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Opposite Regulation of Tyrosine-Phosphorylation of p130^{Cas} by Insulin and Insulin-Like Growth Factor I[1]

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To investigate the difference in signaling between insulin and insulin-like growth factor I (IGF-I), we studied the effects of these hormones on the phosphorylation state of Crk-associated substrate (Cas) in cells expressing human insulin receptor (HIRc). In the basal state, Cas was heavily tyrosine-phosphorylated, and insulin dephosphorylated Cas in a time- and dose-dependent manner. On the other hand, IGF-I phosphorylated rather than dephosphorylated Cas in HIRc cells. In HIRY/F2 cells expressing a mutant insulin receptor lacking a binding site of SHP-2, a protein-tyrosine phosphatase containing src homology 2 (SH2) regions, insulin accelerated phosphorylation of Cas, as did IGF-I. In HIRc cells expressing a mutant SHP-2 lacking a PTPase domain (ΔPTP), which interfered with SHP-2 function, insulin failed to dephosphorylate Cas. In whole cell lysate obtained in the basal state, Cas bound to a glutathione-S transferase fusion protein containing SH2 domains of SHP-2 and dissociated from this GST protein in response to insulin. These results indicate that the opposite regulation of Cas phosphorylation by insulin and IGF-I may be mediated through different properties of their receptors, and that the interaction of the insulin receptor with SHP-2 may play an important role in determining the tyrosine-phosphorylation state of Cas.

Key words: Crk-associated substrate, insulin, insulin-like growth factor I, insulin receptor, SHP-2.

The major effect of insulin is metabolic, while insulin-like growth factor I (IGF-I) is characterized by its growth-promoting effects, although both hormones mediate their specific signals through tyrosine-phosphorylation of insulin receptor substrates (IRSs) (*1, 2*). Several quantitative differences in the molecular mechanisms of insulin and IGF-I signaling have been reported, but reports about their quantitative differences are few. In one such paper, Pillay *et al.* reported that insulin dephosphorylated a focal adhesion kinase, p125^{Fak} (Fak), whereas IGF-I induced phosphorylation of Fak (*3*).

Cas has recently been identified as a 130-kDa protein that is highly phosphorylated on its tyrosine residues and is associated with v-Crk and v-Src in cells transformed by these oncogene products. Cas is a unique signaling molecule having a cluster of multiple Src homology 2 (SH2) binding motifs and a single SH3 domain (*4, 5*). Furthermore, cell adhesion to fibronectin promotes tyrosine phosphorylation of Cas in fibroblasts. Thus, Cas serves as a docking protein and plays important roles in the signaling pathways mediated by cell adhesion as well as transformation (*6*). It has been reported that insulin induced interaction of Cas with Fak (*7*), and insulin regulates phosphorylation states of Fak. Therefore, to clarify the involvement of Cas in the signal transduction pathways of insulin and IGF-I, we investigated the effects of insulin and IGF-I on the tyrosine-phosphorylation states of Cas in the cells overexpressing either a wild-type or a mutant insulin receptor.

MATERIALS AND METHODS

Materials—Purified porcine insulin was a gift from Eli Lilly Company (Indianapolis, IL) and IGF-I was a gift from Fujisawa (Osaka) and Mitsubishi Kagaku (Tokyo). A monoclonal anti-phosphotyrosine antibody (PY69) was from ICN Biomedical (Lisle, IL). Monoclonal antibodies for SHP-2 and Cas and polyclonal antibody against insulin receptor β-subunit (anti-IR) were from Transduction

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Abbreviations: Cas, Crk associated substrate; Fak, Focal adhesion kinase; ΔPTP, cells expressing a mutant SHP-2 lacking a full PTPase domain; IGF-I, Insulin-like growth factor I; GST, glutathione-S-transferase; HIRc, Rat 1 fibroblasts overexpressing human wild-type insulin receptor; IRS, insulin receptor substrate; MAP kinase, mitogen-activated protein kinase; PMSF, phenylmethylsulfonyl fluoride; PTPase, protein-tyrosine phosphatase; SH2, src homology 2 region; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HIRY/F2, a mutant human insulin receptor in which both carboxyl-terminal tyrosine residues (1316 and 1322) were replaced by phenylalanine.

