SH2-B Family Members Differentially Regulate JAK Family Tyrosine Kinases*

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Activation of JAK tyrosine kinases is an essential step in cell signaling by multiple hormones, cytokines, and growth factors, including growth hormone (GH) and interferon-γ. Previously, we identified SH2-Bβ as a potent activator of JAK2 (Rui, L., and Carter-Su, C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7172–7177). Here, we investigated whether the activation of JAK2 by SH2-Bβ is specific to JAK2 and SH2-Bβ or extends to other JAKs or other members of the SH2-Bβ family. When SH2-Bβ was overexpressed with JAK1 or JAK3, SH2-Bβ failed to increase their activity. However, SH2-Bβ bound to both and was tyrosyl-phosphorylated by JAK1. In contrast to SH2-Bβ, APS decreased tyrosyl phosphorylation of GH-stimulated JAK2 as well as Stat3β, a substrate of JAK2. APS also decreased tyrosyl phosphorylation of JAK1, but did not affect the activity or tyrosyl phosphorylation of JAK3. Overexpressed APS bound to and was tyrosyl-phosphorylated by all three JAKs. Consistent with these data, in 3T3-F442A adipocytes, endogenous APS was tyrosyl-phosphorylated in response to GH and interferon-γ. These results suggest that 1) SH2-Bβ specifically activates JAK2, 2) APS negatively regulates both JAK2 and JAK1, and 3) both SH2-Bβ and APS may serve as adapter proteins for all three JAKs independent of any role they have in JAK activity.

The Janus family tyrosine kinases, consisting of JAK1, JAK2, JAK3, and Tyk2, bind to members of the cytokine family of receptors and are activated upon ligand binding to these receptors. This family of receptors consists of more than 20 different proteins that are known to bind to at least 25 different ligands, including growth hormone (GH), prolactin, leptin, interferon (IFN-α, IFN-β, IFN-γ, and most interleukins (1, 2). Of these 25 ligands, more than two-thirds activate JAK2. JAK1 and Tyk2, like JAK2, are ubiquitously expressed and, in general, are activated by a similar, although more limited set of ligands compared with JAK2. In contrast, JAK3 is predominantly expressed in hematopoietic cells and is activated by a different set of ligands, including interleukin (IL)-2, IL-4, and IL-7, which are not potent activators of JAK2 (2, 3). Knockout studies have revealed specific and vital roles for JAK kinases. Mice deficient for JAK2 die by day 12 of embryogenesis from a lack of erythropoiesis (4, 5). JAK1-deficient mice are smaller than their littermates, fail to nurse, and die within 1 day of birth (6). In contrast to JAK1- or JAK2-deficient mice, JAK3 knockout mice survive and develop normally in pathogen-free conditions. However, these mice exhibit severe defects in lymphoid development (7–9). Mice lacking Tyk2 develop normally and exhibit no major abnormalities in fertility or blood cell development. However, Tyk2-deficient mice have induced responses to specific cytokines, including IFN-α, IL-12, and IFN-γ (10). Taken together, studies using cellular models as well as analysis of knockout mice show that activation of JAKs is critical for such diverse responses as growth, lactation, nerve cell differentiation, hematopoiesis, and immune responses (3, 10–12).

Upon ligand binding to cytokine receptors, JAKs phosphorylate themselves and their associated receptors, thereby providing multiple binding sites for signaling proteins containing SH2 or other phosphotyrosine-binding domains. Signaling proteins that bind to receptor-JAK complexes and undergo tyrosyl phosphorylation include STATs (13), Shc (14), insulin receptor substrates (15, 16), and focal adhesion kinase (17). Although activation of JAKs by cytokine receptor ligands is generally rapid and transient, constitutive activation of JAKs has been observed in a variety of cancers, indicating that regulation of JAKs is critical for controlling cell growth and proliferation. For example, leukemic cells from patients with acute lymphoblastic leukemia (18) were shown to have constitutively active JAK2, and specific inhibition of JAK2 in cells derived from an acute lymphoblastic leukemia patient blocked cell growth by inducing apoptosis (19). Similarly, JAK1 and JAK3 were found to be constitutively active in cells from patients suffering from adult T cell leukemia/lymphoma caused by human T cell leukemia/lymphomatis virus type I (20). Furthermore, specific inhibition of JAKs inhibits the growth of multiple breast cancer cell lines and induces apoptosis in MDA-MB-468 breast cancer cells (21). The critical role of JAKs in so many normal physiological responses and their potential role in some cancers make it vitally important to obtain a better understanding of the mechanisms by which JAKs are regulated.

