Phosphorylation controls a dual-function polybasic nuclear localization sequence in the adapter protein SH2B1β to regulate its cellular function and distribution

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Summary
An intriguing question in cell biology is what targets proteins to, and regulates their translocation between, specific cellular locations. Here we report that the polybasic nuclear localization sequence (NLS) required for nuclear entry of the adapter protein and candidate human obesity gene product SH2B1β, also localizes SH2B1β to the plasma membrane (PM), most probably via electrostatic interactions. Binding of SH2B1β to the PM also requires its dimerization domain. Phosphorylation of serine residues near this polybasic region, potentially by protein kinase C, releases SH2B1β from the PM and enhances nuclear entry. Release of SH2B1β from the PM and/or nuclear entry appear to be required for SH2B1β enhancement of nerve growth factor (NGF)-induced expression of urokinase plasminogen activator receptor gene and neurite outgrowth of PC12 cells. Taken together, our results provide strong evidence that the polybasic NLS region of SH2B1 serves the dual function of localizing SH2B1 to both the nucleus and the PM, the latter most probably through electrostatic interactions that are enhanced by SH2B1β dimerization. Cycling between the different cellular compartments is a consequence of the phosphorylation and dephosphorylation of serine residues near the NLS and is important for physiological effects of SH2B1, including NGF-induced gene expression and neurite outgrowth.

Key words: Dimerization domain, Electrostatic interaction, Neurite outgrowth, Nuclear localization sequence, Phosphorylation, Protein kinase C, Urokinase plasminogen activator receptor

Introduction
SH2B1 isoforms have been implicated in a variety of cellular functions, including nerve growth factor (NGF)- and glial-derived nerve growth factor (GDNF)-induced neuronal differentiation (Qian et al., 1998; Rui et al., 1999a; Zhang et al., 2006); growth hormone (GH)-, prolactin- and fibroblast growth factor, PDGF, GH, and prolactin (Maures et al., 2009). Four isoforms of SH2B1 have been identified (α, β, γ and δ) that share 631 amino acids (a.a.) and diverge only in their C-termini (Yousaf et al., 2001). All SH2B1 isoforms, including the ubiquitously expressed SH2B1β that is the focus of these studies, recognize and bind phosphorylated tyrosines in activated, membrane-bound receptor tyrosine kinases or cytokine receptor-associated JAKs, via their SH2 domains (Maures et al., 2007). Even when the SH2 domain of SH2B1β is mutated, a substantial portion of SH2B1β resides at the PM, presumably facilitating SH2B1β recruitment to its PM-localized binding partners (Rui et al., 1999b). SH2B1β lacks hydrophobic membrane-spanning, N-terminal myristoylation, and C-terminal prenylation sequences, all motifs known to localize proteins to the PM (Heo et al., 2006). However, SH2B1β contains a PH domain, a domain that often recruits proteins to phosphatidylinositides in the PM (Lemmon and Ferguson, 2000). We therefore asked whether SH2B1β binds to the PM via its PH domain. We found that SH2B1β bound to the PM, not via its PH domain, but via a polybasic region that we had identified previously as a functional nuclear localization sequence (NLS) (Maures et al., 2009). This polybasic region, therefore, serves the dual function of localizing SH2B1β to both the nucleus and the PM.
Our data suggest that SH2B1β interaction with the PM is most likely mediated via electrostatic interactions that are enhanced by SH2B1β dimerization via its phenylalanine (Phe) zipper. We provide evidence that localization of SH2B1β to the PM versus the nucleus is regulated by phosphorylation of serine (Ser) residues proximate to the polybasic NLS of SH2B1β. Finally, we provide evidence that the ability of SH2B1β to cycle between the PM and other compartments is crucial for the physiological effects of SH2B1β, including enhancement of NGF-induced gene expression and neurite outgrowth.

Results
Polybasic region of SH2B1β is required for its localization to the PM

To identify the PM-binding domain of SH2B1β, we transiently expressed a series of myc-tagged SH2B1β truncations (shown in Fig. 1A) in 2C4-GHR human fibrosarcoma cells and prepared cytosolic and membrane fractions. The wild-type form, myc-SH2B1β(WT), was detected in both cytosolic and membrane fractions (Fig. 1A). However, myc-SH2B1β(1–80), myc-

Fig. 1. The polybasic region within the NLS mediates localization of SH2B1β to the PM. (A) The domain structure of the truncation mutants is indicated in the schematics: DD, a.a. 25–85; NLS, a.a. 146–152; NES, a.a. 224–233; PH, a.a. 247–378; and SH2, a.a. 525–610. Cytosolic (C) and membrane (M) fractions were prepared from 2C4-GHR cells expressing the indicated myc-tagged SH2B1β. Equal amounts of protein (Bradford, 1976) were subjected to SDS-PAGE and immunoblotted with anti-myc (n=2). Because SH2B1β is known to be highly phosphorylated (Rui et al., 1999b), the multiple bands seen in some of the panels are thought to represent differentially phosphorylated forms of SH2B1β. (B, D) Living PC12 cells transiently expressing GFP-tagged SH2B1β(WT), SH2B1β(Δ147–198), SH2B1β[mNLS] [K146P K147LKKR to K146P ALA], SH2B1β(sNLS1) [S145KPKL K150KRF to K145KPKL S151RF], SH2B1β(sNLS2) [S145KPKL K151RF to K145KPKL S151RF], or SH2B1β(S157E) were stained with wheat germ agglutinin Alexa Fluor 594 (PM marker) and imaged. First row: GFP epifluorescence. Second row: overlay of GFP (green) and PM marker (red). Scale bar: 10 μm. Small arrows indicate the PM. (C, E) The green and red signal intensity along the yellow lines in the cells in B and D, respectively, was determined using MetaVue Linescan. Arrows indicate the direction of the linescan; the width of the crossbar on the arrow tail indicates the width of the linescan. (F) 293T cells transiently expressing the indicated GFP–SH2B1β and the phosphatidylinserine biomarker mRFP-Lact-C2 (mRFP-Lact) were fixed and imaged using confocal microscopy. First row: GFP epifluorescence; second row: RFP epifluorescence; third row, overlay of the GFP and RFP signals. Arrows indicate the PM.
SH2B1β(1–105) and myc-SH2B1β(1–150) were localized predominantly to the cytosolic fraction while myc-SH2B1β(1–200) and myc-SH2B1β(1–260) were almost exclusively found in the membrane fraction (Fig. 1A). These results exclude the PH and SH2 domains as being required for PM localization of SH2B1β. Rather, they suggest that the PM localization sequence lies within a.a. 150–200.

