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Both SH2 Domains Are Involved in Interaction of SHP-1 with the Epidermal Growth Factor Receptor but Cannot Confer Receptor-directed Activity to SHP-1/SHP-2 Chimera*

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The previously demonstrated functional and physical interaction of the SH2 domain protein-tyrosine phosphatase SHP-1 with the epidermal growth factor (EGF) receptor (Tomic, S., Greiser, U., Lammers, R., Kharitonenkov, A., Imyanitov, E., Ullrich, A., and Böhmer, F. D. (1995) *J. Biol. Chem.* 270, 21277–21284) was investigated with respect to the involved structural elements of SHP-1. Various mutants of SHP-1 were transiently expressed in 293 or COS-7 cells and analyzed for their capacity to associate with immobilized autophosphorylated EGF receptor *in vitro* and to dephosphorylate coexpressed EGF receptor in intact cells. Inactivating point mutation of the C-terminal SH2 domain reduced the association weakly, point mutation of the N-terminal SH2 domain reduced association strongly and the respective double mutation abolished association totally. The capacity of SHP-1 to dephosphorylate coexpressed EGF receptor was impaired by all point mutations. Truncation of the N-terminal or of both SH2 domains strongly reduced or abolished association, respectively, but the truncated SHP-1 derivatives still dephosphorylated coexpressed EGF receptor effectively.

Various chimeric protein-tyrosine phosphatases constructed from SHP-1 and the closely homologous SHP-2 dephosphorylated the EGF receptor when they contained the catalytic domain of SHP-1. As native SHP-2, the chimera lacked activity toward the receptor when they contained the catalytic domain of SHP-2, despite their capacity to associate with the receptor and to dephosphorylate an artificial phosphopeptide. We conclude that the differential interaction of SHP-1 and SHP-2 with the EGF receptor is due to the specificity of the respective catalytic domains rather than to the specificity of the SH2 domains. Functional interaction of native SHP-1 with the EGF receptor requires association mediated by both SH2 domains.

The phosphorylation level of activated growth factor receptors with endowed tyrosine kinase activity is the net result of tyrosine specific autophosphorylation (1) and a rapid dephosphorylation by phosphotyrosine-specific phosphatases (PTPs).[1] Since important aspects of receptor signaling depend on receptor autophosphorylation, the dephosphorylation reaction is believed to attenuate the receptor signal. Therefore, the identification and characterization of the PTPs involved in growth factor receptor dephosphorylation is of high interest. The SH2 domains containing PTPs SHP-1 and SHP-2 (2, 3) have been shown to interact with multiple growth factor receptors and to modulate their signaling activity. Transient coexpression of SHP-1 with different tyrosine kinase receptors results in complete or partial dephosphorylation of PDGF α- and β-receptor, insulin-like growth receptor-1 receptor, Kit/SCF receptor, insulin receptor, EGF receptor, and HER2 (4). SHP-2 has little activity with respect to dephosphorylation of associated receptors (4, 5) and rather seems to mediate a receptor signal via as yet not fully understood mechanisms (6–9). One important pathway seems to involve tyrosine phosphorylation of receptor-bound SHP-2 at the C terminus, which leads to subsequent association of Grb2 and activation of the Sos/Ras/Raf/MAPK-signaling cascade (10, 11). Depending on the cellular context, however, SHP-2 may be involved in silencing of certain growth factor receptor signals, as demonstrated for the membrane ruffling response to PDGF-BB in stably PDGF receptor expressing 293 cells (12). Also, SHP-2 has been shown to dephosphorylate the autophosphorylated PDGFβ-receptor preferentially at phosphotyrosines 751 and 771 *in vitro* (13).

SHP-2 and SHP-1 enzyme activity is negatively controlled by the N-terminal SH2 domain. This inhibition involves an intra- or intermolecular interaction of the SH2 domain with the C-terminal part of the PTP the detailed mechanism of which is yet unknown (14–18). Activation of SHP-1 and SHP-2 toward macromolecular substrates requires occupation of the SH2 domains, a reaction that can be mimicked *in vitro* by synthetic phosphopeptides (19, 20). An overlapping but not identical substrate specificity of SHP-1 and SHP-2 has been demonstrated with artificial peptide substrates *in vitro* (21). With some low molecular weight substrates *in vitro*, in particular *p*-nitrophenylphosphate at acidic pH, SHP-1 exhibits a higher intrinsic activity than SHP-2 (22, 23).

SHP-1 seems to be involved in negative regulation of receptor signaling and in receptor dephosphorylation in hematopoietic cells, as has been shown for Kit/SCF receptor (24) and CSF-1 receptor (25). Also, receptors without intrinsic tyrosine kinase activity including the erythropoietin receptor (26), the

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[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; hg, hinge; CAT, catalytic part; SCF, stem cell factor; CSF, colony stimulating factor.

