The Involvement of Abl and PTP61F in the Regulation of Abi Protein Localization and Stability and Lamella Formation in Drosophila S2 Cells*

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Most aspects of cellular events are regulated by a series of protein phosphorylation and dephosphorylation processes. Abi (Abl interactor protein) functions as a substrate adaptor protein for Abl and a core member of the WAVE complex, relaying signals from Rac to Arp2/3 complex and regulating actin dynamics. It is known that the recruitment of Abi into the lamella promotes polymerization of actin, although how it does this is unclear. In this study, we found PTP61F, a Drosophila homolog of mammalian PTP1B, can reverse the Abl phosphorylation of Abi and colocalizes with Abi in Drosophila S2 cells. Abi can be translocalized from the cytosol to the cell membrane by either increasing Abl or reducing endogenous PTP61F. This reciprocal regulation of Abi phosphorylation is also involved in modulating Abi protein level, which is thought to affect the stability of the WAVE complex. Using mass spectrometry, we identified several important tyrosine phosphorylation sites in Abi. We compared the translocalization and protein half-life of wild type (wt) and phosphomutant Abi and their abilities to restore the lamellipodia structure of the Abi-reduced cells. We found the phosphomutant to have reduced ability to translocate and to have a protein half-life shorter than that of wt Abi. We also found that although the wt Abi could fully restore the lamellipodia structure, the phosphomutant could not. Together, these findings suggest that the reciprocal regulation of Abi phosphorylation by Abl and PTP61F may regulate the localization and stability of Abi and may regulate the formation of lamella.

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phosphorylation of the various components of WAVE complex. Because the ability of WAVE to reorganize actin is modulated by Abl and the protein stability and activity of WAVE complex is modulated by Abi, it is reasonable to hypothesize that Abl-mediated phosphorylation of Abi regulates its localization and stability, thereby affecting the formation of lamella. In the present study, we first identified PTP61F as a new PTP for Drosophila Abi. We then examined the impact of phosphorylation/dephosphorylation of Abi protein on its location and stability. We found that Abi and PTP61F together cooperated in the modification of the subcellular localization and protein stability of Abi through phosphorylation/dephosphorylation. In addition, a phosphomutant Abi we generated for our experiments was found to have an impaired ability to regulate lamellipodia formation of motile cells. Together, our findings support the notion that reversible tyrosine phosphorylation of Abi may play a critical role in controlling actin cytoskeleton dynamics.

**EXPERIMENTAL PROCEDURES**

**Plasmids, dsRNA Preparations, and Antibodies—**Abi gene was fused with GST, HA tag, or GFP and subcloned into pMT or pAc5.1 vectors (Invitrogen). HA-tagged PTP61F constructs and anti-PTP61F rabbit polyclonal antibodies were provided as gifts from Dr. Tzu-Ching Meng (Academia Sinica, Taiwan). Abi-YF and PTP61F-PA mutants were generated by PCR-based mutagenesis (QuickChange site-directed mutagenesis kit; Stratagene). Targeted gene regions of dsRNA were amplified by PCR, and dsRNA were produced by *in vitro* transcription using T7 polymerase (21). Rabbit and mouse polyclonal anti-Abi antibodies were raised against the fragment of C-terminal amino acids 261–473 and affinity-purified. Purified GST-tagged dAbl (contain N-terminal SH3-SH2 domain) recombinant protein was used to produce rabbit polyclonal anti-dAbl antibody (LTK Biolaboratories, Touyuan, Taiwan). Antibodies to phosphotyrosine (4G10) (Upstate Biotechnology, Inc.), HA (Babco), c-Abl (K12) (Santa Cruz Biotechnology), c-Abl (8E9) (Pharmingen), and β-actin (Chemicon) were used in the detection on proteins according to manufacturer’s instructions.

**Cell Culture and Transient Transfection—**The Drosophila S2 cells were cultured in *Drosophila* expression system medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum at 23 °C. Transient transfection of plasmids into *Drosophila* S2 cells was performed using the liposome-based method with 3 μg of DNA/7.5 × 10^5 cells. To induce metallothionein promoter-driven gene expression, 600 μM CuSO₄ was added to the cells for 24 h. For RNA interference experiments, 1 μg of dsRNA was added to the liposome mix, and the cells were harvested for assay 4 days after transfection.

**GST Pulldown, Immunoprecipitation, and Western Blotting—**Transiently transfected S2 cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Nonidet P-40, 200 μM Na₃VO₄, and protease inhibitor mixture (Complete™; Roche Applied Science)). The lysates were centrifuged at 14,000 × *g* for 20 min at 4 °C to remove insoluble materials. Total protein was measured by the DC protein assay (Bio-Rad). For GST pulldown, the cell lysates were mixed with 30 μl of 1:1 slurry of glutathione-Sepharose (Amersham Biosciences) overnight at 4 °C. After precipitation, proteins bound to the beads were eluted with Laemmli sample buffer for Western blotting. Immunoprecipitations were performed using above-mentioned antibodies, followed by incubation with protein A or G (Calbiochem) beads. Lysates or immunoprecipitates were resolved by SDS-PAGE and electrotransferred to an Immobilon™-P membrane (Millipore). After Western blotting, bounded antibody was visualized with an ECL detection kit (Amersham Biosciences).

