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c-Abl phosphorylates Dok1 to promote filopodia during cell spreading

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Filopodia are dynamic F-actin structures that cells use to explore their environment. c-Abl tyrosine kinase promotes filopodia during cell spreading through an unknown mechanism that does not require Cdc42 activity. Using an unbiased approach, we identified Dok1 as a specific c-Abl substrate in spreading fibroblasts. When activated by cell adhesion, c-Abl phosphorylates Y361 of Dok1, promoting its association with the Src homology 2 domain (SH2)/SH3 adaptor protein Nck. Each signaling component was critical for filopodia formation during cell spreading, as evidenced by the finding that mouse fibroblasts lacking c-Abl, Dok1, or Nck had fewer filopodia than cells reexpressing the product of the disrupted gene. Dok1 and c-Abl stimulated filopodia in a mutually interdependent manner, indicating that they function in the same signaling pathway. Dok1 and c-Abl were both detected in filopodia of spreading cells, and therefore may act locally to modulate actin. Our data suggest a novel pathway by which c-Abl transduces signals to the actin cytoskeleton through phosphorylating Dok1 Y361 and recruiting Nck.

Introduction

Regulated cell migration is important for several biological processes, including development, immune responses, neuron function, and tissue repair. For migration to be productive, a cell must first determine a direction of movement. To this end, a cell explores its environment by extending elongated actin-rich structures referred to as filopodia. Filopodia are extremely dynamic, protruding and retracting from the cell perimeter seeking guidance cues and adhesive proteins in the ECM (Svitkina et al., 2003). Filopodia are abundant at the leading edge of cells that are actively exploring their environment. For example, filopodia are found on migrating sheets of embryonic epithelial cells, spreading fibroblasts, axon growth cones, and neuronal dendritic spines (Wood and Martin, 2002).

Actin polymerization provides the force to drive filopodial membrane protrusions (Mallavarapu and Mitchison, 1999). Current data suggest that filopodia arise from the elongation, convergence, and bundling of preexisting actin filaments in the lamellipod (Svitkina et al., 2003). WASP family proteins regulate filopodia by recruiting the Arp2/3 complex, which nucleates actin polymerization (Pollard and Borisy, 2003). Although activators of WASP have been identified (e.g., Rho family GTPases, PIP_2, and SH3 adaptor proteins), the signaling pathways controlling the formation, maintenance, and termination of filopodia during specific cellular processes remain largely undefined.

Recent observations suggest that c-Abl tyrosine kinase can regulate filopodia formation. c-Abl extends the duration of membrane protrusive activity during the early stages of cell spreading on fibronectin (FN), resulting in increased numbers

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Key words: $Abl^{-/-}Arg^{-/-}$ fibroblasts; fibronectin adhesion; F-actin microspikes; cytoskeleton; Nck

Abbreviations used in this paper: FN, fibronectin; KD, kinase-deficient; MEF, mouse embryo fibroblast; PH, pleckstrin homology; pTyr, phosphotyrosine; SH2, Src homology 2 domain; STI, signal transduction inhibitor; TLC, thin layer cellulose; WT, wild-type.

of filopodia and F-actin microspikes (Woodring et al., 2002), which are precursors to filopodia (Kozma et al., 1995; Svitkina et al., 2003). Active Abl also increases the length of neurites and the number of F-actin–rich branches on embryonic cortical neurons grown on laminin (Zukerberg et al., 2000; Woodring et al., 2002). The effect of c-Abl on neurite branching and persistence of filopodia during cell spreading is dependent on c-Abl tyrosine kinase activity, implicating c-Abl substrates in these processes. Indeed, cell adhesion to ECM components, such as FN, collagen, vitronectin, and laminin, increases c-Abl activity (Lewis et al., 1996; Frasca et al., 2001; Woodring et al., 2001). Several collaborators that synergize with c-Abl in F-actin–mediated processes have been suggested (for review see Lanier and Gertler, 2000; Woodring et al., 2003; Hernandez et al., 2004); however, essential components downstream of c-Abl remain elusive.

Here, we investigated the c-Abl substrates involved in the persistence of F-actin microspikes and filopodia during fibroblast spreading. Using an unbiased biochemical strategy to detect substrates combined with a genetic strategy using fibroblasts from knockout mouse embryos, we found that the p62 docking protein (Dok1) is an essential substrate for c-Abl in the induction of filopodia during FN-stimulated cell spreading. c-Abl and Dok1 are both present in filopodia, supporting a role for both proteins in filopodia function. The effects of c-Abl on filopodia appear to be mediated by the phosphorylation of Y361 of Dok1, which promotes association of Dok1 and Nck, a Src homology 2 domain (SH2)/SH3 adaptor protein that can trigger localized actin polymerization (Campellone et al., 2004; Rivera et al., 2004).

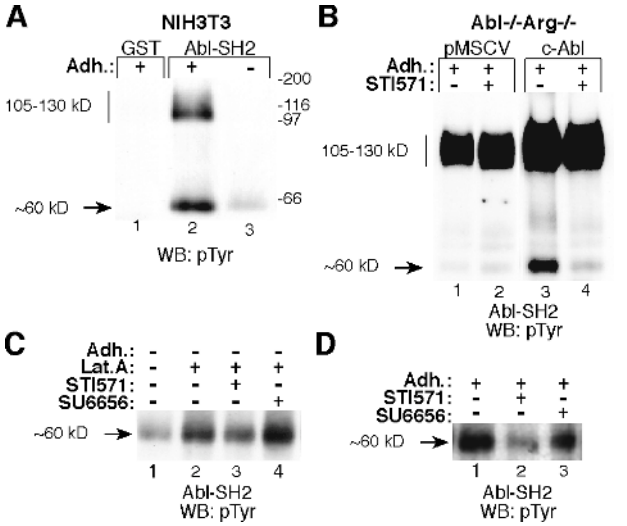

Figure 1. **pTyr-containing proteins associate with Abl-SH2 during FN-stimulated cell spreading.** (A) NIH3T3 fibroblasts were detached from the substratum and held in suspension for 45–60 min, and then half of the cells were plated onto FN for 20 min (Adh.: +) while the other half remained in suspension (Adh.: −). Cell lysates were incubated with GST-agarose (lane 1) or GST-Abl-SH2-agarose (lanes 2 and 3). After washing the beads stringently, bound proteins were subjected to Western blot analysis using pTyr antibodies (WB:pTyr). (B) $Abl^{-/-}Arg^{-/-}$ MEFs stably reconstituted with vector (pMSCV, lanes 1 and 2) or c-Abl (lanes 3 and 4) were pretreated with 5 μM STI571 (lanes 2 and 4) or vehicle (DMSO, lanes 1 and 3) for 8 h. Cell lysates were incubated with Abl-SH2 and processed as in A. (C and D) Abl[$Abl^{-/-}Arg^{-/-}$] MEFs were maintained in suspension for 60 min (C) or replated onto FN (D). As indicated, 1 μM latrunculin A (Lat. A), 1 μM STI571, or 0.5 μM SU6656 was added to culture media before cell detachment (C, lanes 2–4). Cell lysates were incubated with Abl-SH2 and processed as described in A.

Results

An unbiased approach for isolating c-Abl substrates

The Abl-SH2 domain was used as an affinity matrix to isolate c-Abl substrates involved in cell spreading. We chose this strategy because it has been shown previously that the Abl-SH2 domain binds to the phosphotyrosine (pTyr) in known Abl substrates (Songyang et al., 1993; Duyster et al., 1995; Mayer et al., 1995). We detected several pTyr-containing proteins in lysates from spreading NIH3T3 fibroblasts that bound GST-Abl-SH2 (Abl-SH2), but not GST. One group of proteins was in the 105–130-kD range and another in the ~60-kD range (Fig. 1 A). The interaction of these proteins with Abl-SH2 was dependent on cell adhesion, which is consistent with the increase in c-Abl activity observed during fibroblast spreading on FN (Lewis et al., 1996).

