Bcr-Abl induces abnormal cytoskeleton remodeling, β1 integrin clustering and increased cell adhesion to fibronectin through the Abl interactor 1 pathway

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Summary

Hematopoietic cells isolated from patients with Bcr-Abl-positive leukemia exhibit multiple abnormalities of cytoskeletal and integrin function. These abnormalities are thought to play a role in the pathogenesis of leukemia; however, the molecular events leading to these abnormalities are not fully understood. We show here that the Abi1 pathway is required for Bcr-Abl to stimulate actin cytoskeleton remodeling, integrin clustering and cell adhesion. Expression of Bcr-Abl induces tyrosine phosphorylation of Abi1. This is accompanied by a subcellular translocation of Abi1/WAVE2 to a site adjacent to membrane, where an F-actin-enriched structure containing the adhesion molecules such as β1-integrin, paxillin and vinculin is assembled. Bcr-Abl-induced membrane translocation of Abi1/WAVE2 requires direct interaction between Abi1 and Bcr-Abl, but is independent of the phosphoinositide 3-kinase pathway. Formation of the F-actin-rich complex correlates with an increased cell adhesion to fibronectin. More importantly, disruption of the interaction between Bcr-Abl and Abi1 by mutations either in Bcr-Abl or Abi1 not only abolished tyrosine phosphorylation of Abi1 and membrane translocation of Abi1/WAVE2, but also inhibited Bcr-Abl-stimulated actin cytoskeleton remodeling, integrin clustering and cell adhesion to fibronectin. Together, these data define Abi1/WAVE2 as a downstream pathway that contributes to Bcr-Abl-induced abnormalities of cytoskeletal and integrin function.

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Key words: Abi1, Bcr-Abl, WAVE2, Actin cytoskeleton, β1-integrin, Cell adhesion

Introduction

Actin polymerization/depolymerization is a fundamental cellular process that controls cell shape, adhesion and migration. This dynamic cellular process is spatiotemporally regulated in normal cells but its dysregulation is often associated with cellular transformation and tumorogenesis (Pollard and Borisy, 2003; Rao and Li, 2004). The Wiscott-Aldrich Syndrome Protein (WASp) family proteins, which consist of WASp, N-WASP and WAVE/Scar 1, 2 and 3, are crucial components in the signaling network that regulates actin polymerization (Bompard and Caron, 2004; Stradal et al., 2004). By interacting with actin and the Arp 2/3 complex, these proteins act as actin nucleation factors to promote actin polymerization. WASp family proteins are regulated by the small GTP-binding proteins Rac and Cdc42 (Bompard and Caron, 2004). WASp and N-WASP, for example, contain a conserved Cdc42 binding domain. Association of GTP-bound Cdc42 to these proteins is a key step in their activation (Burns et al., 2004; Stradal et al., 2004). WAVE proteins, however, are regulated by Rac through a different mechanism. WAVE proteins do not contain a Rac binding site and therefore do not directly bind to Rac. Instead, these proteins form a complex with Hspc 300, Abl interactor (Abi) proteins, Nck-associated protein 1 (Nap1) and specifically Rac-associated (Sra) protein (Eden et al., 2002; Gautreau et al., 2004; Innocenti et al., 2004; Kunda et al., 2003; Steffen et al., 2004). Among these proteins, Abi plays a central role in holding the complex together, whereas Sra provides a binding site for active Rac (Gautreau et al., 2004; Innocenti et al., 2004; Steffen et al., 2004). Recent studies suggest that the complex formation and subsequent translocation are crucial for WAVE activation (Innocenti et al., 2004; Leng et al., 2005). Still, how these cellular processes are regulated in cancer cells and whether the dysregulation of these cellular processes contributes to cellular transformation and tumorogenesis remain unclear.

Mammalian Abi proteins consist of three members, Abi1 (also known as e3b1), Abi2 and NESH (Biesova et al., 1997; Dai and Pendergast, 1995; Miyazaki et al., 2000; Shi et al., 1995; Wang et al., 1996; Ziemnicka-Kotula et al., 1998). Among these, Abi1 and Abi2 were first identified as the binding partners of c-Abl as well as oncogenic v-Abl and Bcr-Abl tyrosine kinases (Dai and Pendergast, 1995; Shi et al., 1995). Bcr-Abl tyrosine kinases are produced by a reciprocal t(9;22)(q34;q11) chromosomal translocation that fuses

varying amounts of the *bcr* gene on chromosome 22 with sequences upstream of the second exon of the *c-abl* gene on chromosome 9. Depending on the amount of *bcr* sequences fused, three different Bcr-Abl fusion proteins with molecular masses of 185 kDa ($p185^{Bcr\text{-}Abl}$), 210 kDa ($p210^{Bcr\text{-}Abl}$) and 230 kDa ($p230^{Bcr\text{-}Abl}$) may be produced (Melo and Deininger, 2004). Expression of Bcr-Abl is associated with more than 95% of human chronic myelogenous leukemia (CML) and a subset of acute lymphocytic leukemia cases (Melo and Deininger, 2004). Hematopoietic cells isolated from CML patients exhibit multiple abnormalities of cytoskeletal function, such as increased motility, altered adhesion and decreased response to SDF-1α (Bazzoni et al., 1996; Gordon et al., 1987; Kramer et al., 1999; Salgia et al., 1997; Salgia et al., 1999; Verfaillie et al., 1992; Wertheim et al., 2003). These abnormalities may play a crucial role in the progression of CML, as altered adhesion and increased motility may contribute to premature release of CML cells from bone marrow and accumulation of these cells in peripheral hematopoietic tissues such as blood and spleen. The abnormal cytoskeletal function observed in CML cells is caused by the Bcr-Abl oncoprotein, because expression of Bcr-Abl in hematopoietic cell lines is sufficient to induce these abnormalities (Kramer et al., 1999; McWhirter and Wang, 1997; Salgia et al., 1997; Wertheim et al., 2002; Wertheim et al., 2003). Several lines of evidence suggest that Bcr-Abl deregulates β1-integrin function, which may, at least in part, be responsible for altered adhesion and increased motility of CML cells (Bhatia et al., 1996; Salesse and Verfaillie, 2002; Skorski et al., 1998; Verfaillie et al., 1992). The β1-integrin is a subunit of integrin receptors found in hematopoietic cells that link the cytoskeleton to the extracellular matrix (Teixido et al., 1992; Verfaillie et al., 1992; Williams et al., 1991). Although the expression of Bcr-Abl in CML cells did not alter the protein levels of membrane β1-integrin (Bazzoni et al., 1996; Verfaillie et al., 1992; Wertheim et al., 2002), it induced abnormal interactions between β1-integrin and the actin cytoskeleton (Bhatia et al., 1999). Bhatia et al. proposed that abnormal integrin-cytoskeletal interaction restricts the mobility of integrin receptors and results in defective integrin function in CML progenitor cells (Bhatia et al., 1999). However, the precise mechanisms of Bcr-Abl-induced abnormal interaction of integrin and actin cytoskeleton remain largely unknown.

In previous studies we found that a mutant $p185^{Bcr\text{-}Abl}$ with the deletion of the Src homology 3 (SH3) domain and C-terminal proline-rich sequences ($p185^{\Delta SH3\Delta C}$) failed to induce a CML-like disease in a bone marrow transplant (BMT) mouse model (Dai et al., 2001). One pathological difference that distinguishes $p185^{\Delta SH3\Delta C}$ BMT mice from wild-type $p185^{Bcr\text{-}Abl}$ ($p185^{wt}$) BMT mice is the lack of massive splenomegaly, a phenotype that is believed to result from the massive accumulation and retention of both mature and immature myeloid cells in the spleen. Notably, a proportion of $p185^{\Delta SH3\Delta C}$ BMT mice developed high peripheral white blood cell counts with normal spleen weight, suggesting that the $p185^{\Delta SH3\Delta C}$ was capable of stimulating cell proliferation and survival but failed to induce the accumulation and retention of myeloid cells in the spleen (Dai et al., 2001). These observations led us to hypothesize that $p185^{\Delta SH3\Delta C}$ may have a defect in stimulating abnormal cytoskeletal function. The SH3 domain and C-terminal proline-rich sequences are required for Bcr-Abl to bind to Abi1 and Abi2 (Dai et al., 2001; Dai and Pendergast, 1995; Shi et al., 1995). Given that Abi signaling plays a key role in regulating actin polymerization, we sought to test whether this pathway is involved in Bcr-Abl-induced abnormalities of cytoskeletal function. Here we provide evidence that, through direct interaction with the SH3 domain and C-terminal proline-rich sequences of Bcr-Abl, Abi1 mediates Bcr-Abl-induced actin cytoskeleton remodeling and β1-integrin clustering. We also demonstrate that this pathway is required for Bcr-Abl to stimulate hematopoietic cell adhesion on fibronectin-coated surfaces.

