SH2-Bβ is a Rac Binding Protein that Regulates Cell Motility*

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SUMMARY

The Src homology 2 (SH2) domain-containing protein SH2-Bβ binds to and is a substrate of the growth hormone (GH) and cytokine receptor-associated tyrosine kinase JAK2. SH2-Bβ also binds, via its SH2 domain, to multiple activated growth factor receptor tyrosine kinases. We have previously implicated SH2-Bβ in GH and platelet-derived growth factor (PDGF) regulation of the actin cytoskeleton. We extend these findings by establishing a potentiating effect of SH2-Bβ on GH-dependent cell motility and defining regions of SH2-Bβ required for this potentiation. Time-lapse video microscopy, phagokinetic, and/or wounding assays demonstrate reduced movement of cells overexpressing SH2-Bβ lacking an intact SH2 domain due to a point mutation or a C-terminal truncation. An N-terminal proline-rich domain (amino acids 85-106) of SH2-Bβ is required for inhibition of cellular motility by SH2 domain deficient mutants. Co-immunoprecipitation experiments indicate that Rac binds to this domain. GH is shown to activate endogenous Rac and dominant negative mutants of SH2-Bβ are shown to inhibit membrane ruffling induced by constitutively active Rac. These findings suggest that SH2-Bβ is an adapter protein that facilitates actin rearrangement and cellular motility by recruiting Rac and potentially Rac-regulating, Rac effector or other actin-regulating proteins to activated cytokine (e.g. GH) and growth factor receptors.
INTRODUCTION

Cell migration is critical for many vital biological functions, including embryonic development, the inflammatory immune response, wound repair, tumor formation and metastasis, and tissue remodeling and growth. The actin cytoskeleton provides both the protrusive and contractile forces required for cell migration via a combination of actin polymerization and depolymerization, actin filament cross-linking, and the interaction of myosin-based motors with actin filaments (1). The complexity of cell motility and the fact that it is regulated by many hormones, cytokines and growth factors, including growth hormone (GH) (2) and platelet-derived growth factor (PDGF) (3) suggest that multiple signaling mechanisms exist to regulate this process. Multiple signaling proteins have been implicated in the regulation of the actin cytoskeleton and cellular motility. Prominent among these are members of the Rho small GTPase family of proteins (4,5). Activated Cdc42 has been implicated in the formation of filopodia, long thin extensions containing actin bundles. Activated Rac has been implicated in the formation of lamellipodia and membrane ruffles, broader, web-like extensions. Activated Rho is thought to mediate the formation of stress fibers, elongated actin bundles that traverse the cells and promote cell attachment to the extracellular matrix through focal adhesions. A number of other proteins have been implicated in the regulation of the actin cytoskeleton, including Rho family effector (e.g. PAK, N-WASP, ROCK) and regulatory proteins (e.g. GEFs, GAPs and GDIs), adapter or scaffolding proteins (e.g. Nck, Crk, Grb2), and cytoskeleton regulating/binding proteins (e.g. WASP family, Arp2/3 complex, profilin, ADF/cofilin, gelsolin) (6-8). However, how all these molecules work together to enable cells to precisely regulate their actin
Recently, our laboratory implicated the SH2 domain containing protein SH2-Bβ in the regulation of the actin cytoskeleton by GH and PDGF (9) and in nerve growth factor (NGF)-induced morphological differentiation of PC12 cells into neuronal-like cells (10). We demonstrated that SH2-Bβ co-localizes with actin in membrane ruffles. Furthermore, introduction into cells of mutant SH2-Bβ lacking a functional SH2 domain inhibited GH and PDGF-dependent membrane ruffling and pinocytosis and blocked NGF-induced morphological differentiation of PC12 cells. SH2-Bβ is a 670 amino acid, widely-expressed, SH2 domain containing protein that was originally identified by our laboratory in a yeast 2-hybrid screen as a binding partner of the tyrosine kinase JAK2 (11). JAK2 binds to multiple members of the cytokine family of receptors, including the GH receptor and is activated in response to ligand binding to those receptors. This activation of JAK2 is thought to be the initiating step in cytokine receptor signaling (12). SH2-Bβ and/or other isoforms (α, γ) of SH2-B have also been shown to bind, via their SH2 domain, to the activated form of multiple receptor tyrosine kinases, including the receptors for PDGF, NGF (TrkA), insulin, and insulin-like growth factor (11,13-17), suggesting that SH2-Bβ may play a fundamental role in cell function. SH2-Bβ lacks a known enzymatic domain, but contains multiple protein and/or lipid interacting domains, including a pleckstrin homology domain and three proline-rich regions in addition to its SH2 domain (Fig 1). This domain structure suggests that SH2-B isoforms may serve as adapter or scaffolding proteins that recruit proteins to activated membrane receptor complexes. Consistent with this, SH2-B isoforms are also phosphorylated on tyrosines in response to GH, PDGF, and
NGF (10,11,15,18); phosphorylated tyrosines often serve as binding sites for proteins containing SH2 or PTB domains (19). SH2-Bβ has been shown to activate JAK2 (13) and TrkA (20). However, some SH2-Bβ mutants that act as dominant negatives for membrane ruffling and neuronal differentiation do not appear to block kinase activity, suggesting that SH2-Bβ serves a function in cytokine and growth factor signaling in addition to regulation of kinase activity. Taken together, these observations suggest that SH2-Bβ may regulate the actin cytoskeleton by serving as a scaffolding or adapter protein that recruits to activated receptor kinases molecules that regulate the actin cytoskeleton.

