The Phosphotyrosine Phosphatase SHP-2 Participates in a Multimeric Signaling Complex and Regulates T Cell Receptor (TCR) coupling to the Ras/Mitogen-activated Protein Kinase (MAPK) Pathway in Jurkat T Cells

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Summary

Src homology 2 (SH2) domain–containing phosphotyrosine phosphatases (SHPs) are increasingly being shown to play critical roles in protein tyrosine kinase–mediated signaling pathways. The role of SHP-1 as a negative regulator of T cell receptor (TCR) signaling has been established. To further explore the function of the other member of this family, SHP-2, in TCR-mediated events, a catalytically inactive mutant SHP-2 was expressed under an inducible promoter in Jurkat T cells. Expression of the mutant phosphatase significantly inhibited TCR-induced activation of the extracellular-regulated kinase (ERK)-2 member of the mitogen-activated protein kinase (MAPK) family, but had no effect on TCR-ζ chain tyrosine phosphorylation or TCR-elicited Ca^{2+} transients. Inactive SHP-2 was targeted to membranes resulting in the selective increase in tyrosine phosphorylation of three membrane-associated candidate SHP-2 substrates of 110 kD, 55-60 kD, and 36 kD, respectively. Analysis of immunoprecipitates containing inactive SHP-2 also indicated that the 110-kD and 36-kD Grb-2–associated proteins were putative substrates for SHP-2. TCR-stimulation of Jurkat T cells expressing wild-type SHP-2 resulted in the formation of a multimeric cytosolic complex composed of SHP-2, Grb-2, phosphatidylinositol (PI) 3′-kinase, and p110. A significant proportion of this complex was shown to be membrane associated, presumably as a result of translocation from the cytosol. Catalytically inactive SHP-2, rather than the wild-type PTPase, was preferentially localized in complex with Grb-2 and the p85 subunit of PI 3′-kinase, suggesting that the dephosphorylating actions of SHP-2 may regulate the association of these signaling molecules to the p110 complex. Our results show that SHP-2 plays a critical role in linking the TCR to the Ras/MAPK pathway in Jurkat T cells, and also provide some insight into the molecular interactions of SHP-2 that form the basis of this signal transduction process.

The increase in the tyrosine phosphorylation of multiple cellular substrates as a result of the activation and recruitment of Src and Syk/ZAP-70 family tyrosine kinases is the critical, initiating event that couples the TCR to downstream signaling pathways such as calcium mobilization and the Ras–mitogen-activated protein kinase (MAPK)[1] pathway (1, 2). The transmembrane phosphotyrosine phosphatase (PTPase) CD45 positively regulates these events at their origin by dephosphorylating $p56^{lck}$ and $p59^{fyn}$, thereby maintaining these tyrosine kinases in their active conformation (3–6). The actions of CD45-activated $p56^{lck}$ are required for the phosphorylation of the TCR-ζ chain, which is then engaged by Src homology (SH) 2–containing kinases such as ZAP-70 and Syk (1, 6, 7).

The PTPase-mediated dephosphorylation of the numerous proteins that become phosphorylated as a result of TCR ligation remains poorly understood. T cells express the SH2 domain–containing PTPases, SHP-1 and SHP-2, which clearly have distinct roles. Thus, the motheaten mouse pathology, which results from a lack of SHP-1 protein or enzymatic activity, develops in the context of normal levels of SHP-2 expression (8, 9). Beyond their possession of two SH2 domains and a PTPase domain, SHP-1 and SHP-2 have relatively low sequence homology and consequently have the ability to bind to different phosphotyrosine-containing proteins (10) and exhibit quite distinct substrate specificities (11). SHP-1 negatively regulates signaling through

[1]*Abbreviations used in this paper:* AEBSF, 4-(2-Aminoethyl)-benzenesulfonylfluoride.HCl; csw, corkscrew; ERK, extracellular regulated kinase; GST, glutathione *s*-transferase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PI, phosphatidylinositol; PTPase, phosphotyrosine phosphatase; SH2, Src homology 2; SHP, phosphotyrosine phosphatase.

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several hematopoietic receptors (12), and in T cells inhibits TCR-mediated signal transduction, possibly by dephosphorylating ZAP-70 and/or $p56^{lck}$ (13, 14). In addition, the ubiquitously expressed SHP-2 has been proposed to act as a negative regulator of T cell signaling based on its association with the coreceptor CTLA-4 (15). In contrast, SHP-2 and its Drosophila homologue (corkscrew, or csw) have been shown to positively regulate the transduction of receptor-mediated signaling events leading to mitogenesis and differentiation in both hematopoietic and nonhematopoietic systems (16–19). The expression of dominant-negative SHP-2 in a variety of studies has indicated a positive role for this PTPase in regulating receptor coupling to the Ras/MAP kinase pathway in growth factor responsive cells (18, 20). We therefore endeavoured, using a perturbing mutant strategy, to address the role of SHP-2 function in TCR-mediated signaling in Jurkat T cells.

We have previously shown that SHP-2, but not SHP-1, associates with a 110-kD (p110) tyrosine phosphoprotein upon ligation of the TCR in Jurkat T cells (10). The transient tyrosine phosphorylation of p110 after receptor ligation pointed to a possible role for the SHP-2–p110 complex in TCR signal transduction. We now show that SHP-2 selectively regulates TCR-coupling to the Ras-MAP kinase pathway in Jurkat T cells. A catalytically inactive version of SHP-2 is preferentially targeted to membranes, resulting in the increased tyrosine phosphorylation of membrane-associated p110 and p36 proteins. SHP-2 is found in a complex with Grb-2, phosphatidylinositol (PI) 3′-kinase, and a p110 tyrosine phosphoprotein that we distinguish from the recently described signal regulatory protein (SIRP) family of transmembrane proteins (21). This complex becomes membrane associated, a cellular context that may promote the dephosphorylating actions of SHP-2. We propose that SHP-2 promotes coupling of the TCR to the MAPK pathway by dephosphorylating phosphotyrosine residues in p110, a putative docking protein, thereby regulating the Grb-2 and/or PI 3′-kinase content of a multicomponent signaling complex.

Materials and Methods

Cells, Cell Culture, and Stimulation. The E6 Jurkat T cell line was cultured in RPMI medium containing 5% FCS. The L cell fibroblast cell line used was maintained in DMEM with 10% FCS. Jurkat T cells were stimulated by addition of OKT3 for 1 min (for concentrations see figure legends).

