The Src homology-2 (SH2) domain-containing protein SH2-Bβ is a substrate of the growth hormone (GH) receptor-associated tyrosine kinase JAK2. Here we tested whether SH2-Bβ is involved in GH regulation of the actin cytoskeleton. Based on cell fractionation and confocal microscopy, we find SH2-Bβ present at the plasma membrane and in the cytosol. SH2-Bβ colocalized with filamentous actin in GH and platelet-derived growth factor (PDGF)-induced membrane ruffles. To test if SH2-Bβ is required for actin reorganization, we transiently overexpressed wild-type or mutant SH2-Bβ in 3T3-F442A cells and assayed for GH- and PDGF-induced membrane ruffling and fluid phase pinocytosis. Overexpression of wild-type SH2-Bβ enhanced ruffling and pinocytosis produced by submaximal GH but not submaximal PDGF. Point mutant SH2-Bβ (R555E) and truncation mutant ΔC555, both lacking a functional SH2 domain, inhibited membrane ruffling and pinocytosis induced by GH and PDGF. Mutant ΔN504, which possesses a functional SH2 domain and enhances JAK2 kinase activity in overexpression systems, also inhibited GH-stimulated membrane ruffling. ΔN504 failed to inhibit GH-induced nuclear localization of Stat5B, indicating JAK2 is active in these cells. Taken together, these results show that SH2-Bβ is required for GH-induced actin reorganization by a mechanism discrete from the action of SH2-Bβ as a stimulator of JAK2 kinase activity.

Growth hormone (GH) has diverse actions throughout the body. GH stimulates longitudinal bone growth in part from a direct action of GH on epiphyseal chondrocytes, inducing their differentiation into chondrocytes (1). Similarly, GH has a role in adipocyte differentiation. GH-deficient children have a reduced fat cell number, suggesting a role for GH in preadipocyte proliferation and induction of differentiation (2). Indeed, GH is permissive for adipocyte differentiation in tissue culture models (3–5), and GH signaling appears to be required for normal fat deposition in mice (6). GH also stimulates chemotaxis of monocytes (7). A shared requirement for these varied actions of GH is change in cell shape or location. The cytoskeleton is intimately involved in the regulation of cell morphology and movement (8). Hence, it is logical to predict that GH regulates the cytoskeleton. Consistent with this, GH is known to stimulate actin reorganization (9) and microtubule polymerization (10).

Generation of membrane ruffles rich in polymerized actin is one of the earliest detectable events when cells are stimulated with growth factors and hormones such as platelet-derived growth factor (PDGF), insulin, and GH (9, 15, 16). Ruffles are commonly found on the leading edge (lamella) of motile cells and are thought to be required for cell migration (17). Ruffling may also be important for nutrient uptake, since ruffling is linked to fluid phase pinocytosis (18, 19). The signaling pathways involved in the generation of membrane ruffles are emerging. For PDGF and insulin-stimulated ruffling, the Rho family GTPase Rac is clearly required (20, 21). The pathway linking receptor activation to Rac remains elusive, although phosphinositide (PtdIns)3-kinase is known to be involved (22, 23). Numerous downstream effectors of Rac have been identified, but their role in membrane ruffling is uncertain (24).

Our laboratory recently identified the SH2 domain-containing protein SH2-Bβ as a substrate of the GH-activated tyrosine kinase JAK2. Three alternatively spliced forms of SH2-B (α, β, and γ) have been described (25–28) and are considered to belong to a family of adapter proteins that include APS and Lnk (29, 30). In response to GH, SH2-Bβ associates with and is tyrosyl-phosphorylated by JAK2 and is phosphorylated on serines/threonines by unknown kinase(s). In addition to GH, SH2-Bβ is phosphorylated in response to PDGF, epidermal growth factor (31), nerve growth factor (32, 33), and interferon-γ (25) and associates with receptors for insulin-like growth factor-1 and insulin (34). A requirement for SH2-Bβ in nerve growth factor-mediated neuronal differentiation and survival has been demonstrated (32, 33).

Given that neuronal differentiation requires extensive changes in cell shape and that both GH and PDGF stimulate actin reorganization, we hypothesized that SH2-Bβ might play
a role in regulation of the actin cytoskeleton. We show that SH2-Bβ is colocalized with filamentous actin at GH and PDGF-induced membrane ruffles. Introduction of mutant SH2-Bβs into cells inhibits membrane ruffling and pinocytosis stimulated by GH and PDGF. These findings provide evidence that SH2-B is a novel component of the signaling network involved in regulation of cell shape and movement.

