Multiple studies have demonstrated an important role for the Src homology 2-containing tyrosine phosphatase 2 (SHP-2) in receptor tyrosine kinase-regulated cell proliferation and differentiation. Recent studies have identified potential SHP-2 substrates which mediate these effects. SHP-2 also is implicated in several cytokine receptor signaling pathways and in Bcr-Abl transformation. However, its precise role and targets in normal and abnormal hematopoietic cells remain to be determined. We identified two novel tyrosyl-phosphorylated proteins associated with SHP-2 in hematopoietic cells. The first, a 97-kDa cytosolic protein (p97), associates inducibly with SHP-2 upon cytokine stimulation and constitutively in Bcr-Abl-transformed cells. In contrast, p135, a 135-kDa transmembrane glycoprotein, forms a distinct complex with SHP-2, independent of cytokine stimulation or Bcr-Abl transformation. Far Western analysis reveals that SHP-2, via its Src homology 2 domains, can interact directly with either protein. In vitro dephosphorylation experiments, as well as transient transfection studies using wild type and mutant SHP-2 constructs, suggest that p97 and p135 also are SHP-2 substrates. Our results indicate that SHP-2 forms at least two separate complexes in hematopoietic cells and point to new potential SHP-2 targets.

Many signaling pathways are initiated and/or regulated by tyrosyl phosphorylation, controlled by protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). The SH2-containing PTPs (SHPs) comprise a particularly interesting PTP subfamily. SHPs, which include the mammalian proteins SHP-1 and SHP-2 and Drosophila Csw, share the same general structure: two SH2 domains at their N termini, a PTP domain, and a C-terminal region containing two tyrosyl phosphorylation sites, yet subserve distinct biological functions. SHP-1 (previously known as SH-PTP1, PTP1C, HCP, or SHP; Ref. 1) is expressed predominantly in hematopoietic cells, where it acts as a negative regulator of multiple signaling pathways (2–4). SHP-2 (previously known as SH-PTP2, PTP1D, Syp, PTP2C, or SHPTP3; Ref. 1) is expressed ubiquitously and is required to regulate multiple receptor PTK (RTK) signaling pathways (4, 5). Overexpression of catalytically impaired SHP-2 mutants blocks insulin (6–8), EGF (6, 9), and, in some cell types, PDGF-induced (10, 11) mitogenesis. SHP-2 mutants also inhibit basic fibroblast growth factor-induced mesoderm induction and gastrulation in Xenopus (12). Furthermore, csw, the likely homolog of SHP-2, acts downstream of several RTKs (13), including Torso (13, 14) and Sevenless (15), where it is required for proper development of terminal structures (13) and R7 photoreceptor cells (15), respectively.

Much has been learned about how SHP-2 participates in RTK signaling. Via its SH2 domains, SHP-2 binds directly to several RTKs, as well as to accessory molecules such as IRS-1 and Gab (1, 4, 5). In mammalian cells, multiple studies indicate that SHP-2 acts upstream of MAPK (16–18). However, SHP-2 has been reported to act both upstream (17) and downstream (19) of Ras in insulin signaling, and genetic analyses of Sevenless signaling in Drosophila suggest that csw either acts upstream or downstream of Ras and/or in a parallel pathway (15). Since numerous lines of evidence indicate that the PTP domain is required for SHP-2 function, identifying SHP-2 substrates should provide critical insights into its mechanism(s) of action.

Recently, potential substrates for both csw and SHP-2 in RTK signaling have been identified. Genetic and biochemical evidence strongly suggest that Dos (for Daughter of Sevenless) is a substrate for csw in the Sevenless pathway (20, 21). Dos has an N-terminal PH domain, several proline-rich stretches, and 10 potential tyrosine phosphorylation sites. Although Dos is distantly related to mammalian Gab-1 (20, 22), it is not yet clear whether these two molecules are homologs. The recently cloned SHPS-1 (23), which most likely is the 115-kDa protein that was previously reported to associate with SHP-2 in response to insulin or EGF stimulation (16, 17, 24–26), provides a potential target for SHP-2 in growth factor signaling. Unlike Dos, SHPS-1 is a transmembrane glycoprotein with sequence similarity to cell adhesion molecules.

