Nexilin: A Novel Actin Filament-binding Protein Localized at Cell–Matrix Adherens Junction

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Abstract. We isolated two novel actin filament (F-actin)–binding proteins from rat brain and rat 3Y1 fibroblast. They were splicing variants, and we named brain big one b-nexilin and fibroblast small one s-nexilin. b-Nexilin purified from rat brain was a protein of 656 amino acids (aa) with a calculated molecular weight of 78,392, whereas s-nexilin, encoded by the cDNA isolated from rat 3Y1 cells by the reverse transcriptase-PCR method, was a protein of 606 aa with a calculated molecular weight of 71,942. b-Nexilin had two F-actin–binding domains (ABDs) at the NH₂-terminal and middle regions, whereas s-nexilin had one ABD at the middle region because 64 aa residues were deleted and 14 aa residues were inserted in the first NH₂-terminal ABD of b-nexilin, and thereby the first ABD lost its activity. b- and s-nexilins bound along the sides of F-actin, but only b-nexilin showed F-actin cross-linking activity. b-Nexilin was mainly expressed in brain and testis, whereas s-nexilin was mainly expressed in testis, spleen, and fibroblasts, such as rat 3Y1 and mouse Swiss 3T3 cells, but neither b- nor s-nexilin was detected in liver, kidney, or cultured epithelial cells. An immunofluorescence microscopic study revealed that s-nexilin was colocalized with vinculin, talin, and paxillin at cell–matrix adherens junction (AJ) and focal contacts, but not at cell–cell AJ, in 3Y1 cells. Overexpressed b- and s-nexilins were localized at focal contacts but not at cell–cell AJ. These results indicate that nexilin is a novel F-actin–binding protein localized at cell–matrix AJ.

Key words: actin-binding protein • focal contact • stress fiber • adherens junction • integrin

The actin filament (F-actin)–associated adherens junctions (AJs) are subclassed into two types: cell–matrix and cell–cell AJs (Geiger et al., 1985). Cell–matrix AJ includes focal contacts, also referred to as focal adhesions or adhesion plaques, which are the specialized structures at the ends of stress fibers, up to 10 μm in length and 0.5 μm in width (Abercrombie and Dunn, 1975; Izzard and Lochner, 1976; Heath and Dunn, 1978). Originally, focal contacts were described in living or fixed fibroblasts cultured on glass or plastic (Abercrombie et al., 1971). Typical focal contacts are mainly developed in cultured cells and are rarely found in the organism. However, since cultured cells are amenable to microscopic and biochemical analyses and can be experimentally manipulated, the focal contacts in cultured cells have been extensively studied as a model system for cell–matrix AJ. A transmembrane connection between the actin cytoskeleton and the extracellular matrix through the integrin family occurs at these sites of the plasma membrane (Schwartz et al., 1995). At the extracellular surface, integrin interacts with matrix proteins, whereas the cytoplasmic domain interacts directly or indirectly with F-actin–binding proteins, including α-actinin, vinculin, and talin (Jockusch et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996). Moreover, a number of structural and regulatory proteins, such as paxillin (Turner et al., 1990), tensin (Wilkins et al., 1986; Lo et al., 1994; Chuang et al., 1995), zyxin (Beckerle, 1986; Crawford and Beckerle, 1991), profilin (Carlsson et al., 1977), gelsolin (Wang et al., 1984), and FAK (Schaller et al., 1992), have been identified and characterized. Most of these proteins are found not only at cell–matrix AJ but also at cell–cell AJ, but paxillin and talin are restricted to cell–matrix AJ (Geiger et al., 1985; Tidball et al., 1986; Drenkhahn et al., 1988; Turner et al., 1990).

†Abbreviations used in this paper: aa, amino acid(s); ABD, F-actin–binding domain(s); AJ, adherens junction(s); F-actin, actin filament; G-actin, actin monomer; GST, glutathione S-transferase; His6, six histidine residues; S1, subfragment 1.
Molecular linkage between the actin cytoskeleton and the plasma membrane at cell-cell AJ has also extensively been investigated (Geiger, 1983, 1989; Geiger and Ginsberg, 1991; Turner and Burridge, 1991; Luna and Hitt, 1992; Tsukita et al., 1992, 1997; Bretscher, 1993). Many linker proteins have been isolated and characterized at cell-cell AJ, where cadherins interact with each other at the extracellular surface (Takeichi, 1988, 1991; Geiger and Ginsberg, 1991; Tsukita et al., 1992). At the cytoplasmic surface, cadherin is associated with the extracellular surface (Takeichi, 1988, 1991; Geiger and Ginsberg, 1991; Tsukita et al., 1992). The Journal of Cell Biology, Volume 143, 1998 1228