interleukin-3 receptor (27) as well as the FcγIIB1 receptor in B cells (28) are subject to negative regulation by SHP-1. Interaction of the erythropoietin receptor with SHP-1 is mediated by the N-terminal SH2 domain of the PTP recognizing the sequence PHLK(P)Y^{429}LYLV in the phosphorylated cytoplasmic domain of the receptor. Recruitment of SHP-1 to the erythropoietin receptor leads to strong elevation of its PTP activity and dephosphorylation of the receptor-associated tyrosine kinase JAK-2 and possibly other tyrosine phosphorylated targets in the proximity of the receptor. In turn, the erythropoietin signal is attenuated (26). Interaction of SHP-1 with the FcγIIB1 receptor is mediated via the C-terminal SH2 domain of the PTP, recognizing the phosphopeptide sequence ENL(P)YEGLN (28). For the CSF-1 receptor, no direct association of SHP-1 has been detectable (24). In this case, the interaction leading to negative regulation might be mediated by the adapter protein Grb2 and another as yet unidentified M_r 130,000 adapter protein (25). Comparatively little is known about the physiological substrates of SHP-2. SHP-2 potently dephosphorylates its own C terminus, and therefore one function of its PTP activity might be to regulate its own phosphorylation state and thus signaling activity mediated by the C-terminal Grb2-association (29). Recently, the Drosophila protein *daughter of sevenless* (DOS) has been identified as a target of *corkscrew*, the Drosophila homolog of SHP-2 (30). It has, therefore, been speculated that mammalian homologs of DOS do exist and possibly present preferred cellular substrates for SHP-2 (30). Apart from hematopoietic cells, SHP-1 is also expressed in epithelial cells (31, 32) and might be important for modulation of resident receptors in these cells. In an attempt to identify PTPs involved in negative regulation of the EGF receptor, we characterized PTP activity copurifying with the receptor from A431 human epidermoid carcinoma cells (5). Both SHP-1 and SHP-2 were present in the receptor preparations and also associated with the EGF receptor *in vitro* and in intact A431 cells. We found that SHP-1 avidly dephosphorylates autophosphorylated EGF receptor *in vitro* and upon transient coexpression with the receptor in 293 cells and presumably contributes to EGF receptor dephosphorylation in A431 cells. SHP-2 had no detectable activity toward the receptor (5). To better understand the SHP-1/EGF receptor interaction and the structural basis for the strikingly different activity of SHP-1 and SHP-2 toward the receptor, we generated a series of SHP-1 mutants and chimeric PTPs from SHP-1 and SHP-2 and analyzed them with respect to association with the EGF receptor and receptor dephosphorylation. We find that both SH2 domains of SHP-1 are important for interaction with the receptor and that the capacity for receptor dephosphorylation is determined by the catalytic domain.

EXPERIMENTAL PROCEDURES

Constructs for PTP Expression, General—The SHP-1 cDNA used throughout this study was obtained by standard procedures from an SK-BR3 cDNA library and corresponds to the 597-amino acid splice variant of SHP-1, which is predominantly expressed in epithelial cells (32). The N-terminal sequence and numbering is identical to the one depicted in Shen *et al.* (31). Thus, Arg-32 and Arg-138 in our SHP-1 variant correspond to Arg-30 and Arg-136, respectively, of hematopoietic SHP-1 (33). All PCR reactions were carried out with *Pfu* polymerase (Stratagene, Heidelberg) for 25 cycles. All cloning operations were carried out with the respective DNAs in pBluescript KS(−) (Stratagene, Heidelberg). The final constructs were verified by DNA sequencing (T7 sequencing kit, Pharmacia, Uppsala) and subsequently subcloned into the eucaryotic expression vector pRK5 RS (34).

Point and Deletion Mutants of SHP-1—The Arg-32 → Lys (R32K) mutation in the N-terminal SH2 domain was generated by PCR-based mutagenesis, using the primers 1C-E, 5′-AAG AAT TCC CCT ACA GAG AGA TGC TGT CC-3′; RKNSH2, 5′-CAC GGA AGC TTC CTG GCT AAG CCC AGT CGC AAG AAC C-3′; RKNSH2r, 5′-CCA GGA AGC TTC CGT GGA CAC CTC GGC CC-3′; and 2C, 5′-CCG GAA TTC GGC GCC TGA GGC CTC CTC AAT-3′.

The PCR fragments generated with the primer pairs 1C-E/RKNSH2r and RKNSH2/2C and SHP-1 DNA as a template were cloned separately into pBluescript KS (*Eco*RI-*Hin*dIII, *Hin*dIII-*Kpn*I) and subsequently joined. A part of the resulting joined DNA fragment containing the mutated sequence was excised by digestion with *Eco*RI-*Bam*HI and exchanged with the corresponding DNA stretch of native SHP-1 DNA to yield SHP-1 R32K. Mutation of Arg-138 in the C-terminal SH2 domain was carried out similarly, by using primers 1C-E; RKCSH2, 5′-GAG CCA TGG ACG TTT CTT GTG AAG GAG AGC CTC AGC CAG3-′; RKCSH2r, 5′-AAA CGT CCA TGG CTC GCC CTT GGC CTG CAG-3′; and C-HCS, 5′-CAA TGT CAC AGT CCA GGC CC-3′. The two PCR fragments obtained with the primer pairs 1C-E/RKCSH2r and RKCSH2/C-HCS) were cloned into pBluescript, reisolated, and thereafter ligated together. *Bst*EII digestion of this construct yielded a fragment containing the mutated sequence which was used to replace the normal corresponding part in SHP-1 to yield SHP-1 R138K. Exchange of this fragment in SHP-1 R32K yielded the double point mutant SHP-1 R32K,R138K. To obtain SHP-1ΔSH2, SHP-1 DNA was digested with *Bst*EII and *Eco*RI. This treatment removed all DNA 5′ of the codon for Met-172, leaving Met-172 intact. To obtain SHP-1 ΔN-SH2, SHP-1 DNA was digested with *Bam*HI and *Xho*I, and the ends were filled with *Pfu* polymerase. This fragment was ligated into *Eco*RV-digested pRK5 RS. Ligation of the filled *Bam*HI 5′-end of the SHP-1 fragment to the 3′-end of the *Eco*RV-digested vector generated a Met-codon in frame with the remaining sequence of SHP-1 (amino acids 56–597). SHP-1ΔN-SH2, R138K was obtained by the identical procedure from SHP-1 R138K.

Construction of SHP-1/SHP-2 Chimera—The chimera are composed of SH2 domains (*SH2*), the "hinge" part (*hg*), and the catalytic part (*CAT*) derived from SHP-1 or SHP-2 and are designated as depicted in Fig. 1, using raised "1" or "2" to indicate the origin of the respective domain. Chimera SH2^{1}hg^{1}CAT^2, SH2^{2}hg^{2}CAT^1, SH2^{1}hg^{2}CAT^2, and SH2^{2}hg^{1}CAT^1 were obtained by PCR amplification of SHP-1 and SHP-2 fragments with suitable cloning sites at one end and *Eco*RI sites at the internal ends intended for the fusion. The individual fragments were initially separately cloned into pBluescript KS and subsequently joined. Employing this method, an additional Glu-Phe dipeptide sequence corresponding to the *Eco*RI site was introduced at the fusion site of these chimeric molecules.

