Shc has two distinct domains, amino-terminal and SH2 domain, which can interact with activated growth factor receptors. Shc interacts with insulin receptor via Shc-amino-terminal (N) domain, whereas Shc associates with epidermal growth factor (EGF) receptor through both Shc-N and -SH2 domains. In accordance with the different functional roles between insulin and EGF receptors, EGF stimulated tyrosine phosphorylation of Shc faster than insulin. To clarify the functional importance of three distinct Shc domains on insulin and EGF signaling, we microinjected glutathione S-transferase (GST) fusion proteins containing the amino terminus plus collagen homology domain (NCH), collagen homology domain (CH), and Src homology 2 domain (SH2) into Rat1 fibroblasts expressing insulin receptors (HIRc). Bromodeoxyuridine (BrdUrd) incorporation into newly synthesized DNA was subsequently studied to assess the importance of the three distinct domains of Shc. Microinjection of the NCH-GST fusion protein inhibited BrdUrd incorporation induced by both EGF and insulin, whereas microinjection of the SH2-GST fusion protein inhibited EGF, but not insulin stimulation of DNA synthesis. Neither EGF- nor insulin-induced BrdUrd incorporation was inhibited by the CH-GST fusion protein. Following EGF or insulin stimulation, Shc is phosphorylated on single Tyr-317 residue serving as a docking site for Grb2. Microinjection of Shc-N+CH GST fusion protein with Tyr-317 → Phe replacement (Y317F) also inhibited insulin stimulation of DNA synthesis. Next, we stably overexpressed wild-type Shc or Y317F mutant Shc into HIRc cells. Insulin-induced tyrosine phosphorylation of IRS-1 was compared among the transfected cell lines, since IRS-1 and Shc could competitively interact with insulin receptor. Insulin-stimulated tyrosine phosphorylation of IRS-1 was decreased in both WT-Shc and Y317F-Shc cells compared with that in HIRc cells. Furthermore, overexpression of the Shc-SH2 domain or Shc-N+CH domain with Y317F mutation interfered with EGF-stimulated endogenous Shc phosphorylation. These results suggest that the amino terminus domain of Shc is functionally important in insulin- and EGF-induced cell cycle progression and that the phosphorylation of Shc Tyr-317 residue is independent of Shc interaction with these receptors.

After activation of growth factor receptors, the interaction of tyrosine-phosphorylated receptors with various SH2 domain containing effector molecules is responsible for downstream signal transduction leading to biological action (1). One of the major substrates of growth factor receptors is Shc (2). Shc is tyrosine-phosphorylated following growth factors stimulation and then is associated with Grb2-Sos complex to activate p21ras (3–6), which leads to mitogenesis by activation of the mitogen-activated protein kinase (4–9). Therefore, a key to understand the mechanism of how activated growth factor receptors increase the amount of p21ras-GTP lies in the clarification of the mechanisms of linkage between growth factor receptors and Shc. The SH2 domain of Shc has been shown to be a site to bind phosphorylated EGF receptor (10, 11). In this interaction, Shc appears to interact with the amino acid residues amino-terminal to phosphoryrosine of the receptor (12). This interaction is uncommon for SH2 domains that usually specifically interact with the amino acid residues carboxyl-terminal to phosphorytosine (13). In addition, the SH2 domain of Shc does not interact with insulin receptor (3, 4, 7, 8). Instead, it has been reported that amino-terminal region of Shc contains a novel domain called phosphotyrosine-binding (PTB) domain, which is different from that of all members of the known SH2 domain family and is capable of binding to phosphorytosine residues (14–17). Thus, the recent studies suggest a novel mechanism whereby Shc can interact with growth factor receptors. However, the functional significance of the amino-terminal domain of Shc in the interaction with EGF or insulin receptors is unknown. In addition, the functional importance of Shc Tyr-317 phosphorylation for the interaction is also uncertain. Since Shc is phosphorylated on single Tyr-317 residue following EGF or insulin stimulation and the phosphorylated Tyr-317 serves as a docking site for Grb2 (18), phosphorylation of Shc Tyr-317 may also affect the interaction of Shc with these receptors. This study directly addresses these questions, i.e. the functional roles of amino terminus and phosphorylated Tyr-317 of Shc for signal transduction.

