Functional Importance of Shc Tyrosine 317 on Insulin Signaling in Rat1 Fibroblasts Expressing Insulin Receptors

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Shc is phosphorylated on Tyr-317, which serves as a docking site for Grb2. To investigate the specific role of Shc phosphorylation and Shc-Grb2 coupling on insulin signaling, we generated expression vectors for wild-type Grb2 coupling on insulin z-docking site for Grb2. To investigate the specific role of fibroblasts.

Insulin-induced Shc phosphorylation and subsequent association with Grb2 was enhanced in WT-Shc cells. Because of competition between Shc and IRS-1 for interaction with the insulin receptor, insulin-stimulated tyrosine phosphorylation of IRS-1 was decreased in WT-Shc cells compared with that in HIRc cells. Likewise, reduction of endogenous Shc expression by antisense Shc mRNA resulted in increased insulin stimulation of IRS-1 phosphorylation. Although 317Y/F-Shc was also able to interact with insulin receptor, decreased amounts of Shc phosphorylation and Shc association with Grb2 were observed in 317Y/F-Shc cells, indicating that 317Y/F-Shc functions as a dominant-negative mutant. The kinetics of mitogen-activated protein (MAP) kinase activation closely paralleled the kinetics of Shc phosphorylation. Thus, insulin stimulation of MAP kinase activation occurred more rapidly and was prolonged in WT-Shc cells, while the activation was delayed and transient in 317Y/F-Shc cells compared with that in HIRc cells. Importantly, WT-Shc cells displayed enhanced sensitivity to insulin stimulation of thymidine and bromodeoxyuridine incorporation, whereas the sensitivity was decreased in 317Y/F-Shc cells. These results indicate that Shc Tyr-317 phosphorylation plays an important role, via coupling with Grb2 and competition with IRS-1, in signal transduction to MAP kinase by insulin, ultimately leading to mitogenesis in Rat1 fibroblasts.

The activated insulin receptor phosphorylates various cellular substrates on tyrosine residues (1). One of the substrates of the insulin receptor is insulin receptor substrate 1 (IRS-1)1 (2). Tyrosine-phosphorylated IRS-1 functions as a multisite docking protein to interact with the Src homology 2 (SH2) domains of various signal-transducing molecules, including Grb2, the p85 regulatory subunit of PI3-kinase, protein-tyrosine phosphatase SH-PTP2 (also called Syp, PTP1D, and PTP2C), Nck, and Fyn, to propagate the insulin signal downstream (1, 3).

Recently, insulin receptor substrate 2 (IRS-2) was cloned as an alternative substrate of the insulin receptor and appears to play some physiological roles in insulin signal transduction with coupling mechanisms similar to IRS-1 (4). In addition to IRS-1 and IRS-2, another substrate of the insulin receptor termed Shc has been identified (5). Shc has been implicated in mitogenic signaling by a variety of receptors for growth factors such as insulin, epidermal growth factor, platelet-derived growth factor, and nerve growth factor (5–10). Previous studies examined the role of Shc in insulin-induced mitogenic signaling, primarily using the transient expression of wild-type Shc or the microinjection of anti-Shc antibody, and demonstrated the importance of Shc in insulin’s mitogenic signal transduction (11–13).

Shc is composed of three distinct domains: an amino-terminal region called the phosphotyrosine binding domain, a collagen homology domain, and a carboxy-terminal SH2 domain (5, 14–17). Shc has been shown to be involved in the activation of p21ras, which plays a pivotal role in signal transduction initiated by tyrosine kinases of insulin and other growth factor receptors (13, 18, 19). Shc is tyrosine-phosphorylated upon insulin stimulation and subsequently associates with Grb2, which forms a complex with Sos, a p21ras guanine nucleotide exchange factor (5, 18, 20). Shc-Grb2 binding is mediated by the SH2 domain of Grb2 binding to phosphorylated Tyr-317 in the collagen homology domain of Shc suggesting that Tyr-317 plays a key role in insulin signaling (5, 21). Shc has been reported to associate with at least three other downstream signal molecules in addition to Grb2, although the function of these Shc-binding proteins remains to be clarified. First, PEST tyrosine phosphatase has been shown to associate with Shc (22). Two serine residues at positions 5 and 29 in the amino terminus of Shc are suggested to be sites regulating binding to the PEST protein-tyrosine phosphatase. Second, adaptins bind to Shc and are thought to be involved in receptor endocytosis (23). Amino acids 346–355 in the collagen homology domain of Shc are required for adaptin binding. Third, a tyrosine-phospho...

