Insulin-like Growth Factor-I Receptor and Insulin Receptor Association with a Src Homology-2 Domain-containing Putative Adapter*

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Insulin receptor (IR) and the related insulin-like growth factor-I (IGF-I) receptor (IGF-IR) mediate a variety of metabolic and mitogenic cellular responses, some of which may involve unidentified receptor targets. A Src homology-2 (SH2) domain-coding region of a mouse protein was cloned based on its interaction with IR. It was designated mSH2-B based on its high similarity to an earlier reported rat sequence SH2-B. A role of mSH2-B in IGF-I and insulin action was suggested by the interaction of the SH2 domain with activated IGF-IR and IR catalytic fragments but not with an inactive IR catalytic fragment in the yeast two-hybrid system in vivo and by the hormone-dependent association of a glutathione S-transferase (GST) SH2 domain fusion protein of mSH2-B with both receptors in cell extracts. A comparison of IGF-IR and IR mutants lacking individual Tyr autophosphorylation sites for association with GST mSH2-B showed that homologous juxtamembrane (IR960/IGF-IR950) and C-terminal (IR1322/IGF-IR1316) receptor motifs were required. Synthetic phosphopeptides representing IR960 and IR1322 competed for GST mSH2-B binding to the receptor, suggesting that both motifs participate in the association with mSH2-B. Antibodies raised against GST mSH2-B identified a cellular protein of 92 kDa that was not found to be phosphorylated on Tyr. It co-immunoprecipitated with IGF-IR or IR, which was strictly dependent on receptor activation. IR and IGF-IR Tyr phosphorylation motifs were not identified in the complete SH2-B primary structure, suggesting that it may participate as an adapter rather than as a substrate in the IGF-I and insulin signaling pathways.

Insulin-like growth factor-I (IGF-I) has been implicated particularly in mitogenic functions and of insulin more in metabolic cellular functions (1, 2). The insulin receptor (IR) and the closely related IGF-I receptor (IGF-IR) have served as a model system for the elucidation of receptor Tyr kinase-mediated signaling mechanisms (3). The nature for the physiologic differences between the IGF-I and insulin signals is unclear and may involve cellular targets such as Grb10 and pp120 that exhibit a preference for one of the receptors (4, 5). Major cellular targets that are shared by both receptors include the insulin receptor substrates IRS-1, IRS-2, IRS-3, and Shc, most of which also play established roles in other signaling pathways (6–10).

Some of the metabolic responses to insulin must be mediated by mechanisms that parallel PI 3'-kinase, which suggests a role of an undefined alternative IR signaling pathway (11). A role of IRS-1 and PI 3'-kinase has been proposed in the insulin-stimulated glucose uptake by GLUT4 glucose transporter translocation (12, 13); however, an IRS-1- and PI 3'-kinase-independent, unidentified pathway has been described in the insulin stimulation of glucose uptake in independent studies (14, 15). Insulin activates glycogen synthase in CHO cells by an unidentified, Ras- and PI 3'-kinase-independent (wortmannin-independent) mechanism (16). Despite normal Ras binding to Raf-1, truncation of the IR C terminus impairs Raf-1, mitogen-activated protein kinase kinase, and MAPK activities, glucose transport, glycogen synthesis, PI 3'-kinase, and phosphoprotein phosphatase-1 activities, whereas mitogenic responses remain largely unimpaired (17, 18). Overexpression of IRS-1 was shown to restore the mitogenic response of a defective IR (Y960A, which fails to activate IRS-1) including activation of PI 3'-kinase but restored only partial glycogen synthesis and failed to restore MAPK activation (19). IR mutants in the tyrosine kinase region (R1174Q and L1178P) suggest a role of unidentified signaling mediators in the impaired glycogen synthesis, DNA synthesis, and MAPK activation in response to insulin in CHO cells, because IRS-1 appears to be normally activated (20). Mitogen-activated protein kinase and PI 3'-kinase signaling pathways are not sufficient for IGF-I-induced mitogenesis and tumorigenesis, suggesting a role of unidentified signaling mediators in these important IGF-I actions (21). Such mediators may act in parallel to IRS-1 and IRS-2 and may resemble other adapters known to associate with other receptor tyrosine kinases (22). In this study we have characterized the association of a newly identified protein target of 92 kDa, designated mSH2-B, with IGF-IR and IR that is strictly dependent on ligand stimulation for both receptors and was found in a yeast two-hybrid screen (23). We find that the SH2 domain of mSH2-B as well as the juxtamembrane regions and the C-terminal regions of both receptors are required for the association and that synthetic peptides representing these receptor motifs compete with receptor binding to mSH2-B. These characteristics suggest a role of mSH2-B as a Pro-rich and SH2 domain-containing mediator in IGF-I and insulin action.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF036355.

