The SH2 Inositol 5-Phosphatase Ship1 Is Recruited in an SH2-dependent Manner to the Erythropoietin Receptor*

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Ship1 (SH2 inositol 5-phosphatase 1) has been shown to be a target of tyrosine phosphorylation downstream of cytokine and immunoregulatory receptors. In addition to its catalytic activity on phosphatidylinositol substrates, it can serve as an adaptor protein in binding Shc and Grb2. Erythropoietin (EPO), the primary regulator of erythropoiesis, has been shown to activate the tyrosine phosphorylation of Shc, resulting in recruitment of Grb2. However, the mechanism by which the erythropoietin receptor (EPO-R) recruits Shc remains unknown. EPO activates the tyrosine phosphorylation of Ship1, resulting in the interdependent recruitment of Shc and Grb2. Ship1 is recruited to the EPO-R in an SH2-dependent manner. Utilizing a panel of EPO-R deletion and tyrosine mutants, we have discovered remarkable redundancy in Ship1 recruitment. EPO-R Tyr401 appears to be a major site of Ship1 binding; however, Tyr429 and Tyr431 can also serve to recruit Ship1. In addition, we have shown that EPO stimulates the formation of a ternary complex consisting of Ship1, Shc, and Grb2. Ship1 may modulate several discrete signal transduction pathways. EPO-dependent activation of ERK1/2 and protein kinase B (PKB)/Akt was examined utilizing a panel of EPO-R deletion mutants. Activation of ERK1/2 was observed in EPO-RΔ99, which retains only the most proximal tyrosine, Tyr348. In contrast, EPO-dependent PKB activation was observed in EPO-RΔ43, but not in EPO-RΔ99. It appears that EPO-dependent PKB activation is downstream of a region that indirectly couples to phosphatidylinositol 3-kinase.

Erythropoietin (EPO)1 exerts its biological activity by bind-
adapter protein, as when tyrosine-phosphorylated, it can bind Src in a phosphotyrosine-binding domain-dependent manner (20). Proline-rich sequences found at the carboxyl terminus of Ship1 also result in recruitment of the SH3 domain of Grb2 (21). In this study, we show that the EPO-R activates the tyrosine phosphorylation of Ship1 and that Ship1 binds to the EPO-R in an SH2-dependent fashion through multiple phosphotyrosine residues, including EPO-R Tyr401, Tyr429, and Tyr431.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—Ba/F3 cells expressing wild-type or mutant EPO-Rs were maintained in RPMI 1640 medium, 10% (v/v) fetal calf serum, and 50 μM β-mercaptoethanol (RPMI 1640 complete medium) supplemented with 5% (v/v) conditioned medium from WEHI-3B cells or the 1 mM/μg/ml G418. DA-3-EPO-R cells were maintained in RPMI 1640 complete medium and 0.5 units/ml human recombinant EPO. HCD-57 cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 20% fetal calf serum, 50 μM β-mercaptoethanol, and 0.5 units/ml human recombinant EPO.

Various EPO-R constructs were electropropitated into Ba/F3 cells. Individual G418-resistant subclones were isolated by limiting dilution. The expression of the EPO-R was confirmed by Western blotting, and the EPO-dependent growth characteristics of each subclone were examined by performing an XTT assay as described (22).

Generation of EPO-R Mutants—EPO-R tyrosine mutants were generated via overlap extension polymerase chain reaction. These included a series of single tyrosine mutants in which phenylalanine was substituted for different tyrosine residues in the EPO-R and a series of add-back mutants to an EPO-R devoid of tyrosine residues. Oligonucleotide primers were selected that produced a phenylalanine at amino acids 343, 401, 429, and/or 431. Polymerase chain reaction was performed using pBSK-EPO-R or selected pBSK-EPO-R tyrosine mutants to generate an Sphl-EcoRI fragment in PCR. The fidelity of all constructs was confirmed by sequencing both strands of the 440-base pair EcoRI fragment, which was subcloned into Sphl-EcoRI-digested pBSK-EPO-R. The EPO-R cDNA was then subcloned into pcDNA3.1-neo using KpnI and EcoRI.

