunodepletion experiments, and the results were comparable to those obtained with the polyclonal antibody in Fig. 3 and Table I. In addition, we used an anti-phosphotyrosine antibody (pY20) to immunoprecipitate post-anti-Shc antibody supernatants. The major phosphoprotein species in these supernatants are the insulin receptor β-subunit and IRS-1 (Fig. 1A). Immunoprecipitation with pY20 removes more than 90% of phosphorylated IRS-1 (data not shown), and, consequently, any Grb2-Sos associated with IRS-1 would be found in the pY20 precipitates. When the post
The SH2 domain of Grb2 can directly bind to a phosphotyrosine motif in the EGF receptor, forming EGF receptor-Grb2:Sos complexes (22–25). In contrast, Grb2 does not associate directly with the insulin receptor (7, 8). However, it can associate through its SH2 domain with IRS-1 and Shc (7, 8, 26, 27), two proteins that undergo tyrosine phosphorylation upon insulin stimulation (2, 3, 7, 8). Consequently, either IRS-1 or Shc, or both, could couple insulin receptors to Grb2:Sos. In this study, we sought to elucidate the roles of IRS-1 and Shc in linking the insulin signal to Grb2:Sos and ultimately to stimulation of p21G~GTP.

IRS-1 was rapidly phosphorylated after addition of insulin, consistent with its role as a direct substrate of the insulin receptor (2, 3). In contrast, Shc phosphorylation proceeded more slowly, with a lag time before appreciable phosphorylation was detected, consistent with previous observations (7). Shc does not bind to the insulin receptors through SH2 domain interactions (7, 8). However, direct, transient, low affinity association with the receptor is possible. On the other hand, the lag phase in Shc phosphorylation raises the possibility that an intermediary tyrosine kinase mediates the phosphorylation of Shc. This is in contrast to the EGF signaling system, in which the time course of Shc phosphorylation is very rapid, peaking at about 1 min (data not shown), and in which Shc binds directly to phosphorylated EGF receptors (22–25). In any event, the time course of insulin-stimulated Shc phosphorylation corresponds more closely to the kinetics of GNRF activity and p21G~GTP formation than does the time course of IRS-1 phosphorylation, suggesting that Shc plays a more prominent role than IRS-1 in coupling the insulin receptor to p21G~GTP.

We also found that immunoprecipitation of lysates from insulin-stimulated cells with anti-Shc antibody removed 67% of the cellular GNRF activity, whereas anti-IRS-1 antibody precipitation had only a negligible effect to deplete GNRF. Since Sos mediates GNRF activity and it is constitutively bound to Grb2 (16, 22–27), one would expect insulin-induced association of Grb2 with Shc and/or IRS-1 to parallel GNRF activity. This was indeed the case. Thus, the kinetics of Grb2 association with Shc closely paralleled the time course of insulin-stimulated Shc phosphorylation (Fig. 2), and much more of the cellular Grb2 formed complexes with Shc than with IRS-1. For these reasons, we conclude that Shc is the quantitatively dominant pathway linking the activated insulin receptor, through Grb2:Sos, to p21G~GTP activation.

Since the initial identification of the Shc cDNA, increasing evidence has accrued indicating that Shc is an important component of growth signaling pathways (6, 30–33). To specifically identify a functional role for Shc in insulin action, we previously carried out single cell microinjection studies with anti-Shc antibody. Our results demonstrated that microinjection of anti-Shc antibody into HIRc cells led to an 80% inhibition of subsequent insulin-induced DNA synthesis.2 These findings, taken together with the current results, underscore the importance of Shc in the insulin mitogenic signaling pathway and indicate that it is the major molecular connection between the insulin receptor and the Grb2:Sos-p21G~ signaling cascade.

Although it is clear that Shc is a key molecule in the insulin mitogenic signaling pathway, and that IRS-1 is not an upstream regulator of Shc, substantial evidence exists indicating that IRS-1 is also a necessary component of insulin’s growth promoting effects. For example, expression of antisense IRS-1 mRNA leads to inhibition of insulin-stimulated thymidine incorporation into Chinese hamster ovary cells (34). In addition, expression of IRS-1 in the hematopoietic 32D cell line, which lacks endogenous IRS-1, allows these cells to respond to insulin with DNA synthesis (35). Lastly, microinjection of an IRS-1 antibody almost completely inhibits subsequent insulin stimulation of DNA synthesis in HIRc cells (28). Thus, it is evident that IRS-1 is also an important molecule in insulin’s mitogenic signaling actions.

To reconcile the above observations, it is unlikely that all of the involved molecules line up in a linear pathway, and parallel pathways seem possible. For example, IRS-1 and Shc may subserve parallel colinear pathways, leading to activation of p21G~GTP.
with Shc having the major input. Even though the Shc pathway is dominant, both inputs complement each other to generate the necessary stimulatory amount of p21'

Alternatively, since IRS-1-Grb2-Sos complex formation occurs more quickly, albeit to a much lesser extent than Shc-Grb2-Sos formation, it is possible that the same Grb2-Sos molecules which initially bind to IRS-1 subsequently associate with Shc. This might lead to the appropriate translocation or redistribution of Grb2-Sos to facilitate stimulation of membrane bound p21'. Finally, it seems possible that IRS-1 participates in mitogenic signaling through mechanisms largely independent of p21' stimulation. Thus, IRS-1 serves as a multisite docking protein binding to the PI 3-kinase (38), and inhibition of PI 3-kinase action will abrogate insulin's mitogenic effects. Thus, Yamauchi et al. (37) have shown that overexpression of the p85 subunit in Chinese hamster ovary cells inhibits insulin's ability to stimulate c-Fos transcription, and we have found that microinjection of an antibody and Dr. Tahir S. Pillay and S. J. (1993) J. Biol. Chem. 268, 1953-1955

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