In this work, we extend our initial findings implicating SH2-Bβ in the regulation of the actin cytoskeleton by providing strong evidence that SH2-Bβ is required for maximal cell motility using time-lapse video microscopy and two motility assays (phagokinetic and wounding). GH is shown to stimulate cell motility and two regions of SH2-Bβ (the SH2 domain and an N-terminus proline-rich domain [amino acids 85-106]) are implicated in the effect of SH2-Bβ on GH-stimulated cell motility. GH is shown to activate the small GTPase Rac and Rac is shown to bind to SH2-Bβ; the binding requires amino acids 85-106 of SH2-Bβ. Overexpression of dominant negative mutants of SH2-Bβ block the ability of constitutively active Rac to promote membrane ruffling, consistent with intact SH2-Bβ being required for Rac promotion of membrane ruffling. These results suggest that SH2-Bβ acts as an adapter or scaffolding protein that recruits Rac and potentially Rac regulating proteins, Rac effector proteins and/or other actin-regulating proteins to membrane cytokine and growth factor receptors.
EXPERIMENTAL PROCEDURES

**Cells and Reagents --** The stock of murine 3T3-F442A fibroblasts was provided by Dr. H. Green (Harvard University, Cambridge, MA). Recombinant human GH was the gift of Eli Lilly Co. Monoclonal anti-Myc antibody (αMyc; 9E10) and rabbit anti-mouse IgG were obtained from Santa Cruz Biotechnology. Ascites fluid containing 9E10, used for immunoprecipitation experiments (1:200 dilution), was obtained from the Michigan Diabetes Research and Training Center (MDRTC) Hybridoma Core Facility. Polyclonal antibodies raised against a glutathione S-transferase fusion protein containing the C-terminal portion of SH2-Bβ has been described previously (11). Monoclonal αGFP (Living Colors A.v. JL-8) was obtained from Clontech Laboratories, Inc. and monoclonal αRac (clone 238A8) was from Upstate Biotechnology. Goat anti-mouse -Cy5 and Texas Red-phalloidin were from Molecular Probes, Inc.

**Plasmids --** cDNAs encoding GFP-tagged wild-type SH2-Bβ, SH2-Bβ(R555E), SH2-Bβ(1-555) and SH2-Bβ(504-670) have been described previously (13,14). cDNAs encoding GFP-tagged (1-85; 1-106; 504-670 (R555E)) or Myc-tagged (61-670; 118-670; 118-670 (R555E)) truncation mutants of SH2-Bβ were made in our laboratory by introducing appropriate restriction sites or stop codons using Stratagene’s QuikChange kit and SH2-Bβ or SH2-Bβ (R555E) as a template (Gunter D et al., in preparation). cDNAs encoding mutant Rac N17, V12 and L61 (21) were used with the permission of A. Hall (University College, London). cDNA encoding GST-PBD domain of PAK1 was a gift of G. Bokoch (Scripps Research Institute).
Institute, La Jolla, CA). cDNA encoding rat GH receptor (22) was provided by G. Norstedt (Karolinska Institute, Stockholm, Sweden). cDNA encoding JAK2 was a gift of J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN).

Cell Culture and Transfection -- 3T3-F442A and 293T cells were cultured as described (18). 3T3-F442A cells were plated on coverslips and transfected with 2.5 µg (total) cDNA using Transfast (Promega). 293T cells plated in 100 mm dishes were transfected with 10 µg cDNA using calcium phosphate precipitation.

Video Microscopy -- 36 h after transfection, 3T3-F442A cells on coverslips were incubated in serum-free medium overnight. Cells were observed by phase-contrast optics in a Nikon TE300 inverted microscope with shutter-controlled illumination (Uniblitz, Rochester, NY). Cells were recorded for 2 min, treated with GH as indicated and recorded further. Images were collected using a cooled, digital CCD camera (Quantix, Photometrics, Tucson, AZ) and were recorded (1 frame/15 sec) and stored using MetaMorph 2.0 image analysis software (Universal Imaging, West Chester, PA).

To quantify lamellipodia extension and retraction, individual transfected cells were located with a FITC filter set. Binary images of the cell profile were generated from the video frames using MetaMorph. Subtracting a binary image from the subsequent one produced a binary image of areas of extension, and subtracting the second binary image from the first produced a binary image of areas of retraction (23). The area of extension (retraction) was
normalized to the cross-sectional area of the cell. Six cells expressing GFP alone or GFP-SH2-Bβ (R555E), 5 cells expressing GFP-SH2-Bβ, and 3 cells expressing GFP-SH2-Bβ (1-555) (data not shown) were videotaped and analyzed.

**Phagokinetic Assay** -- For the phagokinetic assay (24), cells were plated on colloid gold-covered coverslips 48h after transfection. After 2 or 24h, the coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature and mounted on slides. Individual transfected cells expressing GFP-tagged forms of SH2-Bβ were located with a FITC filter set using a Nikon TE200 microscope. Phase-contrast images were collected and analyzed by NIH Image software. The particle-free area indicating where cells moved was measured for 10 cells each in 3 (3T3-F442A cells) or 4 (293T cells) independent experiments. The phagokinetic index was calculated as a ratio of the particle-free area to the cross-sectional area of the cell.

