Mammalian Enabled (Mena) is a mammalian homologue of Drosophila Enabled (Ena), which genetically interacts with Drosophila Abl tyrosine kinase. The signaling pathway involving c-Abl and Mena (Ena) is not fully understood. To find molecules that participate in the c-Abl/Mena pathway, we searched for Mena-binding proteins using a yeast two-hybrid system. We identified Abl interactor 1 (Abi-1), which is known to interact with c-Abl, as a binding protein for Mena. Binding analysis revealed that the Ena/Vasp homology 1 domain of Mena and the polyproline structure of Abi-1 are necessary for the interaction. The interaction between Mena and Abi-1 was also observed in a mammalian expression system. Importantly, Abi-1 dramatically promoted c-Abl-mediated tyrosine phosphorylation of Mena but not other substrates such as c-Cbl. Mutational analysis demonstrated that the phosphorylation site of Mena is Tyr-296. Our results suggest that Abi-1 regulates c-Abl-mediated phosphorylation of Mena by interacting with both proteins.

Abl interactor 1 (Abi-1) was discovered as a c-Abl-binding protein that can inhibit v-Abl-mediated transformation of cultured cells (14). Abi-1 contains an Src homology 3 (SH3) domain and a polyproline structure. Both domains are involved in the interaction with c-Abl (14). Abl interactor proteins exist as multiple forms in mammalian cells. E3B1 (15) seems to be a splice variant of Abi-1, and Abi-2 (16) is highly related to Abi-1 but encoded by a different gene. It has been reported that E3B1 (Abi-1) is involved in Rac-dependent cytoskeletal reorganization in response to growth factor stimulation (17).

To understand the signal transduction pathway involving c-Abl, Drosophila genetics was applied to obtain genetically interacting molecules (18, 19). Abl mutant flies die at the end of pupation or as young adults (20). Drosophila enabled (ena) was identified as a dosage-sensitive suppressor of mutations in Drosophila Abl (19). Mammalian enabled (mena) has been identified, and its function has been extensively investigated (20–22). Mena contains an Ena/Vasp homology 1 (EVH1) domain, Arg/Leu/Glu-rich region, proline-rich region, and EVH2 domain (20). The EVH1 domain binds to proteins containing consensus sequence (D/E)PPPXX(D/E) (23). The EVH1 ligand motif is found in several proteins including a focal adhesion protein, Zyxin (24), and ActA (25). The proline-rich region of Mena is able to interact with profilin (20, 26), and the EVH2 domain has regions implicated in G- and F-actin binding. In cultured fibroblasts, Mena is localized to focal contacts and the leading edge. Mena is implicated in cell motility through regulation of the actin cytoskeleton (22).

Although the interactions of Mena with actin and actin-binding proteins have been extensively characterized, the signaling pathways involving Mena have not been fully understood. With the hope of finding proteins that regulate the function of Mena, we performed yeast two-hybrid screening. We identified Abi-1 as a Mena-binding protein. Phosphorylation of Mena by c-Abl was greatly increased in the presence of Abi-1 in cultured cells. Tyr-296 of Mena was identified as a c-Abl-binding site for Abi-1. Our results suggest the functional importance of the linkage of these three molecules in signal transduction.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning and Expression Analysis—cDNAs for Mena and Mena(Δ) were amplified by PCR using a mouse spleen cDNA library (Clontech) as a template. An N-terminal primer (5’-AGATCTACCATGAGTGACAGAGTATCTCTG-3’) and a reverse primer (5’-GGGTGGCAG-
GTGGTCCGAGA) were used to amplify the 5' halves. A forward primer (5'-TCTTTGACACCTGACCTC) and a C-terminal reverse primer (5'-AGATCTTTATGCGTGTGATGTGCTGCAG) were used for the 3' halves. The PCR products were subcloned into pCRBlunt (Invitrogen), and then their sequences were determined. Quantitative cDNAs for Mena and Mena(S) were prepared by PCR using the amplified 5' and 3' fragments as templates.