Reagents. The following were gifts: CD3 mAb (OKT3; Cilag, Paris, France), TCR-ζ antiserum (Dr. C. Terhorst, Harvard Medical School, Boston, MA), extracellular regulated kinase (ERK)-2 antiserum (Dr. J. Saklatvala, Babraham Institute, Cambridge, UK), DOS mAb (Dr. T. Raabe, University of Wurzburg, Wurzberg, Germany), and SHP-2 antiserum (Dr. B. Neel, Beth Israel Hospital, Boston, MA). pZEM neo plasmids were provided by Dr. A. Saltiel, Parke-Davis Pharmaceuticals, Ann Arbor, MI. A plasmid containing cDNA encoding glutathione *s*-transferase (GST)–Grb-2 was a gift from Dr. D. Cantrell, ICRF, London, UK. The following commercially available antibodies were used: SHP-2 antiserum (Santa-Cruz Biotechnology, Santa Cruz, CA) PI 3′-kinase p85 antiserum (Upstate Biotechnology, Inc., Lake Placid, NY), SHP-2 and Grb-2 mAbs (Transduction Labs, Lexington, KY), and 4G10 phosphotyrosine mAb (Upstate Biotechnology, Inc.).

Generation of Dominant-negative (PTPase-inactive) SHP-2–expressing Cells. Jurkat T cells were electroporated in the presence of empty expression vector containing the human metallothionein promoter and neomycin resistance gene (pZEM neo) or the same vector containing an insert encoding point mutated ($cysteine^{459}$ to serine) SHP-2 protein (18). Clonal cell lines were isolated by selection in 1.5 mg/ml G418 (Sigma Chemical Co., St. Louis, MO). Expression of mutant SHP-2 (C→S SHP-2) was induced by a 5-h treatment with 10 μM cadmium and assayed by immunoblotting. C→S SHP-2 and vector control clones were matched for CD3 expression by Kolmogorov-Smirnov statistical analysis of FACS® (Becton Dickinson, Mountain View, CA) data.

Immunoprecipitation and Immunoblot Analysis. Before stimulation, Jurkat T cells (10^7) were washed and maintained under serum-free conditions for 5 h. After stimulation cells were lysed in 0.5 ml of lysis buffer (1% NP-40, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 20 mM *tetra*-sodium pyrophosphate, 1 mM sodium orthovanadate, 2 μg/ml aprotinin and leupeptin, 1 mM [4-(2-aminoethyl)-benzenesulfonylfluoride.HCl], or AEBSF, 1 mM EGTA, and 10 mM EDTA).

Electrophoretic mobility shift assays for ERK-2 were performed on postnuclear cell lysates. Samples were separated on 10% gels for 24 h to achieve clear resolution of phosphorylated and nonphosphorylated ERK-2. After transfer to Immobilon-P membrane, the samples were probed with ERK-2 polyclonal antibody.

For immunoprecipitation of SHP-2, TCR-ζ chain, ERK-2, and PI 3′-kinase p85, protein A–precleared cell lysates were mixed with sepharose-bound antibody for 2 h at 4°C. Adsorption of Jurkat T cell lysates to GST fusion proteins was carried out under the same conditions. Wild-type and mutant SHP-2 were independently precipitated as follows: wild-type SHP-2 was removed from mutant-expressing cell lysates using an antibody specific for the COOH terminus, and immunoprecipitates of mutant SHP-2 were then prepared using an antibody that recognized both wild-type and mutant SHP-2 from the depleted lysates. Precipitates were washed rigorously, subjected to SDS-PAGE, and transferred to Immobilon *P* membrane. Immunoblot analysis using phosphotyrosine, SHP-2, Grb-2, p85, and ERK-2 antibodies was performed using the ECL detection system (Amersham Life Sciences Ltd., Little Chalfont, UK). Immunoblots shown are each representative of 3–4 independent experiments. Quantitation of ECL signals was measured using a Bio-Rad chemiluminescence screen (Bio-Rad, Hercules, CA) and GS-525 Molecular Imager System (Bio-Rad).

In Vitro Kinase Assay. ERK-2 immunoprecipitates were washed in PBS and mixed continuously for 30 min at room temperature in 30 mM Tris-HCl, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 5 μM ATP, 8 μg myelin basic protein (MBP), and 5 μCi γ-[^{32}P]ATP. Samples were subjected to SDS-PAGE and analyzed by autoradiography.

Subcellular Fractionation. Control or stimulated Jurkat T cells (2×10^7) were resuspended in 1 ml homogenization buffer (20 mM Hepes, pH 7.5, 5 mM sodium pyrophosphate, 5 mM EGTA, 1 mM Mg_2Cl, 1 mM AEBSF, 10 μg/ml aprotinin and leupeptin, and 1 mM sodium orthovanadate) and sonicated three times at 10% of full power. The resulting homogenate was centrifuged at 12,000 *g* for 1 min to remove unbroken cells and the nuclear pellet. The supernatant was then centrifuged at 100,000 *g*

for 60 min and the membrane pellet and supernatant (soluble fraction) were collected. The pellet was washed, resuspended in 1 ml extraction buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM NaF, 1 mM AEBSF, and 1 mM sodium orthovanadate) and rotated for 60 min. The suspension was then centrifuged at 100,000 *g* for 45 min and the detergent-soluble extract was recovered in the supernatant (membrane fraction). All steps were conducted at 4°C. For subsequent immunoprecipitations, the soluble fraction samples were supplemented with NaCl and detergent to equivalence with membrane fraction samples.

Results and Discussion

SHP-2 Regulates TCR-stimulated ERK-2 Activation in Jurkat T Cells. PTPases contain an essential catalytic cysteine residue, the mutation of which results in a catalytically inactive enzyme, which still binds, but does not hydrolyze, tyrosine phosphate. This inactive PTPase can act as a dominant negative inhibitor of tyrosine dephosphorylation in vitro by binding specific phosphotyrosine residues, subsequently blocking dephosphorylation by the wild-type phosphatase. Mutation of Cys 459 to Ser in SHP-2 is known to ablate catalytic activity of the protein (18). Jurkat T cells were therefore stably transfected with cDNA encoding catalytically inactive SHP-2. Expression of mutant protein (C→S SHP-2) was controlled by a metallothionein promoter and induced by the addition of cadmium. The cadmium-inducible SHP-2 in Jurkat T cells consistently migrated at 69 kD rather than 72 kD (Fig. 1 *A*, *top*). The 69-kD inducible form was no longer recognized by an antibody specific for the SHP-2 COOH terminus (data not shown), suggesting the selective loss of a small COOH-terminal peptide ~30 residues in length from the C→S version of the enzyme. The integrity of the cDNA used in these studies was confirmed by the fact that transfected fibroblasts expressed cadmium-inducible full length C→S SHP-2 (data not shown). Although the truncation resulted in the loss of a putative Grb-2 binding site in the mutant SHP-2, the relevance of this interaction in T cells is nullified by the lack of tyrosine phosphorylation on SHP-2 observed upon TCR ligation in these cells (10). We surmised that the COOH terminus deletion of C→S SHP-2 did not extend into the PTPase domain and therefore should not affect the ability of the protein to bind to its substrate and hence act as a dominant negative.