Recently, our laboratory identified the SH2 domain-containing protein SH2-Bβ (22) as a potent activator of JAK2 (23). Addition of GH stimulates the phosphorylation of JAK2, leading to the association of SH2-Bβ via its SH2 domain to one or

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1 The abbreviations used are: JAK, Janus tyrosine kinase; GH, growth hormone; IFN, interferon; IL, interleukin; STATs, signal transducers and activators of transcription; PDGF, platelet-derived growth factor; αJAK, anti-JAK antibody; αMyc, anti-Myc antibody; αPY, anti-phosphotyrosine antibody; αAPS, anti-APS antibody; GFP, green fluorescent protein; GHR, growth hormone receptor; DMEM, Dulbecco’s modified Eagle’s medium; MAPKs, mitogen-activated protein kinase proteins; ERK, extracellular signal-regulated kinase; SOC, suppressor of cytokine signaling; EPO, erythropoietin.
more phosphorylated tyrosines in JAK2. This latter interaction substantially activates JAK2, thereby increasing the phosphorylation of JAK2 as well as of downstream targets of JAK2 such as Stat5B (23).

SH2-B is a member of a family of adapter proteins, which also includes APS and Lnk (24–26) (Fig. 1). All three contain a pleckstrin homology domain in their amino termini and an SH2 domain near their C termini. The various isoforms of SH2-B (α, β, γ, and δ) also contain at least three proline-rich regions (22, 27, 28). Although we have shown SH2-Bβ to be a positive regulator of JAK2 (23), studies by others suggest that APS and Lnk may be negative regulators of some signaling pathways. Lnk has been shown to play a pivotal role in the regulation of B cell production, as Lnk knockout mice show overproduction of pre-B cells in the spleen and pro-B cells in bone marrow (29). Overexpression of APS suppresses proliferation of NIH-3T3 cells as stimulated by platelet-derived growth factor (PDGF) (30). To date, the effects of APS and Lnk on the kinase activity and tyrosyl phosphorylation of JAKs have not been examined. Furthermore, SH2-Bβ has not been examined as a regulator of JAKs other than JAK2. Because of the importance of the cyto kinase receptor family of ligands and the remarkable ability of SH2-Bβ to activate JAK2, we examined whether the activating ability of SH2-Bβ is specific to JAK2 or extends to other members of the JAK family of tyrosine kinases. We also examined whether the ability of SH2-Bβ to activate JAK2 is shared by APS. Finally, we examined whether SH2-Bβ or APS binds to JAK1, JAK2, or JAK3 and/or is tyrosyl-phosphorylated by any of these JAKs, thereby implicating SH2-Bβ or APS as a signaling molecule for these JAKs.

**Experimental Procedures**

**Cells and Reagents**—The stocks of COS-7, 293T, and 3T3-F442A cells were provided by Drs. M. D. Uhler (University of Michigan, Ann Arbor, MI), O. A. MacDougald (University of Michigan), and H. Green (Harvard University, Cambridge, MA), respectively. Aprotinin, leupeptin, and Triton X-100 were from Roche Molecular Biochemicals. Recombinant protein A-agarose was from Repligen. The enhanced chemiluminescence detection system (ECL) was from Amersham Biosciences, Inc. Anti-JAK2 antisera (αJAK2) was raised in rabbits against a synthetic peptide corresponding to amino acids 758–766 of murine JAK2 (31, 32) and was used at dilutions of 1:500 for immunoprecipitation and 1:15,000 for immunoblotting. Antibody against the Myc tag (αMyc; 9E10) was from Santa Cruz Biotechnology and was used for immunoblotting at a dilution of 1:10,000. For immunoprecipitation, αMyc was used at a dilution of 1:100 with rabbit anti-mouse IgG (1:100) (Upstate Biotechnology, Inc.). Monoclonal anti-phosphotyrosine antibody (aPY; clone 4G10; Upstate Biotechnology, Inc.) was used at a dilution of 1:7500 for immunoblotting. Antibody against murine JAK1 (αJAK1) was kindly provided by Dr. A. C. Larner ( Learner Research Institute, Cleveland Clinic, Cleveland, OH) (33) and was used for immunoprecipitation at a dilution of 1:300. Polyclonal rabbit αJAK1 (Pharmpingen) was used for immunoblotting at a dilution of 1:5000. αJAK3 was raised in rabbits against a synthetic peptide corresponding to amino acids 1104–1124 of human JAK3 (34) and was used at dilutions of 1:300 for immunoprecipitation and 1:3000 for immunoblotting. Anti-green fluorescent protein (GFP) antibody was from CLONTECH and was used at a dilution of 1:5000 for immunoblotting. Polyclonal rabbit anti-phospho Stat5B antibody was from Zymed Laboratories Inc. and was used at a dilution of 1 μg/ml for immunoblotting. Anti-APS antibody (αAPS) was kindly provided by Dr. D. D. Ginty (Johns Hopkins University School of Medicine, Baltimore, MD) (35) and was used at dilutions of 1:100 for immunoprecipitation and 1:1000 for immunoblotting.