To confirm that a.a. 150–200 are required for localization of SH2B1 to the PM, we expressed GFP-tagged forms of SH2B1β and a red fluorescent PM marker in PC12 cells and analyzed their subcellular localization by live-cell confocal microscopy. Although GFP–SH2B1β(WT) is known to undergo continual nucleocytoplasmic shuttling (Chen and Carter-Su, 2004), its steady-state localization is in the cytoplasm and PM, the latter indicated by the yellow color at the cell perimeter in the overlay (Fig. 1B). A linescan across the cell (yellow line in the overlay) illustrates the high degree of positional overlap between the red (PM) and green (GFP–SH2B1β) signals at the cell perimeter (Fig. 1C). By contrast, GFP–SH2B1β did not localize to the PM when a.a. 147–198 were deleted, as indicated by the offset of the red and green signals in the corresponding linescan for cells expressing GFP–SH2B1β(D147–198) (Fig. 1C). Interestingly, a.a. 147–198 (implicated in PM localization) includes the polybasic motif a.a. 146–152 (Fig. 2A) shown previously to enable nuclear localization of SH2B1β (Maures et al., 2009).

Polybasic regions in a number of proteins have been shown to electrostatically interact with the negatively charged inner leaflet of the PM (Heo et al., 2006; Yeung et al., 2008). To determine whether the polybasic sequence within SH2B1 mediates association with the PM, we assessed the localization of SH2B1β(mNLS), a mutant that carries alanine (Ala) substitutions (indicated in bold) at four of the basic residues (K146PKLKKR to K146PALAAA) in the NLS and previously shown to be nuclear localization-defective (Maures et al., 2009). GFP–SH2B1β(mNLS) failed to localize to the PM (Fig. 1B,C). To determine whether binding to the PM requires a specific sequence, we scrambled the order of the basic

Fig. 2. Ser residues proximate to the NLS regulate subcellular localization of SH2B1β. (A) Schematic representation of the domain structure of SH2B1β. The NLS (underlined), basic amino acids in the NLS (blue), and the proximate Ser/Thr (red)-rich region are shown. (B) Living 293T cells transiently expressing the indicated GFP–SH2B1β mutants were imaged and linescans obtained. (C) Relative cytoplasm to PM ratios were calculated from linescans (10–19 cells per condition) using PM and cytoplasm values determined as shown by the colored lines in the linescans in B. PM values were chosen on the basis of the PM peak adjacent to the largest scanned cytoplasmic area to allow direct comparison. Means ± s.e.m. are shown. (D) Living 293T cells transiently expressing the indicated GFP–SH2B1β mutants were imaged. (E,F) Living PC12 (E) or 293T (F) cells expressing the indicated GFP-tagged SH2B1β mutants were treated with or without LMB (20 nM) for 13 (PC12 cells) or 7 (293T cells) hours. Scale bar: 10 μm.
residues in the NLS (S\textsubscript{145}KPKLK\textsubscript{150}K\textsubscript{151}KRF to K\textsubscript{145}KPKLSON\textsubscript{12}KRF [S\textsubscript{NL1S1} or to K\textsubscript{145}KPKLKS\textsubscript{15}KRF [S\textsubscript{NL2S2}]). SH2B1β binding to
the PM was not disrupted (Fig. 1D,E). However, substitution of an
acidic Glu for Ser (S157E) in proximity to the NLS decreased
SH2B1β association with the PM. Together, these results suggest
that the basic residues that comprise the NLS are crucial for
association of SH2B1β with the PM and that association might
depend upon the overall charge of the region.

The anionic PM components, phosphatidylinositol (4,5)-
bisphosphate [PtdIns(4,5)\textsubscript{P}2], phosphatidylinositol (3,4,5)-
trisphosphate [PtdIns(3,4,5)\textsubscript{P}3] (Heo et al., 2006), and
phosphatidylserine (Yeung et al., 2008) are thought to confer the
association of SH2B1β with the PM and that association might
determine whether the NLS localizes SH2B1β to the PM. To gain insight into whether
SH2B1β colocalizes with phosphatidylserine, the predominant
anionic species in the PM (Vance and Steenbergen, 2005), we
used a fluorescent bioprobe (mRFP-Lact-C2) (Yeung et al., 2008)
that binds phosphatidylserine in a charge-independent manner.
GFP–SH2B1β(WT) displayed substantial colocalization with
mRFP-Lact-C2 at the PM (Fig. 1F). GFP–SH2B1β(147–198) was
predominately cytoplasmic and, compared to GFP–
SH2B1β(WT), exhibited substantially reduced colocalization with
mRFP-Lact-C2 at the PM. Together, these results suggest that the
polybasic residues in the NLS that are required for localization of
SH2B1β to the nucleus are also required for SH2B1β localization to the PM, most likely through electrostatic interactions with phosphatidylserine and/or negatively charged phosphoinositides.

Ser residues proximate to the polybasic NLS regulate
the PM localization of SH2B1β

The finding that the NLS serves the dual function of localizing
SH2B1β to both the PM and the nucleus raises the question of
what determines whether the NLS localizes SH2B1β to the PM or
the nucleus. SH2B1β contains an unusually large number of serine or threonine residues (Ser/Thr) in the vicinity of the NLS (Fig.
2A). We hypothesized that phosphorylation of one or more of these
Ser/Thr would disrupt the electrostatic interaction between SH2B1β
and the PM and release SH2B1β from the PM. The NLS would then be available to function as a traditional nuclear localization signal. We predicted that a mutant SH2B1β lacking the regulatory
Ser/Thr phosphorylation sites would be predominately, if not exclusively, PM-bound. To test this, we mutated all 13 of the
Ser/Thr proximate to the NLS to Ala [SH2B1β(S165A)]. In contrast
to SH2B1β(WT), which localizes to both the PM and cytoplasm,
SH2B1β(S165A) localized almost exclusively to the PM (Fig. 2B).
Quantifying linescans from multiple images revealed that the 13SA
mutation reduced the cytoplasm to PM ratio by >90% (Fig. 2C).
A similar reduction was obtained when the four Ser C-terminal to
the NLS were mutated to Ala [SH2B1β(S154,157,161,165A)], suggesting that one or more of these Ser are the most important
modulatory site(s) of phosphorylation.