We therefore assessed the effect of the expression of this inactive enzyme on TCR-stimulated ERK-2 activation. Fig. 1 *A* (*middle*) shows that expression of C→S SHP-2 resulted in an inhibition of TCR-stimulated ERK-2 phosphorylation as measured by an electrophoretic mobility shift assay. This inhibition was observed in four independently isolated clones and was reproducibly greater than the small inhibition of TCR-induced signaling caused by the addition of cadmium alone (Fig. 1, *A* and *C*). The TCR-induced increase in ERK-2 activity, as measured by the phosphorylation of MBP, was similarly affected by expression of mutant SHP-2 (Fig. 1 *A*, *bottom*). Quantitation from four separate experiments on a single clone revealed that ERK-2 activation was inhibited by 39 ± 8% when C→S SHP-2 expression was 50% of total SHP-2. The degree of inhibition observed in each clone correlated well with the amount of mutant SHP-2 protein expressed relative to the endogenous enzyme (Fig. 1 *B*). The modest C→S SHP-2 expression level achieved with this system (18) made it unlikely that the mutant PTPase perturbed pathways unrelated to the actions of SHP-2. Inhibition was selective since tyrosine phosphorylation of the TCR-ζ chain was unaffected by expression of C→S SHP-2 (Fig. 1 *C*), which had no discernible effect on TCR-ζ phosphorylation at either minimal (0.1 μg/ml) or maximal (1 μg/ml) concentrations of TCR agonist (data not shown). Likewise, catalytically-inactive SHP-2 did not affect TCR-stimulated rises in intracellular Ca^{2+} (data not shown).

Despite the inhibition of TCR-induced ERK-2 activation, IL-2 secretion in these cells was not significantly affected by expression of mutant SHP-2 (data not shown). However, the interpretation of such data is confounded by the requirement for phorbol ester and ionomycin in conjunction with CD3 mAb to observe measurable IL-2 secretion in Jurkat T cells. Similarly, this cocktail of stimuli was necessary to observe IL-2 gene induction using a green fluorescent protein-linked reporter in transient transfection assays (data not shown). Since phorbol-ester–activated protein kinase C powerfully drives activation of the Ras/MAPK pathway by a tyrosine kinase–independent mechanism (22), it is possible that the effects of mutant SHP-2 may not be revealed using such protocols. Therefore, a transgenic mouse approach is being used to address the role of SHP-2 in regulating IL-2 secretion in primary T cells, whereas the Jurkat T cell system has been used in the present work to investigate the molecular interactions and potential substrates of SHP-2 and elucidate how SHP-2 is involved in more proximal TCR signaling events.

SHP-2 Acts Primarily at the Membrane. In nonhematopoietic cells in which the PTPase activity of SHP-2 has been demonstrated as necessary for receptor-stimulated MAPK activation, the actions of this PTPase and thereby its substrate(s) have been placed upstream of Ras (23–25). The initiation of the Ras-MAPK pathway occurs at the plasma membrane, where GTP-loaded Ras activates the Raf-1 kinase (26). Interestingly, the activity of the SH2 domain PTPases is also increased by certain phospholipid environments (27). In an attempt to understand how SHP-2, normally a cytosolic protein, might regulate membrane-localized events, we investigated the subcellular distribution of wild-type and catalytically inactive SHP-2. In TCR-stimulated Jurkat T cells, a small percentage (estimated to be 2%) of wild-type PTPase was reproducibly found in the membrane fraction (Fig. 2 *A*). In a C→S SHP-2–expressing clone, in which the ratio of wild-type to mutant SHP-2 was 1:1, fractionation studies revealed that there was approximately twofold more mutant SHP-2 than endogenous enzyme in the membrane fraction, whereas mutant SHP-2 constituted only 34% of total cytosolic SHP-2 (Fig. 2 *A*).

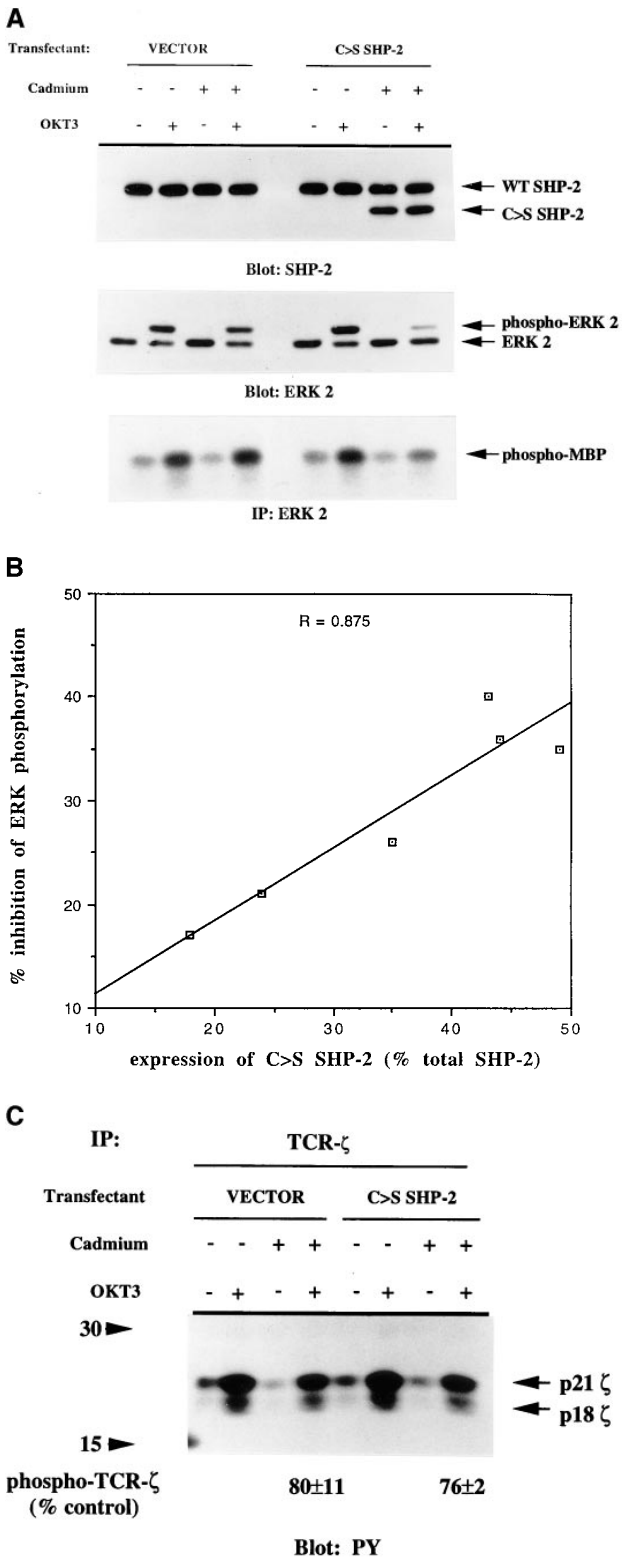
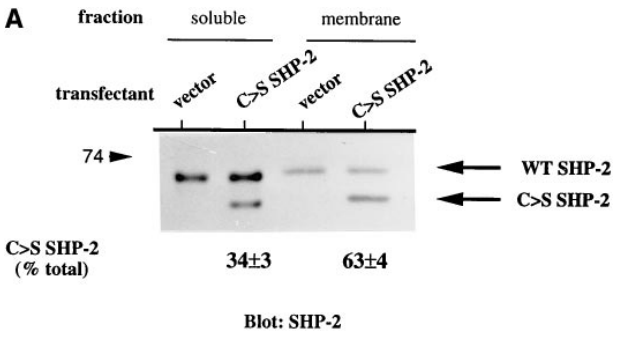


