NESH (Abi-3) is present in the Abi/WAVE complex but does not promote c-Abl-mediated phosphorylation

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Abstract Abl interactor (Abi) was identified as an Abl tyrosine kinase-binding protein and subsequently shown to be a component of the macromolecular Abi/WAVE complex, which is a key regulator of Rac-dependent actin polymerization. Previous studies showed that Abi-1 promotes c-Abl-mediated phosphorylation of Mammalian Enabled (Mena) and WAVE2. In addition to Abi-1, mammals possess Abi-2 and NESH (Abi-3). In this study, we compared the three Abi proteins in terms of the promotion of c-Abl-mediated phosphorylation and the formation of Abi/WAVE complex. Although Abi-2, like Abi-1, promoted the c-Abl-mediated phosphorylation of Mena and WAVE2, NESH (Abi-3) had no such effect. This difference was likely due to their binding abilities as to c-Abl. Immunoprecipitation revealed that NESH (Abi-3) is present in the Abi/WAVE complex. Our results suggest that NESH (Abi-3), like Abi-1 and Abi-2, is a component of the Abi/WAVE complex, but likely plays a different role in the regulation of c-Abl.

Keywords: Abi interactor; c-Abl; Adaptor; NESH; WAVE

1. Introduction

Abl interactor (Abi)-1 and Abi-2 were first identified on yeast two-hybrid screening for proteins that interact with c-Abl tyrosine kinase [1,2], which is the proto-oncogene product of the Abelson murine leukemia virus oncogene v-abl [3]. We previously showed that Abi-1, by linking enzyme and substrate, promotes the c-Abl-mediated phosphorylation of Mammalian Enabled (Mena) and WAVE2, NESH (Abi-3) had no such effect. This difference was likely due to their binding abilities as to c-Abl. Immunoprecipitation revealed that NESH (Abi-3) is present in the Abi/WAVE complex. Our results suggest that NESH (Abi-3), like Abi-1 and Abi-2, is a component of the Abi/WAVE complex, but likely plays a different role in the regulation of c-Abl.

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2. Materials and methods

2.1. Plasmids
cDNAs for human NESH, human Abi-1, and mouse Mena were prepared previously [4,16]. cDNAs for mouse NESH (AK008928) and mouse WAVE2 (AK085488) were obtained from RIKEN. A cDNA for human Abi-2 was amplified by PCR using a human leukocyte cDNA library (CLONTECH). The obtained cDNA was found to be identical to that for abl interactor 2 (accession number BC001439). pcDNA3 c-Abl was a kind gift from Dr. D. Baltimore. Mammalian expression plasmids, pEBG (a gift from Dr. B. Mayer) and pFLAG-CMV-6 (Sigma), were used to express proteins fused with an N-terminal GST and FLAG tag, respectively.

Abbreviations: SH3, Src homology 3; GST, glutathione S-transferase; HUVEC, human umbilical vein endothelial cells

[10], were shown to undergo c-Abl-mediated phosphorylation in an Abi-dependent manner. These results suggest that Abi-1 acts as an adaptor that presents substrates for c-Abl.

Studies from several groups revealed that Abi-1 and Abi-2 are involved in the regulation of actin cytoskeleton. They are present in a macromolecular complex (Abi/WAVE complex), which regulates Arp2/3-mediated actin filament nucleation and actin network assembly, in response to the Rac GTPase [11–14]. This complex includes three other proteins, namely, Nap-1, HSPC300, and PIR121/Sra-1, in addition to Abi and Wave. It has been suggested that Abi-1 directly interacts with WAVE2 in this complex [15].

