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SHPTP2 Serves Adapter Protein Linking between Janus Kinase 2 and Insulin Receptor Substrates

Hiroshi Maegawa, Atsunori Kashiwagi,[1] Toshiki Fujita, Satoshi Ugi, Masaaki Hasegawa, Toshiyuki Obata, Yoshihiko Nishio, Hideto Kojima, Hideki Hidaka, and Ryuichi Kikkawa

The Third Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, 520-21, Japan

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To investigate the role of Janus kinase family (JAK1 and JAK2) in insulin signaling, we assessed their insulin-induced associations with other molecules in the cells overexpressing insulin receptors (HIRc and CHO-IR). After insulin stimulation, pp185 proteins (insulin receptor substrate, IRS) were co-immunoprecipitated with both kinases by αJAK1 and αJAK2 antibodies. However, JAK2 constitutively associated with pp95 protein (IRβ). Moreover, JAK2 also constitutively bound to a protein tyrosine phosphatase containing Src 2 regions (SHPTP2), but JAK1 did not. In HIRc cells expressing PTPase-negative mutant SHPTP2, no association of JAK2 with either pp185 or pp95 was detected. Thus, SHPTP2 might serve as an adapter protein linking between JAK2 and IRS. These results suggest that JAK1 and JAK2 behave differently and they may constitute a new regulatory component in insulin signaling.

Insulin binding to its receptor results in autophosphorylation of the receptor, activation of receptor tyrosine kinase, and then the phosphorylation of cellular endogenous substrate, IRS-1 (1). Recently, newly discovered insulin receptor substrate, IRS-2 was found to be identical with 4PS and phosphorylated by Interleukin 4, suggesting that insulin and cytokine use the same cellular molecule to mediate their signaling (2-4). Furthermore, following interleukin or interferon stimulation, activated Janus kinases (JAK1, JAK2 and Tyk2) associate and phosphorylate IRS-1 as well as IRS-2 in hematopoietic cells (5-7). Moreover, JAK2 kinase also phosphorylates IRS-1 in isolated adipocytes in response to growth hormone stimulation (8). These lines of evidence suggest the presence of crosstalk in interleukin, growth hormone and insulin signal transduction.

To clarify the roles of Janus kinase family (JAK1 and JAK2) in signal transduction of insulin, we assessed the effects of insulin on alteration of their tyrosine-phosphorylated states and association with other molecules in the cells overexpressing insulin receptor (HIRc and CHO-IR cell).

EXPERIMENTAL PROCEDURES

Materials. Purified porcine insulin was a gift from Eli Lilly Company (Indianapolis, IL). A monoclonal anti-phosphotyrosine antibody (αPY69) was from ICN Biochemical Inc.(Costea Mesa, CA). Polyclonal antibodies against JAK1, JAK2 and SHPTP2 (αSyp) were from Upstate Biotechnology Inc.(Lake Placid, NY) and another polyclonal antibody against JAK2 (C-20) was from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against insulin receptor β-subunit (αIR), IRS-1 (αIRS-1) and monoclonal SHPTP2 antibody (αPTP1D) were from Transduction Laboratories (Lexington, KT). Polyclonal antibody against IRS-2 was kindly provided by Dr. T. Kadowaki (Tokyo University)(9). Protein G Sepharose was purchased from Pharmacia PL Biochemical (Uppsala). Aprotinin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). Hygromycin B was from Wako Chemical Inc.(Osaka). All other reagents were from Nakarai Chemicals (Kyoto).

Cell culture. Rat 1 fibroblasts overexpressing insulin receptors (HIRc) provided by Dr. J. M. Olefsky (University of California, San Diego) and Chinese hamster ovary cells overexpressing insulin receptors (CHO-IR) provided by

[1] Corresponding author.

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Dr. M. Kasuga (Kobe University) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (10,11). HIRc cells additionally expressing mutant SHPTP2 (ΔPTP) were also maintained in DMEM medium with 400 μg/ml hygromycin B. Parent HIRc cells transfected with pHyg vector alone were used for control in our study (pHyg) (12).

Association of phosphotyrosine proteins with JAK2 kinase in response to insulin. Cells were starved in serum-free DMEM medium for overnight, then stimulated with 100nM insulin at 37 ℃ for 3-5 min as otherwise described. Thereafter, the cells were lysed in 20 mM Tris-Cl buffer (pH 7.5) containing 1 mM EDTA, 140 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM PMSF, 50 mM NaF, 50μg/ml aprotinin at 4℃ for 20 min. The cell lysates were centrifuged to remove insoluble materials at 15000 g for 20 min, and immunoprecipitated at 4℃ with αJAK1 and αJAK2. The bound proteins were then resolved by SDS-PAGE, electrotransferred to Immobilon-P and immunoblotted with αPY69. The blots were then incubated with horseradish peroxidase-linked second antibody followed by enhanced chemiluminescence detection according to the manufacturer's instructions.

