(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 905 239 A2

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
31.03.1999 Bulletin 1999/13

(51) Int Cl.[6]: **C12N 15/12**, C07K 14/47, C12P 21/02, C07K 16/18

(21) Application number: **98307643.1**

(22) Date of filing: **21.09.1998**

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **22.09.1997 JP 257043/97**

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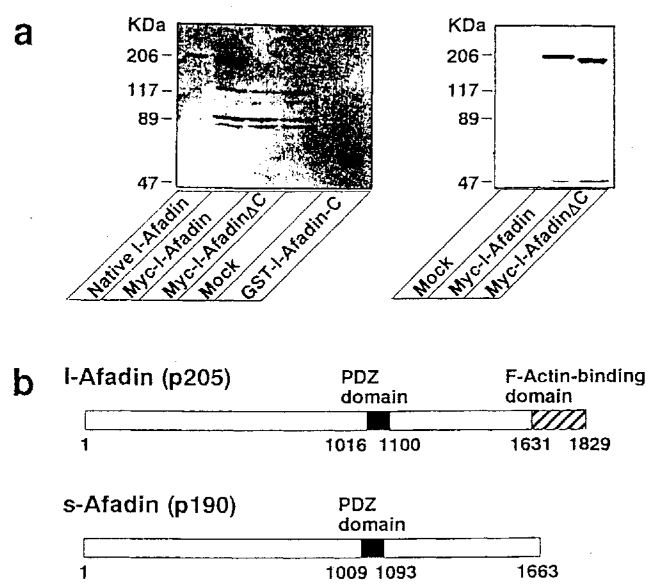
(54) **Gene encoding Afadin-1**

(57) An actin filament-binding protein 1-Afadin having the amino acid sequence of SEQ ID NO: 1 or having an animo acid sequence substantially the same as that of SEQ ID NO: 1, a cDNA sequence encoding the protein, a genomic DNA sequence to which the cDNA sequence or a partial sequence thereof is hybridized, and an antibody specifically recognizing 1-Afadin are provided. The protein is a novel actin filament-binding protein localized at the cadherin based cell-to-cell adherens junction and the other products are useful as the genetic materials for industrially utilizing the protein.

FIG. 3



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Description

[0001] The present invention relates to a novel actin filament binding protein "1-Afadin". More precisely, the present invention relates to a novel animal protein l-Afadin that contributes to the cell-to-cell adherens junction (AJ) which has an important role in individual formation of animals and pathogenesis.

[0002] In various cellular events, such as cell adhesion, cell motility, and cell shape determination, specialized membrane domains are formed with transmembrane proteins, such as cell adhesion molecules, receptors, and channels, and these domains are often associated with the actin cytoskeleton (Biochem. Biophys. Acta 737:305-341, 1983; Curr. Opin. Cell Biol. 1:103-109, 1989; Cell Motil. Cytoskeleton 20:1-6, 1991; Curr. Opin. Cell Biol. 3:849-853, 1991; Science 258:955-964, 1992; Curr. Opin. Cell Biol. 4:834-839, 1992; Curr. Opin. Cell Biol. 5:653-660, 1993; Trends Biochem. Sci. 22: 53-58, 1997). The linkage between the actin cytoskeleton and the plasma membrane plays a crucial role in these cellular events, and proteins linking the actin cytoskeleton to the transmembrane proteins have been identified. However, the molecular basis of the linkage between the actin cytoskeleton and the plasma membrane is not fully understood.

[0003] To understand this molecular linkage, the cell adhesion sites have been studied extensively (Biochem. Biophys. Acta 737:305-341, 1983; Curr. Opin. Cell Biol. 1:103-109, 1989; Cell Motil. Cytoskeleton 20:1-6, 1991; Curr. Opin. Cell Biol. 3:849-853, 1991; Science 258:955-964, 1992; Curr. Opin. Cell Biol. 4:834-839, 1992; Curr. Opin. Cell Biol. 5:653-660, 1993; Trends Biochem. Sci. 22:53-58, 1997). As a result, the actin filament (F-actin)-associated cell adhesion sites are subclassed into two types: cell-to-cell and cell-to-matrix adherens junctions. Many linker proteins have been identified at the cell-to-cell AJ where cadherins interact with each other at the extracellular surface (Development 102:639-655, 1988; Cell Motil. Cytoskeleton 20:1-6, 1991: Science 251: 1451-1455, 1991; Curr. Opin. Cell Biol. 4:834-839, 1992; EMBO J. 8:1711-1717, 1989; Cell 65:849-857, 1991; Science 251:1451-1455, 1991; Curr. Opin. Cell Biol. 4:834-839, 1992). Among these binding proteins, α-catenin interacts directly with F-actin (Proc. Natl. Acad. Sci. USA 92, 8813-8817, 1995). α-Catenin also interacts indirectly with F-actin through α-actinin and/or ZO-1 (J. Cell. Biol. 130:67-77, 1995; J. Cell. Biol. 138:181-192, 1997). Further, Vinculin, another F-actin-binding protein, is concentrated at the cell-to-cell AJ, although the molecules with which it interacts at the cell-to-cell AJ have not yet been identified (Cell Motil. Cytoskeleton 20:1-6, 1991; Curr. Opin. Cell Biol. 4:834-839, 1992). At cell-to-matrix AJ where integrin interacts with matrix proteins at the extracellular surface, the cytoplasmic domain directly or indirectly interacts with F-actin binding proteins, including α-actinin, vinculin, and talin (Ann. Rev. Cell Dev. Biol. 11:379-416, 1995).

[0004] As described above, many F-actin-binding proteins appear to serve as linkers of the actin cytoskeleton to the plasma membrane cadherin and integrin.

[0005] On the other hand, the linkage between the actin cytoskeleton and the plasma membrane is also important for neuron-specific events, such as growth cone pathfinding and subsequent formation and maintenance of synaptic junction (Neuron 1:761-772, 1988; Science 242:708-715, 1988; Curr. Opin. Neurobiol. 4:43-48, 1994; Curr. Opin. Neurobiol. 4:640-647, 1994; Cell 83:171-176, 1995). However, it remains to be clarified which molecules link the actin cytoskeleton to the plasma membrane in these neuron-specific events.

[0006] To address this issue, the inventors of the present patent application have isolated several novel F-actin-binding proteins from rat brain and analyzed the structure of proteins particularly specific to neural tissue and concentrated at the synapse. A patent application for this subject matter has already been filed (Japanese Patent Application No. 9-92615). The protein of the prior invention (hereinafter, referred to as "neurabin" according to the inventor's nomenclature) has one F-actin-binding domain and one PDZ domain. The PDZ domain has been found in a variety of proteins, some of which are localized at cell-to-cell junctions, such as PSD-95/SAP9O at synaptic junctions (Neuron 9:929-942, 1992; J. Biol. Chem. 268:4580-4583, 1993), Dlg at septate junctions (Cell 66:451-464, 1991), ZO-1 and ZO-2 at tight junctions (J. Cell Biol. 193:755-766, 1986; Proc. Natl. Acad. Sci. USA 88:3460-3464, 1991; J. Cell Biol. 121:491-502, 1993; J. Cell Biol. 123:1049-1053, 1993; Proc. Natl. Acad. Sci. USA 90:7834-7838, 1993; J. Cell Biol. 124:949-961, 1994). In addition, recent studies have revealed that the PDZ domain binds to unique C-terminal motifs of target proteins (Trends Biochem. Sci. 21:455-458, 1996), which are found in a large number of transmembrane proteins, such as the N-methyl-D-aspartate receptor and Shaker-type K^+ channel (Nature 378:85-88, 1995; Science 269:1737-1740, 1995; J. Neurosci. 16:2157-2163, 1996).

[0007] From the various findings described above, it is likely that neurabin, the protein of the prior invention found by the present inventors, serves as a linker of the actin cytoskeleton to a transmembrane protein(s) at synapses.

[0008] However, all aspects of the molecular basis for the cell-to-cell adhesion have not yet been clarified. Such clarification is necessary to identify further actin filament-binding proteins.

[0009] In addition, there is a possibility that these proteins will lead to clarification of, for example, the mechanisms of infiltration and metastasis of carcinoma, and it is expected that this will allow the development of diagnostic methods for determining the malignancy of carcinomas, therapeutic methods or agents for treating carcinomas and the like.

[0010] The present invention has been completed with the above in mind. An object of the present invention is to provide a novel actin filament-binding protein contributing to the cell-to-cell adhesion, simultaneously clarifying its structure (amino acid sequence) and its properties.

[0011] Another object of the present invention is to provide suitable material to allow genetic engineering manipulation of the actin filament-binding protein.

