The protein-tyrosine phosphatase SHP-1 binds to and dephosphorylates the epidermal growth factor receptor (EGFR), and both SH2 domains of SHP-1 are important for this interaction (Tenev, T., Keilhack, H., Tomic, S., Stoyanov, B., Stein-Gerlach, M., Lammers, R., Krivtsov, A. V., Ulrich, A., and Böhm, F. D. (1997) J. Biol. Chem. 272, 5966–5973). We mapped the EGFR phosphotyrosine 1173 as the major binding site for SHP-1 by a combination of phosphopeptide activation, phosphopeptide competition, and receptor YF mutant analysis. Mutational conversion of the EGFR sequence 1171–1176 AEY-LRV into the high affinity SHP-1 binding sequence LEY-LYL of the erythropoietin receptor (EpoR) led to a highly elevated SHP-1 binding to the mutant EGFR (EGFR1171–1176EpoR) and in turn to an enhanced dephosphorylation of the receptor. SHP-1 expression interfered with EGF-dependent mitogen-activated protein kinase stimulation, and this effect was more pronounced in case of EGFR1171–1176EpoR. Reduced SHP-1 binding to the EGFR Y1173F mutant resulted in a reduced receptor dephosphorylation by coexpressed SHP-1 and less interference with EGF-dependent mitogen-activated protein kinase stimulation. The effects of receptor mutations on SHP-1 binding were, however, stronger than those on receptor dephosphorylation by SHP-1. Therefore, receptor dephosphorylation may be the result of the combined activity of receptor-bound SHP-1 and SHP-1 bound to an auxiliary docking protein.

Activated and subsequently tyrosine-phosphorylated growth factor receptors are rapidly dephosphorylated, a process that is believed to negatively modulate signaling activity (1–7). The PTPases involved in receptor dephosphorylation are frequently unknown. It is possible that multiple PTPases act in concert on a given receptor, the identity of which may vary depending on the particular cell type or the activation state of cells. The PTPases with SH2 domains, SHP-1 and SHP-2 (8–10), are prone for interaction with tyrosine-phosphorylated proteins and have been shown to bind to multiple receptor species including transmembrane tyrosine kinases (11–15), cytokine receptors (16–18), antigen receptors (19–22), and adhesion molecules (23, 24). SHP-2 is a ubiquitously expressed enzyme and, as largely derived from genetic evidence for the Drosophila SHP-2 homologue Csw, seems to present an essential component of a positive signaling cassette of multiple receptors (25, 26), although negative modulation of receptor signaling by SHP-2 has also been reported (27, 28). SHP-1 is highly expressed in hematopoietic cells, and another closely related isoform of SHP-1 is expressed from an alternative promotor in epithelial cells (29). SHP-1 has been convincingly shown to negatively regulate signaling of multiple receptors in hematopoietic cells, including Kit/SCF-receptor (30, 31), interleukin 3 receptor (14), CSF-1 receptor (32), and the EpoR (17, 33). SHP-1 binds to Kit/SCF receptor (14) but not to the CSF-1 receptor (32) and may thus intercept with receptor signaling by different mechanisms. In case of the EpoR, SHP-1 is recruited via its N-terminal SH2 domain to the receptor subsequent to EpoR phosphorylation by Jak2 kinase (17). A high affinity SHP-1 binding site at Tyr-429 of EpoR has been characterized, and its lack in respective YF mutants of EpoR or truncated receptors leads to an enhanced EpoR signaling activity (17). Negative modulation of EpoR signaling by SHP-1 appears to involve Jak2 dephosphorylation (17). In addition, direct binding of SHP-1 to Jak2 and dephosphorylation of Jak2 by SHP-1 has also been demonstrated (34).