**Wounding Assay** -- Migration of cells into a wound was assessed as described (25,26). 293T cells were transfected with cDNAs encoding epitope-tagged forms of SH2-Bβ, GFP or Myc-tagged Lin-7, with or without cDNA encoding rat GH receptor. Cells were re-plated at both high (5 x 10^5 cells per dish) and low (1.5 x 10^5 cells per dish) density onto 100-mm-diameter dishes. After 2 days, when cells in the high-density dish reached a monolayer, a plastic pipet tip was drawn across the center of some of the plate several times to produce a clean 1-mm-wide wound area of some of the culture (Fig. 5). After a further 24h incubation to permit migration of cells into the empty area, migration of the cells was examined using a phase contrast microscope and the GFP-tagged cells visualized by fluorescence microscopy. In other cells
(70-80% confluent) (Fig 6), cultures were serum-deprived for 24 h before being wounded. Those cells were rinsed with PBS and incubated with DMEM + 0.5% calf serum with GH as indicated. After a further 24 h incubation to permit migration of cells into the empty area, migration of the cells was examined using a phase contrast microscope with a calibrated eyepiece. The distance between the leading edge of the migrating cells from the edge of the wound was measured. The number of tag-positive and tag-negative cells in the wound area were counted (100 cells total /wound). The number of tag-positive and negative cells (100 cells total) were also counted in the low density, non-wounded plate. For Myc-tagged plasmids, cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100 and stained with αMyc. To take into account the efficiency of transfection, a motility index was calculated as a ratio of % tag-positive cells migrating into the wound area to the % tag-positive cells in the low density dish. To confirm that differences in the number of cells present in the wound were due to differences in motility rather than in cell proliferation, serum-deprived, wounded and GH (500 ng/ml) treated cells expressing GFP alone, GFP-SH2-Bβ and GFP-SH2-Bβ(R555E) were analyzed by Fluorescent Activated Cell Sorting (FACS) (Elite, ESP) in parallel with non-wounded but serum-deprived and GH-treated cells. The percentage of GFP positive cells was similar for wounded, GH treated plates and non-wounded, nonhormone-treated plates.

Furthermore, the majority (60-70%) of the cells in all plates were stopped in the G1 phase of the cell cycle (data not shown) in contrast to cells maintained in serum in which >60% of the cells were in S phase as determined by FACS sorting of DNA stained cells (propidium iodide, Hoechst or DAPI). Ten wounds were sampled for each type of transfection. Each experiment was repeated at least 3 times with similar results.
Detection of Activated Rac, Co-Immunoprecipitation and Immunoblotting -- Activated Rac was detected using the PAK effector binding domain (PBD) (27). 293T cells overexpressing GH receptor or 3T3-F442A cells were deprived overnight and treated with 500 ng/ml GH. Cells were lysed and solubilized proteins incubated with GST-PBD and glutathione-agarose beads (Sigma-Aldrich). Bound proteins were immunoblotted with αRac (1:1000 dilution) and analyzed using enhanced chemiluminescence (ECL) (Amersham). Constitutively active Rac L61 expressed in 293T cells was used as a positive control (data not shown). For experiments investigating interaction between forms of GFP-SH2-Bβ and either Myc-Rac N17, Myc-Rac V12 or Myc-Rac L61, Rac was immunoprecipitated with αMyc using rabbit anti-mouse and protein A-agarose. SH2-Bβ was visualized by immunoblotting with αGFP.

Immunocytochemistry -- 3T3-F442A cells on coverslips were co-transfected with cDNAs encoding different forms of GFP-tagged SH2-Bβ and Myc-tagged Rac. 48 h later, cells were fixed for 15 min in 4% paraformaldehyde in intracellular buffer consisting of 30 mM HEPES, pH 7.4, 10 mM EGTA, 0.5 mM EDTA, 5mM MgSO4, 33 mM potassium acetate, 5% PEG400 and 0.02% NaN3. Cells were permeabilized with 1% Triton X-100 in intracellular buffer containing 4% paraformaldehyde for 15 min (28). Coverslips were incubated with αMyc followed by goat anti-mouse Cy5. F-actin was stained with Texas Red-phalloidin. Confocal imaging was performed with a Noran Oz laser scanning confocal microscope (Morphology and Image Analysis Core of the MDRTC). The images presented in the figures are representative of at least 3 separate experiments.
Statistical Analysis—Data from at least three separate transfections were pooled and analyzed using a 2 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. In all imaging experiments, measurements were taken before determining the identity of the cells on the plate/coverslip.
RESULTS

**SH2-Bβ Is Required for GH-induced Lamellipodia Dynamics**-- To examine the role of SH2-Bβ in cell motility, we first made a time-lapse recording of 3T3-F442A fibroblasts overexpressing GFP-tagged wild-type SH2-Bβ or an SH2 domain-deficient version of this protein (SH2-Bβ (R555E)). 3T3-F442A cells are highly responsive to GH (29,30). In SH2-Bβ(R555E), the critical Arg (R) within the FLVR motif of the SH2 domain is mutated to Glu (E). This mutant exhibits substantially reduced binding to JAK2 and fails to serve as a substrate of JAK2 (13). Transfected cells were incubated overnight in serum-free medium. GFP-positive cells were located using a FITC filter set. Recording was initiated and then GH was added. Because these cells did not show directional movement during the time of observation, our study assessed lamellipodia activity. The term "lamellipodia" is used here to refer to dynamic actin-rich extensions at the periphery of the cell (31). Lamellipodia activity was quantified by measuring the percentage of cell retraction and extension.

Addition of 500 ng/ml GH resulted within 10 min in high lamellipodia activity of untransfected cells (Fig 2A, top 2 rows) and cells overexpressing GFP (Fig 2A, top row) or GFP-SH2-Bβ (not shown) (transfected cells are indicated in the leftmost panels). Lamellipodia activity was often accompanied by the appearance and centripetal movement of ruffles. In contrast, cells expressing SH2-Bβ(R555E) (Fig 2A, second row panels) exhibited very little lamellipodia activity. When cells were treated with 25 ng/ml GH (Fig 2A, bottom 2 rows), a concentration of GH reported previously to cause minimal ruffling in untransfected cells and
cells overexpressing GFP (9)), only minimal lamellipodia activity was detected in untransfected cells or cells overexpressing GFP (Fig 2A, bottom rows). In contrast, substantial lamellipodia activity was detected in cells overexpressing full-length SH2-Bβ (Fig 2A, bottom row).

A plot of lamellipodia activity over time is shown (Fig 2B) for single representative cells treated with the higher concentration of GH (500 ng/ml). GH stimulated similar levels of lamellipodia activity in cells expressing GFP alone (Fig 2B, top panel) or GFP-SH2-Bβ (Fig 2B, middle panel); activity was maximal 10 min after GH addition. In contrast, GH stimulated only minimal lamellipodia activity in cells expressing SH2-Bβ(R555E) (Fig 2B, bottom panel). The data in Figs 2A and B show that overexpressing SH2-Bβ enhances and overexpressing SH2-Bβ(P555E) inhibits GH-induced lamellipodia activity, suggesting that SH2-Bβ with an intact SH2 domain is required for maximal GH-induced lamellipodia activity.

