Molecular Mechanism for a Role of SHP2 in Epidermal Growth Factor Receptor Signaling
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The Src homology 2-containing phosphotyrosine phosphatase (SHP2) is primarily a positive effecter of receptor tyrosine kinase signaling. However, the molecular mechanism by which SHP2 effects its biological function is unknown. In this report, we provide evidence that defines the molecular mechanism and site of action of SHP2 in the epidermal growth factor-induced mitogenic pathway. We demonstrate that SHP2 acts upstream of Ras and functions by increasing the half-life of activated Ras (GTP-Ras) in the cell by interfering with the process of Ras inactivation catalyzed by Ras GTPase-activating protein (RasGAP). It does so by inhibition of tyrosine phosphorylation-dependent translocation of RasGAP to the plasma membrane, to its substrate (GTP-Ras) microdomain. Inhibition is achieved through the dephosphorylation of RasGAP binding sites at the level of the plasma membrane. We have identified Tyr992 of the epidermal growth factor receptor (EGFR) to be one such site, since its mutation to Phe renders the EGFR refractory to the effect of dominant-negative SHP2. To our knowledge, this is the first report to outline the site and molecular mechanism of action of SHP2 in EGFR signaling, which may also serve as a model to describe its role in other receptor tyrosine kinase signaling pathways.

The process of protein tyrosine phosphorylation and dephosphorylation is central to growth factor, cytokine, and integrin signal transduction. The enzymes that catalyze phosphorylation reactions are either the receptors themselves with intrinsic tyrosine kinase activity, known as receptor tyrosine kinases (RTKs), or cytoplasmic tyrosine kinases. The substrates for these kinases are the receptors themselves and/or downstream signaling proteins. Some of the most extensively studied RTKs include the epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor (PDGFR), and the fibroblast growth factor receptor (FGF) receptor (13, 21, 22, 27, 29, 37, 42, 49, 55, 57). Upon the binding of a cognate ligand, RTKs dimerize and autophosphorylate tyrosine residues in their cytoplasmic domain or tyrosylphosphorylate downstream substrates (5, 6, 9, 11, 17, 20, 25, 30, 34, 41). More often than not, phosphorylated tyrosine residues serve as binding sites for Src homology 2 (SH2)-domain-containing signaling proteins (38). These interactions mediate the formation of multiprotein signaling complexes by which signals are transduced down the cascade. At least two known functions are effected by these interactions: recruitment of enzymes to substrate microdomains and/or induction of enzyme activity (3, 16, 18, 26, 30, 41, 43; M. Adachi, E. H. Fischer, J. Ihle, K. Imai, F. Jirik, B. Neel, T. Pawson, S. Shen, M. Thomas, A. Ullrich, and Z. Zhao, Letter, Cell 85:15, 1996). The binding of a cytokine to its cognate receptor also elicits similar signaling events. However, tyrosine phosphorylation of proteins, including the receptors themselves, is carried out by associated cytoplasmic tyrosine kinases, since these receptors lack intrinsic tyrosine kinase activity (7, 58, 59).

Recent advances in the area of phosphotyrosylphosphatases (PTPs) indicate that tyrosine dephosphorylation is as important as tyrosine phosphorylation in the transduction of signals elicited by growth factors and cytokines. Among the hundred or slightly more transmembrane and nontransmembrane phosphotyrosine (pY) phosphatases known to date, the ubiquitously expressed mammalian cytoplasmic phosphatase termed SH2 phosphatase 2 (SHP2) is a well-known positive effecter of tyrosine kinase signaling (23, 32, 53, 54). Also, Corkskrew, the Drosophila melanogaster counterpart of SHP2, is an essential positive effecter of the Torso RTK signaling (8). On the other hand, SHP1, which is expressed primarily in hematopoietic cells, is a negative regulator of tyrosine kinase signaling, although it has significant structural similarity to SHP2.

SHP2 possesses two tandemly arranged SH2 domains in its N-terminal region and a phosphatase domain in its C-terminal region (15, 16). It also possesses a stretch of proline-rich sequences and tyrosine phosphorylation sites in its extreme C-terminal region. Both the SH2 domains and the phosphatase domain have been shown to be absolutely essential for the biological activity of SHP2 (10). However, no biological role has been definitively ascribed to the tyrosine phosphorylation sites or the proline-rich region. Deletion of the N-SH2 or mutation of the conserved cysteine residue to serine in the active site of the phosphatase domain eliminates the biological activity of SHP2 (15, 16). Transgenic mice homozygous for the N-SH2 deletion mutation die in the uterus before day E10.5 from multiple defects in mesoderm development (4, 39, 40). Also, microinjection of the N-SH2 deletion or phosphatase-dead mutant mRNA into Xenopus laevis eggs causes abnormal embryonal development (48). These findings have established that SHP2 is a requisite for normal growth and development.