**EXPERIMENTAL PROCEDURES**

Materials—Porcine insulin and human IGF-1 were kind gift from Shimizu Pharmaceutical Co. (Shizuoka, Japan) and Fujisawa Pharmaceutical Co. (Osaka, Japan), respectively. Human EGF was purchased...
was also cloned into Shc cDNA by site-directed mutagenesis. The mutant Shc (Y317F-Shc) point mutation that changed Tyr-317 to Phe was introduced into the was kindly provided by Dr. Allan R. Shatzman (Smith Kline Co.). A cellsexpressing10timesgreateramountsoftheseexpressedexogenous lysates with anti-Shc antibody as described below. Of various cell lines, precipitates were separated by SDS-PAGE and transferred to Immobilon-P. Immunoblots were performed with anti-phosphorysotyrosine antibody. Molecular mass of predominant Shc isoform (52 kDa) is shown by arrows. Results are representative of three separate experiments.

RESULTS

It has been reported previously that both EGF and insulin stimulate tyrosine phosphorylation of Shc (5-7). To compare the time course of Shc phosphorylation between EGF and insulin stimulation, cell lysates were immunoprecipitated with anti-Shc antibody and the precipitates were immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 1A, Shc was tyrosine-phosphorylated following EGF stimulation. The time course of EGF-stimulated Shc phosphorylation was rapid. Peak phosphorylation of Shc was observed by 30 s and declined after 2.5 min following EGF stimulation. In contrast, the time course of insulin-induced Shc phosphorylation was slower than EGF as shown in Fig. 1B. The phosphorylation was first observed at 1 min, reached a maximum at 5 min, and declined thereafter. Thus, it is apparent that EGF and insulin induce tyrosine phosphorylation of Shc with different time kinetics, indicating that Shc interacts with EGF receptor and insulin receptor through different mechanisms.

The Shc protein contains three domains, an amino-terminal region called PTB domain, a collagen homology domain that contains Tyr-317 residue for Grb2 binding, and a carboxy-terminal SH2 domain (2, 21). To evaluate the functional importance of three distinct domains of Shc in EGF and insulin signaling, we performed microinjection studies using Shc-NCH, Shc-CH, and Shc-SH2 GST fusion proteins. A bacterially expressed recombinant GST fusion proteins containing various domains of Shc were purified by glutathione affinity chromatography. Western Blotting Studies—Cell monolayers were starved for 24 h in serum-free Dulbecco’s modified Eagle’s medium. The cells were then treated with 17 nM insulin or 160 nM EGF for the indicated times at 37°C. 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 phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 1 mM Na3VO4, pH 7.4. The cell lysates were centrifuged to remove insoluble materials. The supernatants (100 μg of protein) were used for immunoprecipitation with anti-Shc antibody for 5 h at 4°C, or for 90 min with various Shc-GST fusion proteins, which were then precipitated with glutathione-agarose. The precipitates were separated by SDS-PAGE and transferred to Immobilon-P using a Bio-Rad Transblot apparatus. Western Blotting Studies—Cells were grown on glass coverslips and rendered

Microinjection—Cells were grown on glass coverslips and rendered quiescent by starvation for 24 h in serum-free Dulbecco’s modified Eagle’s medium. GST fusion proteins were solubilized in microinjection buffer consisting of 5 mM NaPO4, 100 mM KCl, pH 7.4. Serum-starved cells were then microinjected with 5 mg/ml GST fusion protein containing 2 mg/ml rabbit IgG using glass capillary needles. 2 h after microinjection, cells were incubated with BrdUrd plus either vehicle, 17 nM insulin, 160 nM EGF, or 10% fetal calf serum for 16 h at 37°C. The cells were then fixed with acid alcohol (90% ethanol, 5% acetic acid) for 20 min at 22°C and then incubated with mouse monoclonal anti-Grb2 antibody followed by enhanced chemiluminescence detection using the ECL reagent according to the manufacturer’s instructions (Amersham Corp.) (5-7).

Microinjection—Cells were grown on glass coverslips and rendered quiescent by starvation for 24 h in serum-free Dulbecco’s modified Eagle’s medium. GST fusion proteins were solubilized in microinjection buffer consisting of 5 mM NaPO4, 100 mM KCl, pH 7.4. Serum-starved cells were then microinjected with 5 mg/ml GST fusion protein containing 2 mg/ml rabbit IgG using glass capillary needles. 2 h after microinjection, cells were incubated with BrdUrd plus either vehicle, 17 nM insulin, 160 nM EGF, or 10% fetal calf serum for 16 h at 37°C. The cells were then fixed with acid alcohol (90% ethanol, 5% acetic acid) for 20 min at 22°C and then incubated with mouse monoclonal anti-Grb2 antibody. The cells were then stained by incubation with rhodamine-labeled donkey anti-mouse IgG antibody and fluorescein isothiocyanate-labeled donkey anti-rabbit IgG antibody for 1 h at 22°C. After the coverslips were mounted, the cells were analyzed with a Microphot-FXA fluorescence microscope (Nikon, Japan) (7). Microinjected cell numbers were 250–300/coverslip. Immunofluorescent stain-
In contrast, phosphorylated EGF receptor was associated with both Shc-NCH and Shc-SH2 GST fusion proteins. No corresponding bands were detected with Shc-CH and control GST fusion protein, and no phosphoprotein bands were observed in lysates from quiescent cells by these GST fusion proteins.