SH2^{1}hg^{1}CAT^2 was constructed using the primers 1C, 5′-AGA TCA AGC TTC CCT ACA GAG AGA TGC TGT CC-3′, and 5C, 5′-CCG GAA TTC GTT CTC TGG CCG CTG CCC-3′, to amplify an SH2^{1}hg^{1}fragment with a 5′ *Hin*dIII and a 3′ *Eco*RI site from SHP-1 DNA. The CAT^2 fragment with a 5′ *Eco*RI site and a 3′ *Xba*I site was generated using the primers 6D, 5′-CCG GAA TTC AAA AAT AGA TAT AAA AAC ATC CTG CCC-3′, and 4D, 5′-CTA GTC TAG ATC ATC TGA AAC TTT TCT GCT GTT G3-′, and SHP-2 DNA as a template. Both fragments were cloned in pBluescript KS, joined at the *Eco*RI site, and subcloned in pRK5 RS.

SH2^{2}hg^{2}CAT^1 was assembled in the same way from SH2^{2}hg^2 (5′ *Hin*dIII, primers 1D, 5′-AGA TCA AGC TTG GGA GGA ACA TGA CAT CGC GG-3′ and 5D, 5′-CCG GAA TTC GTT TTT GTT TTC TTG CCT TTG ACC-3′, template SHP-2), and CAT^1 (primers 6C, 5′-CCG GAA TTC AAG GGC AAG AAC CGC TAC AAG-3′ and 4C, 5′-CTA GTC TAG AGG ACA GCA CCG CTC ACT TCC T-3′, 3′ *Xba*I).

SH2^{1}hg^{2}CAT^2 was assembled from SH2^1 (5′ *H*indIII, primers 1C and 2C) and hg^{2}CAT^2 (primers 3D, 5′-CCG GAA TTC CTT AAC ACG ACT CGT ATA AAT GC-3′, and 4D).

SH2^{2}hg^{1}CAT^{1}was assembled from SH2^2 (primers 1D and 2D, 5′-CCG GAA TTC GGG CTG CTT GAG TTG TAG TAC-3′) and hg^{1}CAT^1 (primers 3C, 5′-CCG GAA TTC GTC TAC CTG CGG CAG CCG TAC-3′, and 4C). To obtain chimera SH2^{2}hg$^{2/1}$CAT^1, a fragment of SHP-2 DNA comprising the sequence corresponding to the SH2 domains and part of the hinge domain was amplified using primers D-Nt, 5′-CCC GAA TTC ATG ACA TCG CGG AGA TGG-3′, and D-int, 5′-CCC GAA TTC TTC CCA AAA GCC TTG TTT-3′, and a fragment of SHP-1 DNA comprising the sequence corresponding to part of the hinge domain and the catalytic part including the C-terminal tail was amplified using the primers C-int, 5′-CCC GAA TTC GAG AGT TTG CAG AAG CAG-3′, and C-Ct, 5′-CCC CTC GAG TCA CTT CCT CTT GAG GGA-3′. The internal primers generated new *Eco*RI sites between Glu-249 and Phe-250 in SHP-1 and correspondingly Glu-250 and Phe-251 in SHP-2 without changing the corresponding amino acid sequence GFWEEF in this part of the hinge region, which is identical in both PTPs. The fragments were cloned into pBluescript KS, fused using the newly created *Eco*RI sites, and subcloned into pRK5 RS.

