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A Common Requirement for the Catalytic Activity and Both SH2 Domains of SHP-2 in Mitogen-activated Protein (MAP) Kinase Activation by the ErbB Family of Receptors

A SPECIFIC ROLE FOR SHP-2 IN MAP, BUT NOT c-Jun AMINO-TERMINAL KINASE ACTIVATION*

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The ErbB family of receptors, which include the epidermal growth factor receptor (EGFR), ErbB2, ErbB3, and ErbB4 mediate the actions of a family of bioactive polypeptides. EGF signals through EGFR, whereas heregulin (HRG) signaling is initiated through binding to either ErbB3 or ErbB4. In this report we studied the role of protein-tyrosine phosphatase SHP-2 in ErbB-mediated activation of mitogen-activated protein kinase (MAPK) by overexpressing SHP-2 mutants in COS-7 cells. We demonstrate that enzymatic activity and both NH_2- and COOH-terminal SH2 domains of SHP-2 are required for EGF-induced MAPK activation, but not for c-Jun amino-terminal kinase stimulation or MAPK activation which occurred in response to myristoylated son of sevenless, activated Ras, or phorbol ester. Dominant-negative forms of SHP-2 had no effect on EGF-stimulated interaction of GRB2 with EGFR or SHC, nor did they influence phosphorylation of SHC and SHC/EGFR association. The same mutant SHP-2 structures that inhibited EGF-mediated stimulation of MAPK also blocked HRG α/β-induced MAPK activation. EGF or HRG β caused SHP-2 SH2 domains to engage multiple phosphotyrosine proteins, and mutation of either domain disrupted these associations. These results demonstrate that SHP-2 performs a common and essential function(s) in ligand-stimulated MAPK activation by the ErbB family of receptors.

The ErbB family of receptors, which include epidermal growth factor receptor (EGFR[1]; ErbB1), ErbB2, ErbB3, and ErbB4, mediate the biological actions of a family of growth factors which are structurally related to EGF (1). This family of bioactive peptides, which includes EGF, transforming growth factor α, amphiregulin, heparin-binding EGF-like growth factor, betacellulin, epiregulin, and heregulin α/β (HRG; neu differentiation factor, neuregulin, acetylcholine receptor-inducing activity, glial growth factor) elicits numerous cellular responses such as mitogenesis, differentiation, trophism, and motility (1). Signaling from ErbBs involves a process of receptor homo- and heterodimerization, which is initiated by engagement of ligand with a specific ErbB receptor (1). EGF and amphiregulin require the presence of EGFR for signaling (2), whereas HRG-induced signal transduction occurs after binding of ligand to either ErbB3 or ErbB4 on cells that co-express ErbB2 (3, 4).

The protein-tyrosine phosphatase SHP-2 (PTP1D, SHPTP2, PTP2C, SHPTP3, or Syp) contains two Src homology 2 (SH2) domains (5) and appears to play a critical role in mitogenic responses to EGF and insulin, but not to platelet-derived growth factor (6–10). Although it is not clear how SHP-2 functions as a positive mediator of EGF signaling, stimulation of cells with EGF has been shown to drive the association of SHP-2 with a number of proteins including a 115-kDa phosphotyrosine (Tyr(P))-containing protein (11), GRB2-associated binder-1 (Gab1) (12), SHP substrate 1 (SHPS-1)/signal-regulatory protein α (SIRPα) (13, 14), and GRB2 via the COOH-terminal SH3 domain of GRB2 (15). In *Drosophila*, membrane targeting of the SHP-2 homologue, *corkscrew*, is sufficient for R7 photoreceptor development in the absence of receptor tyrosine kinase activity (16), and a downstream target called daughter of sevenless (Dos) has been identified (17, 18). In the work reported here, we show that SHP-2 function appears to represent a common point(s) of convergence in signaling downstream of activated EGFR, ErbB2, ErbB3, and ErbB4. Furthermore, enzymatic activity and both NH_2- and COOH-terminal SH2 domains of SHP-2 are required for EGF-induced mitogen-activated protein kinase (MAPK) stimulation.

MATERIALS AND METHODS

Antibodies, Reagents, and cDNAs—Monoclonal antibodies (mAb) against influenza hemagglutinin protein epitope (HA) and c-Jun amino-terminal kinase (JNK) were obtained from Boehringer Mannheim and

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[1] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; HA, influenza hemagglutinin protein epitope; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; JNK, c-Jun amino-terminal kinase; HRG, heregulin; MBP, myelin basic protein; SH2, Src homology domain 2; SHP-2, SH2 domain-containing protein-tyrosine phosphatase 2; PMA, phorbol 12-myristate 13-acetate; myr-Sos, myristoylated cdc25 domain of son of sevenless, WT, wild type; IP, immunoprecipitations.

