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# Identification of SH2-Bβ as a Substrate of the Tyrosine Kinase JAK2 Involved in Growth Hormone Signaling

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**Activation of the tyrosine kinase JAK2 is an essential step in cellular signaling by growth hormone (GH) and multiple other hormones and cytokines. Murine JAK2 has a total of 49 tyrosines which, if phosphorylated, could serve as docking sites for Src homology 2 (SH2) or phosphotyrosine binding domain-containing signaling molecules. Using a yeast two-hybrid screen of a rat adipocyte cDNA library, we identified a splicing variant of the SH2 domain-containing protein SH2-B, designated SH2-Bβ, as a JAK2-interacting protein. The carboxyl terminus of SH2-Bβ (SH2-Bβc), which contains the SH2 domain, specifically interacts with kinase-active, tyrosyl-phosphorylated JAK2 but not kinase-inactive, unphosphorylated JAK2 in the yeast two-hybrid system. In COS cells coexpressing SH2-Bβ or SH2-Bβc and murine JAK2, both SH2-Bβc and SH2-Bβ coimmunoprecipitate to a significantly greater extent with wild-type, tyrosyl-phosphorylated JAK2 than with kinase-inactive, unphosphorylated JAK2. SH2-Bβc also binds to immunoprecipitated wild-type but not kinase-inactive JAK2 in a far Western blot. In 3T3-F442A cells, GH stimulates the interaction of SH2-Bβ with tyrosyl-phosphorylated JAK2 both in vitro, as assessed by binding of JAK2 in cell lysates to glutathione *S*-transferase (GST)–SH2-Bβc or GST–SH2-Bβ fusion proteins, and in vivo, as assessed by coimmunoprecipitation of JAK2 with SH2-Bβ. GH promoted a transient and dose-dependent tyrosyl phosphorylation of SH2-Bβ in 3T3-F442A cells, further suggesting the involvement of SH2-Bβ in GH signaling. Consistent with SH2-Bβ being a substrate of JAK2, SH2-Bβc is tyrosyl phosphorylated when coexpressed with wild-type but not kinase-inactive JAK2 in both yeast and COS cells. SH2-Bβ was also tyrosyl phosphorylated in response to gamma interferon, a cytokine that activates JAK2 and JAK1. These data suggest that GH-induced activation and phosphorylation of JAK2 recruits SH2-Bβ and its associated signaling molecules into a GHR-JAK2 complex, thereby initiating some as yet unidentified signal transduction pathways. These pathways are likely to be shared by other cytokines that activate JAK2.**

Members of the JAK family of tyrosine kinases (JAK1, JAK2, JAK3, and tyk2) play a critical role in cellular responses to ligands that bind to the receptors in the cytokine receptor superfamily. One or more of the JAK kinases is activated upon ligand binding to members of the cytokine receptor superfamily. Multiple cellular proteins are subsequently phosphorylated, including the associated cytokine receptors and the JAKs themselves (reviewed in references 3 and 16). The phosphorylated tyrosines are potential docking sites for proteins containing specific phosphotyrosine binding domains (e.g., Src homology 2 [SH2] and phosphotyrosine binding [PTB] domains). Specific signaling proteins are thereby recruited into the cytokine signaling networks. Thus, identification of signaling molecules which specifically interact with tyrosyl-phosphorylated JAKs is likely to provide an insight into signaling by the cytokines that activate those JAKs.

Of the 25 or so ligands known to activate JAKs, more than two-thirds activate JAK2. These include growth hormone (GH), prolactin, erythropoietin, granulocyte colony-stimulating factor, interleukin-3 (IL-3), IL-5, granulocyte-macrophage colony-stimulating factor, leptin, thrombopoietin, IL-12, gamma interferon (IFN-γ), and ligands whose receptor includes gp130 (IL-6, oncostatin M, leukemia inhibitory factor, IL-11, cardiotropin, and ciliary neurotrophic factor). Some of these ligands show a marked preference for JAK2, whereas others appear to be less discriminating (reviewed in reference 3). Murine JAK2 has 49 potential tyrosyl phosphorylation sites. Little is known about which tyrosines are phosphorylated in JAK2 in response to ligand binding or even whether the same tyrosines are phosphorylated in response to all ligands. However, tryptic digestion of autophosphorylated JAK2 expressed in Sf9 cells suggests that 15 or more tyrosines are phosphorylated (12), with at least 2 of the phosphorylated tyrosines being present in the kinase domain (12). This suggests that activated JAK2 contains multiple phosphorylated tyrosines, each with the potential to bind signaling molecules. GH shows a particularly strong preference for and robust stimulation of JAK2 (2, 26). Indirect evidence suggests that some GH-signaling molecules may interact with phosphorylated tyrosines within JAK2 (e.g., Stat1, Stat3, Shc, insulin receptor substrate 1 [IRS-1] and IRS-2) (1, 4, 26, 28, 29). However, other than GH receptor (GHR), no protein has definitively been shown to associate with JAK2 in response to GH.

In this study, we identified a splicing variant of SH2-B (designated SH2-Bβ) and showed that it binds to activated JAK2. SH2-B was originally cloned from mast cells because of its ability to bind in a yeast tribrid system to the tyrosyl-phosphorylated gamma subunit of the high-affinity immunoglobulin E (IgE) receptor (21). Except for its size, tissue distribution, and possession of an SH2 domain, the protein encoded by that original clone, which we will call SH2-Bα, has remained uncharacterized. The cDNA encoding SH2-Bβ identified in this study contains a 100-nucleotide insert which results in the

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replacement of 126 amino acids at the carboxyl terminus with 39 amino acids. In this report, we also demonstrate that SH2-Bβ is phosphorylated on at least one tyrosine in response to GH and IFN-γ. This suggests that SH2-Bβ is a signaling protein utilized by multiple cytokine receptors that activate JAK2.

## MATERIALS AND METHODS

**Cells and reagents.** *Saccharomyces cerevisiae* EGY48 (*MATα trp1 ura3-52 his3 leu*) and all yeast expression plasmids were provided by Roger Brent and have been described previously (13, 14, 20, 30). The stock of 3T3-F442A cells was kindly provided by H. Green (Harvard University, Cambridge, Mass.), and the stock of Chinese hamster ovary cells expressing full-length rat GHR (CHOA) and truncated GHR (CHO454) was provided by Gunnar Norstedt (Karolinska Institute). Recombinant human GH and porcine insulin were a gift of Eli Lilly and Co. Recombinant murine IFN-γ and glutathione-agarose beads were from Sigma. Recombinant protein A-agarose was from Repligen. The Expand high-fidelity PCR system, alkaline phosphatase, aprotinin, leupeptin, and Triton X-100 were purchased from Boehringer Mannheim. Protein phosphatase 2A (PP2A) was from Upstate Biotechnology Inc. The enhanced chemiluminescence detection system was from Amersham Corp.

**Antibodies.** Antibodies to SH2-Bβ (αSH2-B) were raised by immunizing three rabbits with glutathione *S*-transferase (GST)–SH2-Bβc. Briefly, GST–SH2-Bβc fusion proteins were eluted from the glutathione-agarose beads with elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl [pH 8.0]). The purity was estimated to be >95% as determined by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Antisera to GST–SH2-Bβc were produced at Pocono Rabbit Farm & Laboratory Inc. and used at dilutions of 1:100 for immunoprecipitation and 1:10,000 for immunoblotting. Anti-JAK2 antiserum (αJAK2) was raised in rabbits against a synthetic peptide corresponding to amino acids 758 to 776 (25) and was used at dilutions of 1:500 for immunoprecipitation and 1:15,000 for immunoblotting. Monoclonal anti-phosphotyrosine antibody 4G10 (αPY) was purchased from Upstate Biotechnology Inc. and was used at a dilution of 1:7,500 for immunoblotting. Monoclonal antibody to influenza virus hemagglutinin (HA) tag (αHA; 12CA5) was purchased from Boehringer Mannheim and used at a dilution of 1:100 for immunoprecipitation. Monoclonal antibody against Myc-tag (αMyc; 9E10) was purchased from Santa Cruz Biotechnology, Inc., and used at a dilution of 1:1000 for immunoblotting.

**Plasmid construction.** cDNAs encoding both wild-type murine JAK2 and mutant murine JAK2 in which the critical lysine in the ATP-binding domain is mutated to glutamate (K882E) were previously cloned into mammalian expression vector prk5 and provided by James Ihle and Bruce Witthuhn (St. Jude Children's Research Hospital). *Bam*HI and *Eco*RI sites were added to the 5′ and 3′ ends of the JAK2 cDNAs, respectively, by PCR. Full-length JAK2 cDNAs (both wild type and K882E mutant) were first cloned into pBluescript-SK(−) (Stratagene) and then subcloned in-frame into the LexA bait plasmid (pEG202) at *Bam*HI-*Sal*I sites. An amino-terminally truncated JAK2 (ΔJAK2) bait hybrid plasmid was constructed by subcloning the *Xho*I-*Sal*I fragment of wild-type or mutant JAK2 cDNA into pEG202. The cytoplasmic domain of CD2 was generated by reverse transcription-PCR and cloned into pEG202 at *Eco*RI-*Bam*HI sites. Construction of the bait plasmid containing a portion of *Drosophila* bicoid protein or the cytoplasmic domain of the insulin receptor and construction of the prey plasmid harboring the acidic transcriptional activation domain of B42 fused to human Shc, human IRS-1, or the PTB domain of IRS-1 have been described previously (10, 19, 30). The cDNA encoding the carboxyl-terminal portion of SH2-Bβ (SH2-Bβc) was isolated from the prey plasmid obtained from yeast two-hybrid screening of a rat fat cDNA library (see below) and subcloned in-frame into the GST vector pGEX-5X (Pharmacia Biotech Inc.) by using *Eco*RI-*Xho*I sites and into the pcDNA3 expression vector (Invitrogen) with an HA tag at the amino terminus of SH2-Bβc. The cDNA fragment encoding the entire SH2 domain of SH2-Bβ was produced by PCR with customized primers and cloned in-frame into pGEX-5X. All PCR products and all junctions were verified by DNA sequencing. Detailed cloning strategies are available upon request.