To gain insight into which of these four Ser are essential for
localization of SH2B1β to the PM, we eliminated these Ser in various combinations. Mutating Ser154, Ser157 and Ser165 to Ala
decreased the cytoplasm to PM ratio of the SH2B1β compared to
the wild-type (Fig. 2B,C), but not to the same extent as
SH2B1β(13SA) or SH2B1β(S154,157,161,165A). By contrast,
SH2B1β(S161,165A) localized almost exclusively to the PM
(Fig. 2B,C). The PM localization of SH2B1β(S161A) and
SH2B1β(S165A) was similar to that of SH2B1β(S161,165A), whereas
SH2B1β(S157A) (Fig. 2D; see linescan in supplementary
material Fig. S1A) was retained in the PM to only a slightly greater
extent than SH2B1β(WT).

As predicted for an electrostatic localization mechanism,
conversion of Ser161 or Ser165 to the phospho-mimetic glutamate
(Glu) prevented SH2B1β from localizing to the PM (Fig. 2D,E; see
linescans in supplementary material Fig. S1A,B). Indeed,
introduction of a single Glu to the highly PM-bound
SH2B1β(13SA) shifted SH2B1β(12SA,165E) to the cytoplasm
(Fig. 2D; see linescan in supplementary material Fig. S1A). Taken
together, these results suggest that Ser161, Ser165 and to a lesser extent Ser157, regulate PM versus cytoplasmic localization, most probably by attenuating the positive charge of the polybasic region by a phosphorylation event.

Ser residues proximate to the NLS influence nuclear localization of SH2B1β

We next asked whether Ser161 and Ser165 influence nuclear
localization of SH2B1β. The kinetics of SH2B1β nucleoexocytotic
shuttling are such that steady state levels of nuclear GFP–SH2B1β are generally so low that nuclear SH2B1β is difficult to observe unless nuclear export is inhibited (Chen and
Carter-Su, 2004). Thus, to assess whether phosphorylation of
Ser161 and/or Ser165 affects nuclear localization of SH2B1β,
PC12 cells expressing GFP-tagged forms of SH2B1β were treated
with the nuclear export inhibitor leptomycin B (LMB). Upon LMB
treatment, phospho-mimetic mutants SH2B1β(S161E) and
SH2B1β(S165E), like SH2B1β(WT), accumulate in the nucleus
whereas SH2B1β(S161,165A) does not (Fig. 2E; see linescans in
supplementary material Fig. S1B). Similar results were observed
in 293T cells (Fig. 2F; see linescan in supplementary material Fig.
S1C). Thus, 86% of cells expressing SH2B1β(WT) (n=249)
examined exhibited nuclear accumulation whereas none of the
SH2B1β(13SA) (n=144) or SH2B1β(S161,165A) cells (n=17) did.
These findings suggest that when Ser161 and Ser165 are mutated
to Ala, SH2B1β is unable to undergo nucleoexocytotic shuttling,
presumably because it cannot be phosphorylated on Ser161 and
Ser165 and is therefore unable to be released from the PM. The
phospho-mimetic mutant results also indicate that nuclear shuttling of SH2B1β does not require prior association with the PM.

Phosphorylation of Ser161 and Ser165 is required for
PKC-mediated SH2B1β release from the PM and nuclear
translocation

Ser161 and Ser165 reside in predicted protein kinase C (PKC)
substrate motifs [NetPhos 1.0 server (Blom et al., 1999),
scansite.mit.edu (Obenauer et al., 2003)]. We therefore hypothesized that activation of PKC would phosphorylate Ser161 and/or Ser165 and release SH2B1β from the PM. To test this, we monitored the subcellular localization of GFP–SH2B1β(WT) following treatment
with two PKC agonists, bryostatin-1 (Kortmansky and Schwartz,
2003) and phorbol myristate acetate (PMA). In quiescent PC12
cells, SH2B1β(WT) was at the PM and in the cytoplasm; very little
SH2B1β was detected in the nucleus. Within 3 minutes of treatment
with bryostatin-1, SH2B1β was no longer concentrated at the PM,
and was clearly seen in the nucleus (Fig. 3A, see linescan in
supplementary material Fig. S2A). By contrast, bryostatin-1 did
not alter the localization of GFP–SH2B1β(13SA), which remained
at the PM. Similar effects were obtained in 293T cells using PMA
(Fig. 3B,C; see linescans in supplementary material Fig. S2B).
PMA released SH2B1β from the PM in >90% of the cells (Fig.
3D) and increased it in the nucleus of 68% of cells (Fig. 3E).
PMA-induced redistribution of SH2B1β (WT) from the PM to both the cytoplasm and nucleus was substantially inhibited by pretreating cells with bisindolylmaleimide 1 (BisI), an inhibitor of PKC (Fig. 3C-E). PKC activates extracellular-signal-regulated kinases 1 and 2 (ERK1/2) (Schonwasser et al., 1998). BisI inhibition of PMA-induced ERK1/2 activation (Fig. 3F) illustrates the efficacy of BisI at inhibiting. As predicted from the bryostatin-1-treated cells, PMA had little or no effect on the subcellular distribution of GFP–SH2B1β (S161, 165A) or GFP–SH2B1β (135A), respectively (Fig. 3B; see linescan in supplementary material Fig. S2B), suggesting that Ser161 and Ser165 are the crucial targets of PKC vis-à-vis PM localization. Subcellular fractionation of 3T3-F442A cells treated with or without PMA revealed a similar PMA-dependent shift (decrease in PM and increase in cytoplasmic and nuclear fractions) in the subcellular distribution of endogenous SH2B1 (Fig. 3G). Thus, SH2B1β localization is dynamically regulated by PKC agonists, probably through the phosphorylation of Ser161 and/or Ser165.

