The interaction of plectin with actin: evidence for cross-linking of actin filaments by dimerization of the actin-binding domain of plectin

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SUMMARY

Plectin is a major component of the cytoskeleton and is expressed in a wide variety of cell types. It plays an important role in the integrity of the cytoskeleton by cross-linking the three filamentous networks and stabilizing cell-matrix and cell-cell contacts. Sequence analysis showed that plectin contains a highly conserved actin-binding domain, consisting of a pair of calponin-like subdomains. Using yeast two-hybrid assays in combination with in vitro binding experiments, we demonstrate that the actin-binding domain of plectin is fully functional and preferentially binds to polymeric actin. The sequences required for actin binding were identified at the C-terminal end of the first calponin homology domain within the actin-binding domain of plectin. We found that the actin-binding domain of plectin is able to bundle actin filaments and we present evidence that this is mediated by the dimerization of this domain. In addition we also show that plectin and another member of the plakin family, dystonin, can heterodimerize by their actin-binding domains. We propose a new mechanism by which plectin and possibly also other actin-binding proteins can regulate the organization of the F-actin network in the cell.

Key words: Plectin, Dystrophin, Actin-binding domain, Focal contact

INTRODUCTION

Plectin is a high molecular mass protein (approx. 500 kDa) with versatile binding properties expressed in almost all cells and tissues. It interacts in vitro with intermediate filament (IF) proteins of various types, including lamins, vimentin, keratins, neurofilaments and GFAP (glial fibrillary acidic protein), and it associates with IFs in cultured cells, as demonstrated by immunoelectron microscopy (Pytela and Wiche, 1980; Foisner et al., 1988). Plectin has been shown to be a component of desmosomes (Eger et al., 1997) and hemidesmosomes (Hieda et al., 1992; Gache et al., 1996), two structures that anchor the intermediate filaments to the plasma membrane. Although the exact role of plectin in desmosomes remains to be established, it is likely that it stabilizes IF-desmoplakin bonds. In hemidesmosomes, plectin directly links IFs to the cytoplasmic domain of the β4 integrin subunit (Niessen et al., 1997; Rezniczek et al., 1998; Geerts et al., 1999). In addition to its localization in these junctions, plectin is present in focal contacts and is associated with actin stress fibers (Seifert et al., 1992; Sánchez-Aparicio et al., 1997). More recently, it has been shown that plectin crosslinks IFs with microtubules and microfilaments, which indicates that plectin is a multifunctional cytoskeleton cross-linking molecule (Svitkina et al., 1996; for a review see Steinbock and Wiche, 1999). The physiological importance of plectin is demonstrated by the fact that plectin null-mutant mice and human patients with a mutation in the plectin gene suffer from muscular dystrophy associated with epidermolysis bullosa simplex (MD-EBS) (Gache et al., 1996; McLean et al., 1996; Smith et al., 1996; Pulkkinen et al., 1996; Andrä et al., 1997). Indeed, in MD-EBS, the observed muscular dystrophy and skin blistering are thought to result from fragility of cells when subjected to mechanical stress. In skeletal muscle, plectin probably is involved in connecting IFs with actin filaments, whereas in keratinocytes it connects IFs to the plasma membrane (Gache et al., 1996; McLean et al., 1996; Smith et al., 1996; Pulkkinen et al., 1996; Andrä et al., 1997; Schroder et al., 1997; Schroder et al., 1999; Hijikata et al., 1999).

Sequence analysis of human (McLean et al., 1996) and rat (Wiche et al., 1991) plectin cDNA revealed extensive homology between plectin and other intermediate filament associated proteins (IFAPs), including desmoplakin and the bullous pemphigoid antigen BP230. Based on these sequence homologies, plectin, desmoplakin and BP230 were classified as members of a new family of proteins involved in connecting IFs to the plasma membrane, the plakins (Ruhrberg and Watt, 1997). Each of these proteins consists of a long central α-helical, coiled-coil rod domain flanked by globular end domains. The plectin C-terminal globular domain contains repeated sequences organized in six subdomains (R1-R6). Transfection studies with cDNAs encoding truncated plectin molecules have revealed that the intermediate filament-binding domain (IFBD) of plectin resides in a stretch of 50 amino acids within the R5 repeat that serves as the unique binding site for vimentin and keratin filaments (Nikolic et al., 1996). Extending

these findings we have recently shown, by a yeast two-hybrid assay, that a Gal4-fusion protein containing the IFBD of plectin can efficiently bind GFAP, vimentin and monomeric keratins 14 and 18, but not keratins 5 and 8 (Geerts et al., 1999).

The precise function of the central rod domain of plectin has not yet been established, but there is evidence that this domain mediates plectin dimerization and/or multimerization (Foisner and Wiche, 1987; Uitto et al., 1996). By RT-PCR, mRNA encoding a rod-less plectin was identified in several different rat tissues (Elliott et al., 1997). However, whether this particular mRNA is translated in vivo into a functional variant of plectin is not known. Although it was known for a long time that plectin can be colocalized with actin stress fibers in focal adhesions (Seifert et al., 1992; Sánchez-Aparicio et al., 1997), evidence for a direct binding between plectin and actin has only recently been obtained. Sequence analysis reveals an actin-binding domain (ABD) of the β-spectrin type in the N-terminal globular domain of plectin (McLean et al., 1996). This ABD is also found in other actin-binding proteins, such as dystonin, fimbrin, α-actinin and dystrophin (for a review, see Hartwig, 1994), and is composed of two consecutive calponin homology (CH) domains. Biochemical studies have identified three potential actin-binding sequences (ABS) within this ABD, termed ABS1, ABS2 and ABS3. The ABS1 and ABS3 were first identified by NMR studies of dystrophin, which showed that synthetic peptides corresponding to these two sequences bind to actin. ABS2 was originally identified in ABP120 (a *Dictyostelium* actin-gelation factor) in which it is required to mediate binding to actin (Bresnick et al., 1990).

In plectin-deficient mouse fibroblasts, an N-terminal fragment of plectin that contains the ABD was found to decorate actin stress fibers (Andrä et al., 1998). We have shown that the N-terminal fragment of plectin also interacts with the cytoplasmic domain of the β4 integrin subunit and that binding of β4 to plectin prevents actin from interacting with it (Geerts et al., 1999). Furthermore, plectin regulates actin dynamics and is involved in the reorganization of the actin cytoskeleton in response to activation of small GTPases (Andrä et al., 1998). A role of plectin in actin dynamics is also suggested by the finding that in plectin-deficient fibroblasts, as compared to wild-type cells, the reorganization of the actin cytoskeleton induced by CD95-mediated apoptosis was severely impaired (Stegh et al., 2000). The molecular mechanism by which plectin regulates actin dynamics remains unclear.

The aim of this study was to investigate which domains are involved in the specific interaction of plectin with the β-cytoplasmic actin isoform. To further establish the multifunctional role of plectin as a cytoskeletal linker protein, we have investigated whether plectin can also bind the non-muscular γ-cytoplasmic actin and the α-skeletal muscle actin. Whether plectin-ABD can bind G- and/or F-actin was studied using an in vitro binding assay and the effects of plectin-ABD on actin polymerization were investigated by kinetic analysis and electron microscopy. Our results show that plectin can dimerize by its ABD, and that this leads to the bundling of actin filaments. Furthermore, we identified dystrophin and dystonin as additional actin-binding proteins that can homodimerize by their ABD and show that plectin and dystonin can also form heterodimers by their ABD.

MATERIALS AND METHODS

cDNA constructs

All nucleotide and amino acid positions are numbered with the ATG initiation codon at position 1. Plasmid inserts were generated by polymerase chain reaction (PCR), using the proofreading *Pwo* DNA polymerase (Boehringer) and gene-specific sense and anti-sense primers containing restriction site tags. All plasmid inserts were confirmed by sequence analysis using the ^{T7}Sequencing kit (Pharmacia). The yeast galactose metabolism regulatory gene 4 (GAL4) vectors containing human epithelial plectin (U53204) cDNA subclones and full-length cDNA encoding human α-skeletal muscle actin (J00068), human γ-cytoplasmic actin (M19283) and human β-cytoplasmic actin (AB004047) are described in Figs 1B and 6. Numbers in subscript correspond to the amino acid residues of subclones encoded within the GAL4 activation domain (AD)- or binding domain (BD)-fusion proteins. Vectors used were the yeast GAL4(AD) or GAL4(BD) expression vectors pACT2 or pAS2-1, respectively (Clontech, Palo Alto, CA, USA). The templates used for PCR were cDNAs encoding full-length human actin isoforms, human plectin cDNA (clone GWIM1+2; nucleotides 1-1018, which contains the alternative exon 1c, exons 2-8 and almost the complete exon 9 of epithelial plectin cDNA; a kind gift from Dr E. B. Lane, CRC Cell Structure Group, Department of Anatomy and Physiology, University of Dundee, UK) and human dystrophin cDNA (clone pXJ10, nucleotides 1-2100, which contains exons 1-14; a kind gift from Drs R. Maatman and J. den Dunnen, Department of Human Genetics, Leiden University Medical Center, University of Leiden, the Netherlands). EST clones 41909 and 36254, encoding full-length human γ-cytoplasmic actin and 611141 and 613287, encoding full-length human β-cytoplasmic actin, were from the IMAGE cDNA clone collection, obtained from the Resource Center/Primary Database of the German Human Genome Project (RZPD, Berlin, Germany).

The plectin(1-64)-dystrophin(11-337) chimera was constructed by introducing *Nde*I sites at position 192 of the plectin cDNA, and at position 33 of the dystrophin cDNA using site-directed mutagenesis, followed by the insertion of the appropriate cDNA fragments into pAS2-1.

For use in cell transfection experiments, cDNA fragments encoding plectin$_{1\text{-}339}$ and plectin$_{65\text{-}339}$ were isolated from pAS2-1 constructs (see above) and inserted into pcDNA3HA (Geerts et al., 1999). Similarly, a cDNA fragment encoding plectin$_{1\text{-}339}$ was inserted into the bacterial maltose binding protein (MBP)-fusion protein expression vector pMAL-c2X (New England Biolabs Inc.), for the production of MBP-fusion proteins, or in pRP261, a derivative of pGEX-3x (Amrad Corp. Ltd), for the production of glutathione S-transferase (GST)-fusion proteins. The β4 integrin cDNA expression construct used for the experiments in Fig. 5 encoded β4A integrin cytoplasmic domain from residues 1115-1449 with a single amino acid substitution R1281W (β4^{R1281W}), which abrogates plectin binding, as previously described (Geerts et al., 1999).

