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Relative Involvement of Shc Tyrosine 239/240 and Tyrosine 317 on Insulin Induced Mitogenic Signaling in Rat1 Fibroblasts Expressing Insulin Receptors

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Shc is phosphorylated on Tyr-239/240 and/or Tyr-317, which serves as a docking site for Grb2. To clarify the relative involvement of Shc Tyr-239/240 and Tyr-317 in insulin-induced mitogenesis, we generated expression vectors for Y317F (1F)-Shc, Y239/240F (2F)-Shc, and Y239/240/317F (3F)-Shc, and stably transfected them into Rat1 fibroblasts expressing insulin receptors (HIRc). Insulin-induced Shc phosphorylation and subsequent association with Grb2 was enhanced in wild-type (WT)-Shc cell. In contrast, insulin-stimulated Shc phosphorylation and Shc · Grb2 association were significantly decreased in 1F-Shc and 3F-Shc cells, while these were only slightly affected and almost comparable in 2F cells compared with those in parental HIRc cells. The kinetics of MAP kinase activation closely paralleled the kinetics of Shc phosphorylation and Shc · Grb2 association. Thus, insulin stimulation of MAP kinase activation occurred more rapidly in WT-Shc cells, and the activation was delayed in 1F-Shc and 3F-Shc cells, while it was comparable in 2F-Shc cells compared with that in HIRc cells. Furthermore, WT-Shc cells displayed enhanced sensitivity to insulin stimulation of thymidine incorporation. Importantly, the sensitivity was significantly decreased in 1F-Shc and 3F-Shc cells, while it was almost comparable in 2F-Shc cells compared with that in HIRc cells. These results indicate that Shc Tyr-317 is more predominant insulin-induced phosphorylation site than Tyr-239/240 for coupling with Grb2 leading to MAP kinase activation and mitogenesis in Rat1 fibroblasts.

The activated insulin receptor phosphorylates various cellular substrates on tyrosine residues (1-6). Shc is one of these substrates, and implicated in mitogenic signaling initiated by the insulin receptor (7). Shc is composed of an amino-terminal phosphotyrosine binding (PTB) domain, a central collagen homology (CH) domain, and a carboxyl-terminal SH2 domain (5). Shc binds, via its PTB domain, to the juxtamembrane domain of the activated insulin receptor (8). Then, tyrosine-phosphorylated Shc binds to the SH2 domain of Grb2, which exists as preformed complexes with Ras guanine nucleotide exchange factor Sos (5, 9). Since Shc is an important adaptor protein responsible for linking the activated insulin receptor to the Ras/MAP kinase pathway via Grb2 · Sos complex, elucidation of the mechanisms of how Shc being tyrosine-phosphorylated for Grb2 binding is a key for understanding of insulin's mitogenic signaling. Thus, our previous studies have shown that Shc Tyr-317 residue plays a role, via coupling with Grb2, in insulin-induced mitogenesis (10). However, recent studies have identified novel Shc phosphorylation sites on Tyr-239/240 (11-15). The presence of two Grb2 binding sites in mammalian Shc protein adds the complexity of Shc function. In Drosophila Shc, the tyrosine residues corresponding to Tyr-239/240 residues, but not to Tyr-317 residue, of human and mouse Shc are conserved, indicating key roles of Shc Tyr-239/240 residues (16). Along this line, Shc Tyr-239/240 residues are shown to be involved in c-*myc* induction leading to prevention from apoptosis by IL3 (11). Furthermore, phosphorylation on Shc Tyr-239/240 residues, but not on the Tyr-317 residue, is required for nerve growth factor-induced neurite outgrowth in PC12 cells (12). However, the relative role of Shc Tyr-239/240 residues versus Tyr-317 for Grb2 binding leading to MAP kinase activation is controversial in EGF signaling (12-14). In addition, the role of Shc Tyr-239/240 for insulin-induced Shc phosphorylation and subsequent association with Grb2 is unknown.