In contrast to cadherin-based cell–cell AJ in epithelial and endothelial cells, the molecular mechanism of synaptic junction has not fully been understood, although recent studies have revealed that cadherin is concentrated at synaptic junctions (Fannon and Colman, 1996; Uchida et al., 1996). To identify F-actin–binding proteins, which regulate synaptic junctions, we have been attempting to isolate novel F-actin–binding proteins from rat brain using a blot overlay method with 125I-labeled F-actin. We had isolated and reported four novel F-actin–binding proteins: neurabin-I (Nakanishi et al., 1997), neurabin-II (Satoh et al., 1998), afadin (Mandai et al., 1997), and frabin (Obaishi et al., 1998). Neurabin-I is an F-actin–binding protein specifically expressed in neural tissue and is implicated in both neurite formation and maintenance of synaptic junction (Nakanishi et al., 1997). Neurabin-II is an F-actin–binding protein ubiquitously expressed (Satoh et al., 1998). Neurabin-II is enriched at the postsynaptic density fraction in rat brain and the cell–cell AJ fraction in rat liver. Neurabin-II is identical to the recently reported protein phosphatase 1–binding protein, named spinophilin (Allen et al., 1997). Afadin has two splicing variants: 1-afadin ubiquitously expressed and s-afadin mainly expressed in neural tissue. s-Afadin lacks the F-actin–binding domain. 1-Afadin is localized at cell–cell AJ. Frabin shows a homology to FGD1, which has been identified to be the genetic locus responsible for facioscapulohumeral dystrophy (Pasteris et al., 1994). Frabin is capable of inducing filopodium formation and c-Jun NH2-terminal kinase activation as described for FGD1 (Zheng et al., 1996).

During the purification of these F-actin–binding proteins, we purified the fifth F-actin–binding protein from rat brain, which was localized at cell–matrix AJ. It has two splicing variants that have different physical and functional properties. We named big and small ones b- and s-nexilins, respectively, from a Latin word nexilis meaning “bound together.” We describe here these novel F-actin–binding proteins.

Materials and Methods

125I-Labeled F-Actin Blot Overlay

125I-labeled F-actin blot overlay was done as described (Chia et al., 1991; Pestonjamasp et al., 1995). In brief, purified actin monomer (G-actin) was labeled with 125I– Bolton Hunter reagent. 125I-labeled G-actin (1 mg/ml; average specific activity, 63.3 μCi/mg) was polymerized with 18 μg/ml of gelsolin (Sigma Chemical Co., St. Louis, MO) (molar ratio 100:1) by incubation at 4°C for 10 min in a solution containing 20 mM Pipes at pH 7.0, 50 mM KCl, and 2 mM MgCl2. Phalloidin was then added to give a final concentration of 40 μM. The mixture was then incubated at room temperature for another 15 min and stored at 4°C as 125I-labeled F-actin. The sample to be tested was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in TBS containing 5% defatted powder milk. The membrane was then incubated at room temperature for 1 h with 10 μg/ml of 125I-labeled F-actin in TBS containing 5% defatted powder milk and 4 μM phalloidin. After the incubation, the membrane was washed with TBS containing 0.5% Tween 20, followed by autoradiography using an image analyzer (Fujix BAS-2000; Fuji Photo Film Co., Tokyo, Japan).

For competition experiments, 125I-labeled F-actin was prepared as described. 10 μg/ml of 125I-labeled F-actin was incubated at room temperature for 30 min with 0.66 mg/ml of myosin subfragment 1 (S1) (Sigma Chemical Co.) in a solution containing 20 mM Pipes at pH 7.0, 58 mM KCl, 2 mM MgCl2, 130 mM CaCl2, and 0.4 μM phalloidin. Where indicated, 4 mM MgATP was added to the mixture. After the incubation, the mixture was diluted with an equal volume of TBS containing 0.5% Tween 20 and 10% defatted powder milk, and it was added to the blot membrane, followed by incubation at room temperature for 1 h.

Purification of b-Nexilin

The synaptic soluble fraction was prepared from 480 adult rat brains as described (Mizoguchi et al., 1990) and stored at −80°C until use. 1/12 of the fraction (420 ml, 360 mg of protein) was adjusted to 0.2 M NaCl with 4 M NaCl and applied to a Q-Sepharose FF column (2.6 × 10 cm; Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with buffer A (20 mM Tris·Cl at pH 7.5, 5 mM KCl, and 2 mM MgCl2). The column was washed with 250 ml of buffer A containing 0.2 M NaCl. Elution was performed with 350 ml of buffer A containing 0.5 M NaCl at a flow rate of 5 ml/min. Fractions of 10 ml each were collected. b-Nexilin appeared in fractions 5–19. These fractions (150 ml, 152 mg of protein) were collected, and NaCl was added to give a final concentration of 2 M. The sample was applied to a phenyl-Sepharose column (2.6 × 10 cm; Amersham Pharmacia Biotech) equilibrated with buffer A containing 2 M NaCl. After the column was washed with 250 ml of the same buffer, elution was performed with a 360-ml linear gradient of NaCl (2.0–0 M) in buffer A, followed by 180 ml of buffer A, at a flow rate of 3 ml/min. Fractions of 6 ml each were collected. b-Nexilin appeared in fractions 45–49. The active fractions (30 ml, 3.7 mg of protein) were collected. The two samples were combined and applied to a phenyl-Sepharose column (0.75 × 7.5 cm; Tosoh, Tokyo, Japan) equilibrated with 0.05% trifluoroacetic acid. Elution was performed with a 320-ml linear gradient of acetonitrile (10–80%) in 0.05% trifluoroacetic acid. Fractions of 1.33 ml each were collected. b-Nexilin appeared in fractions 44 and 45. The active fractions of the six phenyl-SPW RP column chromatographies (7.98 ml, 40 μg of protein) were collected, lyophilized, and stored at −80°C.