**Experimental Procedures**

**Cells and Reagents**—The stock of mouse 3T3-F442A fibroblasts was provided by H. Green (Harvard University, Cambridge, MA). Recombinant human GH was the gift of H. Green (Harvard University, Cambridge, MA). Recombinant human PDGF-BB was purchased from Invitrogen. Prestained molecular weight standards were from Life Technologies, Inc., and ECL detection reagents were from Amersham Pharmacia Biotech. All chemicals were reagent grade or better.

**Antibodies and Fluorescent Probes**—c-Myc mouse monoclonal antibody (9E10) was obtained from Santa Cruz Biotechnology. Polyclonal antibody raised against a glutathione S-transferase fusion protein containing the C-terminal portion of SH2-B has been described previously (25). Goat anti-mouse IgG-Oregon Green 488 and Texas Red-phalloidin were from Molecular Probes, Inc. Rabbit anti-mouse IgG-Texas Red was from Jackson ImmunoResearch. Fluorescein-conjugated bovine serum albumin (FITC-BSA) was obtained from Sigma. Antibodies and fluorescent probes used in this study included rabbit anti-mouse α- Myc and anti-mouse IgG-Texas Red antibodies. For immunocytochemistry, Texas Red-phalloidin was from Molecular Probes, Inc.

**Measurement of Fluid Phase Pinocytosis**—Thirty-six h after transfection with cDNA encoding Myc-tagged versions of wild-type or mutant SH2-Bβ, cells were incubated overnight in serum-free medium. As a control, cells were transfected with cDNA encoding Myc-tagged Lin-7, a protein not expected to regulate the actin cytoskeleton (37). To measure fluid phase pinocytosis, cells were incubated with 2 ng/ml FITC-BSA together with GH or PDGF for 10 min, washed, incubated in medium alone for an additional 30 min, fixed with CFA, and permeabilized with CFβ. Nonspecific sites were blocked with 2% goat serum (three incubations) for 15 min at 37 °C. Following two rinses in PBS, nonspecific sites were bound with blocking solution (2% goat serum, three 5-min incubations). Samples were incubated at 37 °C for 45 min at 4 °C. The supernatant was removed, and the pellet was resuspended in lysis buffer. The supernatant and pellet fractions were designated cytosol and membrane fractions, respectively. Samples from each fraction were separated by SDS-polyacrylamide gel electrophoresis (7.5% gels). Proteins were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech) and detected by Western blotting using ECL with antibody to SH2-Bβ (1:15,000). Goat anti-mouse IgG-Oregon Green 488 in blocking solution for 1 h at room temperature. For F-actin staining, samples were incubated with Texas Red-phalloidin (1:40) for 30 min at room temperature. Following staining, the coverslips were rinsed twice with PBS, mounted on slides, and imaged the same day.

**Confocal Fluorescence Microscopy**—Confocal imaging was performed with a Zeiss Axiovert 200 microscope equipped with a 63× oil immersion objective and a cooled charge-coupled device camera. Images were obtained with Metamorph software (Universal Imaging). For localization experiments, a 15-μm slit was used to excite the green (GFP-positive) and red (Texas Red) channels, and the red channel was divided by the green channel. Digital images were converted to TIF files for analysis and presentation using Adobe Photoshop™.

**Subcellular Fractionation and Western Blotting**—Confluent 10-cm plates of cells were rinsed three times with ice-cold PBS supplemented with 1 mM Na3VO4, lysed, and scraped from plates in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EGTA, 5 mM EDTA, 5 mM MgSO4, 33 mM KC2H3O3, and 0.02% NaN3). Cells were permeabilized with CFB (1% Triton X-100, 4% paraformaldehyde, 5% polyethylene glycol 400 in intracellular buffer consisting of 30 mM HEPES, pH 7.4, 10 mM EGTA, 0.5 mM EDTA, 5 mM MgSO4, 33 mM KC2H3O3, and 0.02% NaN3). Cells were permeabilized with CFB (1% Triton X-100, 4% paraformaldehyde, 5% polyethylene glycol 400 in intracellular buffer) for 15 min at 37 °C. Following two rinses in PBS, nonspecific sites were bound with blocking solution (2% goat serum, three 5-min incubations). Samples were incubated with α-Myc (1:200) in blocking solution for 2 h, rinses, and incubation with α-Myc (1:200) and goat anti-mouse IgG incubated overnight, treated with ligands at 37 °C where appropriate, and processed for imaging as described below. Stable lines derived from PC12 cells expressing GFP-SH2-Bβ have been described previously (32).