[0012] Thus, viewed from one aspect the present invention provides an actin filament-binding protein l-Afadin having the amino acid sequence of SEQ ID NO: 1. The invention further extends to corresponding proteins or peptides thereof, derived from any animal, having an amino acid sequence substantially the same as that of SEQ ID NO:1, especially from humans. As referred to herein substantially the same refers to proteins having for example 80% or more sequence identity, e.g. 85 or 90% identity to the amino acid sequence of SEQ ID NO:1. The protein of the invention includes peptide fragments (of 5 or more amino acid residues, e.g. more than 20 or more than 50 residues), e.g. antigenic fragments, containing any partial amino acid sequence in the amino acid sequence of SEQ ID NO: l or from a sequence substantially similar thereto, e.g. derived from a different animal. These peptide fragments can be used as an antigen for producing antibodies. In addition, the protein of the present invention includes fused proteins with any other proteins (for example, fluorescent proteins and the like).

[0013] Furthermore, proteins according to the invention include functionally equivalent variants or precursors of the above defined proteins or peptide fragments. "Functionally-equivalent" is used to define proteins or peptides related to or derived from the native protein, wherein the amino acid sequence has been modified by single or multiple amino acid substitution, addition (e.g the fusion proteins described above) and/or deletion and also sequences where the amino acids have been chemically modified, including by deglycosylation or glycosylation, but which nonetheless retain functional activity, ie which are capable of raising antibodies which bind to proteins of the invention and/or which exhibit similar F-actin binding properties to 1-Afadin described herein. Functionally-equivalent variants include natural biological variants (e.g. from different genera or species or allelic or geographical variants within a species) and derivatives prepared using known techniques.

[0014] The protein of the present invention can be isolated from human or other animal organs, cell lines and so on by the known methods, particularly by methods described herein and such method of preparing proteins of the invention, comprising the steps described in the Examples, form a further aspect of the invention. Proteins obtainable by such methods form a further aspect of the invention. When the protein is used in the form of a peptide, it can also be prepared by chemical synthesis based on the amino acid sequence provided by the present invention. Alternatively, it can be obtained by production in vitro using recombinant DNA techniques with a cDNA fragment provided by the present invention.

[0015] For example, when the protein is obtained by recombinant DNA techniques, nucleic acid fragments, such as a cDNA fragment can be inserted into an appropriate expression vector, and the protein of the present invention can be mass-expressed from the cells (such as Escherichia coli, Bacillus subtilis, yeast, animal cell and the like) which are transformed with the recombinant vector. Specifically, for example, when the protein is expressed in a microorganism such as E. coli, an expression vector is prepared by inserting the cDNA of the present invention into an expression vector having an origin which can be replicated in the microorganism, a promoter, a ribosome-binding site, cDNA cloning site, and a terminator. Host cells are transformed with the expression vector, and then the obtained transformant is cultured so that the protein encoded by the cDNA is mass-produced in the microorganism. Alternatively, the protein can be expressed as a fusion protein with another protein. The simple protein encoded by the cDNA can be obtained by cleaving the obtained fusion protein with an appropriate protease. On the other hand, when it is desired to express the protein of the present invention in animal cells, the cDNA fragment is inserted into an expression vector for animal cells having a promoter for animal cells, a splicing region, a poly (A)-addition site, and then the vector is introduced so that the protein of the present invention is expressed in the animal cells.

[0016] The above mentioned cloning and expression vectors containing nucleic acid molecules of the invention (as defined below), methods for preparing recombinant nucleic acid molecules according to the invention (comprising inserting nucleotide sequences encoding the protein into vector nucleic acid), transformed or transfected prokaryotic or eukaryotic host cells containing such vectors and the synthetic polypeptides expressed by these systems form further aspects of the invention.

[0017] In a further aspect, the present invention provides a genomic DNA sequence (or a nucleic acid molecule containing said sequence) which is a gene of a human or other animal which encodes the above protein, for example to which the cDNA or a partial sequence thereof is complementary or which hybridizes thereto.
Appropriate hybridizing conditions are described below. For example, it can be isolated from any genome library using a cDNA of the present invention or a partial sequence thereof as a probe.

[0018] In a yet further aspect the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding the proteins of the invention. Such nucleic acid molecules may be single or double stranded DNA, cDNA or RNA, preferably DNA, and include degenerate, substantially homologous and hybridising sequences which are capable of coding for the protein/polypeptide/peptide of the ivention.

[0019] For example, the invention provides a cDNA molecule encoding a protein having the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence substantially the same as that of SEQ ID NO:1. In reference to nucleic acid molecules of the invention, "substantially homologous" refers to sequences displaying at least 80%, preferably at least 85 or 90% sequence homology. Hybridizing sequences include within their scope those sequences binding under conditions of high stringency, e.g. 2XSSC, 65°C (where SSC = 0.15M NaCl, 0.015M sodium citrate, pH 7.2) as well as those which but for the degeneracy of the code would hybridize under the above-mentioned conditions.

[0020] A clone of the cDNA of the present invention can easily be obtained by screening a cDNA library prepared from rat by means of an oligonucleotide probe synthesized on the basis of the base sequence of the fragment. Alternatively, using the oligonucleotide as a primer, the desired cDNA can be synthesized by the polymerase chain reaction (PCR) method. Generally, polymorphism is frequently observed in animal genes as a result of individual variation. Therefore, it is to be appreciated that any cDNA having a single or plural addition, deletion and/or substitution of a nucleotide by other nucleotide is included in the present invention. Similarly, as mentioned above, any protein having a single or plural addition, deletion and/or substitution of amino acid by another amino acid is included within the scope of the present invention, insofar as it has an activity of the protein having the amino acid sequence of SEQ ID NO: 1, ie. a functionally equivalent variant thereof.

[0021] The cDNA encoding a partial amino acid sequence, a ie. peptide fragment, of the present invention is a continuous sequence of 10 bps or more (e.g. oligonucleotides with 10-20 base pairs). DNA fragments (sense strand and antisense strand) comprising the continuous sequence are also included in the scope of the present invention. These DNA fragments can be used as probes for gene diagnosis.

[0022] In a yet still further aspect the present invention provides an antibody prepared using the actin filament-binding protein 1-Afadin as an immunogen. Antibodies of the invention include also antigen-binding fragments of the antibodies (e.g. $F(ab)^2$, Fab and Fv fragments ie. fragments of the variable region of the antibody. Antibodies of the invention may be obtained as polyclonal antibodies or monoclonal antibodies by known methods using the above-described protein itself or a partial peptide (ie. fragment thereof) as the antigen.

[0023] The present invention will now be described by way of the following Examples, which should not be construed as a limitation upon the scope of the present invention, with reference to the Figures, in which:-

Figure 1 shows the results of Mono Q column chromatography: (a) absorption at 280 nm (A_{280}); (b) blot overlay with ^{125}I-labelled F-actin; and (c) protein staining after SDS-PAGE;
Figure 2 shows a schematic drawing of the cDNA of l-Afadin (p205) and that of s-Afadin (p190);
Figure 3 shows (a) the F-actin-binding activity of various fragments of recombinant l-Afadin: on the left is the results of the ^{125}I-labelled F-actin blot overlay; on the right is the result of Western blot analysis, and (b) schematic drawings of the structures of l-Afadin and s-Afadin;
Figure 4 shows results of analysis of tissue distribution of l-Afadin: (a) Northern blot analysis, and (b) Western blot analysis with (b1) anti-l-Afadin antibody and (b2) anti-1-Afadin/s-Afadin antibody;
Figure 5 shows the biochemical properties of l-Afadin including (a) the inhibition of F-actin binding activity of l-Afadin by myosin S1, (b) the increase in viscosity of F-actin by l-Afadin, and (c) the binding of His6-l-Afadin-C to F-actin;
Figure 6 shows photographs indicating the localization of l-Afadin, E-cadherin and vinculin in various rat and mouse tissues;
Figure 7 shows photographs indicating the localization of 1-Afadin and ZO-1 in EL cells;
Figure 8 shows photographs indicating the different localizations of l-Afadin, ZO-1 and desmoplakin; and
Figure 9 shows photographs indicating the localization of l-Afadin in rat small intestine.

Example 1: Identification and purification of the actin-binding protein l-Afadin

[0024] Growth cones were isolated from rat fetal brain and subjected to the blot overlay method (Cell Motil. Cytoskeleton, 18:164-179, 1991) with ^{125}I-labelled F-actin to identify a band corresponding to a molecular weight of 205 kDa (p205). The result of the competition experiments showed that the protein bound specifically to F-actin but did not bind to G-actin (actin monomer), indicating that the protein was an F-actin-binding protein.