Determination of Partial Amino Acid Sequence of b-Nexilin

The purified phenyl-SPW RP sample (40 μg of protein) was subjected to SDS-PAGE (8% polyacrylamide gel). A protein band corresponding to b-nexilin was cut out from the gel and digested with a lysyl endopeptidase, and the digested peptides were separated by TSKgel ODS-80Ts (4.6 × 150 mm; Tosoh) reverse-phase high-pressure liquid column chromatography as described (Imazumi et al., 1994). The amino acid (aa) sequences of the eight peptides were determined with a peptide sequencer.

Molecular Cloning of b- and s-Nexilins

Computer homology search revealed that two of the eight peptides were contained within a peptide sequence deduced from the cDNA fragment isolated from a human CDNA library (Elisei et al., 1993). Therefore, a probe was obtained by the PCR method with oligonucleotide primers from the cDNA fragment. To obtain full-length b-nexilin, a rat brain cDNA library in AZIP1 (Stratagene, La Jolla, CA) was screened using the probe.

Reverse transcriptase–PCR was performed to amplify a fragment of...
mRNA encoding s-nexilin expressed in 3Y1 cells. For that purpose, total RNA was prepared from 3Y1 cells using the ISOGEN RNA extraction kit (Nippon Genetics, Tokyo, Japan). Reverse transcription of 0.5 μg of total RNA was carried out using Ready-To-Go You-Prime First-Strand Beads and pd(N6) (Amersham Pharmacia Biotech). For PCR, an aliquot of cDNA was amplified using primers based on the NH2- and COOH-terminal regions of b-nexilin. The primer sequences were 5′-ataagctgtgctgatgcaaccaggca-3′ and 5′-ctagtgcatacatacatactg-3′ for the NH2- and COOH-terminal regions, respectively. DNA sequencing was performed by the dyeoxy nucleotide termination method using a DNA sequencer (ALF express; Amersham Pharmacia Biotech). For coiled-coil prediction, the PAIRCOIL program was used (Berg et al., 1995).

**Expression and Purification of Recombinant b- and s-Nexilins**

Prokaryotic and eukaryotic expression vectors were constructed in pGEX (Amersham Pharmacia Biotech), pCMV5 (Takeuchi et al., 1997), pCIneo (Promega Corp., Madison, WI), and pRSET (Invitrogen Corp., Carlsbad, CA) using standard molecular biological methods (Sambrook et al., 1989). Glutathione S-transferase (GST) fusion constructs of b- and s-nexilins contained the following amino acid (aa) residues: pGEX-b-nexilin-1, aa 1–150; pGEX-b-nexilin-1′, aa 1–240; pGEX-b-nexilin-2, aa 77–240; pGEX-b-nexilin-3, aa 151–283; pGEX-b-nexilin-4, aa 211–330; pGEX-b-nexilin-5, aa 284–450; pGEX-b-nexilin-6, aa 493–656; pGEX-s-nexilin-1, aa 1–100; and pGEX-s-nexilin-1′, aa 1–190. The GST fusion proteins were purified by use of glutathione-Sepharose beads according to the manufacturer’s protocol (Amersham Pharmacia Biotech). Full-length b- and s-nexilins were constructed in pCMV5 and pRSET vectors. Full-length and various truncated mutants of b- and s-nexilins were constructed in pCI-neo-myc vector. pCI-neo-myc vector was constructed by ligating the oligonucleotides, 5′-ctgaccccaaatcgagagaggagactggagagtg-3′ and 5′-aattctgcagaggccccggactggagaggagtgg-3′, into the Nhel and EcoRI sites of pCI-neo. The myc-epitope (MEQKLISEEDL) was inserted at the NH2 termini of full-length b- and s-nexilins; Various myc-tagged truncated mutants of b- and s-nexilins contained the following aa residues: pCI-neo-myc-s-nexilin-M1, aa 1–100; pCI-neo-myc-s-nexilin-M2, aa 1–233; pCI-neo-myc-FC, aa 161–430 for b-nexilin, aa 211–480 for b-nexilin; pCI-neo-myc-ABD, aa 234–400 for s-nexilin, aa 284–450 for b-nexilin; pCI-neo-myc-CC, aa 101–233 for s-nexilin, aa 151–283 for b-nexilin; pCI-neo-myc-b-nexilin-ABD, aa 1–150; and pCI-neo-myc-b-nexilin-CC, aa 1–283. pRSET was designed to express the proteins with the NH2-terminal six histidine residues (His6). The His6-tagged proteins were purified by use of TALON metal affinity beads according to the manufacturer’s protocol (CLONTECH Labs, Palo Alto, CA).

