0888-8809/07/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 21(9):2270–2281
Copyright © 2007 by The Endocrine Society
doi: 10.1210/me.2007-0111

SH2B1 Enhances Leptin Signaling by Both Janus Kinase 2 Tyr^{813} Phosphorylation-Dependent and -Independent Mechanisms

Zhiqin Li, Yingjiang Zhou, Christin Carter-Su, Martin G. Myers, Jr., and Liangyou Rui

Department of Molecular and Integrative Physiology (Z.L., Y.Z., C.C.-S., L.R.), Division of Metabolism, Endocrinology and Diabetes, and Department of Internal Medicine (M.G.M.), University of Michigan Medical School, Ann Arbor, Michigan 48109-0622

Leptin controls body weight by activating its long form receptor (LEPRb). LEPRb binds to Janus kinase 2 (JAK2), a cytoplasmic tyrosine kinase that mediates leptin signaling. We previously reported that genetic deletion of SH2B1 (previously known as SH2-B), a JAK2-binding protein, results in severe leptin-resistant and obese phenotypes, indicating that SH2B1 is a key endogenous positive regulator of leptin sensitivity. Here we show that SH2B1 regulates leptin signaling by multiple mechanisms. In the absence of leptin, SH2B1 constitutively bound, via its non-SH2 domain region(s), to non-tyrosyl-phosphorylated JAK2, and inhibited JAK2. Leptin stimulated JAK2 phosphorylation on Tyr^{813}, which subsequently bound to the SH2 domain of SH2B1. Binding of the SH2 domain of SH2B1 to phospho-Tyr^{813} in JAK2 enhanced leptin induction of JAK2 activity. JAK2 was required for leptin-stimulated phosphorylation of insulin receptor substrate 1 (IRS1), an upstream activator of the phosphatidylinositol 3-kinase pathway. Overexpression of SH2B1 enhanced both JAK2- and JAK2(Y813F)-mediated tyrosine phosphorylation of IRS1 in response to leptin, even though SH2B1 did not enhance JAK2(Y813F) activation. Leptin promoted the interaction of SH2B1 with IRS1. These data suggest that constitutive SH2B1-JAK2 interaction, mediated by the non-SH2 domain region(s) of SH2B1 and the non-Tyr^{813} region(s) in JAK2, increases the local concentration of SH2B1 close to JAK2 and inhibits JAK2 activity. Leptin-stimulated SH2B1-JAK2 interaction, mediated by the SH2 domain of SH2B1 and phospho-Tyr^{813} in JAK2, promotes JAK2 activation, thus globally enhancing leptin signaling. SH2B1-IRS1 interaction facilitates IRS1 phosphorylation by recruiting IRS1 to JAK2 and/or by protecting IRS1 from dephosphorylation, thus specifically enhancing leptin stimulation of the phosphatidylinositol 3-kinase pathway. (*Molecular Endocrinology* 21: 2270–2281, 2007)

THE HYPOTHALAMUS CONTROLS energy balance and body weight by integrating multiple signals, including neuronal, hormonal, and nutritional signals. Leptin, which is produced and secreted by adipocytes proportionally to fat mass, is a key adiposity signal for the hypothalamus. Leptin binds to and activates its long form receptor (LEPRb) in hypothalamic neurons, resulting in weight loss by both inhibiting feeding and promoting energy expenditure. Consequently, genetic deficiency of either leptin or LEPRb results in voracious overfeeding and morbid obesity (1–3).

First Published Online June 12, 2007

Abbreviations: DD, Dimerization domain; HEK, human embryonic kidney; IRS1, insulin receptor substrate 1; JAK2, Janus kinase 2; LEPRb: long-isoform of leptin receptor; PH, pleckstrin homology domain; PI 3, phosphatidylinositol 3; αPY, antiphosphotyrosine antibody; SH2: Src homology 2; STAT, signal transducer and activator of transcription.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

LEPRb is associated with Janus kinase 2 (JAK2), a cytoplasmic tyrosine kinase that binds to multiple members of the cytokine receptor family and mediates cell signaling. Leptin stimulates JAK2, which phosphorylates LEPRb on multiple tyrosines, including Tyr^{985} and Tyr^{1138} (4–6). Phosphorylated Tyr^{985} binds to SHP2 and suppressor of cytokine signaling 3 (4, 6). SHP2 promotes the activation of the ERK1/2 pathway, whereas suppressor of cytokine signaling 3 inhibits leptin signaling (4, 6–8). Phosphorylated Tyr^{1138} binds to the Src homology 2 (SH2) domain of signal transducer and activator of transcription (STAT)3, allowing JAK2 to phosphorylate and activate this transcription factor (6). Genetic disruption of the STAT3 pathway results in severe leptin resistance, hyperphagia, and morbid obesity in mice, indicating that this pathway is required for maintaining normal energy balance and body weight (9–11). Leptin also promotes tyrosine phosphorylation of insulin receptor substrate 1 (IRS1) and IRS2, which activates the phosphatidylinositol (PI) 3-kinase pathway (12–15). Pharmacological inhibition of hypothalamic phosphatidylinositol 3 (PI 3)-kinase abrogates the ability of leptin to inhibit feeding, and genetic deletion of the *IRS2* gene in the brain results in

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

leptin resistance, hyperphagia, and obesity in mice (16–19). These observations indicate that the IRS proteins/PI 3-kinase pathway is also required for leptin regulation of appetite and body weight.

LEPRb promotes JAK2 autophosphorylation on multiple tyrosines, including Tyr^{221}, Tyr^{570}, and Tyr^{1007} (20, 21). Tyr^{570} phosphorylation appears to inhibit JAK2 activity, whereas the role of Tyr^{221} phosphorylation remains unclear (20, 22). Tyr^{1007} phosphorylation is required for cytokines to activate JAK2 (21). Interestingly, GH, the receptor of which also binds to JAK2, stimulates Tyr^{813} phosphorylation in JAK2 (23). Phosphorylated Tyr^{813} binds to the SH2 domain of SH2-B (23–25). Whether leptin similarly stimulates Tyr^{813} phosphorylation and/or Tyr^{813}-mediated interaction of JAK2 with SH2-B is not known.

SH2-B is a member of the SH2B family (SH2-B, APS, and Lnk), which contains a conserved N-terminal dimerization (DD), central pleckstrin homology (PH), and C-terminal SH2 domain. SH2-B, APS, and Lnk were renamed recently by HUGO Gene Nomenclature Committee as SH2B1, SH2B2, and SH2B3, respectively. The *SH2B1 (SH2-B)* gene encodes four isoforms (α, β, γ, and δ) by alternative mRNA splicing; these four forms have identical DD, PH, and SH2 domains, but differ at their C termini (26, 27). SH2B1β was originally identified as a JAK2-binding protein in a yeast two-hybrid screen (28). In cultured cells, SH2B1β not only is tyrosyl phosphorylated by JAK2, but also potentiates JAK2 activation, in response to GH (25, 28, 29). SH2B2 (APS) appears to be involved in insulin signaling (30–35). The *SH2B2* gene encodes two isoforms (α and β) via alternative mRNA splicing (36). SH2B2β, which lacks SH2 domain, binds to both SH2B1β and SH2B2α, thereby attenuating the ability of SH2B1β or SH2B2α to enhance JAK2 activation and insulin signaling (36). These observations suggest that SH2B2β functions as an endogenous inhibitor of SH2B1 and SH2B2α.

The *SH2B1* gene has been disrupted independently by two groups. Yoshimura's group (37) reported that deletion of the *SH2B1* gene impairs reproduction in mice. We demonstrated that genetic deletion of the *SH2B1* gene results in severe leptin resistance, hyperphagia, and morbid obesity (30, 38). SH2B1-deficient mice also develop hyperlipidemia, insulin resistance, and type 2 diabetes (38, 39). Impaired reproduction and type 2 diabetes are associated with leptin resistance and obesity. Neuron-specific restoration of SH2B1 in SH2B1-deficient mice rescues leptin-resistant and obese phenotypes (40). In addition, overexpression of SH2B1 in the brain protects against high-fat diet-induced leptin resistance and obesity in SH2B1 transgenic mice (40). Together, these observations suggest that SH2B1 is a key endogenous enhancer of leptin sensitivity in animals. However, the molecular mechanisms by which SH2B1 enhances leptin signaling remain largely unknown.

In this study, we have identified Tyr^{813} in JAK2 as a leptin-dependent site of JAK2 phosphorylation. Leptin promoted the binding of the SH2 domain of SH2B1 to phosphorylated Tyr^{813}, thereby enhancing JAK2 activation. SH2B1 also bound to IRS1 and promoted leptin-stimulated, JAK2-mediated tyrosine phosphorylation of IRS1.

RESULTS

Leptin Stimulates the Phosphorylation of Tyr^{813} in JAK2

To determine whether leptin stimulates JAK2 phosphorylation on Tyr^{813}, LEPRb, JAK2, and JAK2(Y813F) were stably introduced into γ2A cells. γ2A cells are human fibroblasts genetically deficient of JAK2 (41, 42). JAK2(Y813F) was generated by replacing Tyr^{813} in JAK2 with Phe. γ2A cells were infected with recombinant retroviruses expressing mouse LEPRb to generate $\gamma2A^{LEPRb}$ cells that stably express LEPRb. LEPRb expression was confirmed by immunoblotting with anti-LEPRb antibodies (data not shown). Similarly, $\gamma2A^{LEPRb}$ cells were infected with recombinant retroviruses expressing JAK2 or JAK2(Y813F) to generate $\gamma2A^{LEPRb/JAK2}$ or $\gamma2A^{LEPRb/Y813F}$ cells, respectively.

