The Abl interactor proteins localize to sites of actin polymerization at the tips of lamellipodia and filopodia

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Cell movement is mediated by the protrusion of cytoplasm in the form of sheet- and rod-like extensions, termed lamellipodia and filopodia. Protrusion is driven by actin polymerization, a process that is regulated by signaling complexes that are, as yet, poorly defined. Since actin assembly is controlled at the tips of lamellipodia and filopodia [1], these juxtamembrane sites are likely to harbor the protein complexes that control actin polymerization dynamics underlying cell motility. An understanding of the regulation of protrusion therefore requires the characterization of the molecular components recruited to these sites. The Abl interactor (Abi) proteins, targets of Abl tyrosine kinases [2–4], have been implicated in Rac-dependent cytoskeletal reorganization in response to growth factor stimulation [5]. Here, we describe the unique localization of Abi proteins in living, motile cells. We show that Abi-1 and Abi-2b fused to enhanced yellow fluorescent protein (EYFP) are recruited to the tips of lamellipodia and filopodia. We identify the targeting domain as the homologous N terminus of these two proteins. Our findings are the first to suggest a direct involvement of members of the Abi protein family in the control of actin polymerization in protrusion events, and establish the Abi proteins as potential regulators of motility.

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Results and discussion

Characterization of constructs

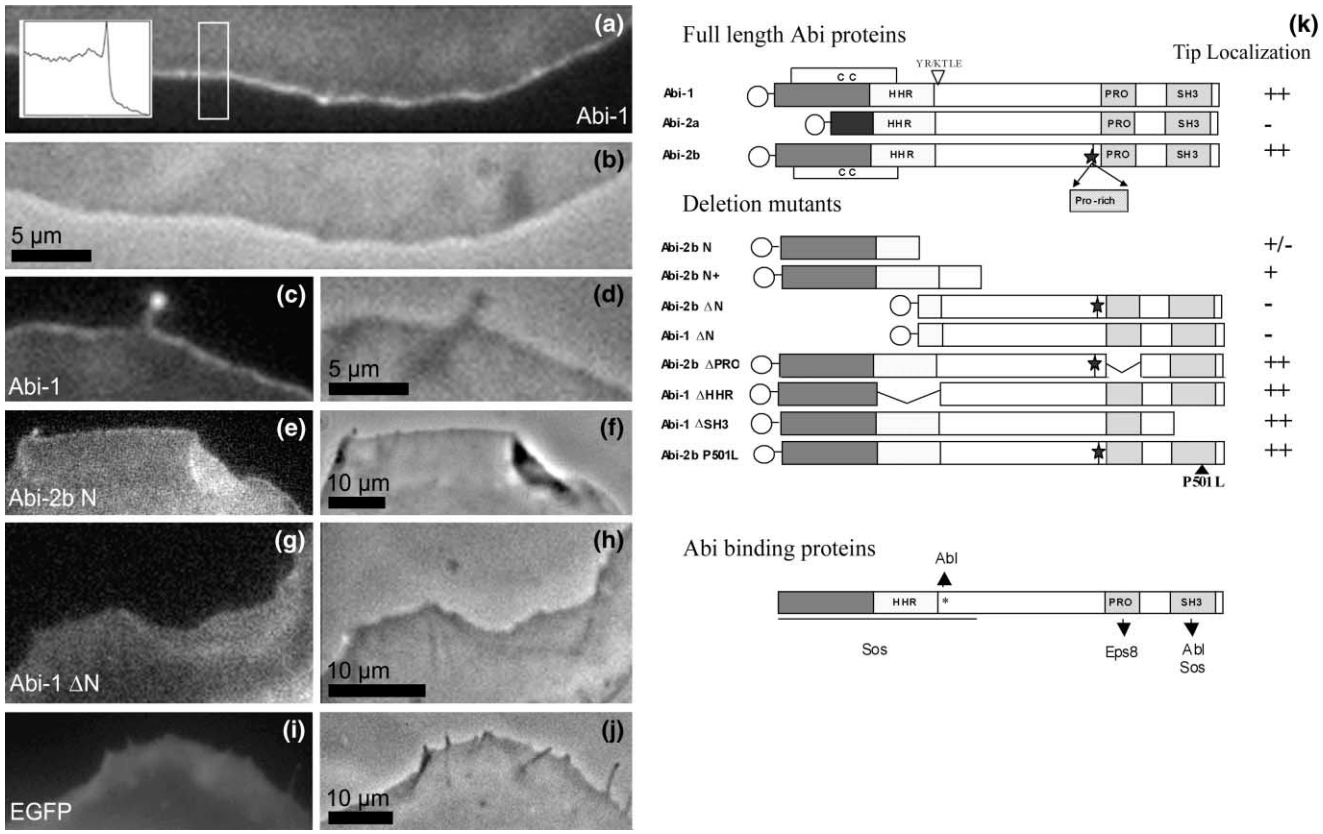
Abi proteins are targets of the Abl kinases and have been linked to Rac-dependent cytoskeletal reorganization [2–5]. Abi-1 (e3B1) associates with Sos-1 and the EGF-receptor substrate Eps8 in a complex that exhibits Rac-GEF activity (Figure 1k) [5]. The depletion of Abi-1 by microinjection of antibodies into fibroblasts blocks membrane ruffling in response to PDGF [5]. These observations implicate Abi proteins in pathways signaling to the actin cytoskeleton. To examine the subcellular localization and live cell dynamics of Abi proteins, we generated EYFP (or EGFP) fusion constructs of Abi-1, Abi-2a, and Abi-2b (Figure 1k). Abi-1 and Abi-2 are the products of different genes, while Abi-2a and Abi-2b are splice variants that differ primarily in their N termini [2, 3, 6]. Deletion constructs were also made of the N terminus (ΔN), the SH3 domain (ΔSH3), the homeobox homology region (ΔHHR), or the polyproline domain (ΔPRO), as indicated in Figure 1k. Further constructs included the N-terminal region alone (N), the N terminus plus the first Abl binding site (N+), and one of Abi-2b carrying a mutation that abrogates binding to Abl through the SH3 domain (P501L).