SHP2 interacts directly with autophosphorylated RTKs such as EGFR and PDGFR or indirectly via tyrosine-phosphorylated adaptor proteins through its SH2 domains (9, 18, 25, 26, 41, 52). These interactions have been shown to be essential for RTK signaling. However, how these interactions contribute...
positively to tyrosine kinase signaling is virtually unknown. Moreover, how PY dephosphorylation by SHP2 drives the signaling message forward and what substrates are acted upon by SHP2 are unknown. Recently, we developed an efficient substrate-trapping mutant of SHP2 and demonstrated that one of its physiological substrates is EGFR (2). In the present report, we describe the molecular mechanism of SHP2 in the mitogenic signaling pathway by using EGFR as a model. We find that SHP2 acts upstream of Ras in the EGFR pathway and functions by increasing the half-life of activated Ras (GTP-Ras) in the cell. It does so by interfering with the process of Ras inactivation (conversion to GDP-bound form) catalyzed by GTPase-activating protein (RasGAP) via inhibition of tyrosine phosphorylation-dependent translocation of RasGAP to the plasma membrane (PM). Furthermore, we demonstrate that Y992 of the EGFR is a negative-regulatory autophosphorylation site that acts as a binding site for RasGAP.

MATERIALS AND METHODS

Cells, cell culture, and antibodies. The cell types used in this study were COS-1, NIH 3T3, and A431. COS-1 and NIH 3T3 were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, whereas A431 cells were grown in Dulbecco’s modified Eagle’s medium containing only 5% fetal calf serum. All cell types were maintained at 37°C with 5% CO2, EGF, PDGF, and FGF-1 were purchased from GIBCO-BRL; anti-phospho-ERK1/2 or pan-ERK2 antibodies were from New England Bioslabs; anti-RasGAP antibodies were from Upstate Biotechnology; anti-PTP1D and anti-Ras monoclonal antibodies were from Transduction Laboratories, anti-pY monoclonal antibody (4G10) was a gift from D. Morrison; and anti-T7 tag monoclonal antibody was purchased from Zymed, whereas the AttoPhos kit from Amersham. For fluorescent quantitation of phosphoprotein band intensities, alkaline phosphatase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Zymed, whereas the AttoPhos fluoroscent substrate was from Promega.

Plasmid construction and site-directed mutagenesis. Construction of plasmids that express wild-type SHP2 (WT-SHP2) or C459S-SHP2 was described previously (36). The R465E-SHP2 and the C459S/R465S-SHP2 mutants, hereafter referred to as R/E and C/S-R/E, respectively, were produced by introducing the indicated point mutation into the WT or the C459S mutant with complementary primers that span the signature motif of the PTP domain. The sense primer for the R/E was 5’-CTGACGTTGCAGTTGGAGCAGGAGCTGTTCTTG-3’ and the antisense primer was 5’-CAACATTACGCGCACTGCGACAC-3’. For the C/S-R/E, the sense primer was 5’-CCAGCTGCGTTACGAGCTGCTGCTGAC-3’ and the antisense primer was 5’-CCAAGCTGTTGAGCAGGAGCTGCTGCTGAC-3’. The Tyr-to-Pho point mutants of the EGFR were produced using the following primers: 5’-GTTGGGTTGATTCTGACAGAGCTCTCCATCCACGAGGGGTCCTC-3’ and 5’-GAAGCCGCCTGTCGAGGAGGAACTCGCTGAG-3’. To study EGF-induced translocation of RasGAP to the PM in the presence or absence of the WT- or the R/E-SHP2, PMFs of COS-1 or A431 cells were prepared as follows. Either cell line was transfected with two different concentrations of WT- or R/E-SHP2, incubated for ~30 h, serum starved for ~12 h, stimulated with 100 ng of EGF/ml for 10 min, and washed once with ice-cold PBS and once with a 10 mM Tris-HCl (pH 7.5) hypotonic buffer. Cells were then washed with a hypotonic buffer (10 mM Tris-HCl [pH 7.5], 1 mM MgCl2, and 1 mM protease inhibitor cocktail) for 10 min, collected by scraping, and lysed by burning (25 strokes with a tight pestle). Lysates were cleaned of cell nuclei and unbroken cells by centrifugation at 10,000 × g for 10 min in a microcentrifuge at 4°C. The supernatant was transferred to polycarbonate tubes and centrifuged at 100,000 × g for 30 min. The pellets were washed once with PBS and then solubilized in the cell lysis buffer described above. PMF lysates were analyzed by direct immunoblotting or subjected to affinity precipitation and immunoblot analysis as described for individual experiments.

Affinity precipitation and far-Western analysis. The SH2 domains of RasGAP fused to GST (kindly provided by Bruce Myers, University of Connecticut) were used for both procedures. Preparation of bacterial lysates and capture of GST-SH2-GAP utilizing alkaline phosphatase-conjugated anti-rabbit or anti-mouse secondary antibody was described above. PMF lysates were analyzed by direct immunoblotting or subjected to affinity precipitation and immunoblot analysis as described for individual experiments.