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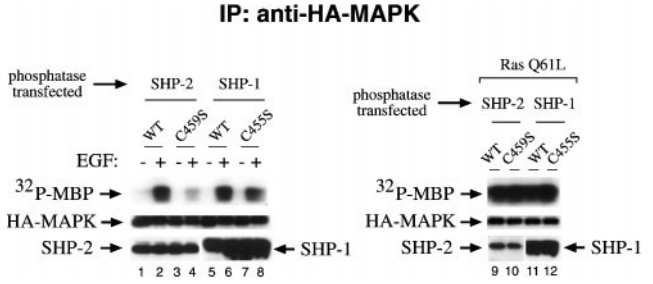

FIG. 1. **Effect of catalytically inactive SHP-1 and SHP-2 on activation of MAPK by EGF and activated Ras in COS-7 cells.** COS-7 cells were transiently transfected with 0.2 μg of HA-MAPK cDNA and 0.8 μg of pCMV5 expression vector encoding either wild type SHP-2 (*WT*; *lanes 1* and *2*), catalytically inactive SHP-2 (*C459S*; *lanes 3* and *4*), wild type SHP-1 (*lanes 5* and *6*), or catalytically inactive SHP-1 (*C455S*; *lanes 7* and *8*) as described under "Materials and Methods." Serum-starved cells were stimulated with 10 nM EGF for 5 min (*lanes 2*, *4*, *6*, and *8*) and lysed, and IPs were performed with anti-HA mAb. Immune complex kinase assays were performed using [^{32}P]ATP and MBP, and incorporation of ^{32}P into MBP was analyzed by autoradiography (*upper panel*), IP of HA-MAPK (*middle panel*), and consistent expression of SHP-2 and SHP-1 (*bottom panel*) were confirmed by Western blotting. In *lanes 9–12*, 0.2 μg of HA-MAPK cDNA, 0.5 μg of activated Ras cDNA (Q61L), and 0.8 μg of either WT SHP-2, C459S SHP-2, WT SHP-1, or C455S SHP-1 cDNA were transfected into cells, serum-starved, and lysed, and HA-MAPK activity was determined.

Pharmingen, respectively. mAbs against MAPK, SHC, GRB2, SHP-1, and SHP-2 were obtained from Transduction Labs. EGF was obtained from PeproTech, Inc. and HRG $\alpha_{177-228}$ and HRG $\beta1_{177-228}$ were provided by Berlex Biosciences. The expression vectors for SHP-1 and SHP-2 structures have been described previously (19). Epitope-tagged SHP-2 cDNAs were generated by subcloning into pcDNA3.1 myc/his (Invitrogen), and DNA sequence was confirmed. Expression vectors for HA-MAPK, HA-JNK, HA-SHC, and myristoylated cdc25 domain of Sos (myr-Sos) have been described by Coso *et al.* (20). ErbB3 and ErbB4 cDNAs were generously provided by Jacalyn Pierce (NCI, National Institutes of Health) and Greg Plowman (Bristol-Myers Squibb), respectively, and were subcloned into pCMV5. The myc-GRB2 cDNA was generously provided by Robert Weinberg (Whitehead Institute).

Transient Transfections—Subconfluent COS-7 cells were transfected with cDNAs as described in the figure legends using the DEAE-dextran/chloroquine technique (21). Cells were allowed to recover for 24 h, serum-starved overnight, and stimulated with 10 nM growth factor or phorbol ester, and cell lysates were generated as described previously (15). Lysate protein concentrations were determined using the Bio-Rad detergent compatible protein assay. In all instances, consistent expression of transfected cDNAs was confirmed by Western blotting analysis of 10 μg of lysate as described previously using enhanced chemiluminescence detection (15).