In addition to its role in RTK pathways, SHP-2 also is implicated in cytokine signaling and in hematopoietic cell transformation by Bcr-Abl. Upon stimulation of factor-dependent cell lines with IL6 or leukemia inhibitory factor (27), IL3/GM-CSF (28), or Epo (29, 30), SHP-2 rapidly becomes tyrosyl-phosphorylated. Catalytically inactive SHP-2 mutants inhibit
prolactin- (31) and interferon-α/β-induced gene activation (32). An EpoR mutant that abolishes interaction with SHP-2 exhibits decreased mitogenic potential (30), whereas cells expressing a mutant p110α that results in elimination of tyrosine phosphorylation of SHP-2 fail to proliferate upon ligand stimulation (33). However, SHP-2 targets in these pathways remain to be identified. SHP-2 is constitutively tyrosyl-phosphorylated in Bcr-Abl-transformed cells and has been reported to bind to Bcr-Abl (34). The role, if any, that SHP-2 plays in Bcr-Abl transformation as well as the identities of SHP-2 targets in Bcr-Abl-transformed cells remain unclear.

To further our understanding of SHP-2’s functions in hematopoietic cells, we searched for tyrosyl-phosphorylated proteins that associate with SHP-2 in response to cytokine stimulation and upon Bcr-Abl transformation. We identified two novel SHP-2-associated tyrosyl phosphoproteins, p97 and p135, in cytokine-induced and Bcr-Abl-transformed BaF3 cells. p97, a cytosolic protein, is the major tyrosyl-phosphorylated protein associated with SHP-2 in response to IL3, GM-CSF, or Epo. Although consistent with previous results (34), we find SHP-2-associated tyrosyl-phosphorylated proteins, p97 and p135, in Bcr-Abl-transformed cells. p135, a membrane-associated glycoprotein, associates constitutively with SHP-2 in a distinct complex. Furthermore, in vitro and in vivo data suggest that p97 and p135 may be SHP-2 substrates. Our results suggest that SHP-2 may regulate the phosphorylation of at least two proteins in hematopoietic cells, and suggest that Bcr-Abl transformation leads to constitutive phosphorylation of one of these proteins.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Stimulations—**BaF3 cells overexpressing the mouse EpoR (BaF3-EpoR) or the human GM-CSFR (BaF3-GM-CSFR) were provided by Dr. Alan D’Andrea (Dana-Farber Cancer Institute, Boston, MA). IL3-dependent cell lines were passaged routinely in RPMI supplemented with 15% WEHI-CM, 20 ng/ml murine IL3 (Biosource, CA), 50 units/ml Epo (Dr. Alan D’Andrea), or 20 ng/ml human GM-CSF (CSF). Scavenging and Metabolism—Staining and flow cytometry were performed with either phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies against mouse IL3/GM-CSF receptors and the murine IL3 receptor α subunit and β subunit. The role, if any, that SHP-2 plays in Bcr-Abl transformation as well as the identities of SHP-2 targets in Bcr-Abl-transformed cells remain unclear.

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**Antibodies, Immunoprecipitation, and Immunoblotting—**Anti-SHP-2 polyethylene glycol (no. 986) were generated against a full-length SHP-2-glutathione S-transferase (GST) fusion protein (36). Immunoglobulins were purified by protein A-Sepharose chromatography (37). Some experiments utilized affinity-purified anti-SHP-2 antibodies, which were gel-purified as described (38). Anti-peptide antibodies against the SHP-2 C terminus and SHP-2 polyclonal antibodies against the β common chain of the IL3/GM-CSF receptors and the murine IL3 receptor β subunit and monoclonal anti-GST antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-phospho-tyrosine antibodies (4G10) were obtained from Upstate Biotechnology, Inc (Lake Placid, NY). The 12CA5 hybrida, which produces anti-HA monoclonal antibodies, was a gift of Dr. Raymond L. Erickson (Harvard University, Cambridge, MA).

