SHP-1 and SHP-2 are two Src homology 2 domain-containing tyrosine phosphatases with major pathological implications in cell growth regulating signaling. They share significant overall sequence identity, but their biological functions are often opposite. SHP-1 is generally considered as a negative signal transducer and SHP-2 as a positive one. However, the precise role of each enzyme in shared signaling pathways is not well defined. In this study, we investigated the interaction of these two enzymes in a single cell system by knocking down their expressions with small interfering RNAs and analyzing the effects on epidermal growth factor signaling. Interestingly, knockdown of either SHP-1 or SHP-2 caused significant reduction in the activation of ERK1/2 but not Akt. Furthermore, SHP-1, SHP-2, and Gab1 formed a signaling complex, and SHP-1 and SHP-2 interact with each other. The interaction of SHP-1 with Gab1 is mediated by SHP-2 because it was abrogated by knockdown of SHP-2, and SHP-2, but not SHP-1, binds directly to tyrosine-phosphorylated Gab1. Together, the data revealed that both SHP-1 and SHP-2 have a positive role in epidermal growth factor-induced ERK1/2 activation and that they act cooperatively rather than antagonistically. The interaction of SHP-1 and SHP-2 may be responsible for previously unexpected novel regulatory mechanism of cell signaling by tyrosine phosphatases.
EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal antibodies against SHP-1, SHP-2, GAPDH, and ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Gab1 and anti-phospho-ERK1/2 were from Upstate Biotechnology, Inc. (Lake Placid, NY) and Cell Signaling, Inc. (Beverly, MA), respectively. Recombinant SHP-1, SHP-2, maltose-binding protein (MBP) fusion protein of SHP-1, and glutathione S-transferase (GST) fusion protein of SHP-2 were expressed and purified as described previously (19–22). Pre-designed SHP-1 and SHP-2 siRNA oligonucleotides were purchased from Ambion Inc. (Austin, TX). Vector-based siRNAs targeted at TGCGGGCT-GACATTGAGAAC of SHP-1 (23) and CAGGAAGCTGAAAT-ACGACG of SHP-2 were made by using the pSUPER-neo vector (OligoEngine, Seattle, WA) according to the manufacturer’s protocol.

Cell Culture, Transient Transfection, Stimulation, and Lysis—We used colorectal adenocarcinoma Caco-2 cells to study the role of SHP-1 and SHP-2 in growth factor signaling. These cells express both of the phosphatases and respond well to EGF stimulation. The cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. The cells were transiently transfected with SHP-1 and SHP-2 siRNAs (Ambion) or pSUPER RNA interference constructs in the presence of Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. After transfection, the cells were cultured for 48 h in a complete medium and then in a serum-free medium for 16 h before stimulation with 50 ng/ml EGF for various periods of time. After washing with cold phosphate-buffered saline three times, the cells were lysed in a cell lysis buffer containing 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, and 100 mM NaCl. To and MBP-SHP-1 at 4 °C for 1 h in a binding buffer containing 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, and 100 mM NaCl. To investigate the interaction of Gab1 with SHP-1 or SHP-2, GST-Gab1CT and GST-pGab1CT were immobilized on glutathione-Sepharose and incubated with purified SHP-1 or SHP-2 in the above buffer system. After extensive washing with the binding buffer, bound and unbound materials were separated on SDS gels and visualized by Coomassie Blue staining or Western blotting analyses.

RESULTS

Suppression of SHP-1 and SHP-2 Expressions by siRNAs—Many previous studies on the role of SHP-1 and SHP-2 in cell signaling have employed overexpressing dominant negative mutants and fibroblast cells derived from knock-out mice. Although overexpression of dominant negative mutants may have nonspecific effects, the knock-out cells express truncated forms of the enzymes (1). The RNA interference technique has been widely used to study the loss-of-function phenotype of genes. In this study, we first employed commercially available double strand RNAs to knock down the expression of SHP-1 and SHP-2 in Caco2 cells. Results are shown in Fig. 1. SHP-1 siRNA specifically suppressed the expression of SHP-1 by 80% but had no effect on that of SHP-2. Conversely, the siRNA of SHP-2 specifically knocked down the expression of SHP-2 by 70% but left SHP-1 intact. We also examined the protein levels

FIGURE 1. Knockdown of SHP-1 and SHP-2 expressions in Caco2 cells by siRNA. Caco2 cells (30–40% confluence) were transfected with the indicated siRNAs in the presence of Lipofectamine 2000 as described under “Experimental Procedures.” Cell extracts containing equal amounts of total proteins were separated on 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and subjected to Western blot analysis with antibodies against SHP-1, SHP-2, ERK1/2, and GAPDH as indicated.
of GAPDH and ERK1/2 to confirm that they were not affected by the siRNAs. The data suggest that these siRNAs worked very efficiently to suppress the expression of SHP-1 and SHP-2. This provides an excellent system to study the function of these enzymes in parallel.