Figure 1. Expression of catalytically inactive (C→S) SHP-2 inhibits TCR-stimulated ERK-2 activation but not TCR-ζ tyrosine phosphorylation in Jurkat T cells. pZEM neo C→S SHP-2 and empty vector transfectants, matched for CD3 expression by FACS® analysis, were incubated for 5 h in the presence or absence of 10 μM cadmium. Cells were then left untreated or were stimulated with TCR agonist (OKT3) at 0.5 μg/ml for 1 min before lysis. (*A*) Aliquots of cell lysates (10^6 cell equivalents) were separated by SDS-PAGE and immunoblotted for SHP-2 (*top*) and ERK-2 (*middle*). Alternatively, ERK-2 immunoprecipitates derived from the indicated cell lysates were subjected to in vitro kinase assay using MBP as a substrate (*bottom*). (*B*) Percentage of inhibition of TCR-stimulated ERK-2 phosphorylation was measured in six different clones expressing different levels of mutant SHP-2 expression. (*C*) TCR-ζ was immunoprecipitated from lysates prepared as above, analyzed by SDS-PAGE, and immunoblotted for phosphotyrosine. Numerical data shown represent phosphorimager quantification of TCR-ζ tyrosine phosphorylation upon TCR ligation in vector and mutant expressing clones and are expressed as percentage of control ± SEM ($n = 4$); control = no addition of cadmium.

These data suggest that the catalytically inactive SHP-2 was being trapped in membranes due to stable association with its substrate(s). Indeed in membrane fractions in which catalytically inactive SHP-2 was present in excess to endogenous PTPase, three hyperphosphorylated proteins, 110 kD, 55/60 kD doublet, and 36 kD, were evident when compared with an equivalent fraction prepared from vector control cells (Fig. 2 *B*). The phosphorylation of these proteins was increased by 1.3-, 1.6-, and 1.5-fold, respectively, when compared with TCR-stimulated cells expressing only endogenous SHP-2. In contrast, the phosphorylation of two other membrane-associated tyrosine phosphoproteins, migrating on either side of the 74 kD marker, was unaffected. The fact that catalytically inactive SHP-2 may affect the phosphorylation state of more than one protein implies that wild-type SHP-2 dephosphorylates several components of a single signaling complex, or that this PTPase regulates the activity of a kinase in this complex which then changes the phosphorylation state of adjacent substrates.

SHP-2–associated p110 Is the Major Putative SHP-2 Substrate in Jurkat T Cells. We have previously shown that SHP-2 associates with a 110-kD tyrosine phosphoprotein (p110) upon ligation of the TCR in Jurkat T cells p110 (10). The COOH-terminal truncation of mutant SHP-2 in the present study has enabled the selective immunoprecipitation of wild-type and catalytically inactive SHP-2 (see Materials and Methods for details). Initial experiments suggested that immunocomplexes of mutant SHP-2 demonstrated stronger p110 phosphotyrosine signals than did immunocomplexes of wild-type SHP-2 (data not shown). These experiments were carried out by sequentially precipitating the two versions of SHP-2 from the same lysate sample using different antibodies. The interpretation of such data are complicated by the use of antibodies raised to distinct regions of SHP-2, which may themselves have intrinsic influences on the nature/quantity of proteins that coprecipitate with SHP-2. We therefore designed an experiment (see Materials and Methods for details), in which wild-type and catalytically inactive SHP-2 were immunoprecipitated, using the same antibody, from vector control and C→S mutant-expressing lysates, respectively (Fig. 3 *A*). In agreement with preliminary findings, Fig. 3 *A* shows that the phosphotyrosine signal derived from SHP-2–asso-



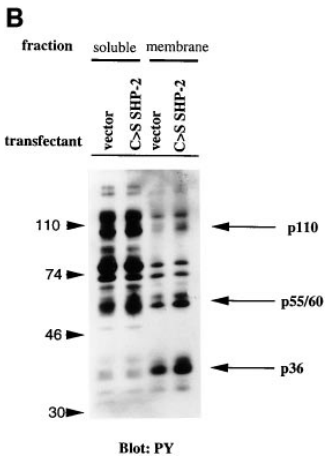
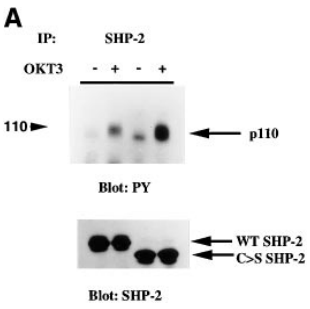


Figure 2. Catalytically inactive SHP-2 locates more effectively to membranes than does wild-type SHP-2, and it increases membrane-associated protein tyrosine phosphorylation. (*A*) pZEM neo C→S SHP-2 and empty vector transfectants were incubated for 5 h in the presence of 10 μM of cadmium and were stimulated for 1 min with OKT3 (0.5 μg/ml). After sonication, the resulting fractions were analyzed by SDS-PAGE and immunoblotted for SHP-2. To achieve similar strength signals for accurate quantitation, 10-fold less cell equivalents of the soluble fraction were loaded. The numerical data shown represent quantitation of the amount of C→S SHP-2 present in each fraction expressed as a percentage of total SHP-2 (wild-type + mutant) ± SEM (n = 3). (*B*) Membrane and soluble fractions prepared from activated (0.5 μg/ml OKT3 for 1 min) vector control or C→S SHP-2–expressing clones were analyzed by SDS-PAGE and immunoblotted for phosphotyrosine.