Cytokine Deprivation and Stimulation—Cells were washed three times in 10 mM HEPES (pH 7.4) and Hanks’ balanced salts; starved in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol for 4 h at 37 °C, and then stimulated in the presence or absence of 1 ng/ml murine recombinant IL-3 or 50 units/ml human recombinant EPO for 10 min at 37 °C. The cells were washed once in 10 mM HEPES (pH 7.4) and Hanks’ balanced salts containing 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 10 μM EDTA, and 1 mM sodium orthovanadate and lysed in ice-cold lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 10 μM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 1 μg/ml pepstatin A. After 5 min on ice, the lysates were centrifuged at 10,000 × g for 5 min at 4 °C.

Antibodies—The anti-phosphoarylamine monoclonal antibody was generously provided by Dr. Brian Druker, and the anti-Ship1 polyclonal antibody was generated as described previously (23). Anti-EPO-R, anti-Grb2, anti-GST, anti-phospho-ERK1/2, and anti-Ship1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Shc polyclonal antibody was obtained from Transduction Laboratories (Lexington, KY). The anti-ERK1/2 antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phospho-PKB (Ser473) and anti-PKB antibodies were purchased from New England Biolabs Inc. (Beverly, MA). A peptide-specific EPO-R antibody has been described previously (22). Horseradish peroxidase (HRP)-conjugated protein A or HRP-conjugated sheep anti-mouse immunoglobulin (Amersham Pharmacia Biotech) was used as the secondary reagent for immunoblotting.

Immunoprecipitations—Antibodies were added to 2 mg of lysates for a 1-h incubation, followed by the addition of a 40-μl volume of protein A-Sepharose 4B beads (Amersham Pharmacia Biotech), and incubation was continued for an additional hour. The beads were washed three times in ice-cold lysis buffer, and elution complexes were eluted by boiling in Laemmli sample buffer containing 100 mM dithiothreitol. Samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane for Western blotting.

Ba/F3 subclones were metabolically labeled with [35S]methionine/cysteine as described previously (22). Labeled proteins were immunoprecipitated with an anti-peptide amino-terminal EPO-R antibody. Labeled proteins were detected by PhosphorImager analysis.

GST Fusion Protein Binding Experiments—Lysates (2 mg) were incubated overnight at 4 °C with GST fusion proteins expressing the SH2 domain of Ship1 immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The beads were washed three times in ice-cold lysis buffer, and proteins were eluted by boiling in Laemmli sample buffer with dithiothreitol. Samples were resolved by SDS-PAGE and analyzed by Western blotting.

Western Blotting—Following the electrophoretic transfer of proteins to nitrocellulose, the membranes were blocked at room temperature with 2.5% BSA in Tris-buffered saline (50 mM Tris (pH 8.0) and 150 mM NaCl) for 1 h. Membranes were then incubated with an optimal concentration of the primary antibody in TBST (Tris-buffered saline containing Tween 20) for 1 h at room temperature, washed four times in TBST, and incubated with the relevant HRP-conjugated secondary antibody (1:5000 dilution in TBST) for 30 min. Membranes were washed four times in TBST and visualized by enhanced chemiluminescence with autoradiographic film (ECL, Amersham Pharmacia Biotech). For reprobing, membranes were stripped in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 0.1 mM β-mercaptoethanol for 30 min at 50 °C; rinsed twice in TBST; and blocked in 2.5% BSA in Tris-buffered saline prior to primary antibody incubation.

Far-Western Blotting—Anti-EPO-R immunoprecipitates from 2 mg of Ba/F3-EPO-R cells were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 2.5% BSA in Tris-buffered saline, membranes were serially incubated with eluted GST fusion proteins (2.5 μg/ml in TBST), anti-GST antibody (0.5 μg/ml in TBST with 0.05% Triton X-100), and HRP-protein A (1:5000 dilution in TBST with 0.05% Triton X-100) and subjected to ECL detection.

Transient Transfection—293T cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Cells at 70% confluence were transfected with LipofectAMINE (Life Technologies, Inc.) with various combinations of wild-type EPO-R (2.5 μg), wild-type Ship1 (2.5 μg), Ship1 R34Q (2.5 μg); generously provided by Dr. Kodimangalam Ravichandran (University of Virginia, Charlottesville, VA), empty vector (pEBG, 2.5 μg), and/or JAK2 (0.1 μg). After incubation for 5 h, the transfection solution was removed, and the cells were cultured in Dulbecco’s modified Eagle’s medium for 24 h. The cells were incubated for 18 h in Dulbecco’s modified Eagle’s medium + 1 mg/ml BSA prior to stimulation with no factor or with 10 units/ml EPO for 10 min. Lysates (0.5 mg) were immunoprecipitated with an anti-Ship1 polyclonal antibody (Santa Cruz Biotechnology).