[0025] Next, the soluble fraction of rat fetal brain was subjected to SDS-PAGE and the protein band with a molecular weight (Mr) of 205 kDa was purified by column chromtographies such as Q-Sepharose, phenyl-5PW, hydroxyapatite, Mono Q. The result of the final Mono Q column chromatography is shown in Figure 1. In Figure 1, (a) shows the absorption at 280 nm, (b) shows the result of blot overlay with ^{125}I-labelled F-actin and (c) shows the protein bands stained with Coomassie brilliant blue. As shown in Figure 1 (c), the purified protein finally gave bands with a Mr of about 205 kDa (p205) and of about 190 kDa (p190) . Then, the two purified proteins were excised from the polyacrylamide gel, subjected to limited digestion with a protease (lysyl endopeptidase) and subjected to peptide mapping. Five peptides common to the two proteins were isolated and partial amino acid sequences thereof were separately determined. As a result of a homology search using a sequence data base, it was confirmed that the five peptide peaks were significantly homologous to those of human AF-6 protein. On the other hand, the amino acid sequence of the two peptide peaks specific to p205 were not found in current protein data base. The results suggested that p205 and p190 were human AF-6 protein-related rat proteins, and p190 was a splicing variant, a homologue, or a degradative product of p205. In addition, since the p205 was localized in the AJ site, the protein was named "a large splicing variant of AF-6 protein localized at adherens junction: l-Afadin" (hereinafter, the protein of the present invention is referred to as l-Afadin or p205).

Example 2: Cloning of a gene for the actin filament -binding protein "l-Afadin"

[0026] Based on the partial amino acid sequences of the 205 kDa protein l-Afadin obtained in Example 1, 7 oligonucleotide probes were prepared and used for screening a rat brain cDNA library. As a result, several overlapping clones as shown in Figure 2 were obtained. The result of sequencing indicated that, among these clones, clone 20 contained a coding region with about 4.9 kbp and the amino acid sequence deduced from this coding region included the whole peptides of p205. In addition, 2 peptides specific to p205 were localized in the C-terminal. Clone 94 contained a coding region of about 4.5kbp encoding p190. However, these clones 20 and 94 did not contain the initiation codon, which was contained in clone 84. Therefore, the full-length cDNA for p205 was constructed from clones 84 and 20, and the full-length cDNA for p190 from clones 84 and 94.

[0027] FISH analysis (Cytogenet. Cell Genet. 61:282-285,1992; Electrophoresis 16:261-272, 1995) using the clones 20, 84 and 94 as probes indicated that these cDNAs were localized on rat chromosome lq12.2.

Example 3: Expression of F-actin-binding protein 1-Afadin in animal cells

[0028] The p205 cDNA prepared in Example 2 was inserted into an expression vector, and transfected into COSI cells. The cell extract was subjected to the blot overlay method with ^{125}I-labelled F-actin. The recombinant protein (myc-l-afadin) showed a mobility on SDS-PAGE and binding activity to ^{125}1-labelled F-actin similar to that of native p205, as shown in Figure 3(a). On the other hand, the deletion mutant of p205 lacking the C-terminal 156 amino acid did not show the F-actin-binding activity. In contrast, a fusion protein of the C-terminal (199 amino acid residues) of p205 and GTS (glutathione-S-transferase) did show the ^{125}I-labelled F-actin-binding activity.

[0029] From the above results, it was confirmed that the p205 gene encodes a protein of 1,829 amino acids as showed in SEQ ID NO: 1, had an estimated molecular weight of 207,667 and had an F-actin-binding domain on 199 amino acid residues in the C-terminal. Further, it was concluded that the p190 gene encodes a protein lacking about 160 amino acid residues in the C-terminal and was a splicing variant of the p205 gene.

[0030] A computer homology search revealed that the amino acid sequence of p190 showed 90% identity over the entire sequence of human AF-6 protein. However, human AF-6 protein and p-190 lacked the C-terminal region of p205. Further, the C-terminal F-actin-binding domain showed no significant homology to any other F-actin-binding protein. Therefore, it was confirmed that, while p190 is likely to be a rat counterpart of human AF-6, p205 is a novel F-actin-binding protein. As shown in Figure 3(b), both p205 and pl90 had one PDZ domain.

Example 4: Preparation of anti-1-Afadin antibody

[0031] According to known methods and using a synthetic peptide corresponding to amino acid residues 1814-1829 of SEQ ID NO:1 as an immunogen, a rabbit polyclonal antibody specifically recognizing l-Afadin was prepared. Also, using a synthetic peptide corresponding to amino acid residues 557-592 of SEQ ID NO: 1 as an immunogen, a rabbit polyclonal antibody specifically recognizing both l-Afadin and S-Afadin was prepared.

Example 5: Confirmation of tissues expressing l-Afadin

[0032] Northern blot analysis using a sequence specific to l-Afadin cDNA as a probe indicated that l-Afadin was ubiquitously expressed in all the rat tissues examined, including heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis, as shown in Figure 4(a).

[0033] Further, it was confirmed, by Western blot analysis using the anti-l-Afadin antibody prepared in Example 4, that l-Afadin was expressed in all the rat tissues, as shown in Figure 4 (bl). However, as shown in Figure 4 (b2), it was confirmed by Western blot analysis using the antibody recognizing both l-Afadin and s-Afadin that of all the organs tested, s-Afadin was only expressed in brain.

[0034] From the above results, it was confirmed that, while s-Afadin was expressed only in brain, l-Afadin of the present invention was ubiquitously expressed.

Example 6: Biochemical characteristic of I-Afadin

[0035] The blot overlay study for the actin-binding ability of the 205 kDa protein (I-Afadin) obtained in Example 1 revealed that the binding of I-Afadin to F-actin was specifically inhibited by myosin S1 (Figure 5(a)), but the inhibition disappeared by the addition of Mg ATP. Since myosin S1 is a protein which is confirmed as binding laterally to F-actin (Science 261:58-65, 1993; Nature 364:171-174, 1993) and Mg ATP is known to dissociate F-actin-myosin complex (Biochemistry 14:2207-2214, 1975), it was confirmed that I-Afadin binds along the side of F-actin.

[0036] Next, a change in viscosity of F-actin by I-Afadin was studied by the falling ball method (Methods Enzymol. 85:211-233, 1982; J. Biol. Chem. 271:31775-31778, 1996). It was found that, I-Afadin increased dose-dependently the viscosity of F-actin, up to a viscosity of about 3 times the maximum, as shown in Figure 5(b).

[0037] In addition, by stoichiometric calculation it was determined that His6-I-Afadin-C binds to F-actin at a ratio of 1 molecule per about 500 molecules of F-actin and that the Kd value was in a order of 10^{-7}M (molar) (Figure 5(c)).

[0038] Further, the effect of I-Afadin on F-actin was examined using pyrene-conjugated actin. It was found that 1-Afadin does not affect the actin polymerisation.

Example 7: Localization of I-Afadin

[0039] Using the anti-I-Afadin antibody, frozen slices of various mouse and rat tissues were observed with confocal microscopy to identify the localization of I-Afadin.

[0040] In liver, I-Afadin was localized in a belt-like junctional complex region along the bile canaliculi (Figure 6(a)). In the small intestine, which was doubly stained with the anti-E-cadherin monoclonal antibody, L-Afadin was detected in a junctional complex region of intestinal absorptive epithelium together with E-cadherin, but was more concentrated in the region than E-cadherin was (Figure 6(bl) - (b3) and (cl) - (c3)). Heart was doubly stained with the anti-vinculin monoclonal antibody. Vinculin is been known as a marker for not only cell-to-cell AJ but also for cell-to-matrix AJ (Cell 18:193-205, 1979; Biochem. Biophys. Acta 737:305-341, 1983). I-Afadin was found to co-localize with vinculin at intercalated discs (Figure 6(dl) - (d3)). However, while vinculin was also periodically located along the lateral border of cardiac muscle cells, I-Afadin was not detected in this region. In addition, when cultured EL cells expressing E-cadherin (Nature 329:341-343, 1987) were doubly stained with the anti-ZO-1 antibody, the localization of I-Afadin was similar to that of ZO-1 (Figure 7(a) and (b)). Since ZO-1 is known to be concentrated at cadherin-based spot-like cell-to-cell AJ in fibroblast (J. Cell Biol. 115:1149-1462, 1991; J. Cell Biol. 121:491-502, 1993), it was suggested that 1-Afadin is also localized at cadherin-based cell-to-cell AJ.

[0041] Further, in order to examine the precise localization of I-Afadin, frozen sections of small intestine were doubly stained with the anti-ZO-1 monoclonal antibody and the anti-I-Afadin antibody. In addition, liver bile canaliculi were doubly stained with the anti-desmoplakin monoclonal antibody and the anti-I-Afadin antibody. ZO-1 is known to be a marker for tight junctions in intestinal absorptive epithelium (J. Cell Biol. 103:755-766, 1986; J. Cell Biol. 121:491-502, 1993) and desmoplakin is known to be a marker for desmosomes (J. Cell Biol. 63:515-523, 1974; Eur. J. Cell Biol. 32: 117-130, 1983; J. Mol. Biol. 163:647-671, 1983; EMBO J. 6:885-889, 1987). The results showed that, in the absorptive epithelia of small intestine, I-Afadin was localized slightly more it the basal side than ZO-1 (Figure 8(al) - (a3)). In the bile duct, the localisation of I-Afadin did not coincide with that of desmoplakin (Figure 8(b1)-(b3)).