Laboratories (Lexington, KT). Monoclonal antibody against α-subunit of the receptor for IGF-I (anti-IGFR) was from Calbiochem (Cambridge, MA). Polyclonal anti-Cas antibody was provided by Dr. H. Hirai (The University of Tokyo). Protein G Sepharose and glutathione-Sepharose were purchased from Pharmacia PL Biochemical (Uppsala, Sweden). Aprotinin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade from Nacalai Chemicals (Kyoto).

Cell Culture—Rat 1 fibroblasts overexpressing either a wild-type (HIRc) or a carboxyl-terminal mutant receptor in which both carboxyl-terminal tyrosine residues (1316 and 1322) were replaced by phenylalanine (HIRY/F2), were provided by Dr. J.M. Olefsky (University of California, San Diego) (*8, 9*) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Effects of Insulin and IGF-I on the Phosphorylation State of Cas in the Cells Expressing a Wild-Type Insulin Receptor—HIRc cells were starved in serum-free medium overnight, then stimulated with either insulin (10 nM) or IGF-I (10 nM) at 37°C for 5 min unless otherwise described. Thereafter, the cells were lysed in 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 140 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM PMSF, 50 mM NaF, and 50 μg/ml aprotinin at 4°C for 20 min. The cell lysates were centrifuged to remove insoluble materials at 12,000$\times g$ for 30 min. The cells were solubilized, and cell lysate was immunoprecipitated with anti-Cas antibody. The bound protein was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to a PVDF membrane, and immunoblotted with anti-phosphotyrosine antibody. The blot was incubated with horseradish peroxidase-linked second antibody followed by an enhanced chemiluminescence detection according to the manufacturer's instructions. The same filter was also re-probed using anti-Cas antibody to quantify the amount of Cas protein.

Effects of Insulin and IGF-I on the Phosphorylation State of Cas in the Cells Expressing a Mutant Y/F2 Insulin Receptor—The change of phosphorylation state of Cas in response to either insulin or IGF-I was also analyzed in the cells expressing a carboxyl-terminal mutant insulin receptor (HIRY/F2). Following either insulin or IGF-I stimulation, the cells were solubilized and cell lysate was immunoprecipitated with anti-Cas antibody, then bound protein was analyzed by Western blotting using anti-phosphotyrosine antibody.

Effects of Insulin and IGF-I on the Association of Their Receptors or IRS-1 with SHP-2—Following either insulin or IGF-I stimulation, HIRc and HIRY/F2 cells were solubilized, cell lysate was immunoprecipitated with anti-SHP-2 antibody, and bound protein was analyzed by Western blotting using anti-IR. In a separate experiment, insulin- or IGF-I-stimulated HIRc cells were solubilized, cell lysate was immunoprecipitated with either anti-IR or anti-IGFR antibody, and bound protein was analyzed by Western blotting using anti-SHP-2 antibody. We also assessed the interaction of IRS-1 with SHP-2 in response to insulin or IGF-1 using anti-IRS-1 antibody and anti-SHP-2 antibody.

Effect of Insulin on the Phosphorylation State of Cas in Cells Expressing a Mutant SHP-2 (ΔPTP)—The effect of insulin on the phosphorylation state of Cas was analyzed in HIRc cells expressing a mutant SHP-2 lacking the full PTPase domain, in which endogenous SHP-2 function was impaired by expression of a dominant negative mutant SHP-2 (*10*). Following insulin stimulation, cell lysate was immunoprecipitated with anti-Cas antibody, then bound protein was analyzed by Western blotting using anti-phosphotyrosine antibody.

Association of GST-SHP-2 Fusion Proteins with Cas Protein—The cellular proteins bound to GST-SHP-2 fusion proteins were analyzed as described (*10*). In brief, GST fusion proteins containing either full-length SHP-2 (GST-SHP-2) or only two SH2 domains of SHP-2 (GST-bi-SH2) were made as described. To prepare whole cell lysate, cells were starved for 24 h, then stimulated with 10 nM insulin for 5 min at 37°C. Following insulin stimulation, the cells were solubilized, and cell lysate (500 μg protein) was incubated with GST fusion proteins coupled to glutathione-Sepharose beads in the presence of phosphatase inhibitors for 90 min at 4°C. After extensively washing, proteins bound to either GST-SHP-2 or GST-bi-SH2 fusion protein were analyzed by Western blotting using anti-Cas antibody.