To examine the expression of mRNAs for Mena and Mena(S) by PCR, a forward primer (5'-TCTTTGACACCTGACCTC) and a reverse primer (5'-CCAGCAGGGCCTCACTTTCC) were used. These primers were designed so as to encompass the region missing in Mena(S), so that the PCR products derived from cDNAs for Mena and Mena(S) could be distinguished. cDNAs derived from mouse 17-day embryos and mouse brains lacking a null mutation in the c-abl gene were purchased from Clontech. cDNAs derived from mouse 17-day embryos and immortalized according to the 3T3 method (29). Deletion mutants of Abi-1 were produced by PCR and verified by DNA sequencing. To generate the Abi-1W426K mutant and mutants in the tyrosine residues of Mena, we replaced the tyrosine residues with phenylalanine by mutagenesis with a QuikChangeTM site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocols.

RESULTS

Cloning and Characterization of an Alternatively Spliced Variant of Mena—To obtain *mena* cDNA, we performed PCR using a mouse spleen cDNA library as a template. The 5' and 3' regions of *mena* cDNA were separately amplified. Amplification of the 3' region yielded two types of PCR products, whereas a single product was obtained for the 5' region (data not shown). Sequence analysis of the two PCR products for the 3' region revealed that one corresponds to reported mouse *mena* and the other to *mena* lacking aa 300–333 (Fig. 1A). Full-length cDNAs for *mena* and its short form, hereafter referred as *mena*(S), were prepared by PCR using the amplified 5' and 3' fragments.

To determine whether or not *mena*(S) mRNA is expressed, we performed PCR analysis using cDNAs from mouse 17-day embryos, mouse brains, and mouse spleens as templates. To distinguish the PCR products for *mena* and *mena*(S), primers were designed to encompass the region corresponding to aa 300–333 of Mena. The PCR products for *mena* (438 bp) and *mena*(S) (336 bp) were observed in all cDNAs examined. The expression of *mena*(S) was predominant in mouse spleen (Fig. 1B).

To examine the expression of Mena and Mena(S) at the protein level, we generated a rabbit polyclonal antibody against bacterially expressed Mena. Lysates of rat brains, two kinds of fibroblasts (Baf3 and LyD9), and 293T cells were transfected with 1 μg of each expression plasmid using LipofectAMINE PLUS reagent (Invitrogen). At 24 h after transfection, the cells were lysed in 300 μl of lysis buffer (25 mM HEPES-KOH (pH 7.2), 150 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.5 μM pepstatin A, 1% Trasylol (aprotinin solution; Bayer), and 1% Triton X-100). The lysate was centrifuged in a microcentrifuge for 10 min at 15,000 rpm to remove insoluble materials. The supernatant was incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 1.5 h with gentle rotation at 4 °C and then washed three times with the lysis buffer. The materials pulled down with the beads were dissociated from the beads by boiling in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The samples were resolved by SDS-PAGE and immunostained with appropriate antibodies using ECL (Amersham Biosciences).

Phosphorylation Analysis—293T cells were transfected with 1 μg each expression plasmid and then lysed in the lysis buffer containing phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM sodium fluoride). The total amounts of plasmas were balanced with pcDNA3 or pFLAG-CMV-2 empty vector. For detection of the phosphorylation of GST-Mena, the supernatant (50 μl) was incubated with glutathione-Sepharose 4B and then incubated at 4 °C for 1.5 h with gentle rotation. The beads were washed with the lysis buffer containing phosphatase inhibitors, and the materials pulled down with the beads were analyzed by Western blotting with anti-phosphotyrosine antibody. For detection of the phosphorylation of HA-c-Bl, anti-HA antibody was added to the supernatant (50 μl), followed by incubation for 3 h at 4 °C. Protein G beads were then added to the mixture, followed by incubation at 4 °C for 1.5 h with gentle rotation. The beads were washed and then analyzed as described above.