**Plasmids**—The cDNA for wild-type murine JAK2 was provided by Drs. J. N. Ihle and B. A. Witthuhn (St. Jude Children’s Research Hospital, Memphis, TN) (31). The cDNA for human JAK3 was described previously (36, 37). The cDNA for murine JAK1 was provided by Drs. X. Yang and C. L. Cepko (Harvard Medical School, Boston MA) (38). cDNA encoding murine JAK1 with a Myc tag at its C terminus was kindly provided by Dr. R. D. Schreiber (Washington University, St. Louis, MO). Construction of the vector encoding SH2-Bβ with a Myc tag at its N terminus has been described previously (23). cDNA encoding Myc-tagged rat APS was kindly provided by Dr. D. D. Ginty (35). cDNA encoding GFP-tagged Stat5B was constructed as described (39). cDNA encoding the rat G protein-coupled receptor (GHR) was provided by Dr. G. Norstedt (Karolinska Institute, Stockholm, Sweden) (40).

**Cell Culture and Transfection**—COS-7, 293T, and 3T3-F442A cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1 mM L-glutamine, 100 units/ml penicillin, 10 μg/ml streptomycin, 0.25 μg/ml amphotericin (supplemented DMEM), and 10% fetal calf serum (COS-7 cells) or 8% calf serum (293T and 3T3-F442A cells). Cells were transiently transfected using calcium phosphate precipitation (41). Transfected cells were assayed 36–48 h after transfection. Before stimulation transfecting cells with hormone, cells were incubated overnight in serum-free medium containing 1% bovine serum albumin and treated with ligands at 37°C. 3T3-F442A fibroblasts were differentiated to adipocytes by treating confluent cells for 48 h with 2 μg/ml insulin, 0.25 μg/ml dexamethasone, 0.5 μg/ml methylisobutylxanthine, and 10% fetal calf serum in supplemented DMEM for 48 h. Cells were treated for an additional 48 h with supplemented DMEM containing 10% fetal calf serum and 1 μg/ml insulin (42). Adipocytes were maintained in supplemented DMEM plus 10% fetal calf serum. Before assays, cells were incubated overnight in serum-free medium containing 1% bovine serum albumin and treated with ligands at 37°C.

**Immunoprecipitation and Immunoblotting**—Immunoprecipitations and immunoblotting were performed as described (43). Thirty (293T cells) to 48 (COS-7 cells) h after transfection, cells were rinsed three times with 10 mM sodium phosphate (pH 7.4), 150 mM NaCl, and 1 mM Na3VO4. Cells were then solubilized in lysis buffer (50 mM Tris (pH 7.5), 0.1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and centrifuged at 14,000 × g for 10 min at 4°C. The supernatant (cell lysate) was incubated with the indicated antibody on ice for 2 h. The immune complexes were collected on protein A-agarose (14–20%) and washed three times with wash buffer (50 mM Tris (pH 7.5), 0.1% Triton X-100, 150 mM NaCl, and 2 mM EGTA) and boiled for 5 min in a 80:20 mixture of lysis buffer and SDS-PAGE sample buffer (250 mM Tris-Cl (pH 6.8), 10% SDS, 10% β-mercaptoethanol, 40% glycerol, and 0.01% bromphenol blue). The solubilized proteins were separated by SDS-PAGE (7.5% or 5–12% gradient), followed by immunoblotting with the indicated antibody and visualization with the ECL detection system.

**In Vitro Kinase Assay**—In vitro kinase assays were performed as described previously (43). JAKs were immunoprecipitated with the
RESULTS

Differential Ability of SH2-Bβ to Activate JAK1, JAK2, and JAK3—We have shown previously that SH2-Bβ is a potent activator of JAK2 (23). To determine whether the activation of JAK2 by SH2-Bβ is specific to JAK2 or shared by other members of the Janus family of tyrosine kinases, we examined whether SH2-Bβ could also activate JAK1. Like JAK2, JAK1 is widely expressed and is activated by an overlapping set of ligands, including GH, leukemia inhibitory factor, and granulocyte colony-stimulating factor (2). We also examined whether SH2-Bβ could activate JAK3, which is activated by ligands that are not potent activators of JAK2 such as IL-7 (3). Initially, we assessed the kinase activity of JAK1 using an in vitro kinase assay. For comparison, the experiment was performed concurrently with JAK2. cDNA encoding the appropriate JAK was transfected alone or with cDNA encoding Myc-tagged SH2-Bβ in COS-7 cells. The expressed JAK was immunoprecipitated with the appropriate αJAK and incubated with [γ-32P]ATP. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose. 32P-Labeled proteins were visualized by autoradiography, and the amount of immunoprecipitated JAK was determined by immunoblotting with the appropriate αJAK. As reported previously (23), JAK2 was constitutively active when overexpressed in COS-7 cells (Fig. 2A, lane 1, upper panel). Furthermore, its in vitro kinase activity was substantially increased by the coexpression of SH2-Bβ (lane 2, upper panel). JAK3 was also constitutively active when overexpressed in COS-7 cells (lane 3, upper panel). However, in contrast to JAK2, JAK3 was not activated by coexpression with SH2-Bβ (lane 4, upper panel). Similar results were obtained when JAK2 and JAK3 were expressed in 293T cells (data not shown).