[0042] These results indicate that I-Afadin is localized at cell-to-cell AJ rather than at tight junctions or desmosomes. Further, according to immunoelectron microscopy, it was observed that I-Afadin was localized in cell-to-cell AJ of absorptive epithelia of small intestine (Figure 9(a) and (b)).

[0043] Accordingly, it was confirmed that I-Afadin of the present invention is a novel protein uniting the actin cytoskeleton and cell-to-cell AJ.

[0044] As described above in detail, the present invention provides a novel actin filament-binding protein I-Afadin localized at the cadherin cell-to-cell adherens junction, the antibody specifically detecting I-Afadin, and the genetic materials for industrially utilizing I-Afadin.

Annex to the description

[0045]

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(F) POSTAL CODE (ZIP):

(ii) TITLE OF INVENTION: Protein

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 257043/1997
(B) FILING DATE: 22-SEP-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1829 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rat
(F) TISSUE TYPE: fetal brain

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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1               5                   10                  15

Ile His His Trp Asn Ala Asn Arg Leu Asp Leu Phe Glu Ile Ser Gln
            20                  25                  30

Pro Thr Glu Asp Leu Glu Phe His Gly Val Met Arg Phe Tyr Phe Gln
        35                  40                  45

Asp Lys Ala Ala Gly Asn Phe Ala Thr Lys Cys Ile Arg Val Ser Ser
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Thr Ala Thr Thr Gln Asp Val Ile Glu Thr Leu Ala Glu Lys Phe Arg
65                  70                  75                  80
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Pro Asp Met Arg Met Leu Ser Ser Pro Lys Tyr Ser Leu Tyr Glu Val
                85                  90                  95

His Val Ser Gly Glu Arg Arg Leu Asp Ile Asp Glu Lys Pro Leu Val
            100                 105                 110

Val Gln Leu Asn Trp Asn Lys Asp Asp Arg Glu Gly Arg Phe Val Leu
        115                 120                 125

Lys Asn Glu Asn Asp Ala Ile Pro Ala Lys Lys Ala Gln Ser Asn Gly
    130                 135                 140

Pro Glu Lys Gln Glu Lys Glu Gly Val Ile Gln Asn Phe Lys Arg Thr
145                 150                 155                 160

Leu Ser Lys Lys Glu Lys Lys Glu Lys Lys Lys Arg Glu Lys Glu Ala
                165                 170                 175

Leu Arg Gln Ala Ser Asp Lys Glu Glu Arg Pro Ser Gln Gly Asp Asp
            180                 185                 190

Ser Glu Asn Ser Arg Leu Ala Ala Glu Val Tyr Lys Asp Met Pro Glu
        195                 200                 205

Thr Ser Phe Thr Arg Thr Ile Ser Asn Pro Glu Val Val Met Lys Arg
    210                 215                 220

Arg Arg Gln Gln Lys Leu Glu Lys Arg Met Gln Glu Phe Arg Ser Ser
225                 230                 235                 240

Asp Gly Arg Pro Asp Ser Gly Gly Thr Leu Arg Ile Tyr Ala Asp Ser
                245                 250                 255

Leu Lys Pro Asn Ile Pro Tyr Lys Thr Ile Leu Leu Ser Thr Thr Asp
            260                 265                 270
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```
Pro Ala Asp Phe Ala Val Ala Glu Ser Leu Glu Lys Tyr Gly Leu Glu
        275                 280                 285

Lys Glu Asn Pro Lys Asp Tyr Cys Ile Ala Arg Val Met Leu Pro Pro
    290                 295                 300

Gly Ala Gln His Ser Asp Glu Arg Gly Ala Lys Glu Ile Ile Leu Asp
305                 310                 315                 320

Asp Asp Glu Cys Pro Leu Gln Ile Phe Arg Glu Trp Pro Ser Asp Lys
                325                 330                 335

Gly Ile Leu Val Phe Gln Leu Lys Arg Arg Pro Pro Asp Tyr Ile Pro
            340                 345                 350

Lys Lys Met Lys Lys His Val Glu Gly Lys Pro Leu Lys Gly Lys Asp
        355                 360                 365

Arg Ala Asp Gly Ser Gly Tyr Gly Ser Ala Leu Pro Pro Glu Lys Leu
    370                 375                 380

Pro Tyr Leu Val Glu Leu Ser Pro Gly Arg Arg Asn His Phe Ala Tyr
385                 390                 395                 400

Tyr Ser Tyr His Thr Tyr Glu Asp Gly Ser Asp Ser Arg Asp Lys Pro
                405                 410                 415

Lys Leu Tyr Arg Leu Gln Leu Ser Val Thr Glu Val Gly Thr Glu Lys
            420                 425                 430

Phe Asp Asp Asn Ser Ile Gln Leu Phe Gly Pro Gly Ile Gln Pro His
        435                 440                 445

His Cys Asp Leu Thr Asn Met Asp Gly Val Val Thr Val Thr Pro Arg
    450                 455                 460
```

Ser Met Asp Ala Glu Thr Tyr Val Asp Gly Gln Arg Ile Ser Glu Thr
465 470 475 480

Thr Met Leu Gln Ser Gly Met Arg Leu Gln Phe Gly Thr Ser His Val
485 490 495

Phe Lys Phe Val Asp Pro Ile Gln Asp His Val Leu Ser Lys Arg Ser
500 505 510

Val Asp Gly Gly Leu Met Val Lys Gly Pro Arg His Lys Pro Gly Ala
515 520 525

Val Gln Glu Thr Thr Phe Glu Leu Gly Gly Asp Ile His Ser Gly Thr
530 535 540

Ala Leu Pro Ala Ser Arg Ser Thr Thr Arg Leu Asp Ser Asp Arg Val
545 550 555 560

Ser Ser Ala Ser Ser Thr Ala Glu Arg Gly Met Val Lys Pro Met Ile
565 570 575

Arg Leu Asp Gln Glu Gln Asp Tyr Arg Arg Arg Glu Ser Arg Thr Gln
580 585 590

Asp Ala Ala Gly Pro Glu Leu Met Leu Pro Ala Ser Ile Glu Phe Arg
595 600 605

Glu Ser Ser Glu Asp Ser Phe Leu Ser Ala Ile Ile Asn Tyr Thr Asn
610 615 620

Ser Ser Thr Val His Phe Lys Leu Ser Pro Thr Tyr Val Leu Tyr Met
625 630 635 640

Ala Cys Arg Tyr Val Leu Ser Ser Gln His Arg Pro Asp Ile Ser Pro
645 650 655

Thr Glu Arg Thr His Lys Ala Ile Ala Val Val Asn Lys Met Val Ser
660 665 670

Met Met Glu Gly Val Ile Gln Glu Val Asp Gln Val Asp Gln Lys Gln
675 680 685

Lys Asn Ile Ala Gly Ala Leu Ala Phe Trp Met Ala Asn Ala Ser Glu
690 695 700

Leu Leu Asn Phe Ile Lys Gln Asp Arg Asp Leu Ser Arg Ile Thr Leu
705 710 715 720

Asp Ala Gln Asp Val Leu Ala His Leu Val Gln Met Ala Phe Lys Tyr
725 730 735

Leu Val His Cys Leu Gln Ser Glu Leu Asn Asn Tyr Met Pro Ala Phe
740 745 750

Leu Asp Asp Pro Glu Glu Asn Ser Leu Gln Arg Pro Lys Ile Asp Asp
755 760 765

Val Leu His Thr Leu Thr Gly Ala Met Ser Leu Leu Arg Arg Cys Arg
770 775 780

Val Asn Ala Ala Leu Thr Ile Gln Leu Phe Ser Gln Leu Phe His Phe
785 790 795 800

Ile Asn Met Trp Leu Phe Asn Arg Leu Val Thr Asp Pro Asp Ser Gly
805 810 815

Leu Cys Ser His Tyr Trp Gly Ala Ile Ile Arg Gln Gln Leu Gly His
820 825 830

Ile Glu Ala Trp Ala Glu Lys Gln Gly Leu Glu Leu Ala Ala Asp Cys
835 840 845

His Leu Ser Arg Ile Val Gln Ala Thr Thr Leu Leu Thr Met Asp Lys
850 855 860

Tyr Val Pro Asp Asp Ile Pro Asn Ile Asn Ser Thr Cys Phe Lys Leu
865 870 875 880

Asn Ser Leu Gln Leu Gln Ala Leu Leu Gln Asn Tyr His Cys Ala Pro
885 890 895

Asp Glu Pro Phe Ile Pro Thr Asp Leu Ile Glu Asn Val Val Ala Val
900 905 910

Ala Glu Asn Thr Ala Asp Glu Leu Ala Arg Ser Asp Gly Arg Asp Val
915 920 925

Gln Leu Glu Glu Asp Pro Asp Leu Gln Leu Pro Phe Leu Leu Pro Glu
930 935 940

Asp Gly Tyr Ser Cys Asp Val Val Arg Asn Ile Pro Asn Gly Leu Gln
945 950 955 960

Glu Phe Leu Asp Pro Leu Cys Gln Arg Gly Phe Cys Arg Leu Val Pro
965 970 975

His Thr Arg Ser Pro Gly Thr Trp Thr Ile Tyr Phe Glu Gly Ala Asp
980 985 990

Tyr Glu Ser His Leu Met Arg Glu Asn Thr Glu Leu Thr Gln Pro Leu
995 1000 1005

Arg Lys Glu Pro Glu Val Ile Thr Val Thr Leu Lys Lys Gln Asn Gly
1010 1015 1020

Met Gly Leu Ser Ile Val Ala Ala Lys Gly Ala Gly Gln Asp Lys Leu
1025 1030 1035 1040