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described previously (30). Briefly, cells plated on fibronectin-coated coverslips were fixed with 4% paraformaldehyde, followed by sequential incubation with primary antibodies and fluorescein isothiocyanate-conjugated or Texas Red-conjugated secondary antibodies. Confocal microscopy was performed with an Olympus Fluoview 300 laser-scanning microscope.

RESULTS

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were analyzed by Western blotting with the anti-Mena antibody. In addition, lysates of 293T cells transfected with an expression plasmid encoding Mena or Mena(S) were also examined (Fig. 1C). In accordance with previous results (20), two protein bands corresponding to 140 and 88 kDa were detected for rat brain lysates. Given its expression only in brain, the former species most likely corresponds to a brain-specific form of Mena, Mena(+/H11001). On the other hand, the latter is probably Mena, because it was expressed in all types of cells examined and exhibited the same mobility on gels as exogenously expressed Mena in 293T cells. (Note that the 88-kDa band for the lysates of 293T cells transfected with the Mena expression plasmid was more conspicuous than that for the lysates of nontransfected cells.) In addition to the 88-kDa protein, an 80-kDa protein was detected in Baf3 and LyD9 lysates. Since the 80-kDa protein exhibited the same mobility on gels as exogenously expressed Mena(S), the 80-kDa protein is most likely Mena(S). These results suggest that Mena(S) is expressed in B-lymphoid cell lines, which is consistent with the fact that the \textit{mena(S)} mRNA is predominantly expressed in spleen.

**Identification of Novel Mena-binding Proteins Using a Yeast Two-hybrid System**—To identify molecules that participate in the c-Abl/Mena pathway in mammalian cells, we searched for Mena-binding proteins using a yeast two-hybrid system. We performed screening with Mena(S) as the bait. We expected that screening with Mena(S) would allow us to obtain new binding proteins, because Mena(S) lacks aa 300–333 of Mena, which contains the profilin-binding motif. We used a human leukocyte cDNA library because Mena(S) appeared to be expressed in lymphoid cell lines (Fig. 1, A and C). In total, 1.6 × 10^7 transformants were screened, and 43 positive clones were obtained. The results of sequence analysis of the positive clones are summarized in Table I. Thirty-six positive clones turned out to be zyxin, which has already been reported to bind to Mena (24). Four clones encoded a novel putative small G protein-interacting protein. Characterization of this protein will be described elsewhere. One clone encoded Abi-1, and two clones encoded its variant, E3B1. Abi-1 was originally identified as a c-Abl-binding protein (14). These results raised the possibility that Abi-1 interacts with Mena.

The EVH1 Domain of Mena Is Involved in the Interaction with Abi-1—We characterized the interaction between Mena and Abi-1 using the yeast two-hybrid system. First, we tested whether or not Mena (a nondeletion form) as well as Mena(S) binds to Abi-1. As shown in the upper panel of Fig. 2, both Mena and Mena(S) bound to Abi-1. To determine the region of Abi-1 that is involved in the binding, we constructed deletion mutants of Mena(S). We divided Mena into four regions, the EVH1 domain (aa 1–111), Arg/Leu/Glu-rich region (aa 112–238), proline-rich region (aa 239–361), and EVH2 domain (aa 362–541). The interactions of Abi-1 with the Mena fragments were investigated. As shown in Fig. 2 (middle panel), only the EVH1 domain bound to Abi-1. A mutant lacking the EVH1 domain did not bind to Abi-1.

Next, we defined the region of Abi-1 that is involved in the
binding to Mena. Abi-1 contains an SH3 domain and a polyproline structure near the C terminus. We constructed C-terminal deletion mutants of Abi-1. Abi-1 (aa 1–391) and (aa 1–363) were SH3 deletion mutants. Abi-1 (aa 1–331) was a mutant lacking both the SH3 domain and the polyproline structure. Abi-1/H9004336–361 was a mutant lacking the polyproline structure. The interactions of these mutants with Mena were examined. As shown in the lower panel of Fig. 2, the two SH3 domain deletion mutants, Abi-1 (aa 1–391) and (aa 1–363), still interacted with Mena. On the other hand, Abi-1 (aa 1–331) and Abi-1/H336–361 did not interact with Mena. We confirmed these results by quantitative $\beta$-galactosidase assays with o-nitrophenyl $\beta$-D-galactopyranoside as an indicator. The wild-type Abi-1 and SH3 deletion mutants exhibited similar levels of interaction with Mena (data not shown). These results suggested that the polyproline structure, but not the SH3 domain, of Abi-1 is critical for the interaction with Mena.