Yeast two-hybrid assay

Yeast strain *S. cerevisiae* PJ69-4A (a gift from Dr P. James, Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI, USA), which contains the genetic markers (trp1-901, leu2-3, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2), was used as the host for the two-hybrid assay. It contains two tightly regulated reporter genes, His and Ade, which makes it suitable for the sensitive detection of protein interactions. The use of PJ69-4A was essentially as described (Schaapveld et al., 1998; Geerts et al., 1999). Equal aliquots of transformed cells were spread out on SC-LT plates containing yeast synthetic complete medium lacking leu and trp (vector markers) or on SC-LTHA plates lacking leu, trp, his and ade (vector and interaction markers). Plates were

incubated at 30°C and growth of colonies was scored after 6 and 10 or 12 days. The plating efficiencies on SC-LTHA plates, as compared with the plating efficiency on SC-LT plates was used as a measure of the strength of the signal generated by the two-hybrid interaction. Cotransformation efficiencies (on non-selective SC-LT plates) for all plasmid combinations were always at least 10^4 cfu/μg plasmid DNA, and the difference in cotransformation efficiencies never varied more than twofold between the various plasmid combinations. Expression of the Gal4-fusion proteins was confirmed by immunoblotting with the mAbs RK5C1 and C-10 (Santa Cruz Biotechnology) directed against the DNA binding domain and the transactivation domain of the Gal4 protein, respectively. Cotransformation of yeast PJ69-4A with an empty pAS2-1 and an empty pACT2 vector, with a derived pAS2-plasmid and an empty pACT2-vector, or with an empty pAS2-vector and a derived pACT2-plasmid, never resulted in the growth of colonies on selective SC-LTHA plates, showing that none of the GAL4-fusion proteins encoded by the recombinant plasmids used could by themselves cause activation of the His and Ade reporter genes. For pAS2-plectin$_{1\text{-}172}$ and pACT2-α skeletal muscle actin, a slight autonomous activation of the reporter genes was found. This could be repressed by the addition of 2 mM of 3-amino-1,2,4-triazole (a His antagonist; A8506, Sigma Chemical Co.) to the medium.

Purification of recombinant fusion proteins

The *E. coli* strain BL21(DE3) (Novagen) was transformed with recombinant plasmids and colonies obtained were used to inoculate Luria Bertani medium containing 100 μg/ml ampicillin; cultures were grown as previously described (Geerts et al., 1999). Bacteria were harvested by centrifugation at 4,000 ***g***, resuspended in PBS containing 1 mM EDTA and 1% (v/v) Triton X-100, and lysed by sonification. Lysates were cleared by centrifugation for 10 minutes at 10,000 ***g*** and 4°C, and the resulting supernatants were incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Beads with affinity-bound proteins were washed three times with PBS containing 1% (v/v) Triton X-100, and equilibrated in 50 mM Tris-HCl (pH 8.0). Bound proteins were eluted in 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione. Recombinant MBP-plectin-ABD fusion-protein was expressed and purified as described above, except that amylose resin (800-21, New England Biolabs) was used for the affinity purification, and that equilibration and elution of the resin was in 20 mM Tris-HCl (pH 7.4), 1 mM β-mercaptoethanol without or with 10 mM maltose, respectively.

Buffers containing the eluted fusion-proteins were exchanged in actin-G buffer (AGB: 2 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM ATP) by dialysis and protein concentration was estimated by the Bradford protein assay (Biorad).

Actin-binding assay

All purified proteins used in this study were clarified by centrifugation at 100,000 ***g*** for 1 hour at 4°C and kept on ice in AGB. Actin cosedimentation assays were performed as follows: rabbit α-skeletal muscle actin (2.5 μM; Cytoskeleton Inc.), premixed or not premixed with fusion proteins, was allowed to polymerize by the addition of 0.1 volume of 10× initiation mix (IM: 2 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 1 M KCl, 5 mM ATP) for 1 hour at room temperature. Actin filaments with bound proteins were pelleted by centrifugation at 100,000 ***g*** for 1 hour at 20°C. Equal amounts of pellet and supernatant were resolved by SDS-PAGE and proteins were visualized by Coomassie Brilliant Blue-staining.

To test the ability of plectin-ABD to bind monomeric actin, MBP-plectin$_{1\text{-}339}$ bound to amylose resin was equilibrated in AGB. Monomeric actin was mixed with immobilized MBP-plectin$_{1\text{-}339}$ to obtain a final concentration of 2.5 μM for actin and 3 μM for plectin. The mixture was incubated for 3 hours at room temperature. After five washes with AGB, samples were boiled in sample buffer and appropriate amounts of bound and unbound protein mixture were resolved by SDS-PAGE. Proteins were visualized by Coomassie Brilliant Blue-staining.

Low-speed sedimentation assays were performed as follows. Actin was polymerized at 24 μM for 3 hours at room temperature, then diluted to 4.5 μM in actin polymerization buffer (APB: AGB supplemented with 0.1 volume of IM) containing 2, 1 or 0.5 μM of MBP-plectin or MBP alone (control). After a 1 hour incubation at room temperature, samples were centrifuged at 14,000 ***g*** for 1 minute, equal amounts of pellet and supernatant were analyzed by SDS-PAGE and proteins visualized by Coomassie Brilliant Blue-staining.

Actin filament assembly assays and electron microscopy

For the pyrene-actin assembly assays (Kouyama and Mihashi, 1981), an actin polymerization kit was purchased from Cytoskeleton Inc. Rabbit α-skeletal muscle actin containing 10% (mol/mol) of pyrene-labeled actin was used at a final concentration of 4 μM. Polymerization was induced by the addition of 0.1 volume of IM and monitored by change in fluorescence at an excitation wavelength of 365 nm and emission wavelength of 407 nm in a fluorimeter. The fluorescence corresponding to 100% of polymerization was measured 3-4 hours after the addition of the IM. Light scattering assays were performed with unlabeled actin (2 μM) by monitoring changes in OD at 300 nm upon the addition of the IM. Electron microscopy was performed on samples after the actin polymerization was complete, as monitored by light scattering. Samples of the polymerization mixtures were fixed by the addition of 0.05% (v/v) glutaraldehyde, spotted on formvar-carbon coated grids, negatively stained with 1% (w/v) aqueous uranyl acetate and examined at 80 kV with a Philips CM 10 electron microscope.

Cell culture, transfection and immunofluorescence microscopy

Rat embryo fibroblasts (REF) and COS-7 cells were maintained in DMEM (Gibco BRL) containing 10% foetal calf serum (FCS) and supplemented with 100 i.u./ml penicillin and 100 U/ml streptomycin. Cells were grown at 37°C in a humidified, 5% CO_2 atmosphere. REFs were transiently transfected with cDNA constructs using Lipofectin (Gibco BRL) according to the manufacturer's instructions. COS-7 cells were transfected using DEAE-dextran as previously described (Schaapveld et al., 1998). Indirect immunofluorescence staining of transfected REF cells with mouse mAb 12CA5 against the haemaglutinin (HA)-epitope (YPYDVPDYA) (Santa Cruz Biotechnology) was performed as described previously (Geerts et al., 1999). Actin filaments were stained with rhodamine-phalloidin from Molecular Probes. Immunfluorescence images were taken using a Leica confocal laser scanning microscope.

In vitro binding assays

The ability of the plectin-ABD to interact with another plectin-ABD was tested in an in vitro binding assay with radiolabeled proteins. Coupled in vitro transcription-translation of 1-2 μg of pcDNA3HA-plectin-ABD$_{1\text{-}339}$ or pcDNA3HA-β4^{R1281W} (negative control) was performed using the TnT rabbit reticulocyte lysate kit (Promega) in the presence of [^{35}S]-methionine/cysteine. Non-incorporated radiolabeled amino acids were removed from the in vitro translation mixture by gel filtration using a PD10 column (Amersham Inc.). Scintillation counting performed on the purified translation mixtures indicated that both proteins were equally labeled, with 9% of the total [^{35}S]-methionine/cysteine incorporated. Purified translation mixtures were then tested for binding to MBP-plectin$_{1\text{-}339}$, or MBP (negative control) immobilized on amylose-agarose beads. For the binding assay, the translation mixtures were diluted in APB containing 2% of heat-inactivated BSA (HI-BSA: BSA heated overnight at 55°C) and incubated for 2 hours at room temperature with 200 pmol of MBP-plectin or MBP immobilized on amylose beads. After incubation, beads were washed 3 times in APB supplemented with 2% (w/v) HI-BSA, then twice in APB. After washing, the beads were boiled in

sample buffer, proteins were subjected to SDS-PAGE and visualized by autoradiography.

A pull-down assay was used to study plectin-ABD interaction in mammalian cells. 48 hours after transfection, COS-7 cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM $MgCl_2$, 1 mM $CaCl_2$) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and soybean trypsin inhibitor and 0.1 U/ml aprotinin). Lysates were clarified by centrifugation at 4°C (20 minutes at 15,000 ***g*)** and diluted five times in buffer A (20 mM Tris-HCl, pH 8.0, 100 mM NaCl). The GST-plectin$_{1-339}$ fusion protein was generated and immobilized on glutathione-Sepharose beads as described previously (Geerts et al., 1999). The beads were incubated in buffer A containing 1% (w/v) BSA to block non-specific binding sites. After washing, 750 pmoles of GST-plectin$_{1-339}$ beads were added to the diluted cell lysates and the mixtures were incubated overnight at 4°C. The Sepharose beads were washed once with buffer A and then sedimented through a sucrose cushion (800 mM sucrose in buffer A). Finally, beads were boiled in SDS-sample buffer and bead-associated proteins were separated by SDS-PAGE and identified by immunoblotting using mAb 12CA5 against HA.