Analysis of ERK1/2 Activation—Lysates (100 μg) were resolved by SDS-PAGE and transferred to nitrocellulose. After blocking with 5% skim milk powder at room temperature, the membrane was incubated with the anti-phospho-ERK1/2 monoclonal antibody (1:200 dilution in 5% skim milk protein in TBST) overnight at 4 °C, washed three times in TBST, and incubated with HRP-conjugated sheep anti-mouse immunoglobulin (1:5000 dilution in 5% skim milk protein in TBST) for 1 h at room temperature. The membrane was washed three times in TBST and visualized by ECL.

Analysis of PKB Activation—Lysates (100 μg) were resolved by SDS-PAGE and transferred to nitrocellulose. After blocking with 5% skim milk powder in TBST for 1 h at room temperature, the membrane was incubated with the anti-phospho-PKB polyclonal antibody (1:1000 dilution in 1% BSA in TBST) overnight at 4 °C, washed six times in TBST, and incubated with HRP-protein A (1:2000 dilution in 2% skim milk powder) for 1 h at room temperature. The membrane was washed six times in TBST and visualized by ECL.

RESULTS

The role of Ship1 in cytokine-mediated signaling was examined in Ba/F3 and DA-3 cells expressing EPO-R as well as in the erythroid cell line HCD-57. Cell lines were depleted of cytokine and then stimulated with no factor or with IL-3 or EPO, and immunoprecipitation with a peptide-specific Ship1 antibody was performed, followed by Western blotting with the anti-phospho-arlyamine monoclonal antibody 4G10 (Fig. 1). IL-3 and EPO stimulated the tyrosine phosphorylation of Ship1 in Ba/F3-EPO-R (lanes 7 and 8) and DA-3-EPO-R (lanes 10 and 11) cells. Stimulation of HCD-57 cells with EPO also resulted in the tyrosine phosphorylation of Ship1 (lane 13). IL-3 stimulation of Ba/F3-EPO-R cells (lanes 7) and IL-3 (lane 10) or EPO (lane 11) stimulation of DA-3-EPO-R cells resulted in the co-
immunoprecipitation of a 52-kDa phosphoprotein with Ship1. Stripping and reprobing of the membrane revealed that this protein was Shc (data not shown). EPO stimulation of DA-3-EPO-R cells also resulted in the co-immunoprecipitation of the 72-kDa EPO-R. On longer exposures, the EPO-R was also shown to co-immunoprecipitate with Ship1 from Ba/F3-EPO-R and HCD-57 cells (data not shown). The ability of the EPO-R to associate with Ship1 from the various cell lines was directly proportional to EPO-R expression. Equal immunoprecipitation of Ship1 was demonstrated by stripping and reprobing the membrane with an anti-Ship1 polyclonal antibody (Ship1 immunoblot).

Ship1 was initially described to interact with Grb2 and Shc (21, 24). To investigate the ability of Ship1 to bind to these adapter proteins, immunoprecipitations were performed using peptide-specific Grb2 and Ship1 antibodies (Fig. 2). DA-3-EPO-R cells were depleted of cytokine and then stimulated with no factor or with IL-3 or EPO. As we have previously shown in CTL-L-EPO-R cells (16), Grb2 co-immunoprecipitated Ship1 (145 and 130 kDa), EPO-R (72 kDa), Shp2 (68 kDa), and Shc (52 and 46 kDa) (lane 6) after EPO stimulation of DA-3-EPO-R cells. IL-3 stimulation resulted in co-immunoprecipitation of Ship1, Shp2, and Shc with Grb2 (lane 5). Since Grb2 assembled a multimolecular complex consisting of Ship1, Shp2, Shc, and EPO-R after EPO stimulation, we were interested in determining whether Shc could be recruited to the EPO-R through Ship1. As shown in Fig. 1, Ship1 co-immunoprecipitated Shc after IL-3 stimulation (Fig. 2, lane 8) and Shc and EPO-R after EPO stimulation (lane 9).