*The SH2 Domain and N-Terminus of SH2-Bβ Are Required for Maximal Cell Motility as Assessed by a Phagokinetic Assay*-- To examine further the effect of SH2-Bβ on cell motility, we performed a gold particle motility (or phagokinetic) assay. This assay measures the disappearance of gold particles from a coverslip, a process requiring both phagocytosis and locomotion. Cells transfected with the desired cDNA were plated on coverslips coated with colloidal gold particles and incubated at 37°C for either 2h (Fig 3A-H) or 24 h (Fig 3I and Fig 4). Cells remove particles while they move, thereby producing areas that are free of colloidal gold. These areas were quantified using NIH Image analysis software.
Initially we measured the motility of 3T3-F442A fibroblasts. Fibroblasts are known to be very motile (32). 3T3-F442A fibroblasts expressing GFP alone (Fig 3A) or GFP-SH2-B\(\beta\) (Fig 3C) and identified as GFP-positive cells using a FITC filter set, removed gold particles from a portion of the coverslip during 2h. Cells expressing GFP-SH2-B\(\beta\)(1-555) lacking a functional SH2 domain removed gold particles from a substantially smaller area (Fig 3E), as predicted from the video microscopy experiments. Like the SH2 domain-deficient mutants of SH2-B\(\beta\), the C-terminus of SH2-B\(\beta\) (SH2-B\(\beta\)(504-670)) was shown previously to inhibit GH-induced membrane ruffling (9). The difference in phagokinetic properties between cells transfected with SH2-B\(\beta\)(504-670) and non-transfected cells is clearly seen in Fig 3G that contains both a transfected and nontransfected cell. The GFP-positive cell (right side) removed very few gold particles while the nontransfected cell (left side) produced clear gold-free areas. The area lacking gold particles after 24h was quantified and normalized for multiple cells. The phagokinetic index was calculated and plotted in Fig 3I. Cells expressing either GFP-SH2-B\(\beta\)(1-555) or GFP-SH2-B\(\beta\)(504-670) demonstrated reduced phagokinesis.

To determine whether SH2-B\(\beta\) is important for cell motility in non-fibroblast cells, we tested cultured human embryonic kidney (293T) cells, monkey embryonic kidney (COS) cells and Chinese hamster ovary (CHO) cells for their ability to phagocytose colloid gold during 24h (Fig 4). These 3 cell lines are all epithelial derived and are more amenable to transfection than 3T3-F442A cells. Although phagokinesis of these 3 cell types was significantly less than that observed for 3T3-F442A fibroblasts (Fig 4E), all formed visible areas that could be measured (Fig 4A-D).
As predicted from the studies using 3T3-F442A fibroblasts, 293T cells expressing GFP alone or GFP-SH2-Bβ or non-transfected cells removed gold particles from similar areas of the coverslips (Fig 4F). Cells expressing SH2-Bβ(1-555), SH2-Bβ(R555E) or SH2-Bβ(504-670) removed substantially fewer particles. These results suggest that both the SH2 domain and N-terminal part of SH2-Bβ are required for maximal cell motility.

Cell Migration in Response to GH Depends on the SH2 Domain and Amino acids 85-106 of SH2-Bβ as Assessed by a Wounding Assay -- To provide further evidence that SH2-Bβ is required for GH-stimulated cell motility, we assessed cell movement with a wounding assay. In initial experiments, migration of 293T cells expressing GFP-tagged SH2-Bβ or SH2-Bβ(R555E) into the wound was assessed after 24 h in 10% calf serum. Fig 5 illustrates that the distance between the leading edge of all migrating cells from the edge of the wound, visualized using a phase contrast microscope, was greater for cells expressing SH2-Bβ than for cells expressing SH2-Bβ(R555E), as predicted from the phagokinetic assay. In addition, the number of GFP positive cells migrating into the wound was significantly greater for cells expressing SH2-Bβ than for cells expressing SH2-Bβ(R555E), as assessed by fluorescence microscopy (Fig 5).

To confirm that SH2-Bβ also contributes to GH-stimulated cell motility, as suggested by the videomicroscopy experiments, 293T cells expressing rat GH receptor and various epitope-tagged forms of SH2-Bβ were tested for their ability to migrate into a wound in response to GH. To ensure that any differences in the number of cells in the wound would be due to
differences in cell motility rather than in rates of cell proliferation, cells were serum-deprived for 24h to establish quiescence. To start migration, cells were wounded and then treated with GH. Cell migration into the wound was assessed after 24h by measuring the distance between the leading edge of all migrating cells from the edge of the wound using a phase contrast microscope with a calibrated eyepiece (Fig 6A). Under these conditions, GH was shown not to stimulate cell proliferation as judged by FACS analysis of DNA stained cells (data not shown). Without GH, cells expressing vehicle alone, SH2-Bβ or SH2-Bβ(R555E) exhibited limited movement (Fig 6A). A submaximally stimulating concentration of GH (25 ng/ml) did not stimulate migration of control cells but cells overexpressing SH2-Bβ exhibited a substantial increase (~3 times the distance in the absence of GH) in their movement into the wound (Fig 6A), consistent with the video microscopy experiments. When cells were treated with 500 ng/ml GH for 24h, control cells moved 5 times the distance seen in the absence of GH. The presence of GFP-SH2-Bβ (R555E) strongly reduced that migration (by 60%). Control experiments established that the percentage of GFP-positive cells did not change over the course of the experiment, indicating that under the conditions of this experiment, ectopic expression of SH2-Bβ or GFP-SH2-Bβ (R555E) did not detectably affect the rate of cell proliferation.