Statistics—The data are expressed as mean$\pm$SE, unless

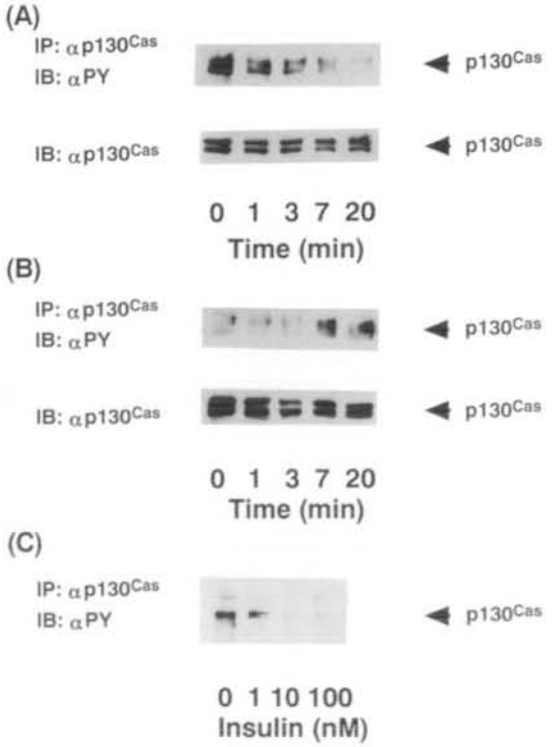

Fig. 1. **Effects of insulin and IGF-I on phosphorylation state of Cas in HIRc cells.** HIRc cells were stimulated with either 10 nM insulin or IGF-I at 37°C for the indicated time and the cell lysate was immunoprecipitated with anti-Cas (αCas) antibody. Bound protein was analyzed by Western blotting using anti-phosphotyrosine antibody (αPY69). The same filters were reproved with anti-Cas antibody to assess the amount of Cas protein. Phosphorylation states of Cas in the basal state should be identical. However, to demonstrate dramatically the effect of each ligand, exposure time was longer for insulin than for IGF-I. A: Time course of the insulin-induced dephosphorylation of Cas. B: Time course of IGF-I-induced phosphorylation of Cas. C: Dose response of insulin-induced Cas dephosphorylation.

otherwise stated. Scheffe's multiple comparison test was used to determine the significance of any differences among more than two groups, and unpaired Student's t-test used to determine the significance of any differences between two groups. $p<0.05$ was considered significant.

RESULTS

In the current study, Cas was heavily phosphorylated in the basal state even though overnight serum starvation, and insulin induced dephosphorylation of Cas in a time- and dose-dependent manner in HIRc cells (41.0±7% of the basal state, $p<0.01$) as shown in Figs. 1 and 2. The effect of insulin became apparent within 5 min, and the maximal effect was achieved at 7–10 min. The half-maximal dephosphorylation of Cas was observed at 1 nM insulin and the maximal dephosphorylation at 10 nM insulin, as shown in Fig. 1C. On the other hand, IGF-I phosphorylated rather dephosphorylated Cas in HIRc cells. IGF-I stimulated phosphorylation levels by 2.4-fold compared with the basal state ($p<0.01$), as shown in Figs. 1 and 2. The maximal IGF-I–induced phosphorylation of Cas was observed at 10 nM IGF-I, and the half-maximal phosphorylation at 1 nM IGF-I.

HIRY/F2 cells expressed a carboxyl-terminal mutant receptor in which both carboxyl-terminal tyrosine residues (1316 and 1322) were replaced by phenylalanine (HIRY/F2), and which is believed to behave like IGF-I receptor as compared with a wild-type receptor (*9, 11, 12*). Thus, we next assessed the alteration in phosphorylation state of Cas