Results

Differential regulation of Abi1 and Abi2 by Bcr-Abl in hematopoietic cells

We have previously shown that Abi proteins undergo ubiquitin-dependent degradation in hematopoietic cells transformed by Bcr-Abl (Dai et al., 1998). Although the antibody used in the previous studies was raised against Abi2 and was immunoreactive preferentially to Abi2, it crossreacted, albeit weakly, with in vitro synthesized Abi1 (supplementary material Fig. S1A). Without a specific antibody to Abi1, our earlier studies were unable to distinguish if the Bcr-Abl-induced degradation of Abi is specific for Abi2 or if it is common to both Abi1 and Abi2 (Dai et al., 1998). To address this question, we made use of an antibody raised against a peptide with sequences unique to Abi1 (Courtney et al., 2000), This antibody specifically recognizes Abi1 with no detectable cross-reactivity to Abi2 (supplementary material Fig. S1A). Using this antibody, we examined the expression of Abi1 in murine pro-B cell line Ba/F3 and myeloid cell line 32D, as well as their derivatives transformed by $p185^{wt}$. Abi1 was readily detected in control Ba/F3 and 32D cells transduced with an empty retroviral vector (Fig. 1A). There was no significant decrease in Abi1 protein level in Ba/F3 and 32D cells transformed by Bcr-Abl, as compared with the control cells (Fig. 1A). This is in contrast to our previous studies using Abi2 antibody, which detected immunoreactive proteins only in control Ba/F3 and 32D cells but not in those transformed by $p185^{wt}$ (Dai et al., 1998) (see also supplementary material Fig. S1B). Thus, the result suggests that Abi2, but not Abi1, is downregulated by Bcr-Abl in hematopoietic cells.

We noticed that the Bcr-Abl transformation induced additional bands with slower mobility on western blots that were recognized by Abi1 antibody (Fig. 1A, middle panel, compare lanes 2 and 4 with lanes 1 and 3). The slower mobility species represent tyrosine-phosphorylated Abi1 as these proteins can be immunoprecipitated by Abi1 antibody and recognized either by Abi1 antibody or the antibody specific to phosphotyrosine proteins on western blots (Fig. 1B, lanes 2 and 4). A kinase-deficient Bcr-Abl mutant ($p185^{K671R}$) was unable to induce Abi1 tyrosine phosphorylation (Fig. 1B, lane 3), suggesting that the tyrosine kinase activity of Bcr-Abl is required for induction of Abi1 tyrosine phosphorylation. In support of this notion, the tyrosine phosphorylation of Abi1 was completely inhibited by imatinib mesylate, a compound that specifically blocks Bcr-Abl kinase activity (Fig. 1B, lane 5). Collectively, these results demonstrated that Abi1 and Abi2 are regulated differently by Bcr-Abl and that Abi1 is a

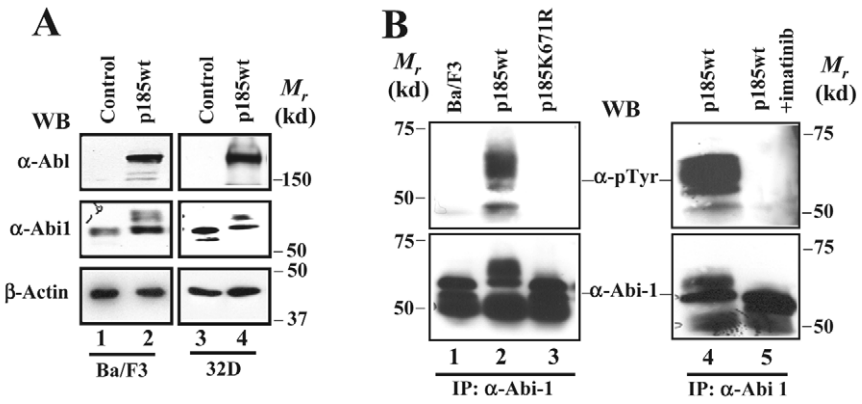

Fig. 1. Abi1 is a downstream substrate of Bcr-Abl tyrosine kinase. (A) Ba/F3 and 32D cells were transduced with empty retroviral vector (control) or retroviral vector expressing $p185^{Bcr\text{-}Abl}$ ($p185^{wt}$). Total lysates from 1×10^6 cells were analyzed by western blot (WB) using indicated antibodies. (B) Tyrosine phosphorylation of Abi1 in Bcr-Abl-transformed hematopoietic cells. Ba/F3 cells and the Ba/F3 expressing either $p185^{wt}$ or $p185^{K671R}$ were treated with or without 1 μM imatinib mesylate, as indicated. The lysates from 2×10^7 cells were immunoprecipitated (IP) with anti-Abi1 antibody, followed by western blot analysis using anti-phosphotyrosine (α-pTyr) antibody (upper panel). The blots were then stripped and reprobed with anti-Abi1 antibody (lower panel). The relative molecular mass (M_r) is indicated and presented as kilodaltons (kd).

downstream substrate of Bcr-Abl tyrosine kinase in hematopoietic cells.

Bcr-Abl stimulates membrane translocation of Abi1 and WAVE2

To determine whether Bcr-Abl signaling affects the interaction between Abi1 and WAVE, we examined complex formation between WAVE and Abi1 in Ba/F3 cells transformed by $p185^{wt}$ and compared it with that in control Ba/F3 cells transduced with an empty retroviral vector. Expression of WAVE2 in control Ba/F3 cells and the Ba/F3 cells transformed by $p185^{wt}$ was readily detected by western blot analysis (Fig. 2A). WAVE2 was coimmunoprecipitated by Abi1 antibody in control Ba/F3 cells as well as in Ba/F3 cells transformed by

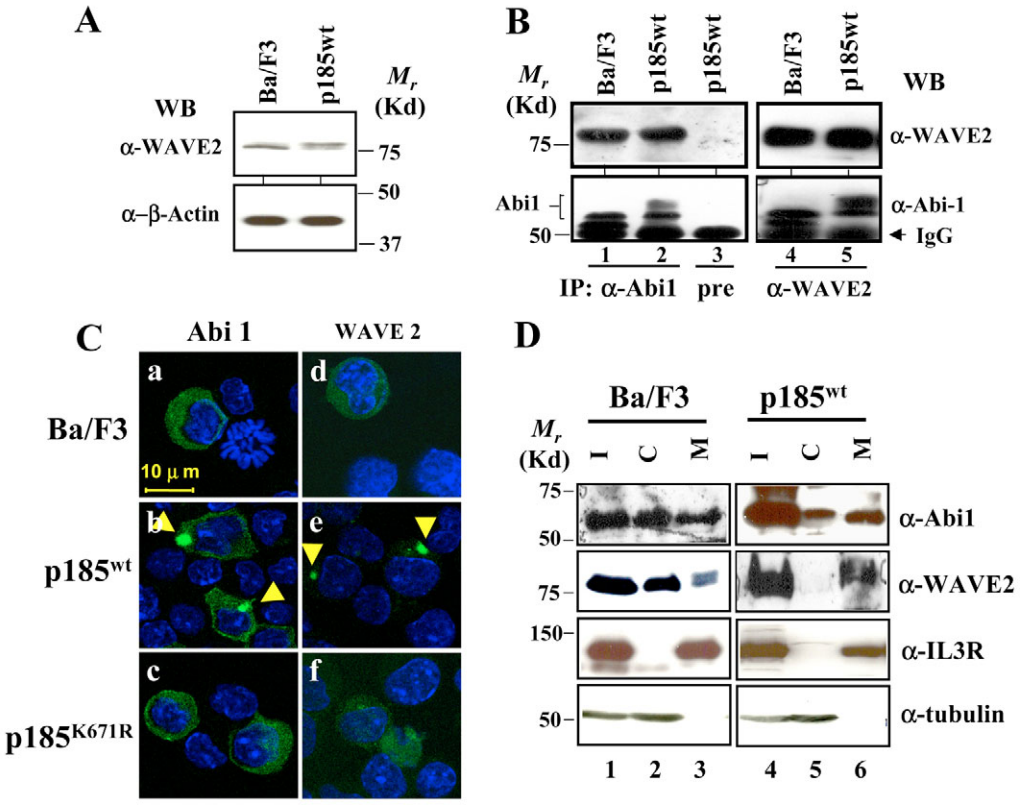

Fig. 2. Complex formation and membrane translocation of Abi1/WAVE2 in Ba/F3 cells transformed by $p185^{wt}$. (A) Expression of WAVE2 in Ba/F3 and $p185^{wt}$-transformed Ba/F3 cells. Total lysates from 2×10^5 cells were analyzed by western blot using indicated antibodies. (B) Complex formation of Abi1 and WAVE2. The lysates from Ba/F3 cells transduced with either an empty retroviral vector (Ba/F3) or the retroviral vector expressing $p185^{wt}$ were immunoprecipitated with anti-Abi1 antibody, anti-WAVE2 antibody or pre-immune rabbit serum (pre), as indicated. The immunoprecipitates were analyzed by western blot using indicated antibodies. (C) Bcr-Abl-induced membrane translocation of Abi1/WAVE2. Retroviral vectors expressing GFP-Abi1 (a-c) and GFP-WAVE2 (d-f) were introduced into Ba/F3 cells or Ba/F3 cells expressing wild type and the mutant form of $p185^{Bcr\text{-}Abl}$, as indicated. The subcellular localization of GFP-Abi1 and GFP-WAVE2 was analyzed by two-photon confocal microscopy. Nuclei were stained by DAPI (blue). Arrowheads indicate the membrane localization of Abi1 and WAVE2. Bar, 10 μm. (D) Subcellular distributions of Abi1 and WAVE2 in Ba/F3 and the Ba/F3 transformed by $p185^{wt}$. The Ba/F3 cells and the Ba/F3 cells transformed by $p185^{wt}$ were lysed and fractionated to separate the cytosol (C) and plasma membrane (M). The equal amounts of total lysate (I, input), cytosol, and membrane fractions were separated on SDS-PAGE and analyzed by western blot using antibodies specific to Abi1 and WAVE2, as indicated. To monitor the quality of the fractionation, the blot was also probed with the antibodies to the β-subunit of IL-3 receptor and α-tubulin, the proteins known to be in membrane and cytosol, respectively.

