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Specificity of the SH2 Domains of SHP-1 in the Interaction with the Immunoreceptor Tyrosine-Based Inhibitory Motif-Bearing Receptor gp49B

Lawrence L. Wang,*† Julie Blasioli,*,‡ David R. Plas,*,‡ Matthew L. Thomas,*† and Wayne M. Yokoyama2a†

Inhibitory receptors on hemopoietic cells critically regulate cellular function. Despite their expression on a variety of cell types, these inhibitory receptors signal through a common mechanism involving tyrosine phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM), which engages Src homology 2 (SH2) domain-containing cytoplasmic tyrosine or inositol phosphatases. In this study, we have investigated the proximal signal-transduction pathway of an ITIM-bearing receptor, gp49B, a member of a newly described family of murine NK and mast cell receptors. We demonstrate that the tyrosine residues within the ITIMs are phosphorylated and serve for the association and activation of the cytoplasmic tyrosine phosphatase SHP-1. Furthermore, we demonstrate a physiologic association between gp49B and SHP-1 by coimmunoprecipitation studies from NK cells. To address the mechanism of binding between gp49B and SHP-1, binding studies involving glutathione S-transferase SHP-1 mutants were performed. Utilizing the tandem SH2 domains of SHP-1, we show that either SH2 domain can interact with phosphorylated gp49B. Full-length SHP-1, with an inactivated amino SH2 domain, also retained gp49B binding. However, binding to gp49B was disrupted by inactivation of the carboxyl SH2 domain of full-length SHP-1, suggesting that in the presence of the phosphatase domain, the carboxyl SH2 domain is required for the recruitment of phosphorylated gp49B. Thus, gp49B signaling involves SHP-1, and this association is dependent on tyrosine phosphorylation of the gp49B ITIMs, and an intact SHP-1 carboxyl SH2 domain. The Journal of Immunology, 1999, 162: 1318–1323.

The role of the ITIM was first elucidated in B cells, where it was shown that the ITIMs of CD22 were responsible for transmitting the CD22 inhibitory signal by engaging and activating SHP-1 (8), a tandem SH2 domain-containing cytoplasmic tyrosine phosphatase (11–14). The importance of the inhibitory signals delivered by SHP-1 is highlighted by the motheaten (me) mouse (15), which has a natural mutation in the SHP-1 locus (16). Mice with this deficiency are characterized by a widespread autoimmune phenomena, caused by an inability to negatively regulate immune responses. Consistent with the pleiotropic defects observed in the motheaten mouse, SHP-1 has also been implicated in negative signaling through many receptors, including receptors for growth factors, cytokines, and Ag, as well as through integrins (17). More recently, an important role for SHP-1 in negative signaling through NK cell inhibitory receptors has emerged. Experiments utilizing phosphorylated ITIM peptides implicated SHP-1 in signaling through the ITIM-bearing receptors in the KIR and Ly-49 family (18) before these associations were verified by coimmunoprecipitation studies (19–22). Another NK cell receptor, the CD94/NKG2A complex, also associates with SHP-1 (23, 24). Thus, SHP-1 plays a prominent and still expanding role in controlling immune responses.

The mechanism of binding and activation of SHP-1 by ITIM-bearing receptors is not yet fully understood. It is clear that tandem ITIMs, either on the same polypeptide (KIR) or as a result of dimerization (Ly-49A), optimally stimulate SHP-1 phosphatase activity (9), suggesting that occupation of both SHP-1 SH2 domains is required for maximal enzymatic activity. However, both SH2 domains are not required for binding of SHP-1 to receptor ITIMs based on binding studies using truncated fusion proteins (10, 19, 25, 26). The binding specificities of the SHP-1 SH2 domains in the context of the full-length protein have not been extensively analyzed. Such studies are...
especially important given that the crystal structure of the related molecule SHP-2 predicts that the phosphatase domain influences the binding of its SH2 domains (27). It is possible that ITIM binding by the SHP-1 SH2 domains is regulated in a similar fashion, although this has not been demonstrated.

The receptor gp49B is expressed on mast cells (28) and NK cells (29–31) and contains two putative ITIMs in its cytoplasmic tail separated by 16 amino acids. These ITIMs appear to be functional in NK cells, as suggested by Rojo et al., who have demonstrated that the gp49B cytoplasmic tail is able to inhibit killing by murine NK cells (30). In this study, we examine the signaling pathway utilized by gp49B and specifically its ability to associate with SHP-1. Additionally, we define the structural requirements of SHP-1 that mediate its interaction with gp49B.