We used four additional criteria to determine if any of these pTyr-containing proteins were indeed c-Abl substrates. First, we performed similar experiments using mouse embryo fibroblasts (MEFs) null for both Abl and Arg ($Abl^{-/-}Arg^{-/-}$). These MEFs were stably reconstituted with c-Abl (Abl[$Abl^{-/-}Arg^{-/-}$]) or pMSCV vector (pMSCV[$Abl^{-/-}Arg^{-/-}$]) using retroviral-mediated gene transfer. Significantly lower levels of the ~60-kD pTyr-proteins were isolated with Abl-SH2 using lysates from spreading pMSCV [$Abl^{-/-}Arg^{-/-}$] MEFs compared with Abl[$Abl^{-/-}Arg^{-/-}$] MEFs (Fig. 1 B, lanes 1 and 3). Second, we pretreated spreading Abl[$Abl^{-/-}Arg^{-/-}$] MEFs with an inhibitor of c-Abl kinase, signal transduction inhibitor (STI)571 (Schindler et al., 2000). This treatment of cells with STI571 also decreased the level of ~60-kD pTyr-proteins isolated with Abl-SH2 (Fig. 1 B, lanes 3 and 4). Third, we used latrunculin A, an inhibitor of F-actin assembly that can activate c-Abl in suspended cells (Woodring et al., 2002). Latrunculin treatment of suspended Abl[$Abl^{-/-}Arg^{-/-}$] MEFs increased the level of ~60-kD pTyr-proteins that were isolated with Abl-SH2 (Fig. 1 C, lanes 1 and 2). Finally, to explore the specificity of the Abl-SH2 affinity method, we used 0.5 μM SU6656, which inhibits the activity of c-Src, Fyn, and Lck (Blake et al., 2000; the IC_{50} for Src kinases is <0.3 μM, whereas the IC_{50} for c-Abl is 1.7 μM). Addition of SU6656 to suspended latrunculin-treated MEFs did not cause a significant decrease in the quantity of ~60-kD pTyr-proteins isolated with Abl-SH2, whereas STI571 did (Fig. 1 C). Also, the Src kinase inhibitor was less effective than the Abl inhibitor at reducing the amount of ~60-kD pTyr-proteins isolated with Abl-SH2 from lysates of spreading cells (Fig. 1 D). Presumably, many of the 105–130-kD pTyr-proteins are not substrates for c-Abl even though they are isolated with Abl-SH2. In contrast, the ~60-kD pTyr-proteins appeared to be good candidates for bona fide c-Abl substrates during FN-stimulated cell adhesion and spreading because

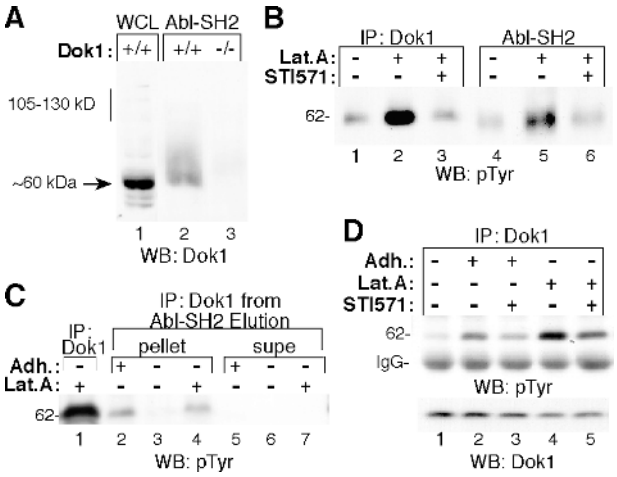

Figure 2. **Dok1 is tyrosine phosphorylated when Abl is active in cells.** MEFs were pretreated with STI571 and latrunculin A as indicated (see Materials and methods), and then detached and either held in suspension (Adh.: −) or replated onto FN for 20 min (Adh.: +). (A) Lysates from latrunculin-treated passage 3 MEFs null for Dok1 (Dok1$^{-/-}$) or littermate control MEFs (Dok1$^{+/+}$) were incubated with Abl-SH2, and the bound Dok1 was detected using Dok1 antibodies in Western blot analysis. Lane 1 contained whole cell lysate as a positional control. (B) Dok1 was immunoprecipitated (α6043) from the same suspended cell lysates (lanes 1–3) as used for the Abl-SH2 affinity analysis (lanes 4–6). Precipitated proteins were subjected to Western blot analysis using pTyr antibodies. Note that the slight variation in mobility of p62 between the immunoprecipitates and the Abl-SH2 pull downs is likely due to the presence of a large quantity of Abl-SH2 domain in lanes 4–6. (C) Abl-SH2 was incubated with the indicated cell lysates, and the bound pTyr-containing proteins were eluted from the agarose with 0.5 M phenylphosphate. Eluates were diluted and subjected to immunoprecipitation with Dok1 antibodies. Equal fractions from the supernatant (lanes 5–7) and pellet (lanes 2–4) of the Dok1 immunoprecipitate were subjected to Western blot analysis using pTyr antibodies. Lane 1 contained a Dok1 immunoprecipitate from latrunculin-treated cells. (D) Dok1 was immunoprecipitated from lysates of the indicated Abl[Abl$^{-/-}$Arg$^{-/-}$] MEFs, and then subjected to sequential Western blot analysis using pTyr antibodies, followed by Dok1 antibodies.

their isolation using Abl-SH2 was increased by both FN and latrunculin, which activate c-Abl, and decreased by both STI571 and deletion of Abl/Arg. Thus, we investigated the identity the ~60-kD pTyr-proteins.

The p62 docking protein Dok1 binds Abl-SH2 when c-Abl is active

Because Dok1 (downstream of tyrosine kinases) is a 62-kD protein that is tyrosine phosphorylated in Bcr-Abl–transformed cells (Wisniewski et al., 1994; Carpino et al., 1997; Yamanashi and Baltimore, 1997), we checked if Dok1 was a component of the ~60-kD pTyr-proteins isolated with Abl-SH2. Using antibodies specific for Dok1 and lysates from latrunculin-treated suspended wild-type (WT; Dok1$^{+/+}$) or Dok1$^{-/-}$ MEFs, we found that Abl-SH2 did isolate Dok1 (Fig. 2 A). The level of ~60-kD pTyr-proteins isolated with Abl-SH2 was significantly decreased using lysates from Dok1$^{-/-}$ MEFs compared with Dok1$^{+/+}$ MEFs (unpublished data), indicating that Dok1 is likely a component of pTyr-p60. To explore this possibility further, we performed side-by-side analysis of Abl-SH2 pull downs and Dok1 immunoprecipitates (Fig. 2 B). The levels of pTyr on Dok1 correlated with the amount of pTyr-p60 isolated with Abl-SH2. Direct evidence that Dok1 was a component of the ~60-kD pTyr-proteins isolated with Abl-SH2 was obtained using Dok1 antibodies to immunoprecipitate Dok1 from proteins that were eluted from Abl-SH2 (Fig. 2 C). We also found that the pTyr on Dok1 was increased during latrunculin treatment and fibroblast spreading, and this increase was largely blocked by STI571 (Fig. 2 D). These data indicate that Dok1 is a component of the ~60-kD pTyr-proteins isolated with Abl-SH2 and support the conclusion that Dok1 is a substrate for c-Abl in spreading MEFs.

Y361 of Dok1 is phosphorylated by c-Abl in response to cell adhesion

To identify residues of Dok1 that are phosphorylated by c-Abl, we stably expressed WT HA-Dok1 and various Y to F HA-Dok1 point mutants in pMSCV[Abl$^{-/-}$Arg$^{-/-}$] and Abl[Abl$^{-/-}$Arg$^{-/-}$] MEFs. We observed constant low levels of pTyr on all HA-Dok1 constructs when expressed in pMSCV[Abl$^{-/-}$Arg$^{-/-}$] MEFs (Fig. 3 A; unpublished data). In the Abl[Abl$^{-/-}$Arg$^{-/-}$] MEFs, the pTyr content of HA-Dok1 was elevated compared with the HA-Dok1 from pMSCV[Abl$^{-/-}$Arg$^{-/-}$] MEFs (Fig. 3 A, lanes 1 and 2). The Y361F Dok1 and Y295F/Y361F Dok1 mutants had decreased pTyr content in Abl[Abl$^{-/-}$Arg$^{-/-}$] MEFs spreading on FN, whereas the pTyr content of the other mutants, including Y295F, was similar to that of WT Dok1. This finding suggests that the phosphorylation of Y361 of Dok1 is induced by c-Abl during cell spreading, so we investigated whether or not c-Abl could directly phosphorylate Y361 of Dok1. In vitro kinase assays were performed with affinity-purified c-Abl (Woodring et al., 2001) and bacterially expressed GST-Dok1 proteins. Tryptic phosphopeptide mapping of WT Dok1 phosphorylated by c-Abl in vitro revealed seven prominent tryptic phosphopeptides, suggesting that c-Abl can phosphorylate multiple tyrosines in Dok1. The map of the Y361F Dok1 mutant was missing two of those tryptic phosphopeptides (Fig. 3 B, 1 and 2). The predicted mobility of the Dok1 tryptic peptide containing Y361 corresponds to the peptide labeled "2". The lysine defining the tryptic cleavage site upstream of Y361 is embedded in acidic residues and, therefore, may be poorly cleaved by trypsin. To determine if peptide 1 was a longer, incompletely digested tryptic peptide containing pY361, peptide 1 was purified from the WT Dok1 map and redigested with trypsin. This procedure yielded some peptide 2 (Fig. 3 C). Peptides corresponding to the long (Fig. 3, 1) and short (Fig. 3, 2) pY361-Dok1 tryptic peptides were synthesized and found to migrate at the same coordinates on our tryptic maps (Fig. 4 A; see Materials and methods). These results indicate that c-Abl can directly phosphorylate Dok1 at Y361 in vitro. Y361 of Dok1 also appeared to be a target for c-Abl in spreading cells because replating MEFs on FN increased pTyr on Dok1 but had little effect on Y361F Dok1 (Fig. 3, A and D).