The functional role of SHP-1 in epithelial cells is much less understood. SHP-1 can bind to the EGF receptor (11, 15, 35) and to HER2 (13) and can dephosphorylate both in transient coexpression systems (13, 15) and in stably transfected epithelial cells (36). Thus, SHP-1 may negatively regulate HER family receptor signaling in epithelial cells. Both SH2 domains of SHP-1 are important for binding of SHP-1 to the EGFR and for receptor dephosphorylation, although the N-terminal SH2 domain contributes to a larger extent to the interaction (35). The catalytic domain of SHP-1 provides the specificity for EGFR dephosphorylation and cannot be replaced by the catalytic domain of SHP-2 in SHP-1/SHP-2 chimeras (35). To further clarify the mechanism of SHP-1 EGFR interaction, we investigated the importance of different EGFR autophosphorylation sites for the interaction with SHP-1. Activation of purified recombinant SHP-1 by EGFR sequence-derived phos-
phosphopeptides, competition of phosphopeptides with binding of autophosphorylated EGFR to immobilized SHP-1 SH2 domains, and binding of SHP-1 to a panel of EGFR YF mutants was employed to identify Tyr(P)1173 as the main binding site for SHP-1 on the EGFR. Introduction of the EpoR high affinity SHP-1 binding site into the EGFR strongly increases SHP-1 binding and also increases receptor dephosphorylation by SHP-1. Introduction of a Y1173F mutation reduces but does not abrogate receptor dephosphorylation by coexpressed SHP-1. Thus, SHP-1 activity toward EGFR is modulated but not exclusively dependent on receptor binding.

**MATERIALS AND METHODS**

**Chemicals and Reagents—**EGF was purchased from Pepro Tech, Inc., (Rocky Hill, NJ). Polyclonal anti-phosphotyrosine antibodies and monoclonal anti-pan-extracellular signal-regulated kinase antibodies were obtained from Transduction Laboratories (Lexington, KY). Monoclonal anti-EGFR antibody 425 was a kind gift from Dr. Luckenbach (Merck AG, Darmstadt, Germany). Polyclonal anti-EGFR and anti-SHP-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Activated MAP kinase was detected with a polyclonal anti-active MAP kinase antibody (Promega Corporation, Madison, WI). Anti-FA 12CA5 monoclonal antibody was purchased from Babco (Berkeley, CA), myelin basic protein was from Sigma. For introduction of point mutations in the EGFR cDNA, an M13 mutagenesis kit (Bio-Rad) was used. [pATP]ATP was purchased from NEN Life Science Products. Factor Xa was obtained from Boehringer Mannheim. Danayl-t-glutamyl-glycyl-arginine chloromethyl ketone was from Calbiochem-Novabiochem.

**Cloning of SHP-1 and SH2 Domain Mutants to pGEX Expression Vector—**The SHP-1 construct in pRK55-SHP-1 (35), which contains the originally cloned cDNA, was sequentially treated with XhoI, Pfu, and EcoRI, and the obtained SHP-1 DNA was cloned into Smal/EcoRI-digested pBluescript KS. The 3′-untranslated sequences were removed by replacing the poly(A)-containing Eco47III-XhoI fragment in this construct with an analogous fragment without poly(A) from the earlier described chimera Sh2h2g5CAT5 (35). 5′-Untranslated sequences were removed by replacing the EcoRI-BamHI fragment of original SHP-1 cDNA with an analogous fragment produced by polymerase chain reaction with SHP-1 cDNA in pBluescript KS as template using primers 5C-E, 5′AGAATTCTTCCACAGAGATGTCCTGC-3′C, and 5′-CCGGATATCTCGGCTAGGCTCTCTAT-3′. The modified SHP-1 DNA was removed from pRK55 using EcoRI/NotI and cloned in pGEX-5X containing the T7 promoter to generate pGEX-SHP-1. The SH2 SH2 domain point mutants with inactivated N-terminal SH2 domain (R32K), with inactivated C-terminal SH2 domain (R138K), or both inactivated SH2 domains with inactivated N-terminal SH2 domain (R32K), with inactivated C-terminal SH2 domain (R138K), or both inactivated SH2 domains (R32K, R138K) were subcloned from the corresponding full-length DNAs in pRK5 (35) to pGEX-5X using EcoRI/XhoI.