We next examined the ability of SH2-Bβ to increase the in vitro kinase activity of JAK1. cDNA encoding JAK1 (or JAK2 for comparison) was transfected into 293T cells alone or with cDNA encoding Myc-tagged SH2-Bβ. 293T cells were used to overexpress JAK1 because we had difficulty overexpressing JAK1 in COS-7 cells. Kinase assays with JAK1 were performed with [γ-32P]ATP as described for JAK3. However, JAK1 incorporated only a small amount of [γ-32P]ATP in vitro compared with JAK2 and JAK3, raising the possibility that our anti-JAK1 antibodies interfere with the in vitro kinase assay. We therefore probed JAK1 (and JAK2) with αPY to examine the effects of overexpressed SH2-Bβ on the tyrosyl phosphorylation of JAK1 (and JAK2). The tyrosyl phosphorylation of JAKs is thought to reflect their autophosphorylation. SH2-Bβ increased the tyrosyl phosphorylation of JAK2 (Fig. 2B, lane 2, upper panel) consistent with increased activity of JAK2. However, SH2-Bβ had no reproducible effect on the tyrosyl phosphorylation of JAK1 (lane 4, upper panel), suggesting that SH2-Bβ has no effect on the activity of JAK1.

JAK1, but Not JAK3, Phosphorylates SH2-Bβ—SH2-Bβ binds to JAK2 (Fig. 3A) as assessed by coprecipitation experiments using overexpressed or endogenous JAK2 and SH2-Bβ (22). Furthermore, we have shown that overexpressed JAK2 tyrosyl-phosphorylates overexpressed SH2-Bβ both in vivo and in vitro and that endogenous SH2-Bβ is phosphorylated on tyrosines in JTK3-F442A cells in response to GH and IFN-γ, two ligands that activate JAK2 (22). This tyrosyl phosphorylation suggests that SH2-Bβ may also function as an adapter protein for ligands that activate JAK2 by recruiting proteins that bind phosphotyrosines to JAK2/SH2-Bβ complexes. To determine whether SH2-Bβ might also serve as a signaling protein for ligands that activate JAK1 and JAK3, even though SH2-Bβ does not appear to activate these JAKs, we examined whether either JAK1 or JAK3 associates with and/or phosphorylates SH2-Bβ. Myc-tagged SH2-Bβ was transiently overexpressed with JAK1 or JAK2 in 293T cells or with JAK3 in COS-7 cells. Myc-SH2-Bβ was immunoprecipitated with αMyc, and precipitated proteins were blotted with the appropriate αJAK. As reported previously (22), SH2-Bβ coprecipitated with JAK1 (Fig. 3A, lane 2, upper panel). It also coprecipitated with JAK1 (Fig. 3B, lane 2, upper panel) and JAK3 (Fig. 3C, lane 2, upper panel), suggesting that SH2-Bβ forms a complex with all three of these JAKs. To determine whether SH2-Bβ is tyrosyl-phosphorylated when overexpressed with JAK1, JAK2, or JAK3, the blots were reprobed with αPY. As shown in Fig. 3A (lane 4), SH2-Bβ was strongly phosphorylated on tyrosines when coexpressed with JAK2. Interestingly, although SH2-Bβ did not seem to stimulate JAK1, SH2-Bβ was phosphorylated on tyrosines in cells overexpressing JAK1 (Fig. 3B, lane 4). No phosphorylation of SH2-Bβ was detected when cells were

In Fig. 2B, murine JAK1 cDNA with a C-terminal Myc tag (provided by Dr. D. R. Schreiber) was used. In Fig. 3B, murine JAK1 cDNA lacking a Myc tag (provided by Drs. X. Yang and C. I. Cepko) was used to avoid directly precipitating JAK1 with αMyc. The bottom band obtained using the latter cDNA (Fig. 3B, lane 2, upper panel; and lane 4) is thought to represent either a degradation product of JAK1 or a truncated form of JAK1 due to a second transcriptional start site encoded in the cDNA.
transfected with SH2-Bβ (data not shown), as reported previously (22), consistent with SH2-Bβ being phosphorylated on tyrosines by JAK1. In contrast, no tyrosyl phosphorylation of SH2-Bβ was detected in cells overexpressing JAK3 (Fig. 3C, lane 4). Thus, the ability of SH2-Bβ to activate appears to be specific to JAK2. However, SH2-Bβ appears to bind to both JAK1 and JAK3 and to serve as a substrate of JAK1. These data suggest that SH2-Bβ may serve as an adapter protein for ligands that activate JAK1 as well as for ligands that activate JAK2 via binding of signaling molecules to phosphorylated tyrosines in SH2-Bβ and possibly to other binding motifs within SH2-Bβ. Although signaling proteins that bind preferentially to phosphorylated tyrosines would not be expected to be recruited to SH2-Bβ complexed to JAK3, SH2-Bβ may still serve as a signaling molecule for ligands that activate JAK3 by recruiting molecules that bind other motifs (e.g. pleckstrin homology and proline-rich regions) within SH2-Bβ to JAK3-SH2-Bβ complexes.

