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Signal Transduction Pathways for Interleukin 4 and Insulin in Human Hepatoma Cells[1]

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IRS-1 has been found to relay the signals from the receptors for insulin, insulin-like growth factor-1, growth hormone, and many cytokines for the downstream effects in the various cell types tested. For interleukin 4 signaling, most studies were performed on hematopoietic cells and cell lines transfected with rat liver IRS-1 cDNA. In a liver cell lineage, IRS-1 expression has been found to be increased in hepatoma cells and hepatocytes in regenerating liver. To elucidate the possible function and the signal transduction pathway for interleukin 4, in comparison with insulin, in liver cells, we used the Hep 3B hepatoma cell line as a model system. Following insulin and interleukin 4 stimulation, rapid tyrosyl phosphorylation of IRS-1 occurred. Interleukin 4, but not insulin, stimulated the tyrosine phosphorylation of JAK1 and, to a lesser extent, JAK2. In contrast to the other cell types, the association of IRS-1 and Grb2 through the SH2 of Grb2 was demonstrated after IL-4 and insulin stimulation of the Hep3B hepatoma cells. Both insulin and interleukin 4 stimulated tyrosine phosphorylation and the enzyme activity of Erk1 kinase. Our results indicate that interleukin 4 and insulin might modulate hepatic cell growth and differentiation through many different or common pathways for the activation of JAK kinases and the usage of IRS-1 as a docking protein. The binding of IRS-1 with Grb2 after IL-4 as well as insulin stimulation may lead to MAP kinase activation, probably through the Grb2/sos/p21^{ras} pathway.

Key words: hepatoma cells, insulin, interleukin 4, JAK kinases, signal transduction.

IRS-1 is a major intracellular substrate for insulin and insulin-like growth factor-1 (IGF-1) receptor beta subunit tyrosine kinases (*1-4*). This protein is rapidly phosphorylated at tyrosine residues on ligand stimulation in almost all cells studied. Following stimulation, IRS-1 serves as a multisite docking protein that binds to certain src homology 2 (SH2)-containing proteins, thus linking insulin binding to multiple downstream signaling pathways (*3-7*). IRS-1 is critical for insulin/IGF-1 actions in cell cycle progression, as demonstrated in *Xenopus* oocyte reconstitution experiments (*8-10*), and for DNA synthesis in and proliferation of mammalian cells, as demonstrated by transfection and anti-sense experiments (*11, 12*).

Recently, IRS-1 has also been shown to be functionally important for many receptors of various ligands, such as interleukin 4 (IL-4), growth hormones and interferon (*13-15*). Most studies on the effects of cytokines were performed on hematopoietic cells or cell lines which had been transfected with rat liver IRS-1 cDNA (*13-16*). As compared to insulin, the mechanism of signal transduction through the IL-4 receptor is only partially understood, although IL-4 can stimulate the tyrosine phosphorylation of a protein, termed 4-PS (IL-4-induced phosphotyrosine substrate), in a myeloid progenitor cell line (*16-18*). This substrate, now known as IRS-2/4PS (*41*), is similar to IRS-1 in terms of sequence homology and the association of p85α of phosphatidylinositol (PI) 3-kinase after factor stimulation, but is not recognized by most anti-IRS-1 antibodies (*2, 16, 17*).

Expression of IRS-1 has been found to be increased in hepatocytes in regenerating liver and hepatoma cells (*19, 20*), and thus IRS-1 may be involved in the control of liver cell growth and differentiation. The role of IL-4 in the hepatocyte function is totally unknown, although the receptor for IL-4 has been demonstrated in hepatoma cells (*21*). In contrast to insulin and other peptide growth factor receptors, there is no intrinsic tyrosine kinase domain, within the IL-4 cytokine receptor (*22*). Further studies have suggested that certain intracytoplasmic tyrosine kinases, the JAK kinase family, may be involved in the IL-4 signaling (*19, 23, 24*). More interestingly, the down-