Since the Ship1 immunoprecipitation experiments demonstrated a ternary complex between the EPO-R, Ship1, and Shc, the possibility that Ship1 could be recruited to the EPO-R in an SH2-dependent manner was next tested. Lysates were prepared from DA-3-EPO-R cells, and in vitro mixing experiments were performed with GST alone and fused with the Ship1 SH2 domain and a mutated Ship1 SH2 domain (GST-Ship1 SH2 R34Q) (Fig. 3). The Ship1 SH2 domain was shown to associate with the IL-3 receptor β3 chain (140 kDa) (lane 8) after IL-3 stimulation, and the 72-kDa EPO-R was shown to bind to the Ship1 SH2 domain after engagement of the EPO-R (lane 9). Stripping and reprobing the membrane confirmed that the 72-kDa phosphoprotein in lane 9 was the EPO-R (lower panel). No phosphoproteins were shown to associate with GST-Ship1 SH2 R34Q after either IL-3 (lane 11) or EPO (lane 12) stimulation. Only small amounts of Shc were observed to associate with Ship1, in contrast to a previous report (25).

To examine whether the SH2-dependent association of Ship1 with the EPO-R was direct or indirect, Far-Western blotting experiments were performed (Fig. 4). Lysates from unstimulated or EPO-stimulated Ba/F3-EPO-R cells were immunoprecipitated with a peptide-specific EPO-R antibody. Following SDS-PAGE and transfer to nitrocellulose, the membrane was cut into strips, and each strip was incubated with GST, GST-Ship1 SH2, or GST-Ship1 SH2 R34Q (20). Following washing, Western blotting was performed using an anti-GST antibody followed by HRP-protein A. GST-Ship1 SH2 (lane 6) was shown to bind to the EPO-R. Neither a GST fusion protein of the Ship1 SH2 domain expressing an inactivating SH2 domain mutation
were immunoprecipitated (IP) with an anti-EPO-R polyclonal antibody (Ab). Immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with 2.5 μg of GST (lanes 4–6), GST-Ship1 SH2 (lanes 7–9), or GST-Ship1 SH2 R34Q (lanes 10–12). The immunoblot was probed with the anti-phosphotyrosine monoclonal antibody (Ab) 4G10 (upper panel) and then stripped and reprobed with an anti-EPO-R polyclonal antibody (lower panel). Lysate controls are shown in lanes 1–3. Molecular mass standards are indicated in kilodaltons. IL-3Rβc, IL-3 receptor β chain.

To examine this possibility in greater detail, we performed a transient transfection in 293T cells (Fig. 5). The EPO-R and JAK2 were expressed with either wild-type Ship1 or the Ship1 SH2 domain R34Q mutant. Tyrosine-phosphorylated wild-type Ship1 was shown to associate with the EPO-R when the cells were stimulated with EPO (lane 2). No tyrosine phosphorylation of Ship1 R34Q was observed when this construct was coexpressed with the EPO-R and JAK2 (lane 4). 293T cells failed to express Ship1 (Ship1 reprobe; lanes 5 and 6), and the association of the EPO-R and Ship1 in this cellular environment was dependent on JAK2 (lane 8). The 72-kDa phosphoprotein in the Ship1 immunoprecipitation was confirmed to be the EPO-R (EPO-R reprobe; lane 2). The experiments in Figs. 3–5 provide strong evidence that Ship1 directly associates with the EPO-R in an SH2-dependent fashion.

To identify the regions of the EPO-R involved in Ship1 recruitment, we next examined (i) EPO-dependent tyrosine phosphorylation of Ship1 and (ii) association of the Ship1 SH2 domain in a panel of EPO-R deletion mutants expressed in Ba/F3 cells. In addition to the wild-type EPO-R, four mutants were selected: EPO-RΔ43 (containing 4 Tyr residues), EPO-RΔ69 (containing 2 Tyr residues), EPO-RΔ99 (containing 1 Tyr residue), and EPO-RΔ99,F1 (containing 0 Tyr residues) (Fig. 6A). All cell lines (with the exception of EPO-RΔ99,F1) were capable of EPO-dependent proliferation as determined by XTT assays (data not shown). Previous studies have indicated that EPO-RΔ99 is capable of EPO-dependent mitogenesis when expressed in Ba/F3 cells (26). The selected subclones were depleted of cytokine and stimulated in the presence or absence of EPO. Immunoprecipitation with Ship1 was performed, followed by anti-phosphotyrosine Western blotting (Fig. 6B). EPO stimulation of Ba/F3-EPO-R (lane 8), Ba/F3-EPO-RΔ43 (lane 10), and Ba/F3-EPO-RΔ69 (lane 12) cells resulted in robust tyrosine phosphorylation of Ship1. Ba/F3-EPO-RΔ99 cells caused a slight stimulation of Ship1 tyrosine phosphorylation in response to EPO (lane 14). Ba/F3 or Ba/F3-EPO-RΔ99,F1 cells showed no increase in Ship1 tyrosine phosphorylation, indicating that the EPO-dependent increase in tyrosine phosphorylation requires EPO-R cytoplasmic tyrosines.