**Assay for Codensation of b- and s-Nexilins with F-Actin**

G-actin was polymerized by incubation at room temperature for 30 min in a polymerization buffer (20 mM imidazole/Cl at pH 7.0, 2 mM MgCl2, 1 mM ATP, 0.5 mM DTT, and 90 mM KCl). His6-b- or His6-s-nexilin in an indicated amount was incubated at room temperature for 30 min with 0.3 mg/ml of F-actin in a solution containing 25 mM imidazole/Cl at pH 7.0, 2 mM MgCl2, 1 mM ATP, 0.05 mM DTT, 27 mM KCl, and 100 mM NaCl, and the mixture (50 μl) was placed over a 50-μl cushion of 30% sucrose in the polymerization buffer. To estimate Kc values, various amounts of His6-b- or His6-s-nexilin were incubated with 0.1 mg/ml of F-actin in a solution containing 32 mM imidazole/Cl at pH 7.0, 2 mM MgCl2, 1 mM ATP, 0.05 mM DTT, 27 mM KCl, and 100 mM NaCl. After the sample was centrifuged at 130,000 g for 20 min, the supernatant was removed from the pellet and the pellet was brought to the original volume in an SDS sample buffer. The total amounts of the supernatant and pellet fractions were subjected to SDS-PAGE, followed by protein staining with Coomassie brilliant blue to quantitate recombinant b- and s-nexilins cosedimented with F-actin using a densitometer. For localization of s-nexilin and other marker proteins in 3Y1 cells, immunofluorescence microscopy was obtained from Chemicon International, Inc. (Temecula, CA).

**Other Procedures**

G-actin was purified from rabbit skeletal muscle as described (Pardee and Spudich, 1982). Protein concentrations were determined with BSA as a reference protein (Bradford, 1976). SDS-PAGE was performed as described (Laemmli, 1970). Protein markers used were either myosin (200
kD), β-galactosidase (116 kD), phosphorylase b (97 kD), BSA (66 kD), and ovalbumin (45 kD), or myosin (206 kD), β-galactosidase (117 kD), BSA (89 kD), and ovalbumin (47 kD).

Results

Purification of b-Nexilin and Molecular Cloning of Its cDNA

During the purification of neurabin-I, -II, afadin, and frabin from rat brain using a blot overlay method with 125I-labeled F-actin (Mandai et al., 1997; Nakanishi et al., 1997; Obaishi et al., 1998; Satoh et al., 1998), we identified a band of 125I-labeled F-actin–binding activity with a molecular mass of about 97 kD (p97). p97 was highly purified by column chromatographies, including Q-Sepharose, phenyl-Sepharose, and phenyl-SP RP column chromatographies. On the final phenyl-SP RP column chromatography, the F-actin-binding protein band closely coincided with a protein with a molecular mass of ~97 kD, which was identified by protein staining with Coomassie brilliant blue (Fig. 1).

When the peptide mapping of the phenyl-SP RP sample of p97 was performed, over 20 peptides were observed. Of these peptides, the aa sequences of the eight peptides were determined. Computer homology search revealed that two of the eight peptides were contained within a peptide sequence deduced from the cDNA fragment (sequence data available from GenBank/EMBL/DDBJ under accession number S67069) isolated from a human cDNA library (Elisei et al., 1993). Therefore, using a probe obtained by the PCR method with oligonucleotide

Figure 1. Phenyl-5PW RP column chromatography. (A) Absorbance at 280 nm (A280). (B) 125I-labeled F-actin blot overlay. An aliquot (33 μl) of each fraction was subjected to SDS-PAGE (8% polyacrylamide gel), followed by 125I-labeled F-actin blot overlay. (C) Protein staining with Coomassie brilliant blue. An aliquot (200 μl) of each fraction was subjected to SDS-PAGE (8% polyacrylamide gel), followed by protein staining with Coomassie brilliant blue. Arrowhead indicates p97 (b-nexilin).