APS Increases the Phosphorylation of Overexpressed JAK2—Because SH2-Bβ activates some but not all JAKs and is tyrosyl-phosphorylated by some but not all JAKs, we hypothesized that other members of the SH2-B family might also activate, bind to, or serve as substrates of specific JAKs. To test this hypothesis, we first examined whether APS increases the kinase activity of JAK2. JAK2 cDNA was cotransfected with cDNA encoding Myc-tagged APS or Myc-tagged SH2-Bβ into 293T cells. JAK2 was immunoprecipitated and incubated with [γ-32P]ATP in an in vitro kinase assay. Like SH2-Bβ, APS increased JAK2 activity (Fig. 4A, upper panel, lane 2 versus lane 3). We also examined whether APS increases the tyrosyl phosphorylation of JAK2 in vivo. Increasing amounts of JAK2 cDNA (0.25–2.0 µg) were transfected with cDNA encoding Myc-SH2-Bβ or Myc-APS in 293T cells. Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotted with an antibody (aPY) to phosphotyrosine (PY). Consistent with the in vitro data shown in Fig. 4A, APS increased the tyrosyl phosphorylation of JAK2 when 2 µg of JAK2 cDNA was used (Fig. 4B, lane 9). When 0.5 or 1 µg of JAK2 was used, APS modestly increased JAK2 phosphorylation (lanes 5 and 7). In contrast to SH2-Bβ, APS had no detectable

![Image](http://www.jbc.org/)

**FIG. 3.** JAK1 and JAK2, but not JAK3, phosphorylate SH2-Bβ. Plasmid (5 µg) encoding Myc alone (lanes 1, 3, and 5) or Myc-SH2-Bβ (lanes 2, 4, and 6) was cotransfected with plasmid encoding JAK2 (1 µg; 293T cells) (A), JAK1 (16 µg; 293T cells) (B), or JAK3 (5 µg; COS-7 cells) (C) as indicated. Proteins from cell lysates were immunoprecipitated (IP) with αMyc. Proteins were resolved by SDS-PAGE and immunoblotted (IB) with the indicated aJAK (lanes 1 and 2, upper panels). Proteins were reprobed with aPY (lanes 3 and 4) and then stripped and reprobed with αMyc (lanes 1 and 2, lower panels). Cell lysates were also immunoblotted directly with aJAK (lanes 5 and 6).

**FIG. 4.** APS stimulates the kinase activity of JAK2 at high but not low levels of JAK2. A, plasmid (5 µg) encoding Myc alone (lane 1), Myc-SH2-Bβ (lane 2), or Myc-APS (lane 3) was cotransfected with plasmid encoding JAK2 (2 µg) into 293T cells. Proteins from cell lysates were immunoprecipitated (IP) with aJAK2. Immunoprecipitated JAK2 was subjected to an in vitro kinase assay with 12.5 Ci of [γ-32P]ATP and resolved by SDS-PAGE. Proteins were visualized by autoradiography (upper panel) and then by immunoblotting (IB) with aJAK2 (middle panel). Cell lysates were immunoblotted directly with αMyc (lower panel). B, plasmid (5 µg) encoding Myc alone (lanes 1, 4, 6, and 8), Myc-SH2-Bβ (lanes 3 and 10), or Myc-APS (lanes 2, 5, 7, and 9) was cotransfected with plasmid encoding JAK2 (as indicated) into 293T cells. Proteins from whole cell lysates were resolved by SDS-PAGE and visualized by immunoblotting with aPY (upper panels) or aJAK2 (lower panels).
effect on JAK2 phosphorylation in cells transfected with 0.25 μg of JAK2 cDNA (lane 2).

APS Decreases GH-stimulated Tyrosyl Phosphorylation of JAK2—Because APS appeared to increase JAK2 phosphorylation in vivo at the higher levels of overexpressed JAK2, but not at the lowest level, we examined whether APS increases the phosphorylation of endogenous GH-stimulated JAK2. cDNA encoding rat GHR was cotransfected with cdNA encoding Myc- or Myc-APS in 293T cells. 24 h after transfection, cells were deprived of serum overnight and stimulated with 50 or 500 ng/ml human GH for 10 min. Protein concentrations were measured in whole cell lysates, and endogenous JAK2 was immunoprecipitated from lysates containing equal amounts of protein. Proteins in the immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-phospho-Stat5B antibody (pSTAT5B; upper panel) and reprobed with anti-GFP antibody (αGFP; lower panel).

Results indicate that SH2-Bβ enhances the tyrosyl phosphorylation (and perhaps serine/threonine phosphorylation) of JAK2, whereas APS, if anything, blocks or inhibits the phosphorylation of JAK2.