In addition to Abi-1 and Abi-2, mammals possess a third family member, ABI gene family member 3 (NESH). ABI gene family member 3 (NESH) was originally identified as a new human gene that possesses a Src homology 3 (SH3) domain [16], and later added to the Abi family because of its amino acid sequence similarity with Abi-1 and Abi-2 [17] (henceforth referred to as NESH). NESH possesses essentially the same domain structure as those of Abi-1 and Abi-2. Ichigotani et al. [18] reported that overexpression of NESH in a metastatic cell line suppresses cell motility and metastasis ability. This suggests that NESH is also involved in the regulation of the cellular cytoskeleton. However, the mechanism by which NESH controls the cytoskeleton is not fully understood.

In this study, we compared the three mammalian Abi family proteins in terms of the regulation of c-Abl kinase and the formation of the Abi/WAVE complex. We found that Abi-1 and Abi-2 promote the c-Abl-mediated phosphorylation of Mena and WAVE2, but that NESH does not. Despite this difference, NESH as well as Abi-1 and Abi-2 participate in the formation of the Abi/WAVE complex.
2.2. Cells

293T and COS7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex and cultured in EGM-2 bulletkit medium (Cambrex).

2.3. Antibodies

The following antibodies were used; anti-Abl (8E9; Pharmingen), anti-FLAG (M2; Sigma), anti-glutathione S-transferase (GST) (Santa Cruz Biotechnology), anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-Abi-1 (1G9; MBL Inc.), anti-WAVE2 (C-14; Santa Cruz Biotechnology), anti-PIR121/Sra-1 (Upstate Biotechnology), and anti-Abi-2 (H-50; Santa Cruz Biotechnology). A rabbit anti-WAVE2 antibody was a kind gift of Drs. T. Takenawa and D. Yamazaki.

Three types of polyclonal antibodies against NESH were produced in this laboratory. The cDNA fragment encoding the C-terminal region (amino acid 201–364) of human NESH was inserted into an expression vector, pGEX-4T-1 (Amersham Pharmacia Biotech). The GST-NESH fusion protein was expressed in *Escherichia coli* cells and purified according to the standard method. Rabbits and mice were immunized with the purified protein. The sera from immunized rabbits were purified using antigen-coupled beads, and used for Western blotting, immunoprecipitation, and immunofluorescence microscopy. The sera obtained from immunized mice were used without purification. A polyclonal antibody against mouse NESH was produced by injecting the C-terminal fragment (amino acid 204–367) of mouse NESH into rabbits and purified as described above. The latter two types of antibodies were used only for Western blotting.

2.4. In vitro binding assay

Transfection and GST pull-down analysis were performed as described previously [4].

2.5. Phosphorylation analysis

293T or COS7 cells were transfected with 1 μg of each expression plasmid using LipofectAMINE PLUS reagent (Gibco BRL) according to the manufacturer’s instructions and then lysed in lysis buffer (25 mM HEPES-KOH (pH 7.2), 150 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.5 μg/ml leupeptin, 2 μM pepstatin A, 1% Transthy (aprotinin solution; Bayer), 1% Triton X-100, 1 mM sodium orthovanadate, and 10 mM sodium fluoride). The pFLAG-CMV-2 empty vector was used to adjust the total amounts of plasmids. The detection of the phosphorylated proteins was performed as described previously [4].

2.6. Immunoprecipitation analysis

Purified rabbit anti-human NESH antibodies (4.3 μg) was added to each HUVEC lysate (500 μg), followed by incubation at 4 °C overnight. Protein G beads were then added to the mixture, followed by incubation at 4 °C for 2 h with gentle rotation. The precipitated proteins were analyzed by Western blotting.

2.7. Immunofluorescence microscopy

Immunofluorescence microscopy was performed essentially as described previously [4].