For in vivo analysis of these constructs, we used B16F1 murine melanoma cells, which are readily transfectable and highly motile when plated on laminin. Western blot analysis of lysates of transiently transfected B16F1 cells confirmed the expression of EYFP- and EGFP-tagged Abi proteins (see Supplementary Figure S1 in Supplementary material published with this article on the Internet). Wild-type and mutant forms of Abi-1, Abi-2a, and Abi-2b fusion proteins migrated at the expected molecular weights (Supplementary Figure S1). Both EYFP-Abi-1 and EYFP-Abi-2b were shown to retain their ability to bind Abl in vivo by immunoprecipitation with antibodies to either GFP or Abl (Supplementary Figure S2).

Abi proteins dynamically localize to protruding lamellipodia and filopodia tips

In motile cells, actin polymerization occurs at three membrane-associated locations: at the tips of lamellipodia and filopodia, and at focal adhesions [1]. The different functions mediated at these locations must be reflected in a molecular heterogeneity in signaling cascades, such as signaling through specific Rho family members [7]. Focal adhesions harbor a complex array of matrix receptors, kinases, adaptors, and structural proteins to serve their func-

Figure 1

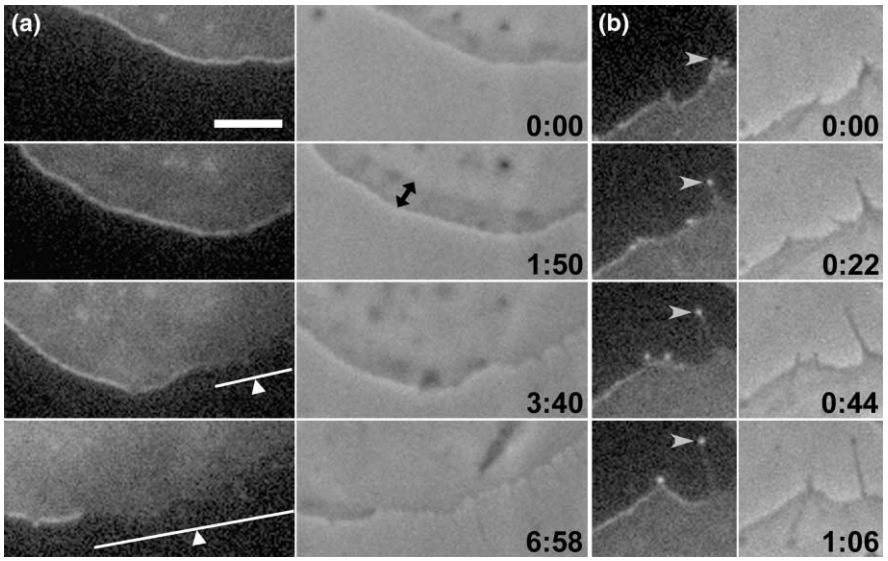


Abi localization in living cells. (a–j) Fluorescence **(a,c,e,g,i)** and phase contrast images **(b,d,f,h,j)** of B16F1 melanoma cells expressing the Abi constructs indicated. Only the region including the lamellipodium is shown. Note the localization of only the full-length (a,c) and N-terminal (e) constructs to the tips of lamellipodia. The dynamic localization of these and other constructs is shown in the supplementary movies. The inset in (a) presents a density scan across the region boxed in white, showing only a single peak at the lamellipodium tip. **(k)** All constructs were tagged with either EYFP or EGFP at the N terminus (indicated by a circle). Abi-1 and Abi-2 derive from separate genes. Abi-2a and Abi-2b arise from alternative splicing of the same gene, and differ only at the N terminus and in a proline-rich insertion in Abi-2b (indicated by a star ⋆). The N termini of Abi-1 and Abi-2b (regions blocked in gray) are identical. Major domains are indicated: CC, coiled-coil region; HHR, homeobox homology region; PRO, polyproline stretch; SH3, SH3 domain. N-terminal constructs (Abi-N) include the coiled-coil region. YR/KTLE is a tyrosine-based signal similar to that (YRSLE) implicated in sorting to the axonal growth cone [25]. "Tip Localization" indicates the frequency and intensity of labeling of the Abi constructs at lamellipodia tips during protrusion: ++, strong signal; +, weak signal; +/−, weak signal in a subset of transfected cells; −, no tip localization. The cartoon at the bottom provides the sites of interaction for relevant Abi binding proteins [2, 17, 18].