In the present study, to directly clarify the relative role of Shc Tyr-239/240 versus Tyr-317 residue in insulin-induced mitogenic signaling, we generated Shc

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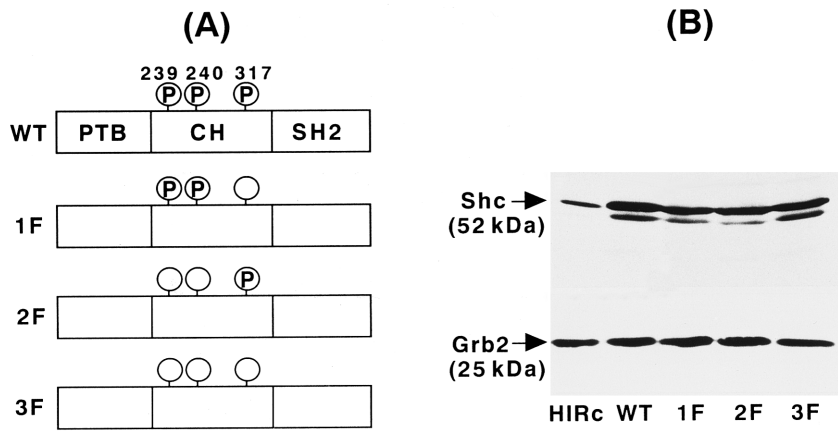

FIG. 1. Constrution of expression plasmids and expression of exogenous wild-type and mutant Shc. *A*, the Shc cDNA, and the mutant Shc encoding 1F, 2F, or 3F mutations were subcloned into EcoRV-digested RLDN. Schematic structures of Shc are shown. The three domains of Shc are a phosphotyrosine binding (PTB) domain, a collagen homology (CH) domain containing the binding sites (Tyr-239/240 and Tyr-317) for Grb2, and a carboxyl-terminal Src homology 2 (SH2) domain, as indicated. *B*, HIRc, WT-Shc, 1F-Shc, 2F-Shc, and 3F-Shc cells were solubilized, and expression of Shc in the cell lysates was analyzed by immunoblotting with anti-Shc antibody. As an internal standard to demonstrate that equivalent amounts of protein were loaded, the membrane was re-immunoblotted with anti-Grb2 antibody. Molecular masses of Shc (52kDa isoform) and Grb2 (25kDa) are shown by *arrows*. Results are representative of three separate experiments.

cDNA with Tyr-317→Phe (1F), Tyr-239/240→Phe (2F), and Tyr-239/240/317→Phe (3F) mutations. These mutant Shc plasmids were stably transfected into Rat1 fibroblasts overexpressing insulin receptors (HIRc), and intracellular insulin's mitogenic signaling was compared among these cell lines.

MATERIALS AND METHODS

Materials. Porcine insulin was a kind gift from Shimizu Pharmaceutical Co., (Shizuoka, Japan). [^{3}H]thymidine (83 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). A polyclonal anti-Shc antibody, a monoclonal anti-Grb2 antibody, a monoclonal anti-phosphotyrosine antibody (pY20) and a monoclonal anti-MAP kinase antibody were from Transduction Laboratories (Lexington, KY). Enhanced chemiluminescence reagents were from Amersham Corp. (Arlington Heights, IL). Electrophoresis reagents were from Bio-Rad (Hercules, CA). All other routine reagents were analytical grade and purchased from Sigma (St. Louis, MO) or Wako Pure Chemical Industries, LTD (Osaka, Japan).