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Abbreviations: GST, glutathione S-transferase; IGF-1, insulin-like growth factor 1; IL-4, interleukin-4; IRS-1, insulin receptor substrate-1; JAK, Janus kinases; MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline; SH2, src homology 2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

stream effects of the stimulation of microtubule-associated protein (MAP) kinase by insulin and IL-4 were different (*25, 26*). How IRS-1 might serve as a common component leading to different signal transduction pathways for insulin and IL-4 in liver cells remains to be elucidated. In this study, we have characterized the signal transduction pathway through the IL-4 receptor in a human hepatoma cell line, Hep 3B. We found that IL-4, as well as insulin, stimulated the tyrosine phosphorylation of endogenous IRS-1. In addition, the tyrosine phosphorylation of JAK1 and JAK2 kinases has been shown to be specifically stimulated by IL-4, but not by insulin. Phosphorylated IRS-1 induced by either insulin or IL-4 can bind to Grb2, which leads to activation of MAP kinase, probably *via* $p21^{ras}$. Thus, tyrosine phosphorylation triggered by JAK tyrosine kinases might be important in the signal transduction for IL-4 in human hepatoma cells.

MATERIALS AND METHODS

Cell Line and Culture—Hep 3B hepatoma cells were cultured in DMEM containing 10% fetal bovine serum in a 37°C humidified incubator under 5% CO_2. The cells were grown to near-confluency before serum-starvation. After 48 h, IL-4 (10 IU/ml) or insulin (0.1 μM) was added to stimulate the cells for different periods of time, as indicated.

Cell Extract Preparation—Cell monolayers were washed with cold PBS after factor stimulation. A whole cell extract was prepared by adding a lysis buffer comprising 1% Nonidet P-40/20 mM Tris, pH 8.0/137 mM NaCl/1 mM phenylmethylsulfonyl fluoride/1 mM Na_3VO_4/0.15 U aprotinin per ml/leupeptin 1 μg per ml to the cells at 4°C. The cell extracts were then clarified by centrifugation at 12,000 × *g* for 10 min (*8*).

Preparation of GST Fusion Proteins and In Vitro Binding Assay—The GST fusion protein of the SH2 of Grb2 was prepared as described previously (*10*). *In vitro* binding of the GST-SH2 fusion protein and cell lysates was carried out at 4°C for 1 h in the presence of glutathione-agarose beads (Sigma). The beads were then washed 4 times with PBS containing 1% Triton X-100 and 0.25 M NaCl. The bound fractions on the agarose beads were then solubilized by boiling in Laemmli's sample buffer containing 100 mM dithiothreitol before gel electrophoresis (*9*).

Immunoprecipitation and Western Blot Analysis—Crude cellular extracts were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) or after immunoprecipitation with the respective antibodies. After separation by SDS-PAGE, the proteins were transferred and immunoblotted as previously described (*8-10*). The signals were detected as to by enhanced chemiluminescence according to the manufacturer (ECL, Amersham). The anti-phosphotyrosine, anti-IRS-1, anti-Shc, anti-Erk1, and anti-JAK1, -JAK2, and -JAK3 antibodies were from UBI.

Erk1 Kinase Activity Assays—Erk1 kinase activity was measured as the phosphorylation of myelin basic protein (MBP) in either the total cell extracts or the anti-Erk1 immunoprecipitates of cells treated with insulin or IL-4 for different incubation times, as indicated (*10*). Mean fold stimulation after factor stimulation was determined in 3 separate experiments, *i.e.* triplicate values *vs.* basal value as the control. Phosphorylation of MBP in the anti-Erk1 immunoprecipitates was analyzed by SDS-PAGE, followed by autoradiography.