**Immunofluorescence Staining—**For immunostaining, S2 cells grown in suspension were plated on poly-L-lysine-coated coverslips (0.1 mg/ml; Biochrome) for 10 min or spread on ConA-coated coverslips (0.5 mg/ml; Sigma) for 40 min. The cells were then fixed with 4% (v/v) paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, washed with phosphate-buffered saline, and permeabilized with 0.1% (v/v) Triton X-100 and 1% (v/v) bovine serum albumin in phosphate-buffered saline for 30 min at room temperature before incubating with primary antibodies overnight at 4 °C. The cells were washed and incubated with fluorophore-labeled secondary antibodies (Jackson Laboratories). Nuclei and F-actin were counterstained with 4′,6-diamidino-2-phenylindole and rhodamine-conjugated phalloidin (Molecular Probes), respectively. The images were obtained with a Leica TCS NT confocal microscope.

**RESULTS**

**Abi Is a Novel Substrate of PTP61F Tyrosine Phosphatase—**Abi was first identified as an evolutionarily conserved SH3 domain-binding partner and substrate of Abl kinase (2–4), although what phosphatase might counteract the Abl phosphorylation is still not known. PTP61F is identified as a *Drosophila* homolog of mammalian PTP1B (22), which is known to be involved in regulation of various signaling pathways, including insulin receptor, platelet-derived growth factor, epidermal growth factor, insulin-like growth factor, P210BCR-ABL, Src, and JAK/STAT (23). Because PTP (Janus kinase/signal transducers and activators of transcription) can interact directly with Dock (24), which binds to Abi (25), and because the PTP61F transcript is primarily observed (26), we speculated that PTP61F might be a candidate phosphatase for the dephosphorylation of Abi. To test this possibility, we disrupted the endogenous PTP61F expression by introducing dsRNA into *Drosophila* S2 cells to determine whether the tyrosine phosphorylation level of endogenous Abi could be affected. Abi immunoprecipitate was calibrated to the comparable amount of protein in each lane for Western blotting with anti-phosphotyrosine antibody. The result showed that the knockdown of PTP61F expression clearly increased the tyrosine phosphorylation level of endogenous Abi (Fig. 1A), suggesting that PTP61F might be an important PTP capable of negatively regulating the tyrosine phosphorylation level of Abi in S2 cells.

To confirm this speculation, we investigated whether Abi would interact with PTP61F. For the substrate trapping assay, we constructed a mutant PTP (D203A) at the active site, one that could stably associate with a phosphorylated substrate but not dephosphorylate it (27). As expected, coexpressing the D203A mutant with GST-Abi did not noticeably affect the Abl and PTP61F Coregulate Phosphorylation of Abi
The phosphorylation level of Abi was compared with the wt PTP61F (Fig. 1B, third panel). Moreover, we found that Abi associated with both the wt and mutant forms of PTP61F, although the latter exhibited a higher affinity for Abi (Fig. 1B, top panel), suggesting a high affinity and active site binding of PTP61F with Abi. Thus, we wanted to identify the domain of Abi required for its interaction with PTP61F. Because PTP61F possesses multiple proline-rich motifs in its C terminus, which might interact with the SH3 domain of Abi, we constructed SH3-deleted and SH3-only (a small fragment only containing the SH3 domain) mutants of Abi for the binding assay. For the GST pulldown assay, we transiently coexpressed the GST-tagged Abi mutants with the full-length PTP61F in S2 cells. We found a strong binding affinity between HA-PTP61F and SH3-only mutant Abi and a weak affinity between HA-PTP61F and the SH3-deleted GST-Abi (Fig. 1C), underscoring its importance of the SH3 domain to the interaction between Abi and PTP61F. However, deletion of the Abi SH3 domain did not completely eliminate PTP61F binding, suggesting that other region(s) might also be involved in this interaction.

To identify the specific proline-rich motif of PTP61F binding to the SH3 domain of Abi, we generated point mutations in those five predicted proline-rich motifs of PTP61F (24) by replacing proline residues with alanine and determined the ability of these mutants (namely PA mutants) to bind GST-Abi-SH3. The first proline-rich motif was found to be the most critical one for the interaction of PTP61F with Abi SH3 domain (Fig. 1D).

After finding that PTP61F associated with Abi, we wanted to determine whether these two proteins colocalize in cells. To characterize the subcellular localization, we ectopically expressed HA-PTP61F in nonadhesive (nonmotile) S2 cells and immunostained the endogenous Abi. Confocal microscopy showed that most Abi colocalized with PTP61F in the cytosol (Fig. 2A). We repeated the same experiment in motile spreading cells by seeding the S2 cells on the ConA-coated coverslips (16). Most endogenous Abi colocalized with HA-PTP61F in the cytosol around the nucleus in motile cells (Fig. 2B, yellow signal of overlay image). A small fraction of Abi