The findings in Figs 2, 4, 5 and 6A that SH2-Bβ(R555E) inhibits cell movement suggest that regions of SH2-B outside of the SH2 domain are required for the potentiating effect of SH2-Bβ on cell movement. Lacking a functional SH2 domain, SH2-Bβ(R555E) would not be expected to bind with high affinity to activated, tyrosyl phosphorylated cytokine receptor-JAK
complexes or growth factor receptors (10,14,17,18). It would thus not be expected to compete with endogenous SH2-Bβ for binding to these receptor complexes. Rather, SH2-Bβ(R555E) seems more likely to be acting as a dominant negative by recruiting critical molecules (e.g. proteins, lipids) away from endogenous SH2-Bβ, thereby rendering endogenous SH2-Bβ less effective. The fact that SH2-Bβ(1-555) also acts as a dominant negative (Figs 3 and 4) suggests that one or more of these critical molecules bind within the first 555 amino acids of SH2-Bβ. To identify the region(s) of SH2-Bβ that bind these accessory molecules, we tested different N- and C-terminal truncations of SH2-Bβ for their ability to inhibit cell motility. Control experiments verified that the different mutants were expressed to similar extents and at levels an estimated 60 fold or greater than endogenous SH2-B (data not shown). Fig 6A illustrates that while SH2-Bβ(1-106) inhibits cell motility to the same extent as SH2-Bβ(R555E), SH2-Bβ(1-85) has no effect on cell motility, suggesting that amino acids 85-106 are required for the dominant negative effect of SH2 domain deficient forms of SH2-Bβ. Consistent with this, R555E(118-670) and R555E(504-670) which also lack both an intact SH2 domain and amino acids 85-106 have no effect on cell motility. Consistent with amino acids 85-106 of SH2-Bβ binding an accessory molecule that needs to be recruited to activated JAK2-cytokine receptor complexes for SH2-Bβ to potentiate cell motility, SH2-Bβ(118-670) and SH2-Bβ(504-670) both act as dominant negatives whereas SH2-Bβ(61-670) does not (Fig 6). Presumably this is because, like wild-type SH2-Bβ and SH2-Bβ(61-670), SH2-Bβ(118-670) and SH2-Bβ(504-670) contain an SH2 domain and can compete with endogenous SH2-Bβ for binding to JAK2. However, in contrast to wild-type SH2-Bβ and SH2-Bβ(61-670), they cannot recruit the accessory molecule(s) that bind to amino acids 85-106 to the activated JAK2-GH receptor complex.
In parallel to the measurements of the migration of the entire population of cells, we also calculated a migration index as the ratio of % tag-expressing cells migrating into the wound to the % tag-expressing cells in the total population (25). Cells expressing control plasmids alone gave a motility index close to 1, as expected if the epitope tag itself has no effect on motility (Fig 6B). Many GFP-positive cells migrate into the wound when cells expressed GFP-SH2-B\(\beta\) (Fig 5A); in the presence of GH, more GFP positive cells migrated into the wound than when the cells expressed GFP alone (Fig 6B). When cells overexpressed GFP-SH2-B\(\beta\)(R555E), only a modest number of GFP-positive cells migrated into the wound (Fig 5C and 6B), whether cells had seen GH or not. The migration index was dramatically reduced when cells expressed epitope-tagged 1-106, 118-670 and 506-670. In contrast, the migration index for cells overexpressing amino acids 118-670 or 504-670 of SH2-B\(\beta\)(R555E) or 1-85 was close to one indicating that these mutants do not affect cell motility (neither mimic wild-type SH2-B\(\beta\) nor act as dominant negatives). Further, cells expressing amino acids 61-670 showed the same elevation in motility as cells overexpressing wild-type SH2-B\(\beta\), consistent with this form of SH2-B\(\beta\) containing all the domains necessary to regulate the actin cytoskeleton. The results of Fig 6 therefore support the notion that amino acids 85-106 and the SH2 domain are required for the potentiating effect of SH2-B\(\beta\) on cell motility.

**GH Activates Rac and SH2 Domain Deficient Mutants of SH2-B\(\beta\) Inhibit Rac-induced Cell Ruffling** -- Activated Rac is one of the major actin-regulating proteins implicated in cell ruffling, lamellipodia formation and cell motility (21,33,34). We therefore considered Rac a good candidate for the molecule that bound to amino acids 85-106 of SH2-B\(\beta\). We first
verified that GH activates Rac by assessing the ability of GH to promote binding of endogenous Rac to the Rac binding domain (PBD) of PAK fused to GST (27). This domain binds only to the active GTP-bound form of Rac (35). As expected, 3T3-F442A cells after overnight serum deprivation had a minimal amount of activated Rac (Fig 7A, lane1). GH (500 ng/ml) led to the activation of endogenous Rac within 5 min (Fig 7A, lanes 2-3).

To determine whether Rac might be one of the binding partners of SH2-Bβ necessary for the potentiating effect of SH2-Bβ on cell motility, we tested whether SH2-Bβ, when overexpressed with constitutively active Rac, would affect cell ruffling. We co-transfected 3T3-F442A cells with cDNA encoding constitutively active mutant RacL61 together with cDNA encoding either wild-type SH2-Bβ or one of the SH2 domain deficient mutants (1-555, R555E). Cells expressing either constitutively active form of Rac showed extensive membrane ruffling (data not shown). Cells co-expressing SH2-Bβ and Rac L61 (Fig 7B, top panel) were as effective as cells expressing RacL61 alone at inducing actin polymerization and formation of lamellipodia, the latter often seen folding back upon themselves to form membrane ruffles (Fig 7B, top right panel, arrow). However, no lamellipodia or membrane ruffles were observed in cells overexpressing Rac L61 and either SH2-Bβ(1-555) (Fig 7B, bottom panel) or SH2-Bβ(R555E) (data not shown).