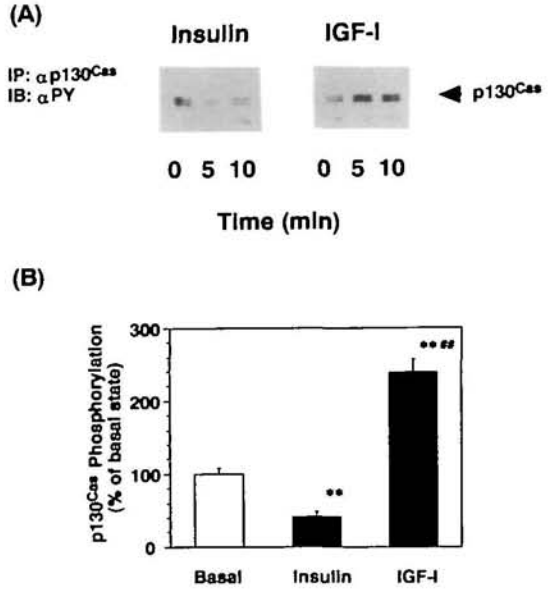

Fig. 2. **Effects of insulin and IGF-I on phosphorylation state of Cas in HIRc cells.** HIRc cells were stimulated with either 10 nM insulin or IGF-I at 37°C for 5 min, and the cell lysate was immunoprecipitated with anti-Cas (αCas) antibody. Bound protein was analyzed by Western blotting using anti-phosphotyrosine antibody (αPY69). A: Effects of insulin and IGF-I on phosphorylation states of Cas. B: Effects of insulin and IGF-I on phosphorylation states of Cas were quantified by Densitometer. Each column shows the mean±SE of 8 separate experiments. $^{**}p<0.01$ *vs.* the basal state. $^{\#}p<0.01$ *vs.* insulin stimulation. Significance was determined by Scheffe's multiple comparison test.

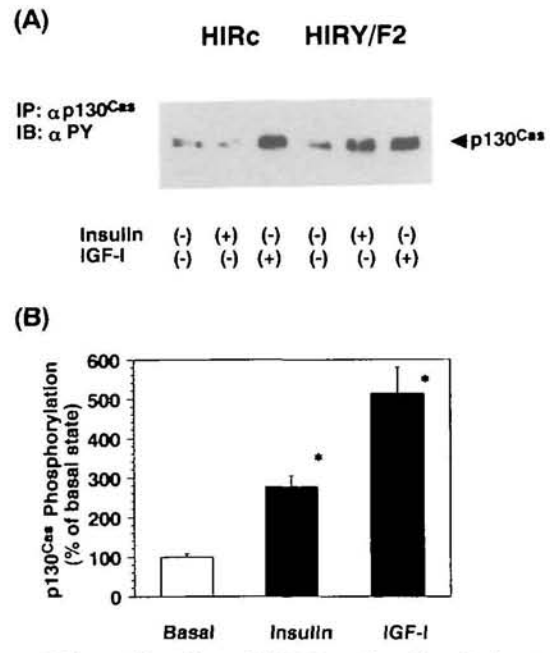

Fig. 3. **Effects of insulin and IGF-I on phosphorylation state of Cas in HIRY/F2 cells.** A: HIRc and HIRY/F2 cells were stimulated with either 10 nM insulin or 10 nM IGF-I at 37°C for 5 min, and the cell lysate was immunoprecipitated with anti-Cas antibody (αCas). Bound protein was analyzed by Western blotting using anti-phosphotyrosine antibody (αPY). B: Intensity of Cas phosphorylation was quantified by Densitometer. Each column shows the mean±SE of 5 separate experiments. $^{*}p<0.05$ *vs.* the basal state. Significance was determined by Scheffe's multiple comparison test.

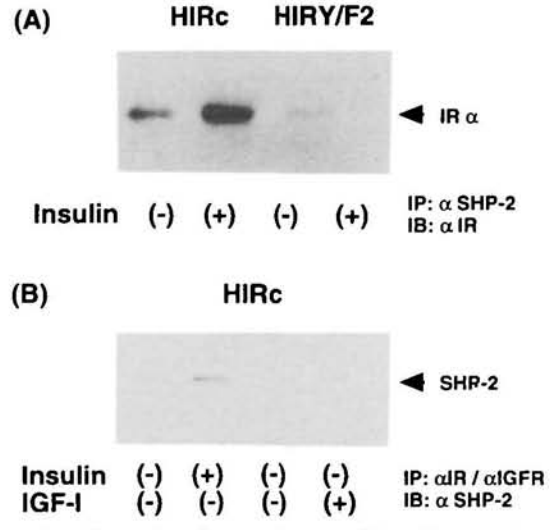

Fig. 4. **Insulin-induced association of insulin receptor with SHP-2 in HIRc and HIRY/F2 cells.** A: HIRc and HIRY/F2 cells were stimulated with 10 nM insulin for 5 min, and cell lysates were immunoprecipitated with anti-SHP-2 antibody (αSHP-2). Bound protein was analyzed by Western blotting using anti-insulin receptor antibody (αIR). B: HIRc cells were stimulated with either 10 nM insulin or 10 nM IGF-I for 5 min, and cell lysates were immunoprecipitated with either anti-insulin receptor antibody (αIR) or anti-IGF-I receptor antibody (αIGF-IR). Bound protein was analyzed by Western blotting using anti-SHP-2 antibody (αSHP-2). Representative results were obtained in at least more than 3 independent experiments.

