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

Laura A. Maile and David R. Clemmons‡

From the Division of Endocrinology, University of North Carolina, Chapel Hill, North Carolina 27599

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-I-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.
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**

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, streptomycin, and 10% fetal bovine serum in 10-cm tissue culture were included 2-base substitutions (TGC-TCA) to encode the Cys 459/H11032 -end to generate an -primer sequence at the Cys . The additional 3 bases are underlined, and the site of the incorporation is marked *.

**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 primer sequence was 5′-ATG GAC TAA GAC GAC GAC AAA ACA TCG CGG AGA AGA TTT CAC CCA AA-3’. The bases encoding the FLAG peptide are underlined. The 3′-primer sequence was complementary to nucleotides 1888–1911 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 CGG CCC ATG GAG CCC-3′), and the 3′-primer sequence was complementary to nucleotides 1502–1524 (5′-CAG ACC AGC 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 Cys459 by Ser in the catalytic site of SHP-2 by using the primer sequence was cloned into the pcDNA 3.1 V5 His vector.

**IGF-I Receptor Dephosphorylation**—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-1 stimulation. However, after a 10-min stimulation 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 IGF-I is determined.
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 has 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 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-I 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-I receptor after IGF-I stimulation. Fig. 5A 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. 5B 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. 5C 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. SHPS-1tr 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 SHPS-1tr**
ligand binding is transient and is followed by a rapid decline in receptor dephosphorylation. In Fig. 7, tagged SHP-2 C-S and that the cells transfected with the empty vector alone do not. 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. 7A 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. 7B, 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. 7B, 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. 7C 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.

**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.

SHP-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, in which the two tyrosine-phosphorylated docking proteins (24) and/or its 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 SHPS-1 which regulate SHP-2 subcellular localization and therefore its binding partners and activation action is important for understanding the role of different splice variants such as α5β3 integrin ligand occupancy in regulating IGF-I signaling.

Acknowledgment—We thank Laura Lindsey for help in preparing the manuscript.

REFERENCES
1. Kato, H., Faria, T. N., Stannard, B., Roberts, C. T., Jr., and LeRoith, D. (1993) J. Biol. Chem. 268, 2655–2661
2. Kato, H., Faria, T. N., Stannard, B., Roberts, C. T., Jr., and LeRoith, D. (1994) Mol. Endocrinol. 8, 40–50
3. Gronberg, M., Wulf, B. B., Rasmussen, J. S., Kjeldsen, T., and Gammeltoft, S. (1993) J. Biol. Chem. 268, 23435–23440
4. Czarno, A., O’Neill, T. J., and Gustafsson, T. A. (1995) J. Biol. Chem. 270, 15630–15635
5. Dey, B. R., Frick, K., Lopacynski, W., Nisley, S. P., and Furlanetto, R. W. (1996) Mol. Endocrinol. 16, 631–641
6. Baserga, R. (1995) Cancer Res. 55, 249–252
7. Blakesley, V. A., Sermingear, A., Episostio, D., and LeRoith, D. (1996) Cytokine Growth Factor Rev. 7, 153–159
8. Freeman, R. M., Jr., Pittzky, J., and Neel, B. G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11239–11243
9. Ahmad, S., Banville, D., Zhao, Z., Fischer, E. H., and Shen, S. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2181–2187
10. Enchi, S., Tartti-Dekker, S., Sawka-Verhelte, D., Gamba, A., and Van Obbergen, E. (1996) Endocrinology 137, 4494–4495
11. Seely, B. L., Reichard, D. R., Staubs, P. A., Hsu, D., Maegawa, H., Milarski, K. L., Saltiel, A. R., and Olefsky, J. M. (1995) J. Biol. Chem. 270, 19151–19157
12. Sun, X. J., Crimmins, D. L., Myers, M. G., Jr., Miralpeix, M., and White, M. F. (1993) Mol. Cell. Biol. 13, 7415–7428
13. Kuhne, M. R., Pawson, T., Lienhard, G. E., and Feng, G. S. (1993) J. Biol. Chem. 268, 11479–11481
14. Stofega, M. R., Wang, H., Ulrich, A., and Carter-Su, C. (1998) J. Biol. Chem. 273, 7112–7117
15. Kim, S. O., Jiang, J., Yu, W., Feng, G. S., and Frank, S. J. (1998) J. Biol. Chem. 273, 2344–2354
16. Stofega, M. R., Herrington, J., Billestrup, N., and Carter-Su, C. (2000) Mol. Endocrinol. 14, 1338–1350
17. Tsuda, M., Matozaki, T., Fukunaga, K., Fujisaki, Y., Imamoto, A., Noguchi, T., Takada, T., Yamato, T., Takeda, H., Ochi, F., Yamamoto, T., and Kasuga, M. (1997) J. Biol. Chem. 272, 13223–13229
18. Fujisaki, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamato, T., Nobuki, T., and Kasuga, M. (1996) Mol. Cell. Biol. 16, 6887–6899
19. Kharitonov, A., Chen, Z., Sures, I., Wang, H., Schilling, J., and Ulrich, A. (1997) Nature 386, 181–186
20. Noguchi, T., Matozaki, T., Fujisaki, Y., Yamato, T., Tsuda, M., Takeda, T., and Kasuga, M. (1996) J. Biol. Chem. 271, 27652–27658
21. Takada, T., Matozaki, T., Takeda, H., Fukunaga, K., Noguchi, T., Fujisaki, Y., Okazaki, I., Tsuda, M., Yamato, T., Ochi, F., and Kasuga, M. (1998) J. Biol. Chem. 273, 9224–9242
22. Stofega, M. R., Argetsinger, L. S., Wang, H., Ulrich, A., and Carter-Su, C. (2000) J. Biol. Chem. 275, 28222–28229
23. Gockerman, A., Jones, J. J., Prevete, and Clemmons, D. R. (1995) Endocrinology 136, 4168–4173
24. Imai, Y., Bubny, W. H., Clarke, J. B., Horwitz, G., Rees, C., and Clemmons, D. R. (1997) J. Clin. Invest. 100, 2596–2605
25. Plaskey, S., Wandle, T. J., Walsh, C. T., and Shoelson, S. E. (1995) J. Biol. Chem. 270, 2897–2900
26. Eck, M. J., Plaskey, S., Trub, T., Harrison, S. C., and Shoelson, S. E. (1996) Nature 379, 277–280
IGF-I Receptor Dephosphorylation