Cell lysates (107 cells/0.35 ml of lysis buffer) were prepared using Nonidet P-40 buffer as described previously (39), and incubated with anti-SHp-2 polyclonal antibodies (1 μg) or anti-peptide antibodies (1 μg of 986 IgG, or 2 μg of affinity-purified antibodies). Immunoprecipitates were collected, washed, and analyzed by SDS-PAGE and immunoblotting as described (39). Blots were probed with anti-phosphotyrosine antibodies (0.5 μg/ml) or monoclonal anti-SHP-2 antibodies (1:1000 dilution) as indicated, incubated with sheep anti-mouse IgG peroxidase-conjugated secondary antibodies (1:8000 dilution), and developed using ECL (Amersham).
Tyrosyl Phosphoproteins Associated with SHP-2 in Response to Cytokine Stimulation and Bcr-Abl Transformation—Previous studies implicated SHP-2 in signaling via receptors (IL3, GM-CSF) that utilize the IL3 receptor β common (βc) chain (28). We examined the participation of SHP-2 in IL3-induced signaling in the IL3-dependent cell line, BaF3. As expected from the results of multiple previous studies, addition of 15% WEHI-CM (as a source of IL3) to factor-starved BaF3 cells induced rapid tyrosyl phosphorylation of multiple proteins (data not shown). We examined SHP-2 phosphorylation and protein-protein interactions in response to IL3 stimulation by anti-SHP-2 immunoprecipitations followed by anti-phosphotyrosine immunoblotting (Fig. 1A). As expected from a previous report (28), SHP-2 became tyrosyl-phosphorylated in response to IL3 addition. Interestingly, however, upon IL3 stimulation, a major tyrosyl phosphoprotein, with an apparent molecular mass of approximately 97 kDa (hereafter termed p97), was found associated with SHP-2. Comparable amounts of SHP-2 were recovered in SHP-2 immunoprecipitates from unstimulated and IL3-stimulated cells, as shown by reprobing the anti-phosphotyrosine blot with anti-SHP-2 antibodies (Fig. 1A). Similar results were obtained using recombinant IL3 (data not shown, but see Fig. 5A). The increased intensity of the p97 band in SHP-2 immune complexes following IL3 stimulation indicates enhanced tyrosyl phosphorylation and/or enhanced association of p97 with SHP-2.

We also noted the presence of a weakly tyrosyl-phosphorylated band with an apparent molecular mass of 135 kDa (hereafter termed p135) in SHP-2 immune complexes (Fig. 1A, but note that p135 is seen more easily in Fig. 1B and later figures). Both p97 and p135 were found to co-immunoprecipitate with SHP-2 using several different SHP-2 antibodies, arguing that they form complexes with SHP-2 rather than sharing SHP-2 epitopes (see also additional experiments below).

We next examined the kinetics of association between SHP-2 and tyrosyl-phosphorylated p97 and p135 in more detail (Fig. 1, B and D). A small amount of tyrosyl-phosphorylated, SHP-2-associated p97 can be detected within 1 min of IL3 addition to factor-starved cells. With time, the amount of tyrosyl-phosphorylated, SHP-2-associated p97 increases, reaching peak levels by about 10 min and remaining at this level for at least 30 min post-stimulation. Conversely, a low level of tyrosyl-phosphorylated p135 co-immunoprecipitated with SHP-2 in the absence or presence of IL3, and its phosphorylation (and/or extent of association) increased minimally, if at all, upon IL3 stimulation.

Transformation of BaF3 cells and many other hematopoietic cell lines with p210 Bcr-Abl or p190 Bcr-Abl renders them factor-independent (43). Previous work (34) indicated that SHP-2 becomes tyrosyl-phosphorylated and associates with p210 Bcr-Abl upon p210 Bcr-Abl transformation. We asked whether p97 or p135 association with SHP-2 was altered upon Bcr-Abl transformation. Lysates from p210 or p190 Bcr-Abl-transformed BaF3 cells were immunoprecipitated with anti-SHP-2 antibodies, and the immune complexes were subjected to anti-phosphotyrosine immunoblotting (Fig. 1C). As in parental BaF3 cells, a small amount of tyrosyl-phosphorylated p135 was associated with SHP-2 in Bcr-Abl-transformed cells. Consistent with the previous report (34), SHP-2 was found to be tyrosyl-phosphorylated constitutively in p210 Bcr-Abl-expressing, as well as in p190 Bcr-Abl-expressing BaF3. A low level of a tyrosyl-phosphorylated band with a size consistent with the respective Bcr-Abl fusion protein also was found in SHP-2 immune complexes from these cells (Fig. 1C, Bcr-Abl). However, a 97-kDa protein (Fig. 1C, p97), was by far the major tyrosyl-phosphorylated protein consistently detected in SHP-2 immune complexes from either p190 Bcr-Abl- or p210 Bcr-Abl-transformed BaF3 cells. SHP-2 also was constitutively tyrosyl-phosphorylated and associated with a major, 97-kDa phosphotyrosyl protein in 32D myeloid cells transformed by either p190 or p210 Bcr-Abl (Fig. 1C). Although association of the 97-kDa phosphotyrosyl protein with SHP-2 was a common feature of transformation with both forms of Bcr-Abl in different cell backgrounds, other tyrosyl phosphoproteins were associated differentially with SHP-2 in Bcr-Abl-transformed cells. For example, a 150-kDa tyrosyl-phosphorylated protein (Fig. 1C, p150) associated with SHP-2 in p210 Bcr-Abl-transformed BaF3 cells, but not in p190 Bcr-Abl-transformed BaF3 cells, whereas a 170-kDa tyrosyl-phosphorylated protein complexed with SHP-2 in p190 Bcr-Abl-transformed 32D cells, but not p210 Bcr-Abl-transformed 32D cells (Fig. 1C, p170).