Association of JAK1 and JAK2 with IRS-1 and IRS-2 and insulin receptors. Reciprocal immunoprecipitation studies were conducted. Cell lysate was immunoprecipitated with either αIRS-1, αIRS-2 or αIR antibodies and then bound proteins were analyzed by Western blotting using αJAK1 or αJAK2 antibodies, respectively. Conversely, cell lysate was also immunoprecipitated with αJAK1 or αJAK2 antibodies, and then bound proteins were analyzed by Western blotting using either αIRS-1, αIRS-2 or αIR antibodies, respectively.

Association of JAK2 kinase with SHPTP2. After insulin stimulation (100 nM for 4 min), cell lysate was immunoprecipitated with αSyp, and bound proteins were then analyzed by Western blotting using either αJAK1 or αJAK2 antibodies. Conversely, cell lysate was also immunoprecipitated with either αJAK1 or αJAK2 antibodies and bound proteins were then analyzed by Western blotting using monoclonal αPTP1D.

RESULTS AND DISCUSSION

Many studies reported that cytokines and growth hormone phosphorylate IRS-1 via activating JAK kinase family. However, there is a few reports that insulin regulates JAK kinase function (13).

In the current study, we found that insulin induced association of pp185 proteins with JAK1 and JAK2 kinases in HIRc cells using anti-phosphotyrosine antibody as shown in Figure 1A. However, we were not able to detect any immunoreactivity of JAK1 or JAK2 in anti IRS-1 or IRS-2 immunoprecipitation regardless of insulin stimulation (data not shown). This discrepancy may be explained by the sensitivity of the detection of small amounts of immunoprecipitation of either IRS-1 or IRS-2 protein using α JAK1 or JAK2 antibody. Thus, we were not able to identify whether pp185 proteins were identical to either IRS-1 or IRS-2.

Regarding association of IRSs with Janus kinase family, it has been reported that JAK1, JAK3 and Tyk2 kinases directly bind to IRS-1 and IRS-2 in response to cytokine stimulation (5-7) and that PH domain of IRS-1 is responsible binding site of Tyk2 (7). In HIRc cells, we did not detect any immunoreactivity of JAK3 and Tyk2. JAK2 kinase is activated following growth hormone stimulation, and phosphorylate IRS-1 in isolated adipocytes (8). However, there is no report about association of either IRS-1 or IRS-2 with JAK2 kinase after cytokine stimulation.

As shown in Figure 1A, in the unstimulated cells, pp90 protein was observed in αJAK1 immunoprecipitation. After insulin stimulation, pp90 protein moved to higher molecular weight (Figure 1). This band was not identical for β subunit of insulin receptors, since this band did not interact with αIR antibody (data not shown). Therefore, the identification of this co-precipitating 90kDa phosphotyrosine protein remains a future challenge. On the other hand, insulin induced the association of pp95 protein with JAK2 kinase. Furthermore, the reciprocal immunoprecipitation study showed that JAK2 kinase and insulin receptors were constitutively associated with each other in HIRc cells as shown in Figure 1B.

SHPTP2 is a ubiquitously expressed protein tyrosine phosphatase containing a pair of Src homology 2 domains (14-18) and also believed to be a positive regulator of insulin signal transduction (19-24). We recently found that overexpression of wild-type and mutant SHPTP2 modulates the phosphorylation state of IRS-1, and led to the impairment in both PI3′-kinase cascade and ras-MAP kinase cascade in HIRc cells (12). To investigate the molecular mecha-