3. Results

3.1. The effects of Abi family proteins on c-Abl-mediated phosphorylation

We first compared the effects of three Abi family proteins, Abi-1, Abi-2, and NESH, on the c-Abl-mediated phosphorylation of Mena. 293T cells were cotransfected with expression

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**Fig. 1.** Abi-1 and Abi-2, but not NESH, promote c-Abl-mediated phosphorylation. (A) 293T cells were transfected with expression plasmids as indicated at the top. To adjust the total amount of plasmids, the pFLAG-CMV-2 empty vector was used (lane 1). Cell lysates were analyzed by Western blotting (WB) with antibodies against phosphotyrosine (z-pY), GST, c-Abl, and FLAG. (B) GST-Mena was pulled down with glutathione beads, and the bound proteins were analyzed by Western blotting with antibodies against phosphotyrosine and GST. (C) COS7 cells were transfected with expression plasmids as indicated at the top. To adjust the total amount of plasmids, the pFLAG-CMV-2 empty vector was used (lane 1). Phosphorylation of GST-WAVE2 was analyzed as described in (B).
plasmids for c-Abl, GST-Mena, and one of the FLAG-tagged Abi family proteins, and phosphorylated proteins were detected by Western blotting. As described previously [4], while expression of c-Abl alone resulted in a limited level of phosphorylation of GST-Mena (Fig. 1A, lane 1), coexpression of FLAG-tagged Abi-1 with c-Abl increased the phosphorylation of GST-Mena (lane 2). When FLAG-tagged Abi-2 was coexpressed with c-Abl, a similar level of phosphorylation of GST-Mena was observed (lane 3), suggesting that Abi-2 is also able to promote the c-Abl-mediated phosphorylation of GST-Mena. In contrast, the phosphorylation of GST-Mena was not increased in the presence of FLAG-tagged NESH (lane 4), although its expression level was higher than those of FLAG-tagged Abi-1 and Abi-2. We confirmed that the phosphorylation of GST-Mena was not increased when FLAG-tagged NESH was expressed at levels similar to those of FLAG-tagged Abi-1 and Abi-2 (data not shown). Double or multiple bands of the expressed Abi family proteins on the blot likely reflect the phosphorylation of proteins. Endogenous Abi family proteins have also been detected as multiple bands [4,18,20]. To confirm that the phosphorylated protein is GST-Mena, GST-Mena was pulled down from the cell lysate and subjected to phosphorylation analysis. Fig. 1B unequivocally demonstrated that FLAG-tagged Abi-1 and Abi-2, but not NESH, promote the phosphorylation of GST-Mena.

Recently, Leng et al. [8] reported that Abi-1 stimulates the c-Abl-mediated phosphorylation of WAVE2. We therefore examined the effects of Abi family proteins on the c-Abl-mediated phosphorylation of WAVE2. Expression plasmids for c-Abl, GST-WAVE2, and one of the FLAG-tagged Abi family proteins were cotransfected into COS7 cells. GST-WAVE2 was pulled down and its phosphorylation state was examined. Consistent with a previous finding [8], coexpression of Abi-1 with c-Abl strongly enhanced the phosphorylation of GST-WAVE2 (Fig. 1C, lane 2). Although coexpression of FLAG-tagged Abi-2 stimulated the phosphorylation of GST-WAVE2

Fig. 2. Three Abi family proteins interact with Mena and WAVE2. GST (lane 1), GST-Abi-1 (lane 2), GST-Abi-2 (lane 3), or GST-NESH (lane 4) was coexpressed with FLAG-Mena (A) or FLAG-WAVE2 (B) in 293T cells. GST-Abi constructs were pulled down with glutathione beads, and then the bound proteins were detected by Western blotting with an anti-FLAG antibody (pull-down). To estimate the amounts of expressed proteins, 2% of each lysate was analyzed by Western blotting with antibodies against FLAG and GST (cell lysates).

Fig. 3. Interaction between c-Abl and Abi family proteins. FLAG-tagged Abi-1, Abi-2, or NESH was coexpressed with c-Abl in 293T cells. Cell lysates were immunoprecipitated (IP) with mouse IgG (A), an anti-c-Abl antibody (A), or an anti-FLAG antibody (B), and the precipitated proteins were analyzed by Western blotting (WB) with antibodies against c-Abl and FLAG. To estimate the amounts of expressed proteins, 2% of each lysate was analyzed (input).
coexpression of FLAG-tagged NESH had no effect (lane 4). These results clearly showed that NESH differs from Abi-1 and Abi-2 in the ability to promote the c-Abl-mediated phosphorylation of Mena and WAVE2.