**Rat adipose cDNA library.** RNA was prepared from epididymal fat pads of Sprague-Dawley rats (10 months old) with TRIZOL reagent (Life Technologies) as specified by the manufacturer. Poly(A)$^+$ RNA was prepared with oligo(dT) cellulose. Oligo(dT)-*Xho*I linker-primed cDNA was synthesized with a kit (Stratagene) as specified by the manufacturer, except that Klenow fragment and mung bean nuclease were used to blunt the cDNA prior to ligation with the *Eco*RI linker. The cDNA was ligated into the *Eco*RI-*Xho*I sites of the pJG4-5 activation domain plasmid and electroporated into XL1-Blue MRF′ (Stratagene). Approximately $10^6$ independent isolates were obtained. Plasmid DNA was purified with the Mega kit (Qiagen).

**Two-hybrid library screening and cloning of full-length SH2-Bβ.** Screening was carried out essentially as described previously (14, 30). Yeast (EGY48) cells were grown at 30°C in YPD medium (1% yeast extract, 2% polypeptone, 2% dextrose). The yeast cells were sequentially transformed with the *LexAop-lacZ* reporter plasmid, pEG202-ΔJAK2 bait plasmid, and cDNA library prey plasmids by the lithium acetate method (23). Triple transformants were grown for 4 h in liquid yeast dropout medium lacking Trp, Ura, and His and subsequently plated on the same dropout medium with the addition of 15% agar. The yeast cells were grown for 4 days at 30°C, and then colonies were collected and replated on yeast dropout medium lacking Trp, Ura, His, and Leu to select for Leu prototrophy. The carbon source was also changed from dextrose to galactose to induce the expression of the activation domain hybrids. After 5 days of growth, colonies were subjected to the filter lift color assay to test for β-galactosidase activity (7, 19). Positive clones were selected, and prey plasmids containing library cDNA inserts were isolated and transformed into bacteria for amplification. The insertion cDNAs were subjected to DNA sequence analysis.

Total RNA was prepared from the kidneys of Sprague-Dawley rats with TRIZOL reagent. Oligo(dT)-primed cDNA was synthesized with a kit (Stratagene) and used as source DNA to obtain full-length SH2-Bβ by PCR with a 5′ primer (5′-GTGGATCCATGAATGGTGCCCCTTCCCCAGAGGATG-3′) flanking the start codon of SH2-Bα and a 3′ primer (5′-CGGAATTCTCAGA CAAATGAGTACTGGTTATTA-3′) flanking the stop codon of SH2-Bα. To facilitate the following cloning process, *Bam*HI and *Eco*RI sites had been integrated into the 5′ and 3′ primers, respectively. The PCR product was cloned into pGEX-KG (provided by Kuan-liang Guan, University of Michigan) to produce GST fusion protein and into mammalian expression vector prk5 with a Myc tag at the amino terminus (6). The entire PCR-produced SH2-Bβ cDNA was sequenced with a set of customized primers by the DNA Sequencing Core, University of Michigan.

**Cell culture, lysis, and transfection.** Mouse 3T3-F442A preadipocytes were grown in Dulbecco modified Eagle medium (supplemented with 1 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin B per ml, and 10% calf serum). CHO cells stably transfected with either full-length rat GHR (CHOA) or truncated GHR lacking half its cytoplasmic domain (CHO454) were cultured in F12 supplemented with 1 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin B per ml, and 10% fetal bovine serum. The confluent cells were deprived of serum overnight. The deprived cells were treated for various times with GH, insulin, or IFN-γ at 37°C at the indicated concentrations and then rinsed three times with ice-cold 10 mM sodium phosphate [pH 7.4]–150 mM NaCl–1 mM $Na_3VO_4$. The cells were solubilized in lysis buffer (50 mM Tris [pH 7.5], 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml) and centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant was used for immunoprecipitation, immunoblotting, or in vitro binding assays.

To lyse yeast cells, exponentially growing cultures (optical density at 600 nm, ~1.0) were washed once with ice-cold $H_2O$, suspended in 50 mM Tris (pH 7.5)–150 mM NaCl–2 mM EGTA–1 mM $Na_3VO_4$–1 mM phenylmethylsulfonyl fluoride–10 μg of aprotinin per ml–10 μg of leupeptin per ml–500 μg of acid-washed glass beads per ml, and vortexed at 4°C for 5 min. The mixtures were centrifuged at 14,000 × *g* for 10 min at 4°C, and the supernatant was used for immunoblotting or immunoprecipitation.

COS cells were transiently transfected by calcium phosphate precipitation (5a) and assayed 48 h after transfection. For each 100-mm culture dish, 5 μg of each construct was used, and the total amount of DNA was adjusted to 10 μg by adding empty pcDNA3 vector.

**In vitro interaction assay with GST fusion proteins.** GST fusion proteins were purified from bacteria by affinity chromatography with glutathione-agarose beads. 3T3-F442A, CHOA, and CHO454 cells were treated with or without GH, and whole-cell lysates were prepared as described above. The immobilized fusion proteins were incubated with the cell lysates at 4°C for 3 h and subjected to extensive washing with lysis buffer. The proteins bound to the immobilized GST fusion proteins were solubilized by boiling for 5 min in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 10% β-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue), separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibody to JAK2 or phosphotyrosine.

**Immunoprecipitation and immunoblotting.** Cell lysates were incubated with the indicated antibody on ice for 2 h. The immune complexes were collected on protein A-agarose (25 μl) during a 1-h incubation at 4°C. The beads were washed three times with washing buffer (50 mM Tris [pH 7.5], 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA) and boiled for 5 min in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer. The solubilized proteins were separated by SDS-PAGE (with a 5 to 12% gradient gel unless noted otherwise) followed by immunoblotting with the indicated antibody by using the enhanced chemiluminescence detection system. Some membranes were then incubated at 55°C for 30 to 60 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]). The membranes were then immunoblotted with the desired antibody. In some cases, the blots were reprobed immediately without stripping with a second antibody.

**Far Western blot.** COS cells were transfected with 5 μg of prk5 expression vector containing cDNA encoding either wild-type or kinase-inactive murine JAK2. JAK2 was immunoprecipitated with αJAK2, separated by SDS-PAGE, and transferred to nitrocellulose. The membrane was incubated with GST–SH2-Bβc (1.5 μg/ml) at 4°C overnight. After extensive washing with lysis buffer, the membrane was immunoblotted with αSH2-B.

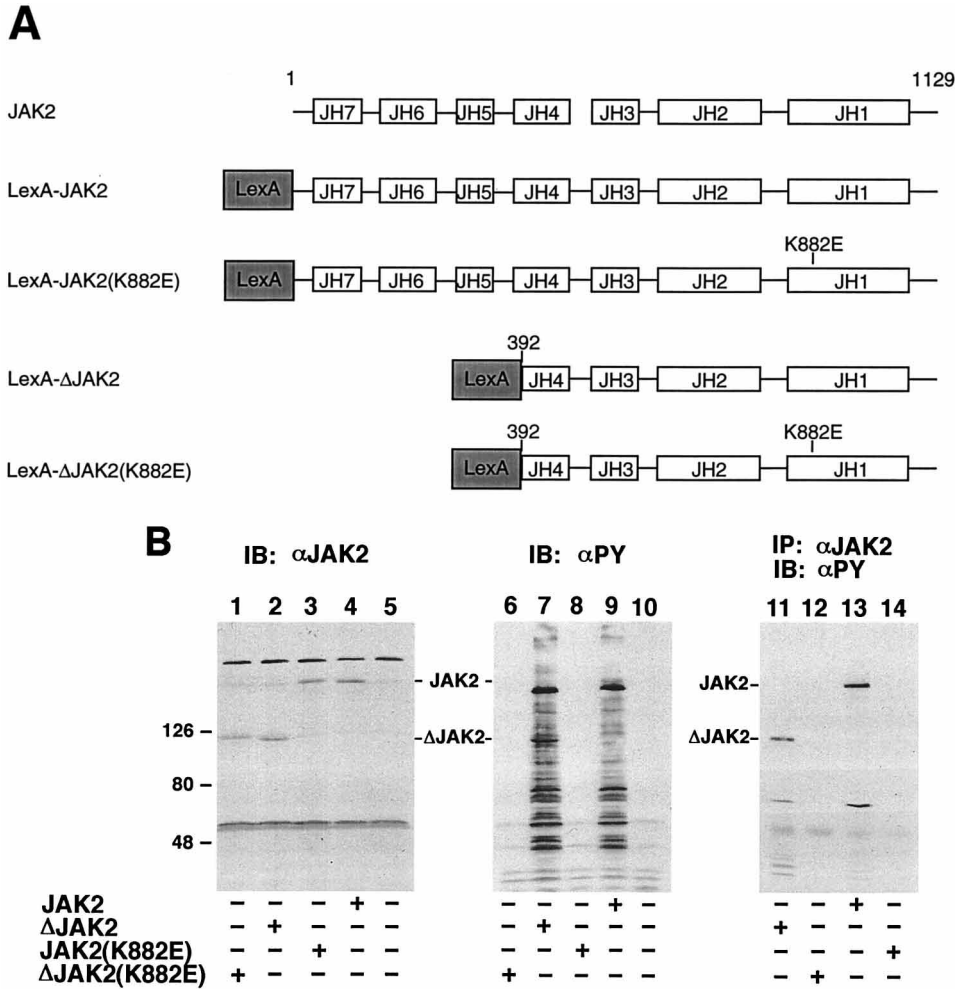



FIG. 1. Expression and tyrosyl phosphorylation in yeast of JAK2 and its mutants fused to the LexA DNA binding domain. (A) Schematic representation of hybrids of JAK2 and JAK2 mutants fused to the LexA DNA binding domain used in the yeast two-hybrid system. The positions of the deletions and point mutations are shown by numbers. The boxes represent subdomains of JAK2. (B) Yeast cells were transformed with the indicated JAK2 bait hybrids. Proteins in whole-cell lysates of the yeast transformants were separated by SDS-PAGE and immunoblotted (IB) with αJAK2 (lanes 1 to 5) or αPY (lanes 6 to 10). In a parallel experiment, proteins in yeast cell lysates were immunoprecipitated (IP) with αJAK2 and immunoblotted with αPY (lanes 11 to 14). The full-length and truncated LexA-JAK2 hybrids reproducibly migrated slightly faster than their K882E counterparts. The slightly slower migration for JAK2(K882E) than for JAK2 observed for ΔJAK2 (lanes 1 and 2, respectively) and to a lesser extent for JAK2 is reproducible and was also observed when wild-type and JAK2(K882E) were expressed in Sf9 cells (4a) and COS cells (see Fig. 5A and 6). The migration of molecular weight standards (in thousands), LexA-JAK2, and LexA-ΔJAK2 is indicated.