**Preparation of GST Fusion Proteins and of Recombinant SHP-1—**pGEX-5X-SHP-1, pGEX-5X-SHP-1 RI, and pGEX-5X-SHP-1 RI2 domain point mutants in pGEX-5X-1 were used to transform Escherichia coli strain BL21 (DE3). A single colony was inoculated into 50 ml of LB medium containing 50 μg/ml ampicillin and cultured overnight. 25 ml of this overnight culture were added to 500 ml of LB/ampicillin and grown at 37 °C to an A600nm of 0.8. Isopropyl-1-thio-galactopyranoside was added to a final concentration of 0.1 m, and the bacteria were kept for 16 h at 25 °C under shaking. Shaker, the bacteria were pelleted at 6,000 × g for 4 °C for 10 min. The pellet was resuspended in 10 ml of a buffer containing 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM β-mercaptoethanol, 2 mM EDTA, 1 mM EGTA, 5 mM β-glycerophosphate, 2 μM aprotinin, 10 μg/ml leupeptin, 1 μg/ml bestatin, 1 μg/ml antipain, 1 μg/ml pepstatin A, and 1 mM phenylmethanesulfonyl fluoride. The suspension was three times frozen and thawed, lysozyme was added (0.5 mg/ml final concentration), and the mixture was incubated at 25 °C for 20 min. Subsequently EDTA and Triton X-100 were added (0.5 mg/ml final concentration), and the mixture was boiled for 5 min. Proteins were separated by SDS-PAGE, and bound proteins were visualized by immunoblotting.

**Peptide Synthesis and Purification—**Six peptides encompassing the full length of EGF receptor tyrosine kinase (EGFR) domain 1 (429-445), (531-547), (563-579), (588-604), (615-631), and (644-660) with T7 promoter were synthesized coupled with a C-terminal C-terminal His6 tag using an Applied Biosystem Model 433 synthesizer. The sequences and the purity of the peptides were verified by complete amino acid analysis. The peptides were purified using the SMART chromatographic system (Amersham Pharmacia Biotech). The elution Fractions were collected and pooled (3 ml). The fractions were analyzed by SDS-PAGE and silver staining. Positive fractions were pooled and concentrated using 10-KDa centrifugal filters. 20% glycerol was added, and the protein solution was stored at −70 °C. The purity of the preparation was 95%; 300 μg of SHP-1 could be obtained from a 500-ml bacterial culture.

**A431 Cell Treatment and Binding of SHP-1 to the EGFR—**A431 cells were grown in 94-mm dishes to about 70% confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The cells were starved for 16 h using serum-free Dulbecco’s modified Eagle’s medium and subsequently stimulated with 100 ng/ml EGF for 5 min and lysed in 700 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 0.5% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM sodium orthovanadate). The lysates were centrifuged at 25,000 g for 20 min. To investigate whether the interaction of SHP-1 and EGFR is direct or not, a sequential immuno- and affinity purification approach was used (37). A431 cell lysates were prepared as described above. EGFR was immunoprecipitated using the monoclonal anti-EGFR antibody 425. The precipitates were denatured in 100 μl of SI buffer (50 mM Hepes, pH 7.5, 1% SDS, 1% β-mercaptoethanol) for 5 min at 95 °C. 100 μl of denatured protein solution was mixed with 900 μl of lysis buffer. The mixtures were incubated with A431 cell lysates for 2 h at 4 °C. The beads were washed three times with HNGT (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM Na3VO4), 60 μl 2× Laemmli buffer were added, and the beads were boiled for 5 min. Proteins were separated by SDS-PAGE, and bound proteins were visualized by immunoblotting.
Phosphopeptide Activation Assay—Activation of SHP-1 by EGFR-related phosphopeptides was carried out in a 50-μl reaction mixture containing the reaction buffer (100 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 10 mM p-nitrophenyl phosphate), 200 μM phosphopeptides, and 500 ng of SHP-1. After 30 min at 25 °C, the reaction was quenched with 100 μl of 1 N NaOH, and the absorbance at 405 nm was measured. Controls with unphosphorylated peptides were carried out in the same way.