To further investigate the phosphorylation of Y361 of Dok1 by c-Abl, we performed two additional sets of experiments: phosphopeptide mapping of cellular Dok1 and immunoblotting using a pY361 phosphospecific Dok1 antibody (Fig. 4). For tryptic phosphopeptide mapping, HA-

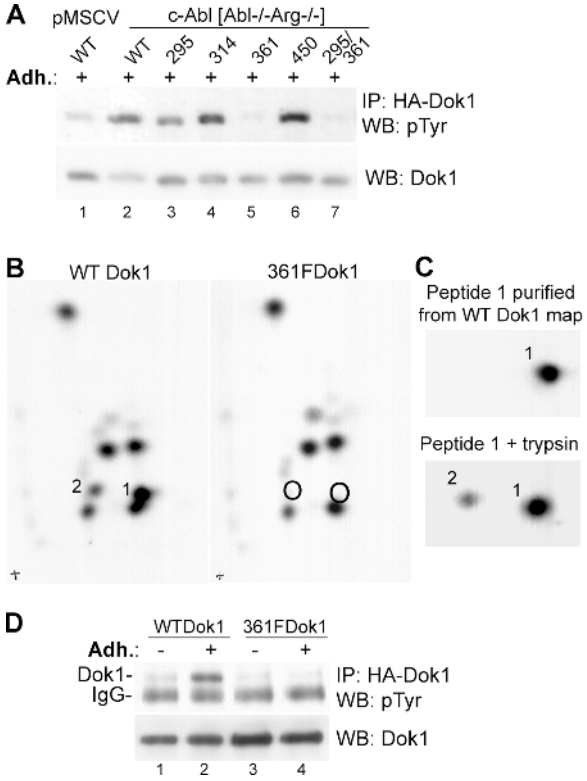

Figure 3. **Expression of c-Abl increases the pTyr on Dok1 but not Y361F Dok1.** (A) HA-Dok1 mutants were expressed stably in $Abl^{-/-}Arg^{-/-}$ MEFs previously reconstituted with either pMSCV empty vector (lane 1) or c-Abl (lanes 2–7). The mutated tyrosines (Y to F) in HA-Dok1 are indicated at the top of each lane. HA-Dok1 mutants were immunoprecipitated from cell lysates of spreading cells and subjected to sequential Western blot analysis using pTyr antibodies, and then Dok1 antibodies. (B) Tryptic phosphopeptide maps of GST-Dok1 and GST-Y361F Dok1. Purified GST-Dok1 proteins were phosphorylated using γ-[^{32}P]ATP and purified c-Abl. GST-Dok1 was isolated with glutathione-Sepharose and subjected to SDS-PAGE. The ^{32}P-labeled Dok1 band was excised from the gel and processed for tryptic phosphopeptide mapping (see Materials and methods). The sample origin is located at the lower left. Circles drawn on the right panel indicate where peptides 1 and 2 would have run if they were present in the map of Y361F Dok1. (C) Peptide 1 was purified from the TLC plate of WT ^{32}P-Dok1 shown in B. The map of purified peptide 1 incubated in buffer without trypsin (top) and the map of purified peptide 1 redigested with trypsin (bottom) are shown. (D) The indicated Abl[$Abl^{-/-}Arg^{-/-}$] MEFs described in A were held in suspension for 45–60 min, and then half of the cells were plated onto FN for 20 min (lanes 2 and 4), while the other half remained in suspension (lanes 1 and 3). HA-Dok1 constructs were immunoprecipitated from cell lysates and subjected to sequential Western blot analysis using pTyr antibodies, and then Dok1 antibodies.

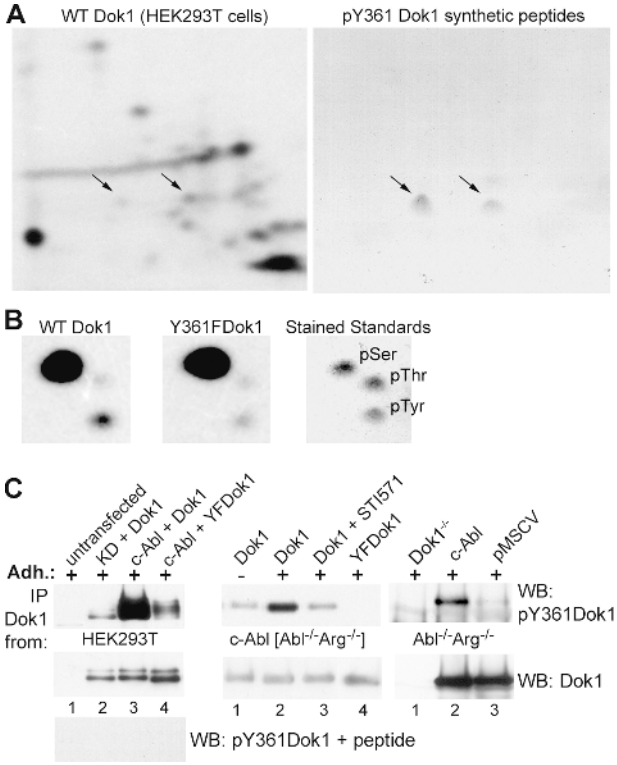

Figure 4. **c-Abl increases the phosphorylation of Y361 of Dok1 in cells.** (A) Tryptic phosphopeptide map of HA-Dok1 from ^{32}P-labeled HEK293T cells that were coexpressing c-Abl. HA-Dok1 was immunoprecipitated and processed for tryptic phosphopeptide mapping. Before spotting onto the TLC plate, trypsin-digested synthetic pY361 Dok1 peptides (see Materials and methods) were added to the ^{32}P-Dok1 sample. Arrows indicate the position of synthetic pY361 Dok1 peptides detected by ninhydrin staining. The autoradiogram (left) was obtained by exposing the ninhydrin-stained TLC plate (right) to film. The mobility of the synthetic pY361 Dok1 peptides corresponds to that of the phosphopeptides labeled "1" and "2" derived from ^{32}P-Dok1 in Fig. 3 B. (B) Phosphoamino acid analysis of WT Dok1 and Y361F Dok1 from ^{32}P-labeled HEK293T cells that were coexpressing c-Abl. Samples were prepared as described previously (Meisenhelder et al., 1999). Right panel shows ninhydrin staining indicating the position of the phosphoamino acid standards that were added to the ^{32}P samples. (C) Dok1 was immunoprecipitated from lysates of the indicated cells, and then subjected to Western blot analysis using the antibodies indicated at the far right of each row of panels. (left) HA-Dok1 was immunoprecipitated from lysates of HEK293T cells that were transfected with empty vector (lane 1); KD Abl and WT Dok1 (lane 2); WT c-Abl and WT Dok1 (lane 3); or WT c-Abl and Y361F Dok1 (lane 4). (bottom left) pY361-Dok1 synthetic peptide 1 was added to the to the αpY361Dok1 immunoblotting solution. (middle) HA-Dok1 was immunoprecipitated from lysates of Abl[$Abl^{-/-}Arg^{-/-}$] MEFs expressing either WT Dok1 (lanes 1–3) or Y361F Dok1 (lane 4). MEFs were held in suspension (lane 1), replated on FN (lanes 2 and 4), or pretreated with STI571 and replated on FN (lane 3). (right) Endogenous Dok1 was immunoprecipitated (α6043) from the following spreading MEFs: lane 1, $Dok1^{-/-}$; lane 2, Abl[$Abl^{-/-}Arg^{-/-}$]; or lane 3, pMSCV[$Abl^{-/-}Arg^{-/-}$]. Because endogenous Dok1 migrates close to the IgG heavy chain, $Dok1^{-/-}$ MEFs were used as a control (lane 1). Note that the intensity level of signals between the left, middle, and right panels are not directly comparable because these results are from three separate experiments.