$p185^{wt}$ (Fig. 2B, left panel), suggesting that these proteins form a complex in vivo and that the complex formation was not affected by Bcr-Abl transformation. Consistent with this notion, the reciprocal immunoprecipitation with WAVE2 antibody demonstrated that both tyrosine-phosphorylated and non-phosphorylated forms of Abi1 were capable of binding to WAVE2 (Fig. 2B, lane 5). Based on these results, we conclude that it is unlikely that the signaling from Bcr-Abl to Abi1 plays a major role in regulation of Abi1/WAVE2 complex formation in Ba/F3 cells.

Next, we tested whether Bcr-Abl signaling plays a role in regulating subcellular localization of Abi1 and WAVE2. To this end, retroviruses expressing either a green fluorescent protein (GFP)-tagged Abi1 or WAVE2 were generated and introduced into Ba/F3 cells as well as the Ba/F3 cells expressing $p185^{wt}$ or $p185^{K671R}$. The subcellular distribution of GFP-Abi1 and GFP-WAVE2 were then examined by two-photon confocal microscopy. Both GFP-Abi1 and GFP-WAVE2 exhibited a diffuse cytoplasmic distribution in control Ba/F3 cells (Fig. 2C,a,d). By contrast, a polarized cytoplasmic localization of GFP-Abi1 and GFP-WAVE2 was observed in Ba/F3 cells transformed by $p185^{wt}$ (Fig. 2C,b,e). In particular, Abi1 and WAVE2 were found concentrated in a spot adjacent to the membrane. This distribution pattern of Abi1 is not an artifact caused by overexpression or by GFP fusion, as the same pattern was observed for endogenous Abi1 in $p185^{wt}$-transformed cells by indirect immunofluorescence staining (see Fig. 3). The fact that a kinase-deficient $p185^{K671R}$ was unable to induce such a pattern (Fig. 2C,c,f) further supports that the polarized cytoplasmic localization of GFP-Abi1 and WAVE2 was indeed induced by Bcr-Abl tyrosine kinase.

To determine whether Abi1 and WAVE2 were associated with plasma membrane in Bcr-Abl-transformed cells, a subcellular fractionation experiment was performed. As shown in Fig. 2D, Abi1 and WAVE2 were found in both cytosol and membrane fractions in parental Ba/F3 cells. However, we observed an increase in membrane-associated Abi1 and WAVE2 in cells transformed by $p185^{wt}$ Bcr-Abl (Fig. 2D, compare lane 6 with lane 5). This finding is consistent with the observation that, upon Bcr-Abl transformation, most Abi1 and WAVE2 were relocated to a site adjacent to the membrane (Fig. 2C). Together, our results indicate that Bcr-Abl transformation induces a translocation of Abi1/WAVE2 to plasma membrane.

Abi1, Bcr-Abl and WAVE2 colocalize with an F-actin-enriched structure in Bcr-Abl-transformed hematopoietic cells

Given that the Abi1/WAVE2 pathway plays a central role in regulating actin polymerization (Stradal et al., 2004), we asked whether the membrane translocation of Abi1/WAVE2 is associated with Bcr-Abl-induced abnormal actin cytoskeleton remodeling. Consistent with previous reports (McWhirter and Wang, 1997; Salgia et al., 1997; Skourides et al., 1999), expression of $p185^{wt}$ in Ba/F3 cells induced a profound actin cytoskeleton remodeling. A spot intensively stained by TRITC-conjugated phalloidin, indicative of an aggregate of filamentous actin (F-actin), was observed in $p185^{wt}$-transformed cells (supplementary material Fig. S2A,b). A similar structure was also observed in hematopoietic cells isolated from Bcr-Abl-positive leukemia patients (Bhatia et al., 1999) (Y.L. and Z.D., unpublished data). It is notable that the

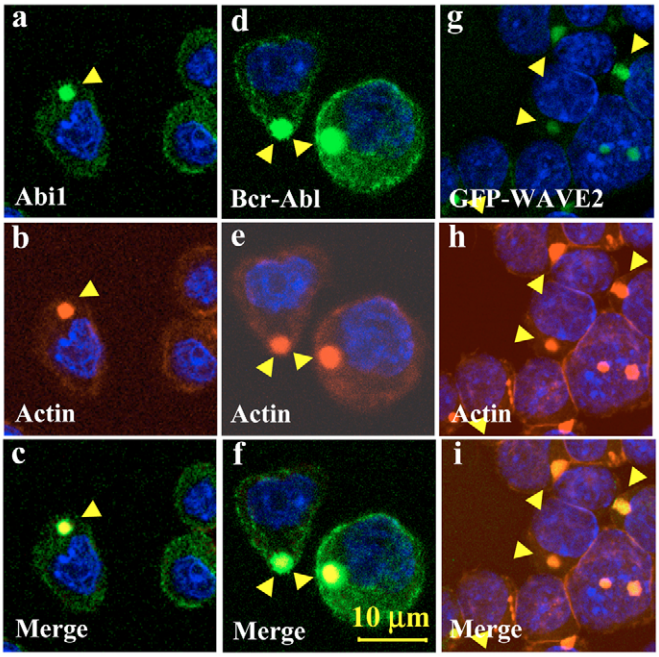

Fig. 3. Colocalization of Abi1, Bcr-Abl and WAVE2 with an abnormal F-actin-rich structure. Ba/F3 cells expressing $p185^{wt}$ were probed with anti-Abi1 (a-c) and anti-Abl (d-f) antibodies, respectively. This was followed by staining with FITC-conjugated secondary antibody. Cells were then counterstained with TRITC-conjugated phalloidin and DAPI to visualize F-actin and nuclei, respectively. In g-i, the Ba/F3 cells expressing $p185^{wt}$ were transduced with retrovirus expressing GFP-WAVE2 and counterstained by TRITC-conjugated phalloidin and DAPI. Subcellular distribution of Abi1 (a, green), Bcr-Abl (d, green), GFP-WAVE2 (g, green) and F-actin structures (b,e,h, red) were visualized by two-photon confocal microscopy, as indicated by arrowheads. The colocalization of these proteins with abnormal F-actin structure is shown by merged images (c,f,i). Bar, 10 μm.

F-actin-enriched structure induced by $p185^{wt}$ appeared similar to the pattern of $p185^{wt}$-induced Abi1 and WAVE2 distribution in both size and localization (compare supplementary material Fig. S2A,b with Fig. 2C,b,e). The indirect immunofluorescence staining of $p185^{wt}$-transformed cells with anti-Abi1 or anti-Abl antibodies followed by counterstaining with TRITC-conjugated phalloidin revealed that Abi1 (Fig. 3a-c) and Bcr-Abl (Fig. 3d-f) colocalize with this F-actin-enriched structure. To determine whether WAVE2 also colocalizes with this abnormal structure, the $p185^{wt}$-transformed Ba/F3 cells were transduced with the retrovirus expressing GFP-WAVE2 and cells were counterstained by TRITC-conjugated phalloidin. The GFP-WAVE2 and F-actin were visualized by two-photon confocal microscopy. As shown in Fig. 3, WAVE2 (Fig. 3g-i) also colocalizes with the F-actin-enriched structure.