RESULTS

Tyrosine Phosphorylation of IRS-1 and Shc by Insulin and IL-4—In Hep 3B hepatoma cells, IL-4, as well as insulin, stimulated the tyrosine phosphorylation of several proteins migrating at M_r = 165-185 kDa (Fig. 1A). The time course for maximal stimulation was slightly longer and the degree of phosphorylation of IRS-1 was less prominent for IL-4 stimulation. In contrast to the stimulation by insulin, there was no phosphorylation of the insulin receptor β subunit following IL-4 stimulation (Fig. 1A). Immunoblotting with the anti-IRS-1 antibody revealed multiple IRS-1 protein bands, with the majority of the

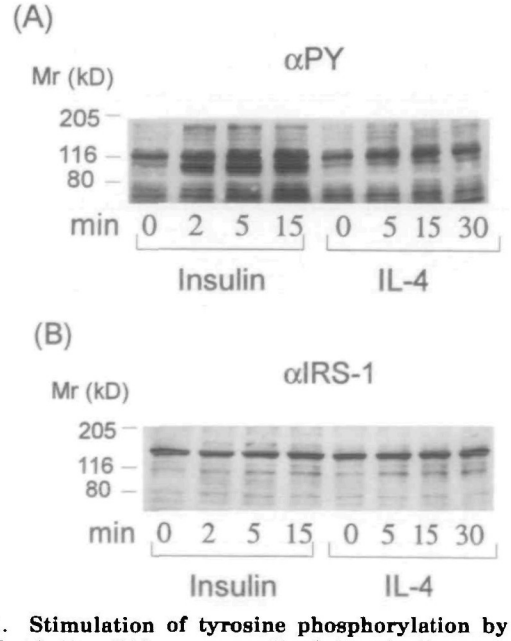

Fig. 1. **Stimulation of tyrosine phosphorylation by insulin and IL-4 in Hep 3B hepatoma cells.** Cultured cell monolayers were treated with insulin (0.1 μM) or IL-4 (10 IU/ml) for different time periods, as indicated in min. Cell extracts were then fractionated by SDS-PAGE and immunoblotted with anti-phosphotyrosine (A) and anti-IRS-1 (B) antibodies.

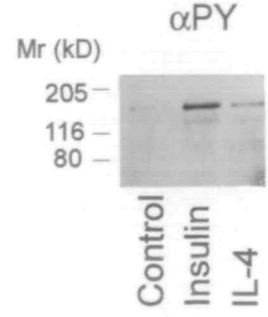

Fig. 2. **Stimulation of tyrosine phosphorylation of IRS-1 by insulin and IL-4 in Hep 3B hepatoma cells.** Lysates of control and insulin or IL-4 stimulated cells (stimulated for 5 min) were immunoprecipitated with anti-IRS-1 antibodies and then immunoblotted with anti-phosphotyrosine antibodies.

unphosphorylated form migrating in lower molecular size ranges (Fig. 1B). The expression of IRS-1 in Hep 3B cells was relatively high, as demonstrated by anti-IRS-1 immunoblotting (Fig. 1B). To confirm the nature of the phosphorylated bands stimulated by insulin and IL-4, immunoprecipitation with the anti-IRS-1 antibody was performed. As shown in the Fig. 2, tyrosine phosphorylation of IRS-1 was found in anti-IRS-1 immunoprecipitates after either insulin or IL-4 stimulation. As the 4PS protein is only weakly recognized by the anti-IRS-1 antibody (*11*), our data suggested that IL-4 could stimulate the tyrosine phosphorylation of endogenous IRS-1. In Hep3B cells, there were 3 Shc proteins migrating in the 50–66 kDa region (lower panel in Fig. 3). Interestingly, only insulin, *i.e.* not IL-4, was found to be able to cause a mild increase in the tyrosine phosphorylation of two of the Shc proteins in Hep3B cells (upper panel in Fig. 3).