tions in signaling and anchorage [8]. Similarly, and in line with results emerging on pathogen motility [9], we can expect a complex of signaling and adaptor molecules to be strategically concentrated at sites where actin polymerization is transduced into protrusion. As shown in Figures 1a and 1c, EYFP-Abi-1 dynamically localized to lamellipodia and filopodia tips, where new actin subunits add to growing filament ends [1]. Abi-2b also showed this localization and, like Abi-1, was specifically recruited to the tips of lamellipodia and filopodia that were undergoing protrusion. Significantly, the label was absent from retracting processes (Figure 2; Supplementary Movies 1 and 5–7). This localization was observed in cells plated on either laminin (Figures 1, 2, and 3) or fibronectin (Supplementary Movie 11), demonstrating that it was independent of substrate type. EYFP-Abi-2a, which lacks homology with Abi-1 and Abi-2b in its N-terminal region, showed no recruitment to lamellipodia tips (Supplementary Movie 10). In some cells, highly mobile dot-like structures, often in rosette-like arrays and reminiscent of small surface ruffles [10], were observed; these harbored Abi proteins as well as the components of the Arp2/3 complex (Supplementary Figure S3; Supplementary Movies 12 and 13).

Comparison of lamellipodia in cells transfected with Abi or Arp3 that were fixed and labeled with phalloidin clearly demonstrates Abi localization to the tip of the lamellipodium, in contrast to Arp3 localization across the breadth of the lamellipodium (Figures 1a, 2a, and 3; Supplementary

Figure 2



Abi proteins are recruited only to the tips of protruding, but not retracting, lamellipodia and filopodia. Images show selected frames of videos of B16F1 melanoma cells transiently transfected with EYFP-Abi-2b. The time scale is provided in minutes and seconds. The scale bar represents 5 μm. (For corresponding movies, see Supplementary material.) **(a)** A region of a lamellipodium is visualized in the fluorescence channel (left) and in phase contrast (right). The phase-dense lamellipodium region is indicated between the arrows in the phase image. The white bar with an arrowhead in the lower two frames in the fluorescence channel indicates regions of lamellipodia retraction. **(b)** A region showing filopodia and including one filopodium that protruded during the imaged sequence (arrowhead) is provided.

material) [11]. The localization of Abi proteins solely to the tips of protruding lamellipodia and filopodia contrasts with that of VASP and Profilin, proteins that are both additionally recruited to sites of substrate anchorage (Figure 3; Supplementary Movie 7) [12–14]. This unique localization of Abi proteins earmarks them as putative regulatory components engaged in protrusion phenomena.