Production of NIH 3T3 cells stably expressing R/E-SHP2. A retroviral vector (SH2/1AIRE/SHP2) was used to produce viruses that express SHP2 proteins. Production of NIH 3T3 cells expressing the WT or the R/E protein was as described previously (1).
RESULTS

In this work, we investigated the mechanism of action of SHP2 in the EGFR signaling pathway. Initially, we examined if C459S-SHP2, frequently referred to as dominant-negative SHP2, inhibits EGF-induced ERK1/2 activation in our system. To narrow our area of focus in the EGFR-Ras-ERK signaling cascade, we also assessed the effect of this mutant on constitutively active Ras (V12–H-Ras)-induced ERK1/2 activation. For the purpose of simplicity, we refer to C495S-SHP2 as the C/S protein and to WT-SHP2 as the WT protein hereinafter.

The C/S protein inhibits EGFR-induced ERK1/2 activation. It was previously shown that the C/S protein inhibits growth factor- or cytokine-induced ERK1/2 activation (10, 28, 31, 44). Here, we wanted to reproduce this observation in our system as a starting point for our investigation of the mechanism of action of SHP2 in EGFR signaling. Thus, we expressed various amounts of the WT or C/S protein in COS-1 cells and assessed EGF-induced ERK1/2 phosphorylation with a phosphospecific antibody. The C/S protein inhibited ERK1/2 phosphorylation in a concentration-dependent manner. Maximum inhibition was observed at an expression level approximately five times the endogenous level. On the other hand, expression of the WT protein caused no inhibition but rather a slight enhancement (Fig. 1A, top). Reprobing with anti-pan-ERK2 antibody showed comparable amounts of protein in all lanes, which also confirmed the level of ERK activation in the context of phosphorylation-dependent band shift (Fig. 1A, middle). Further reprobing the membrane with anti-SHP2 antibody showed similar expression levels of both the WT and C/S proteins (Fig. 1A, bottom). Therefore, these findings are consistent with previous reports that the C/S protein inhibits EGF-induced activation of the ERK1/2 mitogen-activated protein kinase.

The results presented in Fig. 1A showed inhibition of EGF-induced ERK1/2 activation by the C/S protein. However, they did not show whether the inhibition was due to blockade of signal transduction through the cascade or to premature termination of the signal before it reached a level sufficient to cause cellular responses. We reasoned that time course studies with EGF stimulation might provide an answer to these questions. We overexpressed a constant amount of the WT or the C/S protein in COS-1 cells and then stimulated the cells with EGF for various periods ranging from 2 min to 4 h; the vector alone was used as a control. Total-cell lysates prepared from these cells were analyzed for ERK1/2 phosphorylation as described for Fig. 1. In cells transfected with the C/S protein, phosphorylation of ERK1/2 occurred at 2 min, but this phosphorylation was short-lived and reached a basal level before 10 min (Fig. 1B, top). In cells transfected with the vector, the level of ERK1/2 phosphorylation was the same for about 60 min but declined after that. On the other hand, in cells transfected with the WT protein, the duration of ERK1/2 phosphorylation was increased (at least 4 h) and the intensity of the signal was slightly enhanced. Reprobing the membrane with anti-pan-ERK2 antibody showed that there were comparable amounts of protein in all lanes (Fig. 1B, middle). Further reprobing the membrane with anti-SHP2 antibody showed comparable expression levels of the WT and C/S proteins (Fig. 1B, bottom).

In order to better characterize ERK activation, we quanti-
tated ERK1/2 phosphorylation by employing the immunofluorescence quantitation technique (see Materials and Methods). At 2 min, the level of ERK1/2 phosphorylation was five, six, and four times the basal level in the vector and the WT- and the C/S protein-expressing cells, respectively (Fig. 1C).

And at 10 min, phosphorylation reached maximum levels in the vector and the WT-expressing cells, approximately 6.0 and 7.0 times the basal level, respectively. This continued for at least 4 h in the WT-expressing cells but gradually declined in the control. On the other hand, the level of ERK1/2 phosphorylation in cells expressing the C/S protein was only 1.8 times the basal level at 10 min, suggesting that the signal was prematurely terminated. The results with the C/S protein indicated that ERK1/2 phosphorylation does occur but becomes rapidly down regulated. Thus, SHP2 increases the duration of EGFR-induced ERK1/2 activation with slight enhancement.