To examine the ability of the truncated EPO-R mutants to associate with the Ship1 SH2 domain, in vitro mixing experiments were performed (Fig. 6C). Lysates identical to those described above were incubated with GST-Ship1 SH2, and
tyrosine-phosphorylated proteins were detected. EPO stimu-
lated the association of the wild-type EPO-R (lane 4), EPO-
RΔ43 (lane 6), and EPO-RΔ69 (lane 8) with the Ship1 SH2
domain. From these experiments, we conclude that EPO-R
Tyr401 is sufficient to couple to EPO-dependent Ship1 tyrosine
phosphorylation and binding of Ship1 to the EPO-R.

All of the EPO-R deletion mutants were capable of activating
JAK2, and all mutants (with the exception of EPO-RΔ99,F1)
activate STAT5, indicating that EPO-RΔ43, EPO-RΔ69, and
EPO-RΔ99 activate similar downstream signaling pathways.
compared with the wild-type EPO-R (Fig. 6D). Metabolic labeling experiments confirmed expression of each of the EPO-R deletion mutants in Ba/F3 cells (Fig. 6E).

Previous studies have demonstrated that the binding specificity of the Ship1 SH2 domain is pY(Y/D)2X(L/V)3 (27). Scanning the EPO-R cytoplasmic tyrosine residues, it is evident that there are four potential Ship1-binding sites, with Tyr401 potentially serving as the optimal binding site (Fig. 7).

To investigate the specific tyrosine residues that couple to Ship1 recruitment, a series of EPO-R tyrosine mutants was expressed in Ba/F3 cells. We initially examined a series of single tyrosine-to-phenylalanine substitutions at Tyr343 (EPO-R-Y343), Tyr401, Tyr429, and Tyr431 are shown as well as the consensus Ship1 SH2 domain-binding motif. The EPO-R consensus sequence is shown at the top, and similar residues are shown in boldface.

FIG. 7. EPO-R expresses several consensus binding motifs for the Ship1 SH2 domain. The sequences at EPO-R Tyr343, Tyr401, Tyr429, and Tyr431 are shown as well as the consensus Ship1 SH2 domain-binding motif. The EPO-R consensus sequence is shown at the top, and similar residues are shown in boldface.

Next, Far-Western blotting was utilized to discriminate Ship1 binding to the various EPO-R tyrosine mutants (Fig. 8D). Lysates from each cell line were immunoprecipitated with an anti-EPO-R polyclonal antibody. Following electrophoretic transfer, the membrane was incubated with GST-Ship1 SH2, followed by anti-GST Western blotting. Wild-type EPO-R (lane 2) and EPO-R-Y5,F1F2 (lane 6) and EPO-R-Y5,F1F2F3 (lane 8) showed strong binding of Ship1 SH2 domain was observed in EPO-R-Y6,F1F2 (lane 6) and EPO-R-Y5,F1F2F3 (lane 8). No binding was observed in EPO-R-Y4,F1F2F3F4 (lane 10) and EPO-R-F8 (lane 12). No binding to GST alone was observed in any of the EPO-R constructs (data not shown). Reprobing the membrane with an anti-EPO-R antibody revealed equal expression of all subclones, except for EPO-R-Y6,F1F2, which was slightly lower.

Ship1 is a complex protein due to its ability to serve as an adaptor protein in binding to the phosphotyrosine-binding domain of Shc (20) and to the SH3 domain of Grb2. In addition, its catalytic activity has been shown to modulate the activation of protein kinase B/AKT in response to IL-2 (28) and to regulate stem cell factor-dependent mast cell degranulation (29). To begin to understand the function of Ship1, we examined EPO-dependent ERK and PKB activation in the panel of EPO-R deletion mutants described earlier (Fig. 9A). For this analysis, we included an additional mutant, EPO-RΔ221, which is unable to bind and activate JAK2 (22). Phosphorylation of ERK1/2 was observed in all EPO-R deletion mutants, with the exception of EPO-RΔ221 (lane 15). EPO-RΔ99, which displayed weak Ship1 tyrosine phosphorylation in response to EPO stimulation, nevertheless was capable of ERK1/2 activation (lane 12).