**FIGURE 1.** Abi is a novel substrate of PTP61F tyrosine phosphatase. A, repression of PTP61F expression enhances phosphotyrosine level of endogenous Abi. Drosophila S2 cells transfected with control (GFP) or PTP61F dsRNA were immunoprecipitated by anti-Abi antibody for immunoblotting (IB) with anti-phosphotyrosine (pTyr) antibody 4G10. Equal amount of the precipitated Abi protein was loaded for Western blot. B, Abi interacts with PTP61F in cells. Western blot analysis shows GST-Abi strongly interacted with a substrate trapping mutant (DA) but weakly with the wild type (WT) PTP61F. Expression of D203A mutant of PTP61F with GST-Abi does not affect the phosphorylation level of Abi. C, SH3 domain of Abi is critical for binding to PTP61F. GST pulldown assay reveals a strong interaction between HA-PTP61F and SH3-only Abi and a marked decrease of interaction between HA-PTP61F and SH3-deleted GST-Abi (ΔSH3). D, the first proline-rich motif of PTP61F is important for binding to Abi. The left panel is a schematic representation of sequences of PTP61F mutants used in this study. The underlined letters indicate amino acids mutated from proline to alanine. The right panel shows GST pulldown assay for interactions between Abi SH3 domain and different PTP61F PA mutants.

**FIGURE 2.** PTP61F colocalizes with Abi in the perinuclear cytosol but not in the peripheral lamella. A, the suspension cells are plated on poly-L-lysine-coated coverslip. Ectopically expressed HA-PTP61F and endogenous Abi are immunofluorescence labeled with anti-HA (green) and anti-Abi antibody (red), respectively. The nucleus is marked with 4',6-diamidino-2-phenylindole (blue). B, the attached cells spread on ConA-coated coverslip. Actin was marked with rhodamine-phalloidin (blue). Scale bar, 5 μm.
Abl Is the Major Kinase of Abi in Drosophila S2 Cells—Although endogenous Abi was tyrosine-phosphorylated in S2 cells (Fig. 1A), it is unclear whether Abl is the major kinase involved in the tyrosine phosphorylation of Abi there. To find out, we first optimized the knockdown efficiency of dAbl RNAi in S2 cells (Fig. 3A) and then sought to determine whether the reduction of endogenous Abl suppressed the anti-phosphotyrosine signal of Abi. Western blotting showed that the tyrosine phosphorylation level of Abi was markedly lowered (0.3-fold) by the treatment of Abl RNAi as compared with that in the control RNAi-treated cells (Fig. 3A). We also overexpressed GST-tagged Abi for the similar assay and found that the reduction of endogenous Abi also greatly diminished the Abi tyrosine phosphorylation level (Fig. 3B, lane 2). These results clearly suggest that Abl is the major tyrosine kinase of Abi in the steady-state S2 cells.

PTP61F Dephosphorylates Abl-mediated Tyrosine Phosphorylation of Abi—We next wanted to test whether or not the Abl-mediated phosphorylation of Abi could be counteracted by the dephosphorylation of PTP61F. We transfected GST-tagged Abi with Abl RNAi, PTP61F RNAi, or both in S2 cells to determine the effect of endogenous Abl and PTP61F on Abi phosphotyrosine level. Our Western blot analysis showed that a reduction in Abl markedly decreased the Abi phosphorylation level, whereas a reduction in PTP61F increased it (Fig. 3B, lanes 2 and 3). Moreover, when we knocked down both Abl and PTP61F together in cells, the Abi phosphorylation signal was also dramatically decreased (Fig. 3B, lane 4). These results suggest that Abl and PTP61F are the key regulators of Abi and that PTP61F actively reversed Abl-mediated phosphorylation of Abi in cells. To determine the requirement of the catalytic activity of PTP61F for the dephosphorylation of Abi, we generated a catalytically inactive CS mutant in which the cysteine 237 residue of PTP61F was substituted by serine. We coexpressed Abi with c-Abl or with both c-Abl and PTP61F (wt, DA, or CS) in cells. Western blotting showed that Abl-induced Abi phosphorylation was dramatically abolished by coexpressing Abi with the wt PTP61F (Fig. 3C, lanes 2 and 3). In contrast, coexpression of Abi with either inactive form of PTP61F (DA or CS) could only moderately reduce Abi phosphorylation in cells, which retained a high level of tyrosine phosphorylation (Fig. 3C, lanes 4 and 5). To investigate whether the Abl–PTP61F interaction is essential for the dephosphorylation of Abi, we coexpressed an Abi binding-defective mutant of PTP61F (PA1) with Abi and Abi in S2 cells to assess the effect on dephosphorylation of Abi. As shown in Fig. 3D, PTP61F-mediated dephosphorylation of Abi was greatly attenuated by the disruption of Abi–PTP61F interaction (Fig. 3D, lane 4). Together, these results suggest that the Abl-mediated tyrosine phosphorylation on Abi was counteracted by PTP61F in S2 cells.