The C/S protein has no effect on V12–H-Ras-induced ERK1/2 activation. The results in Fig. 1 showed that SHP2 modulates EGFR-induced ERK1/2 activation. However, the site of action of SHP2 in the Ras-ERK signaling cascade was not clear. With the aim of pinpointing the site of action, we assessed the effect of the C/S protein on V12–H-Ras-induced ERK1/2 activation. It was reasoned that inhibition would put SHP2’s site of action downstream, whereas the absence of inhibition would put it upstream of or parallel to Ras in the RTK-Ras-ERK signaling cascade. To address these questions, we transiently co-expressed various amounts of the C/S protein and a constant amount of V12–H-Ras in COS-1 cells (Fig. 2, middle and bottom) and examined ERK1/2 phosphorylation by using the phosphospecific antibody as described above for Fig. 1. In contrast to its effect on ligand-induced activation, the C/S protein showed no inhibitory effect on V12–H-Ras-induced ERK1/2 activation even at the highest concentration used (Fig. 2, top). Reprobing the membrane with anti-T7 (for Ras) antibody showed that equivalent amounts of the Ras protein were expressed in all transfectants. Further reprobing with anti-SHP2 antibody showed that the C/S protein was expressed at an increasing level. These results suggested that the site of action of SHP2 in the Ras-ERK signaling cascade is upstream of or parallel to Ras.

The C/S protein inhibits EGF-induced Ras activation. Experiments with V12–H-Ras suggested that SHP2 acts at the level of Ras in the Ras-ERK signaling cascade, but they could not differentiate between the possibilities, upstream of or parallel to Ras. Thus, we investigated the effect of the C/S protein on Ras activation. The RBD of Raf-1 fused to GST (51) was used to assay for EGF-induced Ras activation (see Materials and Methods for details). Two different concentrations of the WT or the C/S protein and a constant amount of WT–H-Ras were coexpressed in COS-1 cells, and lysates prepared from

**FIG. 2.** Effect of C/S protein on V12–H-Ras-induced ERK1/2 phosphorylation. COS-1 cells were transfected with a constant amount of an expression vector for V12–H-Ras and a varying amount for the C/S protein. Following transfection, cells were incubated for 36 h in 10% serum-containing medium and for a further 12 h in the absence of serum. Total-cell lysates containing equivalent amounts of protein were separated on a 10% polyacrylamide gel and then analyzed by immunoblotting with the indicated antibodies. IB, immunoblot.

**FIG. 3.** Effect of the C/S protein on Ras activation. (A) COS-1 cells were cotransfected with two different concentrations of an expression vector for SHP2 (WT or the C/S protein) and H-Ras. (B) Time course studies of Ras activation. COS-1 cells were cotransfected with a constant amount of WT–H-Ras and the WT or C/S protein. In either case, cells were incubated for 36 h in 10% serum-containing medium, serum starved for 12 h, and then stimulated with 100 ng of EGF/ml for 10 min in panel A or the indicated time points in panel B. Total-cell lysates (TCL) prepared from these cells were subjected to affinity precipitation (AP) with GST-RBD of Raf-1, resolved on a 10% polyacrylamide gel, and then analyzed by immunoblotting (IB) with T7 tag antibody for Ras. To examine expression of SHP2 and Ras proteins, total-cell lysates were directly analyzed with the indicated antibodies.
these cells were assayed for GTP-Ras levels. Overexpression of the C/S protein inhibited, whereas the WT protein enhanced, EGF-induced Ras activation (GDP-Ras to GTP-Ras) in a concentration-dependent manner (Fig. 3A, top). Probing total-cell lysates with anti-T7 tag (for Ras) and anti-SHP2 antibodies showed that all the proteins were expressed as expected (Fig. 3A, middle and bottom, respectively). Similar results were obtained when the EGF-induced endogenous GTP-Ras level was determined (data not shown). These results suggested that SHP2 functions upstream of Ras.

Data presented in Fig. 1B and C show that the C/S protein did not block initial activation of ERK1/2 but shortened its duration. These results led us to conduct time course studies of EGF-induced Ras activation (GDP-Ras to GTP-Ras) in a concentration-dependent manner (Fig. 3A, top). Probing total-cell lysates with anti-T7 tag (for Ras) and anti-SHP2 antibodies showed that all the proteins were expressed as expected (Fig. 3A, middle and bottom, respectively). Similar results were obtained when the EGF-induced endogenous GTP-Ras level was determined (data not shown). These results suggested that SHP2 functions upstream of Ras.

Data presented in Fig. 1B and C show that the C/S protein did not block initial activation of ERK1/2 but shortened its duration. These results led us to conduct time course studies of EGF-induced Ras activation to see if it correlated with ERK activation. We coexpressed the WT or C/S protein with WT–H-Ras in COS-1 cells and determined EGF-induced GTP-Ras levels at the same time points used in the ERK time course studies. In the control cells, the level of GTP-Ras reached the maximum attainable in 2 min and declined after 10 min. On the other hand, the level of GTP-Ras in WT cells was elevated throughout the time points tested. In contrast to the control and WT cells, the level of GTP-Ras in the C/S cells was approximately 30 to 40% of that of the control cells at 2 and 10 min and declined to basal level after that. Immunoblot analysis of corresponding total-cell lysates with anti-SHP2 and anti-T7 tag (Fig. 3A, top and bottom, respectively) showed that all transfected proteins were expressed as expected. These results demonstrated two aspects that were not apparent in the results presented in Fig. 3A: initial Ras activation occurred in the C/S cells but became down regulated rapidly due to lack of SHP2 activity and GTP-Ras accumulated in WT-SHP2-transfected cells possibly due to an increase in its half-life. Thus, ERK1/2 and Ras activations were highly correlated.