1 The abbreviations used are: IRS, insulin receptor substrate; MAP, mitogen-activated protein; WT, wild-type; SH2, Src homology 2; BrdUrd, bromodeoxyuridine; PAGE, polyacrylamide gel electrophoresis.
rylated 145-kDa protein has been shown to bind to Shc (14). Recently, this has been cloned and named SHIP, for SH2-containing inositol phosphatase (24). The amino terminus of Shc (amino acids 46–232) appeared to be responsible for SHIP binding. The Drosophila Shc homologue lacks the tyrosine residue corresponding to the mammalian Grb2 binding site and does not interact with the Drosophila Grb2 homologue (Drek) (25). This finding suggests that, at least in Drosophila, Shc may play a role other than p21ras activation via Shc-Grb2 association. Therefore, although the functional role of Shc as the signal-transducing molecule has been elucidated, the importance of the phosphorylation of Shc Tyr-317 for insulin’s biological action has not been clearly demonstrated.

In the present study, to directly clarify the specific role of Shc Tyr-317 phosphorylation and Grb2 association via Shc Tyr-317 residue on insulin signaling, we generated an Shc cDNA with a Tyr-317 → Phe point mutation (317Y/F-Shc). The mutant 317Y/F-Shc plasmids were stably transfected into Rat1 fibroblasts overexpressing insulin receptors, and intracellular insulin signaling leading to the mitogenic effect of insulin was examined in these cell lines.

**EXPERIMENTAL PROCEDURES**

**Materials**—Porcine insulin was a kind gift from Shimizu Pharmaceutical Co. (Shizuoka, Japan). [3H]Thymidine (83 Ci/mmol) was purchased from DuPont NEN. A polyclonal anti-Shc antibody, a monoclonal anti-Grb2 antibody, and a monoclonal anti-phosphoryrosine antibody (pY20) were from Transduction Laboratories (Lexington, KY). Bromodeoxyuridine (BrdUrd), a monoclonal anti-BrdUrd antibody, and enhanced chemiluminescence reagents were from Amersham Corp. Rhodamine-conjugated anti-mouse IgG antibody was from Jackson Laboratories (West Grove, NY). A polyclonal anti-IRS-1 antibody was kindly provided by Dr. Hiroshi Maegawa (Shiga University of Medical Science, Omaezaka, Shiga, Japan). Human phosphorosine reagents were from Bio-Rad. All other reagents were analytical grade and purchased from Sigma or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Plasmid Construction**—The human Shc cDNA was isolated from human fibroblasts. Two overlapping Shc clones were synthesized by reverse transcription-polymerase chain reaction from mRNA of human fibroblast cells. Primers used in the polymerase chain reaction were: 5'-GGCACTGGAGCATGAAACG-3' (sense strand), 5'-GTACTGGCT-3' (antisense strand), 5'-ATGCAATCTATCTCCATTGC-3' (sense strand), and 5'-TGAATTCAGAATCTTGC-3' (antisense strand). The nucleotide sequences of these clones were verified with various internal primers and were totally consistent with the published sequence (5), and then these clones were ligated at the BamHI site. The resulting fragment containing the entire coding region was filled with Klenow fragment and inserted into the EcoRV site of the mammalian expression vector Rldn to yield RldnWT-Shc. Rldn was kindly provided by Dr. Allan R. Shatzman (SmithKline Beecham). To generate a mutant Shc cDNA encoding the Tyr-317 → Phe point mutation, polymerase chain reaction was performed with a mutagenic oligonucleotide (5'-CTGGAGCCTTGAGAAAAAGGGATT-3', antisense strand) including the native HincII site and primers listed above. The mutant 317Y/F-Shc cDNA was also cloned into the EcoRV site of Rldn (RldnMT-Shc).