The effect of these GST fusion proteins on EGF- or insulin-stimulated DNA synthesis was assessed by microinjection studies. BrdUrd incorporation was monitored to study the functional importance of the distinct domains of Shc. Comparison of the BrdUrd incorporation rates between injected and un.injected quiescent cells indicated that the all three GST fusion proteins had no effect on the basal rate of DNA synthesis (Fig. 3A). In addition, the three distinct domains of GST fusion proteins were not toxic, since the stimulatory effect of serum was not inhibited by microinjection of these GST fusion proteins (data not shown). Insulin or EGF stimulation led to an increase in BrdUrd incorporation. Microinjection of control GST fusion protein did not show any inhibitory effect on both insulin and EGF signaling. Shc bound to the phosphorylated EGF receptor via, at least in part, its SH2 domain, but Shc did not interact with insulin receptor through its SH2 domain (Fig. 2) as the previous studies reported (3, 7, 10, 11). Furthermore, the microinjection of Shc-SH2 GST fusion protein effectively inhibited EGF-stimulated BrdUrd incorporation by 74% but did not affect insulin action. Shc has a second domain in its amino terminus, distinct from the SH2 domain, that can interact with tyrosine-phosphorylated growth factor receptors (Fig. 2) (14–17). Importantly, the microinjection of Shc-NCH GST fusion protein effectively inhibited both EGF- and insulin-stimulated BrdUrd incorporation by 56 and 87%, respectively. Since the microinjection of Shc-CH GST fusion protein did not show any inhibitory effect on these growth factors’ signaling, the inhibitory effect of Shc-NCH GST fusion protein appears to arise from the important role of Shc amino-terminal domain for the interaction with these receptors (Fig. 3, B and C).

Following EGF or insulin stimulation, Shc is phosphorylated on single Tyr-317 residue serving as a docking site for Grb2 (18). We next assessed the involvement of Tyr-317 phosphorylation in the interaction with EGF or insulin receptor. The cell lysates were affinity precipitated with Shc-NCH GST fusion protein carrying Tyr-317 → Phe mutation, and association of the mutant Shc with EGF or insulin receptor was examined. Interestingly, the Shc-NCH GST fusion protein with Y317F mutation also interacted with both phosphorylated EGF and insulin receptors as shown in Fig. 2. In accordance with the result of interaction, the microinjection of Shc-NCH GST fusion protein containing Y317F mutation inhibited both EGF- and insulin-stimulated BrdUrd incorporation by 49 and 76%, respectively, as shown in Fig. 3.

To further demonstrate Tyr-317 involvement in the interaction of Shc with activated insulin receptor, transfected HIRc cell lines overexpressing wild-type Shc (WT-Shc) or Y317F-mutant Shc (Y317F-Shc) were studied. Insulin stimulation of IRS-1 phosphorylation was compared among original HIRc, WT-Shc, and Y317F-Shc cells. Since PTB domain of both Shc and IRS-1 competitively interact with juxtamembrane domain around Tyr-960 of insulin receptor (14, 22–24), examination of the amounts of tyrosine phosphorylated IRS-1 by overexpression of wild-type Shc or Y317F-Shc could clarify the involvement of Shc Tyr-317 residue in the interaction with the insulin receptor. IRS-1 phosphorylation was decreased by overexpression of wild-type Shc as shown in Fig. 4A, indicating competitive interaction of insulin receptor with IRS-1 or Shc. Interestingly, insulin-induced IRS-1 phosphorylation was also reduced by Y317F-Shc overexpression. These results were summarized in Fig. 4B. The relative amount of IRS-1 phosphorylation in WT-Shc or Y317F-Shc cells versus that in HIRc cells was quantified by densitometry. Insulin-stimulated tyrosine phosphorylation of IRS-1 was decreased to 42.7 ± 3.0% in WT-Shc cells and 44.8 ± 5.3% in Y317F-Shc cells compared with that in HIRc cells. Furthermore, to clearly elucidate the importance of Shc subdomain interactions for EGF stimulation of Shc phosphorylation, cell lines overexpressing Shc-SH2 domain or Shc-N + CH domain with Y317F mutation were studied next. EGF stimulation of endogenous Shc phosphorylation was compared among the transfected cell lines. As shown in Fig. 5A, EGF stimulation of endogenous Shc phosphorylation was reduced by overexpressing the Shc-SH2 domain or Shc-N + CH domain with Y317F mutation compared with that in HIRc cells. By the analysis using densitometry, EGF stimulation of endogenous Shc phosphorylation was decreased to 39.1 ± 4.8% and 47.6 ± 2.9% by the Shc-SH2 domain and Shc-N + CH domain with Y317F mutation overexpression, respectively, as shown in Fig. 5B.