27. Hof, P., Pluskey, S., Dhe-Paganon, S., Eck, M. J., and Shoelson, S. E. (1998) *Cell* **92**, 441–450
28. Myers, M. G., Jr., Mender, R., Shi, P., Pierce, J. H., Rhoads, R., and White, M. F. (1998) *J. Biol. Chem.* **273**, 26968–26974
29. Milarski, K. L., and Saltiel, A. R. (1994) *J. Biol. Chem.* **269**, 21239–21243
30. Xiao, S., Rose, D. W., Sasaoka, T., Maegawa, H., Burke, T. R., Jr., Roller, P. P., Shoelson, S. E., and Olefsky, J. M. (1994) *J. Biol. Chem.* **269**, 21244–21248
31. Feng, G.-S., Hui, C. C., and Pawson, T. (1993) *Science* **259**, 1607–1611
32. Vogel, W., Lammers, R., Huang, J., and Ulrich, A. (1993) *Science* **259**, 1611–1614
33. Bennet, A. M., Tang, T. L., Sugimoto, S., Walsh, C. T., and Neel, B. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7335–7339
34. Schaeper, U., Gehring, N. H., Fuchs, K. P., Sachs, M., Kempkes, B., and Birchmeier, W. (2000) *J. Cell Biol.* **149**, 1419–1432
35. Quing, Z.-Q., Yu, D.-H., Park, M., Marshall, M., and Feng, G.-S. (2000) *Cell. Biol. Biol.* **20**, 1526–1536
36. Tauchi, T., Feng, G.-S., Marshall, M. S., Shen, R., Mantel, C., Pawson, T., and Broxmeyer, H. E. (1994) *J. Biol. Chem.* **269**, 25206–25211
37. Miyamoto, S., Toramoto, H., Gutkind, J. R., Burbelo, R. D., Akiyama, S. K., and Yamada, K. M. (1995) *J. Cell Biol.* **131**, 791–805
38. DeMali, K. A., Balciunute, E., and Kazlauskas, A. (1999) *J. Biol. Chem.* **274**, 19551–19559
39. Zheng, B., and Clemmons, D. R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11217–11222