To obtain the chimera SHP-1 tail^2, an existing *Pst*I site in the DNA

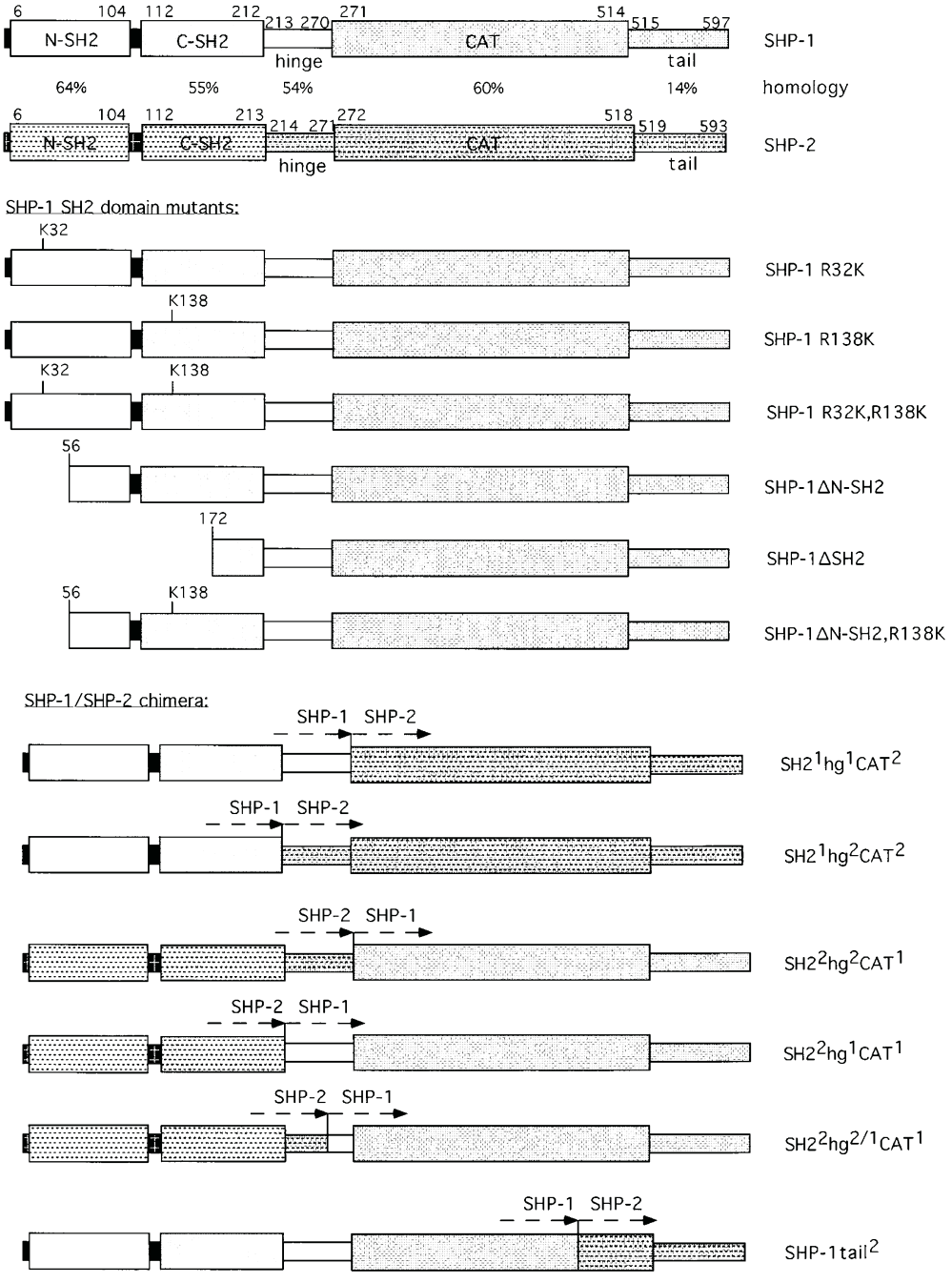

FIG. 1. **Schematic representation of the structure of SHP-1 and SHP-2 and of various SHP-1 mutants and chimeric molecules derived from SHP-1 and SHP-2.** Amino acid numbering is according to Shen *et al.* (31) for SHP-1 and Vogel *et al.* (4) for SHP-2.

corresponding to the highly conserved amino acid sequence VHCSAG (SHP-1 AS 453–458 or SHP-2 AS 457–462) was used. A DNA fragment corresponding to the last 135 amino acids of SHP-2 was excised with *Pst*I from the previously generated CAT^2 DNA and used to replace the respective fragment in SHP-1 DNA.

Transfections—Transfections were carried out using the cytomegalovirus promotor-driven expression vector pRK5 RS, 293 human embryonal kidney cells, or COS-7 cells (German Collection of Microorganisms and Cell Cultures, DSM ACC 60) by a Ca^{2+} precipitation method, as described previously (34).

PTP Assays—293 cells were transiently transfected with the various PTP constructs. Cell lysates were obtained in the absence of PTP inhibitors and directly assayed for PTP activity using [^{32}P]Raytide as a substrate as described previously (5). The amount of lysate protein was titrated in each assay to fall into the linear range of the method. Data from different assay series were normalized by computing the ratio from the activity in lysates of PTP expression plasmid-transfected cells and the activity in the lysate of cells mock-transfected in parallel.

Antibodies—Anti-SHP-1 CT was obtained from Santa Cruz Biotechnology (Santa Cruz, CA; catalogue no. sc-287). Anti-SHP-1 SH2 polyclonal rabbit antiserum was described by D'Ambrosio *et al.* (28) and kindly provided by Dr. J. C. Cambier (Denver, CO). Monoclonal anti-SHP-2 SH2 and polyclonal anti-phosphotyrosine antibodies were from Transduction Laboratories (Lexington, KY; catalogue nos. P17420 and P11230, respectively). The EGF receptor was detected with a polyclonal anti-EGF receptor antibody from Santa Cruz Biotechnology (catalogue no. sc-03) or in some experiments with a monoclonal antibody from Transduction Laboratories (catalogue no. E12020). For association assays, the monoclonal anti-EGF receptor antibody mab425, which is directed against the extracelullar domain of the receptor, was used. The latter antibody was kindly provided by Dr. A. Luckenbach (Merck, Darmstadt).

Association of PTP Derivatives with Autophosphorylated EGF Receptor—Mab425 anti-EGF receptor antibodies were covalently coupled to

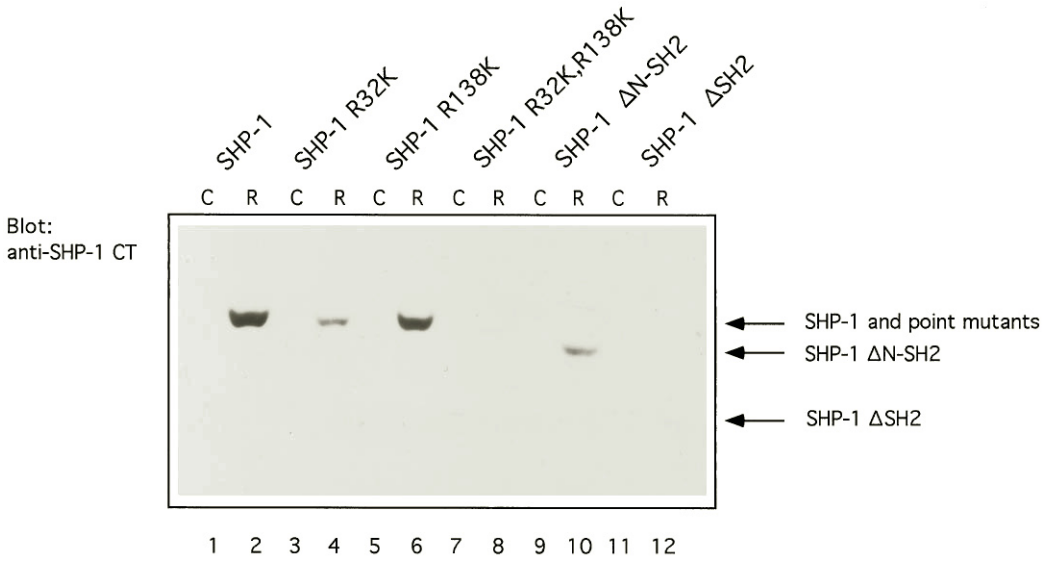

FIG. 2. **Association of SHP-1 SH2 domain mutants with autophosphorylated EGF receptor *in vitro*.** Lysates of EGF receptor overexpressing 293 cells were incubated in the presence of EGF, ATP and kinase buffer to allow autophosphorylation of EGF receptors (*R*), control incubations were performed in the absence of either agent (*C*). The treated lysates were exposed to anti-EGF receptor mab425 beads (see "Experimental Procedures") to immobilize the EGF receptors. The beads were washed and subsequently incubated with lysates of 293 cells overexpressing the SHP-1 SH2 domain mutants, as indicated. Identical amounts of the different SHP-1 mutants were included in the incubation reactions. Bound SHP-1 mutants were visualized by SDS-polyacrylamide gel electrophoresis and immunoblotting using anti-SHP-1 CT antibody. Note that this antibody recognizes the C-terminal end of SHP-1 and therefore reacts with all mutants with the same sensitivity.