Because the level of expression of transfected proteins varies within cells of a population, we scored the formation of ruffles seen with overexpression of constitutively active Rac V12 or Rac L61 and SH2-Bβ(1-555) (Fig 7C). Membrane ruffling was observed in around 90% of
cells expressing constitutively active Rac. However, membrane ruffling was observed in only
20% of the cells that overexpressed Rac V12 and SH2-Bβ(1-555). Cells overexpressing the
more potent, in terms of membrane ruffling, constitutively active form of Rac (Rac L61) and
SH2-Bβ(1-555) also showed decreased ruffling compared to cells expressing Rac L61 alone,
although to a lesser extent (40% of cells showed no ruffles). From these experiments we
conclude that intact SH2-Bβ is required for the stimulatory effect of Rac on membrane ruffling.

**SH2-Bβ Binds Directly to Rac Independent of the Activation State of Rac and the
Tyrosine Kinase JAK2** -- Before using 293T cells to examine whether Rac binds to SH2-Bβ,
we confirmed that GH activates endogenous Rac in 293T cells transiently expressing GH
receptor. Activation of endogenous Rac was observed after 10 min incubation with 500 ng/ml
GH (Fig 8A). To determine whether SH2-Bβ binds to Rac, 293T cells were co-transfected with
cDNA encoding GFP-SH2-Bβ and either constitutively active Myc-Rac V12 or dominant
negative Myc-Rac N17. Rac was immunoprecipitated using αMyc and SH2-Bβ visualized by
blotting with αGFP. Fig 8B shows that SH2-Bβ co-immunoprecipitated with both Rac V12 and
Rac N17. In some experiments, JAK2 (Fig 8B, lanes 2, 4) was included to see if Rac association
with SH2-Bβ is affected by JAK2 which both forms a complex with SH2-Bβ and tyrosyl
phosphorylates it. The association of Rac with SH2-Bβ was not altered by the presence of
JAK2. However, the slower migrating tyrosyl phosphorylated form of SH2-Bβ was visible in
the αRac immunoprecipitates when JAK2 was present (verified by reprobing with antibody to
phosphotyrosine, data not shown). Taken together, these results indicate that both inactive and
active forms of Rac bind to SH2-Bβ and equally well to the tyrosyl phosphorylated and non-
phosphorylated forms of SH2-Bβ.

To identify the region of SH2-Bβ to which Rac binds, we co-expressed in 293T cells Myc-Rac L61 with various truncated forms of GFP-SH2-Bβ. Fig 8C shows that Rac L61 binds to full length SH2-Bβ and SH2-Bβ(1-106) but not to SH2-Bβ(1-30) or SH2-Bβ(1-85), suggesting that amino acids 85-106 contain a binding site for Rac.
DISCUSSION

Cell motility is a complex process involving the coordinated extension of lamellipodia, subsequent formation of new focal contacts to the underlying substratum at the leading edge, generation of traction and tension within the cell cortex, and release of focal contacts at the rear of the cell (1). Each of these events depends on the dynamics of the actin cytoskeleton that are orchestrated by a host of signaling molecules and actin-binding proteins. Many of the molecules that organize and regulate the actin cytoskeleton have been identified (for review: (7,8,36)), and attention is shifting to understanding how those molecules interact to produce movement and are regulated by growth factors, hormones and cytokines. In a previous study (9), we provided evidence that SH2-Bβ is required for induction of membrane ruffling and fluid phase pinocytosis by GH. Here we provide strong evidence that SH2-Bβ is an adapter or scaffolding protein that plays a role in cell motility. We also provide evidence that SH2-Bβ may regulate the actin cytoskeleton and cell motility by binding Rac (and perhaps other Rac interacting proteins or actin regulating proteins) and recruiting it (them) to activated receptor tyrosine kinases or cytokine receptor-JAK complexes in the plasma membrane.

A role for SH2-Bβ in cell motility was established using three independent assays (time-lapse video microscopy, phagokinetic, and wounding assays). Each of these assays has its distinct advantages. Time-lapse video microscopy allows one to quantify in individual unfixed cells relatively rapid changes in lamellipodia extension and retraction. However, it is labor intensive and not amenable to analyzing the results of a population of cells, in part because every
cell has slightly different dynamics. Recording cellular movement by measuring gold particle
free areas of coverslips in the phagokinetic assay permits one to study movement of a larger
number of single cells. However, this assay has the disadvantage that it measures a combination
of two processes that are dependent upon changes in the actin cytoskeleton: cellular movement
and phagocytosis. The wounding assay has the advantage of assessing directed cell movement.
Interestingly, similar results were obtained in the wounding assay whether we measured the
distance of the leading edge or the percentage of migrating SH2-Bβ expressing cells. This
suggests that either a higher percentage of migrating cells than we can detect express a level of
tagged SH2-Bβ sufficient to alter cell migration or movement of SH2-Bβ overexpressing cells
affects movement of non-transfected cells (e.g. cells move as a group).