SHP2 regulates ligand-induced RasGAP translocation to the PM. The results presented in Fig. 3 demonstrate that SHP2 was required for sustained Ras activation. Based on these findings, we postulated that SHP2 was modulating either the activation of Ras catalyzed by son of sevenless (SOS) or the down regulation catalyzed by RasGAP. To discriminate between these possibilities, we determined EGF-induced endogenous GTP-Ras level was determined (data not shown). These results suggested that SHP2 functions upstream of Ras.

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reasoned that the C/S protein might not be an ideal dominant-negative mutant to assay subtle biochemical changes since it has some substrate-trapping ability. It was thus necessary to produce dominant-negative mutants of SHP2 devoid of the substrate-trapping effect. Previously, it was reported that the R residue in the signature motif (R...HCGXXGR/T/S...) is essential for substrate binding by PTPs (59). Hence, we replaced the R residue (positively charged) with E (negatively charged) in the WT and the C/S backgrounds to produce R465E-SHP2 and C459S/R465E-SHP2, hereinafter referred to as the R/E and C/S-R/E proteins, respectively. We evaluated these mutants in several ways: their effect on ERK1/2 activation, their ability to autodephosphorylate (46), and their capacity for PTP domain-mediated interactions. Both the R/E and the C/S-R/E proteins inhibited EGF-induced ERK1/2 activation comparably to the C/S protein (Fig. 4A, top). Anti-pan-ERK2 immunoblotting showed approximately equal amounts of protein in all lanes (Fig. 4A, second panel). Also, anti-SHP2 blotting showed that the different SHP2 proteins were expressed at approximately five times the level of the endogenous protein (Fig. 4A, third panel). Furthermore, anti-pY blotting of total-cell lysates indicated that, as in the C/S protein, the R/E and the C/S-R/E proteins were unable to autodephosphorylate (Fig. 4A, bottom), suggesting that the R-to-E mutation had disabled the PTPase activity of SHP2.

We chose the R/E protein for further characterization simply because the R/E protein was a single mutant with an inhibitory effect equal to that of the C/S-R/E protein. First, we expressed the WT, C/S, or R/E protein in COS-1 cells and then conducted immunoprecipitation with anti-SHP2 and immunoblotting with anti-EGFR to see whether the R/E protein could have PTP domain-mediated interactions; the C/S protein partially traps the EGFR (2). Although expression of each SHP2 protein was comparable, only the C/S protein showed some trapping of the EGFR. The amount of EGFR that precipitated with the R/E protein was identical to that with the WT protein, reflecting only SH2 domain-mediated interactions (data not shown). To further characterize the R/E protein, we expressed the WT or R/E protein in NIH 3T3 cells that respond to PDGF and FGF in ERK1/2 activation. Time course studies showed that the R/E protein shortened the duration of PDGF- and FGF-induced ERK1/2 activation in a manner similar to that of the C/S protein for EGF-induced ERK1/2 activation in COS-1 cells (Fig. 4B and C, top panel). Reprobing with anti-pan-ERK antibody showed comparable amounts of protein in all lanes (middle panels, Fig. 4B and C). Further reprobing with anti-SHP2 antibody indicated that the WT and the R/E proteins were expressed about four to five times more than the endogenous protein (bottom panels, Fig. 4B and C). Thus, the R/E protein is a better dominant-negative mutant since it lacks the partial trapping ability of the C/S protein while preserving an inhibitory effect on ERK1/2 activation induced by the indicated growth factors.

Using the newly developed dominant-negative mutant of SHP2, the R/E protein, we investigated its effect on EGF-induced translocation of RasGAP to the PM. PMFs were prepared from COS-1 cells transfected with the vector, the WT, or the R/E protein (A) or from A431 cells infected with a retrovirus expressing the same set of proteins (B). In both panels A and B, samples of PMFs containing equivalent amounts of protein were separated on a 10% polyacrylamide gel and then analyzed with anti-RasGAP antibody (top), anti-EGFR antibody as a loading control (middle), or anti-SHP2 antibody to show expression (bottom). IB, immunoblot.