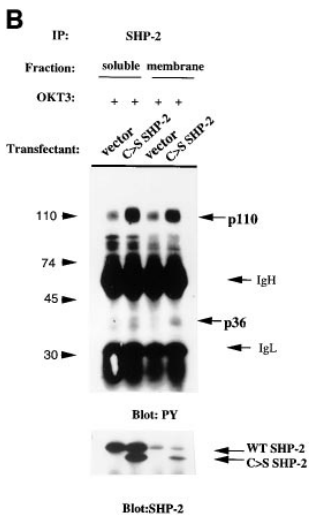


Figure 3. Expression of catalytically-inactive SHP-2 increases the coprecipitation of two tyrosine phosphorylated proteins, p110 and p36, with SHP-2. (*A*) pZEM neo C→S SHP-2 and empty vector transfectants were incubated for 5 h in the presence of 10 μM of cadmium and were left untreated or were stimulated for 1 min with OKT3 (0.5 μg/ml) and lysed. Wild-type (lanes *1* and *2*) or mutant (C→S) SHP-2 (lanes *3* and *4*) was immunoprecipitated from the relevant lysates (see Materials and Methods for details), analyzed by SDS-PAGE and immunoblotted for phosphotyrosine (*top*) and SHP-2 (*bottom*). (*B*) pZEM neo C→S SHP-2 and empty vector transfectants were incubated for 5 h in the presence of 10 μM of cadmium and then stimulated for 1 min with OKT3 (0.5 μg/ml). After sonication and fractionation, total SHP-2 was immunoprecipitated from the indicated fractions. The samples were analyzed by SDS-PAGE and immunoblotted for phosphotyrosine (*top*) and then reprobed for SHP-2 (*bottom*).

ciated p110 was threefold greater when in complex with catalytically inactive SHP-2 compared with wild-type. This was evident under both basal and stimulated conditions. The simplest explanation for the increased immunoprecipitation of p110 in complex with mutant SHP-2 is that p110 is a substrate for SHP-2 and becomes trapped by the catalytically inactive mutant, preventing it from being dephosphorylated by the wild-type PTPase. In the absence of an antibody to p110 we cannot assume that p110 bound to inactive SHP-2 is hyperphosphorylated, although this is an attractive possibility.

To determine if the membrane-associated increases in tyrosine phosphorylation illustrated in Fig. 2 *B* were specifically SHP-2–related, we immunoprecipitated total SHP-2 from soluble and membrane fractions prepared from TCR-stimulated control or mutant-expressing cells and analyzed the samples for phosphotyrosine content (Fig. 3 *B*, *top*). In agreement with the findings using whole fractions (Fig. 2 *A*), membranes contained disproportionately high levels of catalytically inactive SHP-2 (Fig. 3 *B*, *bottom*). Importantly, immunocomplexes containing mutant SHP-2 from membrane fractions had increased levels of two coprecipitating proteins, p110 and p36, when compared with those prepared from control cells. The p55/p60 tyrosine phosphoprotein observed in whole membrane fractions from mutant-expressing cells (Fig. 2 *B*) was not observed in SHP-2 immunoprecipitates, although it may have been obscured by the immunoglobulin heavy chain signal.

SHP-2–associated p110 Is Not a SIRP. The SHP-2–associated p110 tyrosine phosphorylated protein in Jurkat cells has not yet been identified. An immunodepletion strategy of likely candidates has excluded c-cbl, cas, HRS-1, SLAP-130, and PYK2. We have been unable to detect the expression of GAB-1, another likely candidate, in Jurkat cells (data not shown). In terms of its ability to associate with SHP-2 and its characteristic broad electrophoretic mobility (10), Jurkat p110 is strikingly similar to a member of a recently discovered family of novel docking proteins known as SHPS-1 or SIRPs (signal-regulatory proteins) (21, 28). This family has been shown to have negative regulatory effects on cellular responses by growth factors and oncogenes. SIRPs are thought to serve as substrates for SHP-2 and have been proposed to act by sequestering the positively acting PTPase away from critical signaling complexes. The members of this family are all glycosylated, receptor-like transmembrane proteins. In contrast, Fig. 3 *B* shows that SHP-2–associated p110 was detected, in its tyrosine phosphorylated form, in both cytosolic and membrane fractions of Jurkat cells. Furthermore, unlike members of the SIRP family, the migration of p110 upon SDS-PAGE analysis was unaffected by in vitro deglycosylation or upon treatment of cells with tunicamycin in vivo (data not

shown). It is possible that the p110 protein found in T cells is related to the recently reported SHP-2–associating proteins p97 and p100 found in other (non–T cell) hematopoietic cell lines (29, 30).

Since SHP-2–associated p110 is not glycosylated, the broad electrophoretic nature of this protein is therefore likely to be due to multiple states of tyrosine phosphorylation, suggesting that it may act as a 'docking protein' similar to IRS-1/2, GAB-1, or Dos (31–33). Dos is the 115-kD substrate for the Drosophila homologue of SHP-2, known as corkscrew (csw; reference 34) and contains proline-rich sequences, an NH_2-terminal pleckstrin homology domain, and multiple potential tyrosine phosphorylation sites. Dos is thought to be responsible for the activation of SHP-2, its translocation to membranes, and its subsequent interaction with components of the Ras-MAPK pathway. Although the Jurkat p110 was not recognized by an authentic Drosophila Dos mAb (data not shown), our biochemical data suggest that p110 may be the mammalian equivalent of Dos and this possibility is currently under investigation.