Plasmid construction. Shc cDNA was subcloned into the mammalian expression vector Rldn to yield RldnWT-Shc (10). We utilized Transformer Site-Directed Mutagenesis Kit (Clontech, Palo Alto, CA) to engineer Rldn1F-Shc, Rldn2F-Shc, and Rldn3F-Shc by introducing Tyr-239/240→Phe mutations and/or Tyr-317→Phe mutation into RldnWT-Shc. The mutagenic oligonucleotides were 5'-CAT CAG TTC TTT AAT GAC TTC CCG-3′ for Tyr-239/240→Phe mutations and 5′-CTG GAC GTT GAC AAA GGA GGG AT-3′ for Tyr-317→Phe mutation, and selection oligonucleotide was 5′-GGT GCT GGT CCC CCC AAT CCT GCT-3′ which eliminated the *Apa* I site. The nucleotide sequences of these Shc constructs were verified using dye-terminator cycle sequence method.

Cell Culture, DNA transfection, and establishment of cell lines. Rat1 fibroblasts expressing 1×10^6 human insulin receptors (HIRc) were kindly supplied by Dr. J.M.Olefsky, (University of California, San Diego) and were maintained in Dulbecco's modified Eagle / F-12 medium supplemented with 10% fetal calf serum (FCS) (17). HIRc cells (5×10^5 cells per dish) were transfected with 20 μg of RldnWT-Shc, Rldn1F-Shc, Rldn2F-Shc, or Rldn3F-Shc in the presence of 2 μg pcDEB carrying a hygromycin-resistant gene for 4 h using TransIT LT1 polyamine (Pan Vera Corp., Madison, WT). Hygromycin B (400 μg/ml) was then added to the medium to select the resistant cells. Cells expressing these Shc constructs were isolated by limiting dilution and then the cells expressing comparable level of WT-, 1F-, 2F-, and 3F-Shc were identified by immunoblot analysis 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 (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 160 mM NaF, pH 7.4 for 15 min at 4°C. Lysates obtained from the same cell number of each cell line were centrifuged to remove insoluble material, and the supernatants were used for immunoprecipitation with anti-Shc antibody for 2 h at 4°C. The immunoprecipitates or whole cell lysates were separated by 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane by electroblotting. The membranes were blocked with 2.5% BSA 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 multi-well culture plates and serum-starved for 24 h. After stimulation of the cells with various concentrations of insulin for 20 h, 1 μCi of [^{3}H]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 1N NaOH and neutralized with 1N HCl, and counted in a liquid scintillation counter (18).

RESULTS

Expression of exogenous mutant Shc in HIRc cells. Fig. 1A shows the schematic structures of mutant Shc encoding 1F, 2F, or 3F mutations designed to be used in this study. Expression plasmids containing wild-type (WT) and these mutant Shc were transfected into

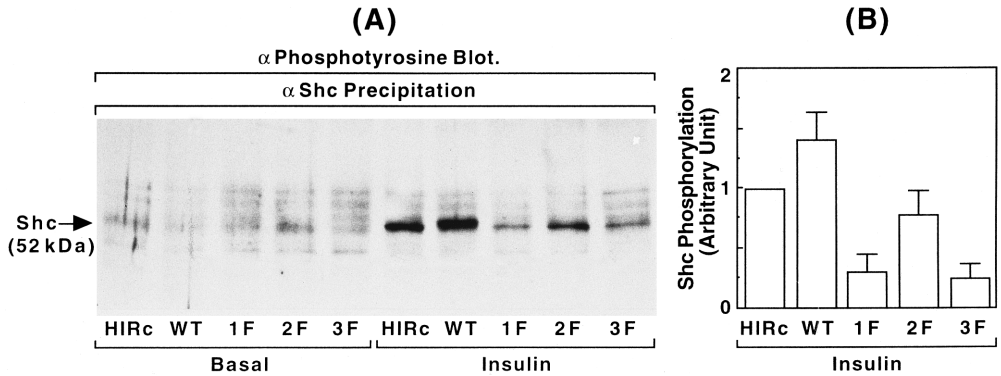

FIG. 2. Insulin-induced tyrosine phosphorylation of Shc in the transfected cell lines. HIRc, WT-Shc, 1F-Shc, 2F-Shc, and 3F-Shc cells were serum-starved for 24 h and then incubated with 17 nM insulin for 10 min. The cells were solubilized, and the cell lysates were immunoprecipitated with anti-Shc antibody. The immunoprecipitates were then subjected to SDS-PAGE and were analyzed by immunoblotting with anti-phosphotyrosine antibody. *A*, representative results are shown. Molecular mass of Shc (52kDa isoform) is shown by an *arrow*. *B*, the amount of Shc phosphorylation was quantitated by densitometry. Results are the mean ± S.E. of three separate experiments.