Competition of the EGFR-SHP-1 Interaction by Phosphopeptides—Solubilized A431 membranes were prepared as described earlier (15). For 15 binding reactions, the following mixture was prepared: 100 μl of solubilized membranes (25 μg of protein) in buffer 5 (50 mM Hepes, pH 7.4, 1% Triton X-100) were supplemented with 3 μM MnCl₂, 0.1 mM Na₃VO₄, and 2 μg/ml EGF (final concentrations). This reaction was incubated on ice for 20 min. Autophosphorylation was started by adding 100 μM ATP and 38 μCi of [γ-³²P]ATP. After a 5-min incubation on ice, the reaction mixture was applied onto a 0.5-ml WGA-agarose column (Amersham Pharmacia Biotech) equilibrated with WGA buffer (50 mM Hepes, pH 7.4, 1 mM Na₂VO₄, 0.1% Triton X-100). The column was washed with 3 ml of WGA buffer. The radioactively labeled EGFR was eluted with 2 ml of WGA buffer containing 0.3 M N-acetylglucosamine, 100-μl fractions were collected, and aliquots were analyzed by scintillation counting. The peak fractions were pooled (500 μl). 50 μg of GST-fused SHP-1 SH2 domains (GST-SH2) were coupled to 30 μl of glutathione-Sepharose beads per reaction. 30 μl of the radiolabeled EGFR (∼20,000 cpm) supplemented with 200 μM phosphopeptides or the unphosphorylated analogues were added to the immobilized GST-SH2. The binding reactions were incubated with shaking for 2 h at 4 °C. Subsequently, the beads were washed three times with HNGT, 60 μl of 2% Laemmli buffer was added, and the samples were incubated for 5 min at 95 °C. The extracted proteins were resolved by SDS-PAGE (7.5% gels), and the gels were analyzed using a GS250 Molecular Imager (Bio-Rad).

Mapping of an SHP-1 Binding Site on the EGFR—Signaling molecules possessing SH2 domains can bind to autophosphorylated growth factor receptors in a direct or indirect manner. An example of this is the adaptor protein Grb2, which binds to the EGFR directly via phosphorylated Tyr-1086. In contrast, Grb2 binds indirectly to the phosphorylated PDGFβ-receptor Tyr-1009 site mediated by SHP-2 (37). We wished to know whether SHP-1 is capable of a direct binding to EGFR. To test this, EGFR immunoprecipitates from A431 cells were denatured by boiling in the presence of 1% SDS. Under these conditions any possible adaptor protein should be inactivated; however, the phosphotyrosine motifs in the autophosphorylated EGFR are expected to retain at least part of their binding affinity to corresponding SH2 domains (37). The denatured immunoprecipitates were diluted to reduce the SDS concentration to a nondenaturing level and were subjected to a binding reaction with a SHP-1 GST fusion protein. As demonstrated in Fig. 1, EGFR can be recovered on GST-SHP-1 when the cells had been stimulated by EGFR. From nonstimulated cells, much less EGFR is bound to GST-SHP-1, and no EGFR is detectable on GST beads in either case. Although the amount of EGFR bound to GST-SHP-1 recovered from denatured immunoprecipitates is only a fraction of what can be recovered with GST-SHP-1 from nondenatured lysates (not shown), this experiment indicates that SHP-1 is capable of a direct interaction with autophosphorylated EGFR.