Tyrosine Phosphorylation of p36 Grb-2 Binding Protein Is also Regulated by SHP-2. Fig. 3 *B* illustrates a SHP-2–associated p36 tyrosine phosphoprotein signal that was detectable only when mutant SHP-2 was present in the immunocomplex. This interaction was particularly striking in membranes considering the relatively low levels of SHP-2 precipitated from this fraction. A hyperphosphorylated p36 tyrosine phosphoprotein was also observed in whole membrane fractions containing mutant SHP-2 (Fig. 2 *B*). p36 was identified as the Grb-2–binding protein (35) since it bound effectively to a GST–Grb-2 fusion protein in vitro (data not shown). Although formal identification is necessary, p36 is very likely to be the recently cloned linker for activation of T cells (LAT). LAT is a putative transmembrane protein with nine potential phosphotyrosine residues (36), and is proposed to act as a binding module for SH2 domain–containing proteins such as Grb-2, PLCγ1, and PI 3′-kinase in TCR-stimulated T cells (35–37). Its state of phosphorylation is critical to the propagation of signals leading to transcriptional activation of nuclear factor of activated T cells and activation protein 1 (36). The relatively small proportion of total cellular p36 that coprecipitates with mutant SHP-2 suggests that SHP-2 regulates the tyrosine phosphorylation of a select pool of this protein. Site

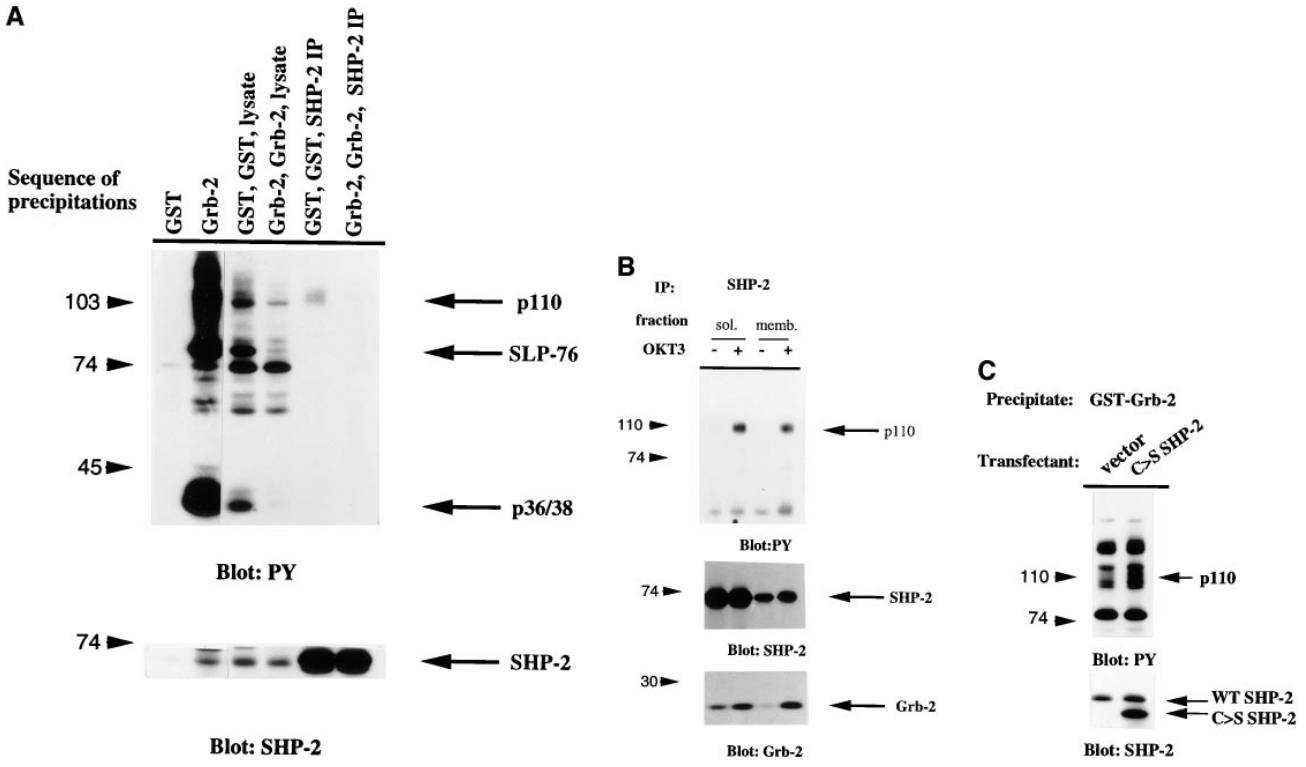


Figure 4. Grb-2, SHP-2, and p110 tyrosine phosphoprotein form a complex in TCR-activated Jurkat T cells. (*A*) Parental Jurkat T cells were stimulated for 1 min with 0.5 μg/ml OKT3 and were lysed. The resulting lysates were subjected to two rounds of precipitation with GST or GST–Grb-2 fusion proteins followed by immunoprecipitation of SHP-2. The resulting precipitates and sample aliquots of fusion protein-treated lysates (10^5 cell equivalents) were analyzed by SDS-PAGE and immunoblotted for phosphotyrosine (*top*) and then reprobed for SHP-2 (*bottom*). (*B*) Parental Jurkat T cells were left untreated or stimulated for 1 min with OKT3 (1 μg/ml) before sonication and fractionation. SHP-2 was immunoprecipitated from soluble and membrane fractions, analyzed by SDS-PAGE and immunoblotted for phosphotyrosine (*top*) and reprobed for SHP-2 (*middle*) and Grb-2 (*bottom*). (*C*) pZEM neo C→S SHP-2 and empty vector transfectants were incubated for 5 h in the presence of 10 μM cadmium and were stimulated for 1 min with OKT3 (0.5 μg/ml) and lysed. The resulting lysates were mixed with immobilized GST–Grb-2 and the resulting precipitates then analyzed on SDS-PAGE and immunoblotted for phosphotyrosine (*top*) and SHP-2 (*bottom*).

mapping and follow-up mutagenesis-function studies on p36 will be necessary to elucidate the consequences of SHP-2 action on this protein.