### Table I

| Number | Name                        |
|--------|-----------------------------|
| 36     | Zyxin                       |
| 4      | A putative small G-protein-binding protein |
| 2      | E3B1                        |
| 1      | Abi-1                       |

### Fig. 2. Two-hybrid analysis of the interaction between Mena and Abi-1

Deletion mutants of Mena, Mena(S), and Abi-1 are schematically represented. Mena or one of its deletion mutants was cloned into a GAL4 DNA-binding domain vector, and Abi-1 or one of its deletion mutants was cloned into a GAL4 activation domain vector. Reporter yeast cells were transformed with the two kinds of vectors. $\beta$-Galactosidase activity was measured on a filter. +, colonies turned blue within 1 h; −, colonies remained white after 24 h.

### Binding of Mena to Abi-1 in Mammalian Cells

The interaction between Mena and Abi-1 was confirmed using a mammalian cell system. GST or GST-Abi-1 was coexpressed with FLAG-Mena in 293T cells. Lysates of the transfected cells were incubated with glutathione beads, and then the bound proteins were detected by immunoblotting with anti-GST antibody (Pull-down). For estimation of the amounts of expressed proteins, 2% of each of the lysates was separated and immunoblotted with anti-FLAG or anti-GST (Cell lysates). The positions of FLAG-Mena, GST, and GST-Abi-1 are indicated. B, GST (lane 1) or GST-Mena (lane 2) was coexpressed with FLAG-Abi-1 in 293T cells. Lysates of the transfected cells were analyzed as described for A. The positions of FLAG-Abi-1, GST, and GST-Mena are indicated. C, GST (lane 1) or GST-Abi-1 (lane 2) was expressed in 293T cells. Lysates of the transfected cells were incubated with glutathione beads, and then the precipitated endogenous proteins were detected by immunoblotting with anti-Mena, anti-Abi, anti-Cbl, and anti-Src antibodies (Pull-down). For comparison, 2% of each lysate was separated and immunoblotted with the same antibodies (Cell lysates). For estimation of the amounts of GST proteins, 2% of each lysate was separated and immunoblotted with anti-GST antibody. The positions of GST and GST-Abi-1 are indicated.

### Binding of Mena to Abi-1 in Mammalian Cells—The interaction between Mena and Abi-1 was confirmed using a mammalian cell system. GST or GST-Abi-1 was coexpressed with FLAG-tagged Mena in 293T cells. GST or GST-Abi-1 was pulled-down with glutathione beads, and then the coprecipitated FLAG-tagged Mena was detected. As shown in Fig. 3A, FLAG-tagged Mena was coprecipitated with GST-Abi-1 but not with GST. Next, GST-Mena and FLAG-tagged Abi-1 were coexpressed in 293T cells, and lysates were subjected to coprecipitation analysis. As shown in Fig. 3B, FLAG-tagged Abi-1 was coprecipitated with GST-Mena. These results showed that exogenously expressed Mena and Abi-1 interact with each other in 293T cells.

To determine whether GST-Abi-1 interacts with endogenous proteins, GST-Abi-1 alone was expressed in 293T cells, and
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Fig. 4. Abi-1 promotes the phosphorylation of Mena by c-Abl kinase. 293T cells were transfected with the expression plasmids indicated at the top. WT, wild-type c-Abl; KD, K290M c-Abl mutant. GST-Mena (A) and HA-c-Cbl (B) were precipitated as described under “Experimental Procedures.” The precipitated proteins were analyzed by immunoblotting with anti-phosphotyrosine (α-pY), anti-GST (α-GST), or anti-HA (α-HA) antibody.