Dok1 was immunoprecipitated from ^{32}P-labeled 293T cells coexpressing c-Abl (Fig. 4 A, left; technical obstacles prohibited us from ^{32}P labeling spreading MEFs). Two ^{32}P-labeled tryptic peptides generated from Dok1 comigrated with the synthetic pY361 synthetic peptides (Fig. 4 A, arrows). These tryptic phosphopeptides were absent in maps of Y361F Dok1 (Fig. 3 B), implying that WT Dok1 is phosphorylated at Y361 in cells. Using phosphoamino acid analysis, we detected enhanced pTyr levels on ^{32}P-WT Dok1 relative to that of ^{32}P-Y361F Dok1 when each was immunoprecipitated from cells coexpressing c-Abl (Fig. 4 B). A phospho-specific antibody directed against pY361 of Dok1 (αpY361) was also used to examine the pY361 content of Dok1 in cells (Fig. 4 C). A prominent signal was detected using αpY361

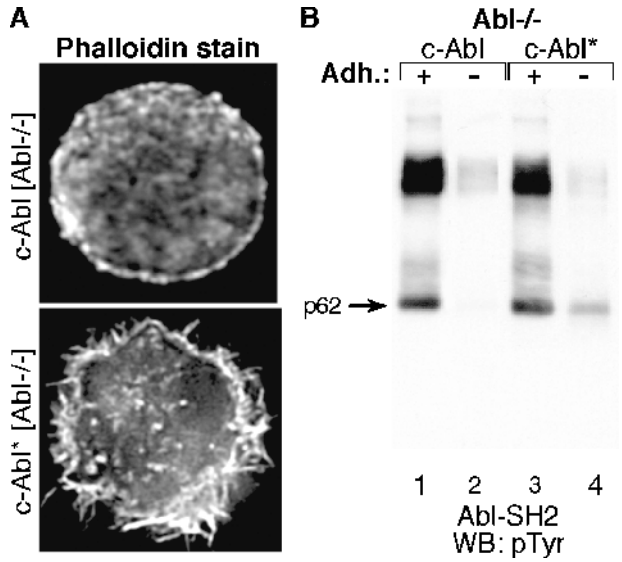

Figure 5. **Stable expression of activated c-Abl (c-Abl*) in nonadherent fibroblasts increases the pTyr content on Dok1 pulled down with Abl-SH2.** (A) c-Abl or activated c-Abl* (c-Abl missing the last four amino acids; Woodring et al., 2002) was stably expressed in $Abl^{-/-}$ MEFs. The deconvolution microscopy images shown highlight the differences observed in cells that were fixed after 60 min in suspension (Woodring et al., 2002). The F-actin cytoskeleton was stained using TRITC-phalloidin. (B) Cell lysates from FN-replated (lanes 1 and 3) and suspended (lanes 2 and 4) MEFs were incubated with Abl-SH2, and the bound proteins were subjected to Western blot analysis using pTyr antibodies. Arrow indicates the p62 kD band we identified as Dok1 (Fig. 2).

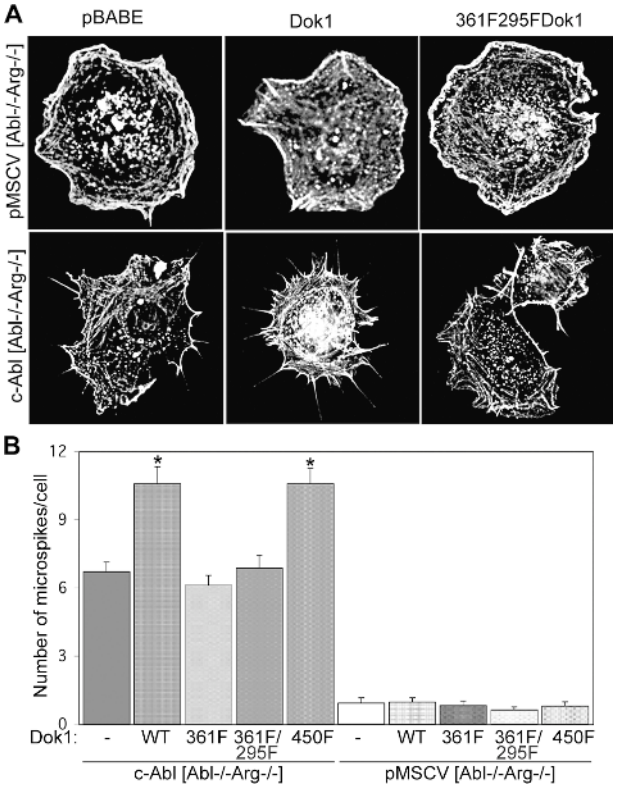

Figure 6. **Dok1 expression increases Abl-dependent filopodia during cell spreading, whereas Y361F Dok1 expression does not affect filopodia.** (A) The indicated $Abl^{-/-}Arg^{-/-}$ MEF lines were held in suspension for 45–60 min, and then replated onto FN-coated coverslips for 20 min before fixation. Fixed cells were stained with TRITC-phalloidin, and images were obtained using deconvolution microscopy. Images shown highlight the differences between the populations of cells. (B) The number of F-actin microspikes/filopodia were counted on 200–1,000 randomly selected cells from each indicated cell line as described in Materials and methods. Error bars represent SEM; *, $P < 0.001$ compared with the parent cell line not expressing Dok1.

in immunoblot analysis of Dok1 immunoprecipitated from HEK cells coexpressing Dok1 and c-Abl (Fig. 4 C, top left, lane 3). Addition of the synthetic pY361 Dok1 peptide to the αpY361 immunoblotting solution effectively reduced this signal (Fig. 4 C, bottom left). The signal was significantly less for Dok1 immunoprecipitated from cells coexpressing either WT Dok1 and kinase-deficient (KD) Abl or Y361F Dok1 and WT c-Abl (Fig. 4 C, left). Thus, c-Abl is capable of phosphorylating Y361 of Dok1 both in vitro and in cells.

Results obtained with the Y361F Dok1 mutant suggested that Y361 is phosphorylated by c-Abl in MEFs spreading on FN (Fig. 3, A and D). Using the αpY361 Dok1 antibody, we found that FN stimulation increased the pY361 content of Dok1 relative to that of Dok1 from suspended cells (Fig. 4 C, middle). STI571 largely blocked the stimulatory effect of FN, and pY361 was not detected on Y361F Dok1. We also detected pY361 on endogenous Dok1 from spreading Abl[$Abl^{-/-}Arg^{-/-}$] MEFs, whereas very little pY361 was detected on Dok1 from pMSCV[$Abl^{-/-}Arg^{-/-}$] MEFs, and no signal was detected in an immunoprecipitate from $Dok1^{-/-}$ cells, as expected (Fig. 4 C, right). Based on the results shown in Figs. 3 and 4, we conclude that c-Abl induces phosphorylation of Dok1 at Y361 in MEFs spreading on FN.

Tyrosine phosphorylation of Dok1 at Y361 coincides with F-actin microspike formation

c-Abl activity prolongs the persistence of exploratory filopodia and F-actin microspikes during the early stages of cell spreading (Woodring et al., 2002). In addition, deletion of four amino acids at the extreme COOH terminus generates a form of Abl (c-Abl*) that remains active in suspended cells (Woodring et al., 2001). Consistent with these two observations, suspended $Abl^{-/-}$ cells expressing WT c-Abl contained few to no microspikes (Fig. 5 A, top) and basal c-Abl activity, whereas suspended cells expressing c-Abl* contained numerous F-actin microspikes (Fig. 5 A, bottom). To explore Abl substrates involved in c-Abl–induced microspike formation in suspended cells, we used Abl-SH2 to isolate Abl substrates from each of these cell lines (Fig. 5 B). After FN stimulation, Abl-SH2 isolated similar amounts of pTyr-containing proteins from the lysates of c-Abl– and c-Abl*–expressing cells (Fig. 5 B, lanes 1 and 3). In suspended cells, Abl-SH2 isolated more pTyr-p62 from lysates of cells expressing c-Abl* than from lysates of cells expressing WT c-Abl (Fig. 5 B, lanes 2 and 4). Thus, there was an increase in F-actin microspikes in detached cells expressing active Abl and a corresponding increase in the quantity of pTyr-p62 (Dok1) isolated using Abl-SH2.