Phosphorylation of PKB/Akt was detected using an activation-specific antibody that detected PKB when phosphorylated at Ser473 (Fig. 9B). EPO activated PKB in Ba/F3 cells expressing wild-type EPO-R (lane 3), EPO-RΔ43 (lane 6), and EPO-RΔ69 (lane 9). No PKB activation was seen in EPO-RΔ99 (lane 12) and EPO-RΔ221 (lane 15).

DISCUSSION

Cytokine receptors recruit a multitude of SH2 effector proteins after receptor engagement. Although the precise phosphorylation events have not been mapped, mutagenesis studies have established the binding location of many intracellular signaling proteins. The EPO-R has been shown to activate the tyrosine phosphorylation of Ship1 (21), thus inducing its association with the SH2 domain of Ship1 (20) and constitutive binding to the Grb2 adaptor protein (21). Using a panel of EPO-R deletion and tyrosine mutants, we have demonstrated that Ship1 is recruited to the EPO-R in an SH2-dependent manner.

Several studies have shown that Ship1 is recruited in an SH2-dependent manner to immune tyrosine inhibition motifs expressed on inhibitory receptors, and Ship1 has also been shown to be recruited to the IL-4 receptor α chain (30). Earlier
studies had suggested that a 145-kDa phosphoprotein competed with Grb2 for binding to Shc Tyr(P) 317 (31). The initial studies demonstrated that a pYXN phosphopeptide competed for 145-kDa protein binding to Shc. Once the 145-kDa protein was identified as Ship1, these investigators demonstrated that GST-Ship1 SH2 bound to Shc in vitro and that the Ship1 SH2 domain bound to a Shc Tyr 317 phosphopeptide in BIAcore experiments (25). They were also unable to demonstrate co-immunoprecipitation of Ship1 and Grb2 (25). In this study, we have shown that Grb2 can co-immunoprecipitate a ternary complex that contains Shc and Ship1 (Fig. 2). The Ship1 SH2 domain selectively binds to either the IL-3 receptor βc chain or the EPO-R after cytokine stimulation with the corresponding ligand (Fig. 3). Furthermore, the Ship1 SH2 domain binds directly to the EPO-R in Far-Western experiments (Fig. 4). We have shown that in cell lines derived to express various EPO-R deletion and tyrosine mutants, the SH2 domain of Ship1 binds to a region of the EPO-R that is involved in negative regulation.

There is considerable redundancy in EPO-R residues Tyr401, Tyr429, and/or Tyr431, as shown in Fig. 7. Although Tyr429 is sufficient to activate the tyrosine phosphorylation and DNA binding of STAT5, other tyrosines are involved in STAT5 recruitment, most notably Tyr401 (7). We chose to focus on this region of the EPO-R to identify the Ship1-binding domain, as all of these sequences represent candidate Ship1 SH2-binding sites (Fig. 7). Ship1 is tyrosine-phosphorylated in Ba/F3 cells expressing full-length EPO-R, EPO-RΔ43, EPO-RΔ69, EPO-R-Y7,F1, and EPO-R-Y6,F1F2 after EPO stimulation. However, the level of tyrosine phosphorylation is dramatically reduced in EPO-R-Y5,F1F2 and EPO-RΔ99. In vitro mixing experiments indicated that the Ship1 SH2 domain associates with the EPO-R, EPO-RΔ43, EPO-RΔ69, EPO-R-Y7,F1, and EPO-R-Y6,F1F2. Far-Western blotting showed that the Ship1 SH2 domain binds directly to the wild-type EPO-R and EPO-R-Y7,F1. In sum, this indicates that the preferred binding sites of the Ship1 SH2 domain are tyrosines 401 and/or 429. Shp2 has been shown to bind to Tyr401 (9), whereas Shp1 associates with Tyr429 (11). It is interesting that EPO-R Tyr401 is a central site in the recruitment of several SH2 effector proteins. For example, it is now known that Cis (10), Ship1 and Shp2 (9), and STAT5 (7, 8) all are capable of binding to this site. Another example of distinct tyrosine sequences recruiting multiple effector proteins has been shown in c-Met Tyr1356, which recruits phosphatidylinositol 3-kinase, phospholipase Cγ, Ship2, and Grb2 (32, 33). In addition, Tie2 recruits Ship2, phosphatidylinositol 3-kinase, phospholipase Cγ, and Grb2 (32, 33).
nositol 3-kinase, Grb2, and Grb7 to Tyr1101 (34, 35).2