**Dephosphorylation.** 3T3-F442A preadipocytes were treated with 500 ng of GH per ml for 15 min, and the cell lysates were immunoprecipitated with αSH2-B (1:100 dilution). The immunoprecipitates were incubated for 60 min at 37°C in 100 μl of dephosphorylation buffer (50 mM Tris-HCl, 0.1 mM EDTA [pH 8.5], 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 40 U of alkaline phosphatase) with or without 5 mM $Na_3VO_4$. The reaction was terminated, and proteins were eluted by boiling in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer. As controls, the immunoprecipitates were treated identically, except that no alkaline phosphatase was added. Similarly, αSH2-B immunoprecipitates were treated with 0.5 U of PP2A at 37°C for 40 min, and the resultant dephosphorylated proteins were resolved by SDS-PAGE and immunoblotted with αSH2-B.

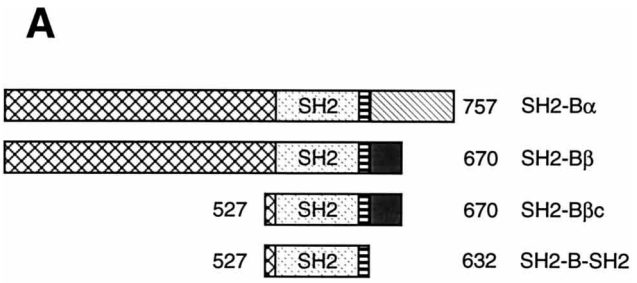
## RESULTS

**Isolation of a JAK2 binding protein with the yeast two-hybrid system.** Amino-terminally truncated JAK2 expressed in insect cells is reported to have higher tyrosine kinase activity and increased autophosphorylation than full-length JAK2 has (11). In addition, several studies suggest that the JAK2 kinase domain alone may be sufficient to initiate some critical signaling pathways. For example, when the kinase domain of JAK2 is fused to the extracellular and transmembrane regions of the epidermal growth factor (EGF) receptor and expressed in an IL-3-dependent cell line (32D) which does not express endogenous EGF receptor, EGF stimulates tyrosyl phosphorylation of the EGF receptor/JAK2 chimera and Stat5, stimulates cell proliferation, and suppresses apoptosis (18). Similarly, a JAK2 chimera composed of a CD16 external domain, a CD17 transmembrane region, and a JAK2 kinase domain mediates the expression of pim-1 and bcl-2 mRNA, delays cell death, and maintains the viability of growth factor-dependent cells (Ba/F3) (22). For these reasons, an amino-terminally truncated JAK2 (amino acids 392 to 1129 [ΔJAK2]) was used as a bait to screen for JAK2-interacting proteins in the yeast two-hybrid system. ΔJAK2 was fused to the DNA binding portion of LexA (Fig. 1A). Its expression in yeast was verified by immunoblotting yeast lysates with αJAK2 (Fig. 1B, lane 2). To test whether LexA-ΔJAK2 is catalytically active in yeast, yeast lysates were immunoblotted with αPY. In contrast to untransformed yeast, which exhibited undetectable levels of tyrosyl phosphorylation (lane 10), yeast expressing LexA-ΔJAK2 exhibited substantial

tyrosyl phosphorylation of multiple yeast proteins (lane 7). This result indicates that LexA-ΔJAK2 expressed in yeast is catalytically active. To determine if LexA-ΔJAK2 itself is tyrosyl phosphorylated, yeast lysates were immunoprecipitated with αJAK2 and immunoblotted with αPY. LexA-ΔJAK2 was found to be tyrosyl phosphorylated (lane 11). Thus, LexA-ΔJAK2 expressed ectopically in yeast has phosphotyrosine-containing motifs that could serve as binding sites for SH2 or PTB domain-containing proteins.

We screened a rat fat tissue-derived cDNA library with LexA-ΔJAK2 as bait. The activation of two reporter genes (*leu* and *lacZ*) was used as an indicator of protein-protein interactions. About 200 positive clones were isolated from about $3 \times 10^6$ yeast transformants. By DNA sequence analysis, the cDNA inserts of library plasmids from eight positive clones were found to encode the SH2 domain of SH2-B previously cloned from rat mast cells (21). However, there is an insert of 100 bp after the sequence encoding the SH2 domain, which is not present in the cDNA encoding SH2-B. For clarity, this new SH2-B isoform encoded in part by this cDNA is designated SH2-Bβ. The carboxyl-terminal SH2-Bβ obtained by yeast-two hybrid screening is designated SH2-Bβc and the originally described SH2-B is designated SH2-Bα in this paper (Fig. 2A). The term "SH2-B" is used to denote both isoforms collectively. To verify that the peptide obtained in the yeast two-hybrid screen, SH2-Bβc, interacts specifically with the ΔJAK2 portion of the bait when fused to the B42 acidic transactivation domain (prey hybrid), SH2-Bβc prey hybrid was coexpressed with bait hybrids that contained the identical LexA DNA binding domain fused to a portion of the *Drosophila* bicoid protein, the cytoplasmic domain of CD2, or the cytoplasmic domain of the insulin receptor. As summarized in Fig. 2B, SH2-Bβc prey hybrid interacted with the ΔJAK2 hybrid but not with the bicoid protein hybrid or the CD2 cytoplasmic domain hybrid, indicating a specific interaction between JAK2 and SH2-Bβc. Interestingly, the SH2-Bβc prey hybrid also bound to the cytoplasmic domain of the insulin receptor, suggesting the potential involvement of SH2-Bβ in insulin signaling.

The originally described SH2-B, referred to as SH2-Bα, was identified as an IgE receptor binding protein in a yeast tribrid system (21). SH2-Bα from rat mast cells consists of 757 amino acids (Fig. 2A) with a calculated molecular weight of 79,631. Because the 100-bp insertion present in the cDNA encoding SH2-Bβc but absent in the cDNA encoding SH2-Bα changes the reading frame, full-length cDNA for SH2-Bβ is predicted to encode a protein that is smaller than SH2-Bα by 87 amino acids. We obtained the cDNA encoding full-length SH2-Bβ from kidney tissue by PCR as described in Materials and Methods and subsequently cloned it into an expression vector (prk5) with a Myc tag at the amino terminus. Sequence analysis revealed that the carboxyl-terminal portion of kidney SH2-Bβ is identical to that obtained from fat tissue by yeast two-hybrid screening. As predicted, SH2-Bβ consists of 670 amino acids with a calculated molecular weight of 70,898. Except for a difference of two base pairs, one of which changes the encoded amino acid from Arg to Lys, and the 100-nucleotide insert just 3′ to the SH2 domain encoding sequence, the nucleotide sequence of SH2-Bβ is identical to that of SH2-Bα (Fig. 2A). Therefore, it is likely that SH2-Bα and SH2-Bβ are alternatively spliced isoforms of the same gene rather than products of different genes. SH2-Bβ, like SH2-Bα, has one SH2 domain near the carboxyl terminus, which is 68% identical to the SH2 domain of Lnk (15) and 56% identical to the SH2 domain of Shc. SH2-Bβ contains nine tyrosine residues, an unusually large number of serines (82 residues) and threonines (29 residues), and several proline-rich regions. Outside the SH2 domain, SH2-Bβ has no significant homology to any known proteins in the EMBL database except for SH2-Bα. A computer-aided search for the corresponding DNAs in the expressed-sequence tag database (EMBL database) revealed that SH2-B is widely expressed (e.g., in the brain, lung, muscle, kidney, heart, fat, breast, liver and ovary) and is evolutionarily conserved in *Drosophila*, suggesting that it is functionally important. However, no function has been reported for SH2-B to date.




B

| prey | bait | reporter LacZ | reporter Leu |
|---|---|---|---|
| SH2-Bβc | LexA-ΔJAK2 | + | + |
| | LexA-CD2 | - | - |
| | LexA-bicoid | - | - |
| | LexA-IR | + | + |

C

| prey | bait | reporter LacZ | reporter Leu |
|---|---|---|---|
| SH2-Bβc | LexA-JAK2 | + | + |
| | LexA-JAK2(K882E) | - | - |
| | LexA-ΔJAK2 | + | + |
| | LexA-ΔJAK2(K882E) | - | - |

D

| bait | prey | reporter LacZ | reporter Leu |
|---|---|---|---|
| LexA-JAK2 | SHC | - | - |
| | PTB | - | - |
| | IRS-1 | - | - |
| LexA-IR | SHC | + | + |
| | PTB | + | + |
| | IRS-1 | + | + |

FIG. 2. Interaction of SH2-Bβ with JAK2 in the yeast two-hybrid system. (A) Schematic representation of full-length and truncated SH2-B. (B to D) The indicated LexA DNA binding domain hybrids and the B42 activation domain hybrids were introduced into yeast with the *lacZ* reporter gene. The interaction of a LexA hybrid with an activation domain hybrid was assessed by examining the activation of reporter *lacZ* and leu by a filter lift color assay or by growth of the transformants in selective media lacking Leu, respectively. IR, cytoplasmic domain of the insulin receptor; CD2, cytoplasmic domain of CD2; PTB, PTB domain of IRS-1.