We examined if Dok1 could enhance the c-Abl–induced increase in the number of filopodia in spreading MEFs. Sta-

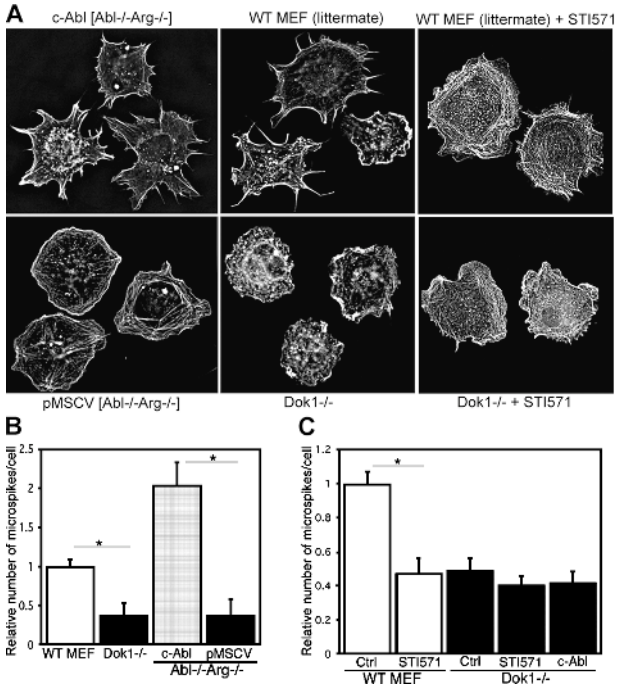

Figure 7. **Dok1$^{-/-}$ primary MEFs have fewer filopodia.** Spreading passage 3 Dok1$^{-/-}$ MEFs (bottom row, middle and right) and the littermate Dok1$^{+/+}$ MEFs (top row, middle and right) were analyzed side-by-side with spreading Abl[Abl$^{-/-}$Arg$^{-/-}$] and pMSCV[Abl$^{-/-}$Arg$^{-/-}$] MEFs (left panels, top and bottom, respectively). TRITC-phalloidin staining (A), and quantification of the number of F-actin microspikes/filopodia in spreading cells and statistics (B and C) were performed as described in Fig. 6. Error bars represent SEM; *, $P < 0.001$ when the values (represented by the columns) on either side of the horizontal line are compared. (C) As indicated, MEFs were pretreated with STI571 or were stably infected with c-Abl or control (pMSCV) retrovirus and selected for 4 d. Note that data for the histograms was normalized to WT MEFs (set to 1) so that experiments performed on separate occasions could be compared.

ble expression of WT Dok1 (3–5-fold endogenous levels) in Abl[Abl$^{-/-}$Arg$^{-/-}$] MEFs increased the number of filopodia present at the cell surface (Fig. 6). This increase was dependent on the presence of c-Abl because Dok1 expression did not enhance membrane protrusions in pMSCV[Abl$^{-/-}$Arg$^{-/-}$] MEFs (Fig. 6, A and B). The stable expression of Dok1 together with c-Abl in Abl$^{-/-}$Arg$^{-/-}$ cells caused a 1.4–2.2-fold increase ($P < 0.001$) in the number of F-actin–rich membrane protrusions over the number observed with c-Abl expression alone (Fig. 6 B). Like WT Dok1, Y450F Dok1 also increased the number of filopodia in spreading Abl-reconstituted cells. In contrast, expression of Y361F or Y361F/Y295F Dok1 did not increase the number of filopodia induced by c-Abl during cell spreading (Fig. 6 B).

We also investigated whether or not Dok1 is required for filopodia formation in spreading cells using early passage primary MEFs null for Dok1 (Fig. 7, A and B). Compared with MEFs of the same passage number from littermate controls, the MEFs lacking Dok1 exhibited 2.1–3.2-fold fewer filopodia during spreading on FN. The number of filopodia on Dok1$^{-/-}$ MEFs was similar to that found on Abl$^{-/-}$Arg$^{-/-}$ MEFs (Fig. 7 B). c-Abl expression did not increase F-actin microspikes on Dok1$^{-/-}$ MEFs, and STI571 treatment decreased the number of filopodia on WT MEFs but not on Dok1$^{-/-}$ MEFs (Fig. 7 C). In summary, c-Abl expression was required for Dok1 to enhance filopodia formation (Fig. 6), and Dok1 was required to observe effects of c-Abl on filopodia during cell spreading (Fig. 7 C). These data implicate Dok1 as a mediator for c-Abl in the formation or maintenance of filopodia during cell spreading. Because c-Abl and Dok1 are required for one another's effects, they most likely are components of the same signaling pathway in modulating filopodia.

Dok1 and Abl are localized to filopodia in spreading fibroblasts

Because spatial organization of intracellular signaling proteins is of critical importance for achieving the proper biological output of a signaling pathway, we examined the localization of Dok1 and c-Abl during cell spreading. Dok1 was localized in filopodia of spreading MEFs as well as, more generally, at the plasma membrane (Fig. 8 A). These observations are consistent with the presence of an NH_2-terminal pleckstrin homology (PH) domain of Dok1, which may promote its association with phosphoinositides in the plasma membrane (Zhao et al., 2001). Higher magnification of individual filopodia (Fig. 8 B, top) revealed that Dok1 was present along and at the tips of filopodia. This spatial analysis suggests that Dok1 is most likely acting locally at the cell perimeter to affect filopodia. Similar staining of filopodia was observed using pTyr antibodies and Abl antibodies (Fig. 8 B, middle). Furthermore, Abl and Dok1 were detected in the same filopodia (Fig. 8 B, bottom). The localization of both c-Abl and Dok1 in filopodia is consistent with Dok1 acting as a local substrate for c-Abl in modulating filopodia protrusion.

Staining of suspended MEFs expressing c-Abl* with pTyr antibodies revealed discrete brilliant staining at the tips of peripheral actin microspikes (Fig. 8 C). Pretreatment with STI571 eliminated the microspikes and therefore eliminated the pTyr staining at the tips of the microspikes. Dok1 was localized to the tips of peripheral F-actin microspikes in suspended cells expressing c-Abl* (Fig. 8 C, right), suggesting a role for Dok1 in formation of these F-actin structures.

Phosphorylation of Y361 can promote Dok1 binding to the SH2 domains of p120RasGAP (p120) and the Nck adaptor protein (Tang et al., 1997; Murakami et al., 2002; Shah and Shokat, 2002). Whereas association with Nck involves the phosphorylation of Y361 of Dok1, association with p120 appears to involve phosphorylation of five additional tyrosines (Murakami et al., 2002). We did not detect an association between p120 and Dok1 in lysates of spreading MEFs, nor did we detect any change in the pTyr content of p190RhoGAP, which associates with p120 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200312171/DC1). In contrast, we did detect endogenous Nck in immunoprecipitates of Dok1 using lysates from spreading Abl[Abl$^{-/-}$Arg$^{-/-}$] MEFs (Fig. 9 A, right). WT Dok1 was present in Nck immunoprecipitates, whereas Y361F Dok1 was not (Fig. 9 A, left). Correspondingly, the Dok1 that coimmunoprecipitated with Nck was phosphorylated at Y361 (Fig. 9 A). Coimmunoprecipitation of endogenous Dok1 and Nck was detected in spreading MEFs (Fig. 9 B,