Immunoprecipitations (IP) and Kinase Assays—IPs were performed as described previously (15). For HA-MAPK kinase assays the immune complex was washed once with 20 mM Hepes (pH 7.4) containing 2 mM EGTA and 10 mM $MgCl_2$ (kinase buffer) prior to assay. Kinase buffer (20 μl) containing 20 μM ATP, 2 μCi of [^{32}P]ATP and 20 μg of myelin basic protein (MBP) was added to the immune complex and incubated for 30 min at room temperature. Reaction was terminated by adding 10 μl of 4× SDS-polyacrylamide gel electrophoresis sample buffer and boiling for 4 min. Reaction mixture was resolved in an 8–16% SDS-polyacrylamide gel electrophoresis gel, and proteins were transferred to a polyvinylidene difluoride membrane. Autoradiography was performed followed by Western blotting to confirm consistent IP of kinases. JNK activity was determined using 100 μM SKAIPS peptide substrate (22) exactly as described above, and reaction was terminated by the addition of 10 μl of 8.5% H_3PO_4. Reaction mixture was spotted onto P81 phosphocellulose paper and washed, and radioactivity was quantitated in a scintillation counter.

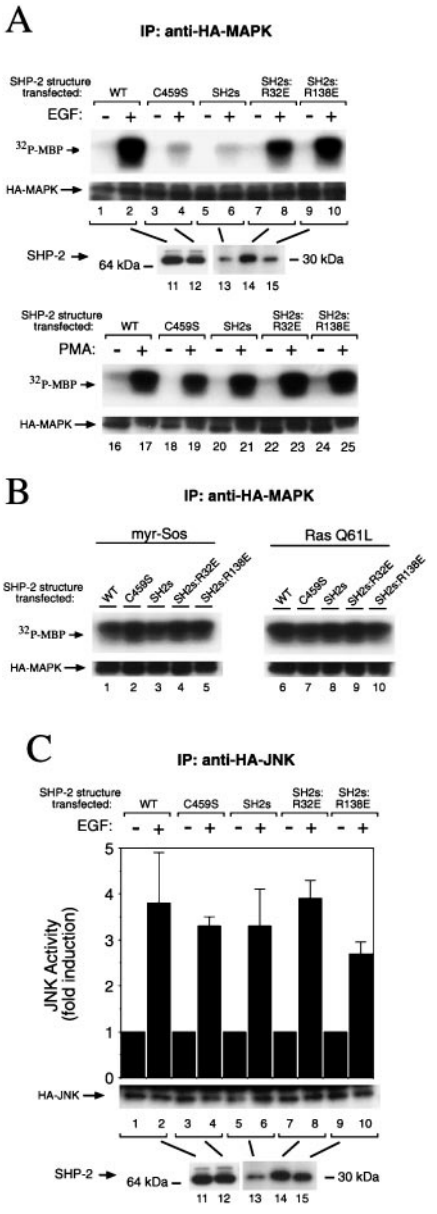

FIG. 2. **Evaluation of role of SHP-2 structures on MAPK and JNK activation by EGF.** In *A*, COS-7 cells were transfected with 0.2 μg of HA-MAPK cDNA along with 0.8 μg of cDNA encoding either WT SHP-2, C459S SHP-2, residues 1–244 of SHP-2 (*SH2s*), residues 1–244 with R32E mutation (*SH2s:R32E*), or residues 1–244 with R138E mutation (*SH2s:R138E*). Cells were stimulated with 10 nM EGF for 5 min (*lanes 2*, *4*, *6*, *8*, and *10*) or with 10 nM PMA for 10 min (*lanes 17*, *19*, *21*, *23*, and *25*) and lysed, and HA-MAPK activity was determined. To confirm expression of SHP-2s pooled aliquots of lysates derived from identically transfected dishes were analyzed by Western blotting (*lanes 11-15*). In *B*, cells were transfected exactly as in *A*, except that 0.5 μg of cDNA encoding either myr-Sos or Ras Q61L were co-transfected with HA-MAPK and SHP-2 cDNAs. In *C*, cells were transfected exactly as in *A*, except 0.2 μg of cDNA encoding HA-JNK was transfected instead of HA-MAPK, and cells were treated with EGF for 15 min. Immune complex kinase assays were performed using [^{32}P]ATP and SKAIPS peptide (22). The results are expressed as fold induction relative to unstimulated cells and represent means ± S.E. of duplicate experiments.