The above results suggested that at least one common SHP-2-binding protein (the 97-kDa species) is targeted by both isoforms of Bcr-Abl, independent of cell background. The size of the 97-kDa band from Bcr-Abl-transformed cells suggested that it might represent the same protein as p97, the IL3-induced SHP-2-binding protein (Fig. 1, A and B). To explore this possibility, we performed V8 partial peptide mapping (40) of the respective 97-kDa proteins. BaF3 and BaF3 p210 Bcr-Abl cells were labeled with [35S]methionine. Cell lysates from the labeled cells were immunoprecipitated with anti-SHP-2 antibodies, resolved by SDS-PAGE, and fluorographed. In starved BaF3 cells, there is no detectable labeled 97-kDa protein in SHP-2 immune complexes, indicating that p97 is not associated with SHP-2 basally. Upon IL3 stimulation, a major, 97-kDa species was found to associate with SHP-2 (Fig. 1D). Since tyrosyl-phosphorylated p97 becomes associated with SHP-2 in IL3-stimulated cells (Fig. 1, A and B), we conclude that association of p97 with SHP-2 in response to IL3 stimulation correlates with tyrosyl phosphorylation of p97. Comparison of the relative intensities of the SHP-2 and 97-kDa bands suggests (assuming that the percentage of methionine in both proteins is comparable) that a substantial fraction (perhaps as much as 20–50%) of SHP-2 is likely to be associated with p97 upon cytokine stimulation. As expected, a band of similar molecular size was associated constitutively with SHP-2 in Bcr-Abl-transformed BaF3 cells (Fig. 1D). The two 97-kDa bands were excised, digested with different amounts of V8 protease, and resolved by SDS-PAGE. The partial peptide maps of the two proteins are essentially identical (Fig. 1E), indicating that they are identical or at least highly related proteins. These results suggest that upon transformation of BaF3 or 32D cells with either p190 or p210 Bcr-Abl, p97, a phosphotyrosyl protein that normally associates inducibly with SHP-2 upon cytokine stimulation, becomes constitutively associated with SHP-2.

Inducible association of SHP-2 with p97 is not restricted to IL3 signaling and BaF3 cells. When BaF3 cells that express the human GM-CSF receptor were treated with GM-CSF, tyrosyl-phosphorylated SHP-2 and a 97-kDa protein were detected in SHP-2 immune complexes (Fig. 1A). Likewise, p97 was found to associate inducibly with SHP-2 upon stimulation of BaF3 cells ectopically expressing the EpoR with Epo (Fig. 1A). A 97-kDa tyrosine-phosphorylated protein also co-immunoprecipitates with SHP-2 upon stimulation of B cell lines by antigen receptor cross-linking, or stimulation of Mo7e cells (a human erythro-megakaryoblastic cell line) with IL3 or GM-CSF (data not shown). Several Stat family members (44), as well as the...
SHP-2-binding Phosphotyrosyl Proteins in Hematopoietic Cells

**FIG. 1**

**A**

![Western blot of SHP-2-binding phosphotyrosyl proteins in hematopoietic cells.](image)

**B**

![Western blot analysis of SHP-2 expression in hematopoietic cells.](image)

**C**

![Western blot analysis showing the effect of BCR-ABL on SHP-2 expression in hematopoietic cells.](image)

**D**

![Western blot analysis of SHP-2 expression in hematopoietic cells with different concentrations of V8 protease.](image)

**E**

![Western blot analysis of SHP-2 expression in hematopoietic cells with varying concentrations of V8 protease.](image)
SHP-2 Can Bind p97 and p135 directly via its SH2 domains—Since SHP-2 contains two SH2 domains, which can bind specific tyrosyl-phosphorylated peptides (46), we used Far Western analysis to ask whether these SH2 domains could interact directly with p97 and/or p135. A GST-fusion protein containing the SH2 domains of SHP-2 was used to probe blots of total cell lysates and SHP-2 immune complexes from starved BaF3 cells, IL3-stimulated BaF3 cells, or BaF3 cells transformed by p210 Bcr-Abl. Proteins on the blot that interacted directly with the GST-fusion protein were visualized by using anti-GST antibodies (Fig. 2). In IL3-stimulated or Bcr-Abl-transformed BaF3 cells strong bands of 97 kDa were detected by the GST-SH2 fusion protein (Fig. 2, arrowhead). Unlike p97, p135 reactivity with GST-SH2 was independent of IL3 stimulation. The SHP-2-associated 150-kDa protein found only in p210 Bcr-Abl-transformed cells was also recognized by GST-SH2 fusion protein (Fig. 2, p150). Probing similar blots with GST alone revealed no reactive bands. Moreover, probing with a GST fusion protein containing the SH2 domain of Grb2 resulted in easy detection of SHP-2, consistent with previous reports that tyrosyl-phosphorylated SHP-2 binds Grb2 (28, 47, 48) but showed no reactivity with p97, p135, or p150 (data not shown). These results indicate that the ability of the SH2 domains of SHP-2 to detect these proteins reflects specific binding properties of these SH2 domains, and suggest that SHP-2 directly interacts with tyrosyl-phosphorylated p97, p135, and p150.