Fig. 5. The SH3 domain and polyproline structure of Abi-1 contribute to promotion of the phosphorylation of Mena. A, 293T cells were cotransfected with expression plasmids for GST-Mena and c-Abl (lanes 1–6) with an expression plasmid for FLAG-Abi-1 (full-length) (lane 1), FLAG-Abi-1 (aa 1–381) (ΔSH3) (lane 2), FLAG-Abi-1 (aa 1–331) (ΔSH3, Δpolyproline) (lane 3), FLAG-Abi-1Δ336–361 (Δpolyproline) (lane 4), or Abi-1W426K (lane 5). GST-Mena was pulled down from the cell lysates as described in the legend to Fig. 3, and the precipitated proteins were analyzed by Western blotting with anti-phosphotyrosine (α-pY) and anti-GST (α-GST) antibodies (Pull-down). Ten percent of each cell lysate used for the pull-down assays was analyzed by Western blotting with anti-Abl (α-Abl), anti-GST (α-GST), and anti-FLAG (α-FLAG) antibodies (Cell lysates). B, quantitation of the Western blot with NIH Image.

then coprecipitation of endogenous proteins with GST-Abi-1 was examined. As shown in Fig. 3C, endogenous Mena and c-Abl were coprecipitated with GST-Abi-1. However, c-Cbl, which is known to be a substrate of c-Abl (10), was not coprecipitated. Another nonreceptor tyrosine kinase, c-Src, was not coprecipitated with GST-Abi-1, either. Overall, these results suggest a specific interaction between Mena and Abi-1 in mammalian cells.

Abi-1 Promotes c-Abl-mediated Phosphorylation of Mena—Ena is reported to be a substrate of Drosophila Abi (19). The finding that Mena interacts directly with Abi-1 prompted us to examine whether or not Abi-1 affects the phosphorylation of Mena by c-Abl (Fig. 4A). 293T cells were cotransfected with expression plasmids for c-Abl, FLAG-tagged Abi-1, and GST-Mena. GST-Mena was pulled down from the cell lysates, and its phosphorylation state was investigated by Western blotting with anti-phosphotyrosine antibody. Phosphorylation of GST-Mena was barely detectable when GST-Mena was coexpressed with c-Abl alone (Fig. 4A, lane 3). A dramatic increase in the phosphorylation of GST-Mena was observed when GST-Mena was coexpressed with FLAG-tagged Abi-1 in addition to c-Abl (Fig. 4A, lane 4). A kinase-deficient mutant of c-Abl, K290M, did not phosphorylate GST-Mena even in the presence of FLAG-tagged Abi-1 (Fig. 4A, lane 5), indicating that the kinase activity of c-Abl is required for the phosphorylation of Mena. Reprobing of the filter used for the detection of the phosphorylation state of GST-Mena with an anti-GST antibody showed that comparable levels of GST-Mena existed in all samples (Fig. 4A, bottom panel). These results demonstrate that FLAG-tagged Abi-1 promoted c-Abl-mediated phosphorylation of GST-Mena.

To assess the specificity of this effect, we used c-Cbl as a substrate for c-Abl (10). Expression plasmids encoding c-Abl, FLAG-tagged Abi-1, and HA-tagged c-Cbl were cotransfected into 293T cells. HA-tagged c-Cbl was immunoprecipitated from the cell lysates with anti-HA antibody, and then its phosphorylation level was examined. The level of phosphorylation of HA-tagged c-Cbl by c-Abl did not significantly change, regardless of the presence or absence of FLAG-tagged Abi-1 (Fig. 4B, lanes 3 and 4). We examined the phosphorylation of p130Cas using the same system. Phosphorylation of p130Cas by c-Abl was not promoted in the presence of FLAG-tagged Abi-1 (data not shown). These results support the idea that Abi-1 specifically promotes the phosphorylation of Mena by c-Abl kinase.