To estimate the levels of recombinant JAK2 and JAK2(Y813F), cell extract was immunoblotted with anti-JAK2 antibody (αJAK2) and anti-β-actin (α-Actin). Parental γ2A cells did not express endogenous JAK2 as expected, whereas JAK2 in $\gamma2A^{LEPRb/JAK2}$ cells and JAK2(Y813F) in $\gamma2A^{LEPRb/Y813F}$ cells were expressed at a similar level (Fig. 1A).

To determine whether $\gamma2A^{LEPRb/JAK2}$ and $\gamma2A^{LEPRb/Y813F}$ cells respond to leptin, cells were deprived of serum overnight and treated with 100 ng/ml leptin for 10 min. JAK2 in cell extract was immunoprecipitated with αJAK2 and immunoblotted with antiphosphotyrosine antibodies (αPYs). Basal tyrosine phosphorylation of JAK2 and JAK2(Y813F) was undetectable, whereas leptin robustly stimulated tyrosine phosphorylation of JAK2 in $\gamma2A^{LEPRb/JAK2}$ cells (Fig. 1B). Leptin also stimulated tyrosine phosphorylation of JAK2(Y813F) in $\gamma2A^{LEPRb/Y813F}$ cells, but to a lesser extent (Fig. 1B).

To provide direct evidence that leptin stimulates Tyr^{813} phosphorylation, $\gamma2A^{LEPRb/JAK2}$ cells were treated with 100 ng/ml leptin for 10 min, and JAK2 was immunoprecipitated with polyclonal αJAK2 and immunoblotted with antiphospho-JAK2 [$pTyr^{813}$ antibodies (αpY813)]. αpY813 has been previously characterized to recognize specifically phosphorylated Tyr^{813} in JAK2 (23). Leptin stimulated Tyr^{813} phosphorylation in $\gamma2A^{LEPRb/JAK2}$ cells (Fig. 1C). In contrast, αpY813 was unable to detect JAK2(Y813F) in $\gamma2A^{LEPRb/Y813F}$ cells treated with or without leptin (Fig. 1C), even though JAK2(Y813F) was phosphorylated on other tyrosines (Fig. 1B). Phosphorylation of Tyr^{813} was detectable by 5 min and sustained for at least 60 min after leptin stimulation (Fig. 1D). Leptin-stimulated overall tyrosine phosphorylation of both JAK2 and JAK2(Y813F) was

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

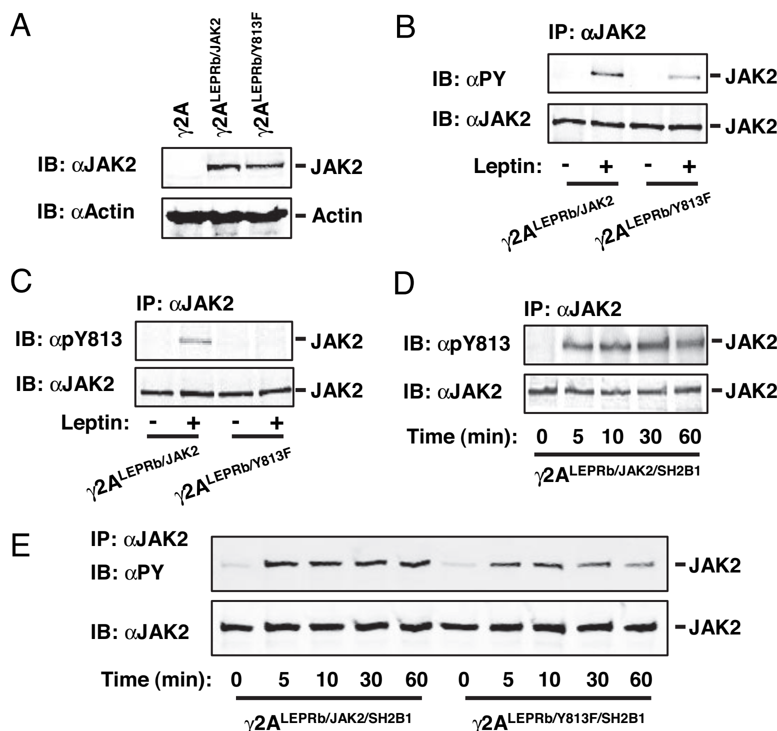

Fig. 1. Leptin Stimulates Phosphorylation of JAK2 on Tyr^{813}

A, Cell extract was prepared from γ2A, $\gamma 2A^{LEPRb/JAK2}$, and $\gamma 2A^{LEPRb/Y813F}$ cells and immunoblotted with αJAK2 or α-actin. B and C, $\gamma 2A^{LEPRb/JAK2}$ and $\gamma 2A^{LEPRb/Y813F}$ cells were treated with 100 ng/ml leptin or vehicle for 10 min. JAK2 in cell extract was immunoprecipitated with αJAK2 and immunoblotted with αPY (B) or αpY813 (C). The same blots were reprobed with αJAK2. D, $\gamma 2A^{LEPRb/JAK2/SH2B1}$ cells were treated with 50 ng/ml leptin for 0, 5, 10, 30, or 60 min. JAK2 in cell extract was immunoprecipitated with αJAK2 and immunoblotted with αpY813. The same blot was reprobed with αJAK2. E, $\gamma 2A^{LEPRb/JAK2/SH2B1}$ and $\gamma 2A^{LEPRb/Y813F/SH2B1}$ cells were treated with 100 ng/ml leptin for 0, 5, 10, 30, or 60 min. JAK2 in cell extract was immunoprecipitated with αJAK2 and immunoblotted with αPY. The same blot was reprobed with αJAK2. IB, Immunoblotting; IP, immunoprecipitation.

prolonged to a similar degree (Fig. 1E), suggesting that the binding of SH2B1 to phosphorylated Tyr^{813} does not protect JAK2 from dephosphorylation.

Phosphorylated Tyr^{813} in JAK2 Mediates Leptin-Stimulated Association of JAK2 with SH2B1

To determine whether phosphorylated Tyr^{813} mediates the interaction of JAK2 with SH2B1, $\gamma 2A^{LEPRb/JAK2}$ and $\gamma 2A^{LEPRb/Y813F}$ cells were infected with recombinant retroviruses expressing rat SH2B1β to generate $\gamma 2A^{LEPRb/JAK2/SH2B1}$ and $\gamma 2A^{LEPRb/Y813F/SH2B1}$ cells, respectively. To compare SH2B1 levels in the different cell lines, cell extract was immunoblotted with αSH2B1. Recombinant SH2B1 protein levels were similar between $\gamma 2A^{LEPRb/JAK2/SH2B1}$ and $\gamma 2A^{LEPRb/Y813F/SH2B1}$ cells, but much higher than the levels of endogenous SH2B1 in parental $\gamma 2A^{LEPRb/JAK2}$ and $\gamma 2A^{LEPRb/Y813F}$ cells (Fig. 2A).

To examine the interaction of SH2B1 with JAK2 or JAK2(Y813F), $\gamma 2A^{LEPRb/JAK2/SH2B1}$ and $\gamma 2A^{LEPRb/Y813F/SH2B1}$ cells were stimulated with 100 ng/ml leptin for 10 min, and SH2B1 in cell extract was immunoprecipitated with αSH2B1 and immunoblotted with polyclonal αJAK2. Leptin promoted coimmunoprecipitation of SH2B1 with JAK2 but not JAK2(Y813F) (Fig. 2B). Moreover, tyrosyl-phosphorylated JAK2, but not tyrosyl-phosphorylated JAK2(Y813F), was easily detected in αSH2B1 immunoprecipitates from leptin-treated cells (Fig. 2B). Interestingly, SH2B1 was undetectable in polyclonal αJAK2 immunoprecipitates (data not shown), suggesting that this polyclonal αJAK2 disrupts the interaction of JAK2 with SH2B1, and/or binds to a site in JAK2 that overlaps with SH2B1-binding site(s). To confirm that JAK2 coimmunoprecipitates with SH2B1, $\gamma 2A^{LEPRb/JAK2/SH2B1}$ cells were treated with or without leptin, and JAK2 was immunoprecipitated with monoclonal αJAK2. Leptin stimulated the coimmunoprecipitation of JAK2 with SH2B1 (Fig. 2C).

To determine whether the SH2 domain of SH2B1 mediates leptin-induced interaction of SH2B1 with JAK2, SH2 domain-defective SH2B1(R555E), which has a replacement of the conserved Arg^{555} within its SH2 domain with Glu, was stably introduced into $\gamma 2A^{LEPRb/JAK2}$ cells via retroviral-mediated gene transfer to generate $\gamma 2A^{LEPRb/JAK2/R555E}$ cells. $\gamma 2A^{LEPRb/JAK2/SH2B1}$ and $\gamma 2A^{LEPRb/JAK2/R555E}$ cells