**Immunocytochemistry and Actin Staining**—Cells were rapidly rinsed twice with PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl), fixed for 15 min at 37 °C in CFA (4% paraformaldehyde, 5% polyethylene glycol 400 in intracellular buffer consisting of 30 mM HEPES, pH 7.4, 10 mM EGTA, 0.5 mM EDTA, 5 mM MgSO4, 33 mM KC2H3O3, and 0.02% NaN3). Cells were permeabilized with CFB (1% Triton X-100, 4% paraformaldehyde, 5% polyethylene glycol 400 in intracellular buffer) for 15 min at 37 °C. Following two rinses in PBS, nonspecific sites were bound with blocking solution (2% goat serum, three 5-min incubations). Samples were incubated with α-Myc (1:200) in blocking solution for 2 h, rinses, and incubation with α-Myc (1:200) and goat anti-mouse IgG-Oregon Green 488 in blocking solution for 1 h at room temperature. For F-actin staining, samples were incubated with Texas Red-phalloidin (1:40) for 30 min at room temperature. Following staining, the coverslips were rinsed twice with PBS, mounted on slides, and imaged the same day.

**Quantification of Membrane Ruffling**—To measure the effect of SH2-Bβ on membrane ruffling, cells expressing wild-type or mutant GFP-tagged SH2-Bβs were deprived of serum overnight, treated with GH or PDGF, or left untreated, fixed, and processed for immunocytochemistry as described above. Filamentous actin was labeled with Texas Red-phalloidin and imaged by epifluorescence microscopy (Carl Zeiss). The number of ruffles per transfected cell was determined. Transflection efficiency was generally low (<5%) and different between constructs, limiting the number of transfected cells that could be studied per condition in each experiment. All of the transfected cells that could be found on the coverslip were counted. Each experiment was repeated at least three times, and the data were pooled and analyzed.

**SH2-Bβ Localization**—To confirm that SH2-Bβ localizes to membrane ruffles, we identified the localization of SH2-Bβ on GFP/Texas Red vector ratios according to the method proposed by T. Howard (36). A fluorescently labeled probe was localized in the F-actin cytoskeleton. The two-dimensional projection of a three-dimensional object, localized variations in fluorescence intensity may be due to regional differences in cell thickness (36). GFP- or GFP-SH2-Bβ-expressing cells were treated with GH or PDGF or left untreated, fixed, and permeabilized as described above. Cells were labeled with 25 μg/ml Texas Red-sulfonil chloride for 30 min. Digital images were taken using a microscope equipped with a cooled CCD camera (Spot-2, Diagnostic Instrumentation, Inc.) using a one-channel filter set, allowing simultaneous imaging of GFP and Texas Red fluorescence. This method protects against possible shifts during the overlay of two images obtained separately. Using Adobe Photoshop™, the two-color image was split into separate black-and-white images corresponding to the green and red channels. These images were thresholded to create binary masks using Metamorph software. Ratio images were obtained by dividing the green (GFP-positive) by the red (general staining) images. Parts of the cells where GFP fluorescence is concentrated, relative to cytoplasmic proteins, appear white on the ratio images. Where increased GFP fluorescence results from increased cell thickness, the ratio images are black. As expected, cells expressing GFP alone have black ruffles in the ratio images.

**Statistical Analysis**—Data from three or four separate transfections were pooled and analyzed using one-tailed unpaired t test. When individual experiments were analyzed, the results were indistinguishable from those obtained from the pooled data. Differences were considered to be statistically significant at p < 0.05. Results are expressed as the mean ± S.E.
within single cells. Since our SH2-B antibody does not specifically recognize SH2-B within 3T3-F442A cells by immunofluorescence procedures, we expressed Myc-tagged SH2-B in 3T3-F442A cells and performed immunocytochemistry with antibody to the Myc tag. SH2-Bb is detected at the plasma membrane and in the cytosol of unstimulated cells (Fig. 1C, left panel). No staining with the Myc antibody was observed in untransfected cells (Fig. 1C, right panel). Similar membrane and cytosol localization of SH2-Bβ was seen with GFP-tagged SH2-Bβ in 3T3-F442A cells, 2C4 human fibrosarcoma cells (data not shown), and PC12 cells (Fig. 1B). Thus, SH2-Bβ is present at the plasma membrane and in the cytosol of unstimulated cells.