Indeed, SH2B1β is heavily phosphorylated upon stimulation with PMA. When 3T3-F442A pre-adipocytes transiently expressing FLAG–SH2B1β were treated with PMA for 15 minutes, an upward shift in SH2B1β migration on SDS-PAGE gels was detected (Fig. 3H). Previous work studying endogenous SH2B1 indicated that this upward shift in mobility is due to increased Ser/Thr phosphorylation (Rui et al., 1999b). An αSH2B1 immunoblot of a two-dimensional electrophoretic gel [isoelectric focusing (IEF) followed by SDS-PAGE] revealed at least six regularly spaced spots in a horizontal line migrating with a relative molecular mass appropriate for GFP–SH2B1β (Fig. 3I). The regularly spaced horizontal spots suggest multiply phosphorylated forms of SH2B1β, with each spot to the left representing an SH2B1β with an additional phosphate. The observed pattern indicates that even in unstimulated cells, SH2B1β is variably phosphorylated, with the most highly phosphorylated form containing five or more phosphorylated amino acids. PMA treatment shifted the migration of those spots to a more acidic pH, consistent with an overall increase in negative charge, indicative of increased numbers of phosphorylated amino acids in SH2B1β. Together, these results suggest that phosphorylation of SH2B1β by PKC; by another kinase with similar substrate, activator and inhibitor specificity; or by a downstream kinase not only releases SH2B1β from the PM, but also enhances nuclear localization of SH2B1β.

**Phosphorylation of Ser154 and Ser161 in the vicinity of the polybasic NLS in SH2B1β is detected by mass spectrometry**

To determine whether Ser residues proximate to the NLS are phosphorylated in PMA-treated cells, we added PMA to 293T cells expressing FLAG–SH2B1β. FLAG–SH2B1β was immunoprecipitated, resolved by SDS-PAGE (supplementary material Fig. S3A), eluted and digested with trypsin. The tryptic peptides were analyzed by liquid chromatography-mass spectrometry (LC/MS) or tandem MS (MS/MS). Doubly-charged peptides corresponding to RF(P)S154LR and monophosphorylated LRS15VGRS16VR were identified. Analysis of the MS/MS spectra using Mascot followed by manual inspection confirmed that RF(P)S154LR was phosphorylated at Ser154 (supplementary material Fig. S3B). Partial sequence data were obtained from the MS/MS spectrum for LRS15VGRS16VR, with a phosphorylated residue detected at Ser161 (supplementary material Fig. S3C). Manual inspection supported this assignment. The measurement
error for the parent mass was also very good at 5 ppm. Therefore, of the Ser residues (161 and 165) strongly implicated by our biologic data in regulating release of SH2B1\(b\) from the PM, the MS/MS data support Ser161 being phosphorylated. We did not detect Ser165-\(P\). However, the tryptic peptide containing Ser165 is four amino acids long, too small to be easily detected by MS/MS. Even though mutating Ser154 to Ala in combination with Ser157 and Ser165 did not shift the subcellular localization of SH2B1\(b\) towards the PM as effectively as mutating Ser161 and Ser165, Ser154-\(P\) was detected. This finding raises the possibility that phosphorylation of Ser154 occurs only after SH2B1\(b\) is released from the PM or that mutation of Ser154 to Ala causes a conformational change in SH2B1\(b\) that overrides any change in electrostatic charge.

We also identified (data not shown) phosphorylation at Ser96, Ser124, Ser126, Ser127, Ser453 and Ser613 (\(P<0.00002\), from Mascot Ion scores) and Thr218 (\(P<0.05\)). Of these, phosphorylation has previously been reported at Ser96 and Ser126 (PhosphoSitePlus, 2009; Rui et al., 1999b). In contrast to mutating Ser157, Ser161 and Ser165, mutating Ser96 (Rui et al., 1999b), Ser124, Ser126/127, or Thr218 (data not shown) to Ala did not alter the subcellular localization of SH2B1\(b\). The first 60 amino acids of SH2B1\(b\) contain a proline-rich domain (a.a. 5–24). The localization of SH2B1\(b\)(23–670) that lacks the proline-rich domain was not significantly different from that of SH2B1\(b\)(WT) (Fig. 4D). Together, these results suggest that the polybasic NLS region is required, but not sufficient, to drive PM localization of SH2B1\(b\); a.a. 23–60 are also important.

**The polybasic region is not sufficient for PM localization of SH2B1\(b\)**

To establish whether the NLS is sufficient for localizing SH2B1\(b\) to the PM, we examined the subcellular localization of GFP–SH2B1\(b\)(118–170), which contains the polybasic NLS. GFP–SH2B1\(b\)(118–170) did not localize to the PM (Fig. 4A). To determine additional region(s) of SH2B1\(b\) that are necessary for localization to the PM, we expressed various SH2B1\(b\) truncations in PC12 cells. SH2B1\(b\)(1–105) and SH2B1\(b\)(1–150), both of which lack an intact polybasic region, failed to localize to the PM (Fig. 4B), consistent with the subcellular fractionation experiments in Fig. 1A. By contrast, SH2B1\(b\)(1–200), which contains the polybasic NLS region, is observed at the PM (Fig. 4C). When N-terminal truncations were tested, neither SH2B1\(b\)(60–670) nor SH2B1\(b\)(118–670) localized to the PM despite containing the polybasic NLS region (Fig. 4C). These results indicate that, in addition to the polybasic NLS, amino acids within the N-terminal region (a.a. 1–60) are required for PM localization of SH2B1\(b\).