β1-integrin, paxillin and vinculin are clustered and associated with F-actin-enriched structures in Bcr-Abl-transformed cells

Actin cytoskeleton organization plays a key role in regulation of integrin distribution and function (Schwartz et al., 1995). To determine whether the abnormal F-actin-enriched structures induced by $p185^{wt}$ affects membrane distribution of integrin,

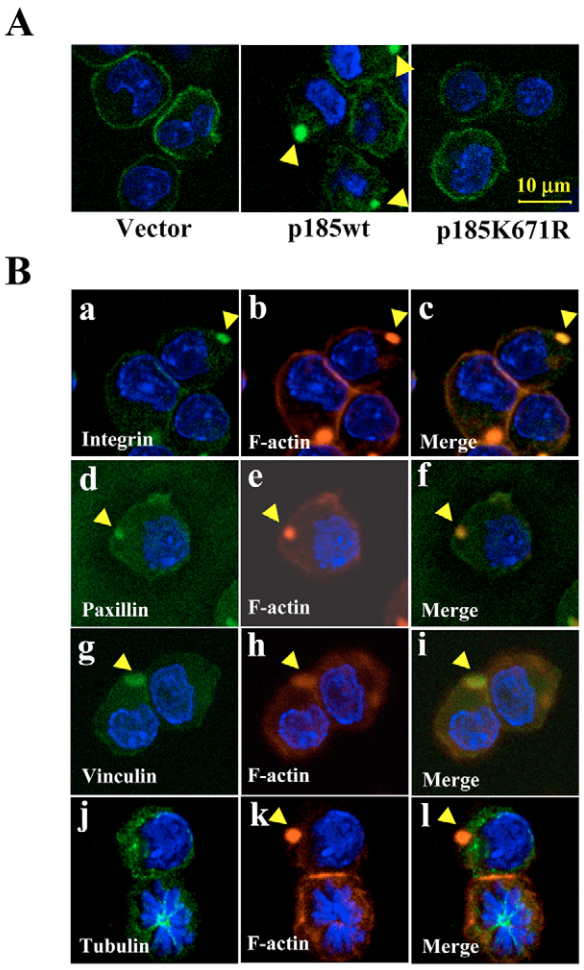

Fig. 4. The expression of $p185^{Bcr\text{-}Abl}$ induced integrin clustering. (A) β1-integrin clustering in $p185^{wt}$-transformed Ba/F3 cells. The Ba/F3 cells transduced with indicated retroviruses were starved, fixed and stained with FITC-conjugated monoclonal antibody against β1-integrin. Cells were then stained with DAPI to visualize nuclei. Images were captured by two-photon confocal microscopy and the β1-integrin clustering is indicated by arrowheads. Bar, 10 μm. (B) Colocalization of β1-integrin, paxillin and vinculin with abnormal actin-enriched structure. The Ba/F3 cells expressing $p185^{wt}$ were starved, fixed, permeabilized and probed with FITC-conjugated antibodies against β1-integrin, paxillin, tubulin and vinculin (green), as indicated. The cells were then counterstained with TRITC-conjugated phalloidin (red) and DAPI (blue) to visualize F-actin and nuclei, respectively. Images were captured by two-photon confocal microscopy and the colocalization of integrin, paxillin and vinculin with abnormal actin-enriched structure is shown in merged images, as indicated by arrowheads. Bar, 10 μm.

we stained control Ba/F3 cells and the Ba/F3 cells expressing either $p185^{wt}$ or $p185^{K671R}$ with FITC-conjugated antibody specific for β1-integrin, a common subunit of integrin receptors found in hematopoietic cells (Teixido et al., 1992; Verfaillie et al., 1992). As shown in Fig. 4A (left panel), the control Ba/F3 cells displayed even membrane distribution of β1-integrin. By contrast, β1-integrin in Ba/F3 cells transformed by $p185^{wt}$ was clustered (Fig. 4A, middle panel). The β1-integrin clustering was Bcr-Abl tyrosine kinase dependent, as the kinase-deficient $p185^{K671R}$ failed to induce such clustering (Fig. 4A, right panel). To determine whether the clustered β1-integrin is associated with abnormal actin-enriched structure, cells were counterstained with TRITC-conjugated phalloidin following the immunofluorescence staining with anti-β1-integrin antibody. As shown in Fig. 4B,a-c, the clustered β1-integrin was found to colocalize with the abnormal actin-enriched structures. Furthermore, paxillin (Fig. 4B,d-f) and vinculin (Fig. 4B,g-i), the adhesion proteins known to link integrin to the cytoskeleton (Burridge et al., 1988), were also found in association with the F-actin-enriched structures in $p185^{wt}$-expressing cells. The association of the F-actin-enriched structures with adhesion proteins and β1-integrin appeared to be specific, as α-tubulin, a cytoplasmic protein, was not found to colocalize with this abnormal structural complex (Fig. 4B,j-l). Together, these results suggest that $p185^{wt}$-induced F-actin-enriched structures interacted with β1-integrin, possibly through adhesion-associated molecules such as paxillin and vinculin.

PI3K pathway is not required for Bcr-Abl-induced Abi1 translocation and F-actin remodeling

Innocenti et al. recently reported that Abi1 interacts with the p85 subunit of phosphoinositide 3-kinase (PI3K) to form a multimolecular complex localized in membrane ruffle (Innocenti et al., 2003). The formation of this multimolecular complex is crucial for activation of Rac and Rac-dependent actin remodeling (Innocenti et al., 2003). To test whether the PI3K pathway is also involved in Bcr-Abl-induced Abi1 translocation and F-actin remodeling, three approaches were explored. First, immunoprecipitation followed by western blotting was performed to determine whether Abi1 forms a complex with the p85 subunit of PI3K. As shown in Fig. 5A (left panel, lanes 3 and 4), under the condition in which WAVE2 was coimmunoprecipitated by Abi1 antibody, we were unable to detect p85 in anti-Abi1 immunoprecipitates from Ba/F3 cells and $p185^{wt}$-transformed cells. Similarly, the anti-p85 antibodies failed to coimmunoprecipitate Abi1 from Ba/F3 cells and $p185^{wt}$-transformed cells (Fig. 5A, right panel). Second, because the phosphorylation of tyrosine 407 in Abi1 is required for its interaction with p85 as well as for growth factor-stimulated actin remodeling (Innocenti et al., 2003), we asked whether the mutation of this tyrosine affects the Bcr-Abl-induced Abi1 translocation and F-actin remodeling. A substitution of tyrosine 407 with phenylalanine in Abi1 ($Abi1^{Y407F}$) did not reduce the overall tyrosine phosphorylation of $Abi1^{Y407F}$ in $p185^{wt}$-transformed cells, as compared with wild-type Abi1 (Fig. 5B, compare lane 3 with lane 2 in left panel). The mutant Abi1 displayed a similar pattern of subcellular distribution as that of wild-type Abi1 when expressed as a GFP-fusion protein in $p185^{wt}$-transformed Ba/F3 cells (Fig. 5B and supplementary material Fig. S2B). Further, the expression of $Abi1^{Y407F}$ had no effects on $p185^{wt}$-induced F-actin remodeling (supplementary material Fig. S2B,h). Together, these results suggest that the tyrosine 407 of Abi1 is not a major phosphorylation site required for Bcr-Abl-induced Abi1 translocation and F-actin remodeling. Finally, we made use of a specific PI3K inhibitor, LY294002, to test whether the pharmacological inhibition of PI3K has any effect on Bcr-Abl-induced Abi1 membrane translocation and F-actin remodeling. The $p185^{wt}$-transformed Ba/F3 cells were transduced with retrovirus expressing GFP-Abi1 and treated

with either LY294002 or the Bcr-Abl inhibitor imatinib. In contrast to imatinib, which effectively inhibited the Bcr-Abl-induced Abi1 translocation (Fig. 5C, middle panel), LY294002 had no effects on the membrane translocation of GFP-Abi1 (Fig. 5C, right panel). Similarly, the formation of F-actin-enriched structures in $p185^{wt}$-transformed Ba/F3 cells was inhibited by imatinib (supplementary material Fig. S2A,c), but not LY294002 (supplementary material Fig. S2A,d). Collectively, the results from these studies support that Bcr-Abl induces Abi1 translocation and F-actin remodeling independently of the PI3K pathway.

Abi1 pathway is required for Bcr-Abl-induced actin cytoskeleton remodeling and integrin clustering

To determine whether the Abi1 pathway is required for Bcr-Abl-induced abnormal actin remodeling and integrin clustering, we made use of $p185^{\Delta SH3\Delta C}$, a mutant Bcr-Abl with the deletion of the SH3 domain and C-terminal proline-rich sequences (supplementary material Fig. S3A). In previous studies we and others have shown that the SH3 domain and C-terminal proline-rich sequences are required for Bcr-Abl to bind to Abi1 and Abi2 (Dai et al., 2001; Shi et al., 1995). Deletion of these sequences did not affect tyrosine kinase activity of $p185^{\Delta SH3\Delta C}$ (Dai et al., 2001), but completely abolished its ability to bind to Abi1 and to induce Abi1 tyrosine phosphorylation (supplementary material Fig. S3B). Deletion of these sequences also caused the inability of $p185^{\Delta SH3\Delta C}$ to induce membrane translocation of Abi1 (Fig. 6A,b) and WAVE2 (Fig. 6A,d) in Ba/F3 cells. Together, these results indicate that $p185^{\Delta SH3\Delta C}$ is defective in signaling to the Abi1 pathway. Correlated with this defect, $p185^{\Delta SH3\Delta C}$ failed to induce abnormal F-actin-rich structures (Fig. 6B,h) and β1-integrin clustering (Fig. 6B,f) when expressed in Ba/F3 cells. These results are consistent with a role for the Abi1 pathway in Bcr-Abl-induced abnormal actin remodeling and integrin clustering.