**Cell Culture, DNA Transfection, and Establishment of Cell Lines**—Rat1 fibroblasts expressing 1 × 10⁶ human insulin receptors per cell (HIRc) were kindly supplied by Dr. J. M. Olefsky (University of California, San Diego) and were maintained in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal calf serum (26). To establish antisense Shc-expressing cells, WT-Shc cDNA was cloned in the negative orientation into Rldn (RldnAS-Shc). HIRc cells (5 × 10⁶ cells per dish) were transfected with 20 μg of RldnWT-Shc, Rldn317Y/F-Shc, or RldnAS-Shc and 2 μg of pcDEB carrying a hygromycin-resistant gene using calcium phosphate methods for 72 h. Hygromycin B (400 μg/ml) was then added to the medium to select for resistant cells. Cells expressing high levels of the wild-type Shc, the 317Y/F mutant Shc, or the antisense Shc were isolated by limiting dilution and then chosen by immunoblotting with anti-Shc antibody.

**Immunoprecipitation and Western Blotting**—Cells were serum-starved for 24 h and then incubated with 17 nm insulin for the indicated times. The cells were lysed in a solubilizing buffer containing 30 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM NaVO₄, 160 μM NaF, pH 7.4, for 15 min at 4 °C. Lysates obtained from the same number of cells for each cell line were centrifuged to remove insoluble material, and the supernatants were used for immunoprecipitation with the specified antibodies for 3 h at 4 °C. The immunoprecipitates, the remaining supernatants, or whole cell lysates were separated by 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes by electroblotting. The membranes were blocked with 2.5% bovine serum albumin and probed with the specified antibodies. Enhanced chemiluminescence detection was performed according to the manufacturer’s instructions (Amersham Corp.).

**Thymidine Incorporation**—Cells were grown to confluence in 24 multwell culture plates and serum-starved for 24 h. After stimulation of the cells with various concentrations of insulin for 20 h, 1 μCi of [3H]thymidine was added for 4 h. The cells were washed twice with ice-cold phosphate-buffered saline, twice with ice-cold 10% trichloroacetic acid, and once with 95% ethanol. The cells were dissolved in 1 N NaOH, neutralized with 1 N HCl, and counted in a liquid scintillation counter (27).

**BrdUrd Incorporation**—Cells were grown on glass coverslips and rendered quiescent by starvation for 24 h. Serum-starved cells were incubated with BrdUrd plus various concentrations of insulin for 16 h at 37 °C. The cells were fixed with acid alcohol (90% ethanol, 5% acetic acid) for 20 min at 22 °C and then incubated with mouse monoclonal anti-BrdUrd antibody for 1 h at 22 °C. The cells were then stained by incubation with rhodamine-labeled donkey anti-mouse IgG antibody for 1 h at 22 °C. After the coverslips were mounted, the cells were analyzed with a Microphot-FXA fluorescence microscope (Nikon, Tokyo, Japan) (12).

**RESULTS**

**Expression of Exogenous Wild-type and Mutant Shc in HIRc Cells**—We generated mutant Shc cDNA encoding a Tyr-317 → Phe mutation by primer-directed in vitro mutagenesis (Fig. 1A). Both the wild-type Shc and the mutant 317Y/F-Shc expression plasmids were transfected into HIRc cells, and clones resistant to hygromycin B were selected. From hygromycin-resistant colonies selected, five independent cell lines overexpressing the wild-type Shc (WT-Shc) or the mutant Shc (317Y/F-Shc) were obtained by immunoblotting the cell lysates with anti-Shc antibody. Each cell line chosen for further study expressed a 10 times greater amount of wild-type Shc or 317Y/F-Shc compared with endogenous Shc in HIRc cells (Fig. 1B).