Interestingly, the amount of p97 and p150 remaining in the supernatant after immune depletion with anti-SHP-2 antibodies was reduced by more than 50%. Under our conditions, essentially all of the SHP-2 was depleted by anti-SHP-2 immune depletions (Fig. 2). Control depletions with non-immune antibodies did not result in reduction of the level of p97 or p150 (Fig. 2). These results suggest that the majority of tyrosyl-phosphorylated p97 (and p150) in cells is associated with SHP-2, supporting the physiological importance of this complex.

Subcellular Localization of p97 and p135—To determine the intracellular location of p97 and p135, we performed subcellular fractionation experiments on IL3-stimulated (Fig. 3A) or Bcr-Abl-transformed (Fig. 3B) BaF3 cells. The cells were lysed in hypotonic buffer, and the crude nuclear (P1), microsomal (P100), and cytoplasmic (S100) fractions were adjusted to lysis buffer conditions (see “Experimental Procedures”) and immunoprecipitated with anti-SHP-2 antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies. All of the SHP-2-associated p97 from IL3-stimulated (Fig. 3A) or Bcr-Abl-transformed (Fig. 3B) cells was found in the S100 fraction (with recovery >50%). The SHP-2-associated p150 protein from Bcr-Abl-transformed cells also fractionates with cytosolic proteins. Conversely, p135 was present only in the P100 fraction (Fig. 3A). Virtually all of the SHP-2 fractionated to the S100, with a small amount present in the other two fractions. If p135 and SHP-2 have comparable numbers of phosphorylation sites, these data raise the possi-

**FIG. 1.** Tyrosyl-phosphorylated proteins associated with SHP-2 in hematopoietic cells. A, 97-kDa tyrosyl-phosphorylated protein associates with SHP-2 in response to stimulation with various cytokines. BaF3, BaF3-GM-CSFR, or BaF3-EpoR cells (10⁷) were starved and either left unstimulated (-), stimulated for 10 min with 15% WEHI-CM (IL3), 20 ng/ml human GM-CSF (GM), or 50 units/ml Epo (EPO), respectively. Cells were lysed and immunoprecipitated with anti-SHP-2 peptide antibodies (I) or preimmune antibodies (PI). Immune complexes were subjected to anti-phosphotyrosine immunoblotting (upTyr). The blot was stripped and reprobed with anti-SHP-2 monoclonal antibodies (α-SHP-2). Note the inducible association of a 97-kDa phosphotyrosyl protein (p97) and the constitutive association of a weakly phosphorylated 135-kDa band (p135); this band is more easily seen in longer exposures/other figures. B, kinetics of association of SHP-2 and tyrosyl-phosphorylated proteins in response to IL3 stimulation. BaF3 cells were starved and stimulated with 15% WEHI-CM for the indicated times. Lysates from each time point were subjected to anti-SHP-2 immunoprecipitation, followed by anti-phosphotyrosine and anti-SHP-2 immunoblotting, as in panel A. C, tyrosyl-phosphorylated proteins associated with SHP-2 in Bcr-Abl-transformed cells. 32D and BaF3 cells (10⁷) transformed by either p190 Bcr-Abl or p210 Bcr-Abl were lysed, immunoprecipitated with preimmune (PI) or anti-SHP-2 peptide antibodies (I), and subjected to anti-phosphotyrosine and anti-SHP-2 immunoblotting as above. D and E, V8 partial peptide mapping. BaF3 and BaF3 p210 Bcr-Abl cells (2.5 × 10⁸) were labeled with [¹⁵S]methionine. Prior to lysing the cells, 1/6 (IX) of the BaF3 cells were retained as unstimulated (-), and 5/6 (IX) of the BaF3 cells were stimulated with 20 ng/ml IL3 for 10 min. Labeled cell lysates were immunoprecipitated with anti-SHP-2 antibodies, fractionated by SDS-PAGE, and fluorographed (D). Note the appearance of a prominent 97-kDa co-immunoprecipitating band (p97) upon IL3 stimulation. This band co-migrates with a constitutively associated band in the Bcr-Abl-transformed cells. The labeled 97-kDa bands from IL3-stimulated or p210 Bcr-Abl-transformed BaF3 cells were excised, subjected to digestion with 20 or 100 ng of V8 protease, as indicated, and resolved by 15% SDS-PAGE. The gel was fluorographed and exposed to X-Omat film for 8 days (F). IX and 2X below the gel in panel D indicate the relative amount of lysate used for each immunoprecipitation.
bility that all of the SHP-2 in the P100 fraction is associated with p135. We conclude that p97 is a cytosolic protein, whereas p135 is a membrane protein, at least when these proteins are tyrosyl-phosphorylated and associated with SHP-2. Moreover, given their disparate intracellular locations and the finding that both proteins interact directly with SHP-2 via the latter’s SH2 domains, p97 and p135 must form distinct, presumably mutually exclusive, signaling complexes with SHP-2 in hematopoietic cells.