HIRc cells, and clones resistant to Hygromycin B were selected. Among Hygromycin B-resistant clones, more than 10 independent cell lines overexpressing these exogenous Shc were obtained by immunoblotting the cell lysates with anti-Shc antibody. Each cell line expressed 5 times the amount of WT-, 1F-, 2F-, or 3F-Shc compared with endogenous Shc in HIRc cells was chosen for the further studies (Fig. 1B). Comparable amount of exogenous Shc expression was confirmed by re-immunoblotting with anti-Grb2 antibody to demonstrate that equivalent amounts of protein were loaded.

Insulin-induced tyrosine phosphorylation of Shc in the transfected cell lines. The transfected cells were analyzed for the induction of Shc phosphorylation in response to insulin. The transfected cells were incubated with 17 nM insulin for 10 min, and the cell lysates were subjected to immunoprecipitation with anti-Shc antibody. The immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine antibody as shown in Fig. 2A. Overexpression of any Shc construct had no apparent effect on tyrosine phosphorylation of Shc in the basal state. Insulin mainly stimulated tyrosine phosphorylation of 52kDa Shc isoform in HIRc cells. Insulin-stimulated Shc phosphorylation was increased by 42 ± 19 % in WT-Shc cells compared with that in HIRc cells. Overexpression of 2F-Shc did not significantly affect insulin-stimulated tyrosine phosphorylation of Shc and it was almost comparable to that in HIRc cells. In contrast, insulin-stimulated Shc phosphorylation was significantly decreased by 68 ± 15 % and 72 ± 11 % in 1F-Shc and 3F-Shc cells, respectively (Fig. 2B).

Effects of Shc overexpression on Grb2 with Shc. Insulin stimulates Shc association with Grb2 (14). Phosphorylated Tyr-239/240 and/or Tyr-317 residue(s) of Shc is reported to serve as a binding site(s) for Grb2 (15, 17-21). Therefore, we next assessed the effect of these mutant Shc overexpression on Shc association with Grb2 as shown in Fig. 3A. Shc · Grb2 association was barely detected at the basal state in any trans-