Insulin-induced Tyrosine Phosphorylation of Shc in the
Both Shc and IRS-1 bind to the juxtamembrane domain of IRS-1. Overexpression of wild-type or 317Y/F mutant Shc had no effect on tyrosine phosphorylation of Shc in the basal state. Insulin stimulated tyrosine phosphorylation of 52-kDa Shc in a time-dependent manner in HIRc cells. In cells overexpressing wild-type Shc, insulin-stimulated Shc tyrosine phosphorylation was faster, and a greater amount of Shc was phosphorylated compared with HIRc cells. In contrast, insulin-stimulated tyrosine phosphorylation of Shc was decreased in 317Y/F-Shc cells compared with that in HIRc cells. These results are summarized in Fig. 2B. Following 10 min of insulin stimulation, Shc phosphorylation was increased to 137 ± 23% in WT-Shc cells and decreased to 27 ± 14% in 317Y/F-Shc cells compared with that in HIRc cells.

**Effects of Shc Overexpression on Tyrosine Phosphorylation of IRS-1**—Both Shc and IRS-1 bind to the juxtamembrane domain centered on Tyr-960 of the insulin receptor β-subunit (15, 28). Therefore, it can be speculated that Shc and IRS-1 serve as competitive substrates of the insulin receptor. To test this, we examined the effect of Shc overexpression on insulin-induced IRS-1 phosphorylation in the transfected cell lines. The cells were incubated with 17 nm insulin for the indicated times, and the cell lysates were subjected to immunoprecipitation with anti-Shc antibody. The immunoprecipitates were then analyzed by immunoblotting with anti-phosphotyrosine antibody. A, representative results are shown. Molecular mass of Shc (52-kDa isoform) is shown by an arrow. B, IRS-1 phosphorylation was quantitated by densitometry. Results are the mean ± S.E. of four separate experiments.

**Effects of Antisense Shc mRNA on Tyrosine Phosphorylation of IRS-1**—To further assess the competition between Shc and IRS-1 as substrates of the insulin receptor, we transfected HIRc cells with RldnAS-Shc. Following selection, we isolated six individual clones. Of these six, the clones demonstrating that Shc expression decreased to 50 ± 6% of that in HIRc cells. Tyrosine phosphorylation of IRS-1 was also decreased in 317Y/F-Shc cells and comparable to that in WT-Shc cells. These results are summarized in Fig. 3B. Following 1 min of insulin stimulation, tyrosine phosphorylation of IRS-1 was decreased to 46.9 ± 13.8% and 53.3 ± 5.5% by WT-Shc and 317Y/F-Shc overexpression, respectively (results normalized to the amount of tyrosine-phosphorylated insulin receptor β-subunit). Thus, both overexpressed WT-Shc and 317Y/F-Shc appeared to be able to compete with endogenous IRS-1 as substrates of the insulin receptor.

