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Regulation of Insulin-like Growth Factor I Receptor Dephosphorylation by SHPS-1 and the Tyrosine Phosphatase SHP-2*

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Activation of insulin-like growth factor I receptor (IGF-IR) kinase is an important site of control of IGF-I-linked intracellular signaling pathways. One potentially important regulatory variable is IGF-IR dephosphorylation. It has been shown that SHP-2, a tyrosine phosphatase, can bind to the activated IGF-IR *in vitro*; however, its role in IGF-IR dephosphorylation in whole cells is unknown. These studies were undertaken to determine whether SHP-2 was a candidate for mediating IGF-IR dephosphorylation. The IGF-IR in smooth muscle cells was dephosphorylated rapidly beginning 10 min after ligand addition, and this was temporally associated with SHP-2 binding to the receptor. IGF-I stimulated SHPS-1 phosphorylation and the subsequent recruitment of SHP-2. In cells expressing a SHPS-1 mutant that did not bind SHP-2 there was no recruitment of SHP-2 to the IGF-IR. Cells expressing a catalytically inactive form of SHP-2 showed SHP-2 recruitment to SHPS-1, but this did not result in SHPS-1 dephosphorylation, and there was a prolonged IGF-IR phosphorylation response after IGF-I stimulation. These studies indicate that IGF-IR stimulates phosphorylation of SHPS-1 which is critical for SHP-2 recruitment to the plasma membrane and for its recruitment to the IGF-IR. Recruitment of SHP-2 to the receptor then results in receptor dephosphorylation. The regulation of this process may be an important determinant of IGF-IR-mediated signaling.

The insulin-like growth factor I (IGF-I)[1] receptor (IGF-IR) is composed of two extracellular subunits and two transmembrane β subunits. Upon ligand binding, the kinase domain of the receptor autophosphorylates several tyrosine residues that are located in the intracellular domains of the β subunits (1–3). This provides binding sites for other molecules, including insulin receptor substrate 1 (IRS-1) and SHC (4, 5), and the activated IGF-IR transmits downstream signals via tyrosine phosphorylation of these molecules. Activation of these tyrosines is not only critical for the downstream signaling, but also for sustained stimulation of IGF-I-stimulated biologic responses. Mutation of specific tyrosines in the receptor has been shown to result in attenuation of the ability of IGF-I to stimulate DNA synthesis, cell migration, and inhibition of apoptosis (6). Most important is the triple tyrosine motif that is phosphorylated rapidly in response to catalytic activation of the receptor, and mutation of any of these residues results in loss of signaling and IGF-I responsiveness (7).

The tyrosine phosphorylation status of proteins in growth factor signaling pathways is maintained by a balance between the activity of tyrosine kinases and tyrosine phosphatases. Because phosphorylation of these tyrosines in the receptor β subunit is so important for IGF-I signaling it has been assumed that dephosphorylation of the receptor would result in appropriate regulatory control of this signaling stimulus. The time course of phosphorylation of tyrosine kinase-containing growth factor receptors has been shown to be biphasic with an early peak resulting in activation of the signaling intermediates within 0–10 min of ligand occupancy of the receptor followed by a slower phase of receptor dephosphorylation usually proceeding over the next 10–30 min. One candidate phosphatase for dephosphorylation of the IGF-IR is the tyrosine phosphatase SHP-2 (Syp, PTP-1D, SHPTP2). SHP-2 is a nontransmembrane phosphotyrosine phosphatase that contains two SH-2 domains (8, 9). It has been shown to bind to a number of growth factor receptors including the IGF-IR (10, 11), the insulin receptor (10), and also to IRS-1 (12, 13); however, whether it dephosphorylates the IGF-IR in whole cells has not been determined.

Growth hormone (GH) activation of GH receptor has been shown to result in binding of SHP-2 to the GH receptor and tyrosine phosphorylation of SHP-2 (14–16). Mutation of tyrosine 595, which is required for SHP-2 binding, results in prolonged GH receptor phosphorylation, suggesting that SHP-2 activation contributes to dephosphorylation of the GH receptor and GH receptor-mediated signaling (16). In addition to binding and interacting with SHP-2, activated GH, insulin, and growth factor receptors have also been shown to stimulate the phosphorylation of a transmembrane protein termed SHPS-1 (Sirp α, Bit) (14, 17–20). After phosphorylation of two tyrosines that are contained in the intracellular domain of SHPS-1, SHP-2 is recruited from the cytoplasm to the plasma membrane, and its binding to SHPS-1 activates its phosphatase activity (18–20). Mutation of these tyrosines in SHPS-1 results in the failure of SHP-2 to be recruited to the membrane (21). The recruitment of SHP-2 to SHPS-1 has been shown to correlate with the negative effect of SHPS-1 on insulin signaling (19), and in the case of the GH receptor, loss of SHP-2 recruitment to SHPS-1 has been shown to result in prolonged GH receptor phosphorylation (22). Based on these prior observations, we attempted to determine whether SHP-2 dephospho-