RESULTS

Identification of plectin sequences required for actin binding

The ABD of human epithelial plectin, amino acids 65-302, is located at the extreme N terminus and is encoded by exons 2-8 of the *PLEC1* gene. The plectin-ABD comprises two 109-residue CH domains, CH1 (position 69-177) and CH2 (185-293), respectively, juxtaposed in tandem (Fig. 1A). Based on the homology of the plectin-ABD sequence with those of other actin-binding proteins of the β-spectrin family (Hartwig, 1994), three conserved sites (ABS1-3) are identified within the plectin-ABD that, in other β-spectrin family members such as dystrophin, are known to be involved in actin binding (Fabbrizio et al., 1993; Gimona and Winder, 1999). In plectin, the putative ABS1 and ABS2 (residues 72-81 and 144-170, respectively) are both in the CH1 domain, whereas ABS3 (183-198) is the only potential actin interaction site within the CH2 domain.

The unequal distribution of the ABS sequences within the CH1 and CH2 domains and the differences in their sequences (Fig. 1A) raise the possibility that these two CH domains have different functions. In addition, recent studies indicate that the single CH domain of the calponin protein is not necessary for F-actin binding, but that sequences located near the CH domain have a role in its interaction with actin (Gimona and Mital, 1998; Corrado et al., 1994). This prompted us to map the plectin sequences involved in actin binding and to gain more insight into the roles of the plectin CH and ABS sequences in the interaction with actin.

Yeast two-hybrid assays for interactions of plectin with actin were performed by cotransformation of the yeast strain PJ69-4A with pACT2-derived plasmids encoding the transcriptional activation domain (AD) of Gal4 fused to full-length actin, together with pAS2-1-derived plasmids encoding fusions between the DNA-binding domain (DB) of Gal4 and different fragments of the plectin-ABD (Fig. 1B). Expression of the fusion proteins was confirmed by immunoblotting with anti-Gal4(BD) or anti-Gal4(AD) antibodies (not shown). Interactions of plectin with actin were detected by the growth of yeast colonies on selective SC-LTHA plates (see Materials and Methods). A high plating efficiency was observed when plectin$_{1-339}$ was coexpressed in yeast with full-length β- or γ-cytoplasmic actin, or α-actin from skeletal muscle, showing that the His and Ade reporter genes were efficiently expressed as a result of a strong interaction between plectin$_{1-339}$ and the different actin isoforms. Plectin$_{1-339}$ contains unique sequences that are not part of the plectin-ABD (amino acids 1-65, encoded by exon 1c, and amino acids 302-343, encoded by exon 9 of plectin). To localize the region within the ABD domain that interacts with actin and to exclude the possibility that sequences outside of the plectin-ABD are also involved, a series of plectin deletion constructs were tested. C-terminal truncation of amino acids 237-339 does not abolish interaction, but in contrast results in a moderate increase in the binding of the plectin-ABD to all the actin isoforms tested. Further truncation extending to the C-terminal end of the first CH domain (residues 173-236) also does not alter binding, showing that the plectin CH2 domain is not necessary for actin binding (Fig. 1B). In contrast, deletion of the second ABS completely abolished the interaction of plectin with α-, β- and γ-actin, showing that this region is required for binding to β- and γ-cytoplasmic actin, as well as to α-skeletal muscle actin.

Does plectin-ABD bind to G-actin and F-actin filaments?

Although plectin decorates actin stress fibers in cultured cells, suggesting that plectin interacts with polymeric actin, no evidence for direct binding has yet been presented. To test whether the plectin-ABD mediates direct interactions with actin filaments, copolymerization assays were performed using purified monomeric α-actin from skeletal muscle and an MBP-fusion protein containing the first 339 amino acids of human plectin. Prior to the induction of polymerization, monomeric actin at 2.5 μM and MBP-plectin$_{1-339}$ in actin G buffer were mixed at the molar ratio indicated in Fig. 2A. Actin polymerization was induced by the addition of initiation mix and actin filaments were sedimented by centrifugation. As shown in Fig. 2A, MBP-plectin$_{1-339}$ was found associated with the actin filaments recovered in the pellet. Quantification by scanning densitometry of Coomassie Brilliant Blue-stained gels revealed that plectin has no influence on the amount of pelleted actin, thus indicating that MBP-plectin$_{1-339}$ neither promotes nor inhibits actin polymerization (not shown and see below). Scatchard analysis revealed that MBP-plectin$_{1-339}$ binds to actin with a K_d of 0.3 μM and a molecular ratio of 1:1 (Fig. 2B). These estimations were made by assuming that the plectin molecules that remain in the supernatant were not associated with unpolymerized actin: at a low plectin concentration (lane 1) less than 5% of plectin is found in the supernatant although it contains unpolymerized actin. Similar experiments performed with a GST-fusion protein containing the first 339 amino acids of the plectin N terminus, GST-plectin$_{1-339}$, gave similar results (data not shown).

To test whether the plectin-ABD is able to bind monomeric G-actin, pull-down assays were performed with MBP-plectin$_{1-339}$ bound to amylose-beads in AGB to ensure that actin is not polymerized to F-actin. While no G-actin was precipitated with amylose-agarose beads alone (not shown), a small proportion was recovered using immobilized plectin$_{1-339}$, which was prevented by the addition of a fivefold excess of soluble MBP-

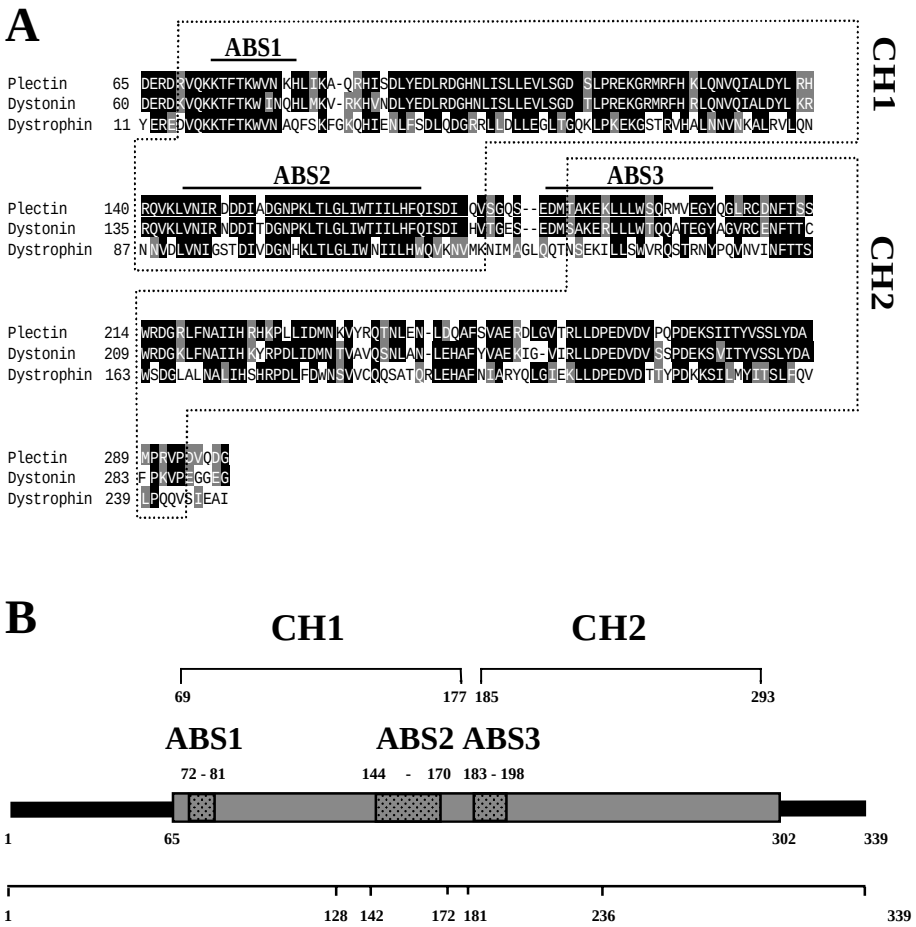

Fig. 1. Comparison of the ABD of plectin, dystonin and dystrophin and interaction of plectin with different isoforms of actin. (A) Alignment of the N termini of human plectin (AAB05427), mouse dystonin (P11277) and dystrophin (P11532). Alignment was performed with the CLUSTAL-W program. Black boxes, identical amino acids; gray boxes, amino acid similarity. The bars above the sequence indicate the ABS1, ABS2 and ABS3 sequences. The CH1 and CH2 domains, identified by sequence homology among the β-spectrin family of proteins, are delineated by dotted boxes. (B) Two-hybrid interaction between the N-terminal part of plectin and different actin isoforms. (Top) Schematic representation of the largest N-terminal construct of plectin (residues 1-339) with its ABD used in this study. (Bottom) Different actin isoforms, α-skeletal muscle (α-actin), β-cytoplasmic (β-actin) and γ-cytoplasmic (γ-actin) actin, were used to determine plating efficiency following cotransformation of yeast host strain PJ69-4A with each of the pAS2-plectin subclones listed together with pACT2-actins. Transformation mixtures were spread on SC-LT and SC-LTHA plates and grown at 30°C. Plating efficiency on selective SC-LTHA plates is expressed as a percentage of plating efficiency on non-selective SC-LT plates of the same transformation, thus: ++, >50%; +, ≥ 50% (slowly growing colonies); ±, 5-25%; –, 0%. ND, not determined. Plates were scored after 6 and 12 days of growth; slowly growing colonies could only be scored after 12 days of growth. Plating efficiencies of <25% always represented slowly growing colonies. All efficiencies listed represent an average of multiple independent transformations on at least two separate occasions.

Gal4(BD)-Plectin fragment (aa)	Gal4(AD) fused to		
	α-actin	β-actin	γ-actin
1-339	**ND**	+	+
1-236	++	++	++
1-181	++	++	++
1-172	++	++	++
1-142	–	–	–
1-128	–	–	–

plectin$_{1\text{-}339}$ (Fig. 2C). These results show that plectin$_{1\text{-}339}$ bound weakly but specifically to G-actin.

Effects mediated by plectin-ABD on the kinetics of actin polymerization

Using two different assays, pyrene-actin fluorescence and light scattering, we next investigated whether plectin can modulate the actin polymerization rate by binding to F-actin. In the pyrene-actin assay the fluorescence signal is directly proportional to the degree of actin polymerization. However, as this test relies on the fact that F-actin is more fluorescent than G-actin because the environment of the labeled residue (Cys$_{374}$) is different in the G and F-actin molecules, an actin ligand that alters the three-dimensional structure of F-actin would also affect fluorescence. On the contrary, light scattering is influenced by the length and bundling of filaments. Although probably less reliable than the pyrene-actin assay, this test has the advantage that unmodified actin is used and that it does not rely on a conformational change of actin.