Tyrosine Kinases Activated by IL-4 Stimulation—Several cytoplasmic tyrosine kinases of the JAK kinase family have been implicated in the IL-4 signaling in various hematopoietic cells (*18, 23, 24*). To test the possibility of the involvement of these tyrosine kinases in IL-4 signaling in liver cells, we assessed the tyrosine phosphorylation of the respective kinases in the immunoprecipitates of anti-JAK1, -JAK2, and -JAK3 after insulin and IL-4 stimulation. To determine the time course of the activation of JAK kinases, as shown in Fig. 4, JAK1 was found to be activated by IL-4 stimulation, as shown on anti-phosphotyrosine immunoblotting of the anti-JAK1 immunoprecipitate, in a time-dependent manner. Among the JAK kinases tested, both JAK1 and JAK2 were stimulated by IL-4 (Fig. 5).

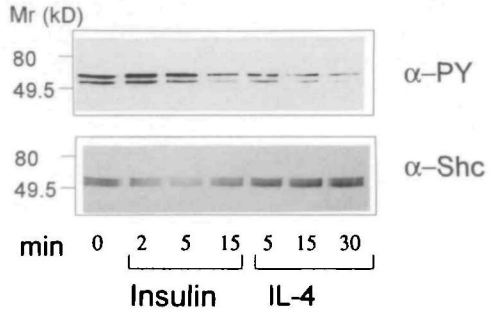

Fig. 3. **Stimulation of tyrosine phosphorylation of Shc by insulin and IL-4 in Hep 3B hepatoma cells.** Lysates of control and insulin or IL-4 stimulated cells were immunoprecipitated with anti-Shc antibodies, and then immunoblotted with anti-phosphotyrosine antibodies (upper panel) or anti-Shc antibodies (lower panel).

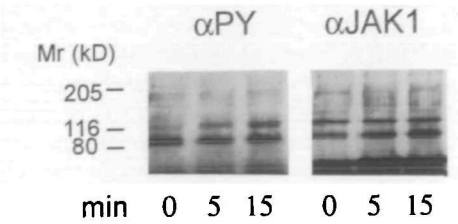

Fig. 4. **Tyrosine phosphorylation of JAK1 kinase by IL-4 in Hep 3B cells.** Cultured cell monolayers were treated with IL-4 at 10 IU/ml for 5 and 15 min, and then extracted for immunoprecipitation with anti-JAK1 and immunoblotted with either anti-phosphotyrosine (left panel) or anti-JAK1 (right panel) antibodies.

However, JAK 3 kinase was not stimulated by IL-4 in this cell line (Fig. 6). No stimulation of JAK kinases was observed on insulin treatment (Figs. 5 and 6).

Stimulation of Erk1 Kinase by IL-4 in Human Hepatoma Cells—Many growth factors and cytokines regulate the cell function through the activation of MAP kinase, and the activation of MAP kinase requires the phosphorylation of tyrosine and threonine residues by the MAP kinase kinase, MEK (*27*, and the references therein). Previously, IL-2, -3, and -5, and GM-CSF, but not IL-4, were shown to activate $p21^{ras}$ and promote the tyrosine phosphorylation of

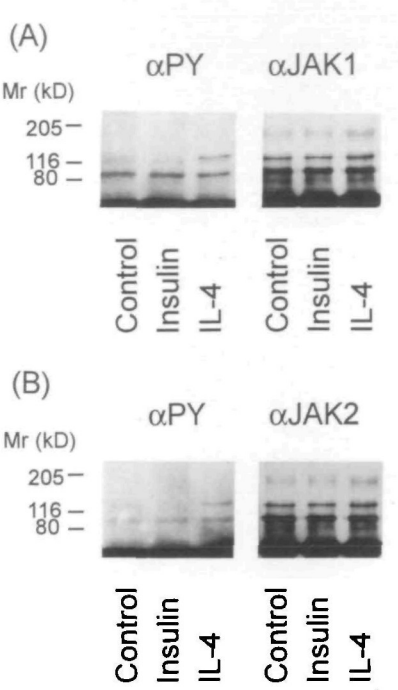

Fig. 5. **Stimulation of JAK1 and JAK2 kinases by insulin and IL-4 in Hep3B cells.** Cultured cell monolayers were treated without or with insulin at 0.1 μM for 5 min or IL-4 at 10 IU/ml for 15 min, and then extracted for immunoprecipitation with anti-JAK1 (A) and anti-JAK2 (B) antibodies, respectively. The precipitated proteins were then fractionated and immunoblotted with anti-phosphotyrosine (left panels) and anti-JAK kinases (right panels) antibodies. Molecular size (in kDa) markers are shown on the left.