```
Gly Ile Tyr Val Lys Ser Val Val Lys Gly Gly Ala Ala Asp Val Asp
                1045                1050                1055

Gly Arg Leu Ala Ala Gly Asp Gln Leu Leu Ser Val Asp Gly Arg Ser
            1060                1065                1070

Leu Val Gly Leu Ser Gln Glu Arg Ala Ala Glu Leu Met Thr Arg Thr
        1075                1080                1085

Ser Ser Val Val Thr Leu Glu Val Ala Lys Gln Gly Ala Ile Tyr His
    1090                1095                1100

Gly Leu Ala Thr Leu Leu Asn Gln Pro Ser Pro Met Met Gln Arg Ile
1105                1110                1115                1120

Ser Asp Arg Arg Gly Ser Gly Lys Pro Arg Pro Lys Ser Glu Gly Phe
                1125                1130                1135

Glu Leu Tyr Asn Asn Ser Ala Gln Asn Gly Ser Pro Glu Ser Pro Gln
            1140                1145                1150

Met Pro Trp Thr Glu Tyr Ser Glu Pro Lys Lys Leu Pro Gly Asp Asp
        1155                1160                1165

Arg Leu Met Lys Asn Arg Ala Asp His Arg Ser Ser Pro Asn Val Ala
    1170                1175                1180

Asn Gln Pro Pro Ser Pro Gly Gly Lys Ser Pro Tyr Thr Ser Gly Thr
1185                1190                1195                1200

Ala Ala Lys Ile Thr Ser Val Ser Thr Gly Asn Leu Cys Thr Glu Glu
                1205                1210                1215

Gln Thr Pro Pro Pro Arg Pro Glu Ala Tyr Pro Ile Pro Thr Gln Thr
            1220                1225                1230
```

Tyr Thr Arg Glu Tyr Phe Thr Phe Pro Ala Ser Lys Ser Gln Asp Arg
1235 1240 1245

Met Ala Pro Val Gln Asn Gln Trp Pro Asn Tyr Glu Glu Lys Pro His
1250 1255 1260

Met His Thr Glu Ser Asp His Ala Ser Ile Ala Ile Gln Arg Val Thr
1265 1270 1275 1280

Arg Ser Gln Glu Glu Leu Arg Glu Glu Lys Val Tyr Gln Leu Glu Arg
1285 1290 1295

His Arg Val Glu Ser Gly Met Asp Arg Lys Cys Asp Ser Asp Met Trp
1300 1305 1310

Ile Asn Gln Ser Ser Ser Val Glu Ser Ser Thr Ser Ser Gln Glu His
1315 1320 1325

Leu Asn His Ser Ser Lys Ser Val Thr Pro Ala Ser Thr Leu Thr Lys
1330 1335 1340

Ser Gly Pro Gly Arg Trp Lys Thr Pro Ala Ala Val Leu Pro Thr Pro
1345 1350 1355 1360

Val Ala Val Ser Gln Pro Ile Arg Thr Asp Leu Pro Pro Pro Pro Pro
1365 1370 1375

Pro Pro Pro Ala His Tyr Thr Ser Asp Phe Asp Gly Ile Ser Met Asp
1380 1385 1390

Leu Pro Leu Pro Pro Pro Pro Ala Asn Gln Ala Ala Pro Gln Ser Ala
1395 1400 1405

Gln Val Ala Ala Ala Glu Arg Lys Lys Arg Glu Glu His Gln Arg Trp
1410 1415 1420

Tyr Glu Lys Glu Lys Ala Arg Leu Glu Glu Glu Arg Glu Arg Lys Arg
1425 1430 1435 1440

Arg Glu Gln Glu Arg Lys Leu Gly Gln Met Arg Thr Gln Ser Leu Asn
1445 1450 1455

Pro Ala Ser Phe Ser Pro Leu Ala Thr Gln Ala Lys Pro Glu Lys Pro
1460 1465 1470

Ser Thr Leu Gln Arg Pro Gln Glu Thr Val Ile Arg Glu Leu Gln Pro
1475 1480 1485

Gln Gln Gln Pro Arg Thr Ile Glu Arg Arg Asp Leu Gln Tyr Ile Thr
1490 1495 1500

Ile Ser Lys Glu Glu Leu Ser Ser Gly Asp Ser Leu Ser Pro Asp Pro
1505 1510 1515 1520

Trp Lys Arg Asp Ala Arg Glu Lys Leu Glu Lys Gln Gln Gln Met His
1525 1530 1535

Ile Val Asp Met Leu Ser Lys Glu Ile His Glu Leu Gln Asn Lys Gly
1540 1545 1550

Asp Arg Thr Ala Glu Glu Ser Asp Arg Leu Arg Lys Leu Met Leu Glu
1555 1560 1565

Trp Gln Phe Gln Lys Arg Leu Gln Glu Ser Lys Gln Lys Asp Glu Asp
1570 1575 1580

Asp Asp Glu Glu Glu Asp Asp Asp Val Asp Thr Met Leu Ile Met Gln
1585 1590 1595 1600

Arg Leu Glu Ala Glu Arg Arg Ala Arg Leu Gln Asp Glu Glu Arg Arg
1605 1610 1615

Arg Gln Gln Gln Leu Glu Glu Met Arg Lys Arg Glu Val Glu Asp Arg
1620 1625 1630

Val Arg Gln Glu Glu Asp Gly Arg His Gln Glu Glu Glu Arg Val Lys
1635 1640 1645

Arg Asp Ala Glu Glu Lys Arg Arg Gln Glu Glu Gly Tyr Tyr Ser Arg
1650 1655 1660

Leu Glu Ala Glu Arg Arg Arg Gln His Glu Glu Ala Ala Arg Arg Leu
1665 1670 1675 1680

Leu Glu Pro Glu Glu Pro Gly Leu Ser Arg Pro Pro Leu Pro Gln Asp
1685 1690 1695

Tyr Glu Pro Pro Ser Gln Ser Ser Ala Pro Ser Ala Pro Pro Pro Pro
1700 1705 1710

Pro Gln Arg Asn Ala Ser Tyr Leu Lys Thr Gln Val Leu Ser Pro Asp
1715 1720 1725

Ser Leu Phe Thr Ala Lys Phe Val Ala Tyr Asp Asp Asp Asp Glu Glu
1730 1735 1740

Glu Asn Tyr Val Pro Ala Gly Pro Asn Ser Tyr Ser Gly Ser Ala Gly
1745 1750 1755 1760

Thr Thr Ala Gly Thr Tyr Asp Ala Pro Arg Asp Thr Arg Glu Lys Leu
1765 1770 1775

Ser Arg Ser Gln Asp Ala Asp Leu Pro Gly Ser Ser Gly Ala Pro Glu
1780 1785 1790

Asn Leu Thr Phe Arg Glu Arg Gln Arg Leu Phe Ser Gln Gly Gln Asp
1795 1800 1805

```
Val Ser Asp Lys Val Lys Ala Ser Arg Lys Leu Thr Glu Leu Glu Asn
    1810                1815                1820