Figure 2. Deduced amino acid sequences of b- and s-nexilins. (A) Deduced aa sequence of b-nexilin. The aa sequences of the eight peptide peaks derived from the purified sample of b-nexilin are indicated by underlining. (B) Aligned sequences of b- and s-nexilins. The deletion and insertion are indicated by a box and black shading, respectively. These sequence data are available from GenBank/EMBL/DDBJ under accession numbers AF056034 for b-nexilin and AF056035 for s-nexilin.
primers from the cDNA fragment, we isolated several clones from a rat brain cDNA library and obtained a full-length clone by connecting two clones among them. The encoded protein consisted of 656 aa and showed a calculated molecular weight of 78,392 (Fig. 2 A). It included all the aa sequences of the eight peptides. The molecular mass value calculated from the predicted aa sequence was slightly less than that estimated by SDS-PAGE. To confirm whether this clone encoded a full-length cDNA of p97, we constructed the eukaryotic expression vector with this cDNA and expressed the protein in COS7 cells. Western blot analysis indicated that the expressed protein showed a mobility similar to that of native p97 on SDS-PAGE (Fig. 3 Aa). Although the reason for the slight difference between the molecular mass value calculated from the predicted aa sequence and that estimated by SDS-PAGE was not known, we concluded that this clone encoded the full-length cDNA of p97.

We named p97 b-nexilin since p97 was the bigger of the two splicing variants described below. To determine the F-actin–binding domain of b-nexilin, we prepared the fusion proteins of several truncated forms of b-nexilin with GST and examined the binding of $^{125}\text{I}$-labeled F-actin to these fusion proteins. Under the conditions where full-length His6-b-nexilin showed $^{125}\text{I}$-labeled F-actin–binding activity, GST-b-nexilin-1 (aa 1–150) and GST-b-nexilin-5 (aa 284–450) showed activity, whereas the other truncated forms of b-nexilin did not show activity (Fig. 3 Ba). This result suggests that b-nexilin has two F-actin–binding domains at the NH2-terminal (aa 1–150) and middle (aa 284–450) regions, and that b-nexilin interacts with F-actin through these domains (Fig. 3 C). This conclusion was supported by two other lines of evidence described below (see Figs. 4 Aa and 7). The two F-actin–binding domains showed no significant homology to any known F-actin–binding protein in current protein database. In addition to the two F-actin–binding domains, b-nexilin had a sequence stretch that had high probability of forming a predicted coiled-coil structure (Fig. 3 C). The coiled-coil probability of the sequence stretch at the middle region (aa 244–281) was calculated to be 0.9–1.0 (data not shown). The NH2- and COOH-terminal regions of b-nexilin did not show any tendency to form a coiled-coil structure.

**Biochemical Properties of b-Nexilin**

In addition to $^{125}\text{I}$-labeled F-actin blot overlay, the binding of b-nexilin to F-actin was examined by cosedimentation of b-nexilin with F-actin. When full-length His6-b-nexilin was incubated with F-actin, followed by ultracentrifugation, His6-b-nexilin was recovered with F-actin in the pellet (Fig. 4 Aa). The stoichiometry of the binding of His6-b-nexilin to actin was calculated to be one His6-b-nexilin molecule per about nine actin molecules (Fig. 4 B). The $K_d$ value was calculated to be 8.0 $\pm$ 0.1 $\times$ 10$^{-7}$ M. It was examined by competition experiments using myosin S1, a well-characterized protein that binds along the sides of F-actin (Rayment et al., 1993; Schröder et al., 1993), whether b-nexilin bound along the sides of F-actin or at the ends. The binding of His6-b-nexilin to $^{125}\text{I}$-labeled F-actin was inhibited by an excessive amount of myosin S1 (Fig. 4 Ca). This inhibition was reversed by the addition of MgATP.

![Figure 3. Molecular characterization of b- and s-nexilins.](image_url)