DISCUSSION

Shc can interact with tyrosine-phosphorylated growth factor receptors in three different ways, either through its SH2 domain, its amino-terminal domain, or both (10, 11, 14–17). Although previous reports have mainly focused on the interactions of the Shc-SH2 domain (10, 11), increasing evidence indicates that the amino-terminal domain of Shc is responsible for the binding to phosphotyrosine residues of the growth factor receptors (14–17). Two-hybrid assay suggested that Shc interacted with the juxtamembrane domain of insulin receptors via the amino-terminal domain of Shc, but not the SH2 domain of Shc (14). Along this line, our findings directly indicate the functional importance of the amino-terminal domain of Shc, but not of the SH2 domain, for insulin stimulation of DNA synthesis in living cells. These results are consistent with the previous study showing that a mutation of Tyr-960 → Phe in the insulin receptor, which is involved in PTB domain interactions, interferes with insulin’s mitogenic signaling (25). As for EGF signaling, although it was previously assumed that the SH2 domain of Shc was responsible for the association of Shc with the EGF receptor (10, 11), the recent studies have shown that the amino-terminal domain of Shc also participates in the interaction independently from the involvement of the SH2 domain (15–17). Thus, our studies demonstrated that both the amino-terminal and the SH2 domain of Shc could interact with EGF receptor. In agreement with these findings, the inhibition of EGF-induced DNA synthesis by microinjecting of either the NCH or SH2 domain of Shc GST fusion protein suggests the functional importance of both domains of Shc in the EGF sig-
Although the association of phosphorylated EGF receptors with Shc provides the mechanism to trigger downstream signals, mutant EGF receptors with the mutation of major phosphorylation sites and truncated EGF receptors lacking carboxyl-terminal autophosphorylation sites are still able to mediate mitogenic signaling (26–29). Since these mutant EGF receptors cannot interact with Shc directly, these results appear to argue against our finding, indicating the importance of Shc interactions. However, erbB2 in the EGF receptor-related family of receptor tyrosine kinase has been demonstrated to function as an alternative factor to transmit EGF signaling in fibroblast cell lines (30–31). Heterodimerization of the EGF receptor with erbB2 and/or transphosphorylation by the EGF receptor are the possible mechanisms of endogenous erbB2 activation following EGF stimulation. Since activated erbB2 phosphorylates tyrosine residue of Shc and erbB2 associates with Shc, Shc is the important factor also in erbB2 signaling (30–31). Taken together with our results showing that overexpression of the Shc N+CH domain with Y317F mutation and ShcSH2 domain interfered with EGF-induced endogenous Shc phosphorylation as shown in Fig. 5, it is logical to speculate that microinjection of the GST fusion proteins of the Shc subdomains would interfere with endogenous Shc interaction with both EGF receptor and erbB2 leading to EGF-induced mitogenic signaling.

The inhibition of BrdUrd incorporation by Shc-SH2 GST fusion protein was greater than that by Shc-NCH GST fusion protein. The result indicates that the SH2 domain of Shc may play the more important role in EGF signaling in vivo. It is not clear whether the amino-terminal and SH2 domains of Shc bind simultaneously to the same receptor or independent to the different receptor. However, it can be speculated that the cooperative effect of the two distinctive domains might explain the high stoichiometry of association between Shc and EGF receptor. Along this line, EGF stimulated Shc phosphorylation much faster than insulin. Since the cooperative effect has been
demonstrated that when the two SH2 domains of p85 regulatory subunit of phosphatidylinositol 3-kinase bind to the platelet-derived growth factor receptor and IRS-1 (32, 33), it is possible that the distinct functional mechanism in the interaction of Shc with EGF or insulin receptors may account for some biological differences between these receptors.