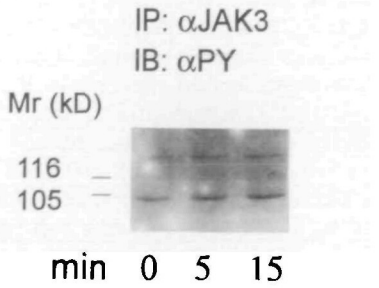

Fig. 6. **Lack of stimulation of JAK3 kinase by insulin and IL-4 in Hep3B cells.** Cultured cell monolayers were treated without or with insulin at 0.1 μM for 5 min or IL-4 at 10 IU/ml for 15 min, and then extracted for immunoprecipitation with anti-JAK3 antibodies. The precipitated proteins were then fractionated and immunoblotted with anti-phosphotyrosine antibodies. Molecular size (in kDa) markers are shown on the left. The JAK3 kinase, with a molecular size of 125 kDa, migrated to the position above the 116-kDa band.

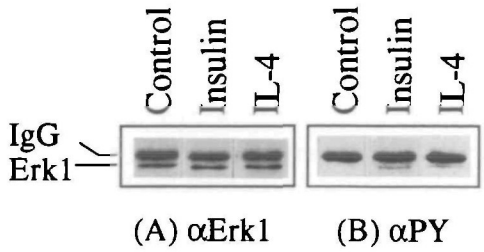

Fig. 7. **Stimulation of tyrosine phosphorylation of Erk1 k nase by insulin and IL-4 in Hep 3B hepatoma cells.** Cultured c monolayers were treated with insulin at 0.1 μM or IL-4 at 10 IU/1 for 5 min, and then extracted for immunoprecipitation with anti-Erk1 antibodies. The precipitated proteins were then immunoblotted with anti-Erk1 (A) and anti-phosphotyrosine (B) antibodies. Arrows indicate the site of the immunoglobulin heavy chain (IgG) and Erk1 kinase.

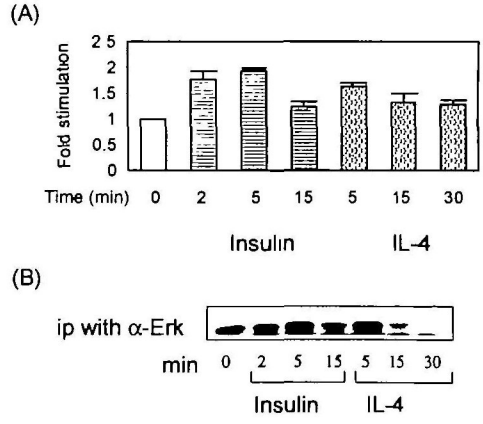

Fig. 8. **Stimulation of kinase activity of Erk1 by insulin and IL-4 in Hep 3B hepatoma cells.** Phosphorylation of myelin basic protein (MBP) was assayed in total cell extracts (A), and in immunoprecipitates with anti-Erk1 antibodies (B) derived from cultured cells treated with insulin at 0.1 μM (striped bars) or IL-4 at 10 IU/ml (stippled bars) for different times, as indicated. The mean fold stimulation and the standard deviations were determined in 3 separate experiments. Phosphorylated MBP, after *in vitro* kinase assaying in the anti-Erk1 immunoprecipitates, was autoradiographed following separation by 15% polyacrylamide gel electrophoresis.