In addition to Abi1, the SH3 domain and C-terminal proline-rich sequences of Bcr-Abl also interact with other signaling molecules. It is therefore possible that the failure of $p185^{\Delta SH3\Delta C}$ to induce actin-enriched structures is because of its inability to activate these pathways, rather than the Abi1 pathway. To directly test the role of the Abi1 pathway in Bcr-Abl-induced abnormal actin remodeling, we generated $Abi1^{PPLL}$, a mutant Abi1 in which the proline residues 180 and 434 in the PXXP motif and the SH3 domain, respectively, are replaced by leucine (Fig. 7A). Previous studies suggest that proline 180 is likely to be involved in the interaction with the Abl SH3 domain (Dai and Pendergast, 1995; Ren et al., 1994), whereas proline 434 is highly conserved among a diverse set of SH3 domains and is thought to be crucial for binding to the Abl C-terminal PXXP motif (Dai and Pendergast, 1995; Musacchio et al., 1992; Ren et al.,

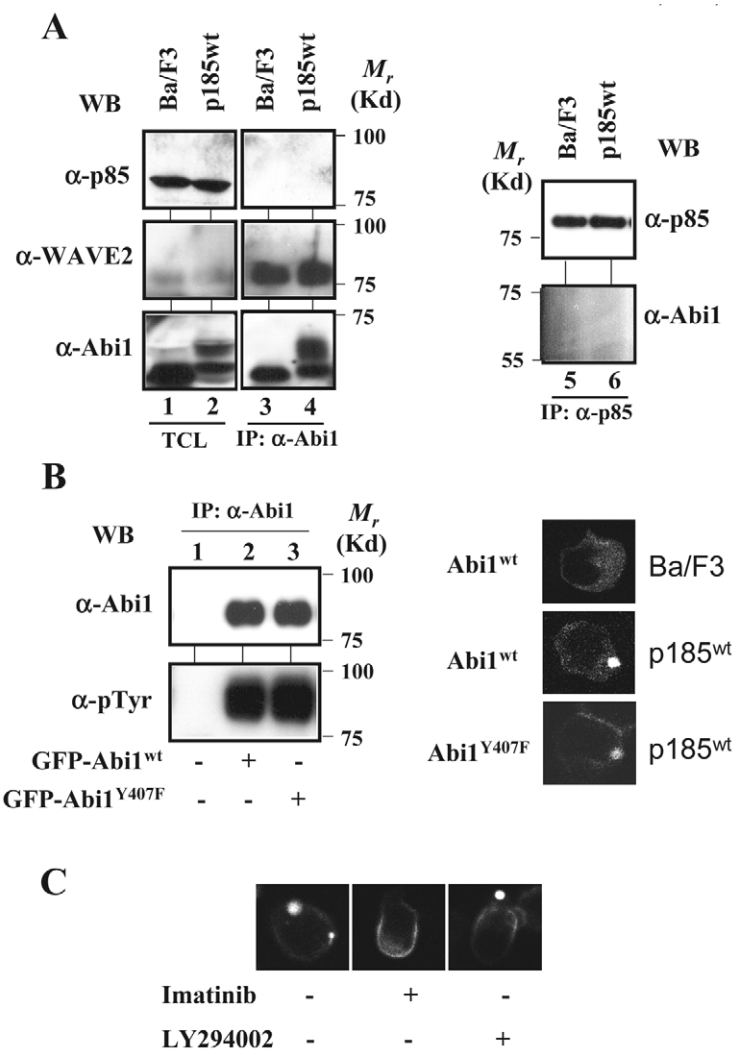

Fig. 5. The PI3K pathway is not required for Bcr-Abl-induced membrane translocation of Abi1. (A) Abi1 does not form a complex with the p85 subunit of PI3K in Ba/F3 cells and the Ba/F3 cells transformed by $p185^{wt}$. The lysates from 2×10^7 Ba/F3 cells and the Ba/F3 cells transformed by $p185^{wt}$ were immunoprecipitated by anti-Abi1 (lanes 3 and 4, left panel) and anti-p85 (lanes 5 and 6, right panel) antibodies, respectively. The immunoprecipitates were analyzed by western blotting using the indicated antibodies. A portion of total cell lysates (TCL) equivalent to 2×10^5 cells (p85 and WAVE2) or 1×10^6 cells (Abi1) was also analyzed by western blotting to show the expression level of Abi1, p85 and WAVE2 (lanes 1 and 2, left panel). (B) The mutation at tyrosine 407 does not affect the Bcr-Abl-induced membrane translocation of $Abi1^{Y407F}$. Left panel: lysates from $p185^{wt}$-transformed Ba/F3 cells (lane 1) and the $p185^{wt}$-transformed Ba/F3 cells expressing either GFP-Abi1 (lane 2) or GFP-$Abi1^{Y407F}$ (lane 3) were immunoprecipitated by anti-Abi1 antibody and analyzed by western blotting using the antibodies indicated. Right panel: Ba/F3 cells and Ba/F3 cells transformed by $p185^{wt}$, as indicated, were transduced with retroviruses expressing either GFP-Abi1 or GFP-$Abi1^{Y407F}$. The subcellular distribution of GFP-fusion proteins was visualized by two-photon confocal microscopy.
(C) LY294002 failed to inhibit Bcr-Abl-induced membrane translocation of GFP-Abi1. The $p185^{wt}$-transformed Ba/F3 cells were transduced with the retrovirus expressing GFP-Abi1. The cells were then left untreated or treated with either 10 μM imatinib or 50 μM LY294002, as indicated, for 8 hours. The cells were fixed and counterstained with TRITC-conjugated phalloidin and DAPI to visualize the F-actin and nuclei, respectively. Images were captured by two-photon confocal microscopy. A color picture is presented in supplementary material Fig. S2B.

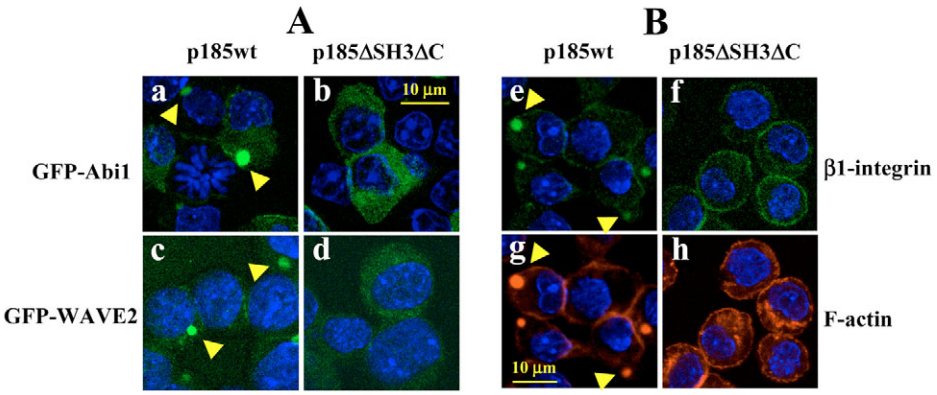

Fig. 6. The $p185^{\Delta SH3\Delta C}$ failed to induce the membrane translocation of Abi1/WAVE2, integrin clustering and abnormal actin remodeling. (A) Ba/F3 cells expressing either $p185^{wt}$ (a,c) or $p185^{\Delta SH3\Delta C}$ (b,d) were transduced with the retroviral vectors expressing either GFP-Abi1 (a,b) or GFP-WAVE2 (c,d). The cells were fixed and GFP-fusion proteins were visualized by two-photon confocal microscopy. (B) Ba/F3 cells transduced with either $p185^{wt}$ (e,g) or $p185^{\Delta SH3\Delta C}$ (f,h) were fixed and incubated with FITC-conjugated monoclonal antibody specific for β1-integrin (e,f). The cells were also stained with TRITC-conjugated phalloidin to visualize F-actin (g,h). The nuclei of the cells were stained by DAPI (blue) and the arrowheads indicate the subcellular localization of GFP-Abi1 (a), WAVE2 (c), clustering β1-integrin (e), as well as abnormal actin-enriched structures (g) in $p185^{wt}$-transformed cells. Bar, 10 μm.