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[1] The abbreviations used are: IGF-I, insulin-like growth factor I; IGF-IR, insulin-like growth factor I receptor; IRS-1, insulin receptor substrate 1; GH, growth hormone; pSMC(s), porcine smooth muscle cell(s); SHPS-1fl and SHPS-1tr, full-length and truncated SHPS-1, respectively; SFM, serum free medium.

rylates the IGF-IR after IGF-I-induced receptor activation, to identify the role of SHPS-1 in recruitment of SHP-2 to the activated receptor, and to determine the consequences of blocking this recruitment using mutagenesis.

EXPERIMENTAL PROCEDURES

Materials

Human IGF-I was a gift from Genentech (South San Francisco, CA). Polyvinylidene difluoride filters were purchased from Millipore Corporation (Bedford, MA). Autoradiographic film was obtained from Eastman Kodak. Fetal bovine serum, Dulbecco's modified Eagle's medium, penicillin. and streptomycin were purchased from Invitrogen. The IGF-IR β chain antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal SHP-2 antibody and the monoclonal, horseradish peroxidase-linked, phosphotyrosine antibody (RC20) were purchased from Transduction Laboratories (Lexington, KY). Two SHPS-1 polyclonal antibodies were used. The first was obtained from Transduction Laboratories and was raised to the entire cytoplasmic domain of SHPS-1 (SHPS-1fl) the second was raised our laboratory in rabbits using a synthetic peptide containing the C-terminal 20 amino acids of the SHPS-1 cytoplasmic tail as an immunogen (SHPS-1tr). All other reagents were purchased from Sigma unless otherwise stated.

Porcine aortic SMCs (pSMCs) were isolated as described previously (23) and maintained in Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum in 10-cm tissue culture plates. The cells were used between passage 5 and 16.

Methods

Generation of Expression Vectors—Full-length human SHP-2 and SHPS-1 were cloned by reverse transcription-PCR from a cDNA library that had been derived from human fibroblast mRNA (GM10, Human Genetic Cell Repository, Camden, NJ). For SHP-2 the 5′-primer sequence used corresponded to nucleotides 133–158 of the human SHP-2 sequence but with the addition, at the 5′-end, of nucleotides encoding the FLAG peptide. The 5′-primer sequence was 5′-ATG GAC TAC AAA GAC GAC GAC GAC AAA ACA TCG CGG AGA TGG TTT CAC CCA AA-3′. The bases encoding the FLAG peptide are underlined. The 3′-primer sequence used was complementary to nucleotides 1882–1911 and was 5′-TCA TCT GAA ACT TTT CTG TTG CAT-3′. After DNA sequencing to confirm that the correct sequence had been amplified the PCR product was subcloned into pMEP4 expression vector (Invitrogen).

To clone SHPS-1 the 5′-primer sequence used corresponded to nucleotides 17–35 of human SHPS-1 (5′-CAG CCG CGG CCC ATG GAG CC-3′), and the 3′-primer sequence was complementary to nucleotides 1502–1524 (5′-CAA ACC ACG GTC CCA TTC TGA-3′). After DNA sequencing to confirm that the correct sequence had been amplified the PCR product was subcloned into the pMEP4 expression vector.

Generation of the Catalytically Inactive SHP-2—PCR was used to incorporate a replacement of Cys^{459} by Ser in the catalytic site of SHP-2 (SHP-2 C-S). This mutation was shown previously to result in a dominant negative catalytically inactive form of SHP-2 (23, 24). Two cDNA fragments encoding the SHP-2 DNA were generated. The first fragment was made using the same 5′-primer as that used to clone full-length SHP-2; the 3′-primer was complementary to nucleotides 1474–1497, but it also contained 3 additional bases at the 3′-end of the DNA sequence which introduced an *Hpa*I cleavage site. The primer sequence was 5′-GTT * AAC GAC CGG CCC TGC ATC CAT GAT-3′. The additional 3 bases are underlined, and the site of the *Hpa*I cleavage is marked *. The fragment was generated using a 5′-primer sequence corresponding to nucleotides 1498–1525, but it also contained a 3-base extension at the 5′-end to generate an *Sna*B1 cleavage site, and it included 2-base substitutions (TGC-TCA) to encode the $Cys^{459} \rightarrow$ Ser mutation. The sequence was 5′-TAC * GTA CAC $TC^{\#}A^{\#}$ AGT GCT GGA ATT GGCCGG A-3′. The additional 3 bases are underlined, and the point of *Sna*B1 cleavage is marked with *. The base changes encoding the Cys → Ser substitution are indicated with #. The 3′-primer was the same as that used to clone the full-length SHP-2.