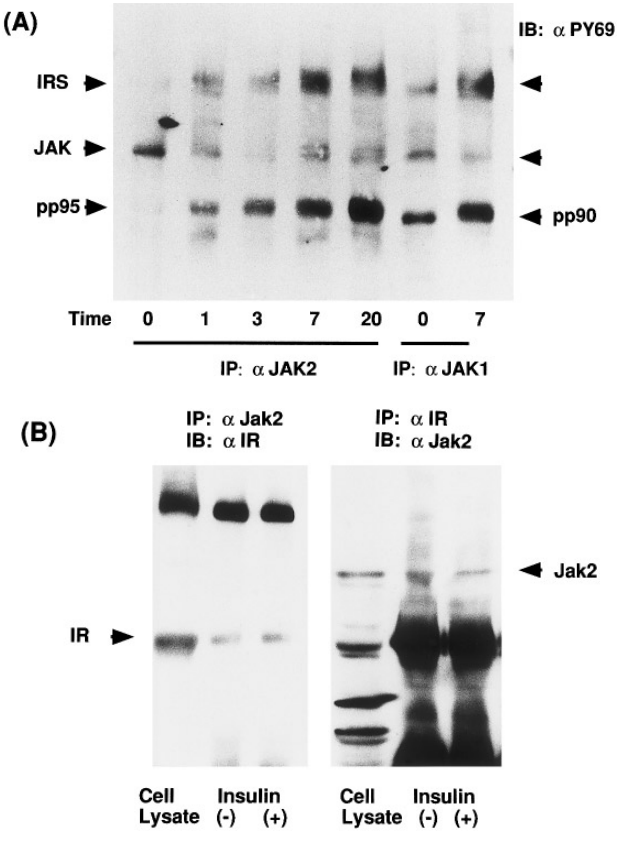

FIG. 1. Time course of insulin-induced association of JAK1 and JAK 2 kinase with phosphotyrosine containing proteins (A) and association of JAK2 with β-subunit of insulin receptors (B). A, HIRc cells were stimulated with 100 nM insulin for 37°C for 0-20 min and the cell lysate was immunoprecipitated (IP) with either αJAK1 or αJAK2 antibodies. Bound proteins were analyzed by Western blotting (IB) using anti-phosphotyrosine antibody (PY69). B, Reciprocal immunoprecipitation studies were performed. Cell lysate was immunoprecipitated with αJAK2 antibody, and immunoblotting was performed using anti-insulin receptor antibody, αIR. Conversely, cell lysate was immunoprecipitated with αIR and bound proteins were analyzed by Western blotting using αJAK2 antibody.

nism of modulating phosphorylation states of IRS-1 by SHPTP2, we searched for SHPTP2 target proteins using GST-SH2 fusion protein binding, and found that SH2 domains of SHPTP2 bound the undefined tyrosine-phosphorylated proteins (pp120 and pp135) as well as IRS-1(12). It has been reported that SHPTP2 plays a positive regulator of the signaling of prolactin or interleukin 11 by physically associating with receptor-JAK2 complex following these stimulation (25-26). Thus, we investigated association of SHPTP2 with JAK2 kinase by the reciprocal immunoprecipitation experiments. Interestingly, even in unstimulated cells, JAK2 kinase physically bound to SHPTP2 in HIRc cells as shown in Figure 2A, B. However, we were not able to detect any SHPTP2 in αJAK1 immunoprecipitation (data not shown). These results showed that some population of JAK2 constitutively associated with SHPTP2. Jak1 and Jak2 kinase interact with similar sets of cytokine receptor and STAT family with some exceptions such as growth hormone signaling (27). In the current study, JAK1 and JAK2 bound to pp185 (possibly IRS) following insulin stimulation. However, JAK1 and JAK2 showed different properties in insulin signal transduction in term of their associations with insulin receptor and SHPTP2. Thus, we speculate that in insulin signal transduction, JAK2 kinase may be one of

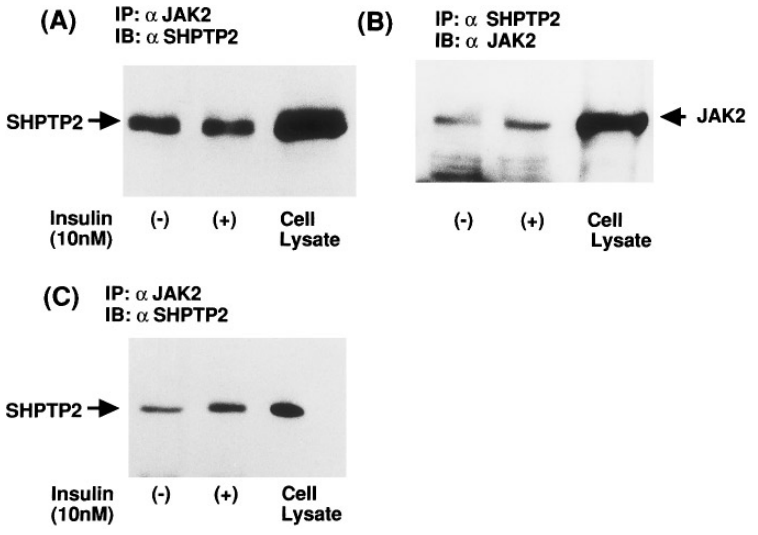

FIG. 2. Association of JAK2 kinase with SHPTP2 in HIRc (A, B) and CHO-IR (C) cells. Reciprocal immunoprecipitation studies were performed. After insulin stimulation, cell lysate was immunoprecipitated with αJAK2 antibody. Bound proteins were resolved by SDS-PAGE and transferred to an Immobilon membrane using the standard procedures. Immunoblotting was performed using monoclonal SHPTP2 antibody, αPTP1D (A, C). Conversely, after insulin stimulation, cell lysate was immunoprecipitated with polyclonal SHPTP2 antibody, αSyp, bound proteins were analyzed by Western blotting using αJAK2 antibody (B).