RESULTS AND DISCUSSION

COS-7 Cells as a Model System to Study the Functional Role of SHP-2 in EGF-induced MAPK Activation—Because COS-7 cells can be easily transfected and used to transiently express proteins, we tested the feasibility of using COS-7 cells to study the role of SHP-2 in activation of MAPK by EGF. cDNAs encoding wild type (WT) or catalytically inactive SHP-2 with a Cys to Ser mutation at residue 459 (C459S) were co-transfected into cells along with an HA epitope-tagged MAPK (extracellular signal-regulated kinase 2). Cells were stimulated with 10 nM EGF for 5 min and lysed, HA-MAPK was immunoprecipitated using anti-HA mAb, and enzymatic activity in the immunoprecipitates was determined using MBP as substrate. As

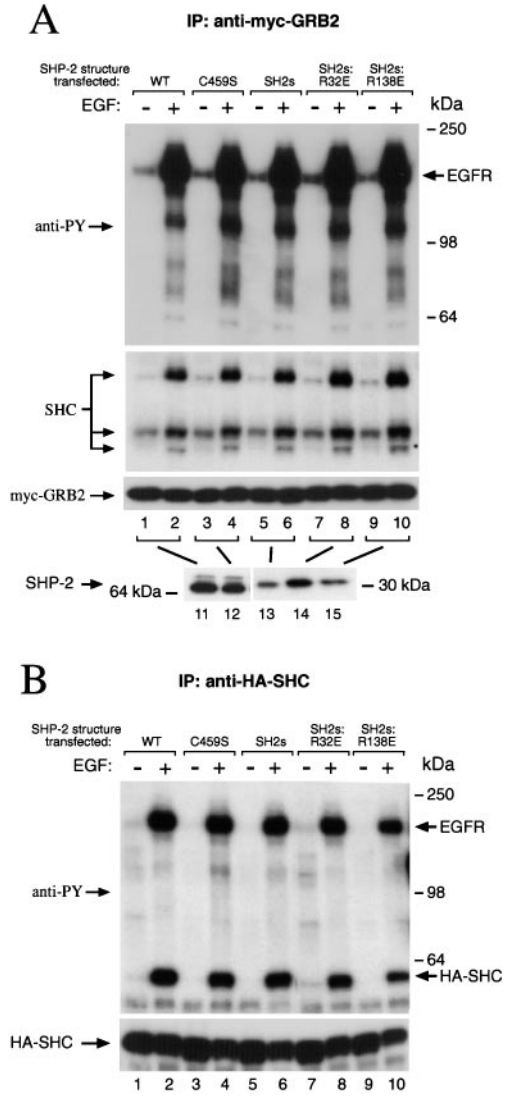

FIG. 3. **Dominant-negative SHP-2 mutants do *not* interfere with GRB2- and SHC-mediated interactions.** In *A*, COS-7 cells were transfected with 0.2 μg of myc-GRB2 cDNA along with 0.8 μg of cDNA encoding either WT, C459S, SH2s, SH2s:R32E, or SH2s:R138E SHP-2s. Cells were stimulated with 10 nM EGF for 5 min (*lanes 2*, *4*, *6*, *8*, and *10*) and lysed, and IPs were performed with anti-myc 9E10 mAb. Immunoprecipitates were analyzed for the presence of Tyr(P), SHC, and GRB2 by Western blotting. To confirm expression of SHP-2s pooled aliquots of lysates derived from identically transfected dishes were analyzed by Western blotting (*lanes 11-15*). In *B*, cells were transfected exactly as in *A*, except 0.2 μg of cDNA encoding HA-SHC was transfected instead of myc-GRB2, and HA-SHC was immunoprecipitated.