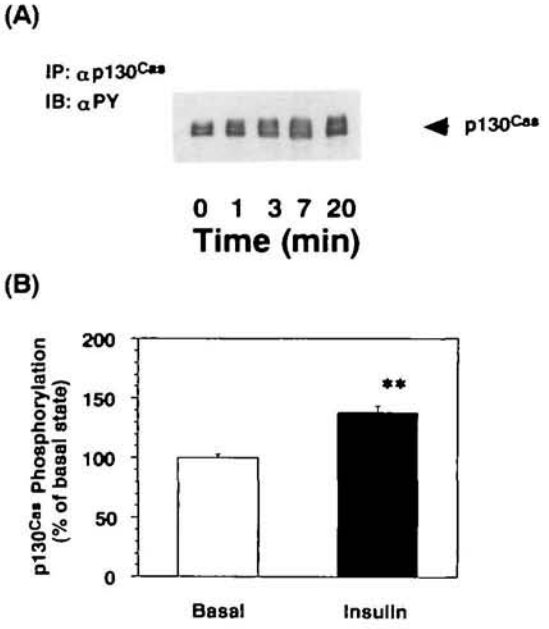

Fig. 5. **Time course of insulin-induced alteration of phosphorylation state of Cas in ⊿PTP Cells.** A: ⊿PTP cells were stimulated with 10 nM insulin at 37°C for 0–20 min, and the cell lysate was immunoprecipitated with anti-Cas (αCas) antibody. Bound protein was analyzed by Western blotting using anti-phosphotyrosine antibody (αPY69). B: ⊿PTP cells were stimulated with 10 nM insulin at 37°C for 5 min, and the cell lysate was immunoprecipitated with anti-Cas (αCas) antibody. Each column shows the mean ± SE of 4 separate experiments. $^*p<0.05$ *vs.* the basal state. Significance was determined by Student's *t*-test.

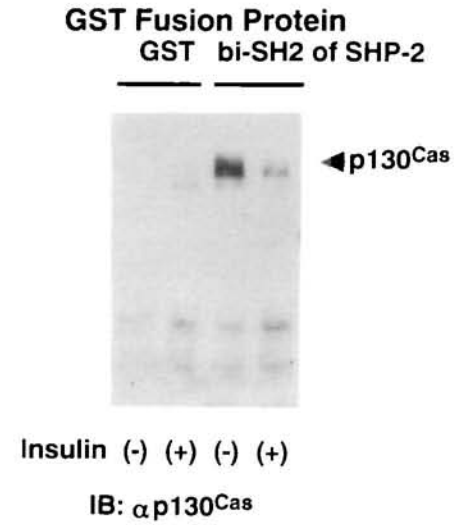

Fig. 6. **Direct association of SHP-2 with Cas in HIRc cells.** After starvation for 24 h, cells were stimulated with 10 nM insulin at 37°C for 5 min, then whole cell lysate (500 μg protein) was incubated with GST-bi-SH2 fusion protein coupled to glutathione-Sepharose beads for 90 min at 4°C. After extensive washing, proteins bound to GST-SHP-2 fusion proteins were analyzed by Western blotting using anti-Cas antibody.

in the HIRY/F2 cells in response to either insulin or IGF-I. In contrast to the HIRc cells expressing the wild-type insulin receptor, insulin phosphorylated rather than dephosphorylated Cas in the HIRY/F2 cells, as shown in Fig. 3. Insulin stimulated the phosphorylation levels by 2.8-fold when compared with the basal state ($p<0.01$).