The two PCR products were then cloned into the pcDNA 3.1 V5 His vector (Invitrogen). After amplification the two fragments were digested and separated from the vector. The first fragment was digested at *Bam*HI in the polylinker of the vector, 5′ to the introduced DNA sequence and at the introduced *Hpa*I site. The second fragment was digested at the *Xho* site in the vector polylinker sequence 3′ to the introduced DNA sequence and at the introduced *Sna*B1 site. The DNA fragments were then ligated at the compatible blunt ends generated by the *Hpa*I and *Sna*B1 digestions, and the DNA sequence encoding the full-length SHP-2 incorporating the Cys → Ser substitution was cloned into the pcDNA 3.1 V5 His vector. After amplification and sequencing the modified sequence was subcloned into the pMEP4 expression vector.

Generation of the Truncated Form of SHPS-1—PCR was used to generate a truncated form of SHPS-1 (SHPS-1tr) that terminated at amino acid 463 resulting in the loss of the two C-terminal tyrosine residues (Tyr^{449}/Tyr^{475}) that had been shown to be required for SHP-2 binding (23). The 5′-primer used was the same as that used to clone the full-length SHPS-1, and the 3′-primer that was used was complementary to nucleotides 1390–1416 except that it contained base substitutions to incorporate a stop codon at the end of the sequence (at position 1414–1416). The sequence was 5′-CTA GGG CTG CGG GCT GGT CTG AAT GCT-3′. The introduced stop site is underlined. The modified DNA sequence was cloned into the pcDNA 3.1 V5 His vector. After sequencing and amplification it was subcloned into the pMEP4 expression vector.

Transfection of pSMCs—The pSMCs (passages 4 and 5) were transfected with the various pMEP4 expression vectors. In all cases transfections were performed using the poly-L-ornithine method as described previously (24). Hygromycin-resistant pSMCs were selected and maintained in Dulbecco's modified Eagle's medium-H containing 15% fetal bovine serum and 100 μg/ml hygromycin. Transfected pSMCs were used in subsequent experiments between passages 6 and 20.

Cell Lysis—Cells were incubated overnight in SFM and then exposed to 100 ng/ml IGF-I for the appropriate length of time before lysis in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The lysates were clarified by centrifugation at 14,000 × *g* for 10 min. Immunoprecipitation was then carried out as described below.

Immunoprecipitation—The supernatants were incubated overnight at 4 °C with the appropriate antibody (IGF-IR, SHP-2, or SHPS-1 using a 1:500 dilution). Immune complexes were then precipitated by adding protein A-Sepharose and incubating for a further 2 h at 4 °C. The samples were then centrifuged at 14,000 × *g* for 10 min and the pellets washed four times with lysis buffer. The pellet was resuspended in 45 μl of reducing Laemmeli buffer, boiled for 5 min, and the proteins separated by SDS-PAGE, 8% gel.

Western Immunoblotting—After SDS-PAGE the proteins were transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 1% bovine serum albumin in Tris-buffered saline with 0.1% Tween (TBST) for 2 h at room temperature. The membranes were incubated with one of four primary antibodies (IGF-IR, SHP-2, SHPS-1, or RC20 all at a dilution of 1:500) overnight at 4 °C and then washed three times in TBST. Binding of the peroxidase-labeled antibody was visualized using enhanced chemiluminescence following the manufacturer's instructions (Pierce) and either exposure to autoradiographic film or detection using the GeneGnome CCD imaging system (Syngene U. K. Ltd.).

Band intensities were measured by scanning densitometry and analyzed using NIH Image, version 1.61. Student's *t* test was used to compare differences between treatments. The results that are shown are representative of at least three separate experiments.