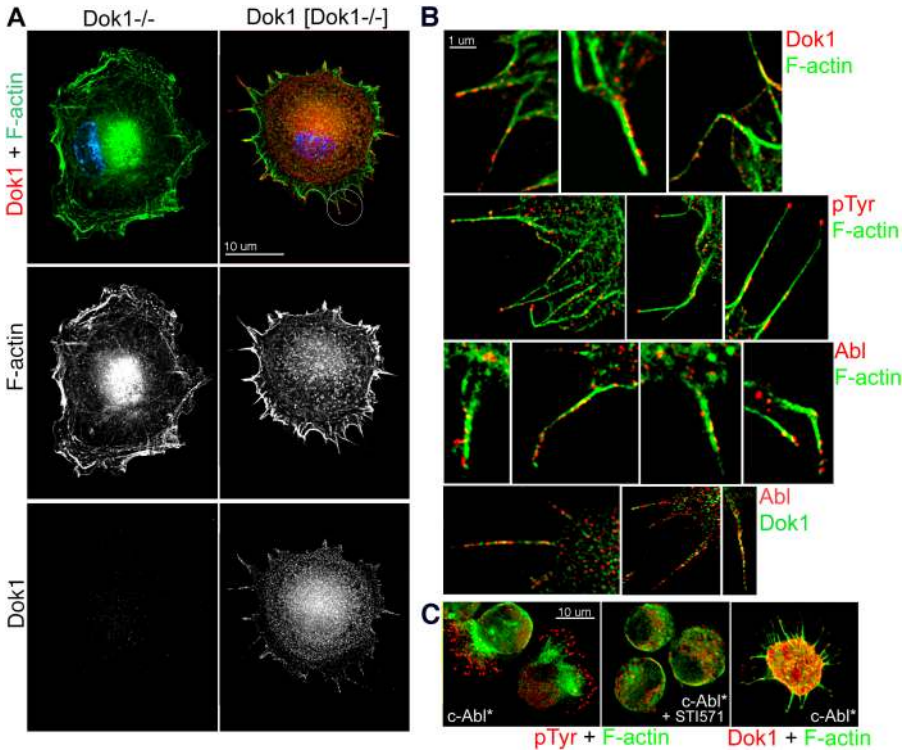

Figure 8. **Dok1, pTyr, and c-Abl localize to the tips of filopodia and peripheral actin microspikes.** (A) Passage 5 Dok1$^{-/-}$ MEFs were infected with pBABE-puro vector (left) or Dok1 retrovirus (right), and then selected for 4 d. MEFs were fixed during spreading on FN (20 min) and stained. FITC-phalloidin was used to detect F-actin (green); Dok1 (A3) + Texas red goat–αmouse antibodies were used to detect Dok1 (red); and Hoechst 33258 (blue) was used to detect nuclei. (B) Spreading MEFs were stained with FITC-phalloidin and the following antibodies: Dok1 (A3, first row), pTyr (4G10, second row), or c-Abl (8E9, third row). Texas red goat–αmouse antibodies were used as secondary antibodies. Abl, Dok costaining (fourth row) was performed sequentially, first staining with αDok (A3) + goat αmouse-Alexa Fluor 488, then with αAbl (8E9, mouse mAb) directly conjugated to Alexa Fluor 546. All panels show images of filopodia protruding from MEFs spreading on FN. The top left panel is a higher magnification of the circled area in A. (C, left and middle) FITC-phalloidin and αpTyr staining of suspended cells stably expressing c-Abl*, which remains active in suspended cells. Cells were pretreated with STI571 as indicated. (right) FITC-phalloidin and αDok1 (A3) staining of a suspended cell stably expressing c-Abl*.

lanes 6 and 7), and this association was enhanced in cell lines stably expressing elevated levels of Dok1 (Fig. 9 B, lanes 4 and 5; note that lanes 7 and 8 were exposed to film longer than lanes 4–6). In addition, the interaction between the Dok1 and Nck was reduced in STI571-treated spreading MEFs (Fig. 9 B, lane 8) and in suspended MEFs (Fig. 9 B, lane 9). Together these data strongly imply that the association between Dok1 and Nck is stimulated by c-Abl.

To investigate if Nck is involved in filopodia formation during cell spreading, we used Nck1$^{-/-}$Nck2$^{-/-}$ MEFs (Gruenheid et al., 2001). The number of filopodia in spreading Nck1$^{-/-}$Nck2$^{-/-}$ MEFs was reduced by threefold relative to cells stably reconstituted with endogenous levels of Nck1 (Fig. 9 C). The number of filopodia observed in MEFs reexpressing Nck1 was reduced with STI571 treatment (Fig. 9 D); however, STI571 did not affect the filopodia of Nck1$^{-/-}$Nck2$^{-/-}$ MEFs, suggesting that Nck is involved in Abl-mediated filopodia formation.

Results from cotransfection experiments suggest that Nck1, Dok1, and Abl may form a ternary complex in cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200312171/DC1). The association of all three proteins was significantly decreased when Y361F Dok1 was used instead of WT Dok1, when KD Abl was used instead of WT Abl, or when cells were treated with STI571. Thus, a signaling complex containing Abl, Dok1, and Nck may form in cells, and its formation is dependent on Abl activity and phosphorylation of Y361 of Dok1. In summary, our data suggest that c-Abl transduces signals to actin at the cell periphery by phosphorylating Y361 of Dok1 and recruiting Nck.

Discussion

We propose a novel c-Abl signaling pathway that promotes formation of filopodial exploratory structures during cell spreading. This pathway involves (a) the activation of c-Abl on integrin interaction with FN, (b) the Abl-dependent phosphorylation of Y361 of Dok1, and (c) the recruitment of the Nck adaptor protein to pY361-Dok1. Our model is consistent with two recent reports demonstrating that clustering of Nck at the cell membrane initiates localized actin polymerization (Campellone et al., 2004; Rivera et al., 2004). Nck can associate with and/or affect proteins that promote assembly of F-actin, such as N-WASP (Rivero-Lezcano et al., 1995; Rohatgi et al., 2001; Benesch et al., 2002), WAVE-1 (Eden et al., 2002), and PAK (p21-activated protein kinase; Zhao et al., 2000). Nck may also directly associate with c-Abl via its NH_2-terminal SH3 domains (Ren et al., 1994). Activation of some of these Nck binding proteins, such as PAK1 and WAVE-1, appears to require the Cdc42 GTPase. Because we did not observe reduction of Abl-induced filopodia formation on expression of dominant-negative Cdc42 (Woodring et al., 2002), we propose that the effect of c-Abl on filopodia is Cdc42 independent. Notably, Nck can increase N-WASP–stimulated actin polymerization in the absence of Cdc42 in vitro (Moreau et al., 2000; Rohatgi et al., 2001; Benesch et al., 2002). Likewise, high concentrations of Nck at the cell membrane stimulates localized actin polymerization through a mechanism that does not require Cdc42 or WAVE-1 but does involve N-WASP (Rivera et al., 2004). Our preliminary studies sug-