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

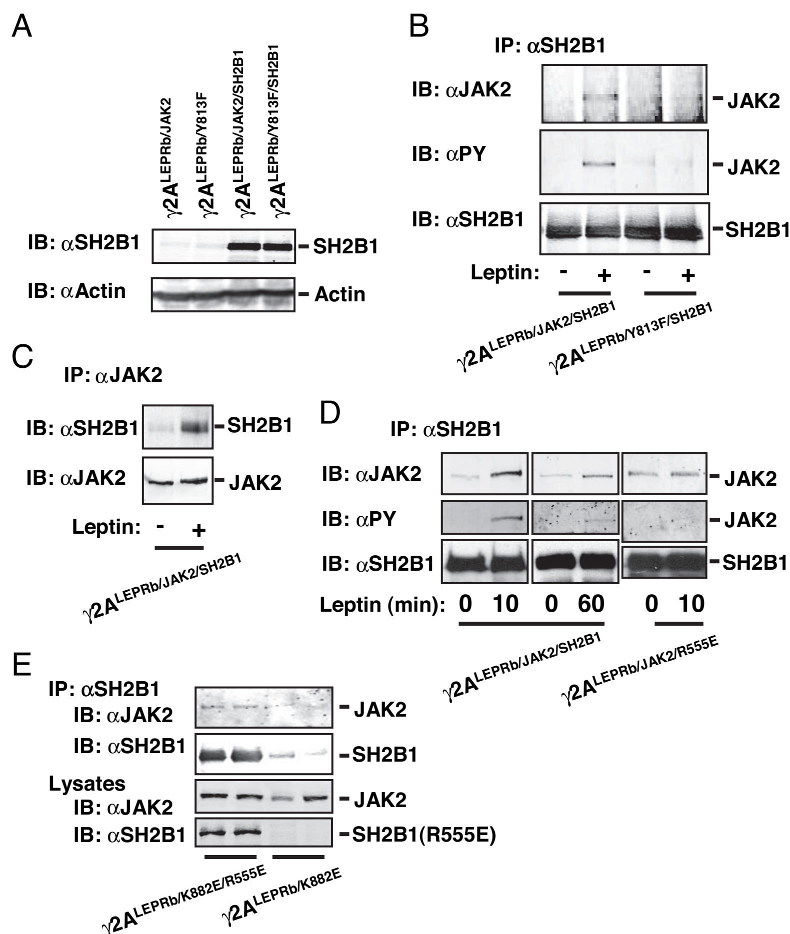

Fig. 2. Phosphorylated Tyr^{813} in JAK2 Mediates Leptin-Stimulated Interaction of SH2B1 with JAK2

A, Cell extract was prepared from γ2A$^{LEPRb/JAK2}$, γ2A$^{LEPRb/Y813F}$, γ2A$^{LEPRb/JAK2/SH2B1}$, and γ2A$^{LEPRb/Y813F/SH2B1}$ cells and subjected to immunoblotting with αSH2B1 or α-actin. B, γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were treated with 100 ng/ml leptin or vehicle for 10 min. SH2B1 in cell extract was immunoprecipitated with αSH2B1 and immunoblotted with αJAK2. The same blots were reprobed with αPY or αSH2B1. C, γ2A$^{LEPRb/JAK2/SH2B1}$ cells were treated with 100 ng/ml leptin for 10 min. JAK2 was immunoprecipitated with the monoclonal αJAK2 and immunoblotted with αSH2B1 or αJAK2. D, γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/JAK2/R555E}$ cells were treated with 100 ng/ml leptin for 0, 10, or 60 min. SH2B1 was immunoprecipitated with αSH2B1 and immuoblotted with monoclonal αJAK2, αPY, or αSH2B1 as indicated. These experiments were performed three times with similar results. E, γ2A$^{LEPRb/K882E/R555E}$ and γ2A$^{LEPRb/K882E}$ cell extracts were immunoprecipitated with αSH2B1 and immuoblotted with monoclonal αJAK2 or αSH2B1. Cell extracts were also immunoblotted with αJAK2 or αSH2B1. IB, Immunoblotting; IP, immunoprecipitation.

were treated with or without leptin, and SH2B1 and SH2B1(R555E) were immunoprecipitated with αSH2B1 and immunoblotted with monoclonal αJAK2. In the absence of leptin stimulation, SH2B1 was constitutively bound to a small amount of JAK2 that was not tyrosyl phosphorylated in γ2A$^{LEPRb/JAK2/SH2B1}$ cells (Fig. 2D). Leptin markedly increased the binding of SH2B1 to JAK2 that was tyrosyl phosphorylated as expected; leptin-stimulated association of SH2B1 with JAK2 was still detectable after 60 min stimulation (Fig. 2D). SH2B1β(R555E) was also constitutively bound to JAK2 in γ2A$^{LEPRb/JAK2/R555E}$ cells; however, leptin was unable to further increase the binding of SH2B1β(R555E) to JAK2 (Fig. 2D). To exclude the possibility that αSH2B1 binds to JAK2 nonspecifically, γ2A$^{LEPRb/K882E}$ and γ2A$^{LEPRb/K882E/R555E}$ cell lines were established via retroviral-mediated gene transfer to stably express kinase-inactive JAK2(K882E) (lacking the ATP-binding site) in γ2A$^{LEPRb/K882E}$ cells and both JAK2(K882E) and SH2B1β(R555E) in γ2A$^{LEPRb/K882E/R555E}$ cells. SH2B1(R555E) was detected in γ2A$^{LEPRb/K882E/R555E}$ but not in γ2A$^{LEPRb/K882E}$ cells as expected; in contrast, JAK2(K882E) was expressed at a similar level between γ2A$^{LEPRb/K882E/R555E}$ and γ2A$^{LEPRb/K882E}$ cells (Fig. 2E, *bottom two panels*). Cell extracts were immunoprecipitated with αSH2B1 and immunoblotted with αJAK2. JAK2(K882E) was detected in αSH2B1 precipitates from γ2A$^{LEPRb/K882E/R555E}$ but not γ2A$^{LEPRb/K882E}$ cells (Fig. 2E, *top two panels*), indicating that αSH2B1 did not bind to JAK2 nonspecifically, and that SH2 domain-defective

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

SH2B1(R555E) constitutively bound to kinase-inactive JAK2(K882E). Endogenous SH2B1-bound JAK2(K882E) was undetectable in $\gamma 2A^{LEPRb/K882E}$ cells, presumably due to very low expression of endogenous SH2B1 (Fig. 2E). Together, these results suggest that before leptin stimulation, SH2B1 constitutively binds to JAK2 independent of the SH2 domain of SH2B1 and phosphorylated-Tyr^{813} in JAK2, thereby increasing the local concentration of SH2B1 close to JAK2. Upon leptin stimulation, Tyr^{813} is phosphorylated and rapidly binds to the SH2 domain of SH2B1 that is preassociated with JAK2.

Interestingly, leptin did not stimulate the autophosphorylation of SH2B1β(R555E)-bound JAK2 in $\gamma 2A^{LEPRb/JAK2/R555E}$ cells (Fig. 2D), suggesting that SH2B1β(R555E) binds to JAK2 and inhibits leptin stimulation of JAK2. Leptin also did not stimulate the autophosphorylation of SH2B1-bound JAK2(Y813F) (Fig. 2B and data not shown), suggesting that SH2B1 binds to JAK2(Y813F) and inhibits leptin stimulation of JAK2(Y813F). Because both SH2B1β(R555E)-JAK2 interaction and SH2B1-JAK2(Y813F) interaction are mediated by a non-SH2 domain region of SH2B1 and a non-Tyr^{813} region of JAK2, this non-SH2 domain region of SH2B1- and the non-Tyr^{813} region in JAK2-mediated constitutive binding of SH2B1 to JAK2 may inhibit basal JAK2 activity.

Phosphorylated Tyr^{813} in JAK2 Mediates SH2B1-Promoted JAK2 Activation

To determine whether SH2B1 promotes JAK2 activation by directly binding to phosphorylated Tyr^{813}, SH2B1β was transiently cooverexpressed with JAK2 or JAK2(Y813F) in human embryonic kidney (HEK)293 cells. Cell extract was immunoblotted with αphospho-JAK2 (αpY1007/1008). αpY1007/1008 detects $Tyr^{1007/1008}$-phosphorylated and active JAK2. SH2B1 robustly stimulated $Tyr^{1007/1008}$ phosphorylation in a dose-dependent manner (Fig. 3A). Similarly, SH2B1 markedly increased the total levels of JAK2 autophosphorylation as measured by immunoblotting JAK2 with αPY (Fig. 3B). In contrast, SH2B1β was unable to increase either $Tyr^{1007/1008}$ phosphorylation or total autophosphorylation of JAK2(Y813F) (Fig. 3, A and B). These results suggest that Tyr^{813} phosphorylation is required for SH2B1 to enhance the activity of overexpressed and constitutively active JAK2.

To determine whether SH2B1 enhances leptin stimulation of JAK2, $\gamma 2A^{LEPRb/JAK2}$ and $\gamma 2A^{LEPRb/JAK2/SH2B1}$ cells were treated with 100 ng/ml leptin for 10 min, and cell extract was immunoblotted with αpY1007/1008. Leptin stimulated $Tyr^{1007/1008}$ phosphorylation to a higher extent in $\gamma 2A^{LEPRb/JAK2/SH2B1}$ cells than in $\gamma 2A^{LEPRb/JAK2}$ cells (Fig. 4A). Phosphorylated JAK2 was quantified and normalized to total JAK2 protein levels. SH2B1 significantly increased leptin-stimulated $Tyr^{1007/1008}$ phosphorylation by 75 ± 21% (mean ± SEM, $n = 5$, $P < 0.05$).

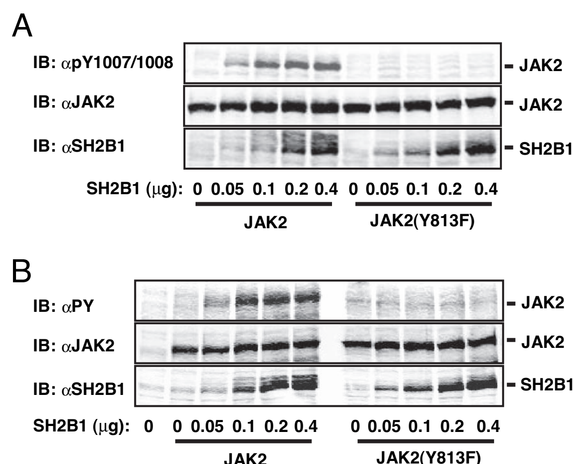

Fig. 3. Tyr^{813} Is Required for SH2B1-Stimulated JAK2 Activation

JAK2 plasmids (0.2 μg) were transiently cotransfected with the indicated amounts of SH2B1 plasmids in HEK293 cells. Cell extract was prepared 22 h after transfection and immunoblotted with the indicated antibodies. IB, Immunoblotting.