RESULTS

Time Course of IGF-IR Phosphorylation in Response to IGF-I—After a 5-min stimulation of quiescent pSMCs with IGF-I there was a 13 ± 1-fold (mean ± S.E., $n = 3$) increase in IGF-IR phosphorylation. After a 10-min treatment with IGF-I there was a further 1.3 ± 0.12-fold ($n = 3$) increase in IGF-IR phosphorylation. By 20 min there was a 2.1 ± 0.25-fold ($n = 3$) decrease in receptor phosphorylation compared with the level of phosphorylation which is detectable at 10 min. A representative experiment in shown in Fig. 1.

Association of SHP-2 with the IGF-IR after IGF-IR Phosphorylation—In Fig. 2 it can be seen that there was little SHP-2 associated with the IGF-IR in the basal state and after 5 min of IGF-I stimulation. However, after a 10-min stimulation there was detectable SHP-2 association, and at 20 min there was a marked 16 ± 4.5-fold increase ($n = 3$) in SHP-2 association with the IGF-IR. These results suggest that there is a correlation between the time at which the phosphorylation state of the

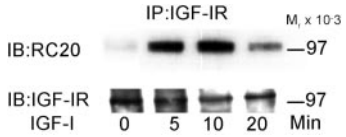

FIG. 1. **IGF-IR phosphorylation time course.** Cells were grown to 90% confluence and then incubated overnight in SFM. Cells were then exposed to 100 ng/ml IGF-I for various lengths of time as indicated. After cell lysis and immunoprecipitation (*IP*) with an anti-IGF-IR antibody, tyrosine phosphorylation of the receptor was visualized by Western immunoblotting (*IB*) with an anti-phosphotyrosine antibody. The results are expressed as arbitrary scanning units: 2,132 (*far left lane*), 25,963 (*second lane*), 30,431 (*third lane*), and 11,699 (*far right lane*). To control for loading inequities the amount of IGF-IR protein in each lane is shown in the *lower panel*.

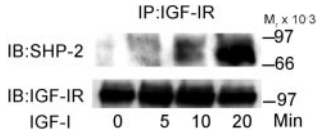

FIG. 2. **SHP-2 association with the IGF-IR.** Cells were grown to 90% confluence and then incubated overnight in SFM. They were then exposed to 100 ng/ml IGF-I for various lengths of time as indicated. After cell lysis and immunoprecipitation (*IP*) with an anti-IGF-IR antibody the association of SHP-2 with the receptor was visualized by Western immunoblotting (*IB*) with an anti-SHP-2 antibody. The results are expressed as arbitrary scanning units: 1,104 (*far left lane*), 8,775 (*second lane*), 15,033 (*third lane*), and 27,114 (*far right lane*). To control for loading inequities the amount of IGF-IR protein in each lane is shown in the *lower panel*.

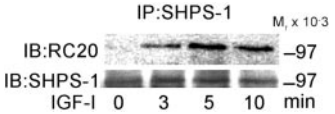

FIG. 3. **SHPS-1 phosphorylation in response to IGF-I stimulation.** Cells were grown to 90% confluence and then incubated overnight in SFM. They were then exposed to 100 ng/ml IGF-I for various lengths of time as indicated. After cell lysis and immunoprecipitation (*IP*) with an anti-SHPS-1 antibody, tyrosine phosphorylation was visualized by Western immunoblotting (*IB*) with an anti-phosphotyrosine antibody. The results expressed as arbitrary scanning units are: 2,961 (*far left lane*), 10,927 (*second lane*), 23,636 (*third lane*), and 18,780 (*far right lane*). To control for loading inequities the amount of SHPS-1 protein in each lane is shown in the *lower panel*.

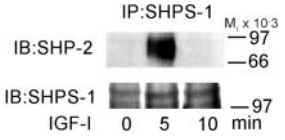

FIG. 4. **SHP-2 recruitment to SHPS-1 in response to IGF-I stimulation.** Cells were grown to 90% confluence and then incubated overnight in SFM. They were then exposed to 100 ng/ml IGF-I for various lengths of time as indicated. After cell lysis and immunoprecipitation (*IP*) with an anti-SHPS-1 antibody association of SHP-2 with SHPS-1 was visualized by Western immunoblotting (*IB*) using an anti-SHP-2 antibody. The results expressed as arbitrary scanning units are: 1,577 (*left lane*), 25,650 (*center lane*), and 2,070 (*right lane*). To control for loading inequities the amount of SHPS-1 protein in each lane is shown in the *lower panel*.