1994; Van Etten et al., 1995). The mutations of these proline residues resulted in the inability of $Abi1^{PPLL}$ to bind to Bcr-Abl (Fig. 7B) and to be tyrosine-phosphorylated when expressed in $p185^{wt}$-transformed cells (Fig. 7C). The mutant protein, however, retains the ability to interact with WAVE2 (supplementary material Fig. S4A) and Hem1 (supplementary material Fig. S4B), a member of Nap1 family proteins specifically expressed in hematopoietic cells (Weiner et al., 2006). We therefore predicted that this mutant Abi1, when overexpressed in $p185^{wt}$-transformed cells, may serve as a dominant-negative molecule to interfere with the signal transduction from Bcr-Abl to endogenous Abi1. To test this, retroviruses expressing GFP-tagged wild-type Abi1 and $Abi1^{PPLL}$ were generated and introduced into Ba/F3 cells expressing $p185^{wt}$. The cells were then stained with TRITC-conjugated phalloidin and subjected to two-photon confocal microscopy analysis. As shown in Fig. 7D, whereas the wild-type Abi1 was found to colocalize with the F-actin-enriched

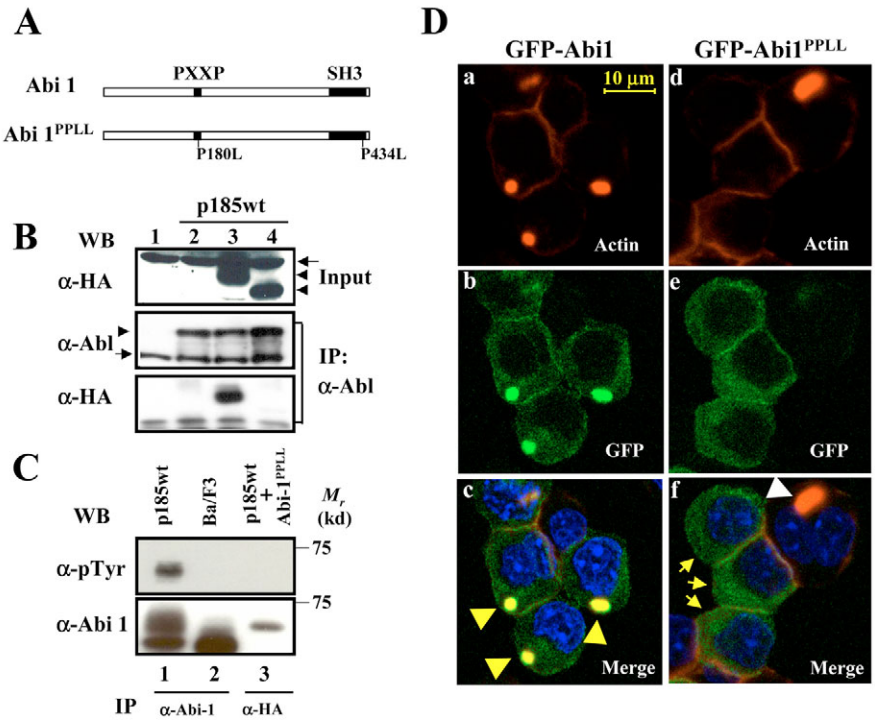

Fig. 7. Expression of $Abi1^{PPLL}$ inhibited Bcr-Abl-induced abnormal actin cytoskeleton remodeling. (A) Schematic diagram of Abi1 and $Abi1^{PPLL}$. (B) $Abi1^{PPLL}$ is defective in binding to Bcr-Abl. Ba/F3 cells (lane 1) and the Ba/F3 cells expressing $p185^{wt}$ alone (lane 2), $p185^{wt}$ plus HA-tagged Abi1 (lane 3) and $p185^{wt}$ plus HA-tagged $Abi1^{PPLL}$ (lane 4) were lysed and immunoprecipitated with anti-Abl antibody. The immunoprecipitates (middle and lower panels) and 1/50 of total cell lysates used for immunoprecipitation (IP) (Input, upper panel) were analyzed by western blot using indicated antibodies. The HA-tagged Abi1 and $Abi1^{PPLL}$ are indicated by arrowheads, whereas a non-specific band crossreacted with anti-HA antibody is indicated by the arrow (upper panel). The anti-Abl antibody recognized both Bcr-Abl and endogenous c-Abl, as indicated by arrowhead and arrow, respectively (middle panel). (C) $Abi1^{PPLL}$ failed to be tyrosine-phosphorylated by Bcr-Abl. Ba/F3 cells (lane 2) and Ba/F3 cells expressing $p185^{wt}$ alone (lane 1) or $p185^{wt}$ plus HA-tagged $Abi1^{PPLL}$ (lane 3) were lysed and immunoprecipitated with indicated antibodies. The immunoprecipitates were analyzed by western blot using indicated antibodies. (D) Inhibition of Bcr-Abl-induced abnormal actin remodeling by $Abi1^{PPLL}$. Ba/F3 cells transformed by $p185^{wt}$ were transduced with retroviruses expressing either GFP-Abi1 (a-c) or GFP-$Abi1^{PPLL}$ (d-f). Cells were fixed and stained with TRITC-conjugated phalloidin and DAPI to visualize F-actin (red) and nuclei (blue), respectively. Subcellular localization of GFP-fusion proteins (b,e, green) and F-actin structure (a,d, red) were analyzed by two-photon confocal microscopy. The merged images were also shown (c,f). The arrowheads in panel c indicate abnormal actin-enriched structures that colocalize with GFP-Abi1, whereas arrows in panel f indicate the $p185^{wt}$-transformed cells that express GFP-$Abi1^{PPLL}$. An open arrowhead in panel f indicates abnormal actin-enriched structure in a cell that did not express $Abi1^{PPLL}$. Bar, 10 μm.

structure, Abi1^{PPLL} displayed a diffuse cytoplasmic distribution in Ba/F3 cells transformed by p185^{wt}. Moreover, no F-actin-enriched structure was observed in p185^{wt}-transformed Ba/F3 cells that express GFP-Abi1^{PPLL} (Fig. 7D, compare panels c-f), indicating that the expression of Abi1^{PPLL} inhibited Bcr-Abl-induced actin cytoskeleton remodeling. This finding, together with the finding that p185$^{\Delta SH3\Delta C}$ failed to induce the F-actin-enriched structure (Fig. 6), strongly supports a role for Abi1 in Bcr-Abl-induced actin cytoskeleton reorganization.

Blockade of the Abi1 pathway or β1-integrin function impairs Bcr-Abl-stimulated hematopoietic cell adhesion on fibronectin-coated surfaces

Actin cytoskeleton structure and integrin function are pivotal for control of cell adhesion (Hynes, 2002; Martin et al., 2002; Schwartz and Ginsberg, 2002). The finding that the Abi1 pathway is required for Bcr-Abl to induce abnormal actin remodeling and integrin clustering raises the question as to whether this pathway is responsible for abnormal adhesion of Bcr-Abl-positive leukemic cells to fibronectin, a phenotype thought to play a role in the pathogenesis of CML (Bazzoni et al., 1996; Gordon et al., 1987; Hemmeryckx et al., 2001; Salesse and Verfaillie, 2002; Wertheim et al., 2003). To address this question, we examined the adhesion of p185^{wt}-transformed Ba/F3 cells to fibronectin-coated surfaces and compared it with those of control Ba/F3 cells as well as the Ba/F3 cells expressing p185$^{\Delta SH3\Delta C}$, the mutant Bcr-Abl defective in signaling to Abi1. Consistent with the previous reports (Bazzoni et al., 1996; Wertheim et al., 2003), expression of p185^{wt} in Ba/F3 cells increased cell adhesion to fibronectin-coated surfaces threefold compared with parental cells (Fig. 8A). However, the ability of Bcr-Abl to stimulate Ba/F3 cell adhesion was greatly reduced by deletion of the SH3 and C-terminal proline-rich sequences (Fig. 8A, left panel), suggesting that the Abi1 pathway is required for Bcr-Abl to stimulate cell adhesion to fibronectin. This is further supported by study of Abi1^{PPLL}, a mutant Abi1 defective in binding to Bcr-Abl (Fig. 7B). We have shown that expression of Abi1^{PPLL} inhibited Bcr-Abl-induced actin cytoskeleton remodeling (Fig. 7C). Correlating with its inhibitory effect on actin cytoskeleton remodeling, expression of Abi1^{PPLL} in p185^{wt}-transformed Ba/F3 cells resulted in a remarkable decrease in cell adhesion to fibronectin, as compared with parental p185^{wt}-transformed cells (Fig. 8A, right panel).

It is unlikely that the increased cell adhesion to fibronectin in p185^{wt}-transformed cells is caused by an elevation of integrin expression, as no significant increase in β1-integrin protein level was observed among BaF3 cells and the Ba/F3 derivatives expressing the wild-type and mutant forms of Bcr-Abl (supplementary material Fig. S5). However, the increased adhesion of p185^{wt}-transformed cells to fibronectin-coated surfaces correlated with the induction of the F-actin-enriched structures. This is demonstrated by analysis of the F-actin-enriched structures in p185^{wt}-transformed Ba/F3 cells grown