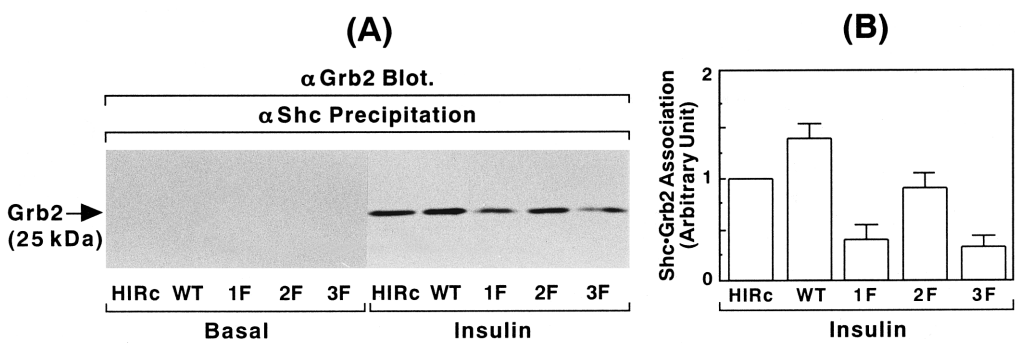

FIG. 3. Effects of Shc overexpression on Shc association with Grb2. HIRc, WT-Shc, 1F-Shc, 2F-Shc, and 3F-Shc cells were serum-starved for 24 h. The serum-starved cells were incubated with 17 nM insulin for 10 min. The cell lysates were immunoprecipitated with anti-Shc antibody. The immunoprecipitates were then subjected to SDS-PAGE and analyzed by immunoblotting with anti-Grb2 antibody. A, representative results are shown. Molecular mass of Grb2 (25kDa) is shown by an *arrow*. *B*, the amount of Grb2 associated with Shc was quantitated by densitometry. Results are the mean ± S.E. of three separate experiments.

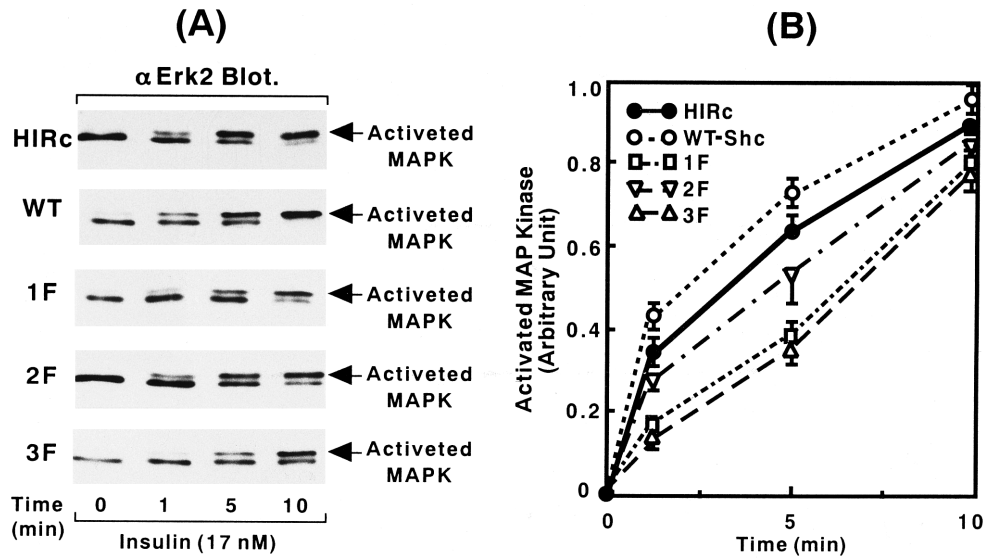

FIG. 4. Effects of Shc overexpression on MAP kinase activation. HIRc, WT-Shc, 1F-Shc, 2F-Shc, and 3F-Shc cells were serum-starved for 24 h and then incubated with 17 nM insulin for the indicated times. The cells were solubilized, and the cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting with anti-Erk-2 antibody. A, representative results are shown. The arrows indicate the activated, slower migrating form of p42 MAP kinase. B, the amount of activated p42 MAP kinase was quantitated by densitometry and normalized for the amount of total p42 MAP kinase. Results are the mean ± S.E. of three separate experiments.

fected cells. Insulin induced Shc association with Grb2 in original HIRc cells. Overexpression of WT-Shc resulted in increased insulin-induced Shc association with Grb2 by 40 ± 13 %. In accordance with the results of Shc phosphorylation, insulin-stimulated Shc · Grb2 association was almost comparable between 2F-Shc and HIRc cells, and it was reduced by 58 ± 15 % and 62 ± 10 % in 1F-Shc and 3F-Shc cells, respectively, compared with that in HIRc cells (Fig. 3B).