Materials and Methods

Cell lines and Abs

A20 (murine B cell) and Jurkat (human T cell) cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in complete DMEM or RPMI 1640 media (10% FCS (Harlan, Indianapolis, IN), l-glutamine, penicillin, streptomycin, and 0.5 U ml-2 ME), respectively. Neither A20 nor Jurkat cells express gp49B (L.L.W., W.M.Y., unpublished observations). The murine NK cell clone KY-2 (32) was maintained in complete RPMI 1640 supplemented with 10 U/ml human rIL-2 (Chiron, Emeryville, CA). Immunoblottling reagents used were rabbit anti-SHP-1 polyclonal antiserum (33), anti-SHP-2 (Transduction Laboratories, Lexington, KY), anti-phosphotyrosine 4G10-biotin (Upstate Biotechnology, Lake Placid, NY), anti-gp49B, and horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, Arlington Heights, IL). For generation of anti-gp49B, rabbits were immunized with the peptide YAQQVKPSRLQKDTAC (Quality Controlled Biochemicals, Hopkinton, MA), corresponding to amino acids 300–314 of gp49B, conjugated to keyhole limpet hemocyanin. Rabbits were injected at 0, 4, 8, and 12 wk with 100 mg of gp49B-Fc in Ribi adjuvant (RIBI ImmunoChem Research, Hamilton, MT) at 0, 3, and 6 wk. To generate antibody against gp49B, rabbits were exsanguinated, and anti-gp49B was purified by peptide affinity chromatography. Anti-gp49B detects a ~55-kDa protein in lysates from KY-2 cells and gp49B-transfected Chinese hamster ovary cells, which is absent in Jurkat and untransfected Chinese hamster ovary cells (data not shown). Immunoprecipitation Abs used were B23.1 (a mouse IgM) (34) purified by MAR18.5 (anti-rat IgG) affinity chromatography, H3 (hamster polyclonal antiserum generated against gp49B-Fc), and isotype control rat IgG (PharMingen, San Diego, CA). For production of H3, Armenian hamsters were injected with 100 mg of gp49B-Fc in Ribi adjuvant (RIBI ImmunoChem Research, Hamilton, MT) at 0, 3, and 6 wk. To construct gp49B-Fc, the extracellular domain of gp49B (amino acids 23–238) was amplified using the PCR primers: forward, 5′-TATATGCTGACGAGGACACGCCAAGCCACGAC-3′, and reverse, 5′-ATATGGGATCCCGGCTAGCGGCTAGCTGATC-3′, which introduced Nhel and BamHI restriction sites at the 5′ and 3′ ends, respectively. The amplified fragment was cloned into Pcd5lneg1 (a generous gift from Brian Seed, Harvard Medical School, Boston, MA) and gp49B-Fc was produced and purified as previously described (35).

Peptides

All peptides were purchased from Quality Controlled Biochemicals and synthesized conjugated to biotin on the amino terminus. Unphosphorylated or tyrosine-phosphorylated peptides with the following sequences were synthesized: SEQEVTSYMVRF (Ly-49A ITIM), GQVYAYVKPS (gp49B proximal ITIM), QDVTVYALCIR (gp49B distal ITIM), VN RHYAKI (ZAP-70 Y474), and GHIYSELIQGF (derivative of CD22-Y5).

Cell stimulation and immunoblot analysis

Cells were washed in PBS and left untreated or stimulated with 10 μM pervanadate for 10 min at room temperature. Pelletted cells were lysed in either 1% Nonidet P-40 lysis buffer (peptide and GST SHP-1 adsorptions) or 1% Triton X-100 lysis buffer (immunoprecipitations) containing 150 mM NaCl, 10 mM Tris, pH 8, PMSF, aprotinin, leupeptin, pepstatin, EDTA, and soybean trypsin inhibitor. For immunoprecipitation and GST SHP-1 studies, sodium fluoride and sodium orthovanadate were also included in the lysis buffer. Insoluble material was removed by centrifugation at 14,000 × g for 20 min at 4°C. Following affinity purification with the indicated reagent, precipitates were washed four times in lysis buffer, separated by SDS-PAGE, and transferred to nitrocellulose, and immunoblot analysis was performed. For membrane stripping, blots were submerged in 100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, for 30 min, 50°C.