GH Stimulates Membrane Ruffling and Lamellipodia Formation in 3T3-F442A fibroblasts—GH is known to stimulate actin reorganization in a variety of cell types (9). However, little is known about the mechanism of action of GH on the actin cytoskeleton. We first characterized the effect of GH on the actin cytoskeleton of 3T3-F442A cells. Subconfluent cultures of cells were deprived of serum overnight and stimulated with 500 ng/ml GH for 0–60 min. Filamentous actin was

FIG. 1. SH2-Bβ is present at the plasma membrane and in the cytosol. A, 3T3-F442A fibroblasts were fractionated into membrane (M) and cytosol (C) compartments as described under “Experimental Procedures.” Proteins (50 μg) were separated by SDS-PAGE and immunoblotted (IB) with αSH2-B. B, living PC12 cells stably expressing GFP-SH2-Bβ (GFP-SH2-B) were imaged by confocal microscopy in physiological saline. C, 3T3-F442A cells transiently transfected with cDNA expression vectors for Myc-tagged SH2-Bβ (Myc-SH2-B) or left untransfected (untransfected) were serum-deprived overnight, fixed, stained with α-Myc followed by α-mouse IgG-Oregon Green, and imaged by confocal microscopy. Scale bars, 10 μm.

FIG. 2. GH and PDGF induce membrane ruffling and lamellipodia formation in 3T3-F442A fibroblasts. 3T3-F442A fibroblasts were serum-deprived overnight, left untreated (−), or treated with either 500 ng/ml GH (GH) for 10 min or 5 ng/ml PDGF (PDGF) for 5 min. Cells were fixed, permeabilized, stained with Texas Red-phalloidin, and imaged by confocal microscopy. The arrows point to membrane ruffles. Scale bar, 30 μm.

FIG. 3. SH2-Bβ specifically localizes to membrane ruffles. A, 3T3-F442A cells transfected with cDNA expression vector encoding Myc-tagged SH2-Bβ were serum-deprived overnight, treated with 500 ng/ml GH or 5 ng/ml PDGF for 10 min, fixed, and stained with α-Myc, α-mouse IgG-Oregon Green, and Texas Red-phalloidin. Localization of Myc-SH2-Bβ and filamentous actin were imaged by confocal microscopy. Scale bar, 20 μm. B, cells transiently expressing GFP alone or GFP-SH2-Bβ were stimulated with GH, stained with Texas Red-sulfon-nyl chloride, and imaged by epifluorescence microscopy. Images were processed as described under “Experimental Procedures.” The arrows in A and B point to membrane ruffles. C, cells expressing GFP alone (GFP) or GFP-SH2-Bβ (WT) were stimulated with GH or PDGF and stained with Texas Red-sulfon-nyl chloride as in B. Bars represent mean ± S.E. percentage of SH2-positive ruffles.

2 J. Herrington and C. Carter-Su, unpublished observations.
stained with Texas Red-phalloidin. Confocal imaging revealed that prior to stimulation, cells possess typical flattened fibroblast morphology with numerous actin stress fibers (Fig. 2, left). GH induces a change in cell shape with the formation of broad, protrusive sheets (lamellipodia) with characteristic membrane ruffles rich in polymerized actin (Fig. 2, center, arrows). The response to GH was transient, peaking within 5–10 min and subsiding by 30–60 min, and was present in approximately 30–40% of the cells. Treatment of cells with 5 ng/ml PDGF for 5–10 min produced more extensive ruffling than GH and was present in the majority of cells (Fig. 2, right, arrows).

**SH2-Bβ Localizes to Membrane Ruffles**—We next examined the localization of SH2-Bβ in cells treated with GH or PDGF. Cells expressing Myc-tagged SH2-Bβ were treated with GH or PDGF for 5–10 min and processed for actin staining and immunocytochemistry against the Myc tag. Confocal imaging revealed that SH2-Bβ colocalizes with polymerized actin in membrane ruffles induced by GH or PDGF (Fig. 3A, arrows).