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1 The abbreviations used are: IGF-I, insulin-like growth factor-I; GST, glutathione S-transferase; IGF-IR, IGF-I receptor; IR, insulin receptor; IRS, insulin receptor substrate; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; SH2, Src homology-2; CHO, Chinese hamster ovary; aa, amino acid; MAPK, mitogen-activated protein kinase.
SH2 Domain Binding to Specific Receptor Domains

EXPERIMENTAL PROCEDURES

Antibodies and Phosphopeptides—Rabbit polyclonal antibodies directed against the IGF-IR or IR β subunit were obtained from Upstate Biotechnology, Inc., monoclonal anti-phospho Tyr antibody PY20 was from Transduction Laboratories, and horseradish peroxidase-coupled anti-IG antibody was from Kirkegaard and Perry Laboratories. mSH2-B antisera was produced by HRP Inc. (Denver, PA) in rabbits against a GST fusion protein containing the SH2 domain of mSH2-B (GST mSH2-B). Phosphopeptides representing py953 (LpYASSNPEY-LSASDV), py960 (SSNPEpYLsASD), py1146 (DlpYEpYDDYRK), py1150 (DlpYEpYDDYRK), py1316 (KRpSYEHHpY), or py1322 (HlpYTHMNGG) were incubated with GST mSH2-B during the immunoprecipitation step as described below (24).

Yeast Two-hybrid Screen and Interaction Specificity—Yeast two-hybrid plasmid pGBT9 (25) containing the C terminus of the IR β subunit (26) was used as a bait to screen a 9.5–10.5-day post conception mouse embryonic cDNA library in plasmid pVP16 (27). Library (prey) plasmids were isolated from His prototroph and sequenced, and re-introduced into Saccharomyces cerevisiae strain L40. Mating with strains (AMR70) carrying various test baits followed by the β-galactosidase-positive yeast colonies (24). Plasmids were introduced into Escherichia coli strain DH5α, sequenced, and re-introduced into Saccharomyces cerevisiae strain L40. Mating with strains (AMR70) carrying various test baits followed by the β-galactosidase color assay evaluated the specificity of the underlying bait-prey interactions (27).

GST Fusion Protein—The cloned cDNA insert encoding the SH2 domain of mSH2-B and surrounding coding sequences was released from the pVP16 library plasmid at its NotI cloning site and inserted into the NotI cloning site of pGEX-4T-1 (Pharmacia Biotech Inc.). GST mSH2-B fusion and control GST protein were expressed in E. coli strain DH5α and purified on a glutathione-agarose column (Pharmacia), eluted in 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, and stored after addition of 10 mM diithiothreitol, 1 mM EDTA as described by the manufacturer.

Cell Culture, Immunoprecipitation, and Immunoblotting—Subconfluent wild-type or mutant IR overexpressing CHO fibroblasts (kindly provided by Drs. Yusuke Ebina, Takashi Kadowaki, and Morris F. White) or wild-type or mutant IGF-IR overexpressing R fibroblasts (kindly provided by Dr. Renato Baserga) were employed in various experiments (28–33). Cells were incubated in serum-free medium for 16 h and stimulated with 100 ng/ml insulin or 100 ng/ml IGF-I for 15 min. Cells were washed twice with phosphate-buffered saline and harvested in ice-cold lysis buffer containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 10 mM NaF, 100 mM Na3VO4, 10 mM sodium pyrophosphate, 10 μM leupeptin, 10 μM aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Proteins were directly subjected to SDS-PAGE or were first mixed with GST mSH2-B fusion protein or antibodies directed against mSH2-B, IR, or IGF-IR and co-precipitated with glutathione-agarose or protein A-Sepharose beads, respectively. Precipitates were washed with lysis buffer, separated by SDS-PAGE, and analyzed by immunoblotting with specific antibodies using the ECL (Amer sham Corp.) detection system (24).