To estimate the effect of SH2B1 on the total level of JAK2 autophosphorylation, JAK2 was immunoprecipitated with polyclonal αJAK2 and immunoblotted with αPY. Leptin-stimulated JAK2 autophosphorylation was higher in $\gamma 2A^{LEPRb/JAK2/SH2B1}$ cells than in $\gamma 2A^{LEPRb/JAK2}$ cells (Fig. 4B). Phosphorylated JAK2 was quantified and normalized to total JAK2 protein levels. SH2B1 significantly increased leptin-stimulated JAK2 autophosphorylation by 58 ± 11% (mean ± SEM, $n = 4$, $P < 0.05$). SH2B1 also enhanced leptin-stimulated phosphorylation of Tyr^{813} in JAK2 (Fig. 4C).

To determine whether Tyr^{813} phosphorylation is required for SH2B1 to enhance leptin-stimulated JAK2 activation, $\gamma 2A^{LEPRb/Y813F}$ and $\gamma 2A^{LEPRb/Y813F/SH2B1}$ cells were treated with 100 ng/ml leptin for 10 min. JAK2(Y813F) activation was examined by immunoblotting cell extract with αpY1007/1008, and the total level of JAK2(Y813F) autophosphorylation was examined by immunoblotting αJAK2 precipitates with αPY. Overexpression of SH2B1 did not increase either $Tyr^{1007/1008}$ phosphorylation ($\gamma 2A^{LEPRb/Y813F}$: 1.10 ± 0.07; $\gamma 2A^{LEPRb/Y813F/SH2B1}$: 1.14 ± 0.05. Arbitrary units, $n = 8$, $P = 0.18$) or total autophosphorylation of JAK2(Y813F) ($\gamma 2A^{LEPRb/Y813F}$: 0.45 ± 0.09; $\gamma 2A^{LEPRb/Y813F/SH2B1}$: 0.42 ± 0.08. Arbitrary units, $n = 4$, $P = 0.67$) (Fig. 4, A and B). In addition, SH2B1 was transiently overexpressed in $\gamma 2A^{LEPRb/JAK2}$ or $\gamma 2A^{LEPRb/Y813F}$ cells via adenoviral-mediated gene transfer. Transient overexpression of SH2B1 enhanced both basal and leptin-stimulated $Tyr^{1007/1008}$ phosphorylation in JAK2, but not in JAK2(Y813F) (Fig. 4D). Phosphorylated JAK2 was quantified and normalized to total JAK2 protein levels. SH2B1 significantly increased leptin-stimulated $Tyr^{1007/1008}$ phosphorylation in JAK2 by 214 ± 65% (mean ± SEM, $n = 4$, $P < 0.05$) in $\gamma 2A^{LEPRb/JAK2}$ cells but not in JAK2(Y813F) in $\gamma 2A^{LEPRb/Y813F}$ cells. Together,

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

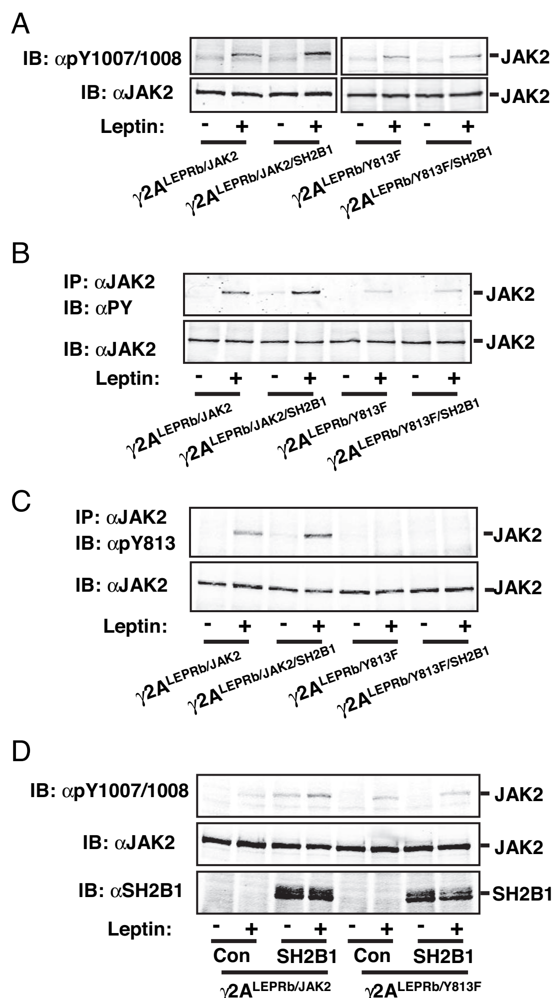

Fig. 4. Leptin-Stimulated Phosphorylation of Tyr^{813} in JAK2 Mediates SH2B1-Promoted JAK2 Activation

A–C, γ2A$^{LEPRb/JAK2}$, γ2A$^{LEPRb/Y813F}$, γ2A$^{LEPRb/JAK2/SH2B1}$, and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were stimulated with leptin (100 ng/ml in both A and B and 50 ng/ml in C) for 10 min, and cell extract was prepared. Cell extract was immunoblotted with αpY1007/1008 or αJAK2 (A). JAK2 in cell extract was immunoprecipitated with αJAK2 and immunoblotted with αPY (B) or αpY813 (C). The same blots were reprobed with αJAK2 (B and C). D, γ2A$^{LEPRb/JAK2}$ or γ2A$^{LEPRb/Y813F}$ cells were infected with SH2B1 or β-galactosidase control adenoviruses. Infected cells were treated with 100 ng/ml leptin for 10 min, and cell extract was immunoblotted with αpY1007/1008, αJAK2, or αSH2B1. IB, Immunoblotting; IP, immunoprecipitation.

these observations suggest that leptin stimulates the phosphorylation of Tyr^{813}. Phospho-Tyr^{813} directly binds to the SH2 domain of SH2B1, thereby enhancing leptin-stimulated JAK2 activation and autophosphorylation.

Phosphorylation of Tyr^{813} in JAK2 Is Not Required for Leptin-Stimulated STAT3 Activation

To determine whether SH2B1 and Tyr^{813} phosphorylation increases leptin-stimulated phosphorylation and activation of STAT3, γ2A$^{LEPRb/JAK2}$, γ2A$^{LEPRb/JAK2/SH2B1}$, γ2A$^{LEPRb/Y813F}$, and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were treated with 100 ng/ml leptin for 10 min. Cell extract was immunoblotted with αphospho-STAT3 (αpSTAT3) or αSTAT3. αpSTAT3 specifically recognizes active STAT3 that is phosphorylated on Tyr^{705}. Surprisingly, leptin stimulated STAT3 phosphorylation to a similar extent in γ2A$^{LEPRb/JAK2}$, γ2A$^{LEPRb/JAK2/SH2B1}$, γ2A$^{LEPRb/Y813F}$, and γ2A$^{LEPRb/Y813F/SH2B1}$ cells (Fig. 5A). To determine whether Tyr^{813} phosphorylation affects the ability of leptin to stimulate STAT3 activation, γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were treated with leptin at various concentrations, and STAT3 activation was estimated by immunoblotting with αpSTAT3. The degree of leptin-stimulated STAT3 phosphorylation was similar between

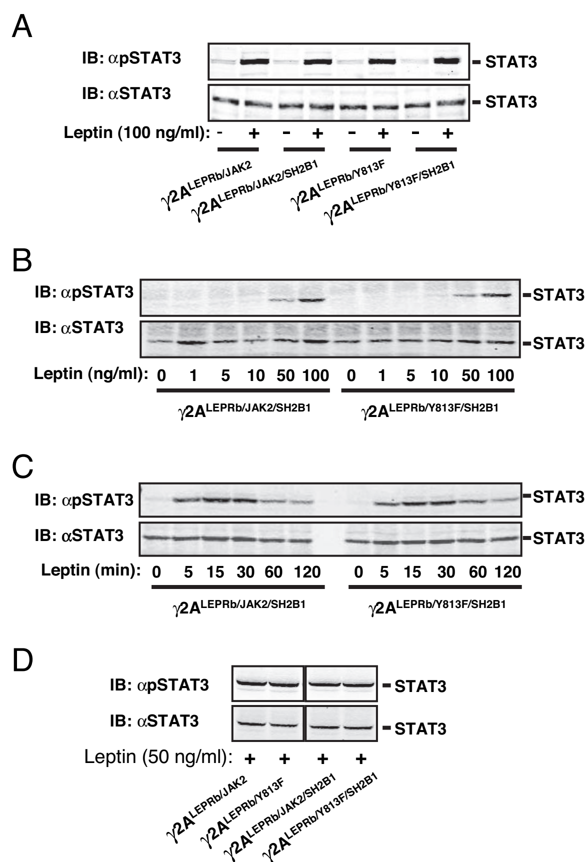

Fig. 5. Phosphorylation of Tyr^{813} in JAK2 Is Not Required for Leptin-Stimulated Activation of STAT3

A, γ2A$^{LEPRb/JAK2}$, γ2A$^{LEPRb/Y813F}$, γ2A$^{LEPRb/JAK2/SH2B1}$, and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were treated with 100 ng/ml leptin for 10 min, and cell extract was immunoblotted with αpSTAT3 or αSTAT3. B, γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were stimulated for 10 min with leptin at various concentrations, and cell extract was immunoblotted with αpSTAT3 or αSTAT3. C, γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were stimulated with 100 ng/ml leptin for various times, and cell extract was immunoblotted with αpSTAT3 or αSTAT3. D, γ2A$^{LEPRb/JAK2}$, γ2A$^{LEPRb/Y813F}$, γ2A$^{LEPRb/JAK2/SH2B1}$, and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were stimulated with 50 ng/ml leptin for 10 min, and cell extract was immunoblotted with αpSTAT3 or αSTAT3. IB, Immunoblotting.