Membrane ruffling causes an increase in the amount of membrane in the light path of the microscope as the cell lifts off the substratum, an event that can complicate interpretation of specific localization to membrane ruffles. Confocal microscopy reduces but does not completely eliminate this problem. To verify that SH2-Bβ is specifically localized at membrane ruffles, we labeled GFP- or GFP-SH2-Bβ-expressing cells with the general cytoplasmic stain Texas Red-sulfonyl chloride and imaged the cells by fluorescence microscopy. The GFP fluorescence image was divided by the Texas Red fluorescence image, effectively normalizing the GFP images to the amount of total cellular protein present (see “Experimental Procedures”). After normalization, GFP-SH2-Bβ but not GFP alone was detectable at GH-induced ruffles (Fig. 3B, arrows). We counted the number of membrane ruffles with more GFP than Texas Red fluorescence (termed SH2-Bβ-positive ruffles). The percentage of total ruffles that were SH2-Bβ-positive is plotted in Fig. 3C. Approximately 80% of ruffles induced by either GH or PDGF have wild-type SH2-Bβ localized there. As expected, few ruffles were positive for GFP alone.

**SH2-Bβ Is Required for GH-induced Actin Reorganization**—To test whether SH2-Bβ participates in GH-stimulated actin reorganization, we overexpressed wild-type or mutant GFP-SH2-Bβ in cells and assayed membrane ruffling (Fig. 4). Cells expressing GFP alone served as a control. Cells overexpressing SH2-Bβ exhibited more pronounced ruffling in response to GH compared with GFP-expressing cells (Fig. 4B). To examine if SH2-Bβ is required for GH-induced membrane ruffling, we tested if mutant forms of SH2-Bβ might act as dominant negatives. Two mutants were assayed: the point mutant SH2-Bβ(R555E), in which the Arg within the FLVR motif of the...
SH2-B Required for GH-induced Actin Reorganization

SH2 domain critical for binding to phosphotyrosine is replaced by Glu, and truncation mutant ΔC555, which lacks a functional SH2 domain (Fig. 4A). Both mutants fail to associate with JAK2 with high affinity and to serve as substrates for JAK2 (35, 39). Both GFP-tagged SH2-Bβ(R555E) and GFP-tagged ΔC555 inhibited membrane ruffling induced by GH (Fig. 4B). Hence these mutants are effective dominant negative proteins and provide further evidence for a requirement of SH2-Bβ in reorganization of the actin cytoskeleton by GH. To better visualize the actions of SH2-Bβ on GH-dependent membrane ruffling, we captured lower magnification images to include both transfected cells and untransfected cells (as judged by GFP fluorescence) within the same field (Fig. 4C). As shown in Fig. 4C, left panel, the cell expressing GFP-SH2-Bβ has pronounced membrane ruffling in response to 50 ng/ml GH, whereas the untransfected cell in the same field does not. In contrast, the cell expressing GFP-tagged SH2-Bβ(R555E) in Fig. 4C, right panel, lacks membrane ruffles when treated with 500 ng/ml GH, whereas the adjacent untransfected cell has a large ruffle.

To quantify the dominant negative activity of mutant SH2-Bβ, we expressed GFP, wild type GFP-SH2-Bβ, or mutant GFP-SH2-Bβ in cells, treated with GH or PDGF, and counted the number of ruffles per cell. GFP-tagged SH2-Bβ(R555E) and ΔC555 inhibited ruffling at all concentrations tested (25–500 ng/ml, Figs. 4B and 5A). Overexpression of wild-type GFP, GFP-SH2-Bβ(R555E), or GFP-SH2-Bβ(ΔC) increased the number of ruffles per cell compared with cells expressing GFP alone at a submaximal concentration of GH (25 ng/ml; Fig. 5A) (GFP-SH2-Bβ, 2.0 ± 0.5 (n = 37); GFP, 1.2 ± 0.2 (n = 44)). Interestingly, the involvement of SH2-Bβ in PDGF-induced ruffling appears subtler, since GFP-tagged SH2-Bβ(R555E) and ΔC555 inhibition of PDGF-induced ruffling were most prominent at low concentrations of PDGF (Fig. 5B). Also, overexpression of SH2-Bβ did not enhance PDGF-induced ruffling when compared with cells expressing GFP alone (Fig. 5B) (GFP-SH2-Bβ, 1.2 ± 0.2 (n = 95); GFP, 1.4 ± 0.2 (n = 88)).