Testing the effect of plectin on the rate of actin polymerization in the pyrene-actin assay revealed that MBP-plectin$_{1\text{-}339}$ increases the rate of actin polymerization in a concentration-dependent manner (Fig. 3A). No differences between the steady-state concentrations of F-actin in the absence or presence of plectin-ABD were detectable by this assay at the concentrations tested (not shown). Thus, the

plectin-ABD does not alter the degree of actin polymerization, which is in agreement with the copolymerization results. As the pyrene-actin assay measures spontaneous polymerization, we conclude that the effects of the plectin-ABD on the rate of fluorescence likely result from an increase in the number of actin nuclei, which increase the amount of polymerizing actin and therefore the fluorescence.

The light-scattering assays provided evidence that plectin can cross-link actin filaments. In our experimental conditions, actin polymerization results in a rapid increase in absorbance during the first 5 minutes, followed by a more moderate increase, ultimately reaching a plateau (Fig. 3B). When MBP-plectin$_{1-339}$ was used at 0.5 or 1 μM concentration, there was a markedly more rapid increase in the OD rate and the OD values were much higher at steady state, as compared to the control. However, only small differences were found between the results obtained with 0.5 and 1 μM MBP-plectin$_{1-339}$, suggesting that these effects were not the result of a direct effect of MBP-plectin$_{1-339}$ on actin polymerization (Fig. 3B). In addition, the results of neither the cosedimentation assays nor the pyrene-actin assays indicate that MBP-plectin$_{1-339}$ increases the amount of polymerized actin.

The data obtained by light scattering in combination with the nucleating effect of the plectin-ABD observed using the pyrene-actin assay led us to assume that MBP-plectin$_{1-339}$ can probably bundle actin filaments by cross-linking at least two actin molecules.

Evidence for a bundling activity mediated by plectin-ABD dimerization

Cross-linking and/or bundling of actin was visualized by electron microscopy of the polymerization mixtures monitored by light scattering. In the absence of plectin, long actin filaments were randomly distributed all over the grid with only some of them organized into bundles (Fig. 4A). At a concentration of 0.5 μM, MBP-plectin$_{1-339}$ notably increased the number of actin bundles (Fig. 4B). These bundles consisted of 4-6 actin filaments in juxtaposition and were partially organized in a branched network. Due to the presence of globular particles, corresponding to the plectin-fusion protein, the bundles have a rather rough appearance. The globular particles were found regularly distributed at 30 nm intervals along the filaments. To confirm that plectin indeed induces bundling of actin filaments, low-speed sedimentation assays were performed using MBP plectin$_{1-339}$ and MBP alone as a control. As expected, we found that only small amounts of polymerized actin can be precipitated by brief centrifugation at 14,000 ***g*** in the presence of 2 μM of MBP (Fig. 5A). By contrast, about 90% of the actin was found in the pellet when the same experiment was done in the presence of various concentrations of plectin, indicating that plectin can induce the formation of large actin complexes. In agreement with the data obtained by light scattering (Fig. 3B), there were no marked differences in the amount of actin sedimented at the various plectin concentrations used.

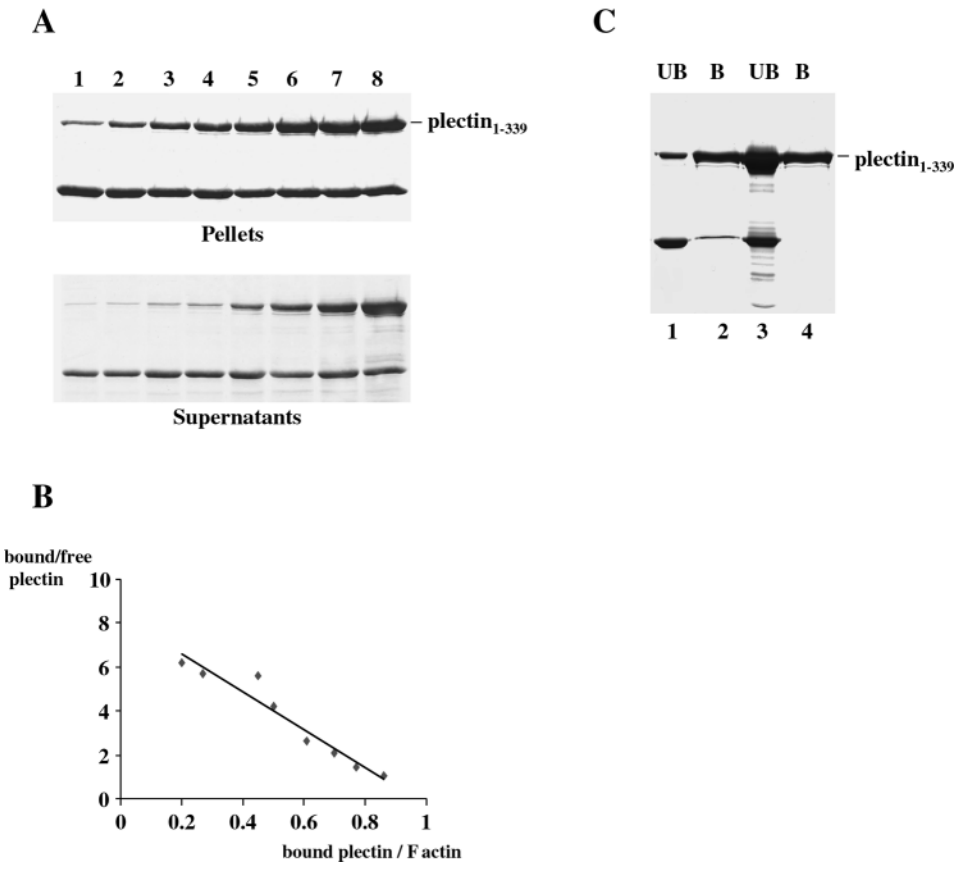

Fig. 2. Biochemical analysis of plectin/actin interactions. (A) Copolymerization assays were performed using bovine α-skeletal muscle actin at 2.5 μM together with different concentrations of MBP-plectin-ABD$_{1-339}$ (lanes 1-8 correspond to 0.2, 0.37, 0.5, 0.6, 1, 1.5, 1.6 and 2.2 μM, respectively). Actin filaments and bound proteins were sedimented by centrifugation and equivalent samples of pellets (upper panel) and supernatants (lower panel) were resolved by SDS-PAGE. (B) Scatchard plot of plectin binding to actin filaments (values are means differing by less than 5% of duplicate experiments). Scatchard plotting indicates that plectin binds to filamentous actin with an apparent K_d of 0.3 μM and a molecular ratio of 1 plectin molecule per actin monomer. (C) Pull-down assay of plectin-ABD$_{1-339}$/G-actin binding. Immobilized MBP-plectin-ABD$_{1-339}$ in suspension (3 μM) was incubated with soluble α-skeletal muscle G-actin (2.5 μM) in the absence (lanes 1 and 2) or presence of a fivefold excess of soluble MBP-plectin-ABD$_{1-339}$ (lanes 3 and 4). After incubation, the beads were pelleted by centrifugation and the supernatants removed. The beads were then washed and the proteins eluted by boiling in sample buffer. Equivalent samples of supernatant (unbound (UB) actin, lanes 1 and 3) and bead eluates (bound (B) actin, lanes 2 and 4) were analyzed by SDS-PAGE. Proteins were visualized by Coomassie Brilliant Blue staining.

These results suggest that the N terminus of plectin is able to dimerize and thus induces the formation of actin bundles. To directly assess the ability of plectin$_{1\text{-}339}$ to interact with the ABD on another plectin molecule, we investigated the association of MBP-plectin$_{1\text{-}339}$ fusion proteins with radiolabeled plectin$_{1\text{-}339}$ (prepared by in vitro transcription/translation of pcDNA3-HA plectin$_{1\text{-}339}$, see Materials and Methods). As a control for the specificity of the interaction, the MBP-plectin$_{1\text{-}339}$ fusion protein was also incubated with a radiolabeled fragment of the cytoplasmic domain of β4, containing a mutation in the second type III fibronectin repeat (β4^{R1281W}) that abrogates binding to plectin (Geerts et al., 1999). The expression and quality of the in vitro translated proteins was confirmed by SDS-PAGE followed by autoradiography. Binding of MBP alone or MBP-plectin$_{1\text{-}339}$ to HA-β4^{R1281W} was barely detectable (Fig. 5B, lanes 1 and 3). There was a weak, but not specific, interaction of MBP alone with HA-plectin$_{1\text{-}339}$ (Fig. 5B, lane 2). In contrast, a strong signal was obtained when plectin$_{1\text{-}339}$ was incubated with HA-plectin$_{1\text{-}339}$ (Fig. 5B, lane 4), suggesting that the plectin N terminus can indeed bind to the N terminus on other plectin molecules. We subsequently confirmed this interaction in a pull-down assay using COS-7 cells, transfected with HA-tagged plectin$_{1\text{-}339}$. Complex formation between the expressed protein and other plectin$_{1\text{-}339}$ molecules, being presented as GST fusion proteins immobilized on glutathione beads, was tested by precipitation, followed by immunoblotting with anti-HA antibodies. As shown in Fig. 5C, the HA-tagged plectin$_{1\text{-}339}$ fragment was precipitated with GST-plectin$_{1\text{-}339}$, but not with GST alone. As expected, an N-terminal truncation mutant (HA-tagged plectin$_{65\text{-}339}$) was also efficiently precipitated with GST-plectin$_{1\text{-}339}$, confirming that the first 64 amino acids of plectin are not required for the interaction between two N-terminal plectin fragments. A negative control using HA-tagged β4^{R1281W} did not bind to GST-plectin$_{1\text{-}339}$. Expression of the HA-tagged proteins was checked by immunoblotting with anti-HA antibody. Taken together these results clearly show that N-terminal fragments of plectin which contain a complete ABD domain can form dimers.