The polybasic region is not sufficient for PM localization of SH2B1\(b\)

Amino acids 23–60 contain most of a Phe zipper, which has been shown by a variety of methods to mediate homo- and heterodimerization of SH2B family members (Dhe-Paganon et al., 2004; Nishi et al., 2005). To gain insight into whether dimerization via this Phe zipper is required for association of SH2B1\(b\) with the PM, we first inserted a charged, bulky amino acid (Asp) at three highly conserved Ala residues {SH2B1\(b\)(A34,38,42D) [SH2B1\(b\)(3AD)]} required for the interdigitating Phe to mediate dimerization (Dhe-Paganon et al., 2004; Nishi et al., 2005). Subsequently, we substituted Ala at two of the crucial interdigitating Phe residues {SH2B1\(b\)(F68,72A) [SH2B1\(b\)(2FA)]}, and then

![Fig. 4. A second region in SH2B1\(b\) between residues 23–60 is required for localization of SH2B1\(b\) to the PM. (A – D) Living PC12 cells expressing the indicated truncations of GFP–SH2B1\(b\) were stained with the PM marker and imaged, and linescans performed as in Fig. 1. The schematics of SH2B1\(b\) indicate the NLS in black and the truncated regions by dotted lines. Scale bars: 10 \(\mu\)m.](image-url)
dimerization, we assessed mCherry–SH2B1 dimerization domain enhance PM binding independent of possibility that the amino acids within the Phe zipper-containing interdigitate into the PM (Heo et al., 2006). To rule out the residues interspersed with bulky hydrophobic residues that SH2B1 with a model in which PM localization requires dimerization of concentrated within the cytoplasm. These results are consistent [SH2B1]SH2B1 with the PM requires dimerization of SH2B1.

We next examined the PM localization of these dimerization domain (DD) mutants in 293T cells. PM localization was modestly reduced for GFP–SH2B1[2FA], and substantially reduced for GFP–SH2B1[3AD] and GFP–SH2B1[2FA+3AD] (Fig. 5B). GFP–SH2B1[3AD] and GFP–SH2B1[2FA+3AD] were diffusely localized throughout the cytoplasm, with a small increase in nuclear localization commensurate with the increased cytoplasmic content. These results strongly suggest that association of SH2B1β with the PM requires dimerization of SH2B1β.

The small GTPase Rit binds to the PM via clusters of polybasic residues interspersed with bulky hydrophobic residues that interdigitate into the PM (Heo et al., 2006). To rule out the possibility that the amino acids within the Phe zipper-containing dimerization domain enhance PM binding independent of dimerization, we assessed mCherry–SH2B1β localization in the absence or presence of GFP–SH2B1β(1–150), which contains the DD but lacks the polybasic NLS. If PM targeting requires dimerization via the DD of SH2B1β molecules, each containing a polybasic region, then GFP–SH2B1β(1–150) should decrease localization of full-length mCherry–SH2B1β to the PM. When expressed alone, mCherry–SH2B1β(1–150) predominately localized to the PM of 293T cells (Fig. 5C). GFP–SH2B1β(1–150) blocked localization of mCherry–SH2B1β(1–150) to the PM, which instead concentrated within the cytoplasm. These results are consistent with a model in which PM localization requires dimerization of SH2B1β molecules, each containing a polybasic NLS.

**Mutation of Ser residues proximate to the polybasic NLS disrupts the ability of SH2B1β to enhance NGF-induced urokinase plasminogen activator receptor expression and neurite outgrowth of PC12 cells**

The above studies indicate that the ability of SH2B1β to bind the PM is regulated by phosphorylation of Ser 154, Ser157 and, especially, Ser161 and/or Ser165. To determine whether phosphorylation of these Ser residues affects cellular function, we turned to PC12 cells. PC12 cells, derived from a rat pheochromocytoma, can be induced to differentiate into sympathetic neuron-like cells by NGF (Greene and Tischler, 1976; Tischler and Greene, 1980). We previously showed that overexpression of SH2B1β enhances both NGF-induced neurite outgrowth and expression of a number of NGF-dependent genes, including the gene encoding urokinase plasminogen activator receptor (uPAR). Knockdown of SH2B1β using shRNA or ectopic expression of the dominant-negative SH2B1β(R555E), which lacks a functional SH2 domain and is not recruited to the NGF receptor TrkA, inhibits both NGF-induced uPAR expression and neurite outgrowth (Chen et al., 2008; Maures et al., 2009; Rui et al., 1999a). uPAR is required for NGF-mediated neuronal differentiation (Farias-Eisner et al., 2000). When SH2B1β(WT) was overexpressed in PC12 cells, NGF induction of uPAR expression was three times that detected in control cells (Fig. 6A). However, mutating Ser154, Ser157, Ser161 and Ser165 to Ala ablated any SH2B1β-dependent increase in uPAR expression [SH2B1β(4SA)]. Similarly, when NGF-dependent neuronal differentiation was investigated by assessing neurite outgrowth, overexpression of SH2B1β(WT) almost doubled the number of cells with neurite outgrowths (Fig. 6B). By contrast, overexpression of SH2B1β[S154,157,161,165A](4SA) had no stimulatory effect on NGF-induced neurite outgrowth. These results suggest that phosphorylation of Ser154, Ser157, Ser161 and/or Ser165 and the consequent release of SH2B1β from the PM is essential for SH2B1β to mediate NGF-induced uPAR expression and neurite outgrowth.