To provide evidence that APS decreases signaling by ligands that activate JAK2, we examined the effect of APS on GH-dependent tyrosyl phosphorylation of the JAK2 substrate Stat5B. 293T cells were cotransfected with cdNA encoding GHR and GFP-Stat5B and either Myc- or Myc-APS. Twenty-four h after transfection, cells were deprived of serum overnight and stimulated with 50 or 500 ng/ml human GH for 10 min. Proteins from whole cell lysates were separated by SDS-PAGE and Western-blotted with antibody recognizing phosphorylated tyrosine 964 in Stat5B. This is the tyrosine whose phosphorylation by JAKs is necessary for activation of Stat5 (44). SH2-Bβ increased GH-dependent tyrosyl phosphorylation of Stat5B at both low and high doses of GH (lanes 6 and 9). In contrast, APS substantially decreased GH-stimulated phosphorylation of Stat5B at both low and high doses of GH (lanes 6 and 9).

APS Inhibits JAK1, but Not JAK3—We next investigated whether APS has any effect on the kinase activity or tyrosyl phosphorylation of JAK1 or JAK3. We first examined the effects of APS on JAK1. As described above, JAK1 had little in vitro kinase activity; therefore, we examined the tyrosyl phosphorylation of JAK1 by immunoblotting with αPY. Myc-tagged APS (or Myc-tagged SH2-Bβ for comparison) was expressed with increasing amounts of JAK1 in 293T cells. JAK1 was immunoprecipitated and immunoblotted with αJAK1 followed by αPY. As shown in Fig. 2B, coexpression of SH2-Bβ had no effect on the tyrosyl phosphorylation of JAK1 (Fig. 6A, lanes 2, 5, and 8). In contrast, coexpression of APS caused a decrease in
the tyrosyl phosphorylation of JAK1 without a corresponding decrease in JAK1 protein (lanes 3, 6, and 9). In two of seven experiments, the APS-induced decrease in the tyrosyl phosphorylation of JAK1 was accompanied by a small decrease in the amount of JAK1 protein (data not shown). However, the observed decrease in the tyrosyl phosphorylation of JAK1 was substantially greater than the decrease in the amount of JAK1.

We next examined the effects of APS on the activity JAK3. JAK3 was expressed with Myc-SH2-Bβ or Myc-APS in COS-7 cells. JAK3 was immunoprecipitated and incubated with [γ-32P]ATP as described above. As shown in Fig. 2B, SH2-Bβ had no effect on JAK3 activity when slight differences in the levels of JAK3 are taken into account (Fig. 6B, lane 2). Like SH2-Bβ, APS had no reproducible effect on the activity of JAK3 (lane 3).

**JAK1 and JAK3 Phosphorylate APS**—To determine whether APS binds to and/or is a substrate of any of the JAKs, JAK1, JAK2, or JAK3 was overexpressed with Myc-APS (or Myc-SH2-Bβ for comparison). Myc-SH2-Bβ and Myc-APS were immunoprecipitated using αMyc, and the immunoprecipitated proteins were immunoblotted first with αPY to detect phosphorylated proteins. The blots were also probed with the appropriate αJAK to detect association of JAK1, JAK2, or JAK3 with APS and then stripped and reprobed with αMyc to compare the levels of expression of Myc-APS and Myc-SH2-Bβ. Additionally, cell lysates were immunoblotted with the appropriate αJAK to verify equal expression of JAK in the APS- and SH2-Bβ-overexpressing cells. As shown in Fig. 3, SH2-Bβ was tyrosyl-phosphorylated when expressed with JAK1 (Fig. 7B, lane 5) and JAK2 (Fig. 7A, lane 5), but not with JAK3 (Fig. 7C, lane 5). In contrast to what was observed with SH2-Bβ, all three JAKs stimulated the tyrosyl phosphorylation of APS (Fig. 7A–C, lane 6). SH2-Bβ and APS appeared to bind similar amounts of JAK1 (Fig. 7B, lane 3, upper panel) and JAK2 (Fig. 7A, lane 3, upper panel). However, APS appeared to bind more JAK3 than was bound by SH2-Bβ (Fig. 7C, lane 3, upper panel). Interestingly, the JAK1 that bound to APS appeared to not be phosphorylated on tyrosines (Fig. 7B, lane 6). Taken together, Figs. 5–7 indicate that APS binds to all three JAKs and that all three JAKs are able to phosphorylate APS. Furthermore, APS is a weak activator of overexpressed JAK2 in vitro and an inhibitor of GH-stimulated JAK2 and of overexpressed JAK1 in vivo.

**APS Is Tyrosyl-phosphorylated in Response to GH in Adipocytes**—To verify that endogenous APS is tyrosyl-phosphorylated in response to cytokines and growth factors that activate JAKs, consistent with APS being a substrate of JAKs, 3T3-F442A adipocytes were deprived of serum overnight and then stimulated for 10 min with human GH (500 ng/ml), murine IFN-γ (10 ng/ml), or human insulin (100 nm) as a positive control. APS was immunoprecipitated and Western-blotted with αPY. We observed that APS was tyrosyl-phosphorylated in response to insulin (Fig. 8A, lane 4, upper panel), as reported previously (45, 46). GH also stimulated the tyrosyl phosphorylation of APS (lane 2, upper panel), as predicted from the activity of JAK2 to tyrosyl-phosphorylate APS. IFN-γ weakly stimulated the phosphorylation of APS (lane 3, upper panel), consistent with the significantly decreased ability of IFN-γ to activate JAK2 compared with GH in these cells (47).