We went on to test the possible involvement of the different autophosphorylation sites of EGFR in the SHP-1 binding. Tyrosines 992, 1068, 1086, 1148, and 1173 have been reported to become tyrosine-phosphorylated by the autokinase activity of the EGFR (for a review see Ref. 43 and references therein). The tyrosine 954 is a potential phosphorylation site and part of a
sequence that resembles the consensus sequence for binding of the N-terminal SH2 domain of SHP-1, hXYPXxh (h = hydrophobic, X = any amino acid) (44–46). We synthesized a panel of phosphopeptides encompassing EGFR sequences around tyrosines 954, 992, 1068, 1086, 1148, and 1173 and the corresponding unphosphorylated peptides. Phosphopeptides with high affinity to the N-terminal SH2 domain of SHP-1 are known to activate the recombinant enzyme in vitro (47, 48). We therefore tested the phosphopeptides for their capacity to activate purified recombinant SHP-1 (Fig. 2, upper panel). The phosphopeptides corresponding to Tyr-954 and Tyr-1173 exerted a strong SHP-1 activation, and the one corresponding to Tyr-1068 had a weaker activation effect. In all cases, the corresponding unphosphorylated sequences had no effect on SHP-1 activity. We then tested the phosphopeptides for their capacity to interfere with binding of SHP-1 SH2 domains to autophosphorylated EGFRs. The unphosphorylated analogs served as internal controls (Fig. 2, lower panel). In these binding assays, we observed competition by the phosphopeptides corresponding to Tyr-954, Tyr-1173, and Tyr-1068. In summary, the phosphopeptide activation and competition experiments suggested that the EGFR tyrosines 954, 1173, and 1068 have the capacity of SHP-1 binding in their phosphorylated form. Next, a panel of mutated EGFRs was generated with replacement of all candidate tyrosines by phenylalanine, including single mutations and some combinations. We also replaced the sequence AEY1173LRV around the candidate binding site Tyr-1173 by a known high affinity binding site for SHP-1, namely the sequence LEY429LYL of the EpoR (17) and thereby generated EGFR1171–1176EpoR. Lysates of 293 cells overexpressing the different receptor mutants were incubated with a GST-SHP-1 SH2 domain fusion protein, and the amount of bound receptor was visualized by immunoblotting. This assay was applied instead of a previously used association assay (35), in particular because it was not hampered by the low level of endogenous wild type EGFR in 293 cells; it was also more sensitive to changes in binding affinity. A representative result for selected mutants is shown in Fig. 3. Despite the potent activating and competing effects of the phosphopeptide 954 in vitro, the Y954F point mutation had little effect on SHP-1 binding, reducing binding in five independent experiments to only 80 ± 10% of the wild type receptor (100%). This finding indicates that tyrosine 954 is most likely only poorly phosphorylated in vivo and therefore is of little importance for SHP-1 binding. In contrast and in agreement with the phosphopeptide data, mutation of Tyr-1173 led to a strong decrease of SHP-1 binding (Fig. 3, mean remaining binding 9 ± 5%). Thus, Tyr-1173 in its phosphorylated form is clearly the main SHP-1 binding site on the EGFR. We also observed some reduction in binding by YF mutation of Tyr-1068 and Tyr-1148 (85 ± 21% and 65 ± 25%, respectively). Although the small effect of the Y1068F mutation would be in agreement with the phosphopeptide activation and competition data and may indicate that Tyr(P)1068 is a minor binding site for SHP-1, the reason for the weak effect of the Y1148F mutation on SHP-1 binding is unclear. The corresponding phosphopeptide neither activated SHP-1 nor showed detectable competition in the binding assay. Possibly Tyr-1148 weakly mediates indirect binding of SHP-1. YF mutation of Tyr-992 and Tyr-1086 had no effect on SHP-1 binding (not shown). Interestingly, EGFR1171–1176EpoR revealed a strongly elevated binding capacity for SHP-1 compared with wild type EGFR (Fig. 3).

We employed GST fusion proteins of SHP-1 with an inactivated N-terminal or C-terminal SH2 domain to clarify the importance of the two SH2 domains for recognition of the binding sites on the EGFR. As shown in Fig. 4, under the conditions of this assay, inactivation of either SH2 domain led to a reduction of EGFR binding to undetectable levels, confirming the earlier finding (35) that both SH2 domains play a role in receptor recognition. When EGFR1171–1176EpoR was analyzed in the same assay, a reduced but substantial binding was detectable employing the SHP-1 construct with inactivated C-terminal SH2 domain, whereas binding became undetectable upon inactivation of the N-terminal SH2 domain (Fig. 4). Thus, as expected, elevation of binding of SHP-1 to EGFR1171–1176EpoR seems mainly caused by increased affinity for the N-terminal SH2 domain. Still, also in this setting, the C-terminal SH2 domain contributes to binding.
We investigated the effect of impaired or enhanced binding of SHP-1 to the EGFR by analyzing the receptor phosphorylation level in EGF-stimulated cells expressing wild type EGFR or mutant receptors in the absence or presence of SHP-1. As shown in Fig. 5, transfection of wild type EGFR with EGFR or EGFR1171–1176EpoR resulted in elevated activity of SHP-1 toward the EGFR variant compared with the wild type; however, not in complete dephosphorylation (Fig. 5). Thus, the changes of binding strength modulated dephosphorylation capacity of SHP-1; however, the changes of receptor-directed PTPase activity were less striking than the changes of SHP-1 binding.

These data suggest that part of the receptor dephosphorylation may be due to a fraction of SHP-1 that is not bound to the receptor. We considered the possibility that another protein that is tyrosine-phosphorylated in response to EGF may serve as a docking protein, bind, and activate a fraction of SHP-1, which in turn would participate in EGFR dephosphorylation. We reasoned that co-overexpression of such a protein may enhance the activity of SHP-1 toward the EGFR. We tested a number of known substrates of activated EGFR for a respective activity, including p85, SHC (66-kDa isoform), phospholipase Cγ, c-Cbl, and SHP-2. In all cases, these proteins were tyrosine-phosphorylated in an EGF-dependent manner, and coexpression of SHP-1 led to a reduced phosphorylation level. However, although the tyrosine phosphorylation level of the EGFR was reduced upon coexpression of phospholipase Cγ in the absence of SHP-1, none of the tested proteins enhanced receptor dephosphorylation by SHP-1 (not shown), making it unlikely that any of these proteins plays the role of an auxiliary docking protein.