MAP kinase in hematopoietic cells (*28, 29*). In Hep 3B cells, we detected a 44-kDa MAP kinase on anti-Erk1 immunoblotting (Fig. 7A). Following stimulation with IL-4 as well as insulin, tyrosine phosphorylation of MAP kinase increased, as shown on anti-phosphotyrosine immunoblotting of anti-Erk1 immunoprecipitates (Fig. 7B). Erk1 kinase activity, determined as myelin basic protein (MBP) phosphorylation activity in both total cell lysates and the anti-Erk1 immunoprecipitates, was stimulated by treatment with insulin and IL-4 in this cell line (Fig. 8). The degree of stimulation of Erk1 kinase by IL-4 was only slightly lower than that by insulin. The maximal fold stimulation of Erk1 kinase by IL4 was about 85% of the level achieved on insulin stimulation (Fig. 8).

Association of Phosphorylated IRS-1 and SH2 of Grb2—The signal transduction process of receptor tyrosine kinase involves the binding of several SH2-containing molecules to the activated receptor itself (*30*) or through a docking protein, IRS-1, in the case of insulin receptor

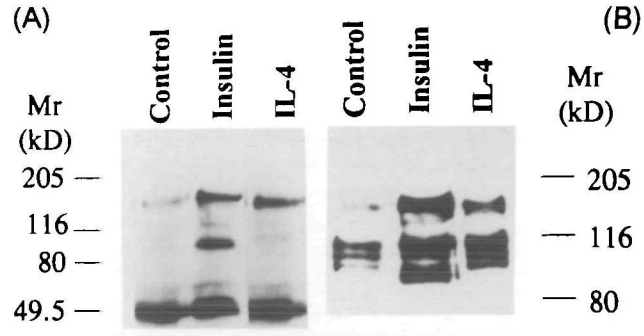

Fig. 9. **Association of IRS-1 and Grb2 through SH2 interaction on insulin and IL-4 stimulation in Hep 3B cells.** (A) Cells after stimulation with insulin (0.1 μM for 5 min) and IL-4 (10 IU/ml for 5 min) were extracted for immunoprecipitation with anti-Grb2 antibodies. The precipitated proteins were then immunoblotted with anti-phosphotyrosine antibodies. (B) *In vitro* binding of cell extracts with the GST-SH2 fusion protein in the presence of glutathione beads incubated at 4°C for one hour. After extensive washing, the bound proteins were solubilized in Laemmli's sample buffer, fractionated and then immunoblotted with anti-phosphotyrosine antibodies.

signaling (*3, 4*). In Hep 3B cells, IL-4, as well as insulin, stimulates the tyrosine phosphorylation of IRS-1 and MAP kinase. To help define the mechanism of MAP kinase activation (*27*), we examined the interaction of IRS-1 and Grb2, which had been shown to trigger the binding of mammalian son of sevenless (mSOS) and the subsequent activation of $p21^{ras}$ by growth factors including insulin (*31* and the references therein). After IL-4 as well as insulin stimulation, phosphorylated IRS-1 was co-immunoprecipitated with the anti-Grb2 antibody (Fig. 9A), suggesting that Grb2 was bound to IRS-1 on insulin and IL-4 stimulation. A phosphorylated band at a M_r of 95,000 corresponding to the β subunit of the insulin receptor was also found in the anti-Grb2 immunoprecipitate after insulin stimulation only (Fig. 9A). Furthermore, phosphorylated IRS-1 bound to the GST fusion protein containing the SH2 of Grb2 *in vitro* following IL-4 and insulin stimulation (Fig. 9B), suggesting the binding of IRS-1 and Grb2 occurred through the SH2 of Grb2. These data suggest that a common component in the signaling pathways *via* the SH2 interaction is present for both cytokine and peptide growth factor receptor signaling.