Peptide adsorption and immunoprecipitation

Cell lysates representing 3 × 106 cells/lane (silver stain or immunoprecipitation) or 1 × 107 cells/lane (immunoblot) were mixed with peptide at the indicated concentration, 2 μg of Ab, or 3 μl of hamster serum. Peptides were precipitated with avidin-agarose (Sigma, St. Louis, MO), rat IgM was precipitated by goat anti-rat IgG agarose (Sigma), and hamster IgG was precipitated with protein G-Sepharose (Pharmacia, Piscataway, NJ). Bound proteins were separated under reducing conditions by 7.5% SDS-PAGE and analyzed by silver stain (Daichii, Tokyo, Japan), or transferred to nitrocellulose, followed by immunoblot analysis.

SHP-1 phosphatase assays

Baculoviral-expressed full-length SHP-1 was purified by conventional chromatography and HPLC. The reaction mixture contained 0–200 μM peptide, 250 ng SHP-1, 50 mM HEPES (pH 7), 5 mM EDTA, 1 mM DTT, and 5 mM p-nitrophenyl phosphate (pNPP) as substrate. Reactions were stopped after 30 min at 37°C, and the relative phosphatase activity was measured by absorbance at 405 nm. Results are expressed as fold increase over basal phosphatase activity of SHP-1 in the absence of peptide. A phosphorylated CD22 peptide was used as a positive control for the phosphatase assay.

GST fusion proteins

For generation of the GST fusion proteins containing the SH2 domains of murine SHP-1 (amino acids 1–219), PCR was used to amplify the tandem SH2 domains from full-length SHP-1 cDNA as template: forward primer, 5′-GTGGAGAACCTCCAGGATGGTGAGGT-3′, and reverse primer, 5′-CAATTAGATCTTCGTTTACCCCGATG-3′, EcoRI and HindIII sites were incorporated into the forward and reverse primers, respectively, and used as the cloning sites for ligation into pGEX-KG (Pharmacia). The mutations of the amino acid and carboxyl SH2 domains were introduced by the PCR site overlap extension method. To clone the amino SH2 domain mutants (R30, 33KE), the internal primers used were forward primer, 5′-GTCCCCGGAGGAGCTCTGGATGAACCGCAGGAAGAACCGG-3′, and reverse primer, 5′-CCCTGTCTTCTCGCTGGCTATAGGTCGCGGCTAC-3′. For the carboxyl SH2 domain (R136K), the PCR primers used were forward primer, 5′-GGGAGGCATGACATTCTTTGGTGAAGAGAAGATGC-3′, and reverse primer, 5′-GACCTCCTTTCACAAAGGAGTCCATG-3′. Site-directed mutagenesis of the full-length SHP-1 cDNA was used to replace Cys853 with Ser in the phosphatase domain. The mutated SH2 domains were cassette-sequencing procedure in a unique restriction enzyme site. All mutations were verified by automated nucleotide sequencing (Applied Biosystems, Foster City, CA). GST fusion proteins were transformed into Escherichia coli strain BL21 (DE3) for protein production. Adsorption experiments were performed with the indicated quantity of GST fusion protein conjugated to glutathione agarose (Sigma).

Results and Discussion

Phosphorylation of the gp49B ITIMs is required for the recruitment of SHP-1 and SHP-2

gp49B contains two putative ITIMs in its cytoplasmic domain. To identify signaling molecules associated with the ITIMs, we synthesized peptides representing each of the two ITIMs of gp49B (membrane proximal and distal) in either the tyrosine-phosphorylated or unphosphorylated state. The Ly-49A ITIM, which has previously been shown to bind to and signal through SHP-1 (18, 22), was also synthesized. These peptides were used to adsorb lysates from A20 cells (Fig. 1A) or KY-2 cells (data not shown), and bound proteins were analyzed by SDS-PAGE and silver stain. The primary product that associated specifically with the gp49B-phosphorylated ITIMs and not the unphosphorylated peptides migrated at 66 kDa (p66). To determine the identity of p66, we performed anti-SHP-1 immunoblot analysis on lysates adsorbed with the ITIM peptides. Each ITIM peptide was able to interact with SHP-1 in a phosphorylation-dependent manner (Fig. 1B, top, lanes 2, 4, and 6). However, a tyrosine-phosphorylated ZAP-70 peptide did not interact with SHP-1, demonstrating the importance of not only the phosphorylated tyrosine but also the presence of the ITIM.
the phosphorylated tyrosine, but also the surrounding ITIM sequence for SHP-1 binding. By our silver stain analysis, only one other protein, migrating at 70 kDa (Fig. 1A), bound specifically to the phosphorylated ITIMs and most likely represents SHP-2 (Fig. 1B, bottom). From these data, we conclude that the phosphorylated gp49B ITIMs interact primarily with SHP-1 from hemopoietic cell lysates.