**Interaction of SH2-Bβc with JAK2 requires JAK2 kinase activity.** To determine whether JAK2 kinase activity and/or tyrosyl phosphorylation of JAK2 is necessary for the interaction of JAK2 with SH2-Bβc, JAK2, ΔJAK2, or their kinase-inactive counterparts [JAK2(K882E) and ΔJAK2(K882E)] were fused to the DNA binding domain of LexA in bait plasmids (Fig. 1A). These four LexA hybrids were expressed individually in yeast. Yeast lysates were immunoblotted with

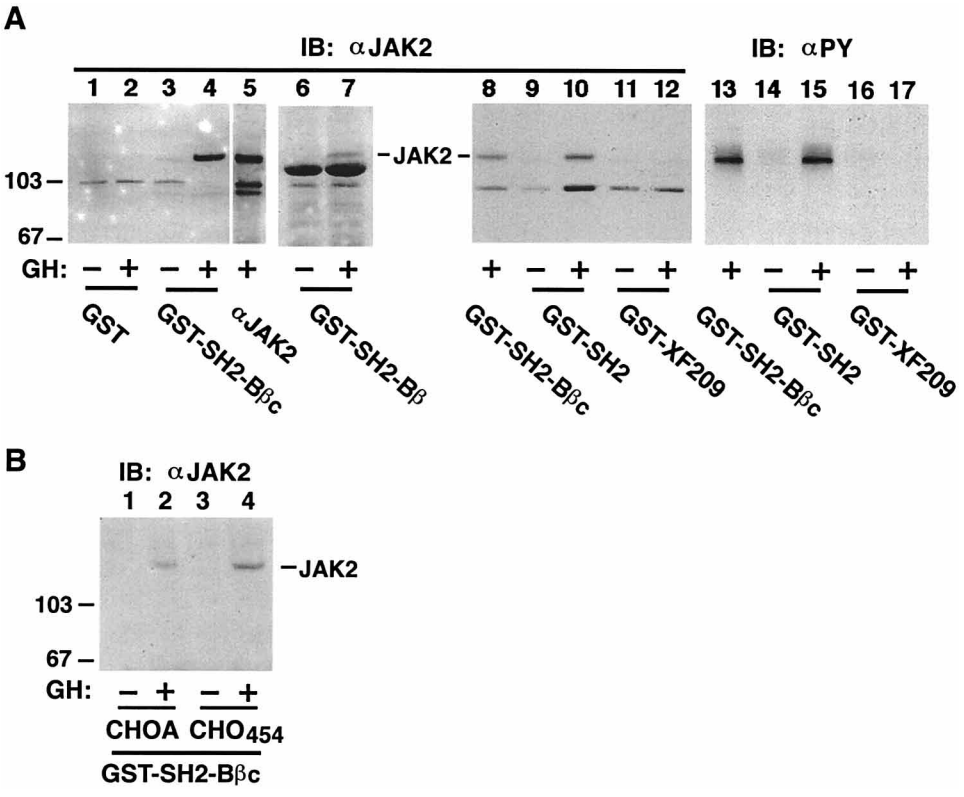



FIG. 3. GH-induced in vitro association of SH2-Bβ with tyrosyl-phosphorylated JAK2 via the SH2 domain of SH2-Bβ. 3T3-F442A cells (A) or CHO cells stably transfected with either full-length (CHOA) or truncated (CHO454) rat GHR (B) were treated for 15 min with vehicle (lanes 1, 3, 6, 9, 11, 14, and 16) or 500 ng of GH per ml (23 nM; lanes 2, 4, 5, 7, 8, 10, 12, 13, 15, and 17). Whole-cell lysates were incubated with either αJAK2 or the indicated GST fusion protein immobilized on agarose beads. Bound proteins were separated by SDS-PAGE and immunoblotted with αJAK2 (A, lanes 1 to 12; B, lanes 1 to 4). The blot corresponding to panel A, lanes 8 to 12, was then stripped and reprobed with αPY (A, lanes 13 to 17). The broad bands below JAK2 in panel A, lanes 6 and 7, are GST–SH2-Bβ. Panel A, lanes 1 to 5, lanes 6 and 7, and lanes 8 to 17, and panel B were from different experiments. The amount of GST–SH2-Bβ was less than one-third the amount of the other GST fusion proteins. The migration of molecular weight standards (in thousands) and JAK2 is indicated.

αJAK2. The expression levels of all four hybrids were comparable (Fig. 1B, lanes 1 to 4). Yeasts expressing LexA-ΔJAK2 and LexA-JAK2 (lanes 7 and 9) but not their kinase-inactive counterparts (lanes 6 and 8) contained multiple tyrosyl-phosphorylated proteins, as judged by immunoblotting yeast lysates with αPY. This verifies that LexA-ΔJAK2 and LexA-JAK2, but not their kinase-inactive counterparts, function as tyrosine kinases in yeast. Immunoprecipitation with αJAK2 and subsequent immunoblotting with αPY revealed that both LexA-ΔJAK2 and LexA-JAK2 are phosphorylated on tyrosines (lanes 11 and 13) whereas LexA-ΔJAK2(K882E) and LexA-JAK2(K882E) are not (lanes 12 and 14). The SH2-Bβc prey hybrid was then coexpressed with each of the four different JAK2 bait hybrids in yeast. As judged by the activation of *leu* and *lacZ* reporter genes, SH2-Bβc was observed to bind to LexA-JAK2 and LexA-ΔJAK2 but not to LexA-JAK2(K882E) or to LexA-ΔJAK2(K882E) (Fig. 2C). These results indicate that SH2-Bβc binds to the carboxyl-terminal portion of JAK2 and that the interaction requires JAK2 kinase activity and most probably the presence of phosphorylated tyrosines in JAK2.

We next investigated whether tyrosyl-phosphorylated JAK2 interacts in the yeast two-hybrid assay specifically with SH2-Bβc or nonspecifically with any protein containing SH2 or PTB domains. Shc, IRS-1 (amino acids 21 to 1242), and the PTB domain of IRS-1 (amino acids 45 to 516) were fused to the B42 acidic transcriptional activation domain in prey plasmids. These prey hybrids were coexpressed in yeast with LexA-JAK2. The interaction of JAK2 with these signaling proteins was assessed by examining the activation of *leu* and *lacZ* reporter genes. We were unable to detect any interaction of JAK2 with Shc, IRS-1, or the PTB domain of IRS-1 (Fig. 2D). As a positive control, the cytoplasmic domain of the insulin receptor β chain (LexA-IR) was used as bait. Shc, IRS-1, and the PTB domain of IRS-1 all interacted strongly with LexA-IR (Fig. 2D). These results demonstrate that tyrosyl-phosphorylated JAK2 selectively binds to SH2-Bβc in the yeast two-hybrid assay. Since both Shc and IRS-1 are believed to be targets of JAK2 (4, 28), these results also raise the possibility that other proteins (e.g., JAK2-associated receptors or adapter molecules) mediate or enhance the association of JAK2 with IRS-1 or Shc.

**The SH2 domain of SH2-Bβ interacts with tyrosyl-phosphorylated JAK2 in vitro.** To examine further the ability of SH2-Bβ to bind to JAK2, the interaction of JAK2 with SH2-Bβc was examined in an in vitro binding assay. SH2-Bβc and full-length SH2-Bβ were fused to a portion of GST, and the GST–SH2-Bβc and GST–SH2-Bβ fusion proteins were each immobilized on glutathione-agarose beads. Murine 3T3-F442A cells, which are well-characterized, GH-responsive preadipocytes (5, 8, 9, 24, 27), were treated with 500 ng of human GH per ml for 15 min, conditions which result in a robust activation and autophosphorylation of JAK2 (2). Cell lysates were incubated with immobilized GST, GST–SH2-Bβc, GST–SH2-Bβ, or GST fused to a novel protein of a size similar to SH2-Bβc (GST-XF209) that was also cloned from the rat fat tissue cDNA library. Proteins that bound to the beads were

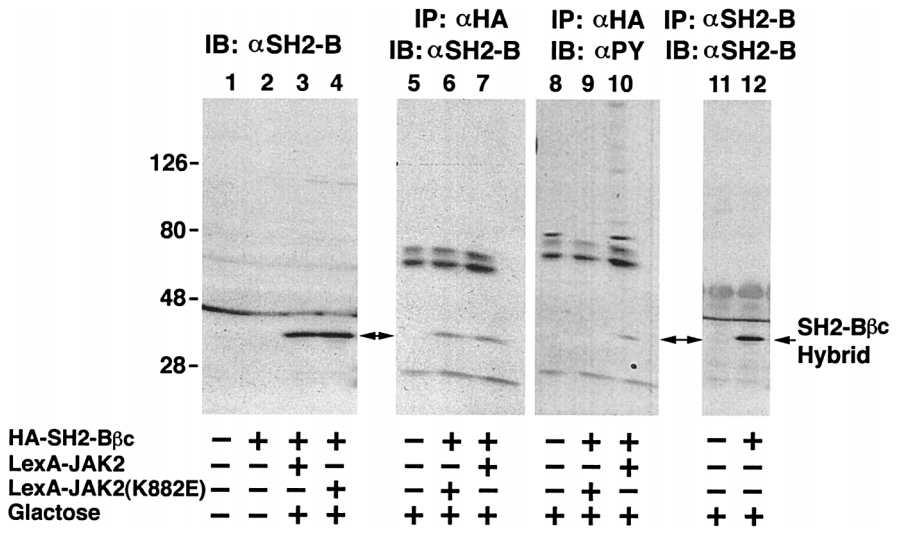



FIG. 4. Immunoblotting (IB) and immunoprecipitation (IP) of SH2-Bβ by αSH2-B. Yeast cells were transformed with plasmids encoding the indicated fusion proteins. Expression of the HA-tagged SH2-Bβc activation domain hybrid was either not induced (lanes 1 and 2) or induced with galactose (lanes 3 to 12). Whole-cell lysates from the yeast transformants were separated by SDS-PAGE and immunoblotted with αSH2-B (lanes 1 to 4). In a parallel experiment, yeast lysates were immunoprecipitated with either αHA (lanes 5 to 7) or αSH2-B (lanes 11 and 12) followed by immunoblotting with αSH2-B (lanes 5 to 7, 11, and 12). The blot corresponding to lanes 5 to 7 was stripped and reprobed with αPY (lanes 8 to 10). The migration of molecular weight standards (in thousands) and HA-tagged SH2-Bβc-activation domain hybrid is indicated.

separated by SDS-PAGE and immunoblotted with αJAK2. Both GST–SH2-Bβc (Fig. 3A, lanes 3, 4, and 8) and GST–SH2-Bβ (lanes 6 and 7) were observed to bind in a GH-dependent manner to a protein that is recognized by αJAK2 in immunoblots. The identity of this protein as JAK2 was further substantiated by its comigration with the protein immunoprecipitated by αJAK2 (lane 5). Neither GST (lanes 1 and 2) nor GST-XF209 (lanes 11 and 12) bound JAK2 from either control or GH-treated cells, indicating that JAK2 interacts specifically with SH2-Bβ. Furthermore, GST–SH2-Bβc was also able to bind to JAK2 from GH-treated but not from untreated CHO cells stably transfected with either full-length rat GHR (CHOA) (Fig. 3B, lane 2) or rat GHR truncated at amino acid 454 (CHO454) (lane 4), indicating that the GH-dependent interaction of JAK2 with SH2-Bβ is not limited to a single cell type. The amount of JAK2 bound to GST–SH2-Bβc is smaller for CHOA cells than that for CHO454 cells, consistent with tyrosyl phosphorylation of JAK2 in GH-treated CHOA cells being less than in GH-treated CHO454 cells in parallel experiments (data not shown).