In this study, we have provided novel evidence that Ship1 is recruited to a region of the EPO-R that has been shown to be correlated with negative regulation. Although the role of Ship1 in erythropoiesis remains to be resolved, several patients suffering from primary familial and congenital polycythemia have truncation mutations of the EPO-R. Of the mutations that have been identified to date, five patients have deletions from amino acids 414 to 427 of the human EPO-R that delete the 6 carboxyl-terminal tyrosines including EPO-R Tyr429 (37–40), whereas one patient has a deletion that results in loss of the EPO-R Tyr401-binding site (41). The functional basis of primary familial and congenital polycythemia has yet to be resolved. Perhaps primary familial and congenital polycythemia arises through the inability of Ship1 to be recruited to the EPO-R in these patients.

The role of Ship1 in hematopoietic function is complex. Gene targeting experiments revealed that mice lacking functional Ship1 suffer from splenomegaly and myeloid infiltration into the lung (42). Increased myeloid progenitors were found in the bone marrow and spleen of knockout animals (42). Bone marrow-derived mast cells from Ship1−/− mice show increased degranulation in response to Steel factor (29). The numbers of colony forming unit-erythroid were observed to be lower in Ship1−/− mice (42).

Ship1 may modulate several signal transduction pathways in response to EPO. First, it serves as an adaptor protein in the SH3-dependent binding of Grb2 (21) and the activation-dependent binding of Shc (20), which may couple Ship1 to Ras activation. Second, through its catalytic activity, Ship1 may regulate PKB/Akt activity (28), as it has been shown that mast cells derived from Ship1−/− mice have elevated PKB activity (23). Third, as has been shown for Steel factor-dependent mast cell degranulation (29), loss of Ship1 may result in increases in calcium influx due to elevated levels of phosphatidylinositol 3,4,5-trisphosphate, which has been shown to be an agonist of calcium channel action.

We have shown that Ship1 recruitment of the EPO-R does not directly correlate with ERK1/2 activation. Deletion mutants of the EPO-R containing only Tyr343 have been shown to be competent for mitigating EPO-dependent mitogenesis, differentiation, and cell survival pathways (26, 43, 44). In this cellular context, EPO stimulates STAT5 tyrosine phosphorylation (7, 8, 45, 46) and ERK activation (47), but abrogates Ship1 recruitment. Several studies have shown that PI 3-kinase is upstream of PKB activation (48). The p85 subunit of PI 3-kinase is recruited to the carboxyl-terminal tyrosine, EPO-R Tyr479 (12). However, activation of PKB does not correlate with binding of PI 3-kinase to the EPO-R (Fig. 9B). PI 3-kinase can be recruited indirectly through other signaling effectors such as Cbl (49). Therefore, it appears that EPO-dependent PKB activation requires more proximal elements located within the carboxyl-terminal 100 amino acids of the EPO-R cytoplasmic tail. Several studies have shown that EPO stimulates a calcium influx through a voltage-independent calcium channel (50–53). We have recently shown that EPO-R Tyr460 couples to EPO-dependent calcium influx (36). Since Ship1 appears to be recruited to a region of the EPO-R distinct from that involved in calcium influx, it would appear that Ship1 may not directly

A

B

FIG. 9. ERK and PKB activation in EPO-R deletion mutants. A, the indicated cell lines were depleted of cytokine for 12 h and stimulated with no factor (lanes 1, 4, 7, 10, and 13) or with 10 ng/ml murine IL-3 (lanes 2, 5, 8, 11, and 14) or 50 units/ml human recombinant EPO (lanes 3, 6, 9, 12, and 15) for 10 min at 37 °C. Lysates (100 μg) were resolved by SDS-PAGE and blotted onto nitrocellulose. The membrane was stripped and reprobed with an antibody that recognizes total ERK1/2 (Ab). The membrane was stripped and reprobed with an antibody that recognizes total PKB (Ab). The membrane was stripped and reprobed with an antibody that recognizes total PKB.