Grb-2, SHP-2, and p110 Tyrosine Phosphoprotein Form a Complex in TCR-activated Jurkat T Cells. Dos has consensus sequence SH2 domain binding sites for a number of signaling proteins besides SHP-2, including Grb-2 and the p85 subunit of PI 3′-kinase (33). It is possible that the dephosphorylating actions of SHP-2 on such a docking protein may affect the localization and effectiveness of other signaling molecules in this complex. Since we believe that p110 may be a mammalian equivalent of Dos the association of Grb-2 with the SHP-2–p110 complex was investigated using a GST fusion protein of full-length Grb-2 (Fig. 4 *A*). Several tyrosine phosphoproteins from TCR-stimulated Jurkat cells, including p36, p76, and a range of tyrosine phosphoproteins migrating between 100 and 130 kD were found to associate with Grb-2, consistent with previous reports (38, 39). TCR-stimulated Jurkat cells were subjected to two rounds of depletion with GST–Grb-2, or GST as a control, and subsequent SHP-2 immunoprecipitates then immunoblotted for phosphotyrosine. As illustrated in Fig. 4 *A* (*top*) no SHP-2–associated p110 was detected in lysates previously depleted of Grb-2 binding proteins, whereas p110 was clearly detected in control GST-treated lysates, indicating that a complex between p110, SHP-2, and Grb-2 exists in TCR-stimulated Jurkat T cells. In accordance with this suggestion, Grb-2 was found to coprecipitate with SHP-2 (Fig. 4 *B*, *bottom*). The amount of Grb-2 detected in SHP-2 immunoprecipitates increased upon TCR ligation (Fig. 4 *B*). Since it was apparent that SHP-2 acted mainly on membrane substrates (Fig. 2 *B*), we investigated the cellular compartmentation of this complex. Fig. 4 *B* shows that despite the fact that between 5- and 10-fold less SHP-2 was precipitated in the membrane versus the cytosolic fraction, the p110 tyrosine phosphorylation and Grb-2 protein levels were strikingly equivalent between the two fractions. The higher stoichiometry of the components of this complex in the membrane compared with the cytosol may indicate that the complex forms in the cytosol and then translocates as a unit to the membrane. This hypothesis is corroborated by the fact that there is an overall increase in the amount of SHP-2 detectable in the membrane fraction upon TCR ligation (Fig. 4 *B*, *middle*).

The presence of Grb-2 in this complex is likely to bring other Grb-2–associated molecules into the microenvironment of SHP-2 and may explain the hyperphosphorylation of p36, the major Grb-2 SH2 domain binding protein, in Jurkat T cells expressing catalytically inactive SHP-2 (Fig. 2 *B*). p36 may prove to be the anchor responsible for recruiting the SHP-2–p110–Grb-2 complex to the membrane. It was notable that we were unable to detect any of the known SH3 domain–mediated Grb-2–binding proteins such as SLP-76, Sos, or c-cbl in SHP-2 immunocomplexes (data not shown). This suggests that the SHP-2–p110 complex contains a select pool of Grb-2 having SH3 domains already occupied by proline-rich sequences in p110, consistent with findings on a similar SHP-2–based signaling complex recently described in IL-3–stimulated BaF3 cells (40). We were interested in determining whether the association of Grb-2 with this complex would be affected by the presence of catalytically inactive SHP-2. Fig. 4 *C* (*top*) shows that the p110 protein precipitated with a GST–Grb-2 fusion protein was hyperphosphorylated in cells expressing the C→S mutant. SHP-2 immunoblot analysis of these precipitates demonstrated that more mutant than wild-type SHP-2 bound to Grb-2 (Fig. 4 *C*, *bottom*). This observation suggests that p110 in complex with mutant SHP-2 binds Grb-2 more efficiently than does p110 associated with wild-type PTPase, and may indicate that the actions of SHP-2 can regulate the Grb-2 content of the complex. The precise consequences of the adaptor function of Grb-2 in TCR-mediated cell signaling pathways have yet to be elucidated.

PI 3′-kinase, SHP-2, and p110 Form a Complex in TCR-activated Jurkat T Cells. By analogy with the Drosophila system, Dos also has a putative binding motif for the SH2 domains of the p85 subunit of PI 3′-kinase (p85). Immunoprecipitation of p85 from TCR-stimulated Jurkat T cells resulted in the coprecipitation of a striking tyrosine phosphoprotein that comigrated with SHP-2–associated p110 (Fig. 5 *A*, *top*). Two other groups have recently reported the interaction of PI 3′-kinase via the SH2 domains of its p85 subunit with a p100 tyrosine phosphoprotein in hematopoietic growth factor signaling (29, 40). Prior depletion of TCR-stimulated Jurkat lysates with p85 antibody resulted in the loss of SHP-2–immunoprecipitable p110 (Fig. 5 *A*, *top*). Fig. 5 *A* (*bottom*) also illustrates that SHP-2 can be detected in p85 immunoprecipitates from TCR-activated cells. The subcellular localization of p85-precipitable p110 correlated well with that of SHP-2–p110 (data not shown). These data indicate that the same p110 tyrosine phosphoprotein coprecipitates with both SHP-2 and p85 and that all three proteins are likely to be in a complex together. Interestingly, a small quantity of p36 was reproducibly observed in p85 precipitates (Fig. 5 *A*, *top*), reinforcing the concept of a single multicomponent signaling complex containing SHP-2, PI 3′-kinase, p36, and p110. Fig. 5 *B* (*top*) shows that the p110 that coprecipitated with p85 from mutant SHP-2 expressing cells gave a considerably stronger phosphotyrosine signal (1.7-fold) than that from cells expressing only wild-type SHP-2. Analysis of these precipitates for SHP-2 content revealed an activation-dependent coprecipitation of wild-type SHP-2 with p85 and, in mutant-expressing cells, an increased propensity for inactive SHP-2 rather than wild-type PTPase to be present in the complex (Fig. 5 *B*, *bottom*). In accordance with the basal phosphorylation of p110 in p85 immunoprecipitates prepared from mutant-expressing cells, a constitutive level of mutant SHP-2 was reproducibly observed in this sample (Fig. 5 *B*, *bottom*). These observations presumably reflect the trapping of inactive SHP-2 in the complex; the effect of this upon PI 3′-kinase content or activity of the com-

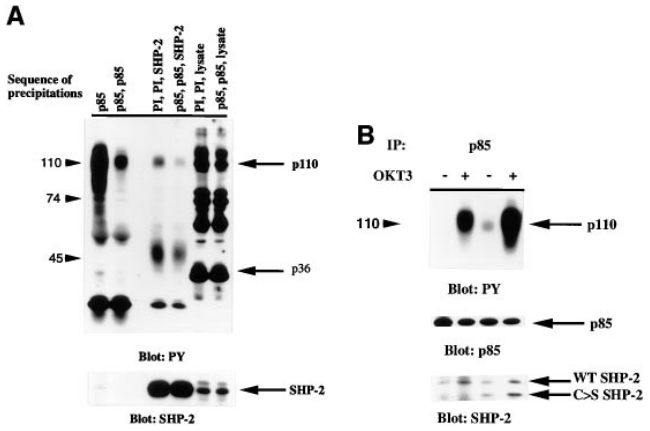


Figure 5. PI 3′-kinase, SHP-2, and p110 tyrosine phosphoprotein form a complex in TCR-activated Jurkat T cells. (*A*) Parental Jurkat T cells were stimulated for 1 min with 1 μg/ml OKT3 and lysed. The resulting lysates were subjected to two rounds of precipitation with preimmune or p85 PI 3′-kinase (p85) antisera followed by immunoprecipitation of SHP-2. The resulting precipitates and sample aliquots of control or p85-depleted lysates (10^5 cell equivalents) were analyzed by SDS-PAGE and immunoblotted for phosphotyrosine (*top*) and then reprobed for SHP-2 (*bottom*). (*B*) pZEM neo C→S SHP-2 (lanes *3* and *4*) and empty vector transfectants (lanes *1* and *2*) were incubated for 5 h in the presence of 10 μM of cadmium and then left untreated or stimulated for 1 min with OKT3 (0.5 μg/ml) and lysed. The resulting lysates were immunoprecipitated for p85 PI 3′-kinase. The precipitates were analyzed on SDS-PAGE and immunoblotted for phosphotyrosine (*top*) and reprobed for p85 (*middle*) and SHP-2 (*bottom*).