Identification of the dimerization domain of plectin-ABD

The role of the CH domains in actin binding remains controversial (Gimona and Mital, 1998). There is stronger homology between the CH1 and CH2 domains, respectively, of different actin-binding proteins (Fig. 1A; Van Troys et al., 1999) than between CH1 and CH2 of the same protein, supporting the current idea that although CH1 and CH2 domains are structurally related, their function is most probably distinct (Van Troys et al., 1999). Here, we have found that the CH1 domain of plectin is necessary for actin binding (see above), while CH2 is not. Altogether these data and the observed bundling mediated by plectin-plectin interactions prompted us to investigate which part of the plectin N terminus is required for this interaction. To this end, full-length and truncated plectin-ABD were tested in a yeast two-hybrid assay and the resulting binding activities were compared to the binding obtained

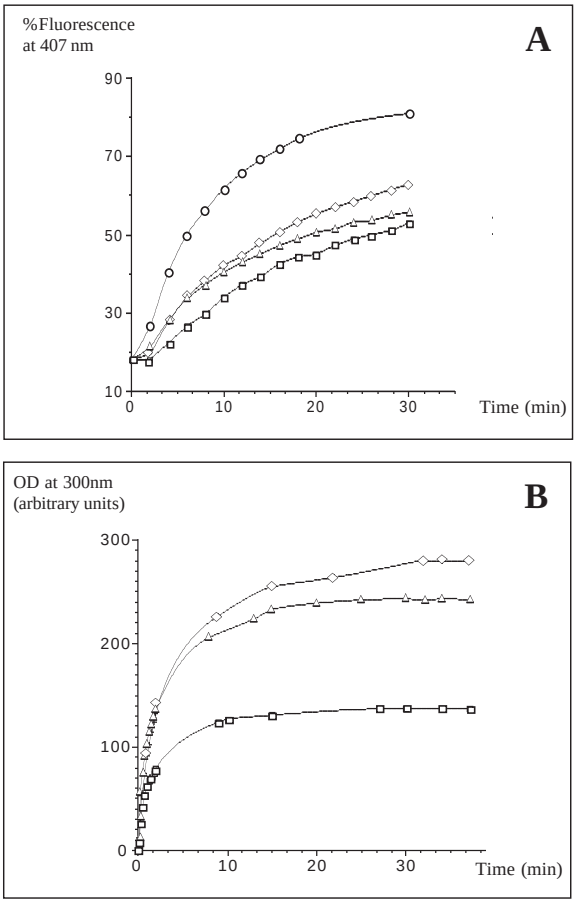

Fig. 3. Effect of plectin-ABD on actin polymerization. (A) α-skeletal muscle actin (4 μM) containing 10% of pyrene-labeled actin was polymerized in the presence of increasing concentrations of MBP-plectin-ABD$_{1\text{-}339}$ (squares, 0 nM; triangles, 30 nM; diamonds, 150 nM; circles, 300 nM). Actin polymerization was initiated at time 0 by the addition of 0.1 volume of 10× initiation mix and fluorescence was recorded at 407 nm using an excitation wavelength of 365 nm. (B) Actin polymerization was monitored by light scattering at 300 nm. Polymerization of 2 μM α-skeletal muscle actin in the absence (squares) and presence of varying concentrations of MBP-plectin-ABD, 0.5 μM (triangles), 1 μM (diamonds) was initiated at time 0 by the addition of 0.1 volume of 10× initiation mix.

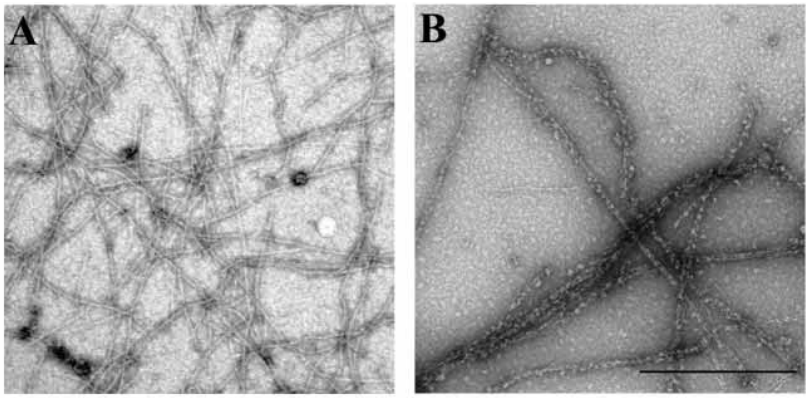

Fig. 4. Plectin-ABD cross-links F-actin into bundles. Electron microscopy examination of polymerized actin mixtures, described in Fig. 3. F-actin alone (A) or in combination with 0.5 μM of purified plectin-ABD (B). Note the numerous bundles of F-actin in the presence of plectin-ABD. Bar, 0.5 μm.

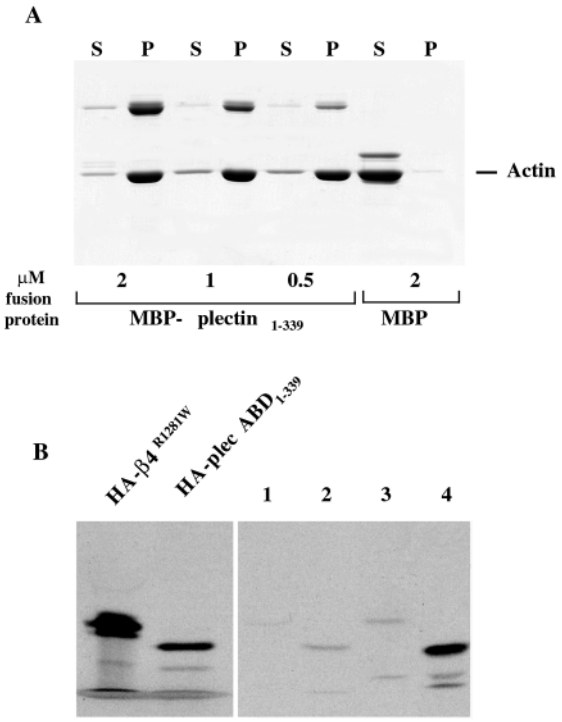

Fig. 5. The plectin-ABD interacts with other plectin-ABD molecules in vitro and bundles actin filaments. (A) Low-speed sedimentation assay. Polymeric actin (4 μM) was incubated with MBP (2 μM) or MBP-plectin (0.5, 1 or 2 μM) for 1 hour at room temperature. Samples were centrifuged at 14,000 ***g*** for 1 minute and equal amounts of pellet (P) and supernatant (S) were subjected to SDS-PAGE and visualized by Coomassie Blue staining. (B) MBP (lanes 1 and 2) and MBP-plectin$_{1-339}$ (lanes 3 and 4) bound to amylose-agarose beads were incubated with ^{35}S-labeled plectin-ABD$_{1-339}$ (lanes 2 and 4) or β4^{R1281W} (lanes 1 and 3), obtained by in vitro translation. After incubation and washing, the beads were boiled in sample buffer and bound proteins were subjected to SDS-PAGE and visualized by autoradiography. Lanes at left show ^{35}S-labeled in vitro-translated plectin-ABD$_{1-339}$ and β4^{R1281W}. (C) Lysates of COS-7 cells, untransfected (lanes 1, 5, 9) or transiently transfected with HA-plectin-ABD$_{1-339}$ (lanes 2, 6, 10), HA-plectin-ABD$_{65-339}$ (lanes 3, 7, 11) or HA-β4^{R1281W} (lanes 4, 8, 12), were incubated with GST (lanes 5-8) or GST-plectin$_{1-339}$ (lanes 9-12), immobilized on glutathione-Sepharose beads. After washing, beads were boiled in sample buffer and associated proteins were visualized by immunoblotting using anti-HA antibody. Lanes 1-4, total COS-7 cell lysates probed by immunoblotting with anti-HA antibody to verify the expression of the HA-tagged proteins. The upper band in lanes 1-4 and 9-10 corresponds to an unidentified protein that is non-specifically recognized by anti-HA antibody.

with β-actin (Fig. 6). We observed that plectin$_{1-339}$ (as a Gal4(BD)-fusion protein) interacted with both plectin$_{1-339}$ (as a Gal4(AD)-fusion) and β-actin. In addition, the plectin mutant in which the 35 N-terminal amino acids had been deleted (plectin$_{36-339}$) still bound to other plectin molecules as well as to β-actin. An N-terminal deletion that removed all 64 amino acids encoded by exon 1c completely abolished the interaction with β-actin, without altering the interaction with other plectin molecules. However, by also deleting residues 65-173 or 65-284, the binding to other plectin$_{1-339}$ molecules was also abolished. Since plectin$_{36-339}$ is able to bind to both β-actin and plectin$_{1-339}$, we tested the effect of C-terminal truncations of this molecule on these interactions. Removal of the CH2 domain enhances binding to β-actin but does not alter binding to plectin$_{1-339}$, whereas deletion of ABS2 abrogates binding to both β-actin and plectin$_{1-339}$. These findings indicate that for dimerization at least one intact CH1 domain in any protein of the dimer is required.