Glu Leu Asn Thr Lys
1825
```

Claims

1. An actin-binding protein 1-Afadin having the amino acid sequence of SEQ ID NO: 1 or fragment, precursor or functionally equivalent variant thereof.

2. An animal protein having an amino acid sequence substantially the same as that of SEQ ID NO: 1 or fragment, precursor or functionally equivalent variant thereof.

3. A nucleic acid molecule comprising a nucleotide sequence encoding the protein or fragment, precursor or functionally equivalent variant thereof as defined in claim 1 or 2.

4. A nucleic acid molecule as claimed in claim 3 wherein said nucleic acid molecule is cDNA.

5. A cloning or expression vector containing a nucleic acid molecule as defined in claim 3 or 4.

6. A host cell transformed with or containing a nucleic acid molecule as defined in claim 3 or 4.

7. A method of preparing a protein, or fragment, precursor or functionally equivalent variant thereof, as defined in claim 1 or 2, wherein said method comprises culturing a host cell containing a nucleic acid molecule encoding said protein, under conditions whereby said protein is expressed and recovering said protein thus produced.

8. A synthetic polypeptide or fragment thereof expressed by a host cell as defined in claim 6.

9. A genomic DNA sequence to which the cDNA of claim 4 or a partial sequence thereof is complementary or hybridizable.

10. An antibody or antigen-binding fragment thereof prepared using the actin-binding protein 1-Afadin of claim 1 or 2 as an immunogen.

FIG. 1

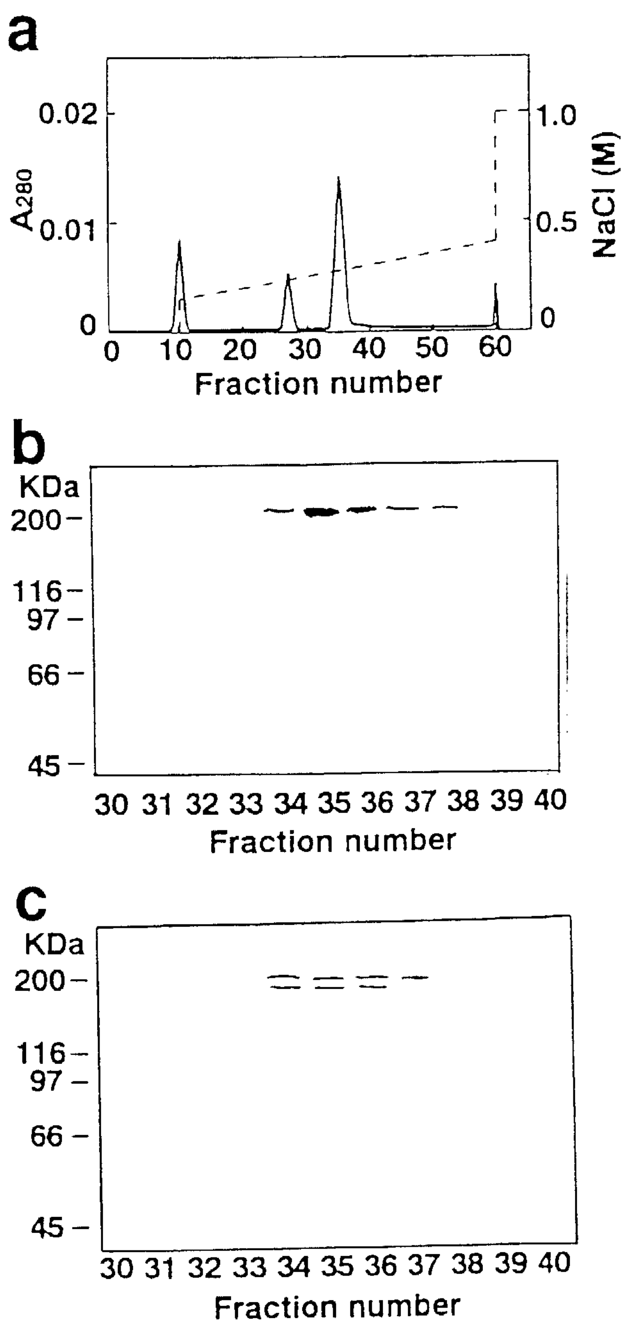

a
0.02
A_{280}
0.01
0
1.0
0.5
0
NaCl (M)
0 10 20 30 40 50 60
Fraction number
b
KDa
200-
116-
97-
66-
45-
30 31 32 33 34 35 36 37 38 39 40
Fraction number
c
KDa
200-
116-
97-
66-
45-
30 31 32 33 34 35 36 37 38 39 40
Fraction number

FIG. 2

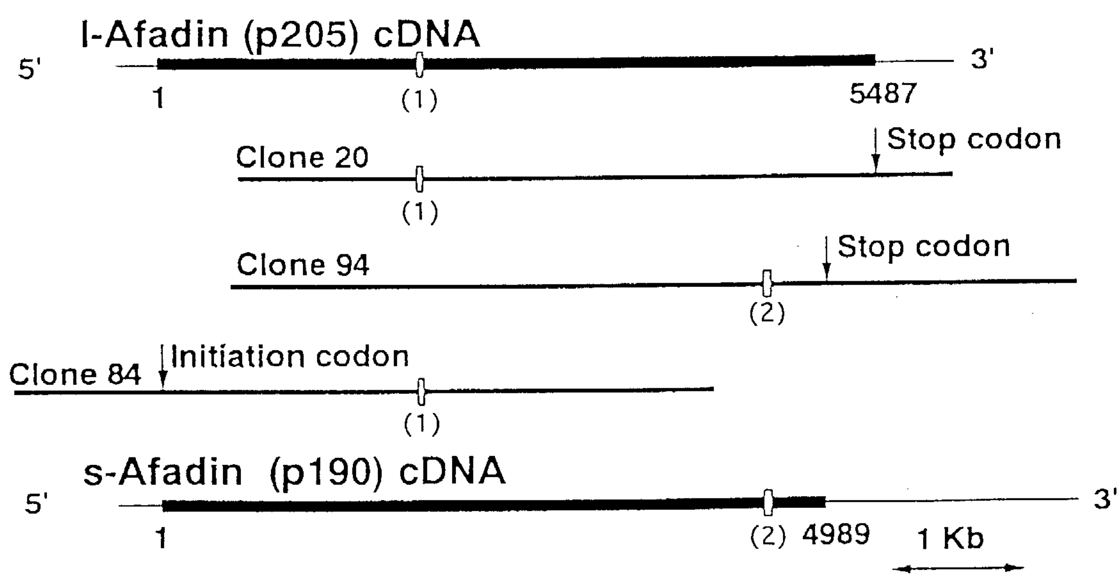

l-Afadin (p205) cDNA
5'
3'
1
(1)
5487
Clone 20
Stop codon
(1)
Clone 94
Stop codon
(2)
Clone 84
Initiation codon
(1)
s-Afadin (p190) cDNA
5'
3'
1
(2)
4989
1 Kb

FIG. 3