**Effects of Shc Overexpression on Grb2 Association with E1**—Insulin-induced tyrosine phosphorylation of IRS-1 in the transfected cell lines, HIRc, WT-Shc, and 317Y/F-Shc cells were serum-starved for 24 h and then incubated with 17 nm insulin for the indicated times. The cells were solubilized, and the cell lysates were immunoprecipitated with anti-Shc antibody. The immunoprecipitates were then subjected to SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibody. A, representative results are shown. Molecular mass of Shc (52-kDa isoform) is shown by an arrow. B, IRS-1 phosphorylation was quantitated by densitometry. Results are the mean ± S.E. of four separate experiments.
The activation of MAP kinase is important for insulin-induced DNA synthesis. Phosphorylation of both tyrosine and threonine residues is required for the activation of MAP kinase (29), and this phosphorylation results in decreased mobility on SDS-PAGE (30). Therefore, we next assessed insulin stimulation of MAP kinase activity using the MAP kinase gel shift assay in transfected cell lines. As can be seen in Fig. 7A, insulin treatment induced a time-dependent phosphorylation of MAP kinase and a time-dependent mobility shift of p42MAPK (ERK-2) in the transfected cell lines. These results are summarized in Fig. 7B. Insulin-stimulated MAP kinase activation was faster and more prolonged in wild-type Shc-overexpressing cells. In contrast, the MAP kinase gel shift was delayed, and only a transient activation was seen in 317Y/F-Shc cells compared with that in wild-type Shc cells. After 10 min of insulin stimulation, Shc-Gerb2 association was increased to 143 ± 24% in WT-Shc cells and decreased to 46 ± 9% in 317Y/F-Shc cells compared with that in HIRc cells as shown in Fig. 5B. To examine the effect of Shc overexpression on IRS-1 association with Grb2, the cell lysates were immunoprecipitated with anti-IRS-1 antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-Grb2 antibody. Compared with insulin-stimulated IRS-1 association with Grb2 in HIRc cells, the amount of IRS-1 association with Grb2 was decreased in both wild-type Shc and 317Y/F-Shc cells (Fig. 6A). Following 1 min of insulin stimulation, IRS-1-Grb2 association was decreased to 56 ± 4% and 58 ± 3% of control in WT-Shc and 317Y/F-Shc cells, respectively (Fig. 6B). These results suggest that 317Y/F-Shc can bind to the insulin receptor upon insulin stimulation, but that it subsequently fails to be phosphorylated or bind to Grb2.
compared with that in HIRc cells.

Effects of Shc Overexpression on Insulin's Mitogenic Action—To study the mitogenic signaling properties of the Shc-overexpressing cell lines, thymidine incorporation was assayed in the transfected cell lines as shown in Fig. 8. Insulin stimulated thymidine incorporation in a dose-dependent manner with an ED50 value of 0.25 ± 0.07 nM in HIRc cells. Expression of WT-Shc led to enhanced insulin sensitivity with a leftward shift of the dose-response curve (ED50 value, 0.09 ± 0.04 nM). In contrast, the insulin sensitivity was decreased with an ED50 value of 0.49 ± 0.11 nM in 317Y/F-Shc cells. We also assessed insulin-induced mitogenesis by the independent approach of BrdUrd incorporation. As shown in Fig. 9, the results of BrdUrd incorporation studies were qualitatively similar to those of thymidine incorporation studies. WT-Shc cells displayed enhanced sensitivity to insulin with an ED50 value of 1.0 ± 0.3 nM compared with 2.8 ± 1.2 nM in HIRc cells, whereas the sensitivity was decreased with an ED50 value of 9.7 ± 1.8 nM in 317Y/F-Shc cells.

**DISCUSSION**

We showed that insulin-stimulated tyrosine phosphorylation of Shc and subsequent Shc association with Grb2 were increased by stable overexpression of wild-type Shc in Rat1 fibroblasts expressing insulin receptors. In addition, the time course of insulin-stimulated MAP kinase activation was more rapid, and more prolonged activation was observed in WT-Shc cells compared with that in parental HIRc cells. Furthermore, insulin sensitivity of both thymidine and BrdUrd incorporation studies was enhanced in wild-type Shc cells compared with that of the parental HIRc cells. Taken together with previous reports showing that Shc-Grb2-Sos, but not IRS-1-Grb2-Sos, is the predominant pathway coupling the activated insulin receptors to p21^waf1 leading to DNA synthesis, and that microinjection of anti-Shc antibody inhibited insulin stimulation of DNA synthesis (12, 13), our results further support the important role of Shc in insulin-induced mitogenic signaling in Rat1 fibroblasts.