2 Jones, N., Master, Z., Jones, J., Bouchard, D., Sasaki, H., Daly, R., and Dumont, D. J. (1999) J. Biol. Chem. 274, 30896–30905.
affect calcium signaling in erythroid progenitor cells. The effects of Ship1 on these and other yet to be identified signaling pathways await to be dissected in erythroblasts isolated from gene-targeted animals.

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REFERENCES

1. D’Andrea, A. D., Lodish, H. F., and Wong, G. G. (1989) Cell 57, 277–285
2. Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Joliffe, L. K., and Wilson, I. A. (1999) Science 283, 877–990
3. Wu, H., Liu, X., Jaenisch, R., and Lodish, H. F. (1995) Cell 83, 59–68
4. Liu, C.-S., Lim, S.-K., D’Agati, V., and Costantini, F. (1996) Genes Dev. 10, 154–164
5. Neuberger, H., Cumano, A., Muller, M., Wu, H., Huffstadt, U., and Pfeffer, K. (1996) Cell 85, 397–408
6. Parganas, E., Wang, D., Stravopodis, D., Topham, D. J., Marine, J. C., Teglund, S., Vanin, E. F., Bodner, S., Colamonici, O. R., van Deursen, J. M., Grosveld, G., and Ile, J. N. (1996) Cell 83, 385–395
7. Klingmuller, U., Bergeisen, S., Hisao, J. G., and Lodish, H. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8324–8328
8. Gebert, S., Chretien, S., Gouilloux, F., Muller, O., Paillard, C., Dusant-Fourt, I., Groen, R., Lacombe, C., Gisselbrecht, S., and Mayeux, P. (1996) EMBO J. 15, 2434–2441
9. Tauchi, T., Damen, J. E., Toyama, K., Feng, G.-S., Broxmeyer, H. E., and Krystal, G. (1996) Blood 87, 4485–4501
10. Verdit, F., Chretien, S., Muller, O., Varlet, P., Yoshimura, A., Gisselbrecht, S., Lacombe, C., and Mayeux, P. (1998) J. Biol. Chem. 273, 23402–23408
11. Barber, D. L., Cutler, R. L., Jiao, H., Yi, T., and Krystal, G. (1995) Blood 85, 2289–2303
12. Barber, D. L., Lacombe, C., and Mayeux, P. (1998) J. Biol. Chem. 273, 28185–28190
13. Barber, D. L., Mason, J. M., Fukazawa, T., Reedquist, K. A., Drayer, D. J., and Roussell, D. J. (1996) Blood 88, 4135–4141
14. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1996) Cell 85, 729–738
15. Damen, J. E., Cutler, R. L., Jiao, H., Yi, T., and Krystal, G. (1996) J. Biol. Chem. 271, 23402–23408
16. Barber, D. L., Mason, J. M., Fukazawa, T., Reedquist, K. A., Drayer, D. J., and Roussell, D. J. (1996) Blood 88, 4135–4141
17. Damen, J. E., Cutler, R. L., Jiao, H., Yi, T., and Krystal, G. (1996) Blood 88, 4135–4141
18. Barber, D. L., Mason, J. M., Fukazawa, T., Reedquist, K. A., Drayer, D. J., and Roussell, D. J. (1996) Blood 88, 4135–4141
19. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1996) Cell 85, 729–738
20. Barber, D. L., Cutler, R. L., Jiao, H., Yi, T., and Krystal, G. (1996) J. Biol. Chem. 271, 23402–23408
21. Barber, D. L., Mason, J. M., Fukazawa, T., Reedquist, K. A., Drayer, D. J., and Roussell, D. J. (1996) Blood 88, 4135–4141
22. Barber, D. L., DeMartino, J. C., Showers, M. O., and D’Andrea, A. D. (1994) Mol. Cell. Biol. 14, 2257–2265
23. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
24. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
25. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
26. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
27. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
28. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
29. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
30. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
31. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
32. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
33. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
34. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
35. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
36. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
37. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
38. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
39. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
40. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
41. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
42. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
43. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
44. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
45. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
46. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
47. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
48. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
49. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791
50. Liu, Q., Sasaki, T., Dumont, D. J., and Penninger, J. M. (1999) Genes Dev. 13, 786–791