RESULTS AND DISCUSSION

Two-hybrid Analysis of IGF-IR and IR Association with the SH2 Domain of mSH2-B in Vivo—We employed the cytokinase-mammalian detergent cell extracts under very different experimental conditions. Because this is unlikely our data suggest that mSH2-B binds directly to the receptors without the involvement of an intermediate protein.

Analysis of Receptor Mutant and Phosphopeptide Association with the SH2 Domain of mSH2-B—Various IR and IGF-IR

![FIG. 1. Two-hybrid prey-bait interaction tests. S. cerevisiae strains (L40) transformed with the indicated individual library (prey) plasmids were crossed with strains of the opposite mating type (AMR70) expressing the eight baits indicated. Diploid strains were selected and transferred to nitrocellulose filters. The underlying prey-bait interactions were assayed by β-galactosidase-mediated blue color and photographed after 1 h. The tested baits are: 1, protein kinase C6; 2, IR cytoplasmic fragment; 3, lamin C test bait; 4, IGF-IR cytoplasmic fragment; 5, Gal4 control bait; 6, IRS-1; 7, catalytically inactive IR cytoplasmic fragment IR K1018A; 8, LexA control bait. The tested library plasmids (preys) contained SH2 domains of the regulatory subunit p85 (C-terminal SH2 domain) of PI 3-kinase, the transforming protein Vav, or mSH2-B.](http://www.jbc.org/content-pdf/269/5/3137/F1.pdf)
mutants in the juxtamembrane or C-terminal receptor regions were employed to test their impact on the association with the SH2 domain of mSH2-B. Cell lines overexpressing individual mutants were mixed with GST mSH2-B. Precipitation with glutathione-Sepharose failed to precipitate juxtamembrane mutants IGF-IR Y950F and IR Y960F as well as C-terminal mutants IGF-IR Y1316F, IR Y1322C, IR Y1316F/Y1322F (YPF2), or dCT lacking 43 aa of the IR C terminus (Fig. 3, A and B). To test whether these motifs are in fact able to bind to mSH2-B, synthetic phosphopeptides representing these regions were tested for competition with normal IR binding to GST mSH2-B. In accordance with the receptor mutant analysis, peptides representing Tyr960 and Tyr1322 abolished IR binding in contrast to peptides representing other receptor regions (Fig. 3C). These data suggest that homologous sites of both receptors at IGF-IR Tyr950 and Tyr960 in the juxtamembrane region and at IGF-IR Tyr1316 and Tyr1322 at the C terminus are involved in the interaction with the SH2 domain of mSH2-B.

Ligand-dependent Association of Cellular SH2-B with IGF-IR and IR—To learn more about the cellular SH2-B protein, a rabbit antiserum was raised against GST mSH2-B that identified a 92-kDa protein in immunoblots of CHO and mouse fibroblast cell extracts (Fig. 4, A and B). To evaluate the association of this protein with the IR and IGF-IR, fibroblast overexpressing either receptor were ligand stimulated, and detergent cell extracts were immunoprecipitated with receptor-specific antibodies followed by SDS-PAGE and immunoblotting with mSH2-B antiserum. A 92-kDa protein of identical mobility was consistently observed in several cell lines, specifically recognized by mSH2-B antiserum, and co-precipitated with IGF-IR and IR in a strictly ligand-dependent fashion. It represents a promising candidate for a pro-rich and SH2 domain-containing mediator involved in the IGF-IR and IR signaling pathways that appears to be shared by both receptors similar to most known IGF-IR and IR targets.

The cloned C-terminal SH2 domain coding fragment of mouse mSH2-B displays high sequence homology to the published rat sequence, designated SH2-B (40), suggesting that the observed changes are explained by species-specific differences. In our efforts to clone the complete mouse mSH2-B coding sequence we have isolated a number of sequence variants that suggest the existence of a family of SH2-B-related mediators.