protein A-Sepharose beads (Pharmacia, Uppsala). For this, 1 mg antibody was incubated with 0.6 ml of phosphate-buffered saline-washed protein A-Sepharose for 1 h at 4 °C with end-over-end rotation. The beads were washed three times with phosphate-buffered saline and twice with 0.1 M sodium borate, pH 9.0 (borate buffer), and the volume was adjusted to 1.2 ml with borate buffer. Dimethyl pimelimidate (Sigma) was added to a final concentration of 20 mM, and the mixture was incubated for 30 min at room temperature. The reaction was quenched by two washes with 0.2 M ethanolamine, pH 8.0, and subsequent incubation in this solution overnight at 4 °C. The beads were sequentially washed with 50 mM glycine, pH 3.0, 1 M Tris-HCl, pH 7.4, and twice with phosphate-buffered saline. The association assays were carried out using these beads ("mab425 beads") as described previously (5). In brief, 20 μl of beads (1:1 suspension) were incubated with lysates of EGF receptor overexpressing or mock transfected 293 cells in the presence or absence of ATP and kinase buffer (see the figure legends). The beads were subsequently washed and exposed to PTP inhibitor-containing lysates of 293 cells overexpressing the different PTP constructs for 1 h at 4 °C. For the direct comparisons made in case of the SHP-1 SH2 domain mutants, the same amounts of expressed PTP were included in the incubations. Thereafter, the beads were washed, and the amount of bound PTP was evaluated by SDS-polyacrylamide gel electrophoresis and immunoblotting.

Effect of PTP Derivatives on EGF Receptor Phosphorylation Level—PTPs and EGF receptor were coexpressed in 293 cells as described previously (5). Lysate aliquots, normalized for EGF receptor amounts, were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with polyclonal anti-phosphotyrosine antibodies, to estimate the EGF receptor phosphorylation level in the absence or presence of the different PTPs. To judge the effect of PTP expression on phosphorylation of endogenous EGF receptors in COS-7 cells, EGF receptors were immunoprecipitated from PTP expression plasmid- or mock-transfected cells using mab425 anti-EGF receptor antibodies and subsequently analyzed by immunoblotting with anti-phosphotyrosine antibodies.

RESULTS

Effects of SHP-1 SH2 Domain Mutations on the Association with the EGF Receptor—To test the requirement of the SH2 domains of SHP-1 for its previously demonstrated association with the EGF receptor, Arg to Lys (RK) point mutants or amino-terminal truncation mutants of SHP-1 were generated as depicted in Fig. 1. Identical RK mutations have been shown before (20, 35) to severely impair the functionality of the concerned SH2 domains. The mutated SHP-1 molecules were expressed in 293 cells and analyzed for their association with immobilized, autophosphorylated EGF receptor. As shown in Fig. 2 (*lane 4 versus lane 2*), mutation of the N-terminal SH2 domain (SHP-1 R32K) strongly reduced the amount of EGF receptor-bound SHP-1, however, some association was still detectable. Very similarly, truncation of the N-terminal SH2 domain reduced but not abolished the association (Fig. 2, *lane 10*). The corresponding inactivating mutation of the C-terminal SH2 domain (SHP-1 R138K) reduced the amount of associated SHP-1 weakly but reproducibly (Fig. 2, *lane 6*). In four experiments the signal of associated SHP-1 R138K was 73 ± 4% compared to native SHP-1 (100%) as revealed by densitometric analysis. The double point mutant SHP-1 R32K,R138K or SHP-1 lacking both SH2 domains (SHP-1Δ SH2) were completely unable to associate with the EGF receptor (Fig. 2, *lanes 8* and *12*). Taken together, the data suggest that both SH2 domains are important for the association of SHP-1 with the EGF receptor; however, the C-terminal SH2 domain contributes to a lesser extent to the association than the N-terminal SH2 domain.

Effects of SHP-1 SH2 Domain Mutations on the Capacity to Dephosphorylate Coexpressed EGF Receptor—All SH2 domain mutants of SHP-1 exhibited catalytic activity. Lysates of 293 cells overexpressing SHP-1 or the various mutants displayed 1.4–2.2-fold PTP activity compared with mock-transfected cells when assayed toward the synthetic peptide substrate [^{32}P]Raytide (not shown). To analyze the effect of SH2 domain mutations on the functional interaction of SHP-1 with the EGF receptor, the various SHP-1 mutants were coexpressed with the EGF receptor in 293 cells. Subsequently, the cells were stimulated with EGF, and the autophosphorylation level of the EGF receptor coexpressed with the PTP mutants was compared to that of cells expressing EGF receptor in the absence of PTP or in the presence of SHP-1. As shown in Fig. 3*A* (*lane 3 versus lane 2*) and demonstrated before (4, 5), coexpression of EGF receptor with native SHP-1 clearly reduced the receptor autophosphorylation level, indicative of receptor dephosphoryl-