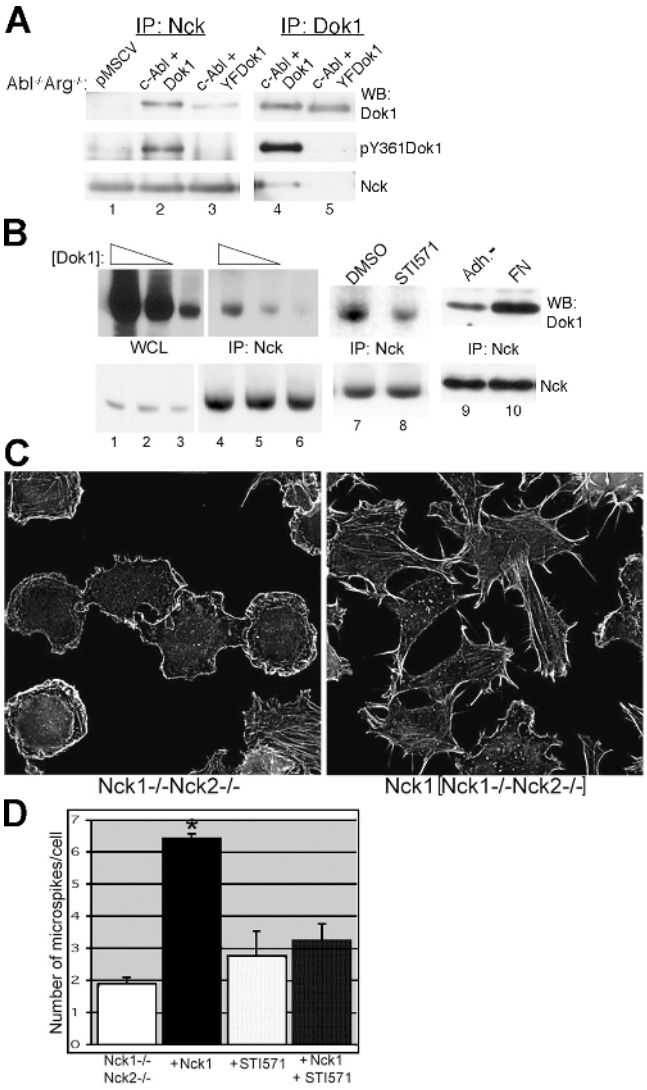

Figure 9. **The Nck adaptor protein is involved in Abl-dependent filopodia formation.** (A and B) $Abl^{-/-}Arg^{-/-}$ MEFs were stably infected with pMSCV empty vector, c-Abl, WT Dok1, and/or Y361F Dok1 retrovirus as indicated. MEFs were held in suspension for 60 min, and then lysed (B, lane 9) or replated onto FN for 25 min (all other lanes in A and B). (A) Endogenous Nck and Dok1 were immunoprecipitated with α5547 (lanes 1–3) and α12CA5 (lanes 4 and 5), respectively. Western blot analysis was performed using antibodies indicated at the right. (B) Lanes 1–3 contain whole cell lysates, and lanes 4–10 contain Nck immunoprecipitates from lysates of Abl[$Abl^{-/-}Arg^{-/-}$] MEFs expressing endogenous Dok1 (lanes 3 and 6–8) or increased levels of Dok1 (lanes 1, 2, 4, 5, 9, and 10). As indicated, MEFs were pretreated with DMSO (lane 7) or 5 μM STI571 (lane 8). Exposure time for lanes 7 and 8 was ~10 times longer than for lanes 4–6. (C and D) MEFs null for Nck1 and Nck2 were stably reconstituted with empty vector (left) or Nck1 (right). Cells were plated on FN for 25 min, fixed, and stained with TRITC-phalloidin. The number of F-actin microspikes and filopodia were counted as described in Materials and methods. *, $P < 0.001$ compared with the cell line lacking Nck.

gest that N-WASP may be involved in Abl-mediated filopodia formation (unpublished data), whereas PAK1 does not appear to be involved (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200312171/DC1). Our results establish that Nck is critical for Abl/Dok1-mediated filopodia formation in spreading MEFs, but the details of the signaling pathway downstream of Nck await further investigation.

Several publications support a role for Abl kinases in regulation of F-actin polymerization in cells (for review see Lanier and Gertler, 2000; Woodring et al., 2003; Hernandez et al., 2004). Overall, it appears that c-Abl stimulates filopodia formation, membrane ruffling, and neurite extension, but inhibits chemotaxis (Frasca et al., 2001; Kain and Klemke, 2001). At first glance, this finding may appear as a paradox: how can c-Abl increase actin polymerization yet inhibit cell movement? This enigma might be resolved by considering the function of filopodia. Filopodia (and membrane ruffles) are present on cells that have paused to explore their environment (Machesky 2000). If a cell remains in the exploratory phase for an extended time, directed migration may progress slower. We (and others) have found that primary cultured Abl-null cells spread faster than the Abl-reconstituted or WT cells (Sheetz, M., personal communication; unpublished data). Thus, c-Abl may slow migration or spreading by promoting formation of actin structures that permit a cell to pause and explore the surroundings before moving toward a target.

Based on overexpression studies, it has been suggested that Dok1 and Dok2 regulate cell motility (Noguchi et al., 1999; Hosooka et al., 2001; Master et al., 2001). Dok1 may also affect plasma membrane ruffling because GFP-Dok1 undergoes a PH domain–dependent translocation to the plasma membrane in response to PDGF (Zhao et al., 2001) and is found in the cytoskeletal and membrane fractions of cells (Noguchi et al., 1999; Hosooka et al., 2001; Zhao et al., 2001). The PH domain of Dok1 is required for tyrosine phosphorylation of Dok1 (Zhao et al., 2001), suggesting that the tyrosine kinases that phosphorylate Dok1 localize at or near the plasma membrane. These observations are consistent with Dok1 regulating localized F-actin–mediated processes.

There are five Dok family members (Dok1–Dok5). We detected expression of Dok1 and Dok2 in the MEFs used in this paper (unpublished data). The COOH-terminal YXXP Abl consensus phosphorylation sites are not conserved in Dok3, Dok4, or Dok5, and these proteins appear to have functions distinct from Dok1 and Dok2 (Cong et al., 1999; Grimm et al., 2001). Recently, it was reported that pTyr-Dok2 can increase Abl kinase activity when coexpressed with c-Abl in HEK293T cells, presumably by binding to and displacing both the SH2 and SH3 domains of c-Abl (Master et al., 2003). This finding raises the interesting possibility that phosphorylation of Dok2 might amplify the effect of c-Abl; however, we did not detect pTyr on Dok2 immunoprecipitated from MEFs plated on FN (unpublished data), so it is unlikely that Dok2 activates c-Abl or mediates the effects of c-Abl in FN-stimulated MEFs. In our analysis, we found that, similar to the overexpression of other adaptor or docking proteins (e.g., Nck, Crk, Abi, and Dok2), Dok1 also increased the pTyr content of c-Abl when coexpressed in HEK293T cells (Woodring et al., 2003; unpublished data). This effect was not dependent on Y361 phosphorylation because the Y361F and Y295F/Y361F Dok1 mutants also increased the pTyr content of c-Abl to a similar level as WT Dok1. This find-

ing suggests that Dok1 may activate c-Abl through an intermediate protein or by a binding mechanism that does not require Y361 of Dok1 in HEK293T cells. Although these overexpression studies are interesting, the results obtained may not be applicable to all systems. For example, the endogenous c-Abl was still activated during FN cell spreading in Dok1$^{-/-}$ MEFs (unpublished data). For these reasons, we believe that neither Dok2 nor activation of c-Abl by Dok1 is responsible for the effect of c-Abl on filopodia in spreading MEFs.

Dok1 has several I/LYXXP consensus sites, including LY_{295}AEP, LY_{314}SDP, IY_{361}DEP, and LY_{376}DLP. Although c-Abl can phosphorylate several tyrosine residues of Dok1 in vitro (Fig. 3 B), it appears that c-Abl predominantly phosphorylates Y361 in spreading MEFs. Y361 of Dok1 is also a substrate for Src in v-Src transformed cells (Shah and Shokat, 2002) and in CHO cells overexpressing the insulin receptor (Noguchi et al., 1999). Src may contribute to the pTyr of Dok1 in MEFs because some Dok1 pTyr remains when cells are treated with STI571 (Fig. 2 D). Src and Abl may even act cooperatively to stimulate pTyr-Dok1, as has been suggested previously in MEFs stimulated with PDGF (Plattner et al., 1999).

The pTyr-Dok1 isolated from spreading cells associated with purified Abl-SH2 domain (Fig. 1 B). The binding of Abl substrates to the Abl-SH2 domain can promote processive phosphorylation of Abl substrates (Duyster et al., 1995; Mayer et al., 1995). It is possible that the tyrosine phosphorylation of Dok1 may allow Dok1 to stably associate with the Abl-SH2 domain to promote further tyrosine phosphorylation of Dok1. Because Dok1 can oligomerize through its pTyr binding domain (Songyang et al., 2001), c-Abl could also promote phosphorylation of Y361 of other Dok1 molecules in an oligomer. However, although Abl-SH2 did bind Dok1 from spreading MEFs, we were unable to detect coimmunoprecipitation of the endogenous proteins, suggesting that their association is transient and/or that minor populations of Dok1 and c-Abl associate, perhaps those molecules localized in filopodia.

Dok1 appears to be a multifunctional signaling protein with reported roles in cell growth, transformation, axonal guidance, and immune response (Holland et al., 1997; Yamanashi et al., 2000; Di Cristofano et al., 2001; Songyang et al., 2001; Zhao et al., 2001; Murakami et al., 2002). We have uncovered a new function of Dok1 in regulating F-actin microspikes and filopodia during fibroblast spreading. Notably, instead of using overexpression and dominant-negative approaches, which most other investigators have used to study Abl or Dok, we have used a genetic strategy with cells from knockout mice reexpressing levels of protein that are comparable to endogenous levels. We have defined a novel signaling pathway downstream of c-Abl leading to F-actin assembly and filopodia formation. Precisely localized c-Abl signaling at the tips of filopodia may be important for regulating the localized dynamics of filopodia extension, retraction, or attachment to the ECM to mediate the exploratory process. Further studies are required to determine if c-Abl, pY361Dok1, and Nck can fine-tune cell guidance by modulating cell exploration in vivo during embryonic development, axon path finding, or wound healing.