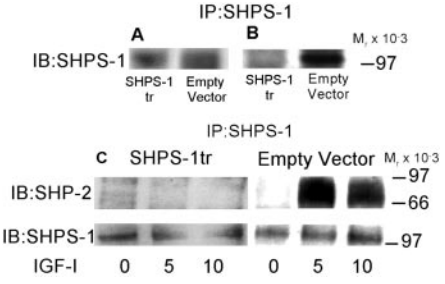

FIG. 5. **SHP-2 recruitment to SHPS-1 in response to IGF-I stimulation in cells expressing SHPS-1tr.** *A* and *B*, expression of SHPS-1 protein in cells transfected with either SHPS-1tr or the empty vector alone. Cells were grown to 90% confluence and then incubated overnight in SFM. After lysis and immunoprecipitation (*IB*) with the anti-SHPS-1fl antibody the level of SHPS-1 protein was visualized by Western immunoblotting (*IB*) with either the anti-SHPS-1fl antibody (*A*) or with the anti SHPS-1tr antibody (*B*). *C*, cells transfected with either SHPS-1tr or the empty vector alone were grown to 90% confluence and then incubated overnight in SFM. The cells were then exposed to 100 ng/ml IGF-I for various lengths of time as indicated. After cell lysis and immunoprecipitation with an anti-SHPS-1 antibody the association of SHP-2 was visualized by Western immunoblotting with an anti-SHP-2 antibody. To control for loading inequities the amount of SHPS-1 protein in each lane is shown in the *lower panels*.

IGF-IR is decreasing and the time at which the SHP-2 tyrosine phosphatase association increases.

SHPS-1 Phosphorylation after IGF-I Stimulation—Because SHP-2 is predominantly a cytosolic protein, it must be recruited to the plasma membrane before it can bind to the IGF-IR. The phosphorylation of SHPS-1 and its subsequent recruitment of SHP-2 from the cytosol have been shown to be stimulated by various growth factors. Fig. 3 shows that as with other growth factors IGF-I stimulated a rapid increase in SHPS-1 phosphorylation. Maximum IGF-I stimulated phosphorylation of SHPS-1 occurred after a 5-min incubation (6.6 ± 0.7-fold; $n = 3$), and this was followed by a 2.6 ± 1.1-fold ($n = 3$) decrease in SHPS-1 phosphorylation by 10 min.

Recruitment of SHP-2 to SHPS-1 after IGF-I Stimulation—We next examined whether SHP-2 was recruited to SHPS-1 after IGF-I-stimulated SHPS-1 phosphorylation. Fig. 4 shows that there was no detectable SHP-2 association with SHPS-1 in the basal state, but SHP-2 was recruited to SHPS-1 after a 5-min exposure to IGF-I. The association of SHP-2 with SHPS-1 decreases rapidly, and association is barely detectable at 10 min.

Loss of SHP-2 Recruitment to SHPS-1 in Cells Expressing a Truncated Form of SHPS-1—The recruitment of SHP-2 to SHPS-1 and then its subsequent rapid disassociation suggested that this was a potential mechanism by which SHP-2 could be recruited to the plasma membrane and then become available to bind to the IGF-IR. The SHP-2 binding site on SHPS-1 has been identified as the two C-terminal tyrosine residues (23). To examine whether SHP-2 recruitment to IGF-IR required that SHP-2 first be recruited from the cytosol to SHPS-1 we expressed a truncated form of SHPS-1 which lacked the two C-terminal tyrosine residues (SHPS-1tr) and examined SHP-2 recruitment to both SHPS-1 and the IGF-IR after IGF-I stimulation. Fig. 5*A* shows that after immunoprecipitation and immunoblotting with the SHPS-1 antibody raised to the entire cytoplasmic tail, comparable levels of SHPS-1 protein were detected in SHPS-1tr cells and in cells transfected with the empty vector alone. However, Fig. 5*B* shows that no SHPS-1 protein can be detected in cells expressing SHPS-1tr when the antibody raised to the C-terminal region of SHPS-1 was used to immunoblot, whereas abundant SHPS-1 protein can be detected in cells transfected with the empty vector alone. This suggests that the truncated form of SHPS-1 is acting in a dominant negative manner.

In Fig. 5*C* it can be seen that despite levels of SHPS-1 protein in the cells expressing SHPS-1tr comparable to levels in cells expressing the empty vector, there was no recruitment of SHP-2 to SHPS-1 in cells expressing the truncated form of the protein, whereas cells transfected with the empty vector showed abundant SHP-2 bound to SHPS-1 after 5 min of IGF-I stimulation. This blot was exposed to film for 24 h to show that there was no SHP-2 association in the cells expressing truncated SHPS-1.