We also analyzed the effects of SHP-1 coexpression on the downstream signaling of the different EGFR variants by evaluating EGF-dependent MAP kinase activation. For this we employed antibodies that specifically recognize activated MAP kinase species (Fig. 5, panel G) or measured the activity of cotransfected, HA epitope-tagged MAP kinase (extracellular signal-regulated kinase 2) after immunoprecipitation in vitro (Fig. 5, panel C) and quantitated the signal by phosphorimaging. SHP-1 coexpression led to a reduced MAP kinase activity in EGF-stimulated cells overexpressing wild type EGFR (59 ±

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Fig. 3. Differential binding of EGFR YF mutants to the SH2 domains of SHP-1. The indicated EGFR YF mutants were transiently expressed in 293 cells from a cytomegalovirus promoter-driven expression plasmid. After transfection, the cells were stimulated with EGF and lysed. Lysates were incubated with 10 μg of immobilized SHP-1 SH2 domain GST fusion protein (GST-SH2) or immobilized GST alone (GST). Beads were washed, and the bound EGFR was visualized by SDS-PAGE and immunoblotting using anti-EGFR antibodies (A). The numbers of the mutants correspond to the EGFR tyrosine residues, which were exchanged for phenylalanine. EGFR EPO corresponds to EGFR1171–1176EpoR, a mutant where the sequence AEY1173LRV was replaced by the known high affinity binding site for SHP-1 LEY429LYL of the EpoR. The expression levels of the EGFR mutants used for the different binding reactions were monitored by immunoblot analyses using lysate aliquots (B). WT, wild type.

Fig. 4. Effect of inactivating point mutations in the individual SH2 domains of SHP-1 on binding to wild type EGFR or EGFR1171–1176EpoR. Wild type EGFR (EGFR WT) and EGFR1171–
1176EpoR (EGFR EPO) were transiently expressed in 293 cells. The cells were stimulated with EGF and lysed, and the lysates were incubated with 50 μg of immobilized GST fusion proteins of wild type SHP-1 (GST-SHP-1), SHP-1 with inactivated N-terminal SH2-domain (RK32) or SHP-1 with inactivated C-terminal SH2-domain (RK138). The beads were washed, and bound EGFR was visualized by SDS-PAGE and immunoblotting (A). Lysate aliquots were analyzed for expression levels by immunoblotting (B).
Effect of mutations in EGFR SHP-1 binding sites on receptor signaling activity in the presence of coexpressed SHP-1. A–E: EGFR mutants (designations as in Fig. 3) were transiently expressed in 293 cells in the absence or presence of SHP-1 as indicated. The cells were stimulated with EGF and lysed, and receptor tyrosine phosphorylation was monitored by immunoblot analyses using anti-phosphotyrosine antibodies (A). The figures above lanes 2, 4, 6, 8, and 14 represent mean values of receptor phosphorylation signal in the presence of SHP-1 compared with receptor phosphorylation in the absence of SHP-1 (100%) as derived from densitometric analysis of two independent experiments with a similar extent of dephosphorylation in the wild type (WT) setting. The same relation of the different mutants was observed in at least three further experiments. The phosphorylation and, therefore, activation of endogenous p42/44 MAP kinases was analyzed in cell lysates of the same experiment by immunoblotting using an antibody, which specifically recognizes the phosphorylated forms of p42/p44 MAP kinases (anti-active MAPK).
16% in presence versus 100% in absence of SHP-1, p = 0.019). The SHP-1-dependent reduction of MAP kinase activity was similar in cells expressing the Y1147F mutant receptor (47 ± 20%), whereas the effect of SHP-1 was less pronounced in cells expressing the Y1173F mutant (77 ± 13%) or the Y1173F,Y954F double mutant (72 ± 17). In contrast, the reduction of MAP kinase activity upon SHP-1 coexpression was clearly more pronounced in case of the EGFR1117–1176EpoR mutant (27 ± 5%, p = 0.033 versus wild type). In summary, the effects of SHP-1 expression on MAP kinase activity in cells expressing the different receptor mutants mirrored the effects on the receptor phosphorylation level.