Comparison of the binding and phosphatase-activating capacities of the gp49B-phosphorylated ITIM peptides

The relative binding capacities of the phosphorylated gp49B ITIMs for SHP-1 were assessed by titrating each phosphorylated ITIM peptide with equivalent amounts of Jurkat cell lysate (Fig. 2). Among the gp49B ITIMs, the distal ITIM appeared to interact more strongly to SHP-1, as it retained binding at 0.16 μM (Fig. 2, lane 11), a concentration at which the proximal ITIM had lost SHP-1 binding (Fig. 2, lane 7). Binding of the distal gp49B ITIM was comparable with that observed for the Ly-49A ITIM, with both peptides losing binding at 0.04 μM.

The ITIM peptides were tested for their ability to activate SHP-1 phosphatase activity in vitro. Purified SHP-1 was incubated with the indicated phosphorylated or unphosphorylated ITIM peptide and the phosphatase substrate pNPP. The relative phosphatase activity was assessed by measuring the absorbance at 405 nm (Fig. 3). As previously published, a phosphorylated CD22 ITIM peptide activates SHP-1 very strongly (8). The gp49B-proximal ITIM demonstrated intermediate levels of SHP-1 activation, comparable with the levels seen with the Ly-49A peptide. In contrast, the distal ITIM of gp49B was a very weak stimulator of SHP-1 activity, but still activated at levels >2-fold above baseline activation with unphosphorylated peptides. Taken together, even though the distal ITIM demonstrates stronger binding to SHP-1 (Fig. 2), it is a far weaker stimulator of the SHP-1 phosphatase than the proximal ITIM (Fig. 3), suggesting that ITIM affinity to SHP-1 does not directly correlate with its phosphatase-activating potential.

Tyrosine phosphorylation of gp49B and recruitment of SHP-1 in pervanadate-treated NK cells

To verify the association between gp49B and SHP-1, we immunoprecipitated gp49B, using either mAb B23.1 or the polyclonal antiserum H3, from untreated or pervanadate-treated KY-2 cells, previously shown to express gp49B (29). Antiphosphotyrosine immunoblotting indicated that gp49B becomes phosphorylated following pervanadate treatment (Fig. 4A, lane 3). Immunoprecipitated proteins were subjected to anti-SHP-1 immunoblotting (Fig. 4B, top), gp49B associated with SHP-1 at very low levels in untreated NK cells (Fig. 4B, top, lanes 2 and 5), an interaction that

![FIGURE 1. Phosphopeptides representing the putative gp49B ITIMs and the Ly-49A ITIM interact specifically with SHP-1 and SHP-2. A, A20 cell (murine B cell) lysates were adsorbed with unphosphorylated (lanes 2 and 4) or phosphorylated peptides (lanes 1, 3, and 5), representing the Ly-49A ITIM and the gp49B membrane-proximal and distal ITIMs. Bound proteins were analyzed by SDS-PAGE and silver stain. The peptides are indicated along the top of the gel and used at 3 μM. Molecular weight markers are indicated in kilodaltons (kDa). B, Representative ITIM peptides in either the unphosphorylated (lanes 1, 3, and 5) or phosphorylated states (lanes 2, 4, and 6) were used to adsorb cell lysates derived from Jurkat cells. A ZAP-70 phosphopeptide (Y474) was included as a control (lane 7). Precipitated proteins were subjected to SDS-PAGE, followed by immunoblotting with Ab against SHP-1 (top) or SHP-2 (bottom).](http://www.jimmunol.org/)

![FIGURE 2. Titration of the phosphorylated gp49B ITIM peptides indicates that the distal ITIM of gp49B interacts more strongly to SHP-1 than the proximal ITIM. Equivalent amounts of Jurkat cell lysates were subjected to affinity chromatography with decreasing levels of phosphorylated gp49B or Ly-49A ITIM peptides. The concentration of each peptide is indicated at the top of the lanes. Bound SHP-1 protein was analyzed by immunoblotting.](http://www.jimmunol.org/)

![FIGURE 3. Activation of SHP-1 by phosphorylated ITIM peptides. The phosphatase activity of purified SHP-1 stimulated by various tyrosine-phosphorylated (pITIM) or unphosphorylated (ITIM) peptides was measured with the substrate pNPP. Phosphatase activity is expressed as fold increase over SHP-1 activity in the absence of peptide.](http://www.jimmunol.org/)
is greatly augmented following pervanadate treatment of cells (Fig. 4B, top, lanes 3 and 6). Anti-gp49B immunoblotting confirmed that comparable levels of gp49B were precipitated in each sample (Fig. 4B, bottom). SHP-1 did not coprecipitate with rat IgM, an isotype control Ab for B23.1, or preimmune hamster serum. Additionally, B23.1 and H3 did not precipitate SHP-1 from pervanadate-treated Jurkat cells that contain SHP-1, but lack gp49B. This establishes that tyrosine-phosphorylated gp49B interacts with SHP-1 in NK cells.