**Discussion**

In this work, we made the intriguing finding that the polybasic NLS of SH2B1 plays a dual role as a nuclear and a PM localization sequence. This conclusion is based on both subcellular fractionation and confocal microscopy of living cells using truncated and/or mutated SH2B1β. The findings were consistent using myc and GFP tags, and in 2C4-GHR, 293T and PC12 cells. Attenuation of the collective positive charge of the polybasic NLS region, by substituting neutral Ala for positively charged Lys, was sufficient to inhibit PM localization of SH2B1β. Scrambling the NLS indicated that the overall charge of the region, not the particular order of the amino acids, controlled localization to the PM. These results suggest that binding to the PM involves an electrostatic interaction between the polybasic NLS and negatively charged moieties in the inner leaflet of the PM (Roy et al., 2000). Binding by electrostatic interactions has been reported for a number of proteins, including PM-localized small GTPase family members.
Fig. 6. Mutation of Ser residues proximate to the polybasic NLS region suppresses the ability of SH2B1 to enhance uPAR gene expression and neurite outgrowth of PC12 cells. GFP (-), GFP–SH2B1(WT) or GFP–SH2B1(S154,157,161,165A/4SA) were transiently expressed in PC12 cells. (A) Cells were incubated in serum-free medium overnight and treated with or without 100 ng/ml NGF for 6 hours. Levels of uPAR mRNA were assessed using QT-PCR. uPAR mRNA levels were normalized to GAPDH mRNA, then to uPAR gene expression in the absence of NGF and NGF-induced expression in GFP control cells. The graph is representative of two experiments. (B) After 4 days in differentiation medium with or without 100 ng/ml NGF, the percentage of PC12 cells with neurite outgrowths at least twice the length of the cell body was scored. Means ± s.e.m. from three experiments are shown. *P<0.05 using a one-tailed, paired Student’s t-test. A combined total of at least 100 cells were counted for each condition.

Fig. 7. Representation of the regulation of subcellular localization of SH2B1. In quiescent cells, SH2B1 binds, via its positively charged polybasic region, to the negatively charged anionic species of the inner leaflet of the PM (p.m.). The strong anionic charge of the inner leaflet is conferred by negatively charged phospholipids such as phosphatidylserine, PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2. Dimerization of SH2B1 facilitates its binding to the PM. Phosphorylation of the Ser residues proximate to the PBR region neutralizes the net positive charge. Once released from the PM, the phosphorylated SH2B1 dimer is free to translocate and signal at other cellular locations (e.g. nucleus).

Phosphorylation controls a polybasic NLS

For most proteins that bind to the PM via polybasic regions, lipid modifications such as N-terminal myristoylation (MARCKS) (McLaughlin and Aderem, 1995) and C-terminal farnesylation (small GTPases such as K-Ras and Rac1) (Bivona et al., 2006; Michaelson et al., 2008) are also required. The lipid is thought to insert into the bilayer of the PM and further stabilize the electrostatic interaction afforded by the polybasic region. Similarly, we observed that the polybasic region of SH2B1 was not sufficient for PM localization. However, because neither the N- nor C-terminus of SH2B1 is required for PM localization, both N-terminal myristoylation and C-terminal prenylation were ruled out. Instead, we found that PM localization was inhibited by mutation of hydrophobic residues (Ala34, Ala38, Ala42, Phe68 and Phe72) required for SH2B1 dimerization via its Phe zipper-containing dimerization domain. Rit and several other proteins bind to the PM through clusters of polybasic residues interspersed with bulky hydrophobic residues that integrate into the PM (Heo et al., 2006). Src family proteins (Kaplan et al., 1990; Sigal et al., 1994), and myristoylated alanine-rich C-kinase substrate (MARCKS) (Taniguchi and Manenti, 1993).

Our data also suggest that phosphorylation of SH2B1β on Ser/Thr residues proximate to the polybasic NLS, especially Ser161 and Ser165, decreases the charge density sufficiently to negate the electrostatic attraction between the polybasic region and the PM, thereby releasing SH2B1β from the PM. Although putting a negative charge on any appropriately positioned amino acid might release SH2B1 from the PM, our studies using Ser to Ala mutations indicated that Ser161 and Ser165 were the most crucial for regulating PM binding. The importance of the four Ser residues N-terminal to the NLS and the NLS is also suggested by the fact that the region containing them (K146PKLKKRF164/S154LR157VGRS161VRG165VRG168) is conserved from humans to zebrafish. The importance of these particular Ser residues among the proximal 13 Ser/Thr residues could stem from their being the only ones undergoing reversible phosphorylation in cells, their phosphorylation being required for phosphorylation of other residues, or the three-dimensional structure of SH2B1 being such that their phosphorylation is best positioned to negate the negatively charged polybasic region.

All four of these Ser residues (Ser154, Ser157, Ser161 and Ser165) are predicted (Blom et al., 1999; Obenauer et al., 2003) to be phosphorylated by PKC, a prediction supported by our finding that PKC agonists induced a rapid release of SH2B1β, but not SH2B1β(S161,165A), from the PM; two-dimensional gel electrophoresis indicated that PMA stimulates the phosphorylation of SH2B1β at multiple sites; and phosphorylation at Ser154 and Ser161 was detected in PMA-treated cells by MS/MS. Mutational studies did not implicate Ser154 as crucial for reversible PM binding of SH2B1β. Perhaps phosphorylation at Ser154 plays a role in some other facet of SH2B1β signaling. Although Ser165 was predicted to be phosphorylated, Ser165-P was not identified, presumably because Ser165 is predicted to reside in a peptide so small (four amino acids) as to elude detection. As we found for SH2B1β, the membrane-targeting region of MARCKS contains a polybasic region flanked by multiple Ser residues spaced 3–4 amino acids apart. The Ser residues are in PKC motifs and are predicted (like Ser154, Ser157, Ser161 and Ser165 in SH2B1) to be in an amphiphilic α-helical motif (Taniguchi and Manenti, 1993). In MARCKS, all four of these Ser residues are phosphorylated by PKC (PhosphoSitePlus; http://www.phosphosite.org), in an ordered fashion (Herget et al., 1995).