To determine whether the plectin CH1 domain alone can support dimerization of the plectin-ABD we performed a similar yeast two-hybrid analysis using a Gal4(AD)-fusion protein containing amino acids 36-181 of plectin. Consistent with the results described above we found that plectin$_{36-181}$ interacts with plectin$_{1-339}$ (Fig. 6) with an efficiency similar to that of plectin$_{1-339}$. In contrast to plectin$_{1-339}$, plectin$_{36-181}$ was unable to interact with plectin$_{65-339}$. This latter finding suggests that sequences in the region of residues 36-65 may also contribute to plectin-ABD binding. Alternatively, these sequences may be required to position the CH1 domain at a sufficient distance from the Gal4 moiety

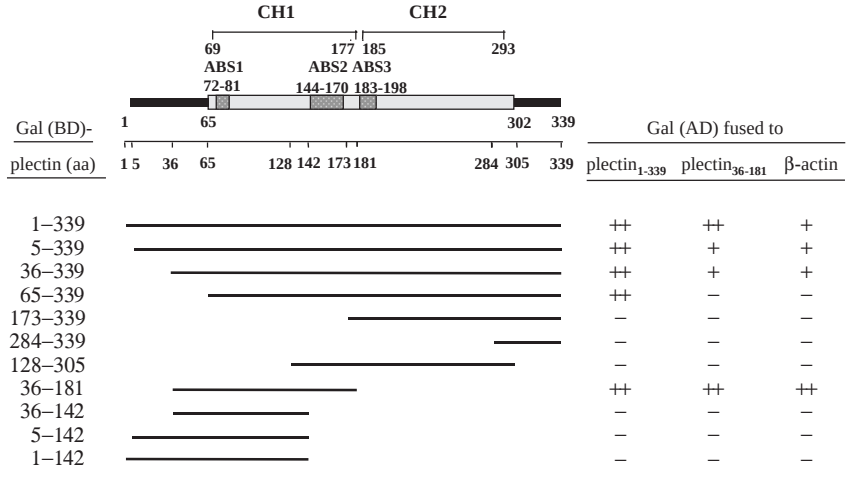

Fig. 6. Mapping of the binding site in the N-terminal part of plectin that is involved in dimerization. Plating efficiency following cotransformation of yeast host strain PJ69-4A with one of each of the pAS2-plectin subclones listed together with pACT2-plectin$_{1-339}$, pACT2-plectin$_{36-181}$ or pACT2-β cytoplasmic actin are shown. Details are as for Fig. 1B.

to make binding possible in yeast. In agreement with the results obtained with plectin$_{1-339}$, we found that deletion of ABS2 (plectin$_{1-142}$, plectin$_{5-142}$, plectin$_{36-142}$) completely abolished plectin$_{36-181}$-binding activity.

Can other ABDs mediate homo- and heterodimerization?

Based on the strong homology in sequences between the ABDs of the β-spectrin family members, we speculate that ABDs other than those of plectin may homodimerize and even heterodimerize. This assumption was tested by yeast two-hybrid assays using Gal4-fusion proteins containing the N-terminal ABDs of dystrophin, dystonin and plectin. We found that plectin$_{1-339}$ binds to other plectin$_{1-339}$ molecules and to dystonin-2$_{1-336}$ with similar efficiencies (Fig. 7). By contrast, there was no binding between plectin$_{1-339}$ and dystrophin$_{1-337}$ or between dystonin-2$_{1-336}$ and dystrophin$_{1-337}$. Dystonin-2$_{1-336}$ can bind to other dystonin-2$_{1-336}$ molecules but this binding appeared to be weaker than that with plectin$_{1-339}$. Binding of dystrophin$_{1-337}$ to other dystrophin$_{1-337}$ molecules proved to be very weak and was barely detectable. Since in the Gal4-fusion protein, the dystrophin-ABD, which starts at residue 10, is located closely to the Gal4 protein, interaction between two of these fusion proteins via their dystrophin-ABDs may not be possible because of steric hindrance by the Gal4-moiety. We therefore tested whether the efficiency of intermolecular binding between two dystrophin-ABDs could be made possible by increasing the size of the linker region between the ABD and the Gal4-moiety. To achieve this, we created a chimeric protein in which the first 65 residues of plectin separated the Gal4-moiety and the dystrophin-ABD. Testing this chimeric protein in the yeast two-hybrid assay indeed revealed efficient intermolecular binding of the chimeric proteins and a somewhat reduced binding of it to dystrophin$_{1-337}$. There were however, still no interactions with either plectin$_{1-339}$ or dystonin-2$_{1-336}$.

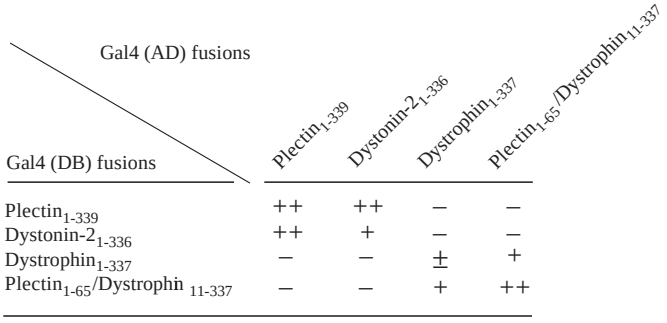

Fig. 7. Yeast two-hybrid analysis of the interactions between the ABD of plectin, dystonin and dystrophin. Plating efficiency following cotransformation of yeast host strain PJ69-4A with the pAS2- and pACT2-plectin, dystonin and dystrophin subclones listed are shown. Details are as for Fig. 1B.

The 64 amino acids encoded by exon 1c are dispensable for actin binding in cells

All our experimental data strongly suggest that the ABD of plectin is sufficient to support binding to actin filaments. However, we have found that the Gal4 or GST moieties fused to the plectin N terminus at amino acid residue 65 abrogate actin binding (see above and unpublished results), presumably because of a steric hindrance of crucial amino acid residues by the tag. Therefore, it was not possible to conclude that the first 64 amino acid residues, which do not form part of the conserved ABD, are unnecessary for actin binding. To address this question, rat embryo fibroblasts (REFs) were transfected with HA-tagged plectin N terminus with or without the first 64 amino acid residues (HA-plectin$_{1-339}$ and HA-plectin$_{65-339}$, respectively). Transfected cells were stained with HA-specific antibodies to detect the HA-tagged protein and rhodamine-phalloidin for staining F-actin. Examination by confocal microscopy reveals that both plectin mutants produce similar intracellular localization patterns with a predominant staining along actin filaments and in focal contacts (Fig. 8A,B). These latter findings clearly demonstrate that the first 64 amino acid of the N terminus of the epithelial variant of plectin are not required for supporting direct and/or indirect binding of plectin to actin.

DISCUSSION

Although plectin contains an ABD that is highly homologous to that of other proteins of the β-spectrin family, its function

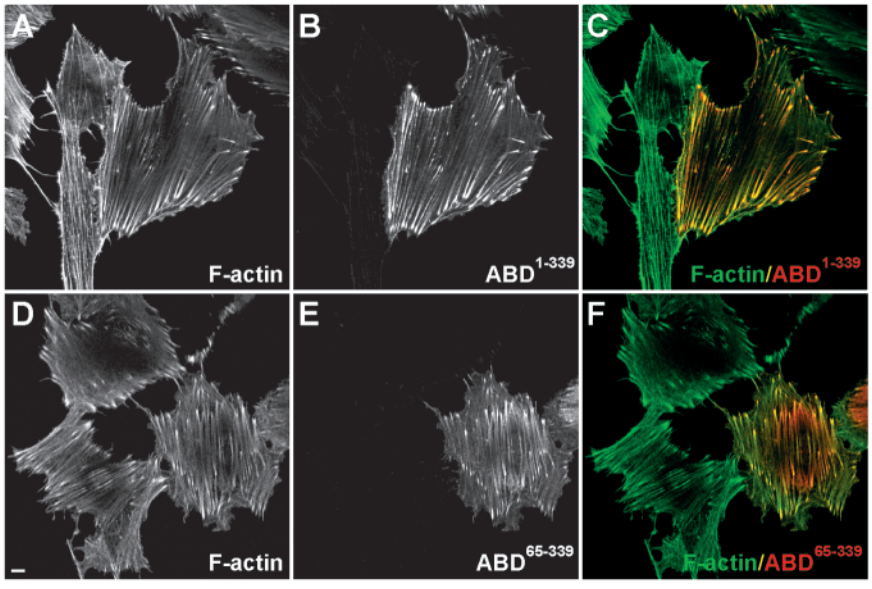

Fig. 8. Codistribution of F-actin and plectin N terminus protein fragments in transfected cells. Rat embryo fibroblasts were transiently transfected with HA-tagged plectin$_{1-339}$ (A-C) or HA-tagged plectin$_{65-339}$ (D-F). The cells were fixed, permeabilized and processed for double labeling using rhodamine-phalloidin to visualize F-actin (A,D) and anti-HA antibodies, followed by FITC-conjugated secondary antibodies to detect the HA-tagged proteins (B,E). Images were obtained using a Leica confocal microscope. (C,F) Composite images. Colocalization appears as yellow. Bar, 10 μm.

as a structural actin-binding protein has not yet been well established. In many cell types plectin is generally found either concentrated in hemidesmosomes or associated with the three cytoskeletal filament systems (Wiche and Baker, 1982; Wiche et al., 1983; Hermann and Wiche, 1987; Errante et al., 1994; Foisner et al., 1994; Bohn et al., 1996; Svitkina et al., 1996; Rezniczek et al., 1998; Andrä et al., 1998; Nievers et al., 1998; Geerts et al., 1999). This protein has been found to be colocalized with actin in focal contacts or along stress fibers, but hardly with cortical actin, suggesting that plectin-actin interactions are tightly regulated or that plectin binds to specific actin isoforms enriched in these structures (for a review, see Herman, 1993). Our findings with the yeast two-hybrid assay indicate that plectin is able to bind to various actin isoforms: α-skeletal muscle, β-cytoplasmic and γ-cytoplasmic actin. Nevertheless, since actin isoforms differ primarily in their N terminus and since in the yeast two-hybrid experiments the actin isoforms are assayed when fused at their N terminus with the Gal4(AD) moiety, we cannot formally rule out that there are minor differences in the interaction of plectin with the various actin isoforms that were not detected using this method.

The actin-binding site in the plectin-ABD is contained within the first CH domain, since truncation at either the N-terminal (Fig. 6) or the C-terminal end of CH1 (Fig. 1B) abolished the binding of plectin to the different actin isoforms. Our yeast two-hybrid assays clearly show that residues 143-172, comprising the ABS2, are required for mediating binding to all the actin isoforms tested. Recently, analyses of the three-dimensional structure of fimbrin and β-spectrin ABDs suggested that the major actin-binding site is localized in the last α-helix of CH1, corresponding to a sequence containing amino acid residues of the ABS2 (Banuelos et al., 1998). Dystrophin, another member of the β-spectrin family, also binds to actin by a conserved ABD. However, in this latter case binding requires both the ABS1 and the ABS2 (Fabbrizio et al., 1993; Corrado et al., 1994). The crystal structure of the first CH domain of human β-spectrin (MMDB Id: 8495) (Carugo et al., 1997) indicates that the first α-helix containing the ABS1 is located in close proximity to the last α-helix, which contains the ABS2. Thus, amino acid residues located in the ABS1 might also participate in actin binding. The observed inability of the complete plectin-ABD fragment to interact with actin (this study and unpublished observations) might be due to steric hindrance of the ABS2 resulting from the fusion to Gal4(BD) and GST. The same mechanism might account for the different results obtained by yeast two-hybrid and cell transfection experiments regarding the involvement of residues 36-65 of plectin in actin-binding. Indeed, we found that fusion proteins (Gal4 or GST) containing plectin$_{65-339}$ were unable to bind actin in yeast two-hybrid and in vitro interaction assays. However, the colocalization of actin and plectin$_{65-339}$ identified in transfected REF cells strongly suggests that the first 64 amino acid residues are not necessary for actin binding.