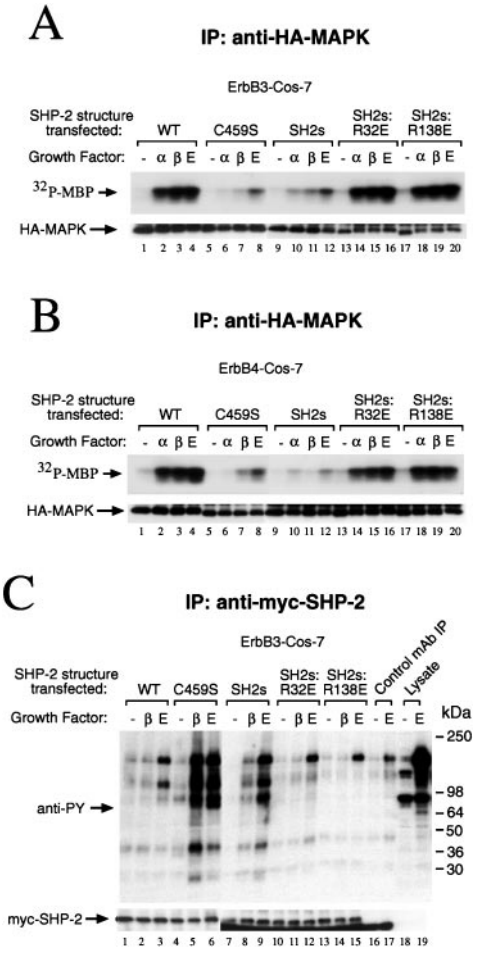

FIG. 4. **Evaluation of role of SHP-2 structures on HRG-induced MAPK activation.** In *A*, COS-7 cells were transfected with 0.2 μg of HA-MAPK cDNA, 0.25 μg of ErbB3 cDNA along with 0.8 μg of cDNA encoding either WT, C459S, SH2s, SH2s:R32E or SH2s:R138E SHP-2s. Cells were stimulated for 5 min with EGF (*E*), HRG α (α), or HRG β (β) and lysed, and HA-MAPK activity was determined. *B* was performed exactly as in *A* except that ErbB4 cDNA was transfected into cells instead of ErbB3 cDNA. *C* was performed exactly as in *A* except that 0.2 μg of myc epitope-tagged SHP-2 cDNAs were transfected instead of HA-MAPK. IPs were performed with anti-myc mAb, and immunoprecipitates were analyzed for Tyr(P) and SHP-2 by Western blotting. A control IP was performed on lysates derived from cells transfected with WT by replacing anti-myc with MOPC.21 control mAb (*lanes 16* and *17*).

shown in Fig. 1, expression of catalytically inactive SHP-2 in COS-7 cells significantly inhibited EGF-induced MAPK activation (*lane 4*), consistent with previous findings in 293 cells (9, 10). SHP-1, like SHP-2, is a protein-tyrosine phosphatase that contains two SH2 domains in tandem (23). Overexpression of catalytically inactive SHP-1 (C455S) did not significantly inhibit EGF-stimulated MAPK activation (*lane 8*) confirming the specificity of inhibition by C459S SHP-2. Further, stimulation of MAPK by expression of an activated form of Ras (Q61L) was unaffected by active or inactive forms of either SHP-2 or SHP-1 (*lanes 9-12*). Thus, COS-7 cells appear to be an excellent model to study the role of SHP-2 in EGF-induced activation of MAPK.

Catalytic Activity and Both SH2 Domains of SHP-2 Are Required for EGF-stimulated MAPK Activation, but Not for JNK Activation—To gain additional insight into which SHP-2 elements are required for EGFR-mediated MAPK activation, COS-7 cells were transfected with a truncated form of SHP-2 that contains both NH_2- and COOH-terminal SH2 domains (residues 1–244), but lacks the COOH-terminal phosphatase domain. This structure was a potent inhibitor of EGF-regulated activation of MAPK (Fig. 2*A*, *lane 6*). In contrast, MAPK activation by phorbol 12-myristate 13-acetate (PMA), myristoylated cdc25 domain of son of sevenless (myr-Sos), or Ras Q61L was not influenced by expression of SHP-2 SH2 domains (Fig. 2*A*, *lane 21*; Fig. 2*B*, *lanes 3* and *8*) demonstrating that attenuation of EGF-stimulated MAPK activity by this structure was highly selective. However, EGF-induced MAPK activation was rescued by mutations that render the SH2 domains incapable of binding Tyr(P) residues (R32E in the NH_2-terminal SH2 domain or R138E in the COOH-terminal SH2 domain) (Fig. 2*A*, *lanes 8* and *10*). These results indicate that both SH2 domains are essential for stimulation of MAPK by EGFR. Expression of C459S SHP-2 had no significant effect on activation of MAPK by PMA (Fig. 2*A*, *lane 19*), myr-Sos, or Ras Q61L (Fig. 2*B*, *lanes 2* and *7*).

To further investigate the role of SHP-2 in mediating downstream signaling events, we tested the ability of C459S SHP-2

and the SH2 domains to interfere with EGF-stimulated activation of JNK (stress-activated protein kinase). The SHP-2 constructs were transfected into COS-7 cells along with HA epitope-tagged JNK (HA-JNK), and JNK enzymatic activity was determined in anti-HA immunoprecipitates using SKAIPS peptide as substrate (22). As shown in Fig. 2*C*, none of the SHP-2 structures had any significant effect on EGF-induced JNK activity, and similar results were obtained when JNK activity was monitored using glutathione *S*-transferase-ATF2 fusion protein as substrate (data not shown). These results demonstrate that the requirement for SHP-2 function is highly specific to MAPK activation by EGF.