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells at all concentrations of leptin (Fig. 5B). To determine whether Tyr^{813} phosphorylation prolongs STAT3 activation, γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were treated with leptin for 0, 5, 15, 30, 60, and 120 min. Leptin-stimulated STAT3 phosphorylation in γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells was similar at each time point (Fig. 5C). To determine whether SH2B1 enhances STAT3 activation at a lower concentration of leptin, γ2A$^{LEPRb/JAK2}$, γ2A$^{LEPRb/JAK2/SH2B1}$, γ2A$^{LEPRb/Y813F}$, and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were treated with 50 ng/ml leptin for 10 min. Leptin-stimulated STAT3 phosphorylation was similar both between γ2A$^{LEPRb/JAK2}$ and γ2A$^{LEPRb/JAK2/SH2B1}$ and between γ2A$^{LEPRb/Y813F}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells (Fig. 5D). Together, these observations suggest that leptin stimulates STAT3 activation independent of Tyr^{813} phosphorylation and subsequent binding of phosphorylated Tyr^{813} to the SH2 domain of SH2B1.

SH2B1 Promotes Leptin-Stimulated IRS1 Phosphorylation Independent of the Phosphorylation of Tyr^{813} in JAK2

IRS protein-mediated activation of the PI 3-kinase pathway is required for leptin to regulate energy balance, body weight, and glucose metabolism (16, 17, 43, 44). We reported previously that genetic deletion of the *SH2B1* gene abrogates leptin-stimulated tyrosine phosphorylation of hypothalamic IRS2 in SH2B1 knockout mice (38). Genetic deletion of SH2B1 also markedly decreases leptin-stimulated tyrosine phosphorylation of endogenous IRS1 in cultured mouse embryonic fibroblasts (13). These findings suggest that endogenous SH2B1 is required for leptin stimulation of the IRS proteins/PI 3-kinase pathway. To determine whether SH2B1 promotes leptin-stimulated tyrosine phosphorylation of IRS1 via binding to phosphorylated Tyr^{813} in JAK2, various γ2A cells were stimulated with 50 ng/ml leptin for 10 min, and endogenous IRS1 was immunoprecipitated with αIRS1 and immunoblotted with αPY. Leptin stimulated IRS1 phosphorylation in γ2A$^{LEPRb/JAK2}$, γ2A$^{LEPRb/Y813F}$, γ2A$^{LEPRb/JAK2/SH2B1}$, and γ2A$^{LEPRb/Y813F/SH2B1}$ but not γ2A^{LEPRb} cells (Fig. 6). SH2B1 markedly enhanced both JAK2- and JAK2(Y813F)-mediated tyrosine phosphorylation of IRS1 in response to leptin (Fig. 6). Phosphorylated IRS1 was quantified and normalized to total IRS1 protein levels. SH2B1 enhanced JAK2-mediated IRS1 phosphorylation by 197 $\pm$ 86% (mean $\pm$ SEM, n = 7; $P < 0.05$) and JAK2(Y813F)-mediated IRS1 phosphorylation by 294 $\pm$ 99% (mean $\pm$ SEM, n = 7, $P < 0.05$). These data indicate that JAK2 is required for leptin to stimulate tyrosine phosphorylation of IRS1, but Tyr^{813} phosphorylation is not required for IRS1 phosphorylation. SH2B1 enhances leptin-stimulated IRS1 phosphorylation independent of Tyr^{813} phosphorylation in JAK2.

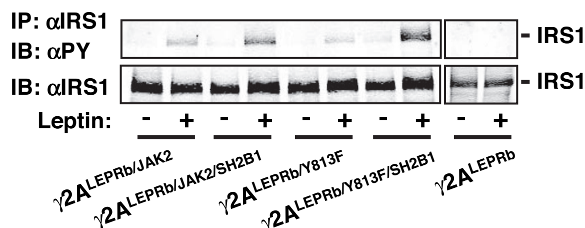

Fig. 6. Tyr^{813} in JAK2 Is Not Required for SH2B1 to Enhance IRS1 Phosphorylation in Response to Leptin

γ2A$^{LEPRb/JAK2}$, γ2A$^{LEPRb/Y813F}$, γ2A$^{LEPRb/JAK2/SH2B1}$, γ2A$^{LEPRb/Y813F/SH2B1}$, and γ2A^{LEPRb} cells were treated with 50 ng/ml leptin or vehicle for 5 min. Cell extract was immunoprecipitated with αIRS1 and immunoblotted with αPY. The same blots were reprobed with αIRS1. IB, Immunoblotting; IP, immunoprecipitation.

Leptin Promotes the Association of SH2B1 with IRS1

SH2B1 could be enhancing leptin-stimulated IRS1 phosphorylation by increasing the accessibility of IRS1 to JAK2. To determine whether SH2B1 forms a multiprotein complex with IRS1 and JAK2, JAK2 and IRS1 were transiently coexpressed with or without SH2B1 in HEK293 cells, and cell extract was immunoprecipitated with αSH2B1 and immunoblotted with αIRS1, αJAK2, or αPY. SH2B1 coimmunoprecipitated with both IRS1 and JAK2 (Fig. 7A, lane 3). Both SH2B1-bound IRS1 and JAK2 were tyrosyl phosphorylated (Fig. 7A, panel 4). Interestingly, SH2B1 also bound to IRS1 in the absence of JAK2 overexpression (Fig. 7A, lane 1); however, JAK2 overexpression slightly increased the coimmunoprecipitation of SH2B1 with IRS1 (Fig. 7A, lanes 1 *vs.* 3). In parallel experiments, IRS1 in cell extract was immunoprecipitated with αIRS1 and immunoblotted with αPY (to detect both phosphorylated IRS1 and JAK2) or αMyc (to detect Myc-tagged SH2B1). IRS1 coimmunoprecipitated with SH2B1 in the absence of overexpressed JAK2; IRS1 was associated with both SH2B1 and JAK2 in the presence of overexpressed JAK2 (Fig. 7B). Consistently, SH2B1 promoted tyrosine phosphorylation of IRS1 (Fig. 7B, *upper panel*).

To determine whether leptin promotes the interaction between SH2B1 and IRS1, γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells were stimulated with 100 ng/ml leptin for 10 min, and endogenous IRS1 in cell extract was immunoprecipitated with αIRS1 and immunoblotted with αSH2B1. Leptin increased the binding of IRS1 to SH2B1 in both γ2A$^{LEPRb/JAK2/SH2B1}$ and γ2A$^{LEPRb/Y813F/SH2B1}$ cells, indicating that leptin promotes the association of SH2B1 with IRS1 independent of Tyr^{813} phosphorylation (Fig. 7C). To determine whether leptin promotes association of endogenous SH2B1 with endogenous IRS1, γ2A$^{LEPRb/JAK2}$ and γ2A$^{LEPRb/Y813F}$ cells were stimulated with 100 ng/ml leptin for 10 min, and endogenous IRS1 in cell extract was immunoprecipitated with αIRS1 and immunoblotted with αSH2B1. Endogenous IRS1 bound to endogenous SH2B1, and the binding was increased in response to leptin stimulation in both γ2A$^{LEPRb/JAK2}$

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

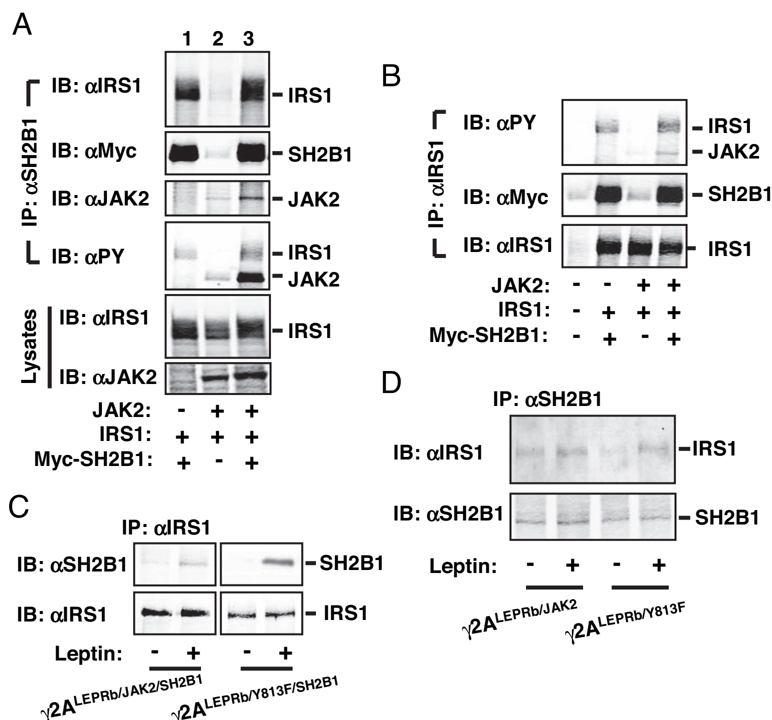

Fig. 7. Leptin Stimulates the Interaction of IRS1 with SH2B1 Independent of Tyr^{813} Phosphorylation

A and B, IRS1 plasmids were transiently cotransfected with SH2B1 plasmids in the presence or absence of JAK2 plasmids in HEK293 cells. Myc-tagged SH2B1 in cell extract was immunoprecipitated with αSH2B1 and immunoblotted with αIRS1, αMyc, αJAK2, or αPY as indicated. Cell extract was also immunoblotted with αIRS1 or αJAK2 (A). In parallel experiments, IRS1 was immunoprecipitated with αIRS1 and immunoblotted with αPY, αMyc, or αIRS1 as indicated (B). C, $\gamma 2A^{LEPRb/JAK2/SH2B1}$ and $\gamma 2A^{LEPRb/Y813F/SH2B1}$ cells were treated with 100 ng/ml leptin for 10 min. IRS1 in cell extract was immunoprecipitated with αIRS1 and immunoblotted with αSH2B1 or αIRS1. D, $\gamma 2A^{LEPRb/JAK2}$ and $\gamma 2A^{LEPRb/Y813F}$ cells were treated with 100 ng/ml leptin for 10 min. IRS1 in cell extract was immunoprecipitated with αSH2B1 and immunoblotted with αIRS1 or αSH2B1. IB, Immunoblotting; IP, immunoprecipitation.

and $\gamma 2A^{LEPRb/Y813F}$ cells (Fig. 7D). Together, these observations suggest that binding of SH2B1 to IRS1 contributes to leptin-stimulated tyrosine phosphorylation of IRS1.