DISCUSSION

The present study demonstrates that (1) IL-4, as well as insulin, stimulates the tyrosine phosphorylation of the endogenous intracellular substrate, IRS-1; (2) only insulin, *i.e.* not IL-4, stimulates the tyrosine phosphorylation of the Shc protein; (3) IL-4, but not insulin, causes activation of cytoplasmic JAK1 and JAK2 tyrosine kinases; and (4) phosphorylation of IRS-1 triggered by IL-4 and insulin results in the association of Grb2 through the binding of SH2 domains; and (5) the subsequent triggering of tyrosine phosphorylation and kinase activity of Erk1 in the human hepatoma cell line Hep3B.

The activation of the IL-4 receptor involves dimerization of the receptor and a common component shared with the IL-2 receptor, termed IL-2R (*32, 33*). Although there is no kinase domain in the IL-4 receptor, cytoplasmic tyrosine

kinases, JAK1, JAK2, JAK3, and FYN kinases, have been reported to be activated on IL-4 stimulation in different cell systems (*18, 23, 24*) as well as hepatoma cells, as shown in this study. Activation of these kinases results in the complex formation of the receptor and 4PS/IRS-1 (*18*). Formation of the signal transducing complexes of the IL-4 receptor, IRS-1, and JAK kinases might be mediated by an I4R motif in the IL-4 receptor sequence (*34*). Whether this sequence involves activation of JAK and other intracellular signaling proteins remains to be examined in liver cells. Compared with other cell systems, we, for the first time, demonstrated that JAK1 and JAK2 kinases, but not JAK3 kinase, were stimulated by IL-4 in the human hepatoma cell line, Hep3B, suggesting that the tyrosine phosphorylation of IRS-1 by IL-4 is mediated by JAK kinases. The activation of JAK kinases by insulin has been controversial (*35*). Our present data and others (*36*) suggested that the insulin receptor tyrosine kinase independent of JAK can trigger tyrosine phosphorylation of IRS-1 directly.

The effect of IL-4 on non-hematopoietic cells is largely unknown, although previous reports showed the effect of IL-4 on the gene regulation of some acute phase proteins and cytochrome P-450 enzymes in a primary hepatocyte culture (*37, 38*). The mechanism for this regulation is not known. In our study, both insulin and IL-4 stimulated the tyrosine phosphorylation of endogenous IRS-1 molecules, suggesting that the divergent pathways from different receptors and different kinases, namely the insulin receptor tyrosine kinase and JAK kinases, converge at the step of tyrosine phosphorylation of IRS-1 in hepatoma cells.

IRS-1 undergoes multisite tyrosine phosphorylation to relay signals from many different receptor and non-receptor tyrosine kinases (*39*). Following phosphorylation, IRS-1 serves to recruit different src homology 2 domain-containing proteins, and to activate downstream pathways leading to activation of phosphatidylinositol 3′-kinase, MAP kinases, and others (*2-7, 9, 10*). So far, the linkage of IRS-1 phosphorylation to the activation of MAP kinases has been established for the stimulation by insulin/IGF-1 in many cell types (*10, 11, 40*). However, the activation of MAP kinase has not been established for IL-4 stimulation (*26, 28, 29*). In the present study, we found the tyrosine phosphorylation of Erk1 was stimulated by both insulin and IL-4. And the mechanisms of the activation of MAP kinase, an important intermediate step in various signaling systems (*27*), seemed to be the same for both insulin and IL-4 in Hep 3B cells, as both insulin and IL-4 stimulate the binding of IRS-1 to Grb2. This interaction occurred through the SH2 of grb2. Our data indicated that IL-4, as well as insulin, could activate MAP kinase *via* the activation of Ras through mSOS. The activation of $p21^{ras}$ and MAP kinase has been observed in the cytokine-stimulated pathways for IL-2, IL-3, and GM-CSF, but not for IL-4, in hematopoietic cells (*28, 29*). The discrepancy between these studies suggests that different signaling pathways might operate in different cells (*30*). The functional implication of this activation of MAP kinase on IL-4 stimulation in the hepatoma cells deserves further investigation.

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