Knockdown of SHP-1 or SHP-2 Reduces EGF-induced Activation of ERK1/2 but Not of Akt—We treated the cells with EGF and analyzed the activation of downstream signaling pathways. We employed phospho-specific antibodies to determine the activation of ERK1/2 and Akt. As shown in Fig. 2, knockdown of either SHP-1 or SHP-2 significantly reduced the activation of ERK1/2. The magnitude of ERK1/2 activation was reduced by about 60%. These data are very similar to those observed with cells overexpressing dominant negative mutants of SHP-1 or SHP-2 (16, 26). Thus, it furthers the positive role of both enzymes in EGF-induced ERK activation. We also analyzed the activation of Akt by using phospho-specific Akt antibodies. Interestingly, knockdown of either enzyme showed no significant effects on Akt. This suggests that SHP-1 and SHP-2 specifically regulate EGF activation of the ERK1/2 pathway but not that of Akt.

To rule out possible nonspecific effects of the siRNA oligonucleotides, we also employed vector-based siRNAs to knock down the expression of SHP-1 and SHP-2. The data shown in Fig. 3 demonstrated that the siRNA constructs also caused specific suppression of SHP-1 or SHP-2 expression and decreases in the EGF-induced activation of ERK1/2.

SHP-1 and SHP-2 Form a Signaling Complex with Gab1—It has been well accepted that signal transduction is carried...
through formation of multiple signaling complexes. This is particularly true for SHP-1 and SHP-2 because both enzymes stay largely inactive in cytosol because of their internal suppressed structures (27, 28). It is believed that they are activated by binding to tyrosine-phosphorylated proteins (1). A major binding protein of SHP-2 involved in growth signaling is Gab1, an adapter protein that undergoes tyrosine phosphorylation upon EGF stimulation (29). Binding of SHP-2 to Gab1 has been well documented. However, whether SHP-1 has a similar property is not clear. We thus performed immunoprecipitation to examine the interaction of SHP-1 and SHP-2 with Gab1. As shown in Fig. 4, SHP-2 was indeed co-immunoprecipitated with Gab1. Interestingly, SHP-1 was also found in the immunocomplex. The association of both SHP-1 and SHP-2 with Gab1 was markedly enhanced upon EGF stimulation. As with the activation of other signaling events, this association is transient. It peaked at 3 min and declined thereafter. Taken together, these biochemical analyses suggest that both SHP-1 and SHP-2 are linked to the EGF signaling pathway through Gab1.

**SHP-2 Directly Interacts with SHP-1 and Mediates the Interaction of SHP-1 with Gab1**—As an SH2 domain-containing protein, it is not surprising that SHP-1 also binds Gab1. The question is whether SHP-1 and SHP-2 affect each other in binding to Gab1. To address this issue, we repeated the above immunoprecipitation experiments with cells that had either SHP-1 or SHP-2 knocked down. Intriguing results emerged that showed distinct functions of SHP-1 and SHP-2. Although knockdown of SHP-1 had no effect on the association of SHP-2 with Gab1, knockdown of SHP-2 totally blocked the interaction of SHP-1 with Gab1 (Fig. 5). This suggests that SHP-2 likely mediates the interaction of SHP-1 with Gab1. The C-terminal portion of Gab1 contains two SHP-2-binding sites that resemble the so-called immunoreceptor tyrosine-based inhibition motifs found in many inhibitory receptors (29). It has been known that both SHP-1 and SHP-2 can bind to these motifs through their SH2 domains (1). To verify that the association of SHP-1 with Gab1 is not through their direct interaction, we performed binding assays with purified proteins. For this purpose, a C-terminal fragment of Gab1 (amino acids 635–724) that carries the two SHP-2-binding sites was expressed in E. coli cells as a GST fusion protein. When co-expressed in E. coli cells with c-Src as described for PZR in our earlier studies (25), the fusion protein was heavily phosphorylated on tyrosine. The phosphorylation presumably occurs on the SHP-2-binding sites because the Gab1 part of the fusion protein contains only two tyrosyl residues, and GST per se could not be phosphorylated (25). After immobilization on glutathione-Sepharose beads, the phosphorylated fusion protein GST-pGab1CT, but not the nonphosphorylated GST-pGab1CT, readily pulled down purified SHP-2. In contrast, neither could pull down SHP-1 (Fig. 6). These data indicate that SHP-1 does not directly bind to Gab1 through the mechanism used by SHP-2. One natural question then is whether SHP-2 carries SHP-1 to Gab1. We thus analyzed the co-immunoprecipitation of SHP-1 and SHP-2. Indeed, SHP-1 and SHP-2 formed a constitutive complex that was not affected by EGF stimulation (Fig. 7A). To prove a direct interaction between these two enzymes, we also performed *in vitro* pulldown assays with purified enzymes. As shown in Fig. 7B, a GST fusion protein of SHP-2 specifically
SHP-1 and SHP-2 in Cell Signaling

**A.**

![Graph showing EGF and SHP-2 interactions](image1)

**B.**

![Graph showing GST-SHP-2 and MBP-SHP-1 interactions](image2)

**FIGURE 7. Direct interaction of SHP-1 with SHP-2.** A, subconfluent Caco2 cells were serum-starved for 16 h and then left untreated or treated with 50 ng/ml EGF for 3 min. Cell extracts were immunoprecipitated (IP) with anti-SHP-1 or anti-SHP-2 antibodies, and the immunoprecipitates were subjected to Western blotting (IB) analyses with antibodies against SHP-1 and SHP-2 as indicated. An irrelevant rabbit IgG was used as controls. B, GST and GST-SHP-2 immobilized onto glutathione-Sepharose beads were incubated with purified MBP or MBP-SHP-1. Proteins bound to the beads and remained in supernatants were analyzed by Western blotting with an anti-SHP-1 antibody.