To determine whether APS and SH2-Bβ are tyrosyl-phosphorylated within the same time period in cells expressing both proteins, 3T3-F442A adipocytes were deprived of serum overnight and stimulated with 25 ng/ml GH for 1, 5, 15, 30, or 60 min. APS (Fig. 8B, upper panel) or SH2-Bβ (lower panel) was immunoprecipitated and Western-blotted with αPY. The tyrosyl phosphorylation of both APS and SH2-Bβ was detectable within 5 min of GH stimulation (lane 3), maximal at 30 min (lane 5), and decreased by 60 min after GH treatment (lane 6).

**DISCUSSION**

**SH2-Bβ Is a Specific Activator of JAK2**—We have demonstrated previously that SH2-Bβ is a potent activator of JAK2 (23). In the present study, we have shown that SH2-Bβ fails to activate JAK1 or JAK3. This conclusion is based on results from *in vitro* kinase assays and/or detection of tyrosyl-phosphorylated JAK by Western blotting with αPY. The simplest explanation for why SH2-Bβ activates JAK2, but not JAK1 or JAK3, would be if JAK2 contains a binding site for SH2-Bβ, whereas JAK1 and JAK3 do not. However, both JAK1 and
SH2-B and APS Regulate JAK Tyrosine Kinases

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Fig. 8. **APS is tyrosyl-phosphorylated in response to GH.** A, 3T3-F442A adipocytes were treated for 15 min with 500 ng/ml GH (lane 2), 25 ng/ml IFN-γ (lane 3), or 100 nM insulin (lane 4). Proteins from cell lysates were immunoprecipitated (IP) with aAPS. Proteins were resolved by SDS-PAGE and immunoblotted with aPY (upper panels) and then re-probed with aAPS (lower panels). B, 3T3-F442A adipocytes were treated with 25 ng/ml GH for the indicated times. Proteins from cell lysates were immunoprecipitated with aAPS (upper panel) or anti-SH2-Bβ antibody (aSH2-Bβ) (lower panel). Proteins were resolved by SDS-PAGE and immunoblotted with aPY.

JAK3 coprecipitated with SH2-Bβ (Fig. 3), indicating that both JAK1 and JAK3 can bind SH2-Bβ. Previous studies revealed two sites of interaction between JAK2 and SH2-Bβ, only one of which is required for activation of JAK2 (43). The interaction site required for SH2-Bβ activation of JAK2 involves the SH2 domain of SH2-Bβ and phosphorylated tyrosines within JAK2. In contrast, the second site of interaction between SH2-Bβ and JAK2 lies within the first 555 amino acids of SH2-Bβ, a region that does not include an intact SH2 domain. Based on this model, one could hypothesize that JAK2 contains both binding sites for SH2-Bβ, whereas JAK1 and JAK3 contain only the non-activating site. However, SH2-Bβ binds better to wild-type JAK3 than to kinase-inactive JAK3 (data not shown), suggesting that SH2-Bβ binds to (presumably via its SH2 domain) phosphorylated tyrosines within JAK3. This suggests that binding of SH2-Bβ to phosphorylated tyrosines within JAK molecules is not sufficient to activate JAKs.

One can envision several alternative scenarios by which JAK2 is activated by binding to SH2-Bβ, but JAK1 and JAK3 are not. For example, SH2-Bβ binding to a specific phosphorylated tyrosine within JAK2 may cause a conformational change that exposes the ATP- or substrate-binding site. The sites that SH2-Bβ binds to in JAK1 and JAK3 may not cause the same conformational change, perhaps because the sites are located on different regions of the JAK molecules. Another possibility is that SH2-Bβ binding relieves inhibition of the kinase activity of JAK2 by another region in JAK2 such as the kinase-like JH2 domain (48). Again, SH2-Bβ could be binding to tyrosines located outside the JH1 or JH2 domains on JAK1 and JAK3 with the result that SH2-Bβ binding does not relieve the inhibition. SH2-Bβ could also be competing for binding to JAK2 with an inhibitor of JAK2 such as a member of the SOCS family or a tyrosine phosphatase. The SH2-Bβ-binding site within JAK1 and JAK3 may be sufficiently different from the JAK2 counterpart such that SH2-Bβ fails to compete with the pertinent inhibitor. Finally, dimerization has been hypothesized to be required for JAK activation (49). SH2-Bβ may enhance the dimerization of JAK2, but not of JAK1 or JAK3. A role for dimerization in the activation of TrkA kinase activity by SH2-B has been hypothesized by Qian and Ginty (50).