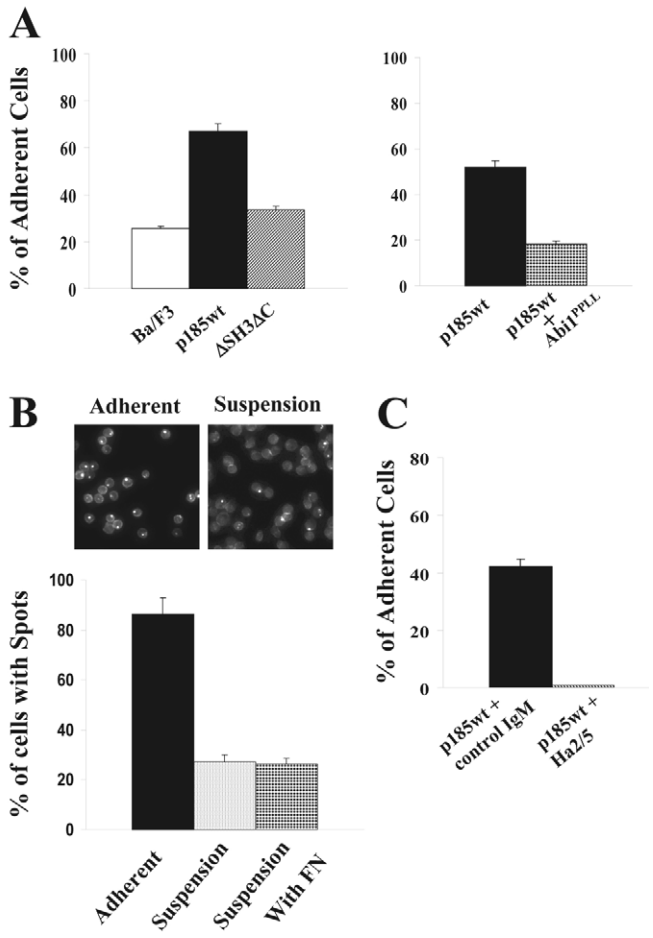

Fig. 8. Blockade of Abi1 pathway or β1-integrin function impaired the ability of Bcr-Abl to stimulate cell adhesion to fibronectin. (A) The Abi1 pathway is required for Bcr-Abl-stimulated cell adhesion to fibronectin-coated surfaces. Left panel: Ba/F3 cells and the Ba/F3 cells expressing either p185^{wt} or p185$^{\Delta SH3\Delta C}$ were grown in fibroncetin-coated six-well plates (2.5×10^5/well) for 16 hours. The total cells and the cells that were adherent to fibronectin-coated surfaces were counted and the percentage of adherent cells calculated. The vertical axis shows the percentage of the adherent cells and is expressed as the mean ± s.d. of duplicate wells. Data are representative of two independent experiments. Right panel: the Ba/F3 cells expressing p185^{wt} alone or p185^{wt} plus Abi1^{PPLL} were grown in fibroncetin-coated plates (2.5×10^5/well) for 16 hours. The total cells and the cells that were adherent to fibronectin-coated surfaces were counted and the percentage of adherent cells calculated. The data represent the mean ± s.d. of triplicate wells. (B) The F-actin-rich structures are enriched in adherent p185^{wt}-transformed Ba/F3 cells. The p185^{wt}-transformed Ba/F3 cells were grown in fibronectin-coated plates with or without fibronectin supplemented in medium (5 μg/ml) for 16 hours. Adherent and non-adherent cells were harvested separately and stained by TRITC-conjugated phalloidin and DAPI to visualize F-actin and nuclei, respectively, by fluorescence microscopy (upper panel). The percentage of the cells containing the F-actin-rich structures (spots) from three randomly selected fields in adherent cells as well as non-adherent cells was statistically calculated and expressed as the mean ± s.d. (lower panel). The data are representative of three independent experiments. (C) Bcr-Abl-stimulated cell adhesion to fibronectin is β1-integrin dependent. The p185^{wt}-transformed Ba/F3 cells were treated with either Ha2/5 or a control hamster IgM (4 μg/2.5×10^5 cells) and plated in fibroncetin-coated plates (2.5×10^5 cells/well) for 16 hours. The total cells and the cells that were adherent to fibronectin-coated surfaces were counted and the percentage of adherent cells calculated. The data represent the mean ± s.d. of triplicate wells and are representative of two independent experiments.

in fibronectin-coated plates. The cells adhered to fibronectin-coated surfaces and those that remained in suspension were collected separately and analyzed for F-actin-enriched structures by fluorescence microscopy. As shown in Fig. 8B, more than 86% of adherent cells displayed abnormal F-actin-enriched structures. By contrast, only 27% of suspension cells were found to have such structures. The enrichment of the F-actin-rich structures in adherent cells is not because of the stimulation by surface-bound fibronectin, as the addition of fibronectin into culture medium did not increase the formation of the F-actin-enriched structures in suspension cells (Fig. 8B). Rather, it is more likely that Bcr-Abl-induced F-actin-enriched structures enable cells to adhere to fibronectin-coated surfaces, possibly by promoting integrin clustering. In support of this notion, β1-integrin was found clustered and colocalized with F-actin-enriched structures in adherent Ba/F3 cells transformed by $p185^{wt}$, but not $p185^{\Delta SH3\Delta C}$ (supplementary material Fig. S6). Furthermore, Bcr-Abl-stimulated cell adhesion to fibronectin is β1-integrin-dependent and is abrogated completely by the treatment with Ha2/5 (Fig. 8C), a specific β1-integrin antibody capable of blocking β1-integrin-mediated cell adhesion.

Discussion

Previous work has shown that deletion of the SH3 domain and C-terminal proline-rich sequences of $p185^{Bcr\text{-}Abl}$ impaired its ability to induce leukemogenesis (Dai et al., 2001). The mechanism involved, however, was not clear. In this study, we demonstrate that the SH3 domain and C-terminal proline-rich sequences are required for Bcr-Abl to induce the tyrosine phosphorylation of Abi1 and membrane translocation of Abi1/WAVE2. Deletion of these sequences not only abolished the tyrosine phosphorylation of Abi1 and membrane translocation of Abi1/WAVE2, but also abrogated the ability of Bcr-Abl to induce actin cytoskeleton remodeling and β1-integrin clustering. Mutant Bcr-Abl with the deletion of these sequences is also deficient in stimulating hematopoietic cell adhesion to fibronectin. Furthermore, expression of a mutant Abi1 defective in binding to the SH3 domain and C-terminal proline-rich sequences of Bcr-Abl inhibited abnormal actin cytoskeleton remodeling and Bcr-Abl-stimulated cell adhesion. Together, these findings place Abi1 signaling as an immediate downstream pathway that links $p185^{Bcr\text{-}Abl}$ to abnormal cytoskeletal function. These results may provide a mechanistic explanation of why $p185^{\Delta SH3\Delta C}$ failed to induce CML-like disease in BMT mice (Dai et al., 2001).

Integrin receptors link the force-generating actin cytoskeleton to extracellular matrix and, therefore, play a central role in the control of cell adhesion and motility. The integrin function is regulated by not only extracellular matrix, but also actin cytoskeleton and other membrane or intracellular signaling molecules (Hynes, 2002; Martin et al., 2002; Schwartz and Ginsberg, 2002). In Bcr-Abl-positive leukemic cells, integrin function is abnormal and this abnormality is believed to be caused by abnormal integrin-cytoskeletal interaction (Bhatia et al., 1999). The pathways that link Bcr-Abl to abnormal integrin-cytoskeletal interaction, however, remain to be defined. We found that β1-integrin was clustered and was associated with an F-actin-enriched structure in $p185^{wt}$-transformed Ba/F3 cells. The constitutive clustering of β1-integrin was independent of extracellular stimulation because it was found in the resting $p185^{wt}$-transformed cells grown in suspension. The interaction of β1-integrin with the abnormal actin-enriched structure, which was induced by $p185^{Bcr\text{-}Abl}$ through the Abi1 pathway, is probably a causative event for β1-integrin clustering. This is supported by the finding that the blockade of the signaling from Bcr-Abl to Abi1 abrogated not only abnormal actin cytoskeleton remodeling but also integrin clustering. Thus, our studies show, for the first time, that the Abi1 pathway is required for Bcr-Abl to induce abnormal integrin-cytoskeletal interaction. Notably, the F-actin-enriched structures observed in $p185^{wt}$-transformed cell lines are also present in hematopoietic cells isolated from a CML patient (Bhatia et al., 1999) (Y.L. and Z.D., unpublished data), suggesting that a similar mechanism may also be utilized by CML cells to deregulate integrin function and cell adhesion.

In addition to abnormal cell adhesion, the Abi1 pathway may also contribute to other cellular processes crucial for leukemic cell homing, such as cell migration and invasion. This is supported by our previous observation that deletion of the SH3 domain and C-terminal proline-rich sequences in Bcr-Abl not only abrogated its signaling to Abi1, but also inhibited its ability to induce spontaneous migration of Ba/F3 cells on fibronectin-coated surfaces (Dai et al., 2001). Consistent with this observation, expression of $Abi1^{PPLL}$ in $p185^{wt}$-transformed cells not only inhibited Bcr-Abl-induced abnormal actin remodeling and cell adhesion, but also impaired the spontaneous cell migration on fibronectin-coated surfaces (supplementary material Fig. S7). Further, ablation of Abi1 in $p185^{wt}$-transformed Ba/F3 cells by short hairpin RNA not only inhibited Bcr-Abl-stimulated actin cytoskeleton remodeling and cell adhesion, but also impaired Bcr-Abl-induced spontaneous cell migration and leukemogenesis (W.Y. and Z.D., unpublished data). Taken together, these results provide the strong evidence that the Abi1 pathway plays an important role in Bcr-Abl-induced leukemogenesis.