SHPTP2 target proteins which potentially modulates the phosphorylation state of IRS-1, but JAK1 kinase not.

SHPTP2 binds to not only phosphorylated IRS-1 (28,29), but also phosphotyrosine motif in C-terminus of insulin receptor in response to insulin, although SHPTP2 does constitutively associate with neither IRS-1 nor insulin receptors (30). In the present study, JAK2 was constitutively associated with SHPTP2. Thus, it is possible that SHPTP2 may serve as an adapter protein between JAK2 and IRS in response to insulin.

Regarding to SHPTP2 binding sites, the N-terminal SH2 domain of SHPTP2 is reported to preferably bind to IRS-1(31). On the other hand, JAK2 kinase has two possible tyrosine-phosphorylation sites (Y^{201}NSV and Y^{1007}YKV) and the C-terminal SH2 domain of SHPTP2 preferably binds to JAK2 kinase and insulin receptors (26). Thus, it is possible that upon insulin stimulation, SHPTP2 acts as a bridge or an adapter between tyrosine-phosphorylated IRS-1 and JAK2 kinase and a possible function of constitutive SHPTP2-JAK2 kinase binding is the localization of SHPTP2 to the membrane to allow easier association with IRS-1.

In the current study, we found that JAK2 kinase itself was phosphorylated at the basal state, and insulin induced dephosphorylation of JAK2 kinase in HIRc cells as shown in Figure 1A. However, in CHO-IR cells, insulin induced phosphorylation of JAK2 kinase as shown in Figure 3A, even though JAK2 kinase constitutively associated with insulin receptors and SHPTP2 as well as in HIRc cells (Figure 2C and 3A). These results suggest that the phosphorylation state of JAK2 kinase is not necessary for association of JAK2 kinase with pp185 proteins and SHPTP2. Phosphorylation state of proteins are regulated by tyrosine kinases and PTPases and these balances may differ in HIRc and CHO-IR cells. To clarify physiological roles of altered phosphorylation states of JAK2 by insulin in HIRc and CHO-IR cells, future study is need.

Finally, we assessed the association of IRS with JAK2 kinase in the HIRc cells expressing PTPase-negative mutant SHPTP2 (ΔPTP). In the cells expressing mutant SHPTP2, insulin

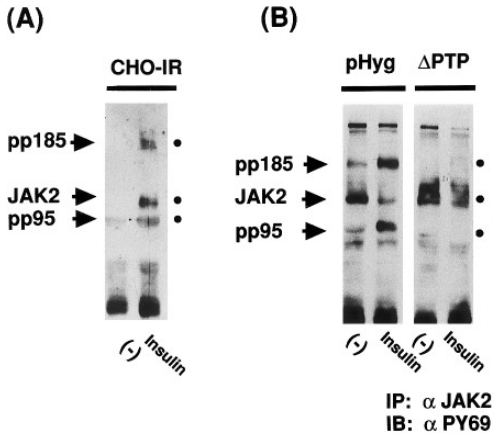

FIG. 3. Effects of insulin on the phosphorylation state of JAK2 kinase in various cells. Cells (A, CHO-IR cells, B, pHyg cells and mutant SHPTP2, ΔPTP cells) were stimulated with 100 nM insulin and cell lysate was immunoprecipitated with αJAK2 antibody. Bound proteins were analyzed by Western blotting using anti-phosphotyrosine antibody (PY69).

failed to induce dephosphorylation of JAK2 kinase. Furthermore, we were not able to detect any association of JAK2 kinase with pp185 (possibly IRS) and pp95 as shown in Figure 3B. In the mutant cells where insulin signaling was attenuated by introduction of dominant negative SHPTP2, we were not able to detect any association of IRSs with JAK2 kinase nor any change of phosphorylation state of JAK2 kinase. Therefore, it is clear that a PTPase domain is essential for association of JAK2 with either pp185 or pp95. Furthermore, it is suggested that SHPTP2 activity may be responsible for dephosphorylation of JAK2 kinase in HIRc cells.

In the current study, JAK1 and JAK2 showed different properties in insulin signal transduction in term of their associations with insulin receptor and SHPTP2. In the case of JAK2, SHPTP2 may serve as an adapter protein between JAK2 and pp185 in response to insulin. Although the physiological meaning of the insulin-induced association of pp185 with either JAK1 or JAK2 remains unclear, we believe that JAK kinase family may constitute of a new regulatory component in signaling transduction pathway of insulin.

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