DISCUSSION

We previously provided multiple lines of genetic evidence demonstrating that SH2B1 is a key endogenous positive regulator of leptin sensitivity in animals. Genetic deletion of the *SH2B1* gene results in severe leptin resistance and morbid obesity in SH2B1 null mice (30, 38). Tissue-specific restoration of SH2B1 in neurons, the main targets of leptin, rescues leptin resistance and obesity in SH2B1 knockout mice (40). Moreover, neuron-specific overexpression of SH2B1 protects against high-fat diet-induced leptin resistance and obesity in SH2B1 transgenic mice (40). In this study, we provide biochemical evidence demonstrating that SH2B1 regulates leptin signaling by multiple mechanisms. In the absence of leptin, SH2B1 constitutively interacted with JAK2 that was not tyrosyl phosphorylated and thus inactive; the SH2 domain of SH2B1 was not required for this constitutive association; however, leptin significantly increased the interaction of SH2B1 with JAK2. Leptin rapidly and robustly stimulated the phosphorylation of Tyr^{813} in JAK2, and phosphorylated Tyr^{813} subsequently bound to the SH2 domain of SH2B1. Consistent with these observations, the PH domain of SH2B1 is both required and sufficient for SH2B1 to bind to a kinase-inactive JAK2 mutant (45). Crystal structural analysis reveals that a phospho-Tyr^{813} peptide of JAK2 physically binds to the SH2 domain of SH2B1 (24). Therefore, SH2B1 appears to bind to JAK2 by multiple sites. A non-SH2 domain region in SH2B1 constitutively binds to a non-Tyr^{813} site in JAK2, whereas the SH2 domain of SH2B1 binds to phosphorylated Tyr^{813} in JAK2.

Surprisingly, leptin was unable to stimulate both SH2B1(R555E)-bound JAK2 and SH2B1-bound JAK2(Y813F). Both SH2B1(R555E)-JAK2 interaction and SH2B1-JAK2(Y813F) interaction are mediated by a non-SH2 domain region of SH2B1 and a non-Tyr^{813} site in JAK2. A C-terminal truncated SH2B1 lacking a functional SH2 domain, which constitutively binds to JAK2 via the interaction of the non-SH2 domain region

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

of SH2B1 and the non-Tyr^{813} site in JAK2, inhibits JAK2 activity (45). Therefore, the interaction of the non-SH2 domain region of SH2B1 with the non-Tyr^{813} site in JAK2 may inhibit basal JAK2 activity and autophosphorylation.

In contrast, SH2B1 significantly enhanced leptin-stimulation of JAK2 that was phosphorylated on Tyr^{813}. The mechanism of SH2B1 stimulation of JAK2 remains unknown. SH2B1 forms homodimers via its N-terminal DD domain (25, 46). SH2B1 dimerization is predicted to induce the dimerization of SH2B1-bound JAK2, thereby promoting JAK2 activation (25). However, overexpression of either N-terminal truncated SH2B1, which lacks the DD domain, or full-length SH2B1, activates JAK2 to a similar extent, and the SH2 domain of SH2B1 is both required and sufficient to activate JAK2 (45, 47). These results suggest that the interaction of the SH2 domain of SH2B1 with phospho-Tyr^{813} stabilizes JAK2 in an active conformation.

We show that leptin stimulates tyrosine phosphorylation of IRS1 in $\gamma2A^{LEPRb/JAK2}$ but not in $\gamma2A^{LEPRb}$ cells, providing strong evidence that JAK2 mediates leptin-stimulated tyrosine phosphorylation of IRS1. Because SH2B1 promotes JAK2 activation, SH2B1 significantly increased leptin-stimulated tyrosine phosphorylation of IRS1, a JAK2 substrate, as expected. In agreement with these findings, genetic deletion of SH2B1 markedly reduces leptin-stimulated tyrosine phosphorylation of IRS1 in mouse embryonic fibroblasts, and this reduction is reversed by the expression of recombinant SH2B1 (13). Surprisingly, SH2B1 also enhanced JAK2(Y813F)-mediated tyrosine phosphorylation of IRS1 in response to leptin, even though SH2B1 did not enhance JAK2(Y813F) activation. SH2B1 directly binds to both IRS1 and IRS2, and the SH2 domain of SH2B1 is sufficient to bind to both tyrosyl-phosphorylated IRS1 and IRS2 (13). Leptin promoted the association of SH2B1 with IRS1 similarly in both $\gamma2A^{LEPRb/JAK2}$ and $\gamma2A^{LEPRb/Y813F}$ cells. These observations suggest that SH2B1 enhances leptin-stimulated tyrosine phosphorylation of IRS proteins by an additional mechanism independent of SH2B1 enhancement of JAK2 activation.

We propose a model of SH2B1 regulation of leptin signaling (Fig. 8). In the absence of leptin, the constitutive binding of SH2B1 (either as monomers or dimers) to JAK2, which is mediated by the non-SH2 domain region(s) of SH2B1 and the non-Tyr^{813} region(s) of JAK2, not only increases the local concentration of SH2B1 close to JAK2 but also inhibits basal JAK2 activity (Fig. 8A). Leptin stimulates JAK2 autophosphorylation on Tyr^{813}, and phosphorylated Tyr^{813} binds to the SH2 domain of SH2B1 (either as monomers or dimers) (Fig. 8B). The physical interaction of the SH2 domain of SH2B1 with phospho-Tyr^{813} in JAK2 robustly and quickly promotes JAK2 activation, thus globally enhancing leptin signaling (Fig. 8B, *left side* of LEPRb). Leptin also stimulates the binding of IRS1 to SH2B1 (either as monomers or dimers). SH2B1-homodimers binds simultaneously to both JAK2 and IRS1, thereby increasing the concentration

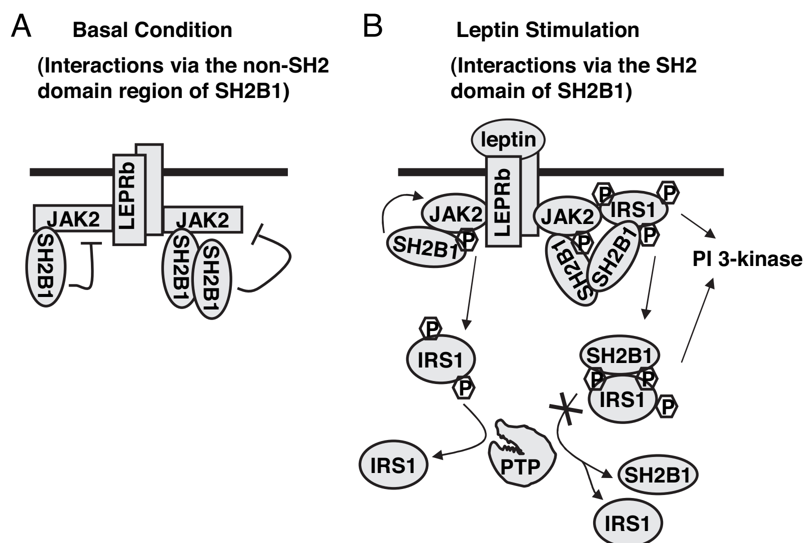

Fig. 8. A Model of SH2B1 Action

A, In the absence of leptin, SH2B1 (either as monomers or dimers) binds via its non-SH2 domain region(s) to inactive and non-tyrosyl-phosphorylated JAK2, thereby both increasing the local concentration of SH2B1 close to JAK2 and inhibiting basal JAK2 activity. B, Leptin stimulates phosphorylation of JAK2 on Tyr^{813}. SH2B1 binds via its SH2 domain to phosphorylated Tyr^{813}, thus enhancing leptin stimulation of JAK2 (*left side* of LEPRb). SH2B1 (either as monomers or dimers) also directly binds to IRS1 or IRS2, thereby recruiting IRS proteins to JAK2 and/or stabilizing JAK2/SH2B1/IRS protein complexes, which facilitates tyrosine phosphorylation of IRS proteins by JAK2 (*right side* of LEPRb). In addition, the binding of SH2B1 to IRS proteins may protect IRS proteins from protein tyrosine phosphatase-mediated dephosphorylation (*low side*). Tyrosyl-phosphorylated IRS proteins activate the PI 3-kinse pathway. PTP, Protein tyrosine phosphatase.