SH2-Bβ Is Required for GH-induced Pinocytosis—Membrane ruffling is closely associated with pinocytic activity (18, 19). To test if SH2-Bβ has a role in fluid phase macropinocytosis, we monitored the uptake of FITC-BSA by cells expressing Myc-tagged wild-type or mutant SH2-Bβ (Fig. 6). Myc-tagged Lin-7, a protein not expected to influence ruffling or pinocytosis, served as a control. In unstimulated cells, pinocytotic activity was very low (<5 vesicles/cell; data not shown). To trigger pinocytosis, GH or PDGF was added to the medium containing FITC-BSA. Fifty ng/ml GH, a concentration that produces strong ruffling (Fig. 5A), produced 17.0 ± 1.0 (n = 44) intracellular vesicles containing FITC-BSA per cell in cells expressing Lin and 17.4 ± 0.7 (n = 88) vesicles per cell in cells expressing SH2-Bβ, indicating a high rate of ongoing pinocy-
tosis (Fig. 6A). In contrast, the number of GH-induced pinosomes per cell in expressing mutant SH2-Bβ was lower by 65–75% (ΔC555, 4.5 ± 0.4 (n = 68); SH2-Bβ(R555E), 6.3 ± 1.0 (n = 86)). At a concentration of GH that produces a relatively small amount of ruffling (25 ng/ml GH), 8.0 ± 0.8 (n = 18) vesicles per cell were present in cells expressing the control Lin protein (Fig. 6A). In contrast, cells overexpressing wild-type SH2-Bβ had 12.9 ± 1.0 (n = 14) vesicles per cell, suggesting that SH2-Bβ enhances GH-induced pinocytic activity. PDGF (0.1 ng/ml) induced 20.4 ± 1.2 (n = 17) vesicles/cell in cells expressing Lin and 23.9 ± 1.2 (n = 16) vesicles/cell in cells expressing SH2-Bβ. Expression of mutant SH2-Bβ modestly inhibited (by 20–25%) pinocytosis induced by 0.1 ng/ml PDGF (ΔC555, 15.6 ± 8 (n = 12); SH2-Bβ(R555E), 16.3 ± 1.3 (n = 15)). At a submaximal concentration of PDGF for ruffling (0.05 ng/ml), wild-type SH2-Bβ did not produce a statistically significant enhancement of pinocytosis (Lin, 9.8 ± 1.0 (n = 12); SH2-Bβ, 11.3 ± 1.3 (n = 11)).

SH2-Bβ Regulates GH-induced Ruffling Irrespective of Modulation of the Kinase Activity of JAK2—One known activity of SH2-Bβ in GH signaling is to bind to JAK2 and enhance the kinase activity of JAK2 (39). Thus, one mechanism by which overexpression of wild-type or mutant SH2-Bβ might influence GH-dependent actin reorganization is through modulation of the activity of JAK2. To examine this possibility, we tested the action of ΔN504, a truncation mutant possessing a functional SH2 domain (Fig. 7A). This mutant protein, like wild-type SH2-Bβ, enhances JAK2 activity when overexpressed with JAK2 (35). Overexpression of ΔN504 inhibited GH-induced membrane ruffling compared with control cells expressing GFP alone (Figs. 7, B and C).

It is possible that overexpression of SH2-Bβ mutants in 3T3-F422A cells might have a general inhibitory action on GH signaling. To verify that this is not the case for ΔN504, we assayed GH-induced nuclear accumulation of GFP-Stat5B, an event dependent on JAK2 activity, in cells expressing wild-type or mutant SH2-Bβ. The nucleus-to-cytosol GFP fluorescence intensity ratio of control and GH-treated cells expressing GFP-Stat5B and Myc-tagged wild-type SH2-Bβ, SH2-Bβ(R555E), or ΔN504 was measured. SH2-Bβ(R555E) but not wild-type SH2-Bβ or ΔN504 inhibited GH-induced Stat5B nuclear accumulation (Fig. 8). SH2-Bβ(R555E) and ΔC555 inhibit JAK2 activity in overexpression systems (35, 39). Hence, the inhibitory action on actin reorganization of SH2-Bβ mutants lacking a functional SH2 domain (SH2-Bβ(R555E) and ΔC555) may be explained, at least in part, by reduced JAK2 activity. More importantly, cells expressing ΔN504 are GH-responsive as assayed by Stat5B nuclear accumulation. Thus, cells overexpressing ΔN504 fail to undergo GH-induced actin reorganization despite the presence of active JAK2, establishing a role for SH2-Bβ in GH-induced actin reorganization discrete from its role as a modulator of JAK2.