Materials and methods

cDNA expression using retroviral-mediated gene transfer

Stable Abl$^{-/-}$Arg$^{-/-}$ polyclonal cell lines reconstituted with murine type IV c-Abl were generated using retroviral-mediated gene transfer and the pMSCV-hyg retroviral vectors (Pear et al., 1993). Stable polyclonal MEF lines (pMSCV[Abl$^{-/-}$Arg$^{-/-}$] and Abl[Abl$^{-/-}$Arg$^{-/-}$]) were established using hygromycin selection (Woodring et al., 2002). Murine Dok1 and Dok1 mutant cDNAs were stably expressed by retroviral-mediated gene transfer using the pBABE-puro retroviral vectors and puromycin for selection. Using retrovirus (without helper virus) for stable expression of proteins resulted in levels of protein from one- to fivefold endogenous levels. Nck1 reexpressing Nck1$^{-/-}$Nck2$^{-/-}$ MEFs were generated in Tony Pawson's laboratory using retroviral-mediated gene transfer (Gruenheid et al., 2001).

Antibodies

pTyr (4G10) and Myc (9E10) mAbs were purchased from Upstate Biotechnology; Dok1 mAb (A3) was purchased from Santa Cruz Biotechnology, Inc.; HA mAb (12CA5) was purchased from Babco; Flag mAb was obtained from Sigma-Aldrich; and Abl mAb (Ab3) was obtained from Oncogene Sciences. Abl mAb (8E9) was a gift from J.Y.J. Wang. Nck (5547) and Dok1 (6043) rabbit antibodies were generated by J. Meisenhelder and N. Carter (The Salk Institute, La Jolla, CA) using GST-Nck1 and GST-Dok1 as immunogens. α5547 immunoprecipitates Nck1/2 but recognizes only Nck1 on immunoblots. Affinity-purified pY361Dok1 rabbit polyclonal antibodies were a gift from M. Lakkis (Biosource International, Hopkinton, MA), and pY412-Abl rabbit polyclonal antibodies were purchased from Biosource International. Secondary HRP-conjugated antibodies used for ECL were obtained from Amersham Biosciences. Secondary antibodies (Texas red, FITC, or Alexa Fluor 488 conjugates) used for immunofluorescence were obtained from Southern Biotechnology Associates, Inc. or Molecular Probes.

Cell culture

The immortal Abl/Arg-deficient fibroblast cell line Abl$^{-/-}$Arg$^{-/-}$ was generated from E9 mouse embryos by A. Koleske (Yale University, New Haven, CT). The Dok1$^{-/-}$ primary MEFs (Di Cristofano et al., 2001) and the Nck1$^{-/-}$ Nck2$^{-/-}$ MEFs (Gruenheid et al., 2001) were gifts from P.P. Pandolfi and T. Pawson, respectively. Cells were maintained in high glucose DME supplemented with 10% FBS (Gemini Bioproducts), L-glutamine, and antibiotics. Early passage MEFs were critical in obtaining the results reported here because MEFs were altered when grown in culture for longer than 4–8 wk. Latrunculin A (Molecular Probes) was added to serum-free media at a concentration of 1 μM for a 2-h pretreatment before cell detachment. Cells were held in suspension in the same media for an additional 40–60 min. The Abl inhibitor STI571 (Gleevec™; Novartis) was added to growth media at a concentration of 1–5 μM, and the Src inhibitor SU6656 (Sugen) was used at a concentration of 0.5–1 μM for 1–8 h. The latrunculin and kinase inhibitors were also present in the media throughout the suspension and reattachment period. Control cells were incubated with DMSO, the vehicle for inhibitors.

Biochemistry

Fibroblast experiments involving cell detachment, FN stimulation, and immunoprecipitation were performed as described previously (Woodring et al., 2001) using 1% Triton X-100 lysis buffer and brief sonication to prepare cellular lysates. GST-Abl-SH2 experiments were performed in the presence of 0.5 M NaCl to reduce nonspecific binding. Purified GST or GST-Abl-SH2 protein was cross-linked to glutathione-Sepharose using dimethyl-pimelimidate (~50 μg of protein coupled to 10 μl of beads per pull down). 200 μg of cell lysate protein were used for pull downs or immunoprecipitation, while 2–3 mg of protein were used for coimmunoprecipitation experiments.

PAK1 kinase assays were performed as described previously (Zhou et al., 2003). For in vitro Abl kinase assays, c-Abl was purified via FLAG antibody chromatography (Woodring et al., 2001) and used to phosphorylate GST-Dok1 protein in vitro. GST-Dok1 was isolated with glutathione-Sepharose before SDS-PAGE, and the ^{32}P-labeled Dok1 was processed for phosphotryptic analysis (Shah and Shokat, 2002).

For in vivo phosphotryptic peptide mapping, Dok1 was coexpressed with c-Abl in HEK293T cells. After transfection, cells were labeled with 1 mCi/ml ^{32}P-orthophosphate overnight, and then lysed in RIPA buffer. Dok1 was immunoprecipitated (αHA) and processed for phosphotryptic peptide mapping and phosphoamino acid analysis as described previously (Meisenhelder et al., 1999). Tryptic phosphopeptides were separated on thin layer cellulose (TLC) plates (EM Science) by electrophoresis (pH 1.9,

1.5 kV, 35 min) and ascending chromatography (phosphochromo buffer, 18 h). Synthetic peptides corresponding to the tryptic peptides containing mouse Dok1 pTyr361 were synthesized on an ABI 432A Synergy peptide synthesizer: (LTDSKEDPIpYDEPEGLAPAPPRGLY (peptide 1 + GLY) and EDPIpYDEPEGLAPAPPRGLY (peptide 2 + GLY). When digested with 1–10 μg TPCK trypsin these peptides yielded peptides 1 and 2 (underlined). Very little peptide 2 was generated from in vitro digestion of synthetic peptide 1. When mixed together and added to the ^{32}P-Dok1 phosphotryptic samples, the purified synthetic peptides were found to comigrate with the two pTyr361 spots in maps of Dok1 isolated from ^{32}P-labeled cells.

Immunofluorescence staining

Cells were prepared as indicated, fixed in suspension, or plated onto coverslips coated with 10 μg/ml purified human FN (Calbiochem) for 20–35 min at 37°C as described previously (Woodring et al., 2002). For Abl/Dok costaining experiments, αAbl (mouse mAb 8E9) was directly labeled with Alexa Fluor 546 according to kit instructions (Molecular Probes). Fluorescent reagents and antibodies are indicated in the figure legends. Widefield microscopy was performed using the 60× objective (Olympus). Applied Precision software (DeltaVision) was used to deconvolve z-section series of images. For quantification of filopodia and peripheral actin microspikes during cell spreading, we counted the number of filopodia and microspikes present on cells visualized with phalloidin, including all visible protruding microspikes >1 μm. Random fields of cells (n = 200–1,000) were selected on coverslips using Applied Precision software. Statistical analysis of the data was performed using Microsoft Excel software.

Online supplemental material

Three supplemental figures address the mechanism by which pY361-Dok1 induces filopodia during cell spreading. Data in Figs. S1 and S3 suggest that p120RasGAP, p190RhoGAP, and PAK1 do not lie downstream of pY361-Dok1, whereas data in Fig. S2 suggest that pY361-Dok1 may form a ternary complex with both c-Abl and Nck1 to promote filopodia in spreading MEFs. Fig. S1 shows that Dok1 did not coimmunoprecipitate with p120RasGAP in spreading MEFs (A) and that tyrosine phosphorylation of p190RhoGAP was similar among Abl$^{-/-}$Arg$^{-/-}$, Abl-, and KD-reconstituted MEFs spreading on FN (B). Fig. S2 shows that pY361-Dok1 coimmunoprecipitated with Nck1 and c-Abl when all three proteins were transiently expressed in 293T cells. Nck1 (A) and c-Abl (B) immunoprecipitates are shown. Fig. S3 shows PAK1 kinase activity was similar among WT, Abl$^{-/-}$Arg$^{-/-}$, and Abl-reconstituted MEFs spreading on FN. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200312171.DC1.

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