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

of IRS1 proximal to JAK2 and facilitating JAK2-mediated phosphorylation of IRS1 (Fig. 8B, *right side* of LEPRb). In addition, SH2B1 binds, via its SH2 domain, directly to phosphotyrosine(s) in IRS1, thereby protecting IRS1 from dephosphorylation by protein tyrosine phosphatase(s) (Fig. 8B, *low side*). Tyrosyl-phosphorylated IRS1 and/or IRS2 mediate leptin stimulation of the PI 3-kinase pathway.

JAK2 Tyr^{813} phosphorylation is not required for leptin-stimulated phosphorylation of STAT3. The time course and dose response of leptin-stimulated STAT3 phosphorylation were indistinguishable between $\gamma 2A^{LEPRb/JAK2/SH2B1}$ and $\gamma 2A^{LEPRb/Y813F/SH2B1}$ cells. Even though leptin-induced JAK2 activation was enhanced by overexpression of SH2B1 in $\gamma 2A^{LEPRb/JAK2/SH2B1}$ cells, SH2B1 did not enhance leptin-stimulated STAT3 phosphorylation. One explanation for these interesting data is that STAT3 is already maximally phosphorylated by $JAK2^{Y813F}$ or by JAK2 at endogenous levels of SH2B1.

In summary, we demonstrated that in the absence of leptin, SH2B1 constitutively interacted with non-tyrosyl-phosphorylated JAK2, inhibiting basal JAK2 activity. Leptin stimulated JAK2 phosphorylation on Tyr^{813}, which subsequently bound to the SH2 domain of SH2B1, resulting in an enhancement of JAK2 activation. JAK2 is required for leptin stimulation of tyrosine phosphorylation of IRS1. SH2B1 bound to IRS1 and enhanced leptin-stimulated tyrosine phosphorylation of IRS1.

MATERIALS AND METHODS

Generation of γ2A Cell Lines Stably Expressing LEPRb, JAK2, JAK2(Y813F), and SH2B1

Murine LEPRb cDNA was inserted into a retroviral vector (pQCXIH) to generate recombinant LEPRb retroviruses. γ2A cells, which are derived from human fibroblasts and do not express endogenous JAK2 (42), were infected with the LEPRb retroviruses and selected by hygromycin. Hygromycin-resistant cells were pooled and designated as $\gamma 2A^{LEPRb}$ cells. $\gamma 2A^{LEPRb}$ cells stably express LEPRb as confirmed by immunoblotting with αLEPRb (data not shown). $\gamma 2A^{LEPRb}$ cells were subsequently infected with recombinant JAK2, JAK2(Y813F), or JAK2(K882E) retroviruses and selected by puromycin to generate $\gamma 2A^{LEPRb/JAK2}$, $\gamma 2A^{LEPRb/Y813F}$, and $\gamma 2A^{LEPRb/K882E}$ cell lines stably expressing JAK2, JAK2(Y813F), or JAK2(K882E), respectively. JAK2(Y813F) and JAK2(K882E) were generated by replacing Tyr^{813} with Phe and Lys^{882} (the ATP-binding site) with Glu using a site-directed mutagenesis kit (Stratagene, La Jolla, CA), respectively. $\gamma 2A^{LEPRb/JAK2}$, $\gamma 2A^{LEPRb/Y813F}$, or $\gamma 2A^{LEPRb/K882E}$ cells were infected with recombinant SH2B1β retroviruses that coexpress green fluorescent protein. Green fluorescent protein-positive cells (top 40%) were sorted by fluorescence-activated cell sorting to generate $\gamma 2A^{LEPRb/JAK2/SH2B1}$, $\gamma 2A^{LEPRb/Y813F/SH2B1}$, and $\gamma 2A^{LEPRb/K882E/SH2B1}$ cell lines stably expressing rat SH2B1β.

Parental γ2A and all derived cells were cultured at 37 C in 5% CO_2 in DMEM supplemented with 25 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 6% fetal calf serum. HEK293 cells were grown at 37 C in 5% CO_2 in DMEM supplemented with 25 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 8% calf serum.

Adenoviral Infection

$\gamma 2A^{LEPRb/JAK2}$ or $\gamma 2A^{LEPRb/Y813F}$ cells were grown to confluence in 100-mm culture dishes. Cells were infected with SH2B1β or β-gal control recombinant adenoviruses (3.8×10^{10} viral particles in 4 ml growth medium per plate) for 4 h, and grown in growth medium. Cells were deprived, 48 h after infection, of serum overnight and then treated with leptin. Cell extract was prepared and used for immunoprecipitation and immunoblotting analysis.

Transfection

HEK293 cells were split at 2×10^5 cells per well in a 12-well culture dish. The next day, JAK2 or JAK2(Y813F) expression plasmids (0.2 μg) were cotransfected with 0, 0.05, 0.1, 0.2, or 0.4 μg SH2B1 expression plasmids using Lipofectamine 2000 reagents (Invitrogen Corp., Carlsbad, CA). The total amount of plasmid DNA was maintained constant for each individual condition by adding empty prk5 vector. Cells were lysed 22 h after transfection and subjected to immunoprecipitation and immunoblotting assays.

Immunoprecipitation and Immunoblotting

Confluent cells were deprived of serum overnight (~16 h) in DMEM containing 0.6% BSA and treated with leptin as indicated in the figure legends. The cells were rinsed two times with ice-cold PBS, solubilized in lysis buffer (50 mM Tris, pH 7.5; 1% Nonidet P-40; 150 mM NaCl; 2 mM EGTA; 1 mM Na_3VO_4; 100 mM NaF; 10 mM $Na_4P_2O_7$; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml aprotinin; 10 μg/ml leupeptin), and centrifuged at $14{,}000 \times g$ for 15 min at 4 C. Protein concentration in the supernatant (cell extract) was determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Cell extract (same amount of protein for each individual condition) was incubated with the indicated antibody on ice for 2 h. The immune complexes were collected on protein A-agarose during a 1-h incubation at 4 C. The beads were washed three times with washing buffer (50 mM Tris, pH 7.5; 1% Nonidet P-40; 150 mM NaCl; 2 mM EGTA; 100 mM NaF; and 10 mM $Na_4P_2O_7$) and boiled for 5 min in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8; 2% SDS; 2% β-mercaptoethanol; 10% glycerol; 0.005% bromophenol blue). The solubilized proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Amersham International Plc, Little Chalfont, Buckinghamshire, UK), and detected by immunoblotting with the indicated antibody using enhanced chemiluminescence or Odyssey detection system. Some membranes were subsequently incubated at 55 C for 30 min in stripping buffer (100 mM β-mercaptoethanol; 2% SDS; 62.5 mM Tris-HCl, pH 6.7) to prepare them for a second round of immunoblotting. Phosphorylated JAK2 was quantified using the Odyssey software and normalized to total JAK2 protein levels. Similarly, phosphorylated IRS1 was quantified and normalized to total IRS1 protein levels. αIRS1, αJAK2, and αphospho-JAK2 were from Biosource (Camarillo, CA) or Upstate Biotechnology, Inc. (Charlottesville, VA). αSTAT3 and αphospho-STAT3 were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) or Cell Signaling Technology, Inc. (Beverly, MA).

Statistical Analysis

The data are presented as the mean ± SEM. Student's *t* tests were used for comparisons between two groups. $P < 0.05$ was considered statistically significant.

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

Acknowledgments

We thank David Morris and Drs. Lawrence Argetsinger and Jason Kurzer for helpful discussion. We thank Dr. George Stark (Cleveland Clinic Foundation, Cleveland, OH) for providing γ2A cells.

Received February 27, 2007. Accepted June 4, 2007.

Address all correspondence and requests for reprints to: Liangyou Rui, Ph.D., Department of Molecular & Integrative Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0622. E-mail: ruily@umich.edu.

This work was supported by Career Development Award (7-03-CD-11) from the American Diabetes Association and RO1 DK 065122 and RO1 DK073601 from National Institutes of Health (NIH) (all to L.R.). This work utilized the cores supported by the Michigan Diabetes Research and Training Center (funded by NIH Grant 5P60 DK20572) and University of Michigan's Cancer Center (funded by NIH Grant 5 P30 CA46592). C.C.S. was supported by NIH Grants DK34171 and DK54222; and M.G.M.J. was supported by NIH Grant DK56731.

Disclosure Statement: All authors have nothing to declare.