The fact that the interaction of SH2-Bβc with JAK2 requires tyrosyl phosphorylation of JAK2 suggests that the SH2 domain within SH2-Bβc may be an important binding motif for JAK2. To test this further, the in vitro binding assay was repeated with only the SH2 domain of SH2-Bβ (amino acids 527 to 632, Fig. 2A) fused to GST. As observed for GST–SH2-Bβc (Fig. 3A, lanes 3, 4, and 8), JAK2 from GH-treated but not untreated cells bound to the GST–SH2-Bβ SH2 domain fusion protein (lanes 9 and 10). When the same blot was stripped and reprobed with αPY, JAK2 that was bound to the GST–SH2-Bβ SH2 domain or GST–SH2-Bβc fusion proteins was found to be tyrosyl phosphorylated (lanes 13 and 15), consistent with tyrosyl phosphorylation of JAK2 being necessary for the interaction of SH2-Bβc with JAK2. These results suggest that SH2-Bβc can bind to tyrosyl-phosphorylated JAK2 via its SH2 domain. Since the SH2 domain is conserved in SH2-Bα and SH2-Bβ, this finding suggests that both SH2-Bα and SH2-Bβ can bind to tyrosyl-phosphorylated JAK2.

**SH2-Bβ associates with and is tyrosyl phosphorylated by JAK2 in vivo in COS cells.** To enable us to examine whether SH2-Bβ associates in vivo with JAK2 in a GH-dependent fashion, antibodies against SH2-Bβ were prepared by immunizing three rabbits with GST–SH2-Bβc. These antibodies will be referred to as αSH2-B because a portion of the antigen is present in both SH2-Bα and SH2-Bβ. The antibodies would therefore be expected to bind to both SH2-Bα and SH2-Bβ. The quality of all three antisera was tested by examining their ability to recognize the SH2-Bβc prey hybrid expressed in yeast. Prey plasmid encoding HA-tagged SH2-Bβc hybrid was cotransformed into yeast with LexA-JAK2 or LexA-JAK2 (K882E), and the expression of SH2-Bβc prey hybrid was induced by growing yeast in galactose-containing medium. Proteins in yeast lysates were resolved by SDS-PAGE and immunoblotted with αSH2-B. αSH2-B, as illustrated in Fig. 4 with antiserum from rabbit 2, recognized a protein of the size appropriate for HA-tagged SH2-Bβc prey hybrid only in galactose-induced yeast transformants harboring HA-tagged SH2-Bβc prey plasmid (Fig. 4, lanes 3 and 4) but not in untransformed yeast cells (lane 1) or noninduced, transformed yeast cells (lane 2). To confirm further that the protein recognized by αSH2-B is indeed the HA-tagged SH2-Bβc prey hybrid, we immunoprecipitated yeast lysates with αHA and immunoblotted them with αSH2-B. A protein of the size appropriate for HA-tagged SH2-Bβc prey hybrid was observed in yeast transformed with prey plasmid encoding HA-tagged SH2-Bβc hybrid (lanes 6 and 7) but not in untransformed yeast (lane 5). Interestingly, when the same blot was reprobed with αPY, HA-tagged SH2-Bβc was observed to be tyrosyl phosphorylated in yeast coexpressing LexA-JAK2 (lane 10) but not in yeast coexpressing LexA-JAK2(K882E) (lane 9) or no bait (lane 8), suggesting that SH2-Bβc is tyrosyl phosphorylated directly by JAK2.

We then tested the ability of αSH2-B to immunoprecipitate SH2-Bβ. Yeast lysates were immunoprecipitated with αSH2-B and subsequently immunoblotted with αSH2-B. A protein of the size appropriate for HA-tagged SH2-Bβc prey hybrid was

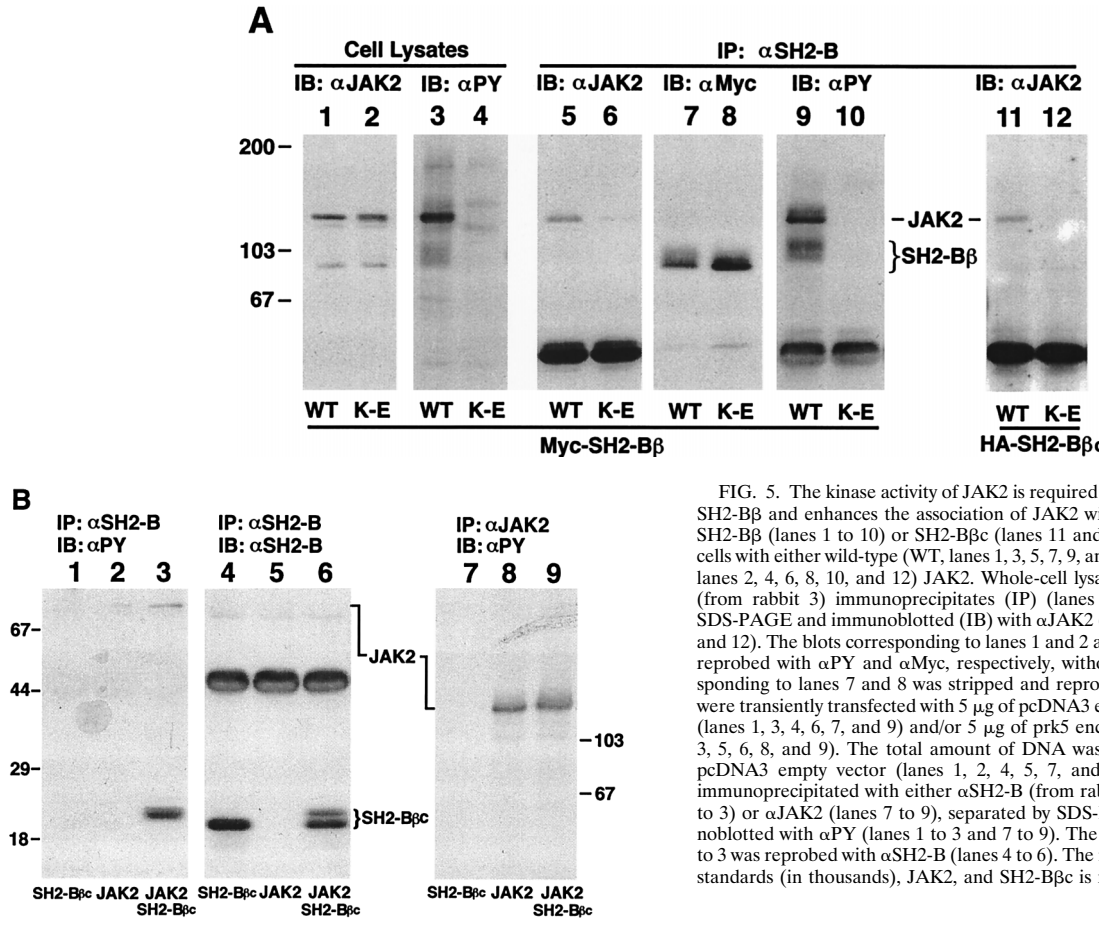



FIG. 5. The kinase activity of JAK2 is required for tyrosyl phosphorylation of SH2-Bβ and enhances the association of JAK2 with SH2-Bβ in COS cells. (A) SH2-Bβ (lanes 1 to 10) or SH2-Bβc (lanes 11 and 12) was coexpressed in COS cells with either wild-type (WT, lanes 1, 3, 5, 7, 9, and 11) or kinase-inactive (K-E, lanes 2, 4, 6, 8, 10, and 12) JAK2. Whole-cell lysates (lanes 1 to 4) or αSH2-B (from rabbit 3) immunoprecipitates (IP) (lanes 5 to 12) were separated by SDS-PAGE and immunoblotted (IB) with αJAK2 (lanes 1 and 2, 5 and 6, and 11 and 12). The blots corresponding to lanes 1 and 2 and lanes 5 and 6 were directly reprobed with αPY and αMyc, respectively, without stripping. The blot corresponding to lanes 7 and 8 was stripped and reprobed with αPY. (B) COS cells were transiently transfected with 5 μg of pcDNA3 encoding HA-tagged SH2-Bβc (lanes 1, 3, 4, 6, 7, and 9) and/or 5 μg of prk5 encoding murine JAK2 (lanes 2, 3, 5, 6, 8, and 9). The total amount of DNA was kept constant at 10 μg with pcDNA3 empty vector (lanes 1, 2, 4, 5, 7, and 8). Whole-cell lysates were immunoprecipitated with either αSH2-B (from rabbit 3 [1:100 dilution], lanes 1 to 3) or αJAK2 (lanes 7 to 9), separated by SDS-PAGE (15% gel), and immunoblotted with αPY (lanes 1 to 3 and 7 to 9). The blot corresponding to lanes 1 to 3 was reprobed with αSH2-B (lanes 4 to 6). The migration of molecular weight standards (in thousands), JAK2, and SH2-Bβc is indicated.

detected by αSH2-B Western blotting in yeast expressing HA-tagged SH2-Bβc prey hybrid (Fig. 4, lane 12) but not in untransformed control yeast cells (lane 11). When SH2-Bβ antiserum was cleared of SH2-Bβ antibody by preincubation with GST–SH2-Bβc, it no longer detected SH2-Bβc prey hybrid (data not shown). Preimmune serum also failed to recognize SH2-Bβc prey hybrid (data not shown). αSH2-B from the other two rabbits is also capable of recognizing SH2-Bβc both by immunoblotting and by immunoprecipitation (data not shown). αSH2-B raised from rabbit 2 was used for all experiments unless indicated otherwise.