Dominant-negative SHP-2 Mutants Do Not Interfere with EGF-induced GRB2- and SHC-mediated Associations—To further confirm that expression of C459S or SHP-2 SH2s inhibit MAPK activation in a specific manner and do not nonspecifically bind Tyr(P) proteins essential to EGFR signaling we studied the effect these mutants had on Tyr(P)/SH2 domain interactions believed to be critical to EGFR function. A myc epitope-tagged GRB2 was expressed in cells along with the various SHP-2s, cells were stimulated with EGF, and myc-GRB2 was immunoprecipitated from lysates (Fig. 3*A*). These data demonstrated that none of the SHP-2 structures had any effect on association of GRB2 with EGFR or with SHC. In a complimentary experiment, cells were transfected with an HA epitope-tagged SHC (p52) and HA-SHC was immunoprecipitated (Fig. 3*B*). Again, none of the SHP-2 mutants influenced tyrosine phosphorylation of SHC or interaction of SHC with EGFR. These results present a strong argument that dominant-negative SHP-2s act to block MAPK stimulation specifically and do not function via the nonspecific sequestration of Tyr(P) proteins.

Catalytic Activity and Both SH2 Domains of SHP-2 Are Required for HRG α- and β-Stimulated MAPK Activation—We next addressed the question of whether SHP-2 plays a role in MAPK activation by HRGs. Western blotting analysis of lysates demonstrated that COS-7 cells do not possess detectable amounts of ErbB3 or ErbB4 and HRGs did not activate MAPK (data not shown). However, ectopic expression of either ErbB3 or ErbB4 in COS-7 cells reconstituted both HRG α- and β-stimulated MAPK activation (Fig. 4*A* and *B*, *lanes 2* and *3*). Expression of C459S SHP-2 or SHP-2 SH2s together with either ErbB3 or ErbB4 abrogated HRG α- or β-induced MAPK activation (Fig. 4, *A* and *B*, *lanes 6*, *7*, *10*, and *11*). Further, ligand-dependent MAPK activation was unaffected by mutated SHP-2 SH2 domains (*lanes 14*, *15*, *18*, and *19*). HRG α- and β-stimulated MAPK activation in these cells was not affected by expression of C455S SHP-1 (data not shown). Thus, SHP-2 function is essential to HRG-stimulated MAPK activation, and the required SHP-2 moieties are identical with those observed for EGF signaling. In addition, cells were co-transfected with ErbB3 and myc epitope-tagged SHP-2 cDNAs and stimulated with EGF or HRG β, and anti-myc immunoprecipitates were evaluated for the presence of Tyr(P) proteins (Fig. 4*C*). These results demonstrated that C459S and SHP-2 SH2s engage several common Tyr(P) proteins in response to EGF and HRG β and bound these proteins to a greater extent than WT SHP-2 (*lanes 2*, *3*, *5*, *6*, *8*, and *9*). Mutation of either SH2 domain inhibited these associations (*lanes 11*, *12*, *14*, and *15*) thereby providing a potential molecular basis for the ability of C459S and SHP-2 SH2s to block ErbB-mediated MAPK activation.

A Common Requirement for SHP-2 in MAPK Activation by the ErbB Family of Receptors—Our findings indicate that SHP-2 represents a common and essential point(s) of convergence in signaling downstream of ErbB receptors, regardless of which receptor combinations are activated. The dominant-negative effects observed by C459S and SHP-2 SH2s were found to be highly specific in that (i) these mutants did not inhibit MAPK stimulation by myr-Sos, Ras Q61L, or activated protein kinase C (PMA), (ii) these mutants did not interfere with GRB2 interaction with SHC and EGFR and SHC/EGFR association, (iii) overexpression of C455S SHP-1 did not block MAPK stimulation, and (iv) these mutants did not inhibit JNK activation. Because both MAPK and JNK stimulation is at least partially mediated by Ras (20), our findings suggest that SHP-2 functions in a Ras-independent pathway which leads to or allows for MAPK activation. The observation that mutation in either SHP-2 SH2 domain rescues ErbB-induced MAPK activation reveals that both domains are required for this response. This finding is important because deletion or simultaneous binding of both SH2 domains by Tyr(P) stimulates SHP-2 enzymatic activity (24, 25). These results suggest that SHP-2 SH2 domains need to simultaneously engage a Tyr(P) protein(s) in order for SHP-2 to act as a positive mediator of ErbB receptor-induced MAPK stimulation.

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