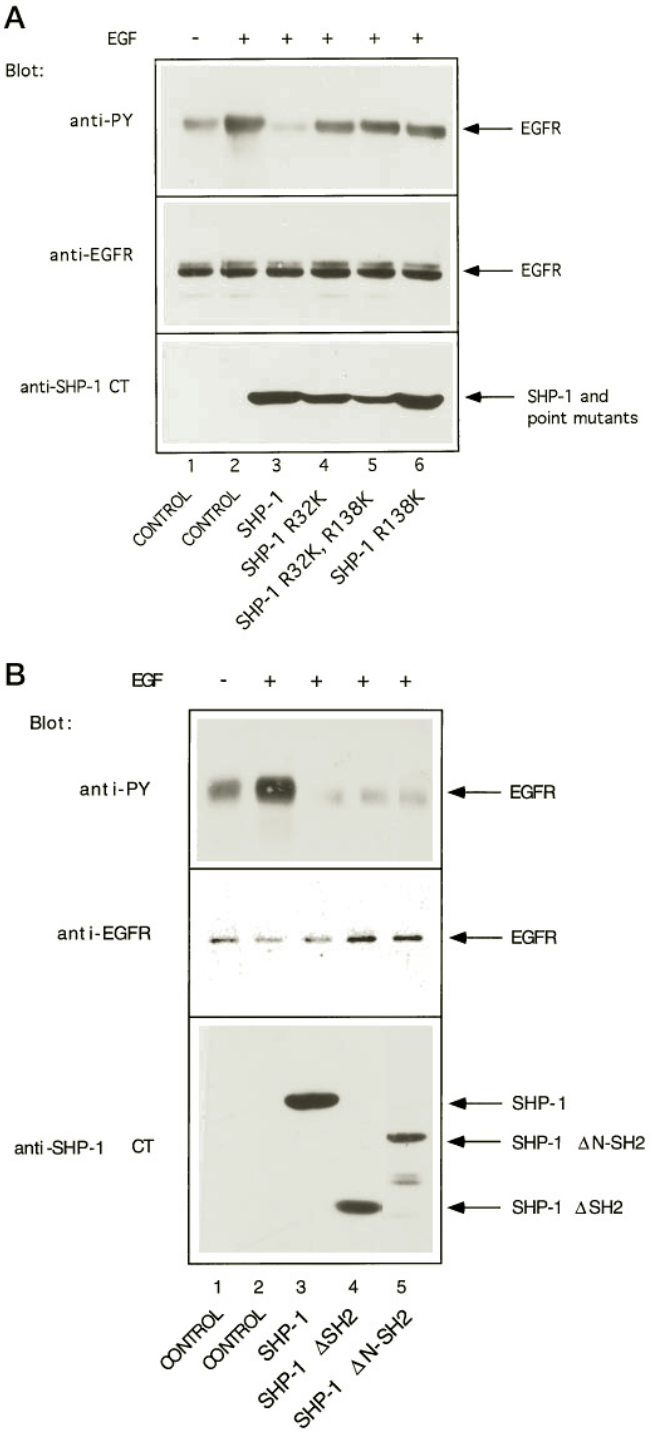

FIG. 3. **Dephosphorylating activity of SHP-1 SH2 domain mutants toward coexpressed EGF receptor.** Various SH2 domain mutants of SHP-1, as indicated, were coexpressed with EGF receptor in 293 cells. The cells were stimulated with EGF or not, as indicated. Cell lysates were prepared and analyzed for the phosphotyrosine level of the EGF receptor (*upper panel*). Control blots are shown to demonstrate comparable expression levels of receptor (*middle panel*) and SHP-1 mutants (*lowest panel*) in the different variants. *A*, analysis of SHP-1 point mutants; *B*, analysis of SHP-1 truncation mutants

ation by SHP-1. Inactivating point mutations in either the N-terminal or the C-terminal or both SH2 domains, strongly impaired the capacity of the respective SHP-1 mutants to dephosphorylate the EGF receptor (Fig. 3*A*, *lanes 4*, *6*, and *5*, respectively) although some residual activity cannot be excluded. Similar results were obtained, when the SHP-1 SH2 domain point mutants were expressed in COS-7 cells, and phosphorylation levels of stimulated endogenous EGF receptors were analyzed. Compared to the reduction of the phosphorylation level caused by expression of SHP-1 (29 ± 18% of control) SHP-1 R32K, SHP-1 R32K,R138K, and SHP-1 R138K reduced the receptor phosphorylation to 54 ± 24, 58 ± 20, and 58 ± 10%, respectively (means of four identical experiments). Thus, in the full-length SHP-1, both SH2 domains seem important for the capacity to dephosphorylate coexpressed EGF receptor.

SH2 domain truncation mutants were likewise analyzed for their activity in the same assay (Fig. 3*B*). Despite their impaired association with the EGF receptor demonstrated above, SHP-1ΔN-SH2 and SHP-1ΔSH2, exhibited activity toward the coexpressed receptor which was comparable to that of the native SHP-1.

To evaluate the possible role of the C-terminal SH2 domain for the activity of SHP-1ΔN-SH2, its derivative with inactivated C-terminal SH2 domain, SHP-1ΔN-SH2,R138K (Fig. 1) was analyzed for activity. SHP-1ΔN-SH2,R138K was fully active with respect to dephosphorylation of coexpressed EGF receptor (not shown). However, expression of the mutant in both, 293 and COS-7 cells gave rise to a degradation product of similar size as SHP-1ΔSH2, complicating interpretation of this result (see "Discussion").

Interaction of SHP-1/SHP-2 Chimera with the EGF Receptor—As demonstrated previously (5), SHP-1 and SHP-2 both associate with activated EGF receptor, however, only SHP-1 has the capacity for receptor dephosphorylation. The structural basis for this difference is unknown. Differential SH2 domain specificity, differential catalytic domain specificity (or both) and the structurally divergent C-terminal tails (see Fig. 1) might be important for the differential activity toward EGF receptor. We, therefore, generated a number of "swap" mutations leading to SHP-1/SHP-2 chimeric PTPs (Fig. 1) and analyzed their activity. All chimeric PTPs exhibited activity toward a synthetic phosphopeptide substrate: PTP activity in lysates of 293 cells overexpressing SHP-1, SHP-2 or the different PTP chimera was 1.3–3.1-fold elevated over the activity detectable in mock-transfected cells (not shown), demonstrating that the catalytic domains of the various constructs are functional. Also, all chimeric PTPs associated with autophosphorylated immobilized EGF receptor *in vitro* (Fig. 4), demonstrating the functionality of the SH2 domains. When coexpressed with EGF receptor in 293 cells, the chimeric molecules fell into two classes with respect to their activity toward the activated receptor: The constructs containing the SHP-2 catalytic domain with the SH2 domain of SHP-1 (with or without the "hinge" domain) failed to dephosphorylate the receptor (Fig. 5, lanes 5, 7 *versus* lane 1) as did native SHP-2 (lane 3). Thus, the SHP-1 SH2 domains cannot confer the capacity to dephosphorylate EGF receptor to SHP-2. All constructs with the catalytic domain of SHP-1, with the SH2 domains of SHP-2 (with or without hinge domain) or with the C-terminal tail of SHP-2 were active toward coexpressed EGF receptor (Fig. 5, *lanes 4*, *6*, *9*, and *11*) similar to native SHP-1 (*lane 2*). Identical results were obtained when the activity of all chimeric PTPs was analyzed toward the endogenous EGF receptor in COS-7 cells (not shown).