Abl and PTP61F Coregulate Abi Subcellular Localization—Several reports have demonstrated that a reduction of the expression of Abi in motile cells severely affects the structure and formation of lamellipodia (29). Because Abi localizes to the lamella regions in motile cells (Fig. 2B), we wanted to investigate whether the phosphorylation of Abi was involved in regulating its subcellular localization. Unlike the ubiquitous cytoplasmic expression pattern of the endogenous Abi (Fig. 2), ectopic expression of GFP–Abi in nonadhesive cells exhibited punctate structures in cytoplasm (Fig. 4A, top panel). The expression of dAbi was uniformly distributed in the cytosol, and that of mammalian c-Abl was uniformly distributed in the cytosol/nucleus (data not shown). In contrast, in cells ectopically coexpressing Abi and dAbi or c-Abl, the punctate pattern of Abi was markedly dispersed, and the protein was recruited to the actin-rich plasma membrane in nonadhesive cells (Fig. 4A, middle and bottom panels). It should be noted that dAbi as well as c-Abl also translocalized from cytosol/nucleus to the cell membrane and colocalized there with cortical F-actin (Fig. 4A). The corecruitment of Abl and Abi to the cell periphery suggests that Abl is crucial for regulation of Abi subcellular localization.
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To determine whether the membrane translocation is Abl kinase activity-dependent, we transfected GFP-Abi with a kinase inactive c-Abl (c-AblKR) in cells. As compared with that with a wt Abl, less Abi was present in the plasma membrane in cells coexpressing c-AblKR, although the Abi punctate structure with a wt Abl, less Abi was present in the plasma membrane in cells expressing c-AblKR, we found a reduced ability for this mutant to disperse the punctate pattern of Abi (69%), compared with that of wt Abl (95%) (Fig. 4B, right panels), suggesting that the kinase activity of Abl plays an important role in modulating the translocalization of Abi from cytosol to cell membrane.

Because the kinase activity of Abl is involved in regulating Abi translocation, the phosphorylation of Abi might be involved. To test the possible involvement, we performed RNAi experiments to find out whether a reduction in endogenous PTP61F, which would increase Abi phosphorylation, would further disperse the punctate pattern of Abi in cells coexpressing GFP-Abi with c-AblKR (Fig. 4B, right panels). The reduction in endogenous PTP61F caused a further significant dispersion of the Abi punctate pattern and increased the relocalization Abi in the cell membrane; no such effect was found in the control RNAi (Luc dsRNA) (Fig. 4C). These results further support the notion that the reciprocal Abl and PTP61F modulation of Abi phosphorylation is critical for regulations of Abi localization.

Abl and PTP61F Regulate Abi Stability—Our previous studies indicated that the overexpressed Drosophila Abi is an unstable protein, and the interaction of Abi with Abl results in an increase of Abi protein level (3). Although proteasome-mediated proteolysis is known to contribute to the instability of Abi (30), the role of Abl in this instability is less clear. To investigate its role in modulating Abi protein level, we performed RNAi-Abi experiments to determine the effect of Abl on Abi protein level. When endogenous Abl was repressed, there was a dramatic reduction in exogenous Abi protein level (Fig. 5A, left panels), indicating that Abl is a crucial positive modulator of Abi protein level.

To test whether kinase activity is involved in Abl control of Abi protein level, we transfected HA-Abi with wt dAbl, c-Abl, or c-AblKR in cells to compare their effects on Abi protein level. An increase in wt Abl proteins markedly increased the expression of Abi to four to five times the levels found in the mock control cells. The kinase inactive form of c-Abl also moderately increased Abi expression (Fig. 5A, right panels), although to a much lesser extent. These findings suggest that kinase activity of Abl is essential for the regulation of Abi protein level, echoing the results of our subcellular localization study.

FIGURE 4. Abl and PTP61F coregulate Abi subcellular localization. A, coexpression of Abl with GFP-Abi relocalizes GFP-Abi from the punctate structure in cytosol to cell periphery. Confocal microscopy showed expression of GFP-Abi alone displayed a punctate pattern in cytosol. GFP-Abi relocalized to cell periphery in cells coexpressing dAbl or c-Abl proteins. GFP-Abi is shown in green, and the localization of dAbl or c-Abl were immunostained by anti-HA or anti-Abl (8E9) antibodies and are shown in blue. Actin counterstained by rhodamine-phalloidin is shown in red. B, Abi relocalization to cell membrane is Abl kinase activity-dependent. Suspension 52 cells were cotransfected with GFP-Abi and dAbl, wt, or kinaseinactive (KR) c-Abl. Confocal microscopy revealed less GFP-Abi relocalizes to cell membrane by KR c-Abl (left panel). The percentage of GFP-positive cells with punctate pattern was quantified following the standard shown in the left panel. More than 100 of GFP-positive cells were counted for each experiment. Each value shown represents the mean of three independent experiments ± S.D. (right panel). C, reduction of PTP61F expression level further disperses the punctate pattern of GFP-Abi in cells coexpressing c-AblKR. The cells were transfected with dsRNA-PTP61F, GFP-Abi, and c-Abl-KR, and GFP fluorescence was visualized by confocal microscope. Histograms shown are the means of three independent experiments ± S.D. The difference between two indicated groups was tested for significance by Student’s t test (*, p < 0.05).
To further test the idea that the extent of Abi phosphorylation affects its protein level, we investigated whether or not the ectopic expression of PTP61F causes a decrease in Abi protein level. As can be seen in Fig. 5B (lanes 2 and 3), wt PTP61F clearly reduced Abi protein level, but inactive PTP61F did not. In addition, we also demonstrated that the PTP61F-PA1 mutant exhibited a defect in both dephosphorylation and destabilization of Abi (Fig. 5C). Together, these results suggest that Abi protein level can be modulated by its phosphorylation status, which is reciprocally regulated by the phosphorylating and dephosphorylating effect of Abl kinase and PTP61F phosphatase.