Effects of Shc overexpression on MAP kinase activation. It is known that the activation of MAP kinase is important for insulin-induced DNA synthesis (10). Phosphorylation of both tyrosine and threonine residues is required for the activation of MAP kinase (19), and phosphorylation of MAP kinase results in a decreased mobility on SDS-PAGE (20). Therefore, we next assessed insulin stimulation of MAP kinase activity using the MAP kinase gel shift assay in the transfected cell lines. As can be seen in Fig. 4A, insulin treatment induced a time dependent mobility shift of p42mapk (ERK-2) in HIRc cells. The time course of insulin-induced mobility shift of MAP kinase was more rapid in WT-Shc cells, and it was not significantly changed in 2F-Shc cells compared with that in parental HIRc cells. In contrast, the time course of the MAP kinase gel shift was significantly delayed in both 1F-Shc and 3F-Shc cells.

Effects of Shc overexpression on insulin's mitogenic action. To study the mitogenic signaling properties of these mutant Shc, thymidine incorporation was assayed in the transfected cell lines as shown in Fig. 5. Insulin stimulated thymidine incorporation in a dose-dependent manner with an ED50 value of 1.3 ± 0.2 nM in parental HIRc cells. Overexpression of WT-Shc led to increased insulin sensitivity with ED50 value of

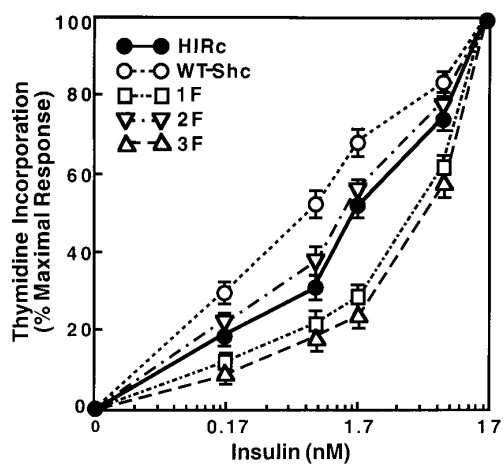

FIG. 5. Effects of Shc overexpression on insulin-induced thymidine incorporation. Thymidine incorporation in HIRc (●), WT-Shc (○), 1F-Shc (□), 2F-Shc (▽), 3F-Shc cells (△) was assayed as described under "Materials and Methods". Dose-response curves for insulin stimulation of thymidine incorporation are shown. Results are expressed as the percent maximum stimulation, and are the mean ± S.E. of four separate experiments. Absolute counts of basal levels (b) and maximal stimulation (m) were as follows: HIRc, b= 18641 ± 2054 dpm/1×10^6 cells and m=1.84 ± 0.29-fold; WT-Shc, b=23925 ± 2355 and m=2.10 ± 0.29; 1F-Shc, b=14924 ± 1778 and m=1.95 ± 0.37; 2F-Shc, b=18005 ± 1769 and m=1.92 ± 0.30; 3F-Shc, b=14350 ± 2035 and m=1.83 ± 0.31.

0.8 ± 0.1 nM. Although insulin sensitivity was almost comparable between 2F-Shc and parental HIRc cells, it was decreased with an ED50 value of 3.4 ± 0.3 nM and 3.8 ±0.4 nM in 1F-Shc and 3F-Shc cells, respectively, compared with that in HIRc cells.

DISCUSSION

The activated insulin receptor phosphorylates on tyrosine residues of Shc and insulin receptor substrate-1 (IRS-1) (1, 7, 21). Interaction of both Shc and IRS-1, via these PTB domains, with the juxtamembrane domain around Tyr-960 of the activated insulin receptor is required for the tyrosine phosphorylation (8). In this regard, our previous studies have shown that Shc and IRS-1 can function as competitive substrates, and that overexpression of Shc inhibited insulin-induced IRS-1 phosphorylation (10, 22). Shc PTB domain alone independent of the tyrosine phosphorylation is suggested to be sufficient for interaction with the activated insulin receptor and for competition with IRS-1 (10, 22). Therefore, we compared insulin-induced tyrosine phosphorylation of IRS-1 among the Shc transfected cell lines to clarify that all Shc constructs similarly function within the cells to affect IRS-1-mediated signaling. Stably overexpressed all mutant Shc constructs similarly inhibited insulin-induced IRS-1 phosphorylation among the transfected cell lines (data not shown). Therefore, comparison among these cell lines is useful to directly examine the role of Shc tyrosine residues on insulin signaling to Shc · Grb2 pathway leading to mitogenesis in Rat1 fibroblasts.