*(A) Western blot analysis of recombinant b- and s-nexilins. The pCMV-b- or pCMV-s-nexilin was transfected into COS7 cells, and the cell lysates were subjected to SDS-PAGE (8% polyacrylamide gel), followed by Western blot analysis using the antinexilin antibody. Arrowheads indicate b- and s-nexilins. (Aa) b-Nexilin and (Ab) s-nexilin. (B) $^{125}\text{I}$-labeled F-actin–binding activity of various truncated forms of b- and s-nexilins. The purified proteins (0.2 $\mu$g of protein each), except for GST-b-nexilin-1, were subjected to SDS-PAGE (10% polyacrylamide gel), followed by $^{125}\text{I}$-labeled F-actin blot overlay. GST-b-nexilin-1 was recovered in the inclusion bodies during the preparation of the supernatant of the cell lysates, and thus an aliquot of the cell lysates was used for the assay. GST-b-Nexilin-1, aa 1–150; GST-b-Nexilin-2, aa 77–240; GST-b-Nexilin-3, aa 151–283; GST-b-Nexilin-4, aa 211–330; GST-b-Nexilin-5, aa 284–450; GST-b-Nexilin-6, aa 493–656; and GST-s-Nexilin-1, aa 1–100. (Ba) b-Nexilin and (Bb) s-nexilin. (C) Schematic drawing of the structures of b- and s-nexilins. ABD, F-actin–binding domain; CC, coiled-coil region.*
because MgATP dissociates the actin–myosin complex (Fraser et al., 1975). β-Nexilin increased the viscosity in a dose-dependent manner, and at 1.0 μM, the viscosity became unmeasurably high as estimated by the falling ball method for low shear viscometry (Fig. 4 D). Preliminary results from electron microscopy confirmed the cross-linking activity of β-nexilin as demonstrated by low shear viscometry (data not shown). All of these results suggest that β-nexilin binds along the sides of F-actin and shows cross-linking activity, presumably because of the two F-actin-binding domains. We confirmed the F-actin-binding domains of β-nexilin shown in Fig. 3 C by the cosedimentation assay using the GST fusion proteins. The purified samples of the GST fusion proteins having the F-actin-binding domains were insoluble under several conditions where full-length β- or s-nexilin was not (data not shown). Therefore, we used the crude samples of E. coli (the supernatant of the cell lysates) expressing the GST fusion proteins. As to GST-β-nexilin-1 (aa 1–150) in Fig. 3 Ba, because it was recovered in the inclusion bodies during the preparation of the supernatant of the cell lysates, we used another GST fusion protein, GST-β-nexilin-1’ (aa 1–240) for the assay. GST-β-nexilin-1’ and GST-β-nexilin-5 (aa 284–450) were cosedimented with F-actin, whereas GST-β-nexilin-6 (aa 493–656) was not (Fig. 4 Ea). GST and GST-β-nexilin-3 (aa 151–283) were not cosedimented with F-actin (data not shown). These results are consistent with those of Fig. 3 Ba and support the conclusion shown in Fig. 3 C that β-nexilin has two F-actin-binding domains at the NH₂-terminal and middle regions, and that β-nexilin interacts with F-actin through these domains.

The molecular mass value of β-nexilin was estimated by sucrose density gradient ultracentrifugation. On sucrose density gradient ultracentrifugation, His6-b-nexilin ap-

Figure 4. Biochemical properties of b- and s-nexilins. (A) Cosedimentation of His6-b- and His6-s-nexilins with F-actin. His6-b- (6.5 μg of protein) or His6-s-nexilin (6.0 μg of protein) was mixed with F-actin, followed by ultracentrifugation. S, supernatant; P, pellet. Arrows indicate actin. Arrowheads indicate His6-b- and His6-s-nexilins. (Aa) His6-b-nexilin and (Ab) His6-s-nexilin. (B) Binding of His6-b- and His6-s-nexilins to F-actin. Various amounts of His6-b- or His6-s-nexilin were mixed with F-actin, followed by ultracentrifugation. Amounts of the free and bound His6-b- or His6-s-nexilin were calculated by determining the protein amounts from the supernatant and pellet fractions with a densitometer. The values are means ± SEM of three independent experiments. (Filled circles) His6-b-nexilin and (open circles) His6-s-nexilin. (C) Inhibition by myosin S1 of the binding of His6-b- and His6-s-nexilins to 125I-labeled F-actin. His6-b- or His6-s-nexilin (50 ng of protein each) was subjected to SDS-PAGE (8% polyacrylamide gel), followed by the blot overlay with 125I-labeled F-actin pretreated with myosin S1 in the presence or absence of ATP. (Ca) His6-b-nexilin and (Cb) His6-s-nexilin. (D) F-actin cross-linking activity of His6-b- and His6-s-nexilins estimated by low shear viscometry. (Filled circles) His6-b-nexilin and (open circles) His6-s-nexilin. (E) Cosedimentation of various truncated forms of b- and s-nexilins having or lacking the F-actin–binding domains. The supernatant (5 μl each) of the lysates of E. coli expressing the GST fusion proteins was mixed with F-actin. After the sample was centrifuged, the supernatant and pellet were subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blot analysis using the anti-GST and antiactin antibodies. GST-b-Nexilin-1’, aa 1–240; GST-b-Nexilin-5, aa 284–450; GST-b-Nexilin-6, aa 493–656; and GST-s-Nexilin-1’, aa 1–190. S, supernatant; P, pellet. Arrowheads indicate actin. (Ea) b-Nexilin and (Eb) s-nexilin.
The molecular mass value of b-nexilin estimated by SDS-PAGE is ~97 kD, this result suggests that b-nexilin exists as a monomer.

**Tissue Distribution of b-Nexilin**

Western blot analysis showed that the antinexilin antibody recognized a protein band with a molecular mass of ~97 kD in brain and testis and a protein band with a slightly smaller size (~95 kD) in testis, spleen, and fibroblasts such as rat 3Y1 and mouse Swiss 3T3 cells (Fig. 5). Neither a big nor small protein was detected in liver, kidney, or epithelial cells, such as MDCK and MTD-1A cells. Because liver and kidney contain abundant epithelial cells, these results suggest that nexilin is expressed in nonepithelial cells but not in epithelial cells.