To provide direct evidence of an association of SH2-Bβ with tyrosyl-phosphorylated JAK2 in mammalian cells, Myc-tagged full-length SH2-Bβ was coexpressed in COS cells with either wild-type or kinase-inactive mutant JAK2. The expression levels of wild-type and kinase-inactive mutant JAK2 were similar (Fig. 5A, lanes 1 and 2). Transient expression of wild-type JAK2 in COS cells produced constitutively tyrosyl-phosphorylated JAK2 (Fig. 5A, lane 3; Fig. 5B, lanes 8 and 9), as observed by others (15a, 17). In contrast, tyrosyl phosphorylation of kinase-inactive JAK2 was undetectable (Fig. 5A, lane 4; Fig. 6, lane 6). When proteins in the cell lysates were immunoprecipitated with αSH2-B and immunoblotted with αJAK2, SH2-Bβ was observed to coimmunoprecipitate with wild-type JAK2 (Fig. 5A, lane 5) but only marginally with kinase-inactive JAK2 (lane 6). Interestingly, reprobing the same blot with αMyc revealed that some Myc–SH2-Bβ migrated slower in cells coexpressing wild-type JAK2 than in cells coexpressing kinase-inactive JAK2 (lanes 7 and 8), suggesting that Myc–SH2-Bβ may be tyrosyl phosphorylated by JAK2. The same blot was then stripped and reprobed with αPY. As expected, tyrosyl phosphorylation of SH2-Bβ was observed only in cells coexpressing wild-type JAK2 (lane 9) and not in cells coexpressing kinase-inactive JAK2 (lane 10). The small amount of kinase-inactive, unphosphorylated JAK2 coimmunoprecipitated with SH2-Bβ (lane 6) raises the possibility of the presence of a second JAK2 binding site within the amino terminus of SH2-Bβ, which might bind to JAK2 in a tyrosyl phosphorylation-independent manner. Consistent with this hypothesis,

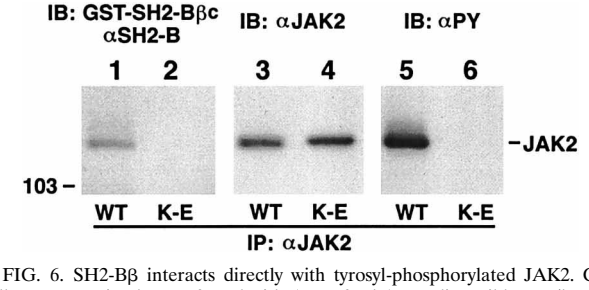



FIG. 6. SH2-Bβ interacts directly with tyrosyl-phosphorylated JAK2. COS cells were transiently transfected with 5 μg of prk5 encoding wild-type (lanes 1, 3, and 5) or kinase-inactive (lanes 2, 4, and 6) murine JAK2. JAK2 was immunoprecipitated (IP) with αJAK2, separated by SDS-PAGE, and transferred onto nitrocellulose. The membrane was incubated with GST–SH2-Bβc at 4°C overnight. After extensive washing, the membrane was immunoblotted (IB) with αSH2-B (lanes 1 and 2). The same blot was sequentially reprobed with αJAK2 (lanes 3 and 4) and αPY (lanes 5 and 6).

no JAK2(K882E) bound to SH2-Bβc (lane 12), which lacks the amino terminus (Fig. 2A).

To gain insight into which tyrosines in SH2-Bβ are targets of JAK2, SH2-Bβc, which contains four tyrosines and has been shown to be tyrosyl phosphorylated in yeast when coexpressed with wild-type JAK2 (Fig. 4, lane 10), was transiently expressed in COS cells either alone or together with JAK2. When SH2-Bβc alone was expressed in COS cells, it migrated as a single protein in SDS-PAGE gels, as determined by immunoprecipitation with αSH2-B and immunoblotting with αSH2-B (Fig. 5B, lane 4). No proteins immunoprecipitated by αSH2-B were recognized by αPY (lane 1), suggesting that SH2-Bβc is not tyrosyl phosphorylated in the absence of JAK2. When SH2-Bβc was coexpressed with JAK2, two proteins were detected by αSH2-B, the protein observed in the absence of JAK2 and a second, slower-migrating SH2-Bβc (lane 6). The slower-migrating SH2-Bβc was recognized by αPY (lane 3). These results strongly suggest that JAK2 directly tyrosyl phosphorylates SH2-Bβ and that one or more of the four tyrosine residues within SH2-Bβc is a target of JAK2.

To verify the result from the yeast two-hybrid system that SH2-Bβc binds to tyrosyl-phosphorylated JAK2 directly rather than indirectly through some unknown mediator(s), far Western blot analysis was performed. COS cells were transiently transfected with either wild-type or kinase-inactive mutant JAK2. Equal amounts of wild-type and kinase-inactive JAK2 were immunoprecipitated by αJAK2 and separated by SDS-PAGE (Fig. 6, lanes 3 and 4). As shown in Fig. 5A, wild-type but not kinase-inactive JAK2 was tyrosyl phosphorylated (Fig. 6, lanes 5 and 6). The blot was incubated first with GST–SH2-Bβc and then with αSH2-B. As shown in Fig. 6, lanes 1 and 2, SH2-Bβc binds directly to wild-type, tyrosyl-phosphorylated JAK2 but not to kinase-inactive, unphosphorylated JAK2.

**GH stimulates association of SH2-B with JAK2 and tyrosyl phosphorylation of SH2-B in 3T3-F442A preadipocytes.** To examine whether endogenous SH2-Bβ associates with JAK2 in mammalian cells, cell lysates from GH-treated 3T3-F442A preadipocytes were immunoprecipitated with αSH2-B and immunoblotted with αJAK2. Significantly more JAK2 was immunoprecipitated by αSH2-B when the cells were stimulated with GH (Fig. 7, lanes 1 and 2). A tyrosyl-phosphorylated protein that comigrates with JAK2 was also immunoprecipitated by αSH2-B only from GH-treated cells (Fig. 7, lane 6; Fig. 8), consistent with JAK2 forming a complex with SH2-Bβ in GH-treated cells. Reprobing the blot with αSH2-B revealed three bands (Fig. 7, lanes 3 and 4). All three bands appear to be SH2-B, since none of them were observed when preimmune serum was used (lane 7).

To examine whether the GH-dependent mobility shift of SH2-B to slower-migrating bands (Fig. 7, lane 4) might be due to tyrosyl phosphorylation of SH2-B, 3T3-F442A cells were treated with 500 ng of GH per ml for 15 min and the cell lysates were immunoprecipitated with αSH2-B and immunoblotted with αPY. There was no detectable tyrosyl phosphorylation of SH2-B in the absence of GH (lane 5). In the presence of GH, all three forms of SH2-B appear to be tyrosyl phosphorylated (lane 6). The amount of phosphorylated tyrosine was routinely largest in the slowest-migrating form and smallest in the intermediate form (Fig. 7, lane 6; Fig. 8, lanes 3, 5 to 8, 13, and 14). The three bands may represent different isoforms of SH2-B (e.g., SH2-Bα and SH2-Bβ), differential phosphorylation states of a single isoform of SH2-B, or a combination of isoforms and phosphorylation states.

GH-induced tyrosyl phosphorylation of SH2-B was found to be rapid and transient (Fig. 8). It was detected within 1 min of GH stimulation (Fig. 8, lane 3), peaked at about 10 min (lane 6), and returned to near basal values by 60 min (lane 9). Tyrosyl phosphorylation of SH2-B was observed at physiological concentrations of GH as low as 5 ng/ml (lane 12).

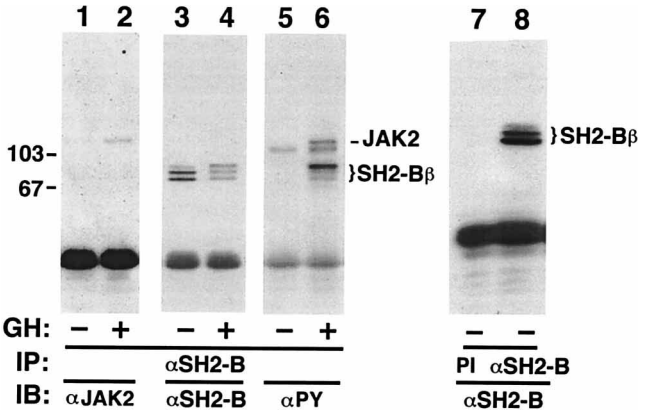



FIG. 7. GH-dependent association of SH2-Bβ with tyrosyl-phosphorylated JAK2 and tyrosyl phosphorylation of SH2-Bβ. 3T3-F442A cells were treated for 15 min with 500 ng of GH per ml (lanes 2, 4, and 6) or with vehicle (lanes 1, 3, 5, 7, and 8). Whole-cell lysates were immunoprecipitated (IP) with αSH2-B (lanes 1 to 6 and 8) or preimmune serum (PI, lane 7) and subsequently immunoblotted (IB) with either αPY (lanes 5 and 6) or αSH2-B (lanes 7 and 8). The blot corresponding to lanes 5 and 6 was stripped and reprobed with αSH2-B (lanes 3 and 4) and then stripped again and reprobed with αJAK2 (lanes 1 and 2). The migration of molecular weight standards (in thousands), JAK2, and SH2-Bβ is indicated.

A constitutively tyrosyl-phosphorylated protein with a molecular weight of about 110,000 (p110) was observed in αSH2-B immunoprecipitates with αSH2-B raised from rabbit 2 (Fig. 8) but not with αSH2-B from the other two rabbits (data not shown), suggesting that αSH2-B from rabbit 2 cross-reacts with this phosphoprotein in immunoprecipitation assays. Alternatively, this p110 phosphoprotein may associate constitutively with a subset of SH2-B which is not recognized by αSH2-B from rabbits 1 and 3.