The results of our study indicate that the plectin CH2 domain is not critically implicated in the binding to actin. However, it appears to have a regulatory function on actin binding since its partial or complete removal increases plectin-actin binding. It is possible that the partial inhibition mediated by the CH2 domain results from hindrance of sequences involved in plectin-actin interactions and thus proteins that interact with the CH2 could potentially modulate the interaction of the plectin-ABD with actin.

In cosedimentation assays, we found that plectin binds to actin with an apparent K_d of 0.3 μM and in a molecular ratio of 1:1. These properties are similar to those of other actin-binding proteins containing the conserved ABD such as dystrophin, α-actinin and dystonin (for a review, see Hartwig, 1994). In addition, in vitro experiments have shown that the plectin-ABD does not modify the amount of polymerized actin, suggesting that the plectin-ABD does not prevent polymerization of G-actin into F-actin. Consistent with this finding, pull-down assays, performed with immobilized plectin-ABD, demonstrate that plectin hardly binds to G-actin. These data are in apparent contrast to those obtained by Andrä et al. (Andrä et al., 1998), who showed that the plectin-ABD binds to monomeric actin using ELISA assays. These authors reported a K_d of 0.32 μM, the same as we established for F-actin. The discordance concerning the binding of plectin to G-actin is probably because Andrä and coworkers used a buffer containing 2 mM Mg^{2+}, with ionic conditions that favor the formation of F-actin. Thus, like other β-spectrin family members (i.e. α-actinin, dystrophin), plectin appears to bind to F-actin with a much higher affinity than to G-actin. Although the yeast two-hybrid assays are normally based on interactions between two monomeric Gal4-fusion proteins, oligomerization of one of the two partners has ocassionally been observed when other cytoskeleton proteins were studied (Meng et al., 1996), suggesting that short F-actin structures can probably be assembled in the yeast nucleus, thereby providing an appropriate ligand for Gal4-plectin-ABD.

The proteins of the β-spectrin family are well-established actin cross-linkers. This property is attributed to protein dimerization mediated by their rod domain. However, recent crystallographic studies showed that in a crystal the ABDs of utrophin and dystrophin are organized in a dimer (Keep et al., 1999; Norwood et al., 2000), which suggests that the ABD of these proteins can also participate in the process of dimerization. Consistent with these findings, we demonstrate that the ABD of plectin is able to form dimers and thereby to bundle actin filaments in vitro. In addition, using pyrene-actin assays, we show that the plectin-ABD increases the rate of actin polymerization and reduces the lag phase, probably by nucleating actin filaments. We assume that this nucleation of actin filaments by the plectin-ABD is also mediated by its dimerization, which might stabilize actin oligomers or even induce their formation. However, we cannot exclude that the plectin-ABD modulates actin polymerization by increasing the rate of monomer addition to barbed ends or by severing actin filaments, thereby creating new barbed ends. By employing the yeast two-hybrid system we have mapped the dimerization domain of the plectin-ABD in the CH1 domain. Thus, the dimerization of the plectin-ABD is different from that of the utrophin-ABD, since the dimerization of the latter is thought to be mediated by CH1-CH2 interaction (Keep et al., 1999). Unexpectedly, we found that the ABS2 is required for both actin binding and for plectin-plectin interaction. Nevertheless, the residues involved in binding are probably different, since the plectin-ABD$_{65-339}$ construct can associate with the plectin-ABD$_{1-339}$ but lacks actin-binding properties. These observations suggest that dimerization of the plectin-ABD does not prevent actin binding, which is supported by our

cosedimentation assays showing that at low concentrations, all of the plectin-ABDs are bound to F-actin. Also, if plectin dimerization inhibited actin binding, then actin-plectin interaction would not have been detected by either the yeast two-hybrid or in vitro binding assays. The distinct binding activities might be explained by the presence of an actin-binding site and a 'dimerization' site located on opposite sides of the first plectin CH domain. Supporting this assumption, Correia et al. (Correia et al., 1999) have shown that the CH1 of fimbrin mediates interactions with actin and vimentin. Using three-dimensional reconstruction a model was proposed by these authors in which actin and vimentin binding sites are located at two opposite sides in the CH1 domain of fimbrin. Besides its role in the bundling of actin filaments, dimerization of the plectin-ABD could also be essential for the binding of actin itself. This is supported by the results of the yeast two-hybrid assays, showing that sequences required for actin binding overlapped with those required for the dimerization of the plectin-ABD. Although the results of the yeast two-hybrid assay suggest a role of the plectin residues 36-65 in dimerization, we assume that, as for binding of plectin to actin, the absence of binding between plectin$_{36\text{-}181}$and plectin$_{65\text{-}339}$ is due to steric hindrance mediated by the Gal4-moiety in plectin$_{65\text{-}339}$.

The in vitro formation of plectin dimers and oligomers has been described (Foisner and Wiche, 1987), and it is believed that they are formed via the rod domain (for reviews, see Wiche, 1989; Foisner and Wiche, 1991). However, while the formation of dimers might be mediated via coiled-coil interactions of the rod domains, oligomerization is likely to require additional binding sites. Our study strongly indicates that the plectin-ABD could provide such additional binding sites. Although it is not yet clear whether the dimerization of the plectin-ABD has physiological relevance, there is evidence that plectin by itself can assemble into a filamentous network in cultured cells (Wiche and Baker, 1982; Wiche et al., 1984).

Using a yeast two-hybrid assay we extended our findings on the dimerization of the plectin-ABD to other β-spectrin family members. We found that dystonin and dystrophin ABDs can also dimerize, but less efficiently than the plectin-ABD. In the case of dystrophin, efficient binding was only obtained with a chimeric molecule containing the first 65 amino acid residues of plectin. Using the same approach we identified the ABD of plectin as a ligand for the dystonin-ABD, a neuronal isoform of the bullous pemphigoid antigen 1 protein (Ruhrberg and Watt, 1997), which strongly suggests that ABDs of different proteins can form heterodimers. By contrast no clear interaction was identified between either the plectin or the dystonin-ABDs with that of dystrophin, which indicates that ABD heterodimerization is specific. The basis of this specificity is probably determined by differences in the sequences, as dystonin and plectin display 88% of homology in their ABD, whereas the homology is 61% in the case of the ABDs of plectin and dystrophin. This heterodimerization of plectin with dystonin further reinforces the role of plectin as a multifunctional cytolinker protein and creates new possibilities for investigating its importance in the regulation of actin dynamics in the cell.

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REFERENCES

Andrä, K., Lassmann, H., Bittner, R., Shorny, S., Fässler, R., Propst, F. and Wiche, G. (1997). Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture. *Genes Dev.* **11**, 3143-3156.

Andrä, K., Nikolic, B., Stocher, M., Drenckhahn, D. and Wiche, G. (1998). Not just scaffolding: plectin regulates actin dynamics in cultured cells. *Genes Dev.* **12**, 3442-3451.

Banuelos, S., Saraste, M. and Carugo, K. D. (1998). Structural comparisons of calponin homology domains: implications for actin binding. *Structure* **6**, 1419-1431.

Bohn, W., Etzrodt, D., Foisner, R., Wiche, G. and Traub, P. (1996). Cytoskeleton architecture of C6 rat glioma cell subclones whole mount electron microscopy and immunogold labeling. *Scan. Microsc. Suppl.* **10**, 285-294.

Bresnick, A., R., Warren, V. and Condeelis, J. (1990). Identification of a short sequence essential for actin binding by *Dictyostelium* ABP-120. *J. Biol. Chem*. **265**, 9236-9240.

Carugo, K. D., Banuelos, S. and Saraste, M. (1997). Crystal structure of a calponin homology domain. *Nat. Struct. Biol.* **4**, 175-179.

Corrado, K., Mills, P. L. and Chamberlain, J. S. (1994). Deletion analysis of the dystrophin-actin binding domain. *FEBS Lett.* **344**, 255-260.

Correia, I., Chu, D., Chou, Y., H., Goldman, R. D. and Matsudaira, P. (1999). Integrating the actin and vimentin cytoskeletons. adhesion-dependent formation of fimbrin-vimentin complexes in macrophages *J. Cell Biol.* **146**, 831-842.

Eger, A., Stockinger, A., Wiche, G. and Foisner, R. (1997). Polarisation-dependent association of plectin with desmoplakin and the lateral submembrane skeleton in MDCK cells. *J. Cell Sci.* **110**, 1307-1316.

Elliott, C. E., Becker, B., Oehler, S., Castañón, M. J., Hauptmann, R. and Wiche, G. (1997). Plectin transcript diversity: identification and tissue distribution of variants with distinct first coding exons and rodless isoforms. *Genomics* **42**, 115-125.

Errante, L. D., Wiche, G. and Shaw, G. (1994). Distribution of plectin, an intermediate filament-associated protein, in the adult rat central nervous system. *J. Neurosci. Res.* **37**, 515-528.

Fabbrizio, E., Bonet-Kerrache, A., Leger, J. J. and Mornet, D. (1993). Actin-dystrophin interface. *Biochemistry* **32**, 10457-10463.

Foisner, R. and Wiche, G. (1987). Structure and hydrodynamic properties of plectin molecules. *J. Mol. Biol.* **198**, 515-531.

Foisner, R. and Wiche, G. (1991). Intermediate filament-associated proteins. *Curr. Opin. Cell Biol.* **3**, 75-81.

Foisner, R., Leichtfried, F. E., Herrmann, H., Small, J. V., Lawson, D. and Wiche, G. (1988). Cytoskeleton-associated plectin: in situ localization, in vitro reconstitution, and binding to immobilized intermediate filament proteins. *J. Cell Biol.* **106**, 723-733.