**DISCUSSION**

The SH2-domain PTPase SHP-1 binds to and dephosphorylates autophosphorylated EGFR and may participate in modulation of EGFR signaling in epithelial cells. Here, we describe mapping of the binding site for SHP-1 on the EGFR. Most important for SHP-1 binding is phosphorylation of Tyr-1173, a prominent autophosphorylation site at the extreme C terminus of the cytoplasmic tail of the receptor. The binding site was assigned based on analysis of YF receptor mutants, competition of binding by a synthetic phosphopeptide corresponding to the sequence around Tyr-Y1173, and strong activation of recombinant SHP-1 by the Tyr-1173 phosphopeptide but not by its unphosphorylated analog. It is likely that Tyr-1173 in its phosphorylated form permits binding of the N-terminal SH2 domain of SHP-1, since the sequence AEY(P)LRV corresponds to the consensus motif hXY(IP)XX derived from phosphopeptide library screens and known binding sites (17, 44–46). Also, activation of recombinant SHP-1 is expected for a binding partner of the N-terminal SH2 domain (47, 48).

Previous studies (35) and data presented in this paper (Fig. 4) clearly show that both SH2 domains are involved in SHP-1 binding to the EGFR. Simultaneous occupation of the tandem SH2 domains with appropriate phosphotyrosine-containing interaction partners is likely to confer much higher affinity binding (49) and more potent activation of the phosphatase than occupation of only the N-terminal SH2 domain. Enhanced activation of SHP-1 with doubly phosphorylated peptides versus monophosphorylated peptides has been reported (46). Similarly, the simultaneous occupation of both SH2 domains of SHP-2 by a doubly phosphorylated peptide leads to an exclusively strong activation (50). The crystal structure of the SH2 domains of SHP-2 revealed steric requirements for simultaneous ligand occupation of both domains with an optimal distance of about 40Å (51). Based on the recent elucidation of the crystal structure of SHP-2, an activation mechanism for SHP-2 has been proposed that involves sequential occupation of first the C-terminal and then the N-terminal SH2 domain by a doubly phosphorylated interaction partner (52). The structure of the SHP-1 SH2 domains may be very similar to the structure of the SHP-2 SH2 domains (44, 45), and activation of SHP-1 is likely to occur similarly as activation of SHP-2. It is currently not clear which EGFR phosphotyrosine binds to the C-terminal SH2 domain of SHP-1. One possible candidate may be Tyr(P)-11148, which could be in approximate distance from Tyr(P)-1173 to allow simultaneous interaction of both sites with both SHP-1 SH2 domains. However, the effect of the Y1147F mutation on SHP-1 binding was only weak and does not compellingly support a role of Tyr(P)-1148 for SHP-1 binding. An alternative possibility would be the interaction of SHP-1 with two EGFR molecules in a receptor dimer, allowing binding of both SH2 domains to Tyr(P)-1173. Such a model has been proposed for interaction of SHP-2 with the PDGFβ receptor via phosphotyrosine 1090 (49). Further work is required to identify the binding partner on the EGFR for the C-terminal SH2 domain of SHP-1.

A phosphopeptide corresponding to the EGFR sequence around Tyr-954 was found to potently activate SHP-1 and to compete with EGFR binding. However, the Y954F mutant receptor was not significantly impaired with respect to SHP-1 binding. Most likely Tyr-954 is not effectively autophosphorylated. Similarly, an EGFR Tyr-954 phosphopeptide was found to bind with high affinity to SHP-2 SH2 domains and to block SHP-2 EGFR interaction; however, the significance of this finding for the in vivo situation is questionable (53). Another binding site of much lower affinity than the one around Tyr-1173 may present Tyr-1068 in its phosphorylated form. As observed for other SH2-domain proteins (54), SHP-1 binding may occur through alternative sites, albeit with very different strength.

Two findings suggest that binding of SHP-1 to EGFR is at least in part direct. First, SHP-1 binding could be observed using recombinant GST SHP-1 and autophosphorylated EGFR from SDS-denatured cell lysate as partners. Second, as mentioned above, the sequence around Tyr-1173 matches a consensus sequence for the N-terminal SH2 domain of SHP-1. Our data do, however, not exclude the possibility that, additionally, direct binding an indirect binding of SHP-1 occurs.