3.2. Interaction of Abi family proteins with Mena, WAVE2, and c-Abl

Our previous study suggested that the interaction of Abi-1 with both c-Abl and Mena is important for the promotion of c-Abl-mediated phosphorylation of Mena. To determine why NESH does not promote the phosphorylation of Mena and WAVE2, the interactions of the three Abi proteins with Mena, WAVE2, and c-Abl were analyzed. GST, GST-Abi-1, GST-Abi-2, or GST-NESH was coexpressed with FLAG-tagged Mena in 293T cells, and the GST constructs were pulled down with glutathione beads. As shown in Fig. 2A, FLAG-tagged Mena was coprecipitated with all GST-Abi constructs, but not with GST. Interestingly, a much larger amount of FLAG-tagged Mena was coprecipitated with GST-Abi-1 or GST-NESH compared with GST-Abi-1 or GST-Abi-2. Similarly, the interactions between Abi family proteins and WAVE2 were examined. As shown in Fig. 2B, FLAG-tagged WAVE2 were pulled down with GST-Abi constructs. These results suggest that all Abi family proteins interact with both Mena and WAVE2.

Next, we examined the interactions between Abi family proteins and c-Abl (Fig. 3). 293T cells were cotransfected with expression plasmids for c-Abl and one of the FLAG-tagged Abi family proteins. The cell lysates were subjected to immunoprecipitation using either anti-c-Abl (Fig. 3A) or anti-FLAG (Fig. 3B). As shown in Fig. 3A, FLAG-tagged Abi-1 and Abi-2, but not NESH, were coprecipitated with c-Abl. Reciprocally, c-Abl was coprecipitated with FLAG-tagged Abi-1 and Abi-2, but not with NESH (Fig. 3B). These results suggest that NESH does not interact with c-Abl or only interacts with it at a level below the sensitivity of this assay.

3.3. NESH is present in Abi/WAVE complexes in HUVEC

Since the interaction of NESH with Mena and WAVE2 was observed in a mammalian overexpression system, we examined whether endogenous proteins are also associated with each
other in cells. As shown in Fig. 4A, a rabbit anti-human NESH antibody only reacted with FLAG-tagged NESH (middle panel) on a blot containing comparable amounts of FLAG-tagged Abi family proteins (left panel). This antibody detected endogenous NESH on immunoblots of lysates of HUVEC (right panel), which were reported to express NESH at a high level [18]. Using this and other antibodies, we investigated the expression of Abi family proteins in human cell lines (HUVEC, HeLa, and 293T) (Fig. 4B). Abi-1 and Abi-2 were detected on immunoblots of lysates of 293T and HeLa cells. The levels of expression of Abi-1 and Abi-2 in HUVEC were much lower than those in 293T or HeLa cells. On the contrary, NESH was abundantly expressed in HUVEC, but not in 293T or HeLa cells. As the expression of Abi family proteins appeared to be differentially regulated, we determined the tissue distribution of NESH in rat. As shown in Fig. 4C, NESH was expressed in several rat tissues, being especially abundant in spleen.

Next, we examined whether or not NESH is associated with WAVE2 in HUVEC (Fig. 4D). Immunoprecipitation results demonstrated that endogenous WAVE2 was coprecipitated with a rabbit anti-human NESH antibody. It is noteworthy that PIR121/Sra-1, a component of the Abi/WAVE complex that does not directly interact with Abi-1 [15], was coprecipitated with NESH. These results suggest that NESH is present in Abi/WAVE complexes containing PIR121/Sra-1. Although NESH interacted with Mena in an overexpression system, Mena was not coprecipitated with the rabbit anti-human NESH antibody under the same condition (data not shown).