Identification of Abi Phosphorylation Sites—To further explore the biological significance of Abi phosphorylation, we sought to identify the tyrosine phosphorylation site(s) of Abi. To enrich Abl-dependent phosphorylation on Abi, we transfected Abl kinase and GST-Abi in S2 cells. The phosphorylated GST-Abi was pulled down for SDS-PAGE analysis to identify the band containing Abi for mass spectrometric analysis (data not shown). Three phosphorylated residues were identified: Tyr155, Tyr248, and Tyr285. Because this assay only completed 56.9% amino acid sequence coverage (data not shown), we used NetPhos (www.cbs.dtu.dk/services/NetPhos/) to search for predicted phosphorylation sites on Abi. Eight tyrosine residues (amino acids 148, 155, 233, 248, 285, 424, 443, and 468) were chosen for verification. Each of the eight residues was individually replaced by phenylalanine (Tyr3Phe) to construct a mutant and was then transfected into S2 cells (without coexpressing exogenous Abl kinase). Using an anti-Tyr(P) antibody in our Western blot, we measured the extent of phosphorylation. As shown in Fig. 6A, the tyrosine phosphorylation level of AbiY248F was markedly attenuated, whereas mutation of other
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FIGURE 7. 4YF mutation disrupts the membrane localization, protein stability, and lamella formation activities of Abi. A, AbiYFF expression leads to less plasma membrane and a more punctate structure in cytosol. wt or 4YF mutant GFP-Abi were cotransfected with dAbi in S2 cells, and patterns of GFP fluorescence were determined by confocal microscopy. The GFP distribution patterns were classified according to the categories shown in the left panel. At least 25 confocal microscopic images were analyzed for each experiment. Shown are the mean of three independent experiments. B, AbiYFF has a shorter half-life than wt Abi in cells. The degradation rate of Abi protein was analyzed by treatment of cells with protein synthesis inhibitor cycloheximide (50 μg/ml). Abi expression was induced by CuSO4 for 12 h and then replaced with fresh medium containing cycloheximide prior to harvest for the determination of the protein levels at the indicated time points. Quantifications are shown after normalization to the level of actin. C, dsRNA targeting Abi 3′-UTR selectively reduces endogenous, but not ectopically expressed Abi protein level in S2 cells. The cells were transfected with different dsRNAs targeting Abi coding sequence (CDS), Abi 3′-UTR (UTR), or luciferase (Luc), in the presence (+) or absence (−) of GFP-Abi. Abi antibody was used to detect the endogenous Abi or ectopically expressed GFP-Abi protein levels in cell lysates. D, the phosphomutant AbiYFF is insufficient to rescue the defects in lamella in cells with reduced endogenous Abi. Suspension cells were transfected with indicated plasmids and dsRNAs. Four days after transfection, the cells were resuspended and plated on ConA-coated coverslips for spreading and stained with rhodamine-phalloidin and visualized by fluorescence microscope to reveal the cell morphology (red in left panels). The phenotypes of lamella structure were quantified following the standard shown in the left panel. Histograms shown are the percentages of three categories in total number of GFP-positive cells observed (right panel). IB, immunoblotting.

days after transfection, the cells were resuspended and plated on ConA-coated coverslips for spreading and protein synthesis inhibitor cycloheximide prior to harvest for the determination of the protein levels at the indicated time points. Quantiﬁcations are shown after normalization to the level of actin.

cycloheximide (50 μg/ml). Abi expression was induced by CuSO4 for 12 h and then replaced with fresh medium containing cycloheximide prior to harvest for the determination of the protein levels at the indicated time points. Quantiﬁcations are shown after normalization to the level of actin.

cytoplasmic intermediate membrane

sites did not notably affect the phosphorylation level (AbiY148F not shown), suggesting that the Tyr248 residue is an important site for endogenous Abl-mediated phosphorylation of Abi in S2 cells. To further investigate whether Tyr248 was also the only key residue phosphorylated by exogenous Abl kinase, we cotransfected c-Abl with GST-AbiY248F. Unexpectedly, all single tyrosine mutation proteins, including AbiY248F, were still heavily phosphorylated by exogenous Abl (Fig. 6B, lane 2; and data not shown), suggesting that there are other tyrosine residues on Abi that also play an important role in Abl-mediated phosphorylation. To generate a mutant with the greatest reduced phosphorylation signal to Abl kinase, we generated a series of Abi mutant constructs in which two, three, or four tyrosine residues were replaced with phenylalanine for assay. We found that a construct carrying four mutated residues (Y148F, Y155F, Y248F, and Y285F), hereafter referred to as AbiYFF, were found to have very weak level(s) (<10% of that of wt Abi) of Abl-mediated phosphorylation (Fig. 6B, compare lane 6 with lanes 2 and 4), as compared with that of wt or Y248F mutant proteins.

We used this AbiYFF mutant to test whether these four tyrosine residues were dephosphorylated by PTP61F. When PTP61F expression was repressed, there was no obvious enhancement of phosphotyrosyl signal of AbiYFF. In contrast, repression of PTP61F could increase the phosphotyrosyl signal of wt Abi and to a minor extent of AbiY248F (Fig. 6C), suggesting that these four tyrosine residues were dephosphorylated by PTP61F.