Effects of Abi-1 Mutants on Phosphorylation of Mena—Next, we attempted to determine the Abi-1 domain involved in the promotion of phosphorylation of Mena by c-Abl kinase. Phosphorylation analysis was performed with the deletion mutants of Abi-1 used for the experiments shown in Fig. 2. In addition, we constructed Abi-1W426K, in which a conserved tryptophan residue in the SH3 domain was replaced by a lysine. Each of the expression plasmids encoding Abi-1 mutants was cotransfected with expression plasmids encoding c-Abl and GST-Mena into 293T cells. GST-Mena was precipitated and immunoblotted with the anti-phosphotyrosine antibody (Fig. 5A). The bands on the blot were quantified with NIH Image (Fig. 5B). The level of phosphorylation of GST-Mena in the lysates of cells cotransfected with Abi-1 (aa 1–391) (ΔSH3) was lower than that with the full-length Abi-1 (Fig. 5B, lane 2). A similar low phosphorylation level was observed when Abi-1Δ336–361 Δpolyproline and Abi-1W426K mutants were expressed (Fig. 5B, lanes 4 and 5). On the other hand, Abi-1 (aa 1–331) (ΔSH3,
Δpolyproline) did not promote the phosphorylation of GST-Mena by c-Abl (Fig. 5C, lane 6). These results suggested that both the SH3 domain and the polyproline structure of Abi-1 contribute to the promotion of phosphorylation of Mena by c-Abl kinase.

Tyr-296 of Mena Is the Sole Phosphorylation Site—Mena contains six tyrosine residues (aa 16, 38, 70, 87, 296, and 505). To determine which tyrosine residue in Mena is phosphorylated by c-Abl kinase, we replaced each one by phenylalanine. Plasmids encoding c-Abl, FLAG-tagged Abi-1, and GST-Mena mutants were cotransfected into 293T cells. GST-Mena mutants were precipitated, and their phosphorylation levels were measured by Western blotting with anti-phosphotyrosine (α-pY) and anti-GST (α-GST) antibodies (Pull-down). Ten percent of each cell lysate was subjected to Western blotting with anti-Abi (α-Abi), anti-GST (α-GST), and anti-FLAG (α-FLAG) antibodies (Cell lysates). The positions of molecular size standards are indicated on the left. B, 293T cells were cotransfected with the expression plasmids for c-Abl, FLAG-Abi-1, and GST-Mena (WT) or one of its mutants, as indicated at the top. Samples were analyzed as described for A. C, summary of the experiments with Mena mutants.

Abi-1 Is Colocalized with Mena at the Leading Edge—Mena is localized to focal adhesions and the leading edge in fibroblasts (20). Although Abi-2 has been reported to be localized to lamellipodia in murine B16F1 melanoma cells (31), its localization has not been precisely compared with that of Mena. We raised a monoclonal antibody against Abi-1, and then the localization of Abi-1 and Mena was compared by double immunostaining. As shown in Fig. 7A, the monoclonal antibody that we raised detected endogenous Abi-1 in lysates of mouse fibroblasts (Abl/H11001/H11001 and Abl/H11002/H11002) and 293T cells as well as ectopically overexpressed FLAG-tagged Abi-1. The two bands observed for the lysates of fibroblasts (Abl/H11001/H11001) may reflect the phosphorylation of Abi-1. Abi-1 is known to be phosphorylated by Abl kinase (32). These bands were occasionally observed as one fuzzy band (data not shown).

The distribution of Mena and Abi-1 in mouse embryo fibroblasts (Abi+/+) was analyzed by immunofluorescence microscopy (Fig. 7B, upper panel). Mena is localized at focal contacts and the leading edge, whereas Abi-1 is localized at the leading edge but not focal contacts. A merged image showed the colocalization of the two proteins at the leading edge. Next, we examined whether or not their localization was affected by depletion of c-Abl. We examined the localization of Abi-1 and Mena in c-Abl-deficient fibroblasts. As shown in the lower panel of Fig. 7B, no significant difference was observed in the
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staining patterns of the two proteins. This indicates that the presence of c-Abl does not affect the localization of either protein. However, the colocalization of Mena and Abi-1 at the leading edge in the fibroblasts is consistent with the present finding that Abi-1 interacts with Mena and may suggest functional collaboration between these molecules for the regulation of the cytoskeleton.