**SH2-Bβ is phosphorylated at multiple sites.** To determine whether the multiple proteins identified by αSH2-B in 3T3-F442A cells represent different isoforms of SH2-B or one isoform with different phosphorylation states, αSH2-B immunoprecipitates were dephosphorylated with alkaline phosphatase. 3T3-F442A cells were stimulated with 500 ng of GH per ml for 15 min, and the cell lysates were immunoprecipitated with αSH2-B. The immunoprecipitates were incubated with 40 U of alkaline phosphatase at 37°C for 60 min and immunoblotted with αPY. GH induced tyrosyl phosphorylation primarily of the slowest-migrating form of SH2-B (Fig. 9, lane 2), consistent with the results shown in Fig. 5 and 6. After dephosphorylation with alkaline phosphatase, no tyrosyl-phosphorylated SH2-B was detected (Fig. 9, lane 4). Alkaline phosphatase treatment reduced the multiple forms of SH2-B from both control (Fig. 9, lanes 5 and 15) and GH-treated (lanes 6 and 10) cells to one form migrating with or slightly below the fastest-migrating form observed in the absence of alkaline phosphatase (lanes 7, 8, 11, and 13). The apparent size of this single band (~71 kDa) corresponds more closely to that predicted for SH2-Bβ (70,898) than to that predicted for SH2-Bα (79,631). In addition, after dephosphorylation by alkaline phosphatase, SH2-Bβ transiently expressed in COS cells migrates slightly slower than SH2-B expressed in 3T3-F442A cells (data not shown). The slightly slower migration of SH2-Bβ expressed in COS cells is thought to reflect the addition of 14 amino acids of Myc tag. These results suggest that the protein recognized by αSH2-B in

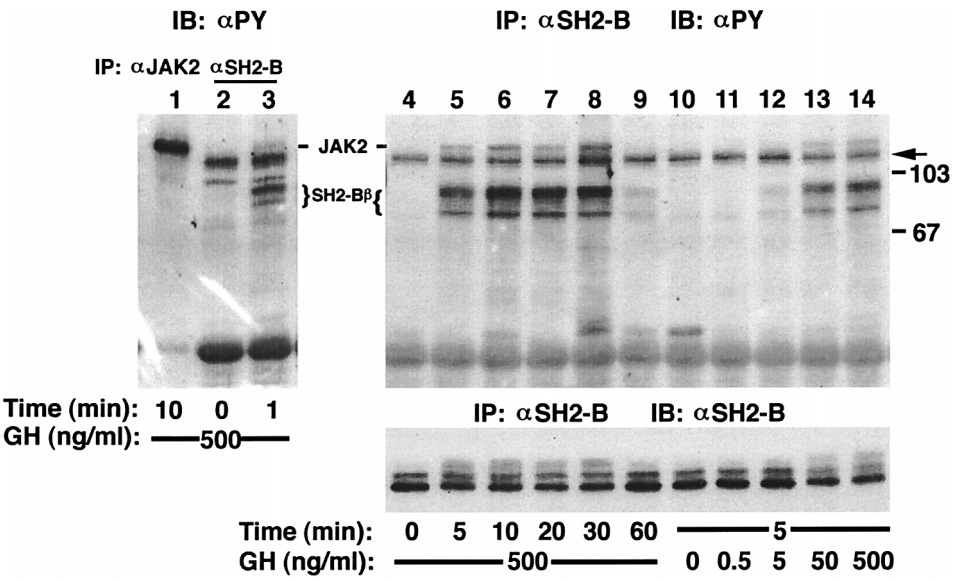



FIG. 8. Time course and dose response of GH-stimulated tyrosyl phosphorylation of SH2-Bβ. 3T3-F442A cells were treated with GH at the indicated concentrations and for the indicated times. Whole-cell lysates were immunoprecipitated (IP) with αSH2-B (lanes 2 to 14), separated by SDS-PAGE (7% polyacrylamide gel), and immunoblotted (IB) with αPY (lanes 1 to 14, upper panel). The blots corresponding to lanes 4 to 14 were reprobed without stripping with αSH2-B (lanes 4 to 14, lower panel). To determine the location of JAK2, a portion of the cell lysates was immunoprecipitated with αJAK2 and immunoblotted with αPY (lane 1). The arrow below JAK2 points to p110. The migration of molecular weight standards (in thousands), JAK2, and SH2-Bβ is indicated.

3T3-F442A cells is SH2-Bβ rather than SH2-Bα. We shall therefore refer to SH2-B in 3T3-F442A cells as SH2-Bβ, with the recognition that isoform-specific antibodies will be needed to verify this conclusion. Alkaline phosphatase treatment substantially increased the amount of the fastest-migrating form of SH2-Bβ (Fig. 9, lanes 7, 8, 11, and 13), indicating a shift of SH2-Bβ from slower-migrating forms to a faster-migrating form as a result of dephosphorylation of SH2-Bβ. Addition of sodium orthovanadate (lanes 12 and 14) or 10 mM EDTA (data not shown), inhibitors of alkaline phosphatase, abolished the effect of alkaline phosphatase on the mobilities of SH2-Bβ, indicating that the shift in SH2-Bβ mobility resulted from dephosphorylation rather than protein degradation. These results suggest that there is one isoform of SH2-B, SH2-Bβ, in 3T3-F442A preadipocytes and that the multiple forms of SH2-B observed in both control and GH-treated cells are attributable to differential phosphorylation of SH2-Bβ. The fact that the three SH2-Bβ bands detected in the absence of GH do not bind αPY suggests that SH2-Bβ is phosphorylated on two or more serines and/or threonines. To provide more direct evidence for serine/threonine phosphorylation of SH2-Bβ, SH2-Bβ from untreated 3T3-F442A cells was dephosphorylated by the serine/threonine-specific phosphatase, PP2A. PP2A treatment also reduced the multiple forms of SH2-Bβ to a single, faster-migrating form (Fig. 9, lanes 16 and 17), further indicating that SH2-Bβ is phosphorylated on serine(s) and/or threonine(s). Okadaic acid, an inhibitor of PP2A, blocks the reduction in bands (data not shown).

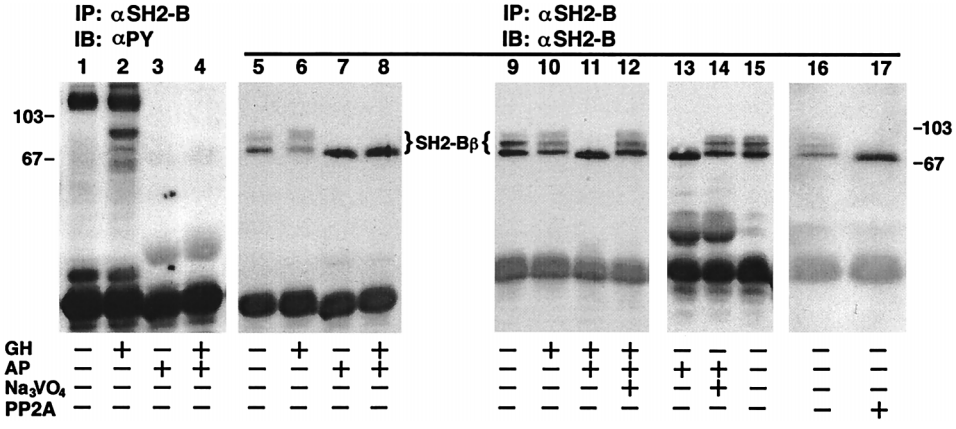



FIG. 9. Phosphorylation of SH2-Bβ at multiple sites. 3T3-F442A cells were treated with 500 ng of GH per ml for 15 min, and whole-cell lysates were immunoprecipitated (IP) with αSH2-B. The immunoprecipitates were incubated at 37°C for 60 min in dephosphorylation buffer with (lanes 3, 4, 7, 8, and 11 to 14) or without (lanes 1, 2, 5, 6, 9, 10, and 15) alkaline phosphatase (AP) and with (lanes 12 and 14) or without (lanes 1 to 11, 13, and 15) $Na_3VO_4$. SH2-Bβ from untreated 3T3-F442A cells was incubated with (lane 17) or without (lane 16) PP2A. The reaction mixtures were subjected to SDS-PAGE and immunoblotted (IB) with αPY (lanes 1 to 4) or αSH2-B (lanes 5 to 17). The blot corresponding to lanes 1 to 4 was stripped and reprobed with αSH2-B (lanes 5 to 8). The migration of molecular weight standards (in thousands) and SH2-Bβ is indicated.

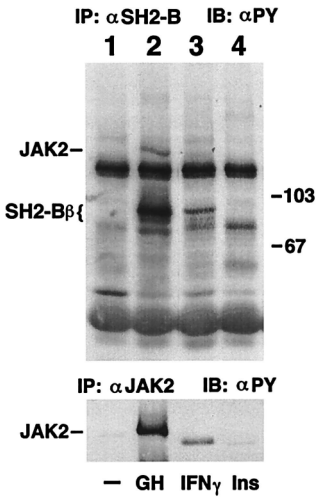



FIG. 10. Ligand-dependent tyrosyl phosphorylation of SH2-Bβ in response to GH, IFN-γ, or insulin. 3T3-F442A cells were treated for 15 min with 500 ng of GH per ml (lane 2), 10 ng of IFN-γ per ml (0.7 nM) (lane 3), or 500 ng of insulin per ml (89.5 nM) (Ins, lane 4). Whole-cell lysates were immunoprecipitated (IP) with αSH2-B (upper panel) or αJAK2 (lower panel) and immunoblotted (IB) with αPY. The migration of molecular weight standards (in thousands), JAK2, and SH2-Bβ is indicated.

**Tyrosyl phosphorylation of SH2-Bβ in response to IFN-γ but not insulin.** Since SH2-Bβ associates with active JAK2 and appears to be tyrosyl phosphorylated by JAK2 in response to GH, we investigated whether SH2-Bβ is tyrosyl phosphorylated in response to IFN-γ, a cytokine that activates JAK2. Since SH2-Bβc was found to interact with the cytoplasmic domain of the insulin receptor β chain in the yeast two-hybrid system, we also examined whether SH2-Bβ is tyrosyl phosphorylated in response to insulin. 3T3-F442A cells were treated individually with GH, IFN-γ, or insulin. Cell lysates were immunoprecipitated with αSH2-B and immunoblotted with αPY. GH and IFN-γ stimulated tyrosyl phosphorylation of SH2-Bβ, whereas insulin did not (Fig. 10, upper panel). As predicted from the much greater stimulation of JAK2 phosphorylation by GH than by IFN-γ (Fig. 10, lower panel), GH was significantly more effective than IFN-γ at stimulating tyrosyl phosphorylation of SH2-Bβ.