Abi Phosphorylation of Abi Is Essential for Abi Relocation, Its Protein Stability, and Its Ability to Modulate the Formation of Lamella—We have demonstrated that the reciprocal regulation of Abi phosphorylation by Abi and PTP61F can modulate the subcellular localization of Abi. To conﬁrm our ﬁndings, we transfected dAbi with GFP-tagged AbiYFF mutant in S2 cells to ﬁnd out whether mutation of these critical tyrosine residues could disrupt the translocalization of Abi to cell membrane. Cells expressing AbiYFF protein were found to have impaired membrane relocation activity. The punctate pattern of Abi was only partly dispersed and did not completely translocalize to the cell membrane (Fig. 7A, middle left panel). We compared the protein localization and punctuate patterns in cells with wt Abi and that in 4YF mutant Abi. Only ~38% of cells expressing AbiYFF pro-

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tein were found to be mainly distributed in the cell membrane, as compared with 70% of cells expressing wt Abi did (Fig. 7A, right panel). This finding concurs with those coexpressing wt Abi and kinase inactive c-Abl as previously shown in Fig. 4.

We next sought to investigate whether the disruption of Abi phosphorylation could affect its protein stability. Using cycloheximide to inhibit cytoplasmic protein synthesis, we compared the protein turnover rate of wt Abi and Abi\(^{4YF}\). Western blot analysis of cell lysates revealed that the Abi\(^{4YF}\) protein had a much shorter half-life (~4 h) than the wt Abi (~9 h) (Fig. 7B), a finding that further supports the results represented in Fig. 5. These findings indicate that the post-translational modification of Abi by tyrosine phosphorylation is critical to protein stability.

Because Abi protein stability and its localization in the cell membrane are both essential for WAVE-mediated actin reorganization, the dysregulation of Abi phosphorylation might cause defects in the formation of lamella in motile cells. To test this hypothesis, we suppressed the expression of the endogenous Abi and then reintroduced it back to the cells with a phosphomutant form of Abi. To do this, we first needed to determine whether or not using a 3' -UTR-targeted RNAi method (31, 32) to knock down the endogenous Abi would affect the ectopic expression of the phosphomutant Abi. We cotransfected a dsRNA, targeting exclusively the 3' -UTR sequence of Abi, with a GFP-Abi containing a vector-derived artificial 3' -UTR resistant to the targeting of Abi\(^{3'-UTR}\) RNAi. The Western blot showed that the endogenous Abi protein level was selectively reduced by the dsRNA, but the exogenous GFP-Abi level was not (Fig. 7C, lane 6). In contrast, a dsRNA targeting the Abi coding sequence (CDS) reduced the levels of both endogenous and exogenous Abi protein (Fig. 7C, compare lane 1 with lane 5 and lane 2 with lane 6).

To explore the biological significance of the phosphomutant Abi\(^{4YF}\) in the formation of lamella, we needed to determine whether treatment with Abi\(^{3'-UTR}\) RNAi could induce phenotypes similar to those induced by treatment with Abi\(^{CDS}\) RNAi (14, 16). By plating on ConA-coated coverslips, both cells treated with Abi\(^{3'-UTR}\) and those treated with Abi\(^{CDS}\) RNAi exhibited stellate lamella, a defect in the lamellipodia-like structures of motile cells (Fig. 7D, top left panel). We then ectopically expressed the phosphomutant GFP-Abi\(^{4YF}\) in cells treated with Abi\(^{3'-UTR}\) to investigate whether these defects in the lamella could be rescued. The lamellipodia defects were only partially rescued, with many cells continuing to show serrate lamella (Fig. 7D, middle left panel). However, cells expressing wt Abi protein were able to efficiently rescue the defects in the lamellipodia and return lamella to normal (Fig. 7D, bottom left panel). Only 28% of rescued with GFP-Abi\(^{4YF}\) experience complete restoration of lamella, in contrast to 51% of those with wt Abi (Fig. 7D, right panel), suggesting that phosphomutant GFP-Abi\(^{4YF}\) was less efficient in modulating the formation of lamella. Taken together, these results indicate that the regulation of Abi tyrosine phosphorylation is essential in modulating its protein stability, membrane relocation, and ability to regulate the formation of lamella.

**DISCUSSION**

Tyrosine phosphorylation/dephosphorylation is a common and important post-translational modification of key signaling proteins (33). Although Abi was originally identified as a kinase substrate of Abl (2, 4), little is known about how phosphorylation contributes to its biological significance. In this study, we demonstrated that the level of Abi tyrosine phosphorylation is balanced between phosphorylation by Abl and dephosphorylation by PTP61F. This conclusion is based on several lines of evidence. First, we showed that RNAi knockdown of PTP61F expression greatly elevated the tyrosine phosphorylation level of endogenous Abi in the *Drosophila* S2 cells. We confirmed that PTP61F is a major physiological phosphatase of Abi by substrate trapping, GST pulldown, and colocalization experiments. Second, our RNAi experiments also suggested that Abi is the major kinase mediator of tyrosine phosphorylation of Abi. Finally and most importantly, Abl-mediated tyrosine phosphorylation of Abi was shown to be counteracted by the dephosphorylation of a wt but not the catalytically inactive PTP61F proteins (DA or CS). Taken together, these results indicate that the coordinated phosphorylating action of Abl and dephosphorylating action of PTP61F together coordinate to regulate the tyrosine phosphorylation level of Abi.