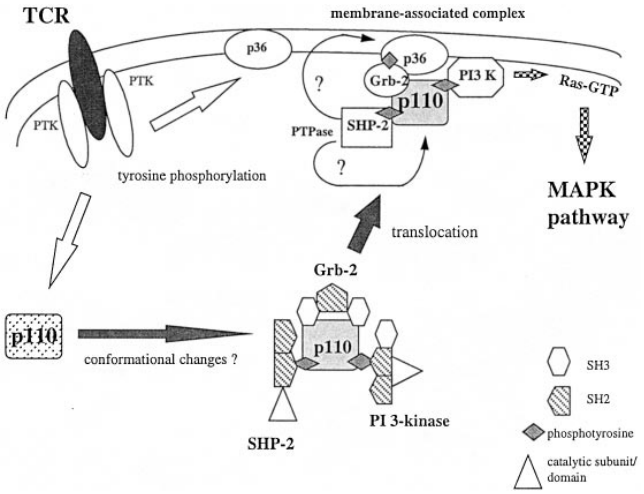


Figure 6. A model to illustrate how SHP-2 regulates coupling of the TCR to the Ras/MAPK pathway. Ligation of the TCR induces the tyrosine phosphorylation of cytosolic p110 and membrane-localized p36. This results in the formation of a cytosolic complex containing SHP-2, PI 3′-kinase, and Grb-2. Grb-2 may then mediate the association of this complex with the membrane via p36 binding. Arrival at the membrane causes full activation of SHP-2, resulting in the dephosphorylation of p110 and p36. Promotion of the Ras/MAPK pathway ensues, possibly due to the release and subsequent activation of PI 3′-kinase.

plex remains to be elucidated. However, it is interesting to note that PI 3′-kinase activation in response to insulin has been shown to be sensitive to the expression of catalytically inactive SHP-2 (41).

We therefore propose that Jurkat p110 acts as a cytosolic docking protein that is responsible for the orchestration of a signaling complex containing SHP-2, Grb-2, and PI 3′-kinase, which in turn regulates the positive actions of SHP-2 on TCR coupling to the MAPK pathway, as illustrated in Fig. 6. The dephosphorylating actions of SHP-2 appear to take place primarily in association with membranes since the C→S SHP-2 mutant localizes there preferentially compared with wild-type PTPase (Fig. 2 *A*) and the effects of mutant SHP-2 on protein tyrosine phosphorylation are more marked in the membrane fraction (Fig. 2 *B*). An attractive model consistent with our results suggests that TCR-induced tyrosine phosphorylation and conformational changes within p110 provide binding sites for SHP-2, PI 3′-kinase, and Grb-2. The tyrosine phosphorylation of the p36 protein may provide a membrane-localized docking site for the complex via its reported interaction with the SH2 domain of Grb-2. By analogy with other docking proteins such as Dos, a putative pleckstrin homology domain in p110 could also be involved in targeting or stabilizing the SHP-2–Grb-2–PI 3′-kinase–p110 complex in the plasma membrane. The occupation of both SH2 domains of SHP-2 is necessary for its full activation (42) yet only the COOH-terminal domain is efficiently occupied by p110 (10). This interaction may serve solely to localize SHP-2 to the plasma membrane where it may then associate with a phosphotyrosine residue that occupies its NH_2-terminal SH2 domain, thereby activating the enzyme. The phospholipid environment of the membrane may also be important for ensuring that SHP-2 PTPase activity is preferentially directed to membrane-associated substrates. Our results suggest that one of these substrates is the SHP-2–associated p110 itself since, first, a hyperphosphorylated 110-kD protein was demonstrated in membrane fractions prepared specifically from PTPase-inactive SHP-2–expressing cells (Fig. 2 *B*) and, second, the phosphotyrosine immunoblotting signal derived from p110 was significantly stronger when the protein was coprecipitated with inactive compared with wild-type SHP-2 (Fig. 3, *A* and *B*). A parallel situation is found in the Drosophila system, in which Dos has been shown to be a substrate for the SHP-2 homologue csw (34).

PTPase actions on p110 may cause the release of signaling molecules from the complex, either by direct dephosphorylation of a binding site or due to a dephosphorylation-related conformational change. Molecules such as PI 3′-kinase may therefore use p110 as a means of transportation from the cytosol to a specific membrane proximal compartment for interaction with their substrates. SHP-2–mediated regulation of the phosphotyrosine content of p110 could then result in the release and/or activation of PI 3′-kinase at its appropriate site of action. A molecular link between PI 3′-kinase and small GTP-binding proteins such as Ras was originally made by Rodriguez-Viciana et al. (43). More recent reports provide evidence for the positive regulation of guanine nucleotide exchange factor proteins by PI 3′-kinase as

a result of the binding of phosphatidylinositol (3,4,5) trisphosphate, the product of PI 3′-kinase action, to their pleckstrin homology domains (44, 45). PI 3′-kinase may therefore provide a link between the dephosphorylating actions of SHP-2 and the positive role played by this PTPase in coupling TCR stimulation to the Ras/MAPK pathway. We have also demonstrated the enhanced phosphotyrosine signal of the Grb-2 binding protein p36 protein in membranes expressing mutant SHP-2 (Figs. 2 *B* and 3 *B*), the significance of which will become apparent with a clearer understanding of p36 structure and function.

In conclusion, we have shown the PTPase SHP-2 to be an important component of a signaling complex that regulates the MAPK pathway in human T cells. The mode of action of SHP-2 in promoting the MAPK pathway is likely to involve the dephosphorylation of at least two membrane-associated tyrosine phosphoproteins, p36 and p110. Future analysis of p36 and identification of p110 will be necessary to define more precisely how SHP-2 is involved in the regulation of TCR-induced MAPK activation and perhaps in other T cell signal transduction pathways.

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