The unique N terminus targets Abi-1 and Abi-2b to the tips of lamellipodia

In order to delineate the domains responsible for the recruitment of Abi-1 and Abi-2b to tips, we generated mutations in a number of potential targeting regions. The first 145 residues of the N terminus, containing a region of homology to the coiled-coil domain of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (t-SNAREs) and a portion of the HHR, were essential for localization to lamellipodia tips (Figure 1e; Supplementary Movie 2). This region alone (Abi-2b-N) was capable of proper localization (Figure 1e), whereas the deletion mutants Abi-1ΔN and Abi-2bΔN were not localized to the tips (Figure 1g; Supplementary Movies 3 and 9). The deletion of the HHR, SH3, or polyproline domains in Abi-2b or Abi-1 did not affect localization to lamellipodia tips (data not shown).

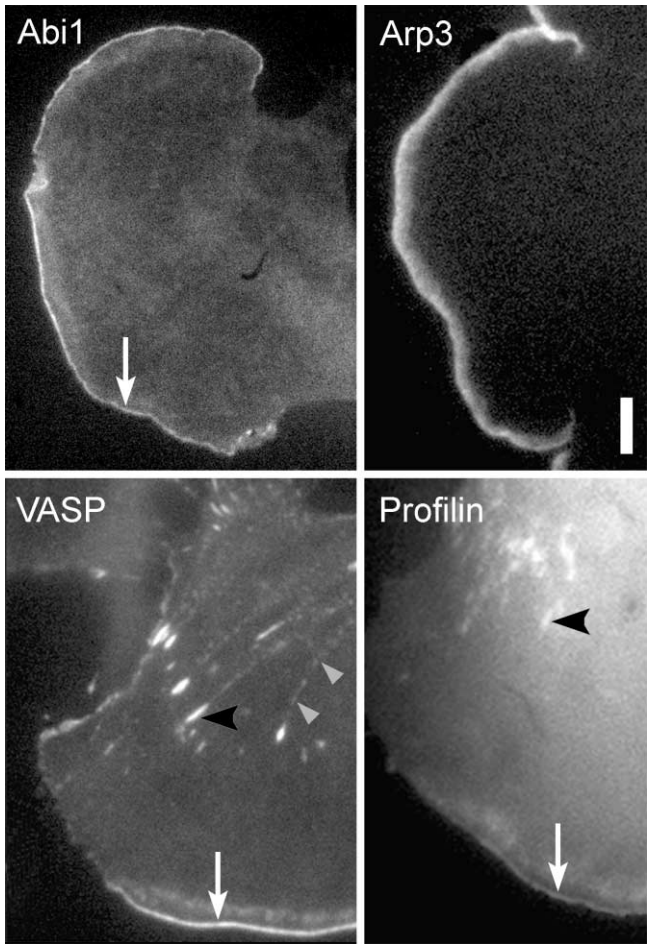
Actin polymerization leading to lamellipodia protrusion is signaled by events downstream of Rac, and involves the activation of the Arp2/3 complex by members of the WASP family, presumably WAVE [11, 15]. The demonstration of a complex between Abi-1, Eps8, and Sos-1 that exhibits Rac-GEF activity places this complex upstream of Rac [5]. Interestingly, the deletion of the polyproline-containing Eps8 binding site in Abi proteins [16, 17] had no effect on Abi localization. The N terminus of Abi-1 was also recently identified as the site responsible for interaction with Sos1/2 in vivo [18]. However, this interaction was mapped to the first 210 residues of Abi-1, and the first 161 residues were insufficient to bind Sos [18]. We show here that the first 145 amino acids of Abi-2b, which are highly conserved in Abi-1, are capable of localizing to tips (Figure 1e), suggesting that this localization can occur independently of Sos binding. It remains to be shown whether Eps8, Sos-1, and Abi are recruited together to lamellipodia tips.

The N terminus of Abi-1 and Abi-2b includes a portion of the HHR and a region of homology with the coiled-coil domain of t-SNAREs that are implicated in vesicle fusion with target membranes [19]. Sequence comparison of mammalian Abi family members, Abi-1/e3B1, Abi-2b, NESH, and to a lesser extent *Drosophila* Abi (Dabi), reveals highest homology in the N-terminal region and the SH3 domain [17, 20–22]. We speculate that the Abi N-terminal domain constitutes a localization determinant in this family of proteins and represents a novel targeting domain for localization to the tips of lamellipodia and filopodia.