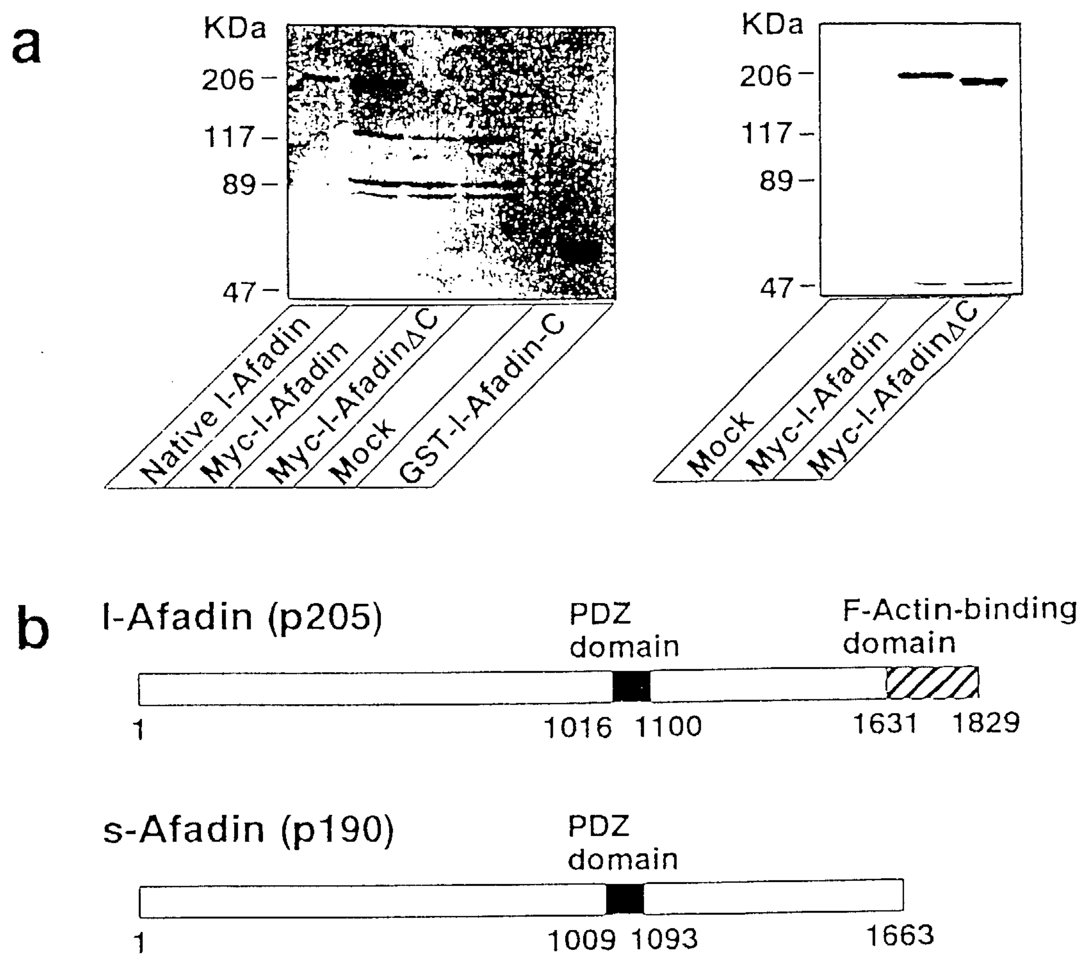

a
KDa
206
117
89
47
Native l-Afadin
Myc-l-Afadin
Myc-l-AfadinΔC
Mock
GST-l-Afadin-C
KDa
206
117
89
47
Mock
Myc-l-Afadin
Myc-l-AfadinΔC
b
l-Afadin (p205)
PDZ domain
F-Actin-binding domain
1
1016
1100
1631
1829
s-Afadin (p190)
PDZ domain
1
1009
1093
1663

FIG. 4

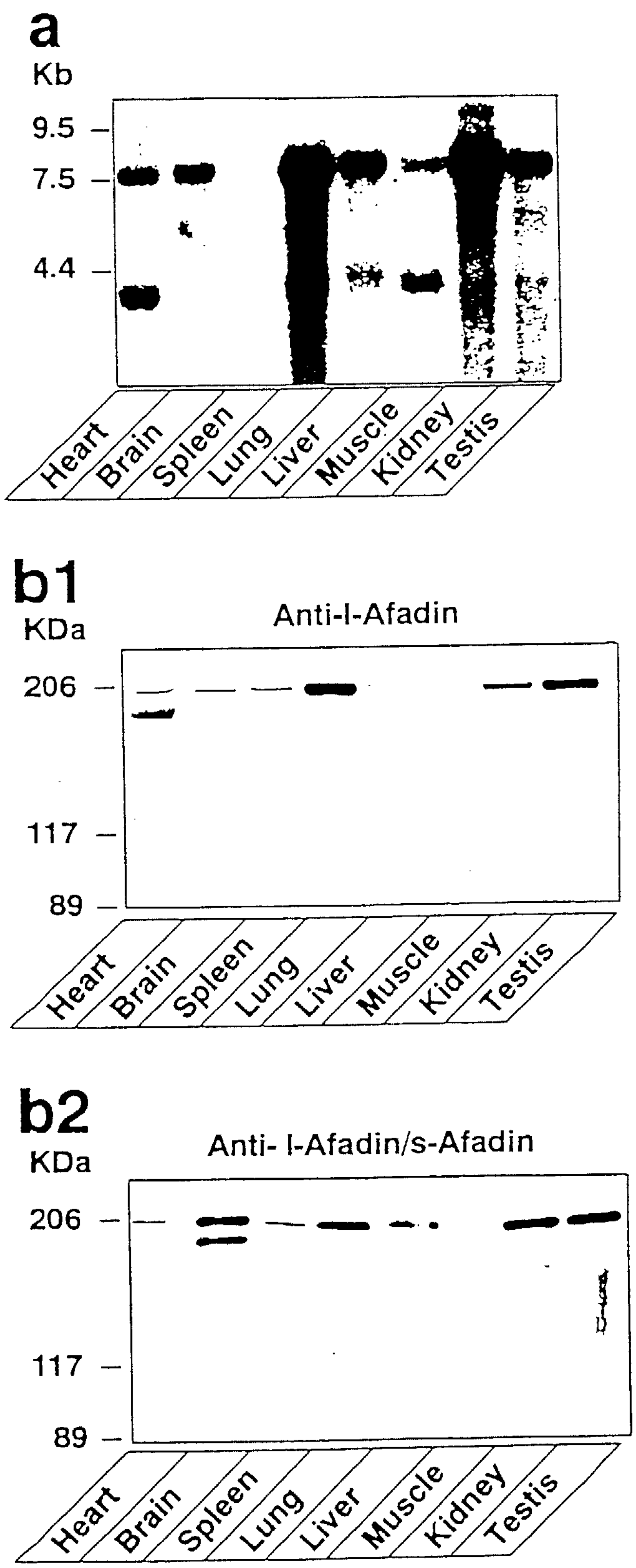

a
Kb
9.5
7.5
4.4
Heart
Brain
Spleen
Lung
Liver
Muscle
Kidney
Testis
b1
KDa
Anti-l-Afadin
206
117
89
Heart
Brain
Spleen
Lung
Liver
Muscle
Kidney
Testis
b2
KDa
Anti- l-Afadin/s-Afadin
206
117
89
Heart
Brain
Spleen
Lung
Liver
Muscle
Kidney
Testis

FIG. 5

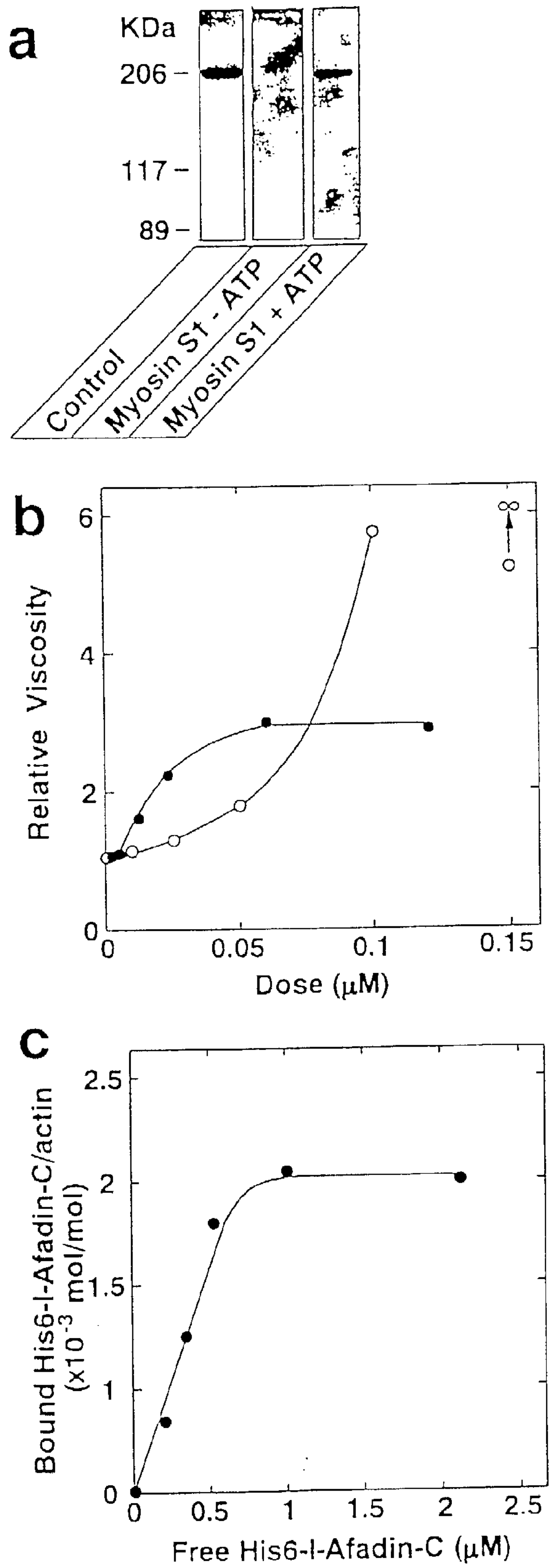
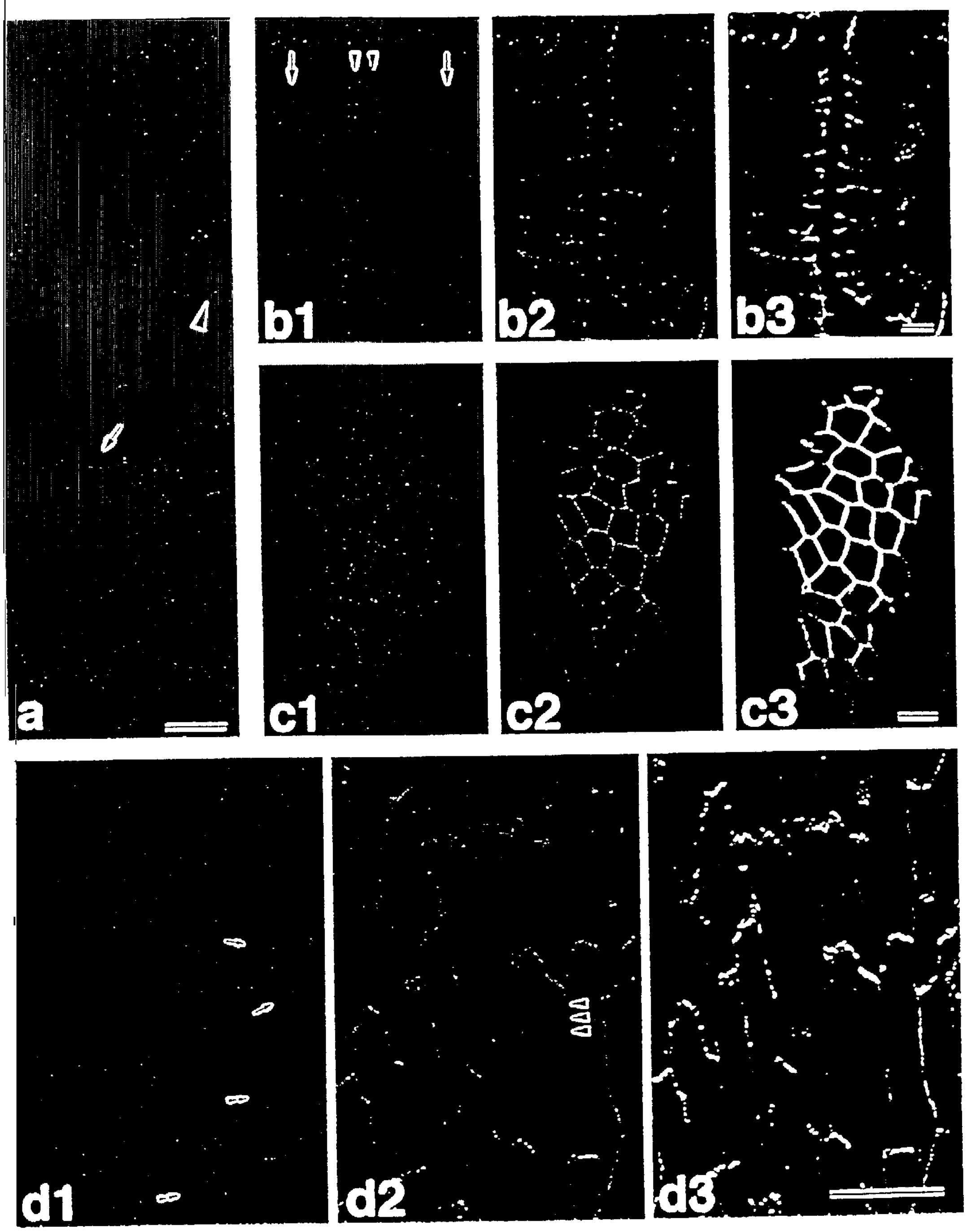

a
KDa
206
117
89
Control
Myosin S1 - ATP
Myosin S1 + ATP
b
Relative Viscosity
0
2
4
6
0
0.05
0.1
0.15
Dose (μM)
∞
c
Bound His6-I-Afadin-C/actin
(x10⁻³ mol/mol)
0
1
1.5
2
2.5
0
0.5
1
1.5
2
2.5
Free His6-I-Afadin-C (μM)

FIG. 6



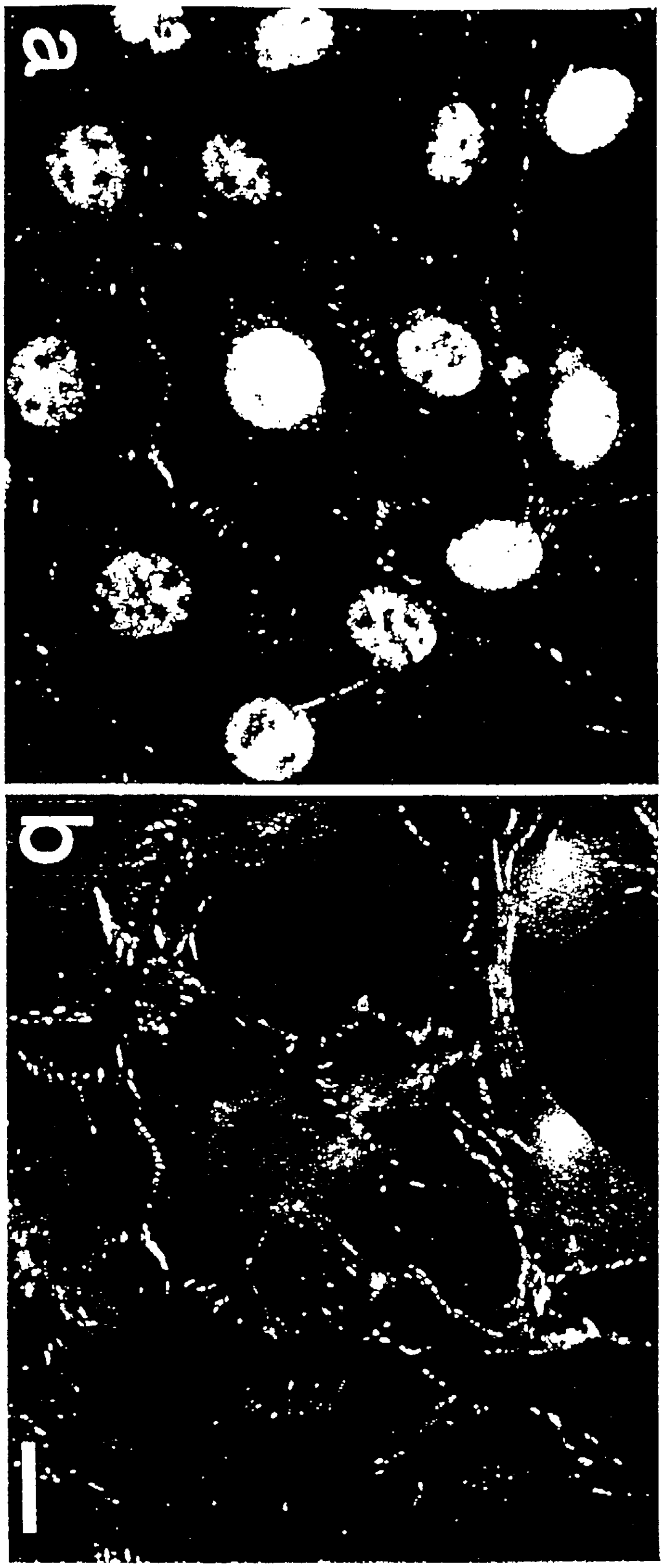


FIG. 7

FIG. 8

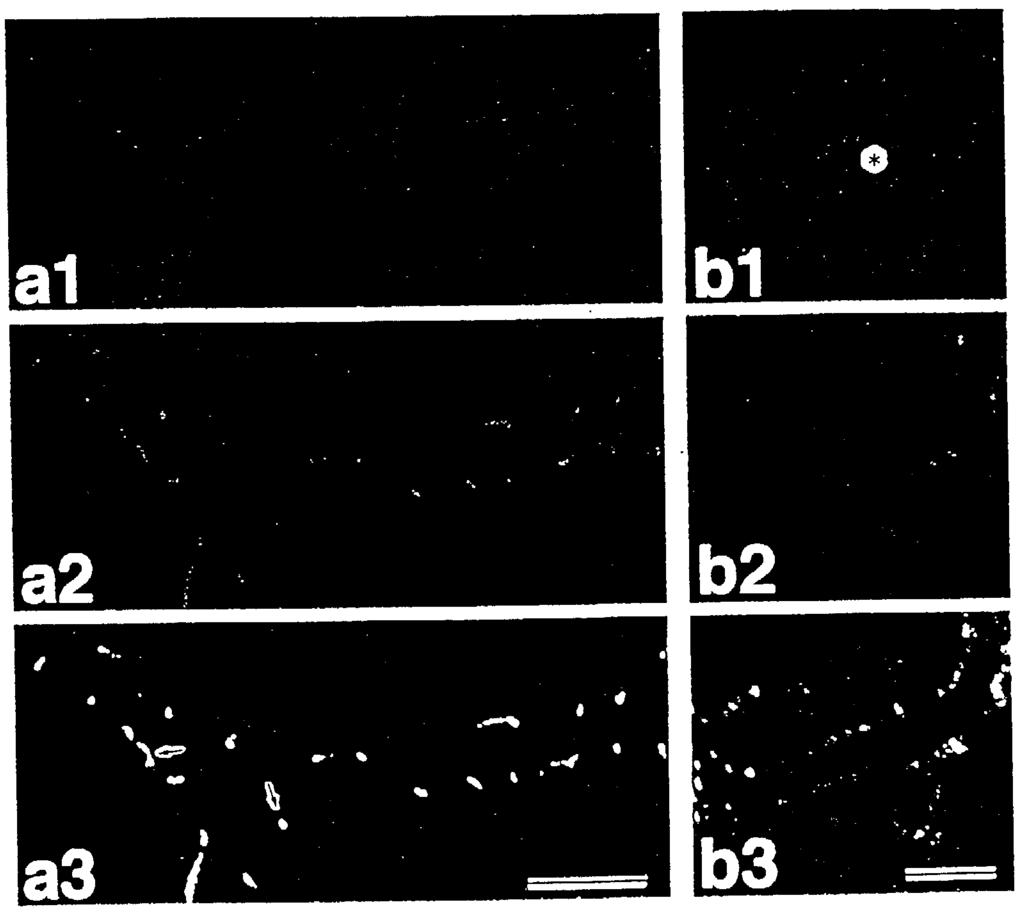


FIG. 9