**Molecular Cloning and Physical Properties of s-Nexilin**

The results described above led us to speculate about the existence of a smaller isoform or a smaller splicing variant of b-nexilin. Therefore, we attempted to isolate it and cloned a small splicing variant from 3Y1 cells by use of the reverse transcriptase-PCR method. The encoded protein had 606 aa and showed a calculated molecular weight of 71,942. The protein showed the aa sequence identical to that of b-nexilin except that it had 64 aa deletion and 14 aa insertion in the first F-actin–binding domain of b-nexilin (Fig. 2 B). We therefore named this variant s-nexilin (a small variant of b-nexilin). To confirm whether this clone encoded a full-length cDNA, we constructed the eukaryotic expression vector with this cDNA and expressed the protein in COS7 cells. Western blot analysis indicated that the expressed protein with a molecular mass of ~95 kD showed two bands with mobilities similar to those of the two bands endogenously detected in 3Y1 cells (Fig. 3 Ab). Although the reason for the slight difference between the molecular mass value calculated from the predicted aa sequence and that estimated by SDS-PAGE was not known, we concluded that this clone encoded the full-length cDNA of s-nexilin. The reason for the presence of the two bands on SDS-PAGE is not known, but the two bands may be caused by posttranslational modifications, such as phosphorylation.

To determine whether the NH2-terminal region of s-nexilin still has its F-actin–binding activity, we prepared a fusion protein of the truncated form of s-nexilin with GST and examined the binding of 125I-labeled F-actin to this protein. Under the conditions where full-length His6-s-nexilin showed 125I-labeled F-actin–binding activity, GST-s-nexilin-1 (aa 1–100) did not show activity (Fig. 3 Bb). This result suggests that s-nexilin has only one F-actin–binding domain at the middle region (aa 234–400), and that s-nexilin interacts with F-actin through this domain (Fig. 3 C). This conclusion was supported by two other lines of evidence described below (see Figs. 4 Eb and 7).

The binding of s-nexilin to F-actin was examined by cosedimentation of s-nexilin with F-actin. When full-length His6-s-nexilin was incubated with F-actin, followed by ultracentrifugation, His6-s-nexilin was recovered with F-actin in the pellet (Fig. 4 Ab). The stoichiometry of the binding of His6-s-nexilin to actin was calculated to be one His6-s-nexilin molecule per about ten actin molecules (Fig. 4 B). The K_d value was calculated to be 9.0 ± 0.2 × 10^{-7} M. s-Nexilin also bound along the sides of F-actin (Fig. 4 Cb). However, s-nexilin did not increase the viscosity estimated by the falling ball method (Fig. 4 D). Consistently, preliminary results from electron microscopy indicated that s-nexilin had no cross-linking activity (data not shown).

We confirmed the F-actin–binding domain of s-nexilin shown in Fig. 3 C. GST-s-nexilin-1' (aa 1–190) was not cosedimented with F-actin (Fig. 4 Eb). This result is consistent with that of Fig. 3 Bb and supports the conclusion shown in Fig. 3 C that s-nexilin has only one F-actin–binding domain at the middle region, and that s-nexilin interacts with F-actin through this domain. Sucrose density gradient ultracentrifugation analysis revealed that His6-s-nexilin appeared at a position corresponding to a molecular mass of ~66 kD (S value, ~4.4) (data not shown), suggesting that s-nexilin exists as a monomer. All of these results suggest that s-nexilin binds along the sides of F-actin but lacks cross-linking activity, presumably because of one F-actin–binding domain.

**Localization of s-Nexilin in 3Y1 Cells**

Since s-nexilin was expressed in 3Y1 cells, we examined the localization of s-nexilin in 3Y1 cells. An immunofluorescence microscopic study revealed that s-nexilin was localized at the ends of stress fibers, which are known to be focal contacts (Fig. 6, A and B). To confirm localization of s-nexilin at focal contacts, we compared the localization of s-nexilin with those of vinculin, talin, and paxillin, which are typical focal contact proteins and thereby known to be good markers for cell–matrix AJ (Geiger, 1979; Burridge and Connell, 1983; Turner et al., 1990). S-Nexilin was colocalized with vinculin, talin, and paxillin (Fig. 6, C–H). However, s-nexilin was not colocalized with P-cadherin, which is known to be a marker for cell–cell AJ (Itoh et al.,
was localized at focal contacts (Fig. 7, A). s-Nexilin was localized at cell–matrix AJ in 3Y1 cells. We examined whether exogenously expressed b- or s-nexilins were consistently localized at focal contacts but not at cell–cell AJ.