FIG. 5. SHP2 regulates EGF-induced translocation of RasGAP to the PM. Immunoblot analysis of PMFs prepared from COS-1 cells transfected with the vector, the WT, or the R/E protein (A) or from A431 cells infected with a retrovirus expressing the same set of proteins (B). In both panels A and B, samples of PMFs containing equivalent amounts of protein were separated on a 10% polyacrylamide gel and then analyzed with anti-RasGAP antibody (top), anti-EGFR antibody as a loading control (middle), or anti-SHP2 antibody to show expression (bottom). IB, immunoblot.
of RasGAP on PMFs prepared from COS-1 cells transfected with the vector or the WT or R/E protein. Captured proteins were separated on a 10% polyacrylamide gel and then analyzed by immunoblotting first with anti-pY and second with anti-EGFR antibodies (see Materials and Methods for details). A prominent phosphoprotein of the size of the EGFR was detected in cells overexpressing the R/E protein (Fig. 6A, top). On the other hand, a significantly smaller amount of this protein was detected in the control or the WT cells. No signal was detected in the unstimulated control cells, suggesting that the interaction required tyrosine phosphorylation. Reprobing the same membrane with anti-EGFR antibody confirmed that it was the EGFR (Fig. 6A, top). On the other hand, a significantly smaller amount of this protein was detected in the control or the WT cells. No signal was detected in the unstimulated control cells, suggesting that the interaction required tyrosine phosphorylation. Reprobing the same membrane with anti-EGFR antibody confirmed that it was the EGFR (Fig. 6A, top). To evaluate if the observed differences were due to differences in total protein levels, total PMFs were analyzed by immunoblotting. Anti-pY blotting showed that the EGFR was highly tyrosine phosphorylated in all stimulated cells with a moderate increase in cells expressing the R/E protein (Fig. 6B, top). Reprobing the membrane with anti-EGFR antibody revealed that the total EGFR protein level was comparable in all lanes (Fig. 6B, middle). Further reprobing with anti-SHP2 antibody showed that expression levels of the WT and R/E proteins were comparable (Fig. 6B, bottom). These results demonstrate that RasGAP interacts with the EGFR via its SH2 domain depending on the presence or absence of specific pY residues and that these pYs could be target substrates of SHP2.

The results presented above cannot rule out the possibility of an indirect interaction via an intermediate that could bind to both RasGAP and the EGFR. To discriminate between these possibilities, we conducted a far-Western analysis of the remaining PMFs using the GST-SH2-GAP (A), or directly separated on a 10% polyacrylamide gel (B and C). In panels A and B, the membranes were probed first with anti-pY antibody and second with anti-EGFR after stripping. Membrane B was further probed with anti-SHP2 antibody to show expression of the different SHP2 proteins. In panel C, total PMFs were analyzed by far-Western blotting with 10 μg of GST-SH2-GAP/ml as described in Materials and Methods. Membrane C was further reprobed with anti-EGFR antibody to show the presence of approximately equal amounts of the EGFR protein in all lanes. IB, immunoblot; AP, affinity precipitation.

Phospho-Y992 (pY992) of EGFR is the target substrate of SHP2. Our recent report (2) and the results presented above suggested that SHP2 targets specific pY residues on the EGFR, which serve as binding sites for RasGAP. However, they did not show which pY residue or residues out of the five or more autophosphorylation sites in the C-terminal region were target substrates of SHP2. To address these questions, we produced single Tyr-to-Phe mutants of EGFR at the five known autophosphorylation sites (Fig. 7A). These constructs were transfected into NIH 3T3 cells, which express undetectable amounts of endogenous EGFR. Immunoblot analysis of total-cell lysates from these cells showed comparable expres-
sion of all constructs (Fig. 7B, top). To identify the target pY residue on the EGFR, we conducted affinity precipitation studies on these lysates using a GST fusion of the PTP domain of the substrate-trapping mutant of SHP2, hereinafter referred to as the DM-PTP (2). The precipitates were resolved on a 10% polyacrylamide gel and then analyzed by immunoblotting with anti-EGFR antibody. As shown in Fig. 7B (middle panel), the DM-PTP precipitated all EGFR constructs except Y992F, suggesting that pY992 could be the target substrate of SHP2. Previous in vitro phosphatase studies have also shown that the recombinant PTP domain of SHP2 preferentially dephosphorylated a phosphopeptide derived from Y992 of the EGFR (33, 47).

To confirm that pY992, which was targeted by SHP2, was a binding site for RasGAP, we conducted affinity precipitation studies using the GST-SH2-GAP on the same set of lysates used in Fig. 6B and C. The SH2 domain of RasGAP precipitated all EGFR proteins except Y992F-EGFR (Fig. 7B, bottom). These results further confirm that pY992, a target substrate of SHP2, is a binding site for RasGAP. Together, these results provide evidence for the first time that the mechanism by which SHP2 positively effects Ras activation involves the inhibition of RasGAP activity on Ras.