DISCUSSION

The SH2 domain PTP SHP-1 associates with autophosphorylated EGF receptor and might contribute to EGF receptor dephosphorylation in epithelial cells. In the present study we investigated structural elements of SHP-1 for their importance in the SHP-1 EGF receptor interaction. Both the N-terminal and the C-terminal SH2 domain of SHP-1 contribute to the

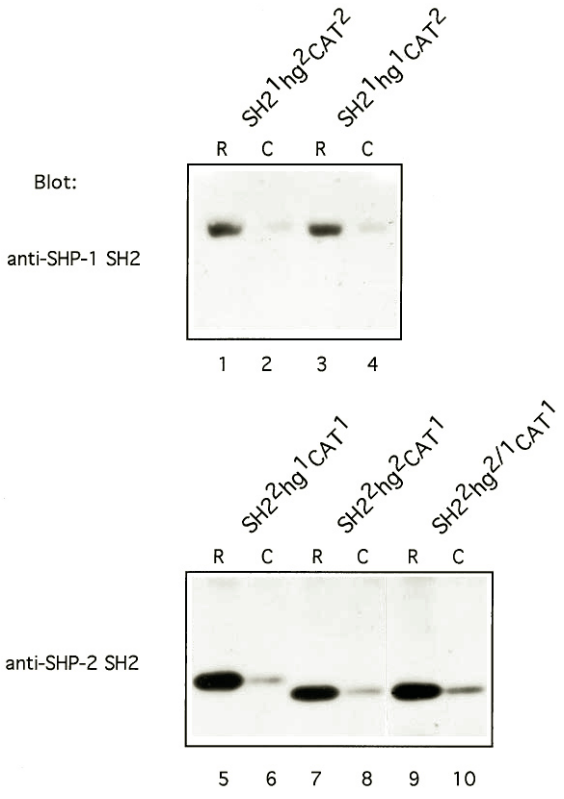

FIG. 4. **Association of SHP-1/SHP-2 chimeric PTPs with the EGF receptor.** The experiments were carried out as described in the legend to Fig. 2, except that for the controls (*C*) anti-EGF receptor mab425 beads were loaded with ATP-treated lysates of nontransfected 293 cells. Low levels of endogenous EGF receptors led to some background binding of the PTPs in this setting. To detect the bound chimera, antibodies specific for SHP-1 or SHP-2 SH2 domains, anti-SHP-1 SH2 and anti-SHP-2 SH2, respectively, were employed.

association with phosphorylated EGF receptor *in vitro*; however, association via the N-terminal SH2 domain seems to occur more efficiently. The two SH2 domains of SHP-1 have been shown to have different phosphopeptide binding specificity (26, 28, 35). Still, the N-terminal SH2 domain can bind to an FcγIIB1 receptor-derived phosphopeptide sequence which has optimal affinity for the C-terminal SH2 domain (35). The phosphopeptide sequences of the EGF receptor involved in SHP-1 binding have not yet been identified. It will be interesting to see whether a common or distinct binding sites are utilized by the two SHP-1 SH2 domains. One might also speculate that the binding involves simultaneous occupation of both SH2 domains, a process leading to particularly efficient activation of SHP-2 (36). From the currently available data it cannot formally be excluded that as yet unidentified further proteins participate in the complex formation of EGF receptor and SHP-1 detected in our assays. However, to mediate dephosphorylation in the high level coexpression setting, a putative mediator for the interaction would be needed at abundant levels, which makes an interaction mechanism involving unidentified third components less likely. Clarifying this point as well as identification of the structural elements of the EGF receptor involved in the SHP-1 interaction are required for a further understanding of the association mechanism. Binding of SHP-1 via the C-terminal part to the insulin receptor has been shown (37). For the EGF receptor, we observed very little if any association of SHP-1 in the absence of intact SH2 domains, rendering an association involving the C terminus unlikely in this case.

Inactivating point mutations of both SH2 domains not only impaired association with the EGF receptor, but also impaired the capacity of SHP-1 to dephosphorylate the coexpressed receptor in intact cells. Due to inherent experimental errors, these assays yielded only qualitative results and did not allow to draw any conclusions about subtle differences between the effects of the different PTP mutants. Therefore, we were unfortunately unable to discern whether inactivation of the N-terminal, of the C-terminal SH2 domain, or of both contributes differently to the impairment of receptor-directed activity. *In vitro* studies with recombinant receptor and PTPs are intended to investigate such likely differences. Truncation of the SH2 domains likewise gradually abrogated association; however, the truncated SHP-1 molecules exhibited activity toward the receptor which was indistinguishable from that of native SHP-1 within the limitations of the experimental design. A mutant with deleted N-terminal SH2 domain and inactivated C-terminal SH2 domain (SHP-1ΔN-SH2,R138K) was likewise fully active, suggesting that the activation by N-terminal truncation overrides the contribution of the C-terminal SH2 domain to activation observed with the full-length SHP-1. However, degradation products resembling SHP-1ΔSH2 in cells overexpressing SHP-1ΔN-SH2,R138K could potentially contribute to the observed dephosphorylation and complicate interpretation of this result. A final clarification of this point has, therefore, to await analysis of activation of the purified recombinant mutant PTP by corresponding EGF receptor phosphopeptides.