For most proteins that bind to the PM via polybasic regions, lipid modifications such as N-terminal myristoylation (MARCKS) (McLaughlin and Aderem, 1995) and C-terminal farnesylation (small GTPases such as K-Ras and Rac1) (Bivona et al., 2006; Michaelson et al., 2008) are also required. The lipid is thought to insert into the bilayer of the PM and further stabilize the electrostatic interaction afforded by the polybasic region. Similarly, we observed that the polybasic region of SH2B1β was not sufficient for PM localization. However, because neither the N- nor C-terminus of SH2B1β is required for PM localization, both N-terminal myristoylation and C-terminal prenylation were ruled out. Instead, we found that PM localization was inhibited by mutation of hydrophobic residues (Ala34, Ala38, Ala42, Phe68 and Phe72) required for SH2B1β dimerization via its Phe zipper-containing dimerization domain. Rit and several other proteins bind to the PM through clusters of polybasic residues interspersed with bulky hydrophobic residues that integrate into the PM (Heo et al., 2006). Thus, the possibility existed that the cluster of Phe residues in the SH2B1 Phe zipper directly interacts with the PM. Our finding that the DD competitively inhibited the localization of SH2B1β(WT) to the PM argues that dimerized SH2B1β molecules, each possessing a polybasic region, are required to localize SH2B1β to the PM (Fig. 7). Consistent with this mechanism, dimerization has been shown to significantly increase the association of proteins with the PM. The soluble head group of PtdIns(4,5)P_2 binds to the
unalivalent PH domain of dynamin-1 with a dissociation constant ($K_d$) estimated in the millimolar range, whereas a dimeric form of the PH domain of dynamin-1 binds PtdIns(4,5)P$_2$-containing vesicles with an effective $K_d$ almost 1000 times lower, in the micromolar range (Klein et al., 1998). We believe that the requirement for dimerization represents a novel means by which non-lipidated proteins with polybasic motifs can be targeted to the PM. Because SH2B1 family members have been shown to form higher-order oligomers (Qian and Ginty, 2001), presumably involving domains in addition to the DD, it is possible that association of SH2B1 with the PM is enhanced even further by oligomerization.

Previously, we showed that mutating the NLS or the NES prevents SH2B1 from promoting NGF-induced neuronal differentiation of PC12 cells. In addition, mutating the NLS prevents NGF-induced transcription of genes important for differentiation, including genes encoding uPAR and matrix metalloproteinases 3 and 10 (Chen and Carter-Su, 2004; Chen et al., 2008; Maures et al., 2009). These findings indicated that an ability to cycle through the nucleus is important for neuronal differentiation. Intriguingly, we provide evidence here that phosphorylation of SH2B1, presumably at Ser161 and Ser165, regulates not only the release of SH2B1 from the PM, but also the ability of SH2B1 to shuttle to the nucleus. Further, mutation of Ser154, Ser157, Ser161, and Ser165 to Ala prevents SH2B1-enhancement of NGF-induced transcription of uPAR and neuronal differentiation. Together, these results provide strong evidence that SH2B1 must be released from the PM and enter the nucleus to promote neuronal differentiation. These results, when combined with our finding here that mutating the NLS prevents SH2B1 from binding to the PM, and previous findings that mutating the NLS or deleting the first 236 amino acids (which contain the NLS) prevents SH2B1-enhancement of NGF-induced neuronal differentiation (Maures et al., 2009; Qian and Ginty, 2001), are also consistent with SH2B1 needing to bind to the PM to promote neuronal differentiation.

The sequence responsible for nuclear import of SH2B1 closely resembles that of a classical monoparticulate NLS (KKKKKRF). SH2B1 is therefore presumed to bind to one or more importin $\alpha$ superfamily members and be transported into the nucleus via an importin $\alpha/\beta$ complex (Yasuhiro et al., 2009). Phosphorylation of Ser near the NLS has been shown to regulate nuclear import of the small GTP-binding proteins, Kir/Gem and Rem, by regulating their binding to importin $\beta$. The Phe zipper-containing dimerization domain is also involved in such interactions. The Phe zipper-containing dimerization domain is also involved in such interactions. The Phe zipper-containing dimerization domain is also involved in such interactions.

In conclusion, we have shown that the polybasic NLS domain of SH2B1 serves a dual function, localizing SH2B1 to both the nucleus and the PM, the latter potentially via an electrostatic interaction. The Phe zipper-containing dimerization domain is also required for PM localization, perhaps because it stabilizes the binding of the polybasic NLS to the PM. Whether SH2B1 is in the PM or nucleus appears to be regulated by the phosphorylation of Ser by PKC and/or other as yet unidentified kinases. The ability to cycle between the PM and other compartments appears to be crucial for functions of SH2B1, including enhancement of NGF-induced uPAR gene expression and neurite outgrowth. These findings not only provide insight into the subcellular localization and function of an important scaffold protein, but also provide further evidence of the use of electrostatic switches to regulate the subcellular localization of signaling proteins. Indeed, a search for other proteins with a stretch of Ser/Thr near a polybasic region revealed adducin. Like SH2B1, adducin has been reported to cycle between the PM, where it regulates the actin cytoskeleton, and the nucleus, where it is thought to regulate cell division (Pariser et al., 2005). Furthermore, phosphorylation of multiple adjacent Ser residues by PKC appears to determine the subcellular localization of adducin. We believe it likely that other proteins that reside either at the PM or the nucleus will be found to cycle between the two compartments by a similar electrostatic switch mechanism.

### Materials and Methods

#### Antibodies

Polyclonal antibody to rat SH2B1 (oSH2B1 (1:1000)) (Duan et al., 2004a) was from Liangyou Rui (University of Michigan, Ann Arbor, MI), oFLAG (M2 (1:2000)) was from Sigma. oMyc (1:5000), oPHSP90a/β (H-114 (1:2000)) and oCEBPβ (H-7) (1:10000) were from Santa Cruz Biotechnology. opERK1/2 (E10) and oERK (1:2000 dilution) and horseradish-peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG (1:5000) were from Cell Signaling (Danvers, MA). IRDye800-conjugated anti-gF (oGF), IRDye700 and IRDye800 conjugated anti-mouse and anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA), and Alexa-Fluor-680-conjugated anti-rabbit IgG (Invitrogen) were used at a dilution of 1:20,000. Protein A-HRP was from Amersham Biosciences.

#### Plasmids

Rat SH2B1 cDNA (NM_001048180) was subcloned into pEGFP (Clontech, Mountain View, CA), pmCherry (Clontech), pCMV-Tag2B (Stratagene) containing a N-terminal FLAG tag, or Prk5 containing an N-terminal myc tag. Mutations were introduced using QuickChange or QuickChange Multi-Site-Directed Mutagenesis kits (Stratagene). To prepare truncation and deletion mutants of SH2B1, BamHI sites were inserted at the desired location(s) and the cDNAs digested with BamHI and re-ligated (Maures et al., 2009). All constructs were sequenced. When expressed in 293T cells, the mutant SH2B1 proteins migrated in SDS-PAGE gels with the correct predicted size (data not shown).