In a recent report, Leng et al. demonstrated that, when stimulated, c-Abl tyrosine kinase induced the tyrosine phosphorylation of Abi1 and promoted Abi1/WAVE2 membrane translocation (Leng et al., 2005). In this study, we have shown that $p185^{Bcr\text{-}Abl}$, a constitutively active tyrosine kinase, stimulated Abi1 tyrosine phosphorylation and membrane translocation of the Abi1/WAVE2 complex. These studies highlight membrane translocation of Abi1/WAVE2 as a crucial regulatory step for WAVE activation and actin cytoskeleton reorganization. Using a melanoma cell line B16F1, Leng et al. also reported that WAVE2 was phosphorylated on tyrosine 150 and that the complex assembly of Abi1 and WAVE2 was enhanced when c-Abl was activated by platelet-derived growth factor (PDGF) stimulation (Leng et al., 2005). In studies described here, we did not observe an increase in Abi1/WAVE2 complex assembly in $p185^{wt}$-transformed Ba/F3 cells (Fig. 2B). In fact, we found that even in resting Ba/F3 cells, most of Abi1 and WAVE2 present as a complex and can be easily coimmunoprecipitated with each other (L.D. and Z.D., unpublished data). This observation is in line with those described by Gautreau et al. (Gautreau et al., 2004) and Innocenti et al. (Innocenti et al., 2004), who demonstrated that Abi, Nap, Sra and WAVE2 were only present as a complex in HeLa cells and that stimulation of cells with epidermal growth factor (EGF) did not affect the complex formation. Although the discrepancy between our observations

and those reported by Leng et al. may be explained by differences in experimental systems, our data and those of Leng et al. raise the possibility that c-Abl and Bcr-Abl may regulate complex formation of Abi1/WAVE2 differently.

The mechanism by which Bcr-Abl induces membrane translocation of Abi1 in hematopoietic cells differs from those utilized by fibroblasts and adherent tumor cells such as HeLa cells and melanoma cells B16F1, in which Abi1 was found to localize to the membrane ruffle and the tip of the lamellipodia (Stradal et al., 2001; Innocenti et al., 2004; Leng et al., 2005). The PI3K pathway, for example, is required for PDGF-stimulated membrane translocation of Abi1 in fibroblasts (Innocenti et al., 2003), but is dispensable for Bcr-Abl-induced Abi1 translocation in hematopoietic cells. Abi1^{PPLL}, a mutant Abi1 deficient in binding to Bcr-Abl, failed to translocate to membrane in p185^{wt}-transformed Ba/F3 cells. However, this mutant Abi1 displayed the same membrane-associated distribution as the wild-type Abi1 in adherent human kidney-derived 293 cells (X.S. and Z.D., unpublished data). Although the tyrosine phosphorylation of Abi1 appears to be crucial for its membrane translocation in both fibroblasts (Innocenti et al., 2003; Leng et al., 2005) and Bcr-Abl-transformed hematopoietic cells, the mechanism involved differs too. The phosphorylation of tyrosine 407 has been shown to be crucial for membrane translocation of Abi1 in fibroblasts, as it provides an anchorage site for PI3K (Innocenti et al., 2003). This tyrosine, however, is not required for Bcr-Abl-induced Abi1 translocation in hematopoietic cells (Fig. 5). It is possible that Bcr-Abl may induce the phosphorylation of other tyrosine residue(s) in Abi1, which may provide an anchorage site for proteins yet to be identified or, alternatively, which may modify the actin polymerization-promoting activity of the Abi1/WAVE2 complex. Further investigation is necessary to map these tyrosine residue(s) and to define the role of tyrosine phosphorylation of Abi1 in the regulation of membrane translocation.

Materials and Methods

Cell lines and reagents

Ba/F3 and 32D cells were grown in RPMI containing 10% fetal bovine serum (FBS) and 15% WEHI3-conditioned medium as a source of IL-3. The Ba/F3 and 32D cell lines expressing wild type and the mutant forms of p185$^{Bcr-Abl}$ were generated by retroviral transduction, as described previously (Dai et al., 2001). Retroviral packaging cell line Bosc 23, kindly provided by W.S. Pear (University of Pennsylvania), was grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. The preparation of rabbit polyclonal antibodies against Abi1 and Abi2 has been described previously (Courtney et al., 2000; Dai et al., 1998). The antibodies against WAVE2, the p85 subunit of PI3K, and phosphotyrosine-containing proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies for Abl and β1-integrin (Ha2/5 and 9EG7) were obtained from BD Pharmingen (San Diego, CA). The other antibodies used were: monoclonal anti-β-actin (Sigma), FITC-conjugated monoclonal anti-β1-integrin (Chemicon International), anti-paxillin (BD Transduction Laboratories), and anti-vinculin (Sigma). The Abl tyrosine kinase inhibitor, imatinib mesylate (Gleevec), was kindly provided by J. DeGregori (University of Colorado Health Sciences Center). The protease inhibitor cocktail was purchased from Sigma.

Retroviral constructs

The full-length cDNA encoding enhanced GFP (eGFP) was generated by polymerase chain reaction (PCR) using pEGFP-C plasmid as template. The resultant cDNA fragment, which contains a 5′ *Bam*HI site and 3′ *Bgl*II/*Xho*I/*Eco*RI sites, was digested by *Bam*HI/*Eco*RI and subcloned into murine stem cell virus (MSCV) at *Bgl*II and *Eco*RI sites. The resultant plasmid, MSCV-GFP, was used to construct the retroviral vectors expressing wild-type and mutant forms of GFP-Abi1 and GFP-WAVE2 fusion proteins. Briefly, cDNAs encoding Abi1 and WAVE2 were generated by PCR, and subcloned in-frame into MSCV-GFP at *Bgl*II/*Eco*RI sites. The cDNAs encoding Abi1^{PPLL} were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and subcloned into MSCV-GFP at *Bgl*II/*Eco*RI sites, as specified by the manufacturer. The mutations were confirmed by sequencing analysis.

Fluorescence microscopy

Cultured Ba/F3 cell lines were starved for 4 hours in RPMI containing 0.5% bovine serum albumin (BSA) before fixation and staining. The starved cells were then fixed in 3.7% formaldehyde in PBS for 10 minutes, permeabilized in 0.2% Triton X-100/PBS for 5 minutes, and stained with 50 μg/ml TRITC-conjugated phalloidin (Sigma) in PBS. After washing extensively with PBS and a brief staining with DAPI (Sigma) to visualize nuclei, 5-10×10^3 cells were loaded per slide by cytospin and mounted with Vectashield mounting medium (Vector, Burlingame, CA). Images were captured and analyzed using a Zeiss two-photon laser scanning confocal microscope with LSM 5 Image software. For indirect immunofluorescence staining of Abi1 and Bcr-Abl, cells stained with TRITC-phalloidin as above were blocked with 2% BSA in PBS for 1 hour, and incubated with appropriate antibodies diluted in 1% BSA/PBS for 2 hours. After extensive washing with PBS, cells were incubated with Alexa-conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR) for 1 hour, washed again with PBS, and mounted on slides, as described above. The immunofluorescence staining for β1-intergrin was performed in both suspension cells and the cells adherent to fibronectin-coated surfaces as described above, except that for suspension cells, they were fixed in 3.7% formaldehyde in PBS for 10 minutes without permeabilization.

Biochemical assays

Subcellular fractionation was performed as described (Wartmann et al., 1997) with modifications. Briefly, cells were disrupted by passing through a $27G^{1/2}$ needle in hypotonic buffer (25 mM HEPES, pH 7.5; 2 mM EDTA, 2 mM $NaVO_4$, 5 mM NaF, and 1% protease inhibitor cocktail), and centrifuged for 15 minutes at 600 ***g*** to separate the nuclei and cytosol/membrane fractions. The supernatant was then centrifuged for 30 minutes at 100,000 ***g*** to yield the cytosol (S100) fraction and the membrane pellet (P100). For coimmunoprecipitation analysis, control Ba/F3 cells and the Ba/F3 cells expressing wild-type and mutant forms of p185$^{Bcr-Abl}$ were lysed in lysis buffer (20 mM HEPES, pH 7.2; 150 mM NaCl, 1% Triton X-100, and 10% glycerol) and incubated with appropriate antibodies bound to Sepharose beads. The immunoprecipitates were separated on SDS-PAGE, transferred to nitrocellulose and immunoblotted with appropriate antibodies.

Cell adhesion assay

Ba/F3 cells and Ba/F3 cells expressing either p185^{wt} alone or p185^{wt} plus Abi1^{PPLL} were suspended in RPMI+10% FBS with (Ba/F3) or without (Ba/F3 expressing p185^{wt} or p185^{wt} plus Abi1^{PPLL}) 15% WEHI3-conditioned medium at a density of 1×10^5 cells/ml. The cells were plated in six-well plates (2.5 ml/well) coated with fibronectin (BD Biosciences, Bedford, MA) and incubated at 37°C/5%CO_2 for 16 hours. Nonadherent cells were removed and adherent cells were washed three times with 1 ml pre-warmed RPMI medium. The adherent cells were then trypsinized and collected. Both nonadherent and adherent cells were counted to determine the percentage of adherent cells. For treatment of p185^{wt}-transformed cells with blocking antibody, either Ha2/5 anti-integrin antibody or control hamster IgM was added to cells (4 μg/2.5×10^5 cells) in fibronectin-coated six-well plates. The cells were incubated at 37°C/5%CO_2 for 16 hours and cell adhesion was determined as described above.

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