Loss of SHP-2 Recruitment to the IGF-IR in Cells Expressing

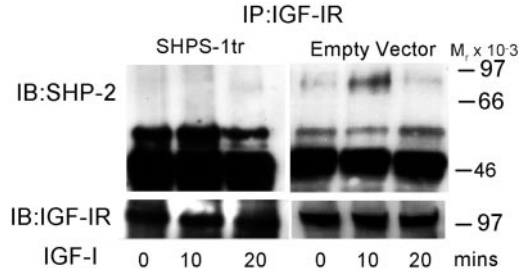

FIG. 6. **SHP-2 association with the IGF-IR in cells expressing SHPS-1tr.** Cells were grown to 90% confluence and then incubated overnight in SFM. The cells were then exposed to 100 ng/ml IGF-I for various lengths of time as indicated. After cell lysis and immunoprecipitation (*IP*) with an anti-IGF-IR antibody the association of SHP-2 with the receptor was visualized by Western immunoblotting (*IB*) with an anti-SHP-2 antibody. To control for loading inequities the amount of IGF-IR protein in each lane is shown in the *lower panels*.

a Truncated Form of SHPS-1—To confirm that loss of SHPS-1 recruitment of SHP-2 would alter SHP-2 association with IGF-IR we utilized the cells expressing the truncated form of SHPS-1. In Fig. 6 it can be seen that after IGF-I stimulation there was no recruitment of SHP-2 to the IGF-IR in cells expressing the truncated form of SHPS-1. This blot was exposed to film for 24 h to confirm that there was no SHP-2 association. Recruitment of SHP-2 to the IGF-IR in cells expressing the empty vector alone is also shown.

Altered IGF-IR Phosphorylation Time Course in Cells Expressing an Inactive Form of SHP-2—The rapid decrease in SHPS-1 phosphorylation and loss of SHP-2 association with SHPS-1 are consistent with phosphorylated SHPS-1 being a substrate for SHP-2 phosphatase (18, 20). To prove that the recruitment of SHP-2 to SHPS-1 and its subsequent release were required for SHP-2 association with and dephosphorylation of the IGF-IR we expressed a catalytically inactive form of SHP-2 (SHP-2 C-S), which cannot dephosphorylate SHPS-1. We then examined the time course of IGF-IR dephosphorylation in these cells.

Fig. 7*A* shows that the transfected cells express FLAG-tagged SHP-2 C-S and that the cells transfected with the empty vector alone do not. In Fig. 7*B*, *top panel*, it can be seen that in cells expressing the catalytically inactive form of SHP-2, SHPS-1 was phosphorylated after 5 min of stimulation with IGF-I; however, unlike cells that contain wild type SHP-2, cells expressing the SHP-2 C-S mutant showed no decrease in SHPS-1 phosphorylation at 10 min. Fig. 7*B*, *middle panel*, demonstrates that in contrast to the cells transfected with the empty vector alone, the cells expressing SHP-2 C-S showed no dissociation of SHP-2 from SHPS-1 over the time course that was examined. This was confirmed further by showing that when IGF-I-R was immunoprecipitated followed by immunoblotting for SHP-2, no SHP-2 could be detected (data not shown). This shows that retention of SHP-2 binding to SHPS-1 was associated with a loss of binding to the IGF-IR.

Fig. 7*C* shows that after a 5-min exposure to IGF-I, cells expressing the C-S mutant or cells transfected with an empty vector showed a marked increase in IGF-IR phosphorylation. After 10 min there is a significant reduction in IGF-IR phosphorylation in the empty vector cells but no change in cells expressing the C-S mutant, and these cells show a further increase in receptor phosphorylation at the 20-min time point. These data suggest that in the absence of SHP-2 release from SHPS-1 there is no recruitment of SHP-2 to the IGF-IR and no receptor dephosphorylation.