The SHP-1 phosphatase domain regulates the interaction between gp49B and SHP-1

To further define the interaction between gp49B and SHP-1, we synthesized GST fusion proteins containing only the tandem SH2 domains of SHP-1 (2SH2). In addition to synthesizing the wild-type SH2 domains of SHP-1, mutations were introduced in either the amino SH2 domain (R30, 33KE) or the carboxyl SH2 domain (R136K), which severely impair the binding of the respective SH2 domain (36). Untreated or pervanadate-treated KY-2 lysates were precipitated with the wild-type SH2 domains or the single mutated SH2 domains. Bound proteins were separated by SDS-PAGE and subjected to gp49B immunoblotting. Wild-type GST SHP-1 bound to gp49B only from pervanadate-stimulated cells (Fig. 5A, top, lane 3). Furthermore, mutation of either the amino or carboxyl SH2 domain of SHP-1 did not appear to affect the association with phosphorylated gp49B (Fig. 5A, top, lanes 6 and 9). Consistent with our phosphopeptide-binding data, binding of each SHP-1 fusion protein to unphosphorylated gp49B was undetectable. SHP-1 immunoblotting was also performed to confirm comparable loading of GST SHP-1 (Fig. 5A, bottom). Thus, the SHP-1 SH2 domains interact with tyrosine-phosphorylated gp49B, and either SH2 domain of SHP-1 is sufficient for this interaction.

Previous studies examining the specificities of the SHP-1 SH2 domains have suggested that the amino SH2 domain of SHP-1 is sufficient for interactions with two other ITIM-containing proteins, CD22 (25) and the erythropoietin receptor (26). In contrast to these data, the ITIMs of human KIR appear to require the carboxyl SH2 domain for binding, while the amino SH2 domain alone was not able to interact with phosphorylated ITIM peptides (19). More recent data, however, demonstrate that both KIR ITIMs can interact with either SHP-1 SH2 domain (10). These studies differed from our current experiments in that they utilized truncated fusion proteins containing only single SH2 domains, and it is possible that...
caused by an intramolecular association with the phosphatase domain. B, the amino SH2 domain is diminished due to a conformational change in SHP-1, which remains in the inactive state. The ITIM-binding capacity of the carboxyl SH2 domain (R136K) completely abrogated binding to gp49B (Fig. 5A, top, lane 9). However, the presence of the phosphatase domain prevents this interaction (Fig. 5B, top, lane 5). Moreover, the carboxyl SH2 domain of SHP-1, even in the context of full-length SHP-1, is sufficient for binding to gp49B (Fig. 5B, top, lanes 2 and 3). These results are consistent with predicted models of SHP-2 regulation (27), and we propose a similar model for SHP-1 association with receptor ITIMs (Fig. 6). In the basal state, the amino SH2 domain forms an intramolecular association with the phosphatase domain, which greatly diminishes the affinity of the amino SH2 domain for phosphorylated ITIM (Fig. 6A). The carboxyl SH2 domain of SHP-1 initiates an interaction by binding one site in a biphosphorylated ligand, thus increasing the local concentration of phosphorylated ITIM (Fig. 6B). This allows the amino SH2 domain to bind phosphorylated tyrosine, leading to release and activation of the phosphatase (Fig. 6C). The crystal structure of SHP-1 may ultimately prove this model.

The results presented in this work support and extend previously published data (31, 37) showing that gp49B represents another member of a growing family of inhibitory receptors that signal through tyrosine phosphatases. Our silver stain and immunoblot analysis suggest that SHP-1 is the primary product that binds specifically to the phosphorylated ITIMs of gp49B. Although others have reported SHIP association with the gp49B ITIMs (37), we failed to see this interaction, although it may be beyond the sensitivity of the silver stain. We verified the interaction between phosphorylated gp49B and SHP-1 in stimulated NK cells by immunoprecipitation studies and showed that the interaction of SHP-1 with the gp49B ITIMs is an orchestrated event. Our experiments argue that SHP-1 is involved in regulating NK cell function through gp49B, and suggest a model by which SHP-1 binds to receptor ITIMs.

Acknowledgments

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