**DISCUSSION**

In this paper, we provide strong evidence that SH2-Bβ is required for GH-induction of membrane ruffling and fluid phase pinocytosis. In support of a requirement of SH2-Bβ for GH-induced actin reorganization, we show that overexpression of wild-type SH2-Bβ enhances membrane ruffling and pinocytosis induced by GH (Figs. 4 and 5A). Since SH2-Bβ is known to enhance the kinase activity of JAK2 when the two proteins are co-expressed (39), it is possible that the enhancement of GH-induced actin rearrangement may simply be a reflection of greater JAK2 activity. However, experiments with truncation mutant ΔN504 suggest a more specific role for SH2-Bβ in GH-induced actin reorganization. ΔN504, which contains a functional SH2 domain, is capable of enhancing the kinase activity of JAK2 in overexpression systems (35). Nevertheless, we find ΔN504 to be a potent inhibitor of GH-induced membrane ruffling (Fig. 7). Thus, the action of ΔN504 on membrane ruffling cannot simply be explained by an effect on JAK2 activity. We also find that cells expressing ΔN504 respond to GH in terms of Stat5B nuclear accumulation (Fig. 8), ruling out a global inhibitory action of this mutant on GH signaling. Therefore, ΔN504 specifically disrupts GH-induced actin rearrangement.

Further support for a role for SH2-Bβ in GH-induced actin reorganization comes from experiments with SH2-Bβ mutants lacking a functional SH2 domain (SH2-Bβ(R555E) and ΔC555). These mutants also potently inhibit GH-induced membrane ruffling and pinocytosis (Figs. 4 and 5A). Unlike ΔN504, SH2-Bβ(R555E) and ΔC555 inhibit JAK2 kinase activity and GH-induced Stat5B nuclear accumulation (Fig. 8; Ref. 35). Thus, the inhibitory effect of these mutants on GH-induced actin reorganization may be explained in part by inhibition of JAK2. However, inhibition of JAK2 activity probably cannot explain all of the inhibitory effect of SH2-Bβ(R555E) and ΔC555 on GH-induced actin reorganization. For example, SH2-
Bβ(R555E) and ΔC555 also inhibit ruffling and pinocytosis produced by PDGF, albeit less effectively than by GH (Figs. 5 and 6). These mutants are not thought to regulate PDGF-induced receptor tyrosine kinase activity (39). Further, SH2-Bβ(R555E) blocks nerve growth factor-induced neurite outgrowth without affecting the apparent kinase activity of nerve growth factor receptor TrkA (32). Taken together, our results with mutant SH2-Bβ favor a specific role of SH2-Bβ as a regulator of the actin cytoskeleton.

The mechanism(s) by which mutant forms of SH2-Bβ inhibit GH-induced actin rearrangement are unknown. We suspect that ΔN504, the C-terminal fragment containing the SH2 domain (35), lacks a binding site for effector molecules that link GH-activated JAK2 to the cytoskeleton. Overexpression of ΔN504 would be expected to bind to JAK2 and sequester JAK2 away from endogenous SH2-Bβ, thereby preventing the initiation of an SH2-Bβ signal to the cytoskeleton. SH2-Bβ mutants lacking a functional SH2 domain (SH2-Bβ(R555E) and ΔC555) are hypothesized to inhibit GH-dependent actin reorganization by a similar mechanism. Overexpression of these mutants may sequester effector molecules, preventing endogenous SH2-B from assembling a signaling complex active at actin rearrangement. We find that mutants SH2-Bβ(R555E) and ΔC555 cannot bind to JAK2 with high affinity and are not phosphorylated by JAK2 (35, 39). Thus, phosphorylation of SH2-Bβ, either on tyrosines or serines/threonines, may also be required for GH signaling to the actin cytoskeleton.