Because many adaptor proteins undergo protein relocalization in response to tyrosine phosphorylation and protein-protein interaction (34), we investigated whether the balance of tyrosine phosphorylation in Abi affected its localization subcellularly. In cells ectopically expressing Abi and Abi, both Abi and Abi proteins relocalize to the same cortex cell regions, where active actin polymerization occurs (Fig. 4). The relocalization appears to be dependent, as least in part, on the kinase activity of Abi because the kinase inactive Abi would exhibit markedly reduced ability to relocalize Abi from a cytosolic punctate position to a plasma membrane position. This finding was further supported by additional experiments showing that a reduction in endogenous PTP61F could increase the relocalization of GFP-Abi from cytosol to the plasma membrane, and when Abi\(^{4YF}\) phosphomutant protein was expressed, there was a decrease in this translocation activity. We believe that the Abl/PTP61F-mediated phosphorylation/dephosphorylation of Abi is critical in regulating Abi functions by relocalizing Abi from the cytoplasm to the cell membrane. The importance of Abi phosphorylation is also evident in our study of Abi protein stability. Using overexpression or RNAi knockdown approach to control Abi or PTP61F protein levels, we demonstrated that both protein level and activity of these two enzymes are essential in the control of Abi protein stability. Furthermore, we showed that the protein turnover rate of Abi\(^{4YF}\) was much faster than that of wt Abi, further supporting the idea that the tyrosine phosphorylation of Abi is critical in modulating its protein stability. Together, these results highlight the importance of the Abi phosphorylation/dephosphorylation in modulating its localization and protein stability.

Although our data indicate that Abi phosphorylation of Abi is critical in modulating Abi subcellular localization, the underlying mechanism behind this process remains elusive. Nevertheless, the Abi protein is known to contain a myristoyl group,
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A common module for anchoring a protein to a cell membrane (35, 36). Moreover, recent structural studies, which found that removal of the myristoyl group activates c-Abl kinase activity, suggest that by binding myristoyl group to the kinase pocket of Abl, an inactive conformation may be promoted (37, 38). Because Abi functions as a positive regulator of Abl kinase, the Abl-Abi interaction might induce a conformational change through which the buried region that bears an N-myristoylation signal is exposed. This would result in targeting the Abl-Abi complex to cell membrane. In fact, our data showed that the interaction of a kinase-defective Abl with Abi was also somewhat to trigger the Abi relocation from the cytosol to the cell membrane (Fig. 4B). We also found that Abl phosphorylation of Abi in the complex further promoted the relocation of Abi to cell membrane (Figs. 4B and 7A). One recent structural and biochemical study has shown that a phosphotyrosine ligand can further convert the Abi protein into a more "open" conformation (37). It might be possible that the phosphotyrosine residues of Abi further opened the semi-unfolded position of myristoyl group in Abi, further promoting the relocation of Abi-Abi complex.

Another intriguing issue is the unique subcellular localization of PTP61F. Our immunocytochemistry experiments showed that most PTP61F colocalized with Abi in the perinuclear region (Fig. 2), presumably where the endoplasmic reticulum (ER) is situated, but not at the leading edge of the spreading cells, where Abi is preferentially localized. Our finding regarding the subcellular localization of PTP61F in S2 cells is similar to those reported for its mammalian counterpart, PTP1B. PTP1B largely resides on the external face of the ER (39). Given our finding that PTP61F counteracts Abl phosphorylation of Abi, the spatial separation of PTP61F from Abi in the leading edge might suggest that the phosphorylation of Abi is less affected by PTP61F in this region and that Abi residing at the leading edge might exert a higher tyrosine phosphorylation signal than Abi in the perinuclear region. In fact, several groups have reported that a number of proteins localized to the leading edge of motile cells are tyrosine-phosphorylated (40, 41). Nevertheless, the substrates of PTP targeting the cell periphery ultimately have to be dephosphorylated to terminate its role in signaling. To elucidate the mechanism underlying this process, three models have been proposed. First, the receptor tyrosine kinases, such as epidermal growth factor receptor and platelet-derived growth factor receptor, can be directed from plasma membrane to ER by vesicle trafficking for dephosphorylation by PTP1B (42). Second, the ER membrane-anchored PTP1B can be cleaved by the calcium-dependent neutral protease calpain to relocate its catalytic domain from membranes to the cytosol (43). Last, by reaching the ER network to the cell periphery, PTP1B at the local attachment site can interact with its substrates in the plasma membrane (44, 45). These models together with our findings suggest that there is a possible link between PTP61F and Abi signaling in the regulation of Abi phosphorylation at the leading edge of the plasma membrane.