We previously reported that SHP-2, a protein-tyrosine phosphatase containing SH2 domain (*13–18*), bound to a carboxyl-terminal of insulin receptor, and HIRY/F2 failed to bind to SHP-2 *in vitro* experiments (*19*), because this receptor lacked this SHP-2 binding motif. To confirm our *in vitro* findings, we studied the association of the insulin receptors with SHP-2 in whole cells. As shown in Fig. 4A, insulin induced the association of β-subunit of the insulin receptors with SHP-2 in HIRc cells, but not in HIRY/F2 cells. In the case of IGF-I receptors, we did not observe any significant association of these receptors with SHP-2, as shown in Fig. 4B. Furthermore, ligand induced association of IRS-1 with SHP-2 was comparable in the HIRc cells stimulated by either insulin and IGF-I (data not shown).

To further clarify the involvement of the interaction of insulin receptor with SHP-2 in regulation of Cas phosphorylation, we studied the effects of insulin in the cells expressing a mutant SHP-2 lacking a full PTPase domain (⊿PTP), where interferes with endogenous SHP-2 function. In these cells, the interaction of endogenous SHP-2 with either insulin receptors or insulin receptor substrate-1 (IRS-1) was inhibited by introduction of mutant SHP-2 (*10*). In the cells expressing a mutant SHP-2 (⊿PTP), insulin also failed to dephosphorylate Cas. As shown in Fig. 5, insulin significantly increased the phosphorylation state of Cas in ⊿PTP cells.

We previously reported that an undefined phosphotyrosine-containing protein with a molecular mass of 130 kDa binds to GST fusion proteins of SHP-2 (*10*), and it is possible, therefore, that SHP-2 interacts directly with Cas. To assess whether this 130-kDa GST-SH2 binding protein is identical to Cas, we characterized the protein by Western blotting using anti-Cas antibody. As shown in Fig. 6, GST-bi-SH2 protein bound to Cas immunoreactivity in the basal state. Furthermore, this association of GST-bi-SH2 protein with Cas was decreased in response to insulin (26.3 ± 6.7% of the basal state).

DISCUSSION

The mechanisms of insulin and IGF-I actions are not completely understood, and reports on qualitative differences at the molecular level are few. Pillay *et al.* reported that insulin dephosphorylated Fak, whereas IGF-I induced phosphorylation of Fak (*3*).

In the current study, we found that insulin dephosphorylated Cas in a dose- and time-dependent manner. In contrast, IGF-I stimulated phosphorylation state of Cas in HIRc cells. Thus, we speculated that the opposite regulations by insulin and IGF-I of both Cas and Fak phosphorylation states may be derived from different properties of their receptors and partly different signaling.

In HIRY/F2 cells, the insulin-stimulated mitogen-activated protein (MAP) kinase activity is enhanced as compared to the cells expressing the wild-type receptor (*9, 11, 12*), and the carboxyl-terminal mutated insulin receptor is thus believed to behave as an IGF-I receptor. Furthermore, the insulin-induced dephosphorylation of Fak is also attenuated in HIRY/F2 cells (*3*). It has been reported that the phosphorylation state of Cas parallels

that of Fak. Thus, it is possible that this structural modification of a carboxyl-terminal portion of the insulin receptor may alter the dephosphorylation state of Cas. To examine this possibility, we assessed the effects of insulin and IGF-I on the phosphorylation state of Cas in HIRY/F2 cells. As expected, insulin induced the phosphorylation rather than the dephosphorylation of Cas in HIRY/F2 cells. These opposite regulations of the phosphorylation state of Cas and Fak in the cells expressing wild-type and mutant Y/F2 receptors may be the result of the different properties of the C-terminal portion of their receptors. Regarding the difference in properties between wild-type and Y/F2 insulin receptors on Cas phosphorylation, we speculate that SHP-2 is involved in insulin-induced Cas dephosphorylation, because SHP-2 was found to be able to bind a carboxyl-terminal portion of the insulin receptor through its SH2 domains in our previous *in vitro* study (*19*), and this binding motif of SHP-2 is removed in the HIRY/F2 mutant receptor (*9, 11*). Thus, we speculate that a lack of interaction between Y/F2 receptor and SHP-2 may lead to the failure of Cas dephosphorylation and result in the enhanced mitogenicity, because the tyrosine-phosphorylated Cas activates $p21^{ras}$ through its complex formation with nucleotide exchange factors (*6*). Nevertheless, SHP-2 is believed to be a positive regulator of the tyrosine kinase pathway and to enhance the $p21^{ras}$ and MAP kinase cascade, and attenuation of SHP-2 function by an introducing a dominant negative mutant led to decreased mitogenic activity of insulin (*10, 20-24*). In the present study, insulin induced phosphorylation rather than dephosphorylation of Cas in the cells expressing a dominant negative mutant SHP-2 (⊿PTP). In terms of phosphorylation of Cas by insulin, these cells were expected to show potentiated mitogenic activity. However, this was not the case (*10*). Normal insulin signaling potentiates mitogenic activity through activation of MAP kinase cascade. Simultaneously, insulin may negatively regulate the mitogenic activity through its Cas dephosphorylation. Therefore, the lack of dephosphorylation of Cas in HIRY/F2 cells may enhance mitogenicity. In contrast, in the ⊿PTP mutant cells, insulin-induced $p21^{ras}$ activation was impaired even though insulin-induced Cas dephosphorylation was attenuated, suggesting that the regulatory site of SHP-2 on the activation of $p21^{ras}$ might be downstream of Cas dephosphorylation.