**SH2-Bβ Is Phosphorylated by JAK1 and JAK2, but Not by JAK3.** SH2-Bβ contains multiple potential protein-protein interaction domains and is differentially phosphorylated on tyrosines and/or serines/threonines in response to a variety of ligands, including GH, nerve growth factor, PDGF, brain-derived neurotrophic factor, and epidermal growth factor (22, 24, 35, 51–54). These observations suggest that SH2-Bβ may play a role as an adapter protein in cell signaling in addition to its role as an activator of JAK2. An adapter role for SH2-Bβ is supported by the observation that SH2-Bβ binds JAK1 and is tyrosyl-phosphorylated by JAK1, but fails to increase the tyrosyl phosphorylation of JAK1. SH2-Bβ may act as an adapter protein for cytokines that activate JAK1 by recruiting proteins containing SH2- or phosphotyrosine-binding domains into receptor-JAK-SH2-Bβ complexes. It is interesting to note that SH2-Bβ is not phosphorylated on tyrosines when coexpressed with JAK3. Thus, even though JAK3 can bind SH2-Bβ, JAK3 does not phosphorylate SH2-Bβ on tyrosines, even under conditions in which the kinase activity of JAK3 is very high. This finding is consistent with reports that the substrate specificities of JAK1 and JAK2 are similar to each other, but differ from that of JAK3 (55).

**APS Decreases the Tyrosyl Phosphorylation of JAK1 and JAK2.** The finding that SH2-Bβ is a potent activator of JAK2, but not of JAK1 or JAK3, led us at one time to hypothesize that APS might activate a different subset of JAKs than SH2-Bβ. The data presented here argue against this hypothesis. In fact, they argue for APS having, under the appropriate conditions, the opposite effect of SH2-Bβ, i.e. inhibiting JAK2. In support of this, APS decreased GH-dependent phosphorylation of JAK2 and a substrate of JAK2, Stat5B. The ability of APS to decrease phospho-JAK2 (and presumably JAK2 activity) may decrease the transcriptional activity of Stat5B because the tyrosyl phosphorylation of Stat5B by JAK2 is required for activation of Stat5B. Stat5B initiates transcription of a variety of genes in response to GH, including 

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overexpressed APS is tyrosyl-phosphorylated in response to activator of JAK1 (47). The phosphorylation of APS appears to in response to GH, a potent activator of JAK2 and a weak F442A adipocytes, endogenous APS is tyrosyl-phosphorylated with this hypothesis, we have demonstrated that in 3T3-L1 fibroblasts, sister cells of 3T3-L1 cells. In contrast, we detected 3T3-F442A cells contain a substantial amount of SH2-B. Second, SH2-Bβ may serve as an adapter for JAK1 and JAK2, but not for JAK3, by providing phosphorylated tyrosine(s) that can recruit other signaling proteins to receptor-JAK/SH2-Bβ complexes. Third, in contrast to SH2-Bβ, APS may provide an equally powerful negative feedback mechanism for ligands that activate JAK1 and/or JAK2. This role may overlap with the ability of APS to function as an adapter protein in that ligand stimulation would cause the association of APS and JAKs and the subsequent phosphorylation of APS. Phosphotyrosines on APS may provide binding sites for downstream molecules that decrease the phosphorylation of JAK1 and JAK2. It is tempting to therefore speculate that relative levels of APS and SH2-Bβ in a cell may titrate the cell’s response to ligands that activate JAK1 and JAK2. Both APS and SH2-Bβ are widely expressed. Northern blot analysis, reverse transcription-PCR, and/or Western blot analysis have revealed the presence of SH2-B transcripts and/or protein in heart, brain, lung, liver, skeletal muscle, kidney, testis, pancreas, spleen, adipose tissue, and thymus (22, 24, 27, 52) (data not shown). Using similar approaches, APS transcripts and/or protein have been detected in some of the same tissues (testis, skeletal muscle, spleen, brain, thymus, and kidney) as well as in some other tissues (prostate, uterus, peripheral leukocytes, small intestine, and bone marrow) (25, 67). Interestingly, Moodie et al. (46) detected significantly higher levels of APS transcript and protein in 3T3-L1 adipocytes than in fibroblasts. We confirmed a higher expression of APS in adipocytes compared with fibroblasts in 3T3-F442A cells, sister cells of 3T3-L1 cells. In contrast, we detected SH2-Bβ in both 3T3-F442A fibroblasts and adipocytes (Fig. 5B) (data not shown). Further studies will be needed to delineate relative levels of APS and SH2-B in different tissues and cell types within tissues, to determine whether levels of either are subject to physiological regulation, and to determine whether relative levels of APS and SH2-Bβ in specific cells titrate a cell’s response to ligands that activate JAK2. It will also be interesting to determine whether SH2-B and APS serve similar or different adapter roles for the different JAK proteins and whether specific phosphorylated tyrosines are important for this adapter role.

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