REFERENCES

1. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM 1994 Positional cloning of the mouse obese gene and its human homologue. Nature 372:425–432
2. Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, Morgenstern JP 1996 Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. Cell 84:491–495
3. Chua Jr SC, Chung WK, Wu-Peng XS, Zhang Y, Liu SM, Tartaglia L, Leibel RL 1996 Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. Science 271:994–996
4. Bjorbak C, Lavery HJ, Bates SH, Olson RK, Davis SM, Flier JS, Myers Jr MG 2000 SOCS3 mediates feedback inhibition of the leptin receptor via Tyr985. J Biol Chem 275:40649–40657
5. Eyckerman S, Broekaert D, Verhee A, Vandekerckhove J, Tavernier J 2000 Identification of the Y985 and Y1077 motifs as SOCS3 recruitment sites in the murine leptin receptor. FEBS Lett 486:33–37
6. Banks AS, Davis SM, Bates SH, Myers Jr MG 2000 Activation of downstream signals by the long form of the leptin receptor. J Biol Chem 275:14563–14572
7. Howard JK, Cave BJ, Oksanen LJ, Tzameli I, Bjorbaek C, Flier JS 2004 Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. Nat Med 10:734–738
8. Mori H, Hanada R, Hanada T, Aki D, Mashima R, Nishinakamura H, Torisu T, Chien KR, Yasukawa H, Yoshimura A 2004 Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. Nat Med 10:739–743
9. Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AW, Wang Y, Banks AS, Lavery HJ, Haq AK, Maratos-Flier E, Neel BG, Schwartz MW, Myers Jr MG 2003 STAT3 signalling is required for leptin regulation of energy balance but not reproduction. Nature 421:856–859
10. Cui Y, Huang L, Elefteriou F, Yang G, Shelton JM, Giles JE, Oz OK, Pourbahrami T, Lu CY, Richardson JA, Karsenty G, Li C 2004 Essential role of STAT3 in body weight and glucose homeostasis. Mol Cell Biol 24: 258–269
11. Gao Q, Wolfgang MJ, Neschen S, Morino K, Horvath TL, Shulman GI, Fu XY 2004 Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. Proc Natl Acad Sci USA 101:4661–4666
12. Carvalheira JB, Ribeiro EB, Folli F, Velloso LA, Saad MJ 2003 Interaction between leptin and insulin signaling pathways differentially affects JAK-STAT and PI 3-kinase-mediated signaling in rat liver. Biol Chem 384: 151–159
13. Duan C, Li M, Rui L 2004 SH2-B promotes insulin receptor substrate 1 (IRS1)- and IRS2-mediated activation of the phosphatidylinositol 3-kinase pathway in response to leptin. J Biol Chem 279:43684–43691
14. Kellerer M, Koch M, Metzinger E, Mushack J, Capp E, Haring HU 1997 Leptin activates PI-3 kinase in C2C12 myotubes via janus kinase-2 (JAK-2) and insulin receptor substrate-2 (IRS-2) dependent pathways. Diabetologia 40:1358–1362
15. Kim Y-B, Uotani S, Pierroz DD, Flier JS, Kahn BB 2000 *In vivo* administration of leptin activates signal transduction directly in insulin-sensitive tissues: overlapping but distinct pathways from insulin. Endocrinology 141: 2328–2339
16. Lin X, Taguchi A, Park S, Kushner JA, Li F, Li Y, White MF 2004 Dysregulation of insulin receptor substrate 2 in β cells and brain causes obesity and diabetes. J Clin Invest 114:908–916
17. Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers Jr MG, Schwartz MW 2001 Intracellular signalling. Key enzyme in leptin-induced anorexia. Nature 413: 794–795
18. Zhao AZ, Huan JN, Gupta S, Pal R, Sahu A 2002 A phosphatidylinositol 3-kinase phosphodiesterase 3B-cyclic AMP pathway in hypothalamic action of leptin on feeding. Nat Neurosci 5:727–728
19. Suzuki R, Tobe K, Aoyama M, Inoue A, Sakamoto K, Yamauchi T, Kamon J, Kubota N, Terauchi Y, Yoshimatsu H, Matsuhisa M, Nagasaka S, Ogata H, Tokuyama K, Nagai R, Kadowaki T 2004 Both insulin signaling defects in the liver and obesity contribute to insulin resistance and cause diabetes in Irs2(−/−) mice. J Biol Chem 279:25039–25049
20. Feener EP, Rosario F, Dunn SL, Stancheva Z, Myers Jr MG 2004 Tyrosine phosphorylation of Jak2 in the JH2 domain inhibits cytokine signaling. Mol Cell Biol 24: 4968–4978
21. Feng J, Witthuhn BA, Matsuda T, Kohlhuber F, Kerr IM, Ihle JN 1997 Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. Mol Cell Biol 17:2497–2501
22. Argetsinger LS, Kouadio JL, Steen H, Stensballe A, Jensen ON, Carter-Su C 2004 Autophosphorylation of JAK2 on tyrosines 221 and 570 regulates its activity. Mol Cell Biol 24:4955–4967
23. Kurzer JH, Argetsinger LS, Zhou YJ, Kouadio JL, O'Shea JJ, Carter-Su C 2004 Tyrosine 813 is a site of JAK2 autophosphorylation critical for activation of JAK2 by SH2-B β. Mol Cell Biol 24:4557–4570
24. Hu J, Hubbard SR 2006 Structural basis for phosphotyrosine recognition by the Src homology-2 domains of the adapter proteins SH2-B and APS. J Mol Biol 361:69–79
25. Nishi M, Werner ED, Oh BC, Frantz JD, Dhe-Paganon S, Hansen L, Lee J, Shoelson SE 2005 Kinase activation through dimerization by human SH2-B. Mol Cell Biol 25:2607–2621
26. Yousaf N, Deng Y, Kang Y, Riedel H 2001 Four PSM/SH2-B alternative splice variants and their differential roles in mitogenesis. J Biol Chem 276:40940–40948
27. Nelms K, O'Neill TJ, Li S, Hubbard SR, Gustafson TA, Paul WE 1999 Alternative splicing, gene localization, and

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024

binding of SH2-B to the insulin receptor kinase domain. Mamm Genome 10:1160–1167
28. Rui L, Mathews LS, Hotta K, Gustafson TA, Carter-Su C 1997 Identification of SH2-Bβ as a substrate of the tyrosine kinase JAK2 involved in growth hormone signaling. Mol Cell Biol 17:6633–6644
29. Rui L, Carter-Su C 1999 Identification of SH2-bβ as a potent cytoplasmic activator of the tyrosine kinase Janus kinase 2. Proc Natl Acad Sci USA 96:7172–7177
30. Li M, Ren D, Iseki M, Takaki S, Rui L 2006 Differential role of SH2-B and APS in regulating energy and glucose homeostasis. Endocrinology 147:2163–2170
31. Liu J, Kimura A, Baumann CA, Saltiel AR 2002 APS facilitates c-Cbl tyrosine phosphorylation and GLUT4 translocation in response to insulin in 3T3-L1 adipocytes. Mol Cell Biol 22:3599–3609
32. Ahmed Z, Smith BJ, Kotani K, Wilden P, Pillay TS 1999 APS, an adapter protein with a PH and SH2 domain, is a substrate for the insulin receptor kinase. Biochem J 341: 665–668
33. Ahmed Z, Smith BJ, Pillay TS 2000 The APS adapter protein couples the insulin receptor to the phosphorylation of c-Cbl and facilitates ligand-stimulated ubiquitination of the insulin receptor. FEBS Lett 475:31–34
34. Moodie SA, Alleman-Sposeto J, Gustafson TA 1999 Identification of the APS protein as a novel insulin receptor substrate. J Biol Chem 274:11186–11193
35. Minami A, Iseki M, Kishi K, Wang M, Ogura M, Furukawa N, Hayashi S, Yamada M, Obata T, Takeshita Y, Nakaya Y, Bando Y, Izumi K, Moodie SA, Kajiura F, Matsumoto M, Takatsu K, Takaki S, Ebina Y 2003 Increased insulin sensitivity and hypoinsulinemia in APS knockout mice. Diabetes 52:2657–2665
36. Li M, Li Z, Morris DL, Rui L 2007 Identification of SH2B2β as an inhibitor for SH2B1- and SH2B2α-promoted Janus kinase-2 activation and insulin signaling. Endocrinology 148:1615–1621
37. Ohtsuka S, Takaki S, Iseki M, Miyoshi K, Nakagata N, Kataoka Y, Yoshida N, Takatsu K, Yoshimura A 2002 SH2-B is required for both male and female reproduction. Mol Cell Biol 22:3066–3077
38. Ren D, Li M, Duan C, Rui L 2005 Identification of SH2-B as a key regulator of leptin sensitivity, energy balance, and body weight in mice. Cell Metabolism 2:95–104
39. Duan C, Yang H, White MF, Rui L 2004 Disruption of the SH2-B gene causes age-dependent insulin resistance and glucose intolerance. Mol Cell Biol 24:7435–7443
40. Ren D, Zhou Y, Morris D, Li M, Li Z, Rui L 2007 Neuronal SH2B1 is essential for controlling energy and glucose homeostasis. J Clin Invest 117:397–406
41. Burfoot MS, Rogers NC, Watling D, Smith JM, Pons S, Paonessaw G, Pellegrini S, White MF, Kerr IM 1997 Janus kinase-dependent activation of insulin receptor substrate 1 in response to interleukin-4, oncostatin M, and the interferons. J Biol Chem 272:24183–24190
42. Kohlhuber F, Rogers NC, Watling D, Feng J, Guschin D, Briscoe J, Witthuhn BA, Kotenko SV, Pestka S, Stark GR, Ihle JN, Kerr IM 1997 A JAK1/JAK2 chimera can sustain α and γ interferon responses. Mol Cell Biol 17:695–706
43. Choudhury AI, Heffron H, Smith MA, Al-Qassab H, Xu AW, Selman C, Simmgen M, Clements M, Claret M, Maccoll G, Bedford DC, Hisadome K, Diakonov I, Moosajee V, Bell JD, Speakman JR, Batterham RL, Barsh GS, Ashford ML, Withers DJ 2005 The role of insulin receptor substrate 2 in hypothalamic and β cell function. J Clin Invest 115:940–950
44. Morton GJ, Gelling RW, Niswender KD, Morrison CD, Rhodes CJ, Schwartz MW 2005 Leptin regulates insulin sensitivity via phosphatidylinositol-3-OH kinase signaling in mediobasal hypothalamic neurons. Cell Metab 2:411–420
45. Rui L, Gunter DR, Herrington J, Carter-Su C 2000 Differential binding to and regulation of JAK2 by the SH2 domain and N-terminal region of SH2-bβ. Mol Cell Biol 20:3168–3177
46. Qian X, Ginty DD 2001 SH2-B and APS are multimeric adapters that augment TrkA signaling. Mol Cell Biol 21: 1613–1620
47. Kurzer JH, Saharinen P, Silvennoinen O, Carter-Su C 2006 Binding of SH2-B family members within a potential negative regulatory region maintains JAK2 in an active state. Mol Cell Biol 26:6381–6394

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Downloaded from https://academic.oup.com/mend/article/21/9/2270/2738650 by guest on 19 April 2024