We previously reported that four tyrosine-phosphorylated proteins (185, 130, 115, and 95 kDa) bound to SHP-2 (*10*). Two major tyrosine-phosphorylated proteins bound to both GST proteins after insulin stimulation. The 185-kDa tyrosine-phosphorylated protein was identified as IRS-1 or IRS-2 (*19, 25, 26*), and the 115-kDa protein might be SHP-2-binding protein as reported (*10, 26-31*). The 95-kDa tyrosine-phosphorylated protein (β subunit of the insulin receptor) also bound to both GST-SHP-2 and GST-SH2 in response to insulin (*10, 32, 33*). To rule out a possibility that the 130-kDa protein was identical to Cas, we assessed the direct interaction between Cas and SHP-2 using GST-SHP-2 fusion proteins and observed that tyrosine-phosphorylated Cas bound to GST-bi-SH2 fusion protein in the basal state and dissociated from GST protein in response to insulin. Therefore, we speculated that tyrosine-phosphorylation states of Cas are critical for the association of Cas with SHP-2.

As mentioned, the association of SHP-2 with β-subunit of the insulin receptors may have a regulatory role in the dephosphorylation of Cas. SHP-2 may be activated by the association of the insulin receptors, then attack phosphorylated Cas in response to insulin. Alternatively, SHP-2 has a pair of SH2 domains, and it may act as an adaptor protein linking to other signaling molecules (*26*). If so, SHP-2 may link the insulin receptor with the Cas protein *via* a pair of SH2 domains in response to insulin stimulation, and this complex formation may be critical for full activation of SHP-2 and lead to Cas dephosphorylation. Further study is necessary to assess the mechanism of the insulin-induced Cas dephosphorylation. On the other hand, the ligand association of IRS-1 with SHP-2 was comparable in the cells stimulated by either insulin or IGF-I. We speculated, therefore that the intracellular distribution of SHP-2 complex with receptors might be different from that of the SHP-2 complexes with IRS-1.

Of the PTPases involved in Cas dephosphorylation, PTP-PEST and PTP1B have been reported to be candidates (*34, 35*). However, we did not observe any significant association of Cas with PTP1B (data not shown). In the current study, GST-bi-SH2 (SH2 domains alone) bound more strongly to Cas than GST-full-length SHP-2 (data not shown), suggesting that GST-full-length SHP-2 might dephosphorylate Cas, but GST-bi-SH2 might not because of the lack of the PTPase domain. Furthermore, Cas dephosphorylation was attenuated in the cells expressing a mutant SHP-2. These lines of evidence strongly suggest that the interaction between SHP-2 and the insulin receptor may be important for dephosphorylation of Cas.

In conclusion, the tyrosine-phosphorylation of Cas is regulated by insulin and IGF-I in the opposite direction, and these regulations may be responsible for the different properties of their receptors. Furthermore, the interaction of the β-subunit of insulin receptor with SHP-2 may play an important role in determining the phosphorylation state of Cas, and may subsequently play a critical role in a negative feedback circuit to reduce exaggerated activation of $p21^{ras}$ and MAP kinase cascade in the insulin signal pathway. Our results clearly indicate that SHP-2 has a unique role in differentiation of insulin signaling from IGF-I signaling, through at least Cas dephosphorylation.

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