All three of the assays revealed that mutating or deleting the SH2 domain of SH2-Bβ
inhibits changes in the actin cytoskeleton, resulting in decreased lamellipodia activity, membrane
ruffling and cell movement. A role for SH2-Bβ in regulation of the actin cytoskeleton is not
limited to a single cell type. It has now been shown here and previously (9) for both 3T3-F442A
fibroblasts (ruffling, video microscopy, phagokinetic assays) and 293T kidney epithelial-derived
cells (phagokinetic, and wounding assays) and for both confluent (wound healing assay) and
non-confluent cells (ruffling, video microscopy and phagokinetic assays). It is also consistent
with both overexpressed (9) and endogenous (Diakonova and Carter-Su, manuscript in
preparation) SH2-Bβ being localized to membrane ruffles. The fact that overexpression of the
same mutant form of SH2-Bβ (R555E) blocks NGF-induced neurite outgrowth of PC12 cells
and SH2-Bβ enhances it (10) is consistent with SH2-Bβ playing a role in growth factor
regulation of the cytoskeleton. SH2 domains in general have been shown to bind preferentially to phosphotyrosine-containing motifs and the SH2 domain of SH2-Bβ has been shown to be required for high affinity binding of SH2-Bβ to the cytokine receptor associated tyrosine kinase JAK2 and the receptors for PDGF and NGF (TrkA) (10,14,17,18). We therefore think it likely that this domain is required for cell motility because it mediates binding of SH2-Bβ to activated forms of receptor-associated tyrosine kinases (e.g. JAK2) or receptor tyrosine kinases (Fig 9). However, it is also possible that SH2-Bβ regulates the actin cytoskeleton at least in part by binding via its SH2 domain to phosphotyrosines within other types of proteins involved in the regulation of the cytoskeleton. In this regard, however, preliminary results indicate that SH2-Bβ does not bind directly to tyrosyl phosphorylated FAK (Herrington and Carter-Su, unpublished data).

Using the wounding assay, we identified a second region of SH2-Bβ required for maximal cellular motility in the presence of GH - amino acids 85-106, a region rich in prolines. Proline-rich regions are often binding sites for SH3 or WW domain containing proteins (19,37). Thus, the simplest explanation for the requirement of this region for cell motility is that it binds to one or more proteins that are required for cytokine or growth factor regulation of the cytoskeleton (Fig 9). One candidate suggested by our studies is the small GTPase Rac. We showed that Rac binding to SH2-Bβ requires this proline-rich region. Furthermore, forms of SH2-B lacking an intact SH2 domain but containing this region block the ability of constitutively active forms of Rac to promote membrane ruffling, consistent with these mutated forms of SH2-Bβ being able to sequester Rac away from endogenous SH2-Bβ, thereby
interfering with the proper localization of Rac. Rac does not contain an obvious WW or SH3 domain, raising the possibility of the existence of a previously unidentified proline-interaction domain within Rac or the existence of an intervening protein linking Rac and SH2-Bβ.

One mechanism whereby SH2-Bβ recruitment of Rac to cytokine receptor-JAK complexes or receptor tyrosine kinases might regulate the cytoskeleton is by bringing Rac into close proximity to the appropriate guanine nucleotide exchange factor(s) (GEF). GEFs are often recruited to activated cytokine receptor-JAK complexes or growth factor receptors (38,39). Recruitment of Rac to plasma membrane receptors as a consequence of binding to SH2-Bβ would also position Rac near the appropriate part of the actin cytoskeleton for regulating cell motility. Previous studies have indicated that although Rac can be found in the cytoplasm, coupling of small GTPases to their effector pathways in cells generally requires their membrane association (40,41). One can also envision SH2-Bβ facilitating ligand activation of the actin cytoskeleton by binding not only Rac but also other proteins important for the actions of Rac on the actin cytoskeleton, for example, Rac effector proteins, such as PAK (42,43) or Rac modulatory proteins, such as DOCK 180 (44).

In summary, we have used three different assays to provide substantial additional evidence that SH2-Bβ plays a role in the regulation of cell motility. We identified the SH2 domain and amino acids 85-106 containing a proline-rich domain as regions of SH2-Bβ required for maximal cell motility. We also show that Rac binds to SH2-Bβ with the interaction requiring amino acids 85-106. Finally, we show that SH2 domain-deficient mutants of SH2-
Bβ block membrane ruffling caused by constitutively active Rac. Based upon these findings, we propose that SH2-Bβ functions as an adapter/scaffolding protein that recruits Rac and perhaps other proteins to activated membrane receptor-JAK complexes or receptor tyrosine kinases where they are then positioned appropriately to regulate the actin cytoskeleton and promote membrane ruffling and cell motility.
FOOTNOTES

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1 The abbreviations used are: GFP, green fluorescent protein; GH, growth hormone; NGF, nerve growth factor; PDGF, platelet-derived growth factor; SH2, Src homology-2
FIGURE LEGENDS

Figure 1. Schematic representation of wild-type and mutant forms of rat SH2-Bβ used in this study. Circles represent proline-rich regions (amino 13-24, 89-103, 469-496). The rectangle represents a pleckstrin homology domain (amino acids 274-376) and the triangle represents an SH2 domain (amino acids 527-620).

Figure 2. The SH2 domain of SH2-Bβ is required for maximal lamellipodia activity assessed using time-lapse video microscopy. (A) 3T3-F442A cells expressing GFP alone, GFP-SH2-Bβ, or GFP-SH2-Bβ(R555E) on coverslips were treated with GH as indicated. Individual transfected cells (leftmost panels) were located with a FITC filter set and then observed by phase-contrast microscopy. Arrows indicate lamellipodia. Scale bar, 20 μm. (B) 3T3-F442A cells expressing GFP alone (top), GFP-SH2-Bβ (middle), or GFP-SH2-Bβ(R555E) (bottom) were incubated with 500 ng/ml GH and analyzed for 3hrs by video microscopy. Results are shown for representative cells.

Figure 3. Maximal phagokinesis of 3T3-F442A fibroblasts requires the SH2 domain and N-terminal part of SH2-Bβ. 3T3-F442A cells expressing the indicated form of GFP-SH2-Bβ were plated on colloid gold-covered coverslips and areas that became free of colloid gold were visualized (outlined). (A-H) Individual transfected cells were located with a FITC filter set (Panels B, D, F, H) and then observed by phase-contrast microscopy (Panels A, C, E, G). Scale bar, 25 μm. (I) 3T3-F442A cells expressing GFP alone or GFP-tagged forms of SH2-Bβ were
plated on colloid-gold covered coverslips. The area of gold-free tracks was determined after 24 h using NIH Image software. A phagokinetic index was calculated as the ratio of the particle-free area to the cross-sectional area of the cell. Bars represent mean ± S.E., n=30.