p135 Is a Novel Transmembrane Glycoprotein—The above results indicate that p135 is a membrane protein, but do not establish whether it is a transmembrane protein. To resolve this issue, we first asked whether p135 can bind to lectins, since many membrane proteins are lectin-binding glycoproteins. For these experiments, we attempted to increase the level of p135 tyrosyl phosphorylation by pretreating BaF3 cells with the nonspecific PTP inhibitor pervanadate. Such treatment leads to increased recovery of p135 in SHP-2 immune complexes (see Fig. 5A). p135 eluted from anti-SHP-2 antibody beads was found to bind well to either ConA or WGA beads, resolved by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibodies. The apparent size of p135 is decreased to about 100 kDa (closed arrowhead) upon N-glycosidase F treatment, which is clearly different from the bands produced upon deglycosylation of βc and IL3Rβ (open arrowhead).

Fig. 3. Subcellular localization of SHP-2-associated proteins. BaF3 cells (10⁷) stimulated with 15% WEHI-CM (clone 5A3) (A) or transformed by p210 Bcr-Abl (B) were homogenized in hypotonic buffer and fractionated into P1, P100, and S100 fractions (see “Experimental Procedures”). Comparable aliquots of cells were lysed directly in 1% Nonidet P-40 lysis buffer (TCL). Each lysate was immunoprecipitated with anti-SHP-2 antibodies and immunoblotted with anti-phosphotyrosine antibodies. p97 is mainly present in the cytosol (S100), whereas p135 is mainly present in the membrane fraction (P100). These blots were stripped and reprobed with anti-SHP-2 monoclonal antibodies.

Fig. 4. p135 is a novel glycoprotein. A, cell lysates from pervanadate-treated BaF3 cells were immunoprecipitated with anti-SHP-2 antibodies (986) cross-linked to protein A-Sepharose beads. SHP-2 immune complexes were loaded directly onto SDS-PAGE (−) or eluted from the beads with 100 mM glycine, pH 2.5, neutralized, incubated with either ConA or WGA beads, resolved by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibodies. B, lysates from pervanadate-treated BaF3 cells were immunoprecipitated with anti-SHP-2 antibodies (986), anti-βc, or anti-IL3 Rβ antibodies. Immune complexes were left untreated (−) or treated (+) with N-glycosidase F (EndoF), resolved by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibodies. The apparent size of p135 is decreased to about 100 kDa (closed arrowhead) upon N-glycosidase F treatment, which is clearly different from the bands produced upon deglycosylation of βc and IL3Rβ (open arrowhead).
Several lines of evidence argue that p135 is neither βc nor IL3Rβ. Both βc and IL3Rβ migrated more slowly than p135 in our gel system (Fig. 4B). The core glycoproteins resulting from N-glycosidase F treatment of βc and IL3Rβ also were substantially larger than that produced from p135 (Fig. 4B). In addition, we could not detect any SHP-2 in either βc or IL3Rβ immunoprecipitates, nor could we detect βc and IL3Rβ by immunoblotting of SHP-2 immunoprecipitates (data not shown). For these reasons, we conclude that SHP-2 does not associate directly with known components of the IL3R complex. Previously, we described an approximately 130-kDa tyrosyl phosphoprotein that is associated constitutively with SHP-1 in primary macrophages and macrophage cell lines (48). More recent studies indicate that the SHP-1-associated p130 also is a transmembrane glycoprotein, and its tyrosyl phosphorylation increases upon GM-CSF treatment.3 However, N-glycosidase F digestion of the macrophage protein yields a product of about 70 kDa, clearly distinct from that produced from p135, indicating that these two proteins also are distinct.3 Instead, we conclude that membrane association of a small amount of SHP-2 is mediated by the novel transmembrane glycoprotein, p135.