IRS-1 and Shc are the major intracellular substrates of the activated insulin receptor (2, 5). Since both IRS-1 and Shc interact with the juxtamembrane domain around Tyr-960 of the insulin receptor (15, 28), one can speculate that Shc and IRS-1 bind competitively to phosphorylated insulin receptors.
Our results showed that stably overexpressed wild-type Shc could compete with endogenous IRS-1 for interaction with insulin receptors, since insulin-stimulated tyrosine phosphorylation of IRS-1 was decreased in WT-Shc cells compared with that in the parental HiRc cells, and reduction of Shc expression by antisense Shc RNA enhanced insulin-stimulated tyrosine phosphorylation of IRS-1. These results clearly indicate that Shc and IRS-1 serve as competitive substrates of the insulin receptor. This idea was further supported by the fact that overexpression of wild-type Shc increased Shc/Grb2 association and decreased IRS-1/Grb2 association. Importantly, overexpression of the mutant 317Y/F-Shc also decreased insulin stimulation of IRS-1 phosphorylation as shown in Fig. 3. This result suggests that 317Y/F-Shc binds to the insulin receptor upon insulin stimulation, but that it subsequently fails to bind to Grb2. Therefore, overexpressed 317Y/F-Shc appears to function as a dominant-negative mutant at the level of Grb2 association. The dominant-negative feature of exogenous 317Y/F-Shc overexpression was further supported by the fact that the time course of insulin-stimulated MAP kinase activation was delayed and transient in 317Y/F-Shc cells compared with that in HiRc cells as shown in Figs. 8 and 9. These findings demonstrate the essential role played by Shc Tyr-317 phosphorylation in insulin signaling to Map kinase activation and mitogenic action in rat1 fibroblasts. In this regard, Shc binding to other signaling molecules, which is not mediated by Shc Tyr-317 phosphorylation and subsequent association with Grb2, is not sufficient to transduce insulin's mitogenic signals.

It was somewhat surprising that insulin-induced typhimidine and BrdUrd incorporation are only mildly impaired by overexpression of 317Y/F-Shc. There are several possible explanations for this finding. First, the expression level of the 317Y/F-Shc may not be sufficient to completely compete with endogenous Shc. Second, the 317Y/F-Shc may be phosphorylated on alternative tyrosines generating low affinity binding sites for Grb2, and this may prevent a major abnormality in the mitogenic effects of insulin. A previous study has indicated that Shc Tyr-240 is a candidate for an alternative tyrosine phosphorylation site (31). These possibilities are both consistent with our observation that insulin stimulation of Shc phosphorylation and subsequent association with Grb2 were not totally inhibited by 317Y/F-Shc overexpression. Third, one can speculate that other signaling pathways independent of Grb2 can partially compensate for the loss of the Shc/Grb2 pathway in p21ras activation. Along this line, it has been shown that SH-PTP2/Syp may activate p21ras through a non-Shc/Grb2 pathway.

Overexpression of catalytically inactive SH-PTP2 inhibited insulin stimulation of p21ras activation without affecting tyrosine phosphorylation of Shc or Shc association with Grb2 (32–34). Furthermore, it is known that mammalian cells contain several p21ras guanine nucleotide exchange factors apart from Sos. C3G is one of these guanine nucleotide exchange factors that may activate p21ras (35). C3G, via its proline-rich region, binds to the amino-terminal domain of the adaptor protein Crk (35). The Crk-C3G pathway may compensate for diminished activity of the Shc/Grb2/Sos pathway in p21ras activation, although Rap1 rather than p21ras was recently identified as a preferred target for C3G in mammalian cells and the function of the Crk-C3G pathway in insulin signaling has not yet been characterized (36).

In summary, although Shc might associate with multiple downstream molecules through various sites, Shc Tyr-317 phosphorylation plays an essential role, via coupling with Grb2 and competition with IRS-1 as an insulin receptor substrate, in insulin-induced cell cycle progression.

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