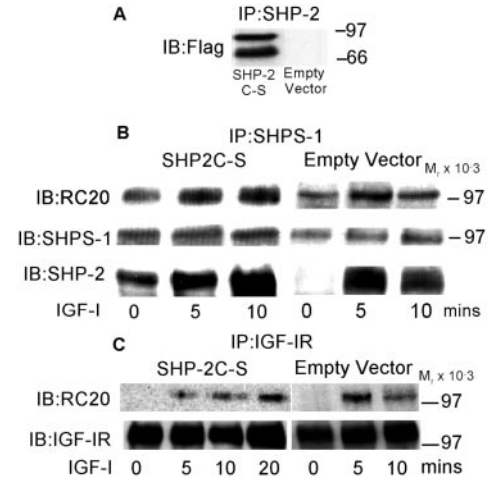

FIG. 7. **IGF-IR phosphorylation time course and SHP-2 recruitment in cells expressing catalytically inactive SHP-2 (SHP-2 C-S).** *A*, cells expressing SHP-2 C-S and cells transfected with the empty vector alone were grown to 90% confluence and then incubated in SFM overnight. After lysis and immunoprecipitation (*IP*) with an anti-SHP-2 antibody the expression of FLAG-tagged protein was visualized by Western immunoblotting (*IB*) with an anti-FLAG antibody. *B*, *top panel*, cells transfected with either SHP2 C-S or the empty vector alone were grown to 90% confluence and then incubated overnight in SFM. Cells were then exposed to 100 ng/ml IGF-I for various lengths of time as indicated. After cell lysis and immunoprecipitation with an anti-SHPS-1 antibody, tyrosine phosphorylation was visualized by Western immunoblotting with an anti-phosphotyrosine antibody. The results expressed as arbitrary scanning units are: 11,378 (*far left lane*), 21,519 (*second lane*), 23,415 (*third lane*), 10,171 (*fourth lane*), 25,709 (*fifth lane*), and 12,890 (*far right lane*). *Middle panel*, to control for loading inequities the amount of SHPS-1 protein in each lane is shown. *Bottom panel*, after immunoprecipitation with an anti-SHPS-1 antibody the association of SHP-2 was visualized by Western immunoblotting with an anti-SHP-2 antibody. The results expressed as arbitrary scanning units are 14,337 (*far left lane*), 22,418 (*second lane*), 28,541 (*third lane*), 1,007 (*fourth lane*), 26,090 (*fifth lane*), 14,944 (*far right lane*). *C*, cells transfected with either SHP2 C-S or the empty vector alone were grown to 90% confluence then incubated overnight in SFM. Cells were then exposed to 100 ng/ml IGF-I for various lengths of time as indicated. After cell lysis and immunoprecipitation with an anti-IGF-IR antibody, tyrosine phosphorylation of the IGF-IR was visualized by Western immunoblotting with an antiphosphotyrosine antibody (RC20). The results expressed as arbitrary scanning units are: 1,547 (*far left lane*), 10,519 (*second lane*), 12,177 (*third lane*), 19,030 (*fourth lane*), 2,999 (*fifth lane*), 26,514 (*sixth lane*), and 8,445 (*far right lane*). To control for loading inequities the amount of IGF-IR protein in each lane is shown in the *lower panels*.

DISCUSSION

The increase in IGF-IR phosphorylation which occurs after ligand binding is transient and is followed by a rapid decline in the level of IGF-IR phosphorylation. Because there are no significant changes in IGF-IR protein levels over this time this suggests that there are activation and recruitment of a tyrosine phosphatase to the receptor. *In vitro* studies have shown that a catalytically inactive form of SHP-2 can bind directly to tyrosine-phosphorylated forms of both the insulin and IGF-IR (10). Those studies suggested indirectly that SHP-2 could dephosphorylate both receptors after it bound because only a phosphatase-inactive form of SHP-2 formed a stable complex and did not dephosphorylate either receptor (10). From the studies presented here we propose that the recruitment of SHP-2 to the IGF-IR via SHPS-1 is responsible for the decrease in IGF-IR phosphorylation after IGF-I-induced receptor activation and is therefore an important regulator of IGF-I signaling. This was first suggested by our data demonstrating an association between the time at which the IGF-IR phosphorylation was decreasing and the time at which the SHP-2 phosphatase associated with the receptor. This conclusion is supported further by our data demonstrating that in cells expressing a truncated form of SHPS-1, SHP-2 is not recruited to the IGF-IR and by the detection of sustained IGF-IR phosphorylation in cells

expressing the catalytically inactive form of SHP-2 which is not released from SHPS-1.