Localization of Overexpressed Full-Length and Truncated Mutants of b- and s-Nexilins in 3Y1 Cells

We isolated and characterized here two novel F-actin–binding proteins, which were likely splicing variants derived from the same gene. We named the big and small ones b- and s-nexilins, respectively. We have concluded that both the proteins are real F-actin–binding proteins on the basis of the following observations: (a) full-length b- and s-nexilins showed 125I-labeled F-actin–binding activity; (b) both full-length b- and s-nexilins were cosedimented with F-actin; (c) full-length b-nexilin showed F-actin crosslinking activity; and (d) when full-length b- or s-nexilin was expressed in COS7 cells, both the proteins were colocalized with F-actin (data not shown).

Discussion

We isolated and characterized here two novel F-actin–binding proteins, which were likely splicing variants derived from the same gene. We named the big and small ones b- and s-nexilins, respectively. We have concluded that both the proteins are real F-actin–binding proteins on the basis of the following observations: (a) full-length b- and s-nexilins showed 125I-labeled F-actin–binding activity; (b) both full-length b- and s-nexilins were cosedimented with F-actin; (c) full-length b-nexilin showed F-actin crosslinking activity; and (d) when full-length b- or s-nexilin was expressed in COS7 cells, both the proteins were colocalized with F-actin (data not shown).

Structural analysis of b- and s-nexilins showed that the nucleotide sequence of the s-nexilin cDNA was identical to that of the b-nexilin cDNA except for the two splicing
regions that were located in the first F-actin–binding domain of b-nexilin. Consequently, the NH₂-terminal region of s-nexilin lost F-actin–binding activity. Thus, the big variant of nexilin has two F-actin–binding domains, whereas the small one has one F-actin–binding domain (Fig. 3 C). Among many F-actin–binding proteins so far identified, some variants lose the F-actin–binding domain. For instance, BPAG1n, the neuronal splicing form, contains the functional F-actin–binding domain at the NH₂-terminal region but BPAG1e, which is mainly expressed in epidermis, lacks the domain (Yang et al., 1996). l-Afadin also has the F-actin–binding domain at the COOH-terminal region, but s-afadin lacks this domain (Mandai et al., 1997).

Among many F-actin–binding proteins having F-actin cross-linking activity, the members of the α-actinin/spec¬trin superfamily have most extensively been studied (Hartwig and Kwiatkowski, 1991; Matsudaira, 1991). Most of them usually form oligomers by association at rod domains and hence show F-actin cross-linking activity, but the members of the fimbrin (plastin) subfamily show F-actin cross-linking activity without oligomerization since they have two F-actin–binding domains in a single
mechanism by which signals at the cell surface are relayed (Keely et al., 1998). However, little is known about the signaling hot spots on the cell surface. For instance, the initial interaction between transmembrane proteins, such as integrins, and extracellular ligands has been shown to turn on a cascade of several signal transduction (Keely et al., 1998). However, little is known about the mechanism by which signals at the cell surface are relayed to the nucleus and vice versa. Recently, several proteins, including c-abl (Lewis et al., 1996), ZO-1 (Gottardi et al., 1996), zyxin (Nix and Beckerle, 1997), and β-catenin (Behrens et al., 1996; Molennar et al., 1996), have been demonstrated to reside both at AJ and in the nucleus. Interestingly, the PSORT protein localization prediction program (Nakai and Kanehisa, 1992) indicates that b- and s-nexilins also partition in the nucleus since these two proteins have the consensus sequences for nucleoplasm-in-like nuclear localization signals (Robbins et al., 1991) (data not shown).

In fact, we observed weak nuclear stainings in fibroblasts as shown in Fig. 6. However, we have not yet succeeded in demonstrating that transfected full-length b- or s-nexilin is translocated into the nucleus (data not shown). The significance of these nuclear localization signals in a variety of proteins that are localized at cell–matrix and/or cell–cell AJs, including nexilin, is currently unknown.

We thank Dr. W. Birchmeier for providing MDCK cells, Dr. Sh. Tsutka for providing rat 3Y1 and MTD-1A cells, Dr. D.W. Russell (University of Texas Southwestern Medical Center, Dallas, TX) for providing the pCMV5 vector, and Dr. Y. Hata (Takai Biotimer Project, ERATO, Kobe, Japan) for providing the pCneo-myc vector. We also thank Drs. Sh. Tsukita and M. Itoh (Kyoto University, Kyoto, Japan) and Drs. Y. Yoneda and T. Tachibana (Osaka University, Osaka, Japan) for helpful discussions. We are grateful to M. Tokunaga, M. Kinoshita, T. Inoue, Y. Hasyi, and H. Nohara for their technical assistance.

The work performed at Osaka University Medical School was supported by grants-in-aid for Scientific Research and for Cancer Research from the Ministry of Education, Science, Sports, and Culture, Japan (1997), by grants-in-aid for Abnormalities in Hormone Receptor Mechanisms and for Aging and Health from the Ministry of Health and Welfare, Japan (1997), and by grants from the Human Frontier Science Program (1997).

Received for publication 9 April 1998 and in revised form 21 September 1998.

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