p97 and p135 Are Potential Substrates of SHP-2—Since p97 and p135 bind to SHP-2, we were interested in determining whether either or both might be SHP-2 substrates. We performed three different types of experiment to address this question. First, we pretreated BaF3 cells with pervanadate to inhibit endogenous PTPs including SHP-2 and examined the association of p97 and p135 with SHP-2 (Fig. 5). When compared with starved BaF3 cells, pervanadate-treated BaF3 cells exhibited a marked increase in the level of tyrosyl-phosphorylated p135 in SHP-2 immune complexes. Treatment of BaF3 cells with pervanadate alone also evoked a slight increase in the level of tyrosyl-phosphorylated p97 found in SHP-2 immunoprecipitates (Fig. 5A). These data raise the possibility that p97, and, in particular, p135, are dephosphorylated at significant rates under basal (i.e. factor-starved) conditions. However, because pervanadate is a non-selective PTP inhibitor, these results do not identify SHP-2 as the specific molecule responsible for p97 and/or p135 dephosphorylation. Treatment with pervanadate plus IL3 did not further increase the level of SHP-2-associated p135 (Fig. 5A), consistent with our earlier finding that the level of p135 associated with SHP-2 is independent of IL3 stimulation (Fig. 1). However, compared with IL3 stimulation alone, pervanadate plus IL3 treatment resulted in enhanced recovery of tyrosyl-phosphorylated p97 in SHP-2 immuno precipitates. These data also are consistent with the hypothesis that the level of tyrosyl-phosphorylated p97 in association with SHP-2 is determined both by an IL3-stimulated PTK(s) and one or more PTPs.

Next, we examined whether SHP-2-associated p97 and p135 can be dephosphorylated in vitro by recombinant SHP-2. To obtain high levels of p97 and p135 (Fig. 5), randomly growing BaF3 cells were pretreated with pervanadate, lysed, and incubated with SHP-2 antibodies. SHP-2 immune complexes containing p97 and p135 were then treated with equivalent amounts (as determined by in vitro PTP assays using the artificial substrate para-nitrophenol phosphate) of GST-SHP-2 and GST-SHP-1 fusion proteins for 1 or 5 min (Fig. 5B). Incubations carried out in assay buffer alone resulted in significant dephosphorylation of p97 and, to lesser extents, p135 and endogenous SHP-2 within 5 min. These dephosphorylation events likely are due to the presence of endogenous SHP-2 in the immune complex and are consistent with the notion that SHP-2 can dephosphorylate both binding proteins, as well as itself. Addition of recombinant GST-SHP-2 resulted in enhanced dephosphorylation of p135, p97, and SHP-2. Together, these data indicate that SHP-2 can dephosphorylate p97 and p135 (as well as itself) in vitro. Interestingly, however, incubation of the SHP-2 immune complex with the GST-SHP-1 fusion protein did not lead to enhanced dephosphorylation of p135, p97, or SHP-2 within the period of the assay. This finding suggests that p135 and p97 may be preferred substrates for SHP-2 (compared with SHP-1).

Finally, we attempted to determine whether p97 and p135 are substrates of SHP-2 in vivo. We exploited the observation that PTPs bearing a cysteine→serine (Cys→Ser) mutation in the signature motif cannot dephosphorylate their targets, but retain the ability to bind (‘‘trap’’) potential targets (4, 49–51). Such mutants behave as biochemical dominant negatives, which, when expressed in vivo, should lead to enhanced phos-

3 J. Timms, H. Gu, and B. G. Neel, unpublished data.

4 J. Timms, H. Chen, and B. G. Neel, manuscript in preparation.
were obtained when p210 Bcr-Abl-transformed BaF3 cells were transfected with the same sets of constructs. Increasing amount of p135, 97, and p150 were associated with SHP-2(Cys → Ser) compared with SHP-2WT (Fig. 6D). In all of these experiments, similar or lower levels of SHP-2(Cys → Ser), compared with wild type SHP-2, were expressed (Fig. 6, A and B). Thus, although other explanations remain possible (see “Discussion”), the finding of higher levels of tyrosyl-phosphorylated p97 and p135 in SHP-2(Cys → Ser)-expressing cells is consistent with the notion that p97 and p135 are substrates for SHP-2.