Endogenous Abi proteins are present at the tips of lamellipodia

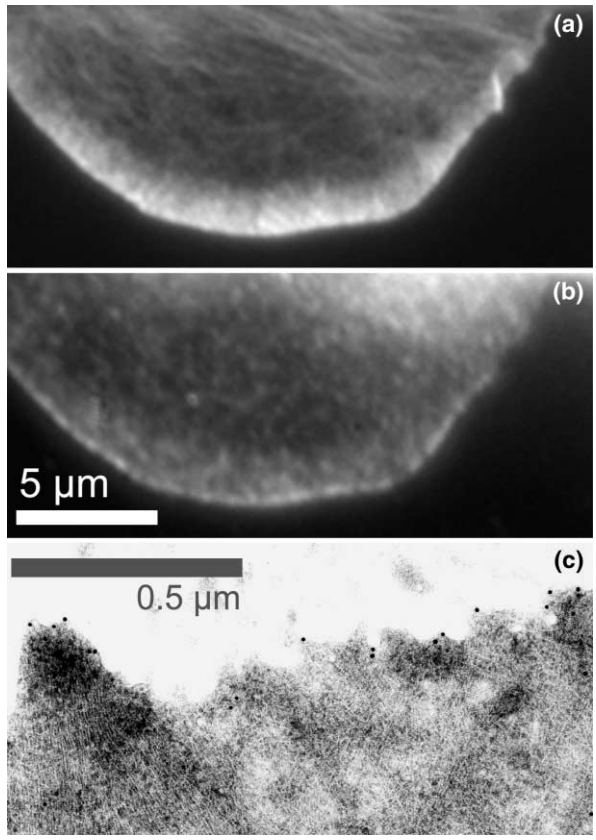
Murine B16F1 melanoma cells express detectable levels of endogenous Abi proteins as determined by Western blot analysis using an antibody that recognizes Abi-1 and Abi-2 (data not shown) [4, 6]. By employing fixation conditions found suitable for preserving lamellipodia in B16F1 cells [12], the endogenous Abi proteins were localized using the polyclonal anti-Abi-1/Abi-2 antibody. Immunofluorescence microscopy revealed essentially the same localization as obtained with the GFP- tagged constructs

Figure 3



Differential localization of Abi-1, VASP, Profilin, and Arp3. Images of live cells transfected with EYFP-Abi-1, EGFP-Profilin II, EGFP-VASP, or Arp3-EGFP show that all proteins except Arp3 are recruited to lamellipodia tips (white arrows), while Arp3 localizes to the entire lamellipodium. In addition to lamellipodia tips, VASP and Profilin also localize to focal adhesions (black arrowheads). The additional, punctate labeling of stress fibers by VASP is indicated by gray arrowheads. The scale bar represents 10 μm.

Figure 4



Endogenous Abi is localized at lamellipodia tips. Double labeling of a B16F1 melanoma cell with fluorescent phalloidin **(a)** and a polyclonal antibody to Abi proteins **(b)**. **(c)** Electron micrograph of the anterior region of a lamellipodium in a B16F1 melanoma cell labeled with the Abi polyclonal antibody and a secondary antibody conjugated to 10 nm gold.

(Figure 4a,b). This characteristic localization has also been observed in other cell types, including mouse embryo fibroblasts (M. Grove and A.M.P., unpublished data). Immunoelectron microscopy performed with the same antibody and fixation conditions showed that the immunolabel was concentrated at the interface between the actin meshwork and the leading membrane front (Figure 4c).

The localization of Abi proteins shown here is consistent with our recent findings that place Abi proteins in neuronal growth cone particles, synaptosomes, and presumptive dendritic spines [6]. Increasing evidence points to a critical role for actin filament dynamics in these structures [23]. We predict that Abi proteins are involved in the regulation of actin dynamics at the tips of neuronal processes.

Conclusions

The protrusion of lamellipodia and filopodia is based on the controlled polymerization of actin filaments via the insertion of actin monomers at their tips. Our findings provide evidence that Abi proteins are dynamically recruited to these sites. We propose that Abi proteins contribute to the regulation of actin assembly at sites of cell membrane protrusion.

Materials and methods

Expression constructs

pEYFP-C1, pEGFP-C1, and pEGFP-N3 were purchased from Clontech. EYFP-Abi-1 and EYFP-Abi-2b were described previously [6]. The cDNA for human Arp3 was provided by L. Machesky. The vectors for EGFP-VASP and Profilin II-EGFP were provided by U. Carl [24] and A. Sechi [14], respectively. Remaining constructs are described in Supplementary material.

Supplementary material

QuickTime videos of phase contrast and fluorescent images of live, motile B16F1 melanoma cells expressing the EYFP/EGFP-tagged constructs described in this paper are available at http://images.cellpress.com/supmat/supmatin.htm. Additional materials and methods are provided as supplementary material.

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