Our data are in line with the current concept of SHP-1 activation. For the full-length enzyme, receptor association is required for activation. However, SHP-1 with truncated SH2 domains is constitutively active and can dephosphorylate the receptor in the absence of any association. The latter finding might partially be related to high level overexpression in our cell system. On the other hand, the intriguing implication arises that SHP-1, once activated by binding to an appropriate phosphoprotein could act on other substrates in proximity without the need of a direct association. Such type of interaction has been proposed for SHP-1 and the CSF-1 receptor (25).

As shown previously (4, 5), SHP-2 exhibits no detectable activity toward coexpressed EGF receptor in 293 cells. This finding raised the interesting question, whether the differential activity of SHP-1 and SHP-2 is mediated by the SH2 domains, the catalytic domain, or the C terminus of the molecules, where the protein sequences are most divergent (Fig. 1). To address this question, we generated chimeric PTPs containing the SH2 domains of one PTP and the catalytic part of the other PTP or vice versa and also exchanged the C-terminal tail of SHP-1 for the one of SHP-2. Such a strategy had been proposed earlier by Sun and Tonks (38) to dissect structure-function relationships in this PTP family, but has to our knowledge not yet been applied. Clearly, the SH2 domains of SHP-1, although mediating association of the respective chimera (SH2^{1}hg^{2}CAT^2 and SH2^{1}hg^{1}CAT^2), are not sufficient to confer activity toward the EGF receptor. Vice versa, molecules with the SH2 domains of SHP-2 and the catalytic part of SHP-1 (SH2^{2}hg^{2}CAT^1, SH2^{2}hg^{1}CAT^1, SH2^{2}hg$^{2/1}$CAT^1) are similarly active as native SHP-1 toward the receptor. In this setting it is possible that the SH2 domains of SHP-2 mediate receptor association (as shown) leading to subsequent activation of the chimeric PTPs. If this assumption is correct, the possibly distinct phosphopeptide specificity of the SHP-2 SH2 domains has no influence on the capacity of the chimera to dephosphorylate the receptor. Alternatively, the SH2 domains of SHP-2 might be unable to participate in a regulatory interaction with the C-terminal part of SHP-1, as occurs in native SHP-1, which would in turn lead to a constitutively active chimeric PTP in a similar manner as does truncation of the SH2 domains. Kinetic

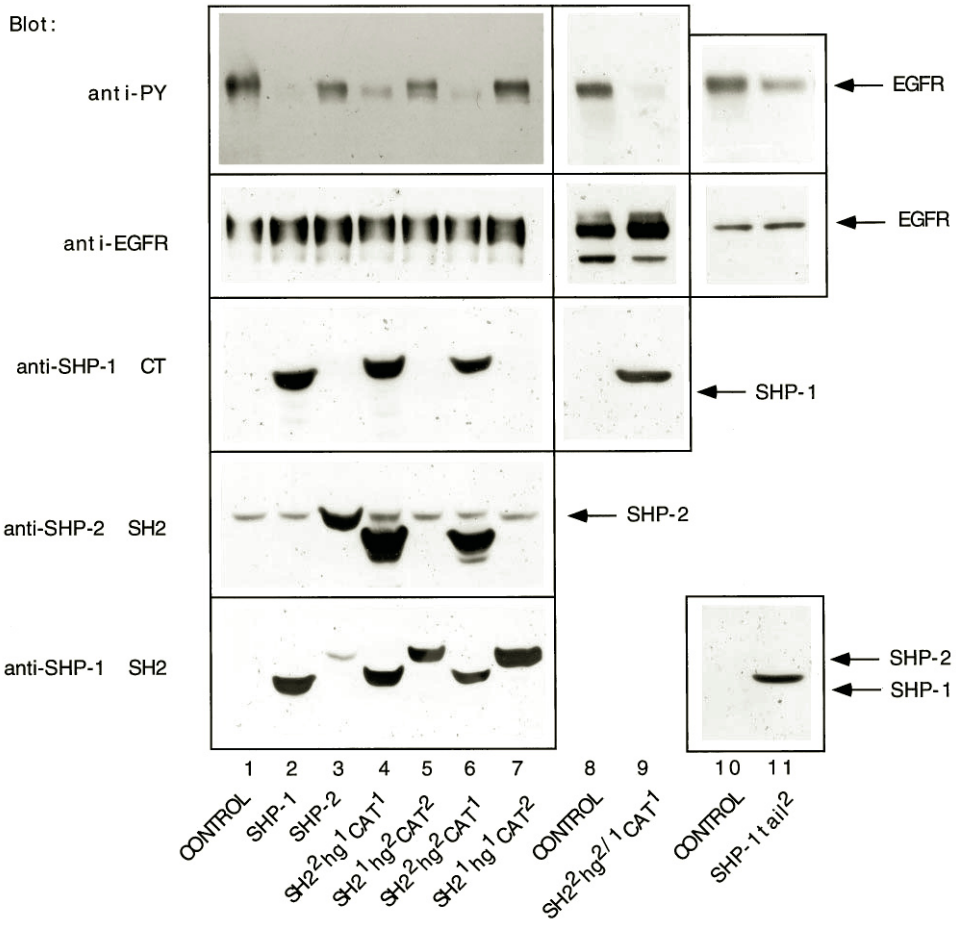

FIG. 5. **Dephosphorylating activity of SHP-1/SHP-2 chimeric PTPs toward coexpressed EGF receptor.** Various SHP-1/SHP-2 chimeric PTPs were coexpressed with EGF receptor in 293 cells and the cells stimulated with EGF. Cell lysates were prepared and analyzed for the phosphotyrosine level of the EGF receptor (*upper panel*). Control blots are shown to demonstrate comparable expression levels of receptor (*second panel*) and PTP mutants (*third* to *fifth panel*) in the different variants.

investigation of the recombinant chimera is required to resolve this issue. Exchange of the C-terminal tail of SHP-1 for that of SHP-2 did not impair the activity toward the EGF receptor. Thus, the distinct C-terminal sequences are not important for the differential enzymatic activity of SHP-1 and SHP-2 in this system.

Taking all data together, the catalytic domain specificity seems to be the prime determinant for the potent activity of SHP-1 toward the EGF receptor, whereas the SH2 domains play a major role in activity regulation rather than in substrate targeting of SHP-1.

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