Protein stability is also a critical factor for regulating Abi functions in actin dynamics (29). When Abi expression is lost in S2 cells, there are severe defects in the formation of lamella (14, 16). However, Abi alone does not affect the formation of lamella. Abi forms a tight complex with WAVE, Hem, and Sra-1 to regulate the formation of lamellipodia (17, 28). Moreover, the lamellipodia formation is dependent on the integrity of a functioning WAVE macromolecular complex (WAVE:Abi:Hem:Sra-1) (14, 16), but the down-regulation of Abi expression impairs the stability of WAVE complex in both mammalian and Drosophila cells (29). Thus, the protein stability of Abi is highly correlated with its regulation of the formation of lamella. One of our previous studies demonstrates that the interaction between Abi and Abl leads to an increased protein level of Abi (3). Herein, we have extended this finding by showing that the impaired Abl-mediated phosphorylation of Abi resulted in protein instability of Abi in S2 cells. We also demonstrated that the ectopic expression of a wt, but not an inactive, PTP61F also dramatically diminished the Abi protein stability. Thus, our results indicate that tyrosine phosphorylation/dephosphorylation by Abi/PTP61F is critical in controlling Abi protein turnover. Because it has been suggested that Abi undergoes an ubiquitin-mediated proteolysis (30), it might be possible that that the Abi phosphorylation of Abi represses the proteolysis degradation. One recent study has demonstrated that the Abl phosphorylation of c-Jun blocks the access of E3 ubiquitin ligase, Itch, to its binding epitope of c-Jun and thus prevents c-Jun from proteosome-mediated proteolysis (46).

Although tyrosine phosphorylation of Abi is important in stabilizing Abi protein level, the protein interaction between Abi and Abl might also attribute to Abi protein stability. We showed that the coexpression of a kinase-defective c-Abl and Abi resulted in a 1.8-fold increase of Abi protein level (Fig. 5A, right panel, compare lanes 1 and 4). Moreover, this protective effect probably is a common feature of both the Abi-Abl and Abi-WAVE:Hem:Sra-1 complexes. Our preliminary data showed that the ectopic coexpression of Abi, Hem, and WAVE could enhance Abi protein level and that the reduction of Hem or WAVE markedly decreased the endogenous Abi protein level, suggesting that a constitutive complex of Abi with WAVE, Hem, and Sra-1 could protect Abi from post-translational degradation. This finding is also comparable with what was documented in the scenario of WAVE/Scar stability (14).

Following this logic, it is reasonable to speculate that a sizeable fraction of the ectopically expressed Abi proteins would be short of match protein members to form the protein complex. Under this circumstance, the Abi would be more dependent on the phosphorylation regulation mechanism to modulate its protein stability. Supporting this view, we showed that the protein stability of exogenous Abi was particularly sensitive to the phosphorylation regulation (Figs. 5 and 7B). Although component proteins of the WAVE/Scar complex are thought to form a constitutive multiprotein complex in cells, a recent study showed that a small population of WAVE2 exists as a monomeric form without forming a complex with other component.
proteins in A431 cells (47). In addition to WAVE2, Hem/Nap1 was also reported to regulate cell motility through a WAVE/Scar-independent pathway in Dictyostelium and human neutrophil (48, 49). These studies suggest that the component proteins of WAVE/Scar complex may present as a “noncomplex” form of protein in regulating cell motility. Accordingly, although exogenous Abi is relatively independent of the physiological state, it is more amenable to the study of protein stability.

Another interesting issue related to the Abi stabilization by Abl is linked to a possibility that the stabilized Abi protein might in return further enhance the Abl kinase activity and Abi phosphorylation, thus establishing a positive feedback loop between Abl activity and Abi phosphorylation/stability. Because Abl kinase activity is tightly regulated in cells, this positive feedback loop should be eventually interrupted by a third molecule. PTP61F might be such a suppressor and function as a brake on the overactivated Abl-Abi signaling pathway by dephosphorylating Abi and facilitating protein degradation.

Finally, this study investigated whether a phosphomutant of Abi, which is not phosphorylated by Abl, could disrupt the lamella formation in motile cells. Our experiments suggest that the expression of Abi44R was unable to rescue the lamella defects that were caused by treatment with Abi3133-UTR RNAi (Fig. 7D), supporting the idea that Abl might act positively in promoting Abi regulation of the lamella formation. Two recent reports have demonstrated that the mutation on a critical Abl phosphorylation residue of WAVE can disrupt its role in actin-dependent cytoskeleton remodeling (10, 20). Our data might suggest that Abl phosphorylation on Abi promotes actin polymerization. Therefore, we asked whether Abl also phosphorylates all the other WAVE members and whether that would in turn modulate their roles in actin dynamics. Our preliminary data showed that Abl, via the linkage of Abi, could tyrosine phosphorylate each protein member of the WAVE complex and that process was also counteracted by PTP61F.3 Although additional experiments are required to determine the physiological significance of the phosphorylation/dephosphorylation of WAVE complex proteins, it is possible that the opposing actions of Abl and PTP61F in all of the other WAVE members might play a role in fine-tuning actin polymerization in cells. To resolve this possibility, it will be important to investigate whether Abl/PTP61F phosphorylation/dephosphorylation of the WAVE complex is occurred in a consecutive or synchronized manner in actin polymerization.

Our work has demonstrated that Abi undergoes protein modification by Abl and PTP61F via reversible tyrosine phosphorylation/dephosphorylation. This process regulates its relocalization to the cell membrane and protein stability, and ultimately it regulates the formation of lamella in motile cells. Therefore, the balance between Abi kinase and PTP61F phosphatase in regulating tyrosine phosphorylation of Abi may mediate the function of WAVE complex in cell periphery. Our study furthers our understanding of how and where the reciprocal regulatory processes of an actin-associated protein function within the cells.

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