Shc is phosphorylated predominantly at single Tyr-317 residue (18). Following EGF and insulin stimulation, the phosphorylation of Shc Tyr-317 serves as a docking site for Grb2 (18). Since Grb2 is directly associated with the p21ras guanine nucleotide releasing factor Sos (34), Tyr-317 phosphorylation of Shc plays an important role for EGF or insulin-induced signal transduction. However, it is unlikely that phosphorylation of Tyr-317 is involved in the interaction of Shc with EGF and insulin receptors, because we have been able to affinity precipitate phosphorylated EGF and insulin receptors using Shc-NCH GST fusion protein carrying Tyr-317 → Phe mutation. Furthermore, microinjection of Shc-NCH GST fusion protein with Tyr-317 → Phe replacement inhibited both EGF and insulin-stimulated BrdUrd incorporation. Although the precise mechanisms of inhibitory effect of microinjection of GST fusion proteins on EGF- or insulin-induced DNA synthesis remain to be defined, the NCH GST fusion proteins containing Tyr-317 → Phe mutation presumably interfere with endogenous Shc functions by binding of the GST fusion protein to phosphorylated EGF or insulin receptors. Taken together, our results suggested that Tyr-317 phosphorylation of Shc is not required for the functional interaction of Shc with EGF and insulin receptors.

IRS-1 and Shc are the major intracellular substrates for the activated insulin receptor (14, 18, 22–24). Previous reports demonstrated that overexpression of Shc resulted in increased mitogen-activated protein kinase activity leading to enhanced insulin-induced cell cycle progression (8). Microinjection of anti-Shc antibody inhibited insulin stimulation of DNA synthesis (7). Thus, all of the previous studies indicate that Shc plays an important role as a positive regulator of insulin induced mitogenesis. On the other hand, IRS-1 is also an important component of insulin’s growth promoting effects. For example, expression of antisense IRS-1 cDNA and microinjection of anti-IRS-1 antibody inhibited insulin-stimulated DNA synthesis (35, 36). However, insulin stimulation of DNA synthesis was attenuated by increased expression of both IRS-1 and insulin receptor (9, 37, 38), but increased expression of IRS-1 alone did not always decrease insulin-induced DNA synthesis (37, 39, 40). These studies suggest that the role of IRS-1 on insulin-induced mitogenesis differs depending on cell context factors. Thus, it may account for this phenomenon that the inhibition of insulin signaling seems to arise from a sequestration of Grb2 from Shc by overexpression of IRS-1 (9). Although both IRS-1-Grb2 and Shc-Grb2 pathways possibly mediate the insulin-induced p21ras activation, Shc-Grb2 pathway rather than IRS-1-Grb2 pathway seems to be a predominant one for p21ras activation leading to DNA synthesis (4, 5, 9). Therefore, the competitive association with Grb2 between Shc and IRS-1 appears to be crucial to determine the signaling direction toward mitogenic activity.

Tyr-960 phosphorylation of insulin receptor is required for tyrosine phosphorylation of endogenous substrates such as IRS-1 and Shc (14, 22, 23). Both IRS-1 and Shc, via these PTB domains, interact with the juxtamembrane domain around Tyr-960 of insulin receptor (14, 22, 23). Therefore, in addition to the competitive association with Grb2 between Shc and IRS-1, Shc and IRS-1 can also be speculated as competitive substrates of insulin receptor. Our results demonstrated that the amount of IRS-1 phosphorylation was decreased in WT-Shc cells as shown in Fig. 4, while the amount of phosphorylated Shc, which was associated with Grb2, was increased in WT-Shc cells (data not shown). Thus, our results indicated that stably overexpressed Shc could compete with endogenous IRS-1 for the interaction with insulin receptors. In addition, overexpression of Y317F-Shc as well as wild-type Shc also decreased insulin-stimulated IRS-1 phosphorylation. Our result is the first report to clearly show the competitive interaction of Shc and IRS-1 with the insulin receptor, to which Shc Tyr-317 phosphorylation is not relevant.

In summary, our studies indicate that the amino-terminal domain of Shc functionally interacts with both EGF and insulin receptors, while the SH2-domain plays an important role for the association with EGF receptor but not with insulin receptor. EGF- and insulin-stimulated phosphorylation of Shc-Tyr-317 is not mandatory for Shc interaction with EGFR and insulin receptor, whereas it is necessary for serving as a docking site for Grb2.

Acknowledgment—We thank Dr. Kodimangalam S. Ravichandran for his gifts of the Shc-GST fusion proteins.

REFERENCES

Shc Amino-terminal Domain Interaction in Mitogenesis

Functional Importance of Amino-terminal Domain of Shc for Interaction with Insulin and Epidermal Growth Factor Receptors in Phosphorylation-independent Manner
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doi: 10.1074/jbc.271.33.20082

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