Binding of SHP-1 to the EGFR was highly elevated upon changing the sequence around Tyr-1173 to the one of the known SHP-1 binding site around Tyr-429 in the EpoR. In this setting the binding of SHP-1 is dominantly mediated via the N-terminal SH2 domain. The observation supports assignment of Tyr-1173 as the SHP-1 binding site; however, it indicates that the binding of SHP-1 to wild type EGFR is weaker than binding to EpoR via Tyr-429 in its phosphorylated form.

An interesting general question with respect to physiological substrates of the SH2 domain PTPases is to what extent the SH2 domains may target the enzymes to substrates. We observed that binding of SHP-1 to the EGFR to some extent correlated with its capacity to dephosphorylate the receptor in that the Y1173F mutant receptor was less effectively and the EGFR1117–1176EpoR mutant was more readily dephosphorylated, respectively. The changes observed in receptor dephosphorylation capacity for Y1173F and EGFR1117–1176EpoR mutants were less striking than the changes in SHP-1 binding to the receptor compared with the wild type. On the other hand, the capacity of SHP-1 to dephosphorylate the EGFR is critically dependent on intact SHP-1 SH2 domains (35). Two conclusions can be derived from these data. First, the fraction of receptor-bound SHP-1 has only moderate activity toward the receptor, which may be due to the steric position of the PTPase catalytic site relative to the SH2-domains. Also, the catalytic site may have sterical access to only some of the autophosphorylation sites when SHP-1 is immobilized at Tyr-1173. It is currently unknown whether SHP-1 displays any selectivity with respect to dephosphorylation of individual EGFR autophosphorylation sites. Second, a part of receptor dephosphorylating activity of SHP-1 is likely to be due to a fraction of PTPase that is not bound to the receptor. To become activated, it should,
however, be bound to a tyrosine-phosphorylated protein. We propose that such an auxiliary docking protein may participate in regulation of EGFR-directed SHP-1 activity. Work is in progress to identify respective candidate proteins. A similar dissociation of SHP-1 binding and dephosphorylation activity as described here has been reported for receptor tyrosine kinases in hematopoietic cells. SHP-1 dephosphorylates the CSF-1 receptor; however, it does not bind to it. A 130-kDa docking protein has been proposed to mediate activity of SHP-1 toward the receptor (32).

Mitogenic signaling of the EGFR seems to critically depend on activation of the MAP kinase cascade. We found that SHP-1 coexpression attenuates MAP kinase activation via EGFR in the 293 cell system. Interference of SHP-1 with MAP kinase activation by Y1173F mutant EGFR was less pronounced, whereas increased SHP-1 binding to the EGFR,1173-1176Ser receptor resulted in a more potent interference of SHP-1 with MAP kinase activation. These findings are in agreement with a reduced MAP kinase activation secondary to a reduced EGFR autophosphorylation in the presence of SHP-1. A lower phosphorylation level of the EGFR is expected to lead to a reduction in the recruitment of Grb2 and SHC, which are believed to be the prime mediators of the MAP kinase response to EGF (55). Indeed, we observed reduced SHC phosphorylation in SHP-1-overexpressing cells (not shown). Alternatively or in addition, SHP-1 binding may compete for SHC binding to the receptor, which has been described to involve primarily phosphoryrosines 1173 and 1148 (55, 56). Finally, SHP-1 may also directly dephosphorylate SHC and thereby reduce complex formation with Grb2 and subsequent activation of the signaling cascade. An attenuation of nerve growth factor-dependent MAP kinase activation in PC12 cells has likewise been observed as a consequence of SHP-1 overexpression (57). In other experimental systems a positive role of SHP-1 for MAP kinase activation has been described (58, 59). Recently, SHP-1 has been demonstrated to have the capacity for activation of the cytoplasmic tyrosine kinase c-Src by dephosphorylation of the inhibitory phosphotyrosine at the C terminus (60), an event that may be related to SHP-1-dependent activation of the MAP kinase cascade observed in the above-quoted studies. It is possible that, depending on the cellular background, i.e., levels and activation state of critical signaling proteins, a positive or negative net effect of SHP-1 activity on signaling cascades is observable. The availability of EGFR mutants with abrogated or elevated SHP-1 binding will help to clarify the role of EGFR-SHP-1 interaction for generation and modulation of receptor signals in appropriate stable cell lines.

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