#### Cell culture and transfection

3T3-F442A preadipocytes (stock from Howard Green, Harvard University, Cambridge, MA) and 293T cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 1 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin (DMEM culture medium) and 8% calf serum. Cells were incubated in serum-free medium containing 1% BSA overnight prior to treatment with PMA. 24A human fibrosarcoma cells stably expressing Gf receptor (G4-CHO cells), from George Stark (Cleveland Clinic, Cleveland, OH) and Ian Kerr (Imperial Cancer Research Fund, London, UK), were maintained in DMEM culture medium, 10% fetal bovine serum (FBS) and 5 mM sodium pyruvate. PC12 cells (stock from ATCC) were grown in DMEM culture medium with heat-inactivated univalent PH domain of dynamin-1 with a dissociation constant ($K_d$) estimated in the millimolar range, whereas a dimeric form of the PH domain of dynamin-1 binds PtdIns(4,5)P$_2$-containing vesicles with an effective $K_d$ almost 1000 times lower, in the micromolar range (Klein et al., 1998). We believe that the requirement for dimerization represents a novel means by which non-lipidated proteins with polybasic motifs can be targeted to the PM. Because SH2B1 family members have been shown to form higher-order oligomers (Qian and Ginty, 2001), presumably involving domains in addition to the DD, it is possible that association of SH2B1 with the PM is enhanced even further by oligomerization.

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Phosphorylation controls a polybasic NLS

5% FBS and 10% horse serum (MP Biomedical) on plates coated with rat tail collagen (BD Biosciences). 3T3-F442A cells were transiently transfected using Amaxa Nucleofector with solution V and setting U24 (Lonza), 2C4-GHR (using 20 μg/mL CalPhos maximizer; Clontech) and 293T cells were transiently transfected using calcium phosphate precipitation (Chen and Okayama, 1987). PC12 cells were transiently transfected using a Gene Pulser Xcellator electroporator (400 V, 500 μF) (BioRad, Hercules, CA). After 5 hours, cells were washed and fresh growth medium was added for 24 hours.

Neuronal differentiation of PC12 cells
PC12 cells transiently expressing the various SH2B1β were plated on six-well, collagen-coated plates and grown in differentiation medium (DMEM, 2% horse serum, 1% FBS) with or without NGF (BD Bioscience). Medium was replaced every 2 days. Differentiation was assessed using a Nikon Eclipse TE200 microscope with a 20× or 40× objective. Transfected (GFP-positive) cells with neurite length at least twice the diameter of the cell body were scored as differentiated. The percentage of differentiated cells was determined by dividing the number of differentiated cells by the total number of GFP-positive cells counted.

Cell Imaging
Cell imaging was performed using an Olympus Fluoview 500 laser scanning confocal microscope and Fluoview version 5.0 software. Cells were plated on 35-mm collagen-coated (PC12) or non-coated (293T) glass bottom dishes (MatTek, Ashland, MA) in Ringer’s buffer (10 mM HEPES, 155 mM NaCl, 2 mM CaCl2, 5 mM KCl, 1 mM MgCl2, 10 mM NaH2PO4 and 10 mM glucose, pH 7.2). The PM was labeled with wheat germ agglutinin-conjugated Alexa Fluor 594 (Invitrogen). Two or more individual experiments were conducted with each mutant SH2B1β. All transfected cells were viewed (≥20 cells per experiment). Generally, 90% of the cells showed the same subcellular distribution for individual forms of GFP-SH2B1β, independent of the expression level. The outliers were either clearly dying or dead cells, or highly expressing cells in which the ratio of cytoplasmic to PM signal was abnormally high, presumably due to saturation of a limited number of binding sites in the PM. Image planes were chosen to allow maximal visualization of the PM and nucleus. Line scan profiles were obtained using MetaVue Software (Universal Imaging, Sunnyvale, CA). Each value represents an average of 15 pixels (1.5 μm) perpendicular to and centered around the scanned line.

For the initial experiments shown in Fig. 2, cells were monitored hourly after 4 hours with 20 nM LMB (Sigma), and imaged when the nuclear to cytoplasmic ratio of cytoplasmic to PM signal showed the same subcellular distribution for individual forms of GFP–SH2B1β, mutants were lysed in 50 mM Tris, 1% Triton X-100, 150 mM NaCl, 2 mM CaCl2, 5 mM MgCl2, 10 mM NaH2PO4 and 10 mM glucose, pH 7.5 (fractionation buffer); and sonicated (Misonix Sonicator 3000). The sonicated lysates were centrifuged at 12,000 × g for 4°C for 2 hours. The supernatant was designated the cytosolic fraction. The pellet, designated the membrane fraction, was washed twice with fractionation buffer and dispersed by sonication. 3T3-F442A cells were fractionated using the ThermoScientific Subcellular Protein Fractionation Kit.

Immunoprecipitation and immunoblotting
293T cells transiently expressing FLAG or FLAG–SH2B1β and the designated GFP-tagged SH2B1β mutants were lysed in 50 mM Tris, 1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 10 mM NaF, 1 mM Na2VO4, 0.1 μM Na3I, and protease inhibitors (1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin), pH 7.5 (fractionation buffer) and sonicated (Misonix Sonicator 3000). The sonicated lysates were centrifuged at 12,000 × g for 4°C for 2 hours. The supernatant was designated the cytosolic fraction. The pellet, designated the membrane fraction, was washed twice with fractionation buffer and dispersed by sonication. 3T3-F442A cells were fractionated using the ThermoScientific Subcellular Protein Fractionation Kit.

RNA preparation and QPCR
Total RNA was isolated using Stat60 (Tel-Test), cDNA was generated using OneStep RT-PCR Kits with SYBR Green (BioRad) and uPAR gene expression assessed in duplicate by QPCR using the iCycler system as described previously (Maures et al., 2009). All readings were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which did not differ between the different transfected cells.

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References