SHP-1 and SHP-2 are tyrosine phosphatases that are involved in regulating cell signaling. However, there is a misconception that SHP-1 is a negative signal transducer simply because it causes dephosphorylation of growth factor and cytokine receptors and associated tyrosine kinases. This misconception may stem from the transient nature of the physiological substrates of SHP-1 and SHP-2, which have not been clearly defined. This may be due to the transient nature of phosphorylation of these substrates that makes their detection and quantification difficult.

**DISCUSSION**

SHP-1 has been generally considered as a negative signal transducer, essentially as an antagonist of SHP-2. This view is based on the relative levels of SHP-1 and SHP-2, but it is not proportional to the level of signal transduction activation. The intensity of signal transduction may not be proportional to the level of tyrosine phosphorylation of upstream receptors at a steady state but to the dynamics of the phosphorylation. Of course, the site of phosphorylation also matters. Therefore, SHP-1 should not be viewed as a negative signal transducer simply because it dephosphorylates growth factor receptors. Our present study suggests SHP-1 and SHP-2 have a similar function in EGF signaling and that their roles are not redundant. They rely on each other to regulate cell signaling. In this regard, the present results may also have therapeutic implications because diseases associated with loss-of-function of one enzyme can be treated by inhibiting the other enzyme. By knocking down the expression of SHP-1 and SHP-2, our study also suggests that SHP-1 and SHP-2 specifically regulate the ERK1/2 pathway but not the Akt pathway. The detailed mechanism by which SHP-1 and SHP-2 regulate ERK activation remains unknown. It may not be a simple dephosphorylation of phosphorylated EGF receptor by SHP-1 or SHP-2. The ERK activation signaling pathway consists of upstream components Grb2, SOS, Ras, Raf, and MEK. Grb2 functions by directly binding to growth factor receptors or other associated adaptor proteins (e.g. Gab1 and SHC) thereby recruiting SOS. In turn, SOS activates Ras, which leads to activation of the downstream cascade Raf/MEK/ERK1/2. SHP-2 is known to be required for maintaining normal levels of Ras activation. Ras is activated by guanine nucleotide exchange factor SOS but inactivated by GTPase-activating protein GAP. One scenario that SHP-2 promotes the ERK activation pathway is to prevent activation of GAP by dephosphorylating the phosphorylation site responsible for recruitment and activation of GAP. As a phosphatase, SHP-1 may play a similar role. In all, SHP-1 and SHP-2 presumably function by dephosphorylating some key components of the signaling pathways thereby facilitating signal transduction. However, their physiological substrates have not been clearly defined. This may be due to the transient nature of phosphorylation of these substrates that makes its detection and quantification difficult.
Signal transduction is carried through formation of multiple signaling complexes involving adaptors and scaffold proteins. The Gab family adaptor proteins are scaffolding adaptor molecules that display sequence similarity with *Drosophila* DOS (daughter of sevenless), which is initially identified as a substrate for Corkscrew, the SHP-2 homolog in *Drosophila*, and they contain a pleckstrin homology domain and binding sites for SH2 and SH3 domains, thereby recruiting various signaling proteins (29). Studies in multiple systems have implicated that Gab1 has a critical role in signaling via many growth factors and cytokine receptors. Gab1-deficient mouse embryos die *in utero* with multiple defects in placental, heart, skin, and muscle development similar to phenotypes observed in mice lacking signals of EGF, platelet-derived growth factor, and hepatocyte growth factor pathways (34). Binding of SHP-2 with Gab1 has been well defined and is believed to be critical for Gab1-mediated signal transduction, and Gab1 mutants lacking SHP-2-binding sites are unable to activate ERK1/2 and downstream transcription factors (24, 29). In fact, a major function of SHP-2 is to dephosphorylate the GAP-binding sites on Gab1 thereby disengaging GAP and sustaining Ras activation (35). Our study suggests that SHP-1 also binds to Gab1 but through SHP-2. Participation of both SHP-1 and SHP-2 in the Gab1 signaling complex presumably provides more versatility and flexibility for cells to control signal transduction. First, interactions of SHP-1 and SHP-2 may affect each other’s catalytic activity. Second, SHP-1 and SHP-2 may have different substrate specificity and thus preferentially act on different proteins. Even with shared substrates, they may have different catalytic efficiencies (19, 20). Taken together, a fine-tuning of cell signaling can be achieved by increasing or decreasing the activity of either SHP-1 or SHP-2.

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