**Y992F-EGFR activates Ras and ERK1/2 independently of SHP2.** As shown in Fig. 7, pY992 of the EGFR is a binding site for RasGAP and a target substrate of SHP2. These results suggest that the role of SHP2 in EGFR signaling is to dephosphorylate negative-regulatory autophosphorylation sites. If this is the case, then Y992F-EGFR should activate the Ras-ERK signaling cascade independently of functional SHP2. To address this point, we first produced NIH 3T3 cells stably expressing the R/E protein by infecting them with a retrovirus (see Materials and Methods). Immunoblot analysis of total-cell lysates showed that the R/E protein expression level was approximately three times that of the endogenous protein (data not shown). NIH 3T3 cells were chosen for this purpose because they express undetectable levels of endogenous EGFR. These cells were then transfected with WT- or Y992F-EGFR. After incubation for ~36 h and serum starvation for 12 h, they were stimulated with 100 ng of EGF/ml for the indicated time points, and lysates prepared from these cells were analyzed for ERK1/2 activation by using the phosphospecific antibody. Similar to the effect of C/S protein on endogenous EGFR in COS-1 cells (Fig. 1B), activation of ERK1/2 occurred at 2 min in WT-EGFR-transfected cells on the background of R/E protein expression. However, the duration of the signal was short, reaching basal levels shortly afterward. In contrast, ERK1/2 activation induced by Y992F-EGFR was higher and more prolonged under the same background (Fig. 8A, top panel). Reprobing the membrane with anti-pan-ERK2 and anti-SHP2 antibodies showed that the amount of each protein in each lane was comparable (Fig. 8A, second and third panels, respectively). Further reprobing the membrane with anti-EGFR antibody indicated that the expression levels of WT-EGFR and Y992F-EGFR were similar (Fig. 8A, third panel).

In order to determine the effect of Y992F-EGFR on Ras activation, WT- and Y992F-EGFR were coexpressed in the same NIH 3T3 cells stably expressing the R/E protein. Transfected cells were incubated and stimulated under identical conditions as in Fig. 8A, and GTP-Ras levels were assayed as in Fig. 3. Similar to the results of ERK1/2 activation, the R/E protein inhibited Ras activation induced by WT-EGFR. This was consistent with the effect of the C/S protein on endogenous EGFR in COS-1 cells (Fig. 3). However, the R/E protein showed no effect on Ras activation induced by Y992F-EGFR at any of the time points tested. There-
fore, Y992F-EGFR prolongs the duration of the activated state of the Ras-ERK signaling cascade independently of functional SHP2.

**DISCUSSION**

Experiments with knockout and transgenic mice, as well as *Xenopus* cells, have shown that SHP2 is absolutely required for normal development. Furthermore, it was shown previously that SHP2 is an essential mediator of cell transformation induced by v-Src (19) and constitutively active FGF receptor 3 (1). Recent reports also show that gain-of-function SHP2 mutations were detected in myeloid leukemias, which may represent an early initiating lesion (50). Therefore, SHP2 is a very important tyrosine phosphatase involved in both biological and disease conditions. Molecular mechanisms by which SHP2 effects its function has remained an enigma. With the aim of defining the molecular mechanism of SHP2 in RTK signaling, we investigated its role in EGF-induced activation of the Ras-ERK cascade. We demonstrate that SHP2 acts upstream of Ras. We specifically show that SHP2 dephosphorylates pY992, which is a RasGAP recruitment site on the EGFR, suggesting that Y992 is a negative-regulatory site. Based on the results reported in this study, we propose that the mechanism by which SHP2 functions is by temporally regulating the level of GTP-Ras via modulating ligand-induced translocation to the PM of RasGAP, the down regulator of GTP-Ras.

Consistent with previous reports, the C/S protein inhibited ERK1/2 activation induced by EGF, whereas the WT protein did not. In fact, the WT protein increased the duration of ERK1/2 activation with slight enhancement of the signal (Fig. 1). As revealed in time course studies, ERK1/2 activation did occur in cells overexpressing the C/S protein but was short-lived and submaximal. These results indicated that EGF-induced signals were initially transduced from the receptor to ERK1/2 in the absence of SHP2 function. Thus, it is apparent that SHP2 modulates the duration of the Ras-ERK cascade in EGFR signaling. On the other hand, overexpression of the C/S protein did not inhibit V12-Ras-induced ERK1/2 activation (Fig. 2), indicating that SHP2 acts upstream of or parallel to Ras in the EGFR signaling cascade. However, further investigation of the effect of the C/S protein on EGF-induced Ras activation revealed that SHP2 acts upstream of Ras. Similar observations were made by Neel’s group using “constitutively active” SHP2 mutants (35).

Based on the above observations, we reasoned that SHP2 might be acting at the level of SOS or RasGAP. It was thus a logical step to study the effect of SHP2 on EGF-induced translocation of SOS or RasGAP to the PM, to their substrate microdomain. To accomplish this task, it was necessary to develop a new dominant-negative mutant of SHP2 since the C/S protein poses technical problems due to its trapping on target substrates, which may block SH2-mediated interactions (12, 32, 53). We thus developed the R/E protein, which showed an inhibitory effect equal to that of the C/S protein but lacked the partial substrate-trapping characteristic. The loss of substrate binding by the R/E protein could be explained by (i) loss of positive charge that coordinates the phosphate moiety on the substrate and (ii) electrostatic repulsion on the phosphate moiety by the acidic group of the substitute, the glutamate. Two observations that support this explanation are that the R/E protein was unable to autodephosphorylate and that it could not have a PTP domain-mediated interaction with the EGFR. Given that the R/E protein also inhibits PDGF- and FGF-induced ERK1/2 activation, it would be a better dominant-negative mutant than the C/S in future SHP2 studies.