Figure 4. Epithelial derived cells, like fibroblasts, exhibit phagokinesis. (A,D) 3T3-F442A fibroblasts (A), COS cells (B), CHO (C), and 293T cells (D) were tested for their ability to phagocytose colloid gold. Representative tracks after 24 h plating on colloid gold are shown. Scale bar, 25 µm. (E) The average phagokinetic index was calculated for 20 cells for each cell line at various times after plating. Bars represent mean ± S.E. (F) 293T cells expressing GFP alone or GFP-tagged SH2-Bβ, SH2-Bβ (1-555), SH2-Bβ(R555E), or SH2-Bβ (504-670) were treated and analyzed after 24h as described for Fig. 3I. Bars represent mean ± S.E., n=40. *, p<0.05 compared to cells expressing GFP.

Figure 5. SH2-Bβ is required for cell migration into a wound. 293T cells expressing GFP-SH2-Bβ (A, B) or GFP-SH2-Bβ(R555E) (C, D). Cells were re-plated and wounded. At 24 h, the edge of the wound was located by phase-contrast microscopy (B, D) and GFP-positive cells that had migrated into the wound were visualized (A, C). Scale bar, 100 µm.

Figure 6. Maximal GH-dependent migration of cells into wound requires the SH2 domain and a proline-rich region of SH2-Bβ. 293T cells were transfected with cDNA encoding the indicated forms of epitope-tagged SH2-Bβ. (A) Cell movement into a wound was assessed as an average migration distance of the leading edge. (B) A motility index was calculated as a ratio of %
transfected cells migrating into the wound to the % transfected cells. Bars represent mean ± S.E., n=30. *, p<0.05 compared to cells expressing vehicle with the same GH treatment; +, p<0.05 compared with vehicle without GH.

Figure 7. Dominant negative forms of SH2-Bβ inhibit membrane ruffling induced by constitutively active forms of Rac. (A) 3T3-F442A cells were serum-deprived overnight and treated with 500 ng/ml GH for 0, 5 or 10 min. Cell lysates were incubated with GST-PBD fusion protein and bound, active GTP-Rac was identified by Western blotting with αRac (top). Cell lysates were probed with αRac (bottom). (B) 3T3-F442A cells were co-transfected with cDNA encoding the indicated forms of GFP-tagged SH2-Bβ and Myc-tagged constitutively active Rac L61. Cells were fixed, permeabilized, stained for Rac (αMyc and αmouse-Cy5) and F-actin (phalloidin-Texas Red) and imaged by confocal microscopy. Scale bar, 20 μm. (C) 3T3-F442A cells overexpressing Rac V12 or Rac L61 with or without GFP-SH2-Bβ(1↑555) were deprived of serum overnight, treated with 500 ng/ml GH for 10 min and imaged. 100 cells positive for GFP-tagged SH2-Bβ and Myc-Rac were scored for the presence of ruffles for each experimental condition. Each transfection was repeated three times with similar results. *, p<0.05 compared to cells expressing the same Rac in the absence of SH2-Bβ(1-555).

Figure 8. SH2-Bβ binding to Rac requires amino acids 85-106 of SH2-Bβ. This binding does not depend on JAK2 or Rac activation. (A) 293T cells overexpressing GH receptor were treated and analyzed as described in Fig 7A. (B) 293T cells were co-transfected with cDNA encoding
GFP-SH2-Bβ and either Myc-Rac V12 or Myc-Rac N17, with or without cDNA encoding JAK2. Proteins were immunoprecipitated with αMyc and immunoblotted with αSH2-Bβ. (C) 293T cells overexpressing the indicated proteins were immunoprecipitated with αMyc and immunoblotted with αGFP (lanes 1-6). Cell lysates were immunoblotted with αGFP (lanes 7-12). The migrations of the various GFP-tagged forms of SH2-Bβ are indicated on the right.

Figure 9. Model of role of SH2-Bβ in regulation of the actin cytoskeleton. Two regions of SH2-Bβ are implicated in the regulation of cell motility: the proline-rich region of amino acids 85-106, and the SH2 domain. The SH2 domain is required for binding to phosphotyrosines in receptor and non-receptor tyrosine kinases. The proline-rich region binds to Rac.
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Fig. 1
**Fig. 2**

A

+500 ng/ml GH

GFP

SH2-Bβ(R555E)

+25 ng/ml GH

GFP

SH2-Bβ

0 min 5 min 10 min 20 min

B

[min after GH]

GFP

SH2-Bβ

SH2-Bβ(R555E)

% of retraction % of extension
Fig. 3-I
Fig. 4
Fig. 6
Fig. 7

A

-active Rac
-total Rac

min with GH: 0 5 10

B

SH2-Bβ

SH2-Bβ(1-555)

GFP-SH2-Bβ  Cy5-Rac  Phalloidin-TR

C

Rac V12

Rac V12+

Rac L61

Rac L61+

percentage cells with ruffles
Fig. 8
Fig. 9

Actin rearrangement
Appendix

A) IP: anti-SH2-Bβ
   IB: anti-SH2-Bβ

B) Cell lysates
   IB: anti-SH2-Bβ

**Fig. 1** Untransfected 293T cells or 293T cells overexpressing myc-SH2-Bβ were immunoprecipitated with anti-SH2-Bβ and immunoblotted with anti-SH2-Bβ antibody (A). Cell lysates were immunoblotted with anti-SH2-Bβ antibody (B). The migration of the SH2-Bβ is indicated on the right.

**Fig. 2** 293T cells overexpressing GFP-SH2-Bβ were immunoprecipitated with anti-SH2-Bβ and immunoblotted with anti-SH2-Bβ antibody. The migration of the endogenous and the GFP-tagged ectopic forms of SH2-Bβ are indicated on the right.
SH2-Bβ is a Rac binding protein that regulates cell motility
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