In the present study, insulin-induced tyrosine phosphorylation of Shc and subsequent association with Grb2 was decreased, the time course of insulin-stimulated MAP kinase activation was delayed, and insulin sensitivity of thymidine incorporation was relatively reduced in 2F-Shc cells compared with that in WT-Shc cells. These results indicate that Shc Tyr-239/240 residues newly identified as alternative tyrosine phosphorylation sites play a certain role in insulin-induced mitogenesis in Rat1 fibroblasts. However, Shc Tyr-317 residue rather than Tyr-239/240 residues seems to be a preferential phosphorylation site, since overexpression of 1F-Shc led to greater reduction of insulin-induced Shc phosphorylation and subsequent association with Grb2 than that of 2F-Shc. These reductions were similarly observed at any time point following insulin stimulation (data not shown). In accordance with decreased Shc phosphorylation and Shc · Grb2 association, insulin-stimulated MAP kinase activation and insulin sensitivity of thymidine incorporation were greatly reduced in 1F-Shc cells than those in 2F-cells. Furthermore, these reductions in 1F-Shc cells were almost comparable to those in 3F-Shc cells. These results further confirmed the essential role played by Shc Tyr-317 phosphorylation in insulin signaling to MAP kinase activation and mitogenesis in Rat1 fibroblasts. These results are in accordance with the recent report showing that Shc Tyr-239/240 contributes little to Ras/MAP kinase activation and that EGF-induced activation of the Ras/MAP kinase pathway appears to be mainly dependent on phosphorylation of Shc Tyr-317 in NIH-3T3 cells (14). Interestingly, Drosophila Shc has tyrosine residues corresponding to Tyr-239/240 residues, but not Tyr-317 residue of mammalian Shc (16). The fact that Shc Tyr-239/240 residues are highly conserved in evolution suggests that the phosphorylation of these residues is of fundamental importance. In spite of the fact, tyrosine-phosphorylated Drosophila Shc does not interact with Drosophila Grb2 in flies expressing the activated Drosophila EGF receptor homologue (DER) (16). These results indicate that signaling pathway from Shc to Grb2 is occurred through evolution mainly via Shc Tyr-317 residue, although Shc develops to utilize also its Tyr-239/240 residues as a minor player to associate with Grb2 during the evolutional process, at least, in EGF signaling. Thus, it is logical to speculate that Shc Tyr-317 residue rather than Shc Tyr-239/240 residues is mainly involved in the coupling with Grb2 leading to MAP kinase activation in insulin signaling. In contrast, our results are argue against the previous reports showing that Tyr-239/240 residues are more important than Tyr-317 residue for EGF-induced Shc phosphorylation and subsequent association with Grb2 in Cos1 and 293T cells (12,13). It is possible that in some systems, such as the insulin signaling system, Tyr-317 may have the role as the major tyrosine phosphorylation and Grb2 binding site, whereas in other systems, Tyr-239/240 is the major player. In this connection, Shc interacts with EGF receptors via its both SH2 and PTB domains, while Shc interacts with insulin receptors only via its PTB domain (22). It is interesting to surmise that importance of the tyrosine residues to be phosphorylated may depend on the specificity of Shc interaction with the membrane receptors. Alternatively, it is also possible that the relative importance of Shc Tyr-317 versus Tyr-239/240 depends on the cell types with evolutional differences.

In summary, although Shc has possible three tyrosine phosphorylation sites within the CH domain, Tyr-317 appears to be more predominant insulin-induced phosphorylation site than Tyr-239/240 for subsequent Grb2 binding leading to MAP kinase activation and cell cycle progression in Rat1 fibroblasts.

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