## DISCUSSION

The SH2 domain-containing molecule SH2-Bα was cloned more than 18 months ago (21), yet surprisingly little is known about its function. Since it was cloned in a yeast tribrid system with phosphorylated cytoplasmic domain of the gamma subunit of the high-affinity IgE receptor as bait, it is reasonable to hypothesize that it serves as a signaling molecule for the IgE receptor. However, its ability to serve in that capacity has not been examined in anything other than the yeast tribrid system. In the present work, we identify a new isoform of SH2-B, SH2-Bβ. Except for two nucleotide differences and an insert of 100 nucleotides in SH2-Bβ that is absent in SH2-Bα, the sequences are identical, indicating that SH2-Bα and SH2-Bβ are two different isoforms derived from RNA alternative splicing rather than products of two different genes. A BLAST search of the expressed-sequence tag database with the insert sequence specific to SH2-Bβ reveals that SH2-Bβ or its homolog(s) containing the insert is widely expressed in human, mouse, and rat in tissues such as embryo, heart, and liver and in fibroblast cells.

The present work provides evidence that SH2-Bβ binds to activated JAK2 and serves as a signaling molecule for GH and IFN-γ, both of which activate JAK2. Multiple lines of evidence indicate that SH2-Bβ binds to kinase-active, tyrosyl-phosphorylated JAK2. First, in the yeast two-hybrid system, both full-length and amino-terminally truncated (amino acids 392 to 1129), tyrosyl-phosphorylated, kinase-active JAK2 interact directly with the carboxyl-terminal portion of SH2-Bβ (amino acids 232 to 670) whereas their unphosphorylated, kinase-inactive counterparts do not. Second, both GST–SH2-Bβ and GST–SH2-Bβc interact with tyrosyl-phosphorylated JAK2 from GH-stimulated cells but not with unphosphorylated JAK2 from untreated cells. Third, SH2-Bβc coimmunoprecipitates with wild-type, tyrosyl-phosphorylated JAK2 but not with kinase-inactive, unphosphorylated JAK2 when SH2-Bβc and JAK2 are coexpressed in COS cells. Similarly, SH2-Bβ associates with wild-type JAK2 to a much greater extent than with kinase-inactive JAK2. Fourth, GST–SH2-Bβc directly binds to wild-type, tyrosyl-phosphorylated JAK2 but not to kinase-inactive, unphosphorylated JAK2 in a far Western blot. Finally, GH promotes the coimmunoprecipitation of JAK2 with SH2-Bβ in 3T3-F442A cells. The specificity of the SH2-B–JAK2 association is supported by several findings. JAK2 did not bind to Shc, IRS-1, or the PTB domain of IRS-1 in the yeast two-hybrid system. JAK2 from GH-stimulated cells did not bind to GST alone or to GST-XF209. SH2-Bβc did not bind to kinase-inactive JAK2 or to any proteins other than wild-type JAK2 present in the αJAK2 immunoprecipitates in the far Western assay. Finally, whereas JAK2 is detectable by αJAK2 Western blotting in αSH2-B immunoprecipitates in a GH-dependent fashion in 3T3-F442A cells, JAK2 has not been detected by αJAK2 Western blotting in immunoprecipitates of antibodies to other SH2 domain-containing proteins that have been shown by other mechanisms to serve as signaling molecules for GH. These proteins include Shc proteins (28), Grb2 (28), and the p85 regulatory subunit of phosphatidylinositol 3′-kinase (4).

Interaction of JAK2 with SH2-B appears to be mediated at least in part via the SH2 domain of SH2-B, since GST–SH2-Bβ SH2 fusion protein bound tyrosyl-phosphorylated JAK2 from GH-treated cells but not unphosphorylated JAK2 from control cells. Furthermore, SH2-Bβc bound only to tyrosyl-phosphorylated JAK2 in a far Western blot. When coexpressed with JAK2 in COS cells, SH2-Bβ bound kinase-active, tyrosyl-phosphorylated JAK2 to a much greater extent than it bound kinase-inactive, unphosphorylated JAK2. These findings suggest that tyrosyl phosphorylation of JAK2 is critical for its association with SH2-Bβ. Since the SH2 domain is the only known phosphotyrosine binding motif present in SH2-Bβ, these results suggest that the SH2 domain may be a major binding site in SH2-Bβ for tyrosyl-phosphorylated JAK2. Since both isoforms of SH2-B contain the SH2 domain, the finding that the SH2 domain binds to JAK2 suggests that both SH2-Bβ and SH2-Bα are likely to bind to JAK2 in response to ligands activating JAK2. Taken together, the experiments performed in yeast, with GST fusion proteins, with overexpression of JAK2, SH2-Bβ, and SH2-Bβc in COS cells, and with endogenous SH2-B and JAK2 in 3T3-F442A cells strongly argue that upon activation and autophosphorylation of JAK2 in response to GH, SH2-B is recruited into GHR-JAK2 complexes at least in part by binding of its SH2 domain to phosphorylated tyrosines within JAK2. Whether other regions of SH2-B contribute to the interaction of SH2-B with JAK2 or whether some SH2-B that is phosphorylated in response to GH is recruited to GHR-JAK2 complexes by binding to phosphorylated tyrosines

within GHR or another GH signaling molecule remains to be determined.

Consistent with SH2-Bβ acting as a signaling molecule for GH, we demonstrate that in 3T3-F442A cells, GH stimulates the rapid (within 1 min) and transient (undetectable by 60 min) tyrosyl phosphorylation of SH2-Bβ at physiologically relevant concentrations of GH. The time course and GH dose response for SH2-Bβ tyrosyl phosphorylation parallel those for JAK2 tyrosyl phosphorylation (2), consistent with SH2-Bβ phosphorylation being dependent upon JAK2 activation and phosphorylation. SH2-B was also observed to be tyrosyl phosphorylated in response to GH in other cell types such as 3T3-L1 and CHO cells stably transfected with GHR (data not shown). Two lines of evidence suggest that SH2-Bβ is tyrosyl phosphorylated by JAK2. First, SH2-Bβc is tyrosyl phosphorylated in yeast coexpressing kinase-active JAK2 but not kinase-inactive JAK2. Second, when transiently expressed in COS cells, SH2-Bβ or SH2-Bβc is tyrosyl phosphorylated only when wild-type (but not when kinase-inactive) JAK2 is coexpressed. The finding that SH2-Bβc is tyrosyl phosphorylated in both the yeast and COS cells by JAK2 suggests that one or more of the four tyrosines within the carboxyl-terminal region of SH2-Bβ is a site of phosphorylation. Taken together, these results indicate that SH2-Bβ is an endogenous substrate of JAK2 and that GH stimulates the association of SH2-Bβ with GHR-JAK2 complexes and the subsequent tyrosyl phosphorylation of SH2-Bβ by JAK2.

Since SH2-Bβ appears to be recruited to tyrosyl-phosphorylated JAK2 and tyrosyl phosphorylated by JAK2, one might expect it to serve as a signaling molecule for other cytokine receptors that activate JAK2. Consistent with this, IFN-γ also stimulates the tyrosyl phosphorylation of SH2-Bβ. GH was significantly more effective than IFN-γ at stimulating the tyrosyl phosphorylation of SH2-Bβ, presumably because it is so much more effective at activating JAK2. The large difference in the level of tyrosyl phosphorylation of SH2-Bβ suggests that SH2-Bβ may play a greater role in the action of GH than in the action of IFN-γ. However, the finding that in 3T3-F442A cells, SH2-Bβ is not tyrosyl phosphorylated in response to insulin even though insulin receptor binds to SH2-Bβc in the yeast two-hybrid system and in an in vitro GST–SH2-Bβc binding assay (data not shown) raises the possibility that SH2-Bβ can be recruited to membrane receptors and can serve as a signaling molecule for some cytokines and/or hormones without being tyrosyl phosphorylated.

Sequence analysis reveals that both SH2-Bα and SH2-Bβ contain an SH2 domain, several proline-rich regions, nine tyrosine residues, and no conserved sequences that would suggest that they possess enzymatic activity. Thus, it seems possible that both SH2-Bα and SH2-Bβ function as adapter molecules that recruit and/or activate one or more downstream signaling molecules. The tyrosine(s) in SH2-B that is phosphorylated by JAK2 in response to ligand binding may form docking sites for other SH2 or PTB domain-containing proteins and enable SH2-B to function as a bridge between JAK2 and these downstream molecules. In addition or alternatively, the proline-rich sequences on SH2-Bβ may recruit SH3 and/or WW domain-containing molecules to JAK2. To gain some insight into a possible function for SH2-Bβ, we examined the possibility of association of SH2-Bβ from 3T3-F442A cells with some known GH-responsive proteins. In preliminary experiments, we were unable to detect GH-dependent association of SH2-Bβ with Shc, IRS-1, Stat1, Stat3, Stat5, Grb2, phospholipase Cγ (PLCγ), or the p85 regulatory subunit of phosphatidylinositol 3′-kinase by coimmunoprecipitation (data not shown). Therefore, SH2-Bβ may not be an adapter molecule that links JAK2 to these molecules but, rather, may initiate novel signaling pathways. Alternatively, any potential interaction between SH2-Bβ and these proteins is likely to be transient and a highly regulated dynamic process. Detection of such protein-protein interactions by coimmunoprecipitation may not be possible with our current antibodies, especially in cells such as 3T3-F442A cells used in this study in which SH2-Bβ and these signaling molecules are present at endogenous levels.

Interestingly, SH2-Bβ appears to be phosphorylated on two or more serines and/or threonines, which is not surprising given the large number of serines (82 residues) and threonines (29 residues) in the protein. We do not yet know which kinase(s) is responsible for the serine/threonine phosphorylation of SH2-Bβ. However, based upon sequence analysis, there are potential phosphorylation sites for cyclic AMP (cAMP)- and cGMP-dependent protein kinases, casein kinase II, ERK1, ERK2, and protein kinase C. The physiological consequence of the serine/threonine phosphorylation of SH2-Bβ is not yet known, but it is reasonable to hypothesize that serine/threonine phosphorylation modulates SH2-Bβ function. The fact that SH2-Bβ is phosphorylated on serine(s) and/or threonine(s) as well as on tyrosine(s) suggests that SH2-Bβ may be subject to regulation by multiple kinases. Thus, SH2-Bβ may be a site of convergence for cross talk among different cytokines and hormones. Taken together, the characteristics of SH2-Bβ suggest that SH2-Bβ may be an important multifunctional adapter molecule for cytokine receptors that activate JAK2. Whether it also serves as a signaling molecule downstream of other members of the JAK family of tyrosine kinases and receptor tyrosine kinases remains to be determined. The identification of signaling molecules that are downstream of SH2-Bβ and the physiological role(s) of SH2-Bβ are under investigation.

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