**DISCUSSION**

We have identified and characterized two novel SHP-2-associated tyrosyl-phosphorylated proteins in hematopoietic cells. One, p97, is a cytosolic protein that inducibly associates with SHP-2 upon stimulation with several different cytokines and is constitutively tyrosyl-phosphorylated and associated with SHP-2 in Bcr-Abl-transformed cells. The other, p135, is a transmembrane glycoprotein that associates constitutively with SHP-2, independent of cytokine stimulation or Bcr-Abl transformation. These proteins appear to interact with the SHP-2 SH2 domains and to form distinct complexes with SHP-2. Since experiments *in vitro* and *in vivo* suggest that p97 and p135 may be SHP-2 substrates, one or both represent likely targets/mediators of SHP-2 actions in hematopoietic cells.

Several lines of evidence indicate that p97 and p135 are potential substrates of SHP-2. Pervanadate treatment of BaF3 cells results in increased co-immunoprecipitation of tyrosyl-phosphorylated p97 and p135 with SHP-2. Pervanadate is a non-selective PTP inhibitor, so these findings alone do not specifically implicate SHP-2 as the PTP that dephosphorylates p97 or p135. Interestingly, however, pervanadate treatment increased recovery of p135/SHP-2 complexes to a greater extent than it increased co-immunoprecipitation of p97 and SHP-2 (Fig. 5). This suggests that p135 is subject to ongoing phosphorylation by one or more cellular PTKs, and the low level of basal p135 tyrosyl phosphorylation/SHP-2 association may be a consequence of active dephosphorylation of p135 by one or more cellular PTPs. Conversely, p97 tyrosyl phosphorylation/SHP-2 association appears to require activation of a cytokine-induced PTK(s). Both p97 and p135 can be dephosphorylated by SHP-2 *in vitro*. There appears to be some specificity in this assay, as SHP-1 exhibits lower activity against these proteins (Fig. 5B).

The strongest evidence that p97 and p135 are targets of SHP-2 comes from transient transfection studies (Fig. 6). When similar amounts of HA-tagged SHP-2WT and SHP-2(Cys → Ser) were expressed transiently in BaF3, more tyrosyl-phosphorylated p97 and p135 co-immunoprecipitated with the Cys → Ser mutant compared with the tagged wild-type protein. Note increased recovery of tyrosyl-phosphorylated p97, and p150 in cells expressing the tagged Cys → Ser mutant at levels comparable to the tagged wild-type protein, consistent with the idea that the associated proteins are substrates for SHP-2 (see “Discussion”).

| kDa | IP: α HA | Vector | IL-3 |
|-----|---------|--------|------|
| 97  | p135    | 97     | p97  |
| 68  | SHP-2-HA| Reprobe: α SHP-2 |

**Fig. 6. Expression of SHP-2(Cys → Ser) mutant increases co-immunoprecipitation of tyrosyl-phosphorylated p97 and p135.** BaF3 (A) or BaF3 p210 Bcr-Abl (B) cells were electroporated with vector alone, HA-tagged SHP-2 wild type (WT) or HA-tagged SHP-2(Cys → Ser) (C → S). Twenty-four hours post-transfection, transfected BaF3 cells were starved for 8 h and left unstimulated (−) or stimulated with 15% WEHI-CM (+). Cell lysates were immunoprecipitated with anti-HA monoclonal antibodies and immunoblotted with anti-phosphotyrosine antibodies. The same blots were stripped and reprobed with anti-SH2 antibodies. The data presented are representative of at least two independent experiments. Similar data were obtained when p210 Bcr-Abl-transformed BaF3 cells were transfected with the same sets of constructs. Increasing amount of p135, 97, and p150 were associated with SHP-2(Cys → Ser) compared with SHP-2WT (Fig. 6D). In all of these experiments, similar or lower levels of SHP-2(Cys → Ser), compared with wild type SHP-2, were expressed (Fig. 6, A and B). Thus, although other explanations remain possible (see “Discussion”), the finding of higher levels of tyrosyl-phosphorylated p97 and p135 in SHP-2(Cys → Ser)-expressing cells is consistent with the notion that p97 and p135 are substrates for SHP-2.

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SHP-2-binding Phosphotyrosyl Proteins in Hematopoietic Cells

Our results suggest that SHP-2 forms distinct complexes with two different substrates in BaF3 cells, suggesting that SHP-2 may regulate more than one signaling pathway in these cells. These findings are consistent with the recent description of two different types of SHP-2 substrates in other systems (see Introduction). The biochemical properties of p97 (cytokine inducibility, location in cytosol) are similar to those of the Daugh-...
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