Using the R/E protein, we showed that SHP2 modulates EGF-induced translocation of RasGAP to the PM with no apparent effect on SOS (Fig. 5). Because the two SH2 domains
were intact in all SHP2 proteins used in this study, the observed changes in RasGAP levels at the PM must reflect the role played by the PTP domain. In unstimulated cells, RasGAP is primarily localized to the cytosol, but upon stimulation, it translocates to the PM. This is mediated through its two SH2 domains, which interact with phosphotyrosyl residues on proteins at the level of the PM. Some RasGAP interacting proteins are RhoGAP, EGFR, and PDGFR (14, 24, 45). Thus, SHP2 PTP activity regulates RasGAP recruitment to the PM most probably by dephosphorylating binding sites on interacting proteins.

We recently showed that the EGFR is one of the target substrates of SHP2 (2). Thus, it was reasonable to hypothesize that SHP2 dephosphorylates pY residues on the EGFR that serve as binding sites for RasGAP. The finding that the SH2 domains of RasGAP efficiently precipitated the EGFR obtained from cells overexpressing the R/E protein supports this hypothesis. The mechanistic explanation is that the R/E protein dominantly interfered with the dephosphorylation of target pY(s) on the EGFR that serves as a binding site for RasGAP. Thus, it is the presence of specific pYs on the EGFR, not the total pY content, that determines RasGAP interaction.

Affinity precipitation studies could not rule out the possibility of an indirect interaction via an intermediate that binds to both RasGAP and the EGFR. However, far-Western analysis showed that RasGAP directly interacts with the EGFR (Fig. 6C). The demonstrations that EGFR is a target substrate of SHP2 and that RasGAP directly interacts with the EGFR only when it comes from cells expressing the R/E protein clearly show that SHP2 is dephosphorylating RasGAP binding sites on the EGFR. These results concur with the findings for Drosophila, where Corkscrew, the Drosophila homologue of SHP2, counteracts RasGAP’s interaction with the Torso RTK by specifically dephosphorylating pY918, which is a binding site for RasGAP (8). Therefore, it is possible that SHP2 might be functioning in a similar manner in EGFR signaling as Corkscrew does in Torso signaling.

The results presented in Fig. 6 could not show which tyrosine residue(s) out of the five major autophosphorylation sites on the cytoplasmic region of the EGFR was a target of SHP2 PTP activity. This question was addressed by employing the recently developed substrate-trapping mutant of SHP2 termed DM-PTP (2). DM-PTP showed remarkable specificity toward pY992 (Fig. 7B, middle), suggesting that it is the target substrate of SHP2. Interestingly, the SH2 domains of RasGAP also showed significant specificity toward pY992 (Fig. 7B, bottom). These results were consistent with Y992 of EGFR being a negative-regulatory site. Thus, we hypothesized that if Y992 of the EGFR is a negative-regulatory autophosphorylation site in EGF-induced activation of the Ras-ERK signaling cascade, then Y992F-EGFR must activate this signaling cascade independently of SHP2. Remarkably, that was exactly what we found, a reciprocal of the results presented in Fig. 1 and 3. Y992F-EGFR was refractory to the effects of the R/E protein as evidenced by prolongation of the activated state of the Ras-ERK cascade in the absence of functional SHP2 (Fig. 8). To our knowledge, this is the first report showing that Y992 of the EGFR is a negative-regulatory site by serving as a binding site for RasGAP, the down regulator of GTP-Ras.

Based on the results reported in this study and other previous reports on the subject, we have proposed a possible molecular mechanism for the biological role of SHP2 in the EGFR-Ras-ERK signaling pathway, which may also serve as a model for SHP2’s action in other RTK pathways. As depicted in Fig. 9, the binding of EGF to EGFR induces receptor dimerization and autophosphorylation on the five sites including Y992. These phosphorylated tyrosines serve as binding sites for SH2 domain-containing proteins including RasGAP. Because RasGAP preferentially binds to pY992 and this site is a target substrate of SHP2, the binding of RasGAP to pY992 is counteracted by the PTP activity of SHP2. The net effect would be an equilibrium shift toward an increased GTP-Ras level that results in prolongation of the signal to cause the desired cellular responses.

In summary, the mechanism by which SHP2 increases the duration and intensity of EGFR signals is by increasing the half-life of activated Ras (GTP-Ras) in the cell by interfering...
with the process of Ras inactivation by RasGAP via inhibition of tyrosine phosphorylation-dependent translocation of Ras-GAP to the PM. Thus, SHP2 is not a relayer but a modulator of EGFR signals at the level of Ras so that these signals can attain levels sufficient to induce corresponding cellular responses. Based on the findings in this report and similar reports for Drosophila (8), we propose that the role of SHP2 in RTK-induced activation of the Ras-ERK cascade is to act on negative-regulatory Pys on the receptors themselves and/or on scaffolding proteins to prolong the duration of the signal.

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