SHPS-1 has recently been identified as a transmembrane glycoprotein with three immunoglobulin-like domains, four potential tyrosine phosphorylation sites, and SH-2 binding motifs in its cytoplasmic domain (17–20). It has been shown to be phosphorylated on tyrosines in response to ligand occupancy of several growth factor receptors and the insulin receptor generating two high affinity binding sites for SHP-2 (17–20). Binding of the two SH-2 domains within SHP-2 to these high affinity binding sites on SHPS-1 increases SHP-2 association and activates the phosphatase by releasing the N-terminal SH-2 domain from constraining the phosphatase active site (25–27). SHPS-1 phosphorylation not only provides a binding site for SHP-2, but it is also a substrate for the phosphatase, resulting in dephosphorylation of SHPS-1 and therefore loss of the SHP-2 binding sites (18, 20). It has been proposed that once SHP-2 has been recruited to SHPS-1, after its activation and the subsequent dephosphorylation of SHPS-1, it is available to associate with other plasma membrane-associated proteins (21, 22). As has been shown for the insulin, platelet-derived growth factor, and the epidermal growth factor receptors (16), we show here that IGF-I can stimulate an increase in SHPS-1 phosphorylation, and this results in an increase in the association of SHP-2. The time course of maximal SHPS-1 phosphorylation and SHP-2 recruitment after IGF-I stimulation precedes maximal IGF-IR phosphorylation and SHP-2 recruitment by the receptor. This further supports the conclusion that recruitment of SHP-2 to the IGF-IR via SHPS-1 is the mechanism by which SHP-2 is recruited to the receptor. This conclusion is strongly supported by our data showing that in cells expressing a truncated form of SHPS-1, in which the two tyrosines (Tyr^{449} and Tyr^{475}) that are known to be the binding site for SHP-2 have been deleted (21), SHP-2 is not recruited to either SHPS-1 or to the IGF-IR. The importance of the release of SHP-2 from SHPS-1 is substantiated further by the data demonstrating a lack of SHP-2 recruitment to the IGF-IR in cells expressing the catalytically inactive form of SHP-2. In these cells SHPS-1 is not dephosphorylated, SHP-2 is not released, and IGF-IR phosphorylation is prolonged. Thus stimulation of SHPS-1 phosphorylation after ligand occupancy of IGF-IR and the subsequent dephosphorylation of SHPS-1 by SHP-2 are critical steps that are required for the IGF-IR dephosphorylation response to be initiated.

SHP-2 has been shown to bind various signaling molecules and depending upon the molecule and the experimental approach taken, it has been shown to have both positive and negative effects on signaling by intracellular mediators that have been activated by tyrosine kinase receptors. Expression of a mutated form of IRS-1 which does not bind SHP-2 results in enhanced insulin-stimulated tyrosine phosphorylation of IRS-1, which in turn increased the association of PI-3 kinase with IRS-1 and enhanced insulin-stimulated protein synthesis (28). Similarly, mutation of the SHP-2 binding site in the GH receptor prolongs GH-stimulated tyrosine phosphorylation of the GH receptor and the signaling proteins JAK2 and STAT 5B (29). Overexpression of SHPS-1 has been shown to result in a decrease in insulin-stimulated DNA synthesis, and this result was attributed to its phosphorylation and recruitment of SHP-2 (19). However, other studies using dominant negative mutants and anti-SHP-2 antibodies have suggested that SHP-2 may play an important positive role in IGF-I and other growth factor-stimulated cell proliferation through activation of mitogen-activated protein kinase, although the signaling components that are dephosphorylated by SHP-2 which mediate these effects have not been identified (20, 21, 29–33). This appears to occur upstream of RAS activation and at least for some growth factors involves SHP-2 recruitment to GAB-1-like tyrosine-phosphorylated docking proteins (24) and/or its binding to Grb2 (34, 35, 36). It would seem therefore that SHP-2 can play a dual role in growth factor signaling, and its activity would appear to be dependent upon its subcellular localization and binding partners. However, as demonstrated in our studies the IGF-IR and a previously published study for the GH receptor (22), SHP-2 recruitment to SHPS-1 clearly contributes to the ability of SHP-2 to effect receptor dephosphorylation.

Because it has been shown previously that integrin ligand occupancy can regulate SHP-2 localization and activation (17, 18, 37, 38), our findings suggest that its association with the IGF-IR may also be regulated by integrin occupancy. The regulation of SHP-2 association with the IGF-IR and hence IGF-IR phosphorylation levels by $\alpha_V\beta_3$ ligand occupancy is an attractive hypothesis to explain our observation that blocking $\alpha_V\beta_3$ ligand occupancy reduces IGF-IR phosphorylation (39). Identification of factors other than SHPS-1 which regulate SHP-2 subcellular localization and therefore its binding partners and activation action will be important for understanding the role of other variables such as $\alpha_V\beta_3$ integrin ligand occupancy in regulating IGF-I signaling.

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