Membrane ruffling is closely associated with fluid phase pinocytosis, and ruffles may pinch off to form vesicles that are internalized (19). Fluid phase pinocytosis includes both macropinocytosis, by pinosomes greater than 0.2 μm in diameter, and micropinocytosis, by clathrin-coated vesicles and small uncoated vesicles (40). Macropinocytosis requires a functional actin cytoskeleton and is sensitive to cytochalasins, while micropinocytosis is actin-independent (41). We could not distinguish in our assay between these two types of pinosomes. Considering that SH2-Bβ is required for GH-induced membrane ruffling, it seems more likely that SH2-Bβ would be required for macropinocytosis but not actin-independent micropinocytosis. If so, some of the pinosomes detected in cells at “subruffling” concentrations of ligand and in cells expressing mutant SH2-Bβ (Fig. 6) may reflect SH2-Bβ-independent micropinocytosis. Thus, our data on the effects of mutant forms of SH2-Bβ are likely to be underestimates of the role of SH2-Bβ in actin-dependent pinocytosis.

The mechanism by which SH2-Bβ regulates actin reorganization is unknown. One potential mechanism is by intersecting with the cascade of Rho family G-proteins. Membrane ruffling and lamellipodia formation in response to PDGF is dependent on Rac (20). One could envision SH2-Bβ directly influencing ligand-induced Rac activity. However, SH2-Bβ lacks a Dbl (or DH) domain characteristic of guanine nucleotide exchange factors for Rho family GTPases (42), suggesting that SH2-Bβ is probably not a Rac guanine nucleotide exchange factor. Also, SH2-Bβ lacks a CRIB motif, the domain that often mediates interaction of target proteins with Cdc42 and Rac (43). SH2-Bβ may act upstream or downstream of Rac and other Rho GTPases. PI 3-kinase is required for actin reorganization induced by PDGF and insulin, acting upstream of Rac (22, 23). GH-induced membrane ruffling and actin reorganization is inhibited by wortmannin (9), suggesting a requirement for PI 3-kinase. GH activation of PI 3-kinase is thought to involve the insulin receptor substrate proteins, including insulin receptor substrate-1 and -2 (44–46). It will be important to determine if SH2-Bβ lies upstream or downstream of PI 3-kinase and Rho family GTPases in the regulation of the actin cytoskeleton by GH.

We find SH2-Bβ at the plasma membrane and in the cytoplasm of unstimulated cells (Fig. 1). When cells are treated with either GH or PDGF, SH2-Bβ specifically localizes to membrane ruffles (Fig. 3). The mechanism of SH2-Bβ localization to membrane ruffles is unknown. We have found that the SH2 domain is not required. The binding of the pleckstrin homology domain of SH2-Bβ to a local accumulation of phosphoinositides at ruffles may be responsible for SH2-Bβ localization.

3 M. Diakonova, J. Herrington, and C. Carter-Su, manuscript in preparation.
at these sites. Alternatively, SH2-Bβ may be recruited to ruffles by binding to another protein. Membrane ruffles are active centers of signal transduction containing several signaling molecules (17). Several potential candidates exist. One candidate is JAK2, which has been shown to interact with focal adhesion kinase in Chinese hamster ovary cells (13). However, localization of JAK2 at sites of actin reorganization has not been reported. JAK2 is generally found in the cytoplasm and nucleus (47, 48). Another candidate is Grb2. SH2-B, presumably by binding to the Src homology-3 (SH3) domain of Grb2 (33). Glutathione-S-transferase-Grb2 fusion protein localizes to membrane ruffles when injected into cells (49). Other SH3 domain-containing proteins have been localized to membrane ruffles, including the actin-binding proteins cortactin, myosin I, and fodrin/spectrin (50–53). SH2-Bβ may localize to membrane ruffles by one of its proline-rich regions binding to the SH3 domain of one of these proteins or an as yet to be identified protein.

In summary, our data suggest that SH2-Bβ is required for GH-dependent actin reorganization. How SH2-Bβ localizes to membrane ruffles and regulates the actin cytoskeleton awaits identification of more binding partners for SH2-Bβ. Understanding the role of SH2-Bβ in GH regulation of the cytoskeleton should provide important insight into how GH regulates diverse processes such as cell proliferation and differentiation.

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