Foisner, R., Feldman, B., Sander, L., Seifert, G., Artlieb, U. and Wiche, G. (1994). A panel of monoclonal antibodies to rat plectin: distinction by epitope mapping and immunoreactivity with different tissues and cell lines. *Acta Histochem.* **96**, 421-438.

Gache, Y., Chavanas, S., Lacour, J.-P., Wiche, G., Owaribe, K., Meneguzzi, G. and Ortonne, J. P. (1996). Defective expression of plectin/HD1 in epidermolysis bullosa simplex with muscular dystrophy. *J. Clin. Invest.* **97**, 2289-2298.

Geerts, D., Fontao, L., Nievers, M. G., Schaapveld, R. Q. J., Purkis, P. E., Wheeler, G. N., Lane, E. B., Leigh, I. M. and Sonnenberg, A. (1999). Binding of integrin α6β4 to plectin prevents plectin association with F-actin but does not interfere with intermediate filament binding. *J. Cell Biol.* **147**, 417-434.

Gimona, M. and Mital, R. (1998). The single CH domain of calponin is neither sufficient nor necessary for F-actin binding. *J. Cell Sci.* **111**, 1813-1821.

Gimona, M. and Winder, S. J. (1999). Single calponin homology domains are not actin-binding domains. *Curr. Biol.* **8**, R674-675.

Hartwig, J. (1994). Actin-binding proteins 1: spectrin superfamily. *Protein Profile* **1**, 711-778.

Herman, I. M. (1993). Actin isoforms. *Curr. Opin. Cell Biol.* **5**, 48-55.

Hermann, H. and Wiche, G. (1987). Plectin and IFAP-300K are homologous proteins binding to microtubule-associated proteins 1 and 2 and to the 240-kilodalton subunit of spectrin. *J. Biol. Chem.* **262**, 1320-1325.

Hieda, Y., Nishizawa, Y., Uematsu, J. and Owaribe, K. (1992). Identification of a new hemidesmosomal protein, HD1: a major, high molecular mass component of isolated hemidesmosomes. *J. Cell Biol.* **116**, 1497-1506.

Hijikata, T., Murakami, T., Imamura, M., Fujimaki, N. and Ishikawa, H. (1999). Plectin is a linker of intermediate filaments to Z-discs in skeletal muscle fibers. *J. Cell Sci.* **112**, 867-876.

Keep, N. H., Winder, S. J., Moores, C. A., Walke, S., Norwood, F. L. M. and Kendrick-Jones, J. (1999). Crystal structure of the actin-binding region of utrophin reveals a head-to-tail dimer. *Structure* **7**, 1539-1546.

Kouyama, T. and Mihashi, K. (1981). Fluorimetry study of N-(1-pyrenyl)iodoacetamide-labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. *Eur. J. Biochem.* **114**, 33-38.

McLean, W. H. I. Pulkkinen, L., Smith, F. J. D., Rugg, E. L., Lane, E. B., Bullrich, F., Burgeson, R. E., Amano, S., Hudson, D. L., Owaribe, K., McGrath, J. A., McMillan, J. R., Eady, R. A. J., Leigh, I. M., Christiano, A. M. and Uitto, J. (1996). Loss of plectin causes epidermolysis bullosa with muscular dystrophy: cDNA cloning and genomic organization. *Genes Dev.* **10**, 1724-1735.

Meng, J. J., Khan, S. and Ip, W. (1996). Intermediate filament protein domain interactions as revealed by two-hybrid screens. *J. Biol. Chem.* **271**, 1599-1604.

Niessen, C. M., Hulsman, E. H. M., Rots, E. S., Sánchez-Aparicio, P. and Sonnenberg, A. (1997), Integrin α6β4 forms a complex with the cytoskeletal protein HD1 and induces its redistribution in transfected COS-7 cells. *Mol. Biol. Cell* **8**, 555-566.

Nievers, M. G., Schaapveld, R. Q. J., Oomen, L. C. J. M., Fontao, L., Geerts, D. and Sonnenberg, A. (1998). Ligand-independent role of the β4 integrin subunit in the formation of hemidesmosomes. *J. Cell Sci.* **111**, 1659-1672.

Nikolic, B., Mac Nulty, E., Mir, B. and Wiche, G. (1996). Basic amino acid residue cluster within nuclear targeting sequence motif is essential for plectin-vimentin network junctions. *J. Cell Biol.* **134**, 1455-1467.

Norwood, F. L. M., Sutherland-Smith, A. J., Keep, N. H. and Kendrick-Jones, J. (2000). The structure of the N-terminal actin-binding domain of human dystrophin and how mutations in this domain may cause Duchenne or Becker muscular dystrophy. *Structure* **8**, 481-491.

Pulkkinen, L., Smith, F. J. D., Shimizu, H., Murata, S., Yaoita, H., Hachisuka, H., Nishikawa, T., McLean, W. H. I. and Uitto, J. (1996). Homozygous deletion mutations in the plectin gene (PLEC1) in patients with epidermolysis bullosa simplex associated with late-onset muscular dystrophy. *Hum. Mol. Genet.* **5**, 1539-1546.

Pytela, R. and Wiche, G. (1980). High molecular weight polypeptides (270,000-340,000) from cultured cells are related to hog brain microtubule-associated proteins but copurify with intermediate filaments. *Proc. Natl. Acad. Sci. USA* **77**, 4808-4812.

Rezniczek, G. A., de Pereda, J. M., Reipert, S. and Wiche, G. (1998). Linking integrin α6β4-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the β4 subunit and plectin at multiple molecular sites. *J. Cell Biol.* **141**, 209-225.

Ruhrberg, C. and Watt, F. M. (1997). The plakin family: versatile organizers of cytoskeletal architecture. *Curr. Opin. Genet. Dev.* **7**, 392-397.

Sánchez-Aparicio, P., Martínez de Velasco, A. M., Niessen, C. M., Borradori, L., Kuikman, I., Hulsman, E. H. M., Fässler, R., Owaribe, K. and Sonnenberg, A. (1997). The subcellular distribution of the high molecular mass protein, HD1, is determined by the cytoplasmic domain of the integrin β4 subunit. *J. Cell Sci.* **110**, 169-178.

Schaapveld, R. Q. J., Borradori, L., Geerts, D., van Leusden, M. R., Kuikman, I., Nievers, M. G., Niessen, C. M., Steenbergen, R. D. M., Snijders, P. J. F. and Sonnenberg, A. (1998). Hemidesmosome formation is initiated by the β4 integrin subunit, requires complex formation of β4 and HD1/plectin, and involves a direct interaction between β4 and the bullous pemphigoid antigen 180. *J. Cell Biol.* **142**, 271-284.

Schroder, R., Mundegar, R. R., Treusch, M., Schlegel, U., Blumcke, I., Owaribe, K. and Magin, T. M. (1997). Altered distribution of plectin/HD1 in dystrophinopathies. *Eur. J. Cell Biol.* **74**, 165-171.

Schroder, R., Warlo, I., Herrmann, H., van der Ven, P. F., Klasen, C., Blumcke, I., Mundegar, R. R., Furst, D. O., Goebel, H. H. and Magin, T. M. (1999). Immunogold EM reveals a close association of plectin and the desmin cytoskeleton in skeletal muscle. *Eur. J. Cell Biol.* **78**, 288-295.

Seifert, G. J., Lawson, D. and Wiche, G. (1992). Immunolocalization of the intermediate filament-associated protein plectin at focal contacts and actin stress fibers. *Eur. J. Cell Biol.* **59**, 138-147.

Smith, F. J. D., Eady, R. A. J., Leigh, I. M., McMillan, J. R., Rugg, E. L., Kelsell, D. P., Bryant, S. P., Spurr, N. K., Geddes, J. F., Kirtschig, G., Milana, G., de Bona, A. G., Owaribe, K., Wiche, G., Pulkkinen, L., Uitto, J., McLean, W. H. I. and Lane, E. B. (1996). Plectin deficiency results in muscular dystrophy with epidermolysis bullosa. *Nat. Genet.* **13**, 450-457.

Stegh, A. H., Herrmann, H., Lampel, S., Weisenberger, D., Andrä, K., Seper, M., Wiche, G., Krammer, P. H. and Peter, M. E. (2000). Identification of the cytolinker plectin as a major early in vivo substrate for caspase 8 during CD95- and tumor necrosis factor receptor-mediated apoptosis. *Mol. Cell Biol.* **15**, 5665-5679.

Steinbock, F. A. and Wiche, G. (1999). Plectin: a cytolinker by design. *Biol. Chem.* **380**, 151-158.

Svitkina, T. M., Verkhovsky, A. B. and Borisy, G. G. (1996). Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. *J. Cell Biol.* **135**, 991-1007.

Uitto, J., Pulkkinen, L., Smith, F. J. D. and McLean, W. H. I. (1996). Plectin and human genetic disorders of the skin and muscle. The paradigm of epidermolysis bullosa with muscular dystrophy. *Exp. Dermatol.* **5**, 237-246.

Van Troys, M., Vandekerckhove, J. and Ampe, C. (1999). Structural modules in actin-binding proteins: towards a new classification. *Biochim. Biophys. Acta* **1448**, 323-348.

Wiche, G. (1989). Plectin: general overview and appraisal of its potential role as a subunit protein of the cytomatrix. *Crit. Rev. Biochem. Mol. Biol.* **24**, 41-67.

Wiche, G. and Baker, M. A. (1982). Cytoplasmic network arrays demonstrated by immunolocalization using antibodies to a high molecular weight protein present in cytoskeletal preparations from cultured cells. *Exp. Cell Res.* **138**, 15-29.

Wiche, G., Becker, B., Luber, K., Weitzer, G., Castañón, M. J., Hauptmann, R., Stratowa, C. and Stewart, M. (1991). Cloning and sequencing of rat plectin indicates a 466-kD polypeptide chain with a three-domain structure based on a central alpha-helical coiled coil. *J. Cell Biol.* **114**, 83-99.

Wiche, G., Krepler, R., Artlieb, U., Pytela, R. and Denk, H. (1983). Occurrence and immunolocalization of plectin in tissues. *J. Cell Biol.* **97**, 887-901.

Wiche, G., Krepler, R., Artlieb, U., Pytela, R. and Aberer, W. (1984). Identification of plectin in different human cell types and immunolocalization at epithelial basal cell surface membranes. *Exp. Cell Res.* **155**, 43-49.