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**Fig. 2. Interaction of IR and IGF-IR with the SH2 domain of mSH2-B in vitro.** Cell lines overexpressing insulin receptors (CHO/IR(A)) or insulin-like growth factor-I receptors (R/IGF-IR(B)) were incubated with the respective ligand (+) or were left untreated (−). Detergent cell extracts were immediately separated by SDS-PAGE (dash) or were first immunoprecipitated with purified GST mSH2-B fusion proteins (GST-SH2) or control GST. Complexes were precipitated with glutathione-Sepharose beads, separated by SDS-PAGE, and immunoblotted with specific antibodies directed against the respective receptors. The positions of the IR and IGF-IR are marked on the right.

**Fig. 3. Analysis of the IR and IGF-IR association with the SH2 domain of mSH2-B.** Cell lines overexpressing wild-type IR (WT), IR mutations Y953F, Y960F, Y972F, Y1250F, Y1316F/Y1322F (YPF2), or IR lacking 43 aa of the C terminus (dCT) (A), or wild-type IGF-IR, IGF-IR mutations Y950F, Y1250F, Y1251F, or Y1316F (B), or wild-type IR (C) were stimulated with the respective ligand (+) or were left untreated (−). Detergent extracts were immediately separated by SDS-PAGE (dash) or were first incubated for immunoprecipitation (IP) with GST mSH2-B alone (A and B) or in addition with phosphopeptides representing the major phosphotyrosine autophosphorylation motifs of the IR (as indicated by aa numbers) (C). Complexes were precipitated with glutathione-Sepharose, separated by SDS-PAGE, transferred to nitrocellulose, and identified by immunoblotting with antibodies directed against the respective receptor. The positions of the IR and IGF-IR are marked on the right.

**Fig. 4. Association of cellular SH2-B with IR or IGF-IR.** Cell lines overexpressing IR (A) or IGF-IR (B) were stimulated with the respective ligand (+) or were left untreated (−). Detergent extracts were immediately separated by SDS-PAGE (dash) or were first immunoprecipitated (IP) with mSH2-B-specific (mSH2-B) or receptor-specific (IR or IGF-IR) antiserum prior to SDS-PAGE. Proteins were transferred to nitrocellulose and identified by immunoblotting (BLOT) with specific antiserum directed against mSH2-B, IR, or IGF-IR. Molecular markers in kDa are shown on the right.
including APS, some of which may be generated by differential splicing (41, 42).

Because many IR and IGF-IR targets including IRS-1 (6), IRS-2 (8), and She (7) are receptor substrates that are phosphorylated on Tyr by the ligand-activated receptor Tyr kinase, we began to investigate whether mSH2-B may be a new member of this group. The analysis of its primary structure did not reveal any consensus target sites YXXM or YXXYM for the IGF-IR or Tyr IR Tyr kinases (6), which strongly suggested against a role of mSH2-B as an IGF-IR and IR substrate. This was confirmed in biochemical experiments to evaluate whether SH2-B was phosphorylated on Tyr upon IGF-I or insulin stimulation and recognized by phosphotyrosine-specific antibodies in immunoblots. With these antibodies we consistently failed to detect SH2-B directly in cell lysates or after prior immunoprecipitation with mSH2-B antisera. Similarly, immunoprecipitation with phosphotyrosine antibodies failed to bring down SH2-B based on subsequent immunoblots with mSH2-B antisera. IR and IGF-IR autophosphorylation in these experiments confirmed appropriate experimental conditions and ligand stimulation (not shown). Given that an insulin-stimulated IR-associated Ser kinase has been implicated in insulin action (43) we also addressed the putative phosphorylation of SH2-B (Ser or Thr). However, preliminary experiments failed to identify SH2-B as a phosphoprotein after 32P labeling of various fibroblasts (not shown), independent of ligand stimulation (44). We consider that SH2-B may not be phosphorylated in response to IGF-I or insulin stimulation or a weaker SH2-B phosphorylation may not have been detected due to limitations in the sensitivity of the assay (42).

Possible functions of mSH2-B may include a positive role in any of the pleiotropic responses to IGF-I and insulin. Alternatively, mSH2-B may be a negative regulator by counter balancing IRS-1, IRS-2, IRS-3, Shc-mediated, or other signals. There is some evidence that mSH2-B may function like other signaling adapter proteins such as Grb2 by forming complexes with other proteins and by directing their intracellular localization (22). It may undergo phosphorylation in response to other stimuli that remain to be defined (42).

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