Given that NESH is a component of the Abi/WAVE complex, we investigated the subcellular localization of NESH in HUVEC. HUVEC were plated on fibronectin-coated

![Fig. 5. NESH as well as Abi-1 is localized to the edge of lamellipodial protrusions. HUVEC plated on fibronectin-coated coverslips were fixed and double-stained with antibodies against WAVE2 (A and J) and Abi-1 (B) or c-Abl (K), or human NESH (rabbit antibody) (D) or Abi-2 (G) and Abi-1 (E and H). Merged images are shown on the right (C, F, I, and L). In (E), double asterisk (**) and single asterisk (*) indicate Abi-1-positive lamellipodial protrusions, where NESH is colocalized and not colocalized, respectively.](image-url)
coverslips, and the distribution of the three Abi family proteins, WAVE2, and c-Abl was analyzed by immunofluorescence microscopy. As reported previously [8,13,14,21], Abi-1 was found to be colocated with WAVE2 at the edge of lamellipodial protrusions (Fig. 5A–C). Because we do not have mouse antibodies against WAVE2 and NESH for immunofluorescence analysis, we could not observe the colocalization of NESH and WAVE2 in double-stained cells. Instead, we compared the localization of NESH with that of Abi-1. As shown in Fig. 5D–F, NESH staining overlapped with that of Abi-1 at the edge of lamellipodial protrusions. However, it is of note that NESH was not always observed at the edge of lamellipodial protrusions positive for Abi-1. NESH was detected at about half of the Abi-1-positive protrusions. In contrast to previous studies using B16F1 melanoma cells [8,21], Abi-2 (G–I) and c-Abl (J–L) were seen predominantly in the cytosol but not significantly at the edge of lamellipodial protrusions. Localization of Abi family proteins and c-Abl may slightly differ according to cell types and/or may be regulated by signals such as growth factors.

4. Discussion

In this study, we showed that Abi-1 and Abi-2, but not NESH, stimulate the c-Abl-mediated phosphorylation of MenA and WAVE2. Although the three family members bind to MenA and WAVE2, Abi-1 and Abi-2, but not NESH, interact with c-Abl in an overexpression system. Thus, it is most likely that the inability of NESH to promote the c-Abl-mediated phosphorylation is due to a lack of or reduced binding affinity as to c-Abl. These results are surprising, because NESH possesses the proline-rich region and the SH3 domain, both of which have been proposed to be involved in the interaction of Abi-1 with c-Abl [2]. Despite the lack of the ability to promote the c-Abl-mediated phosphorylation, NESH, like Abi-1 and Abi-2, is associated with WAVE2. As Sra-1/PIR121, another Abi/WAVE complex component that appears not to directly interact with the Abi component, was coprecipitated with anti-NESH, NESH likely forms a complex similar to Abi-1 and Abi-2. In line with this, NESH, like Abi-1, was found to be localized to the leading edge of lamellipodial protrusions where WAVE2 was localized.

At present, the physiological significance of the differences among Abi family proteins is not clear. Leng et al. [8] reported that the c-Abl-mediated phosphorylation of WAVE2 is stimulated by Abi-1 and that a mutation of Tyr-150, the major Abl-mediated phosphorylation site of WAVE2, impairs the induction of WAVE2-driven actin polymerization. Their results suggest that Abi-1 is a key molecule that enables WAVE2 to serve as a link between activated Rac and cortical actin. Our study shows that NESH, like Abi-1 and Abi-2, interacts with WAVE2, whereas it does not promote c-Abl mediated phosphorylation. It is possible that the WAVE complex containing NESH differs from that containing Abi-1 or Abi-2 in the regulation by c-Abl.

In summary, we have shown differences between Abi family proteins, Abi-1, Abi-2, and NESH, in the regulation of c-Abl kinase. Our results suggest that individual members play different roles in the signaling pathway involving c-Abl kinase.

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