DISCUSSION

Abi-1 was first discovered as an Abi-interacting protein that can suppress v-Abl-mediated transformation of cultured cells (14). In the present study, we have demonstrated that Mena also interacts with Abi-1. The EVH1 domain of Mena and the polyproline structure of Abi-1 are involved in the protein-protein interaction. Our results suggest a biochemical linkage between c-Abl, Abi-1, and Mena. Indeed, the phosphorylation of Mena by c-Abl was dramatically stimulated by Abi-1. The fact that the phosphorylation of c-Cbl and p130Cas by c-Abl is not stimulated by Abi-1 unequivocally rules out the possibility that Abi-1 merely activates c-Abl kinase activity for all substrates. The present results are not only in accord with the previous finding that Drosophila Abi and Abi-2 enhance the phosphorylation of Drosophila Enabled by Abi (33) but also provide a molecular basis for the finding.

The interaction between c-Abl, Abi-1, and Mena would predict the presence of a ternary complex of the three proteins. We sought to detect one using a yeast three-hybrid system. However, no such complex was obtained (data not shown). One plausible explanation for why a stable ternary complex was not observed is that the binding site for c-Abl on Abi-1 is partially occupied by Mena. Our binding studies revealed that the polyproline structure of Abi-1 is critical for the binding to Mena. On the other hand, the SH3 domain of Abi-1 is essential for the binding to c-Abl, and the polyproline structure facilitates the binding (14). Thus, c-Abl may bind to Abi-1 only through the interaction with the SH3 domain when the polyproline structure is occupied by Mena. These interactions may not be enough for the formation of a stable ternary complex but are probably sufficient for the promotion of the phosphorylation of Mena by c-Abl.

It is interesting that the phosphorylation of Mena by c-Abl is promoted to some extent in the presence of Abi-1 mutants lacking either the SH3 domain or the polyproline structure. One explanation for this observation is that Abi-1 may induce conformational changes in c-Abl and/or Mena upon binding to these proteins. The binding of Abi-1 to c-Abl may endow c-Abl with high affinity for the substrate Mena but not other substrates. The binding of Abi-1 to Mena may also affect the conformation of the phosphorylation site of Mena. In this sense, Abi-1 is not just a scaffold for Mena and c-Abl but a regulator that mediates the presentation of Mena to c-Abl by binding to both a substrate and kinase.

It is reasonable to assume that the phosphorylation of Tyr-296 regulates the function of Mena. Given that Tyr-296 is located in the middle of the proline-rich region, the phosphorylation may affect the interaction of Mena with proteins that bind to this region, such as profilin. Phosphorylation of Tyr-296 may also cause a gross structural change in Mena, which affects the functions of domains (EVH1 and EVH2) distant from the phosphorylation site.

The biological significance of Mena(S) is not clear at present. Mena(S) lacks the profilin-binding motif that is located in the proline-rich region of Mena. Mena(S) is expressed in B-lymphocytes predominantly, suggesting that its function may be limited to specific tissues. It has been reported that the proline-rich region of Mena is dispensable for random cell motility but required for intracellular Listeria movement (34). Mena(S) may regulate reorganization of the actin cytoskeleton in certain circumstances. Alternatively, since Mena(S) is probably not able to bind to profilin, it may block Mena/profilin-mediated pathways by competing with the interaction of Mena with Mena-binding proteins. It is noteworthy that the region deficient in Mena(S) is adjacent to the phosphorylation site of Mena. Phosphorylation may have different effects on Mena and Mena(S).

In summary, we have shown that Abi-1 binds to Mena and c-Abl and specifically promotes the phosphorylation of Mena by c-Abl. Our results point to the biochemical significance of the interaction of the three molecules, c-Abl, Abi-1, and Mena, reflecting a signaling pathway possibly downstream of c-Abl kinase.

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