Biochemical and Biophysical Research Communications **273,** 183–187 (2000)
doi:10.1006/bbrc.2000.2916, available online at http://www.idealibrary.com on IDEAL®

The 300-kDa Intermediate Filament-Associated Protein (IFAP300) Is a Hamster Plectin Ortholog

Bryan H. Clubb,* Ying-Hao Chou,* Harald Herrmann,† Tatyana M. Svitkina,‡ Gary G. Borisy,* and Robert D. Goldman*,[1]

**Department of Cell and Molecular Biology, Northwestern University, Chicago, Illinois 60657; †Division of Cell Biology, German Cancer Research Center, Heidelberg, Germany; and ‡Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706*

Received May 15, 2000

Plectin is a high-molecular-weight cytoskeleton-associated protein that was initially identified in intermediate filament (IF)-enriched fractions of rat C6 glioma cells. At the cellular level, plectin has been found to associate with IF networks and IF-associated structures that are involved in cell–cell and cell–substrate adhesions. IFAP300 is an IF-associated protein that was initially identified in hamster cells by a monoclonal antibody directed against a high molecular weight protein present in IF-enriched cytoskeletal preparations. Plectin and IFAP300 display similar distribution patterns within cells as determined by immunofluorescence. Based upon this and the finding that their biochemical properties are similar, it has been suggested that they may actually be orthologous proteins. In this paper we demonstrate that this is the case. Cloning and sequencing of most of the hamster plectin cDNA demonstrates that plectin is found in hamster cells and that its sequence is highly conserved between species. Using immunological cross-reactivity, epitope mapping, and immunoelectron microscopy, we show that IFAP300 is actually the hamster ortholog of plectin. © 2000 Academic Press

Key Words: **cytoskeleton; plectin; intermediate filament-associated protein.**

Plectin is a high-molecular-weight cytoskeleton-associated protein that was initially identified in rat C6-glioma cells (1), but was later shown to be widely expressed in different tissues and cell types (2, 3). At the cellular level, plectin associates with vimentin and keratin IF networks and structures that are involved in cell-cell and cell-substrate adhesions such as desmosomes and hemidesmosomes (2–6). Cloning and sequencing of human and rat plectin cDNAs has demonstrated that this protein is composed of multiple structural and functional domains, including an amino-terminal actin-binding domain, a central α-helical rod and six highly homologous repeats in the carboxyl-terminus (7, 8). Using a variety of biochemical and molecular techniques it has been shown that plectin can bind directly to several cytoskeletal proteins other than IF and actin, including α-spectrin, β4 integrin and the microtubule-associated proteins, MAP1 and MAP2 (9, 10). While the precise nature of the interactions between plectin and other cytoskeletal proteins remains unknown, a putative IF binding domain has been mapped to ~50 amino acids following the fifth repeat in the carboxyl-tail (10, 11). Recent genetic analysis of patients suffering from the severe skin blistering diseases epidermolysis bullosa simplex (EBS)-Ogna and EBS with associated muscular dystrophy suggests that plectin plays an essential role in mechanically strengthening cells, probably by linking IF networks either directly or indirectly to cellular junctional complexes (5, 12–14).

Following its discovery, several findings suggested that the 300 kDa IF-associated protein, IFAP300, was not plectin. IFAP300 was initially characterized as a protein in IF-enriched preparations from the baby hamster kidney fibroblastic cell line, BHK-21 (15, 16). Immunofluorescence studies using a monoclonal antibody (mAb) directed against this protein co-localized with IF in BHK cells before and after colchicine treatment, in spreading cells and co-cycled with IF in an *in vitro* assembly/disassembly assay (15). Based on similarities in size and immunocytochemical staining patterns, several studies have attempted to determine whether IFAP300 and plectin are the same or different proteins. After it became clear that plectin and MAP2 were not related as originally proposed (1), biochemical

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. AF260753.

[1] To whom correspondence should be addressed at Department of Cell and Molecular Biology, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago IL 60657. Fax: (312) 503-0954. E-mail: r-goldman@northwestern.edu.

0006-291X/00 $35.00

TABLE 1

Comparison of Trout Plectin Peptides Prepared from IF-Preparations of RTG-2 Cells with Human Sequence

Peptide No.		Sequence	Location of human peptide (aa#)	Identity
Pep1	Trout	LQNVQIALDFLR	Globular N-terminus	92%
	Human	LQNVQIALD**Y**LR	127–138	
Pep2	Trout	LLFNDVQTLK	Globular N-terminus	100%
	Human	LLFNDVQTLK	478–487	
Pep3	Trout	YAQDLLTLVEE	Globular N-terminus	73%
	Human	Y**L**QDLL**AW**VEE	551–561	
Pep4	Trout	YSELMTLTSQYIK	α-Helical rod	92%
	Human	YSEL**T**TLTSQYIK	1350–1362	
Pep5	Trout	FFDPNTLDNLTY	Globular C-terminus	84%
	Human	FFDPNT**EE**NLTY	4117–4128	

Note. Sequence letters represent single-letter amino acid code. Bold letters denote differences in amino acid sequence.

studies using peptide mapping and immunological cross-reactivity, suggested that IFAP300 and plectin were the same (10). However, while polyclonal antibodies directed against IFAP300 recognize plectin, monoclonal antibodies against IFAP300 do not cross-react with rat plectin and monoclonal antibodies against rat plectin do not cross-react with hamster IFAP300, respectively (17). Moreover, two-dimensional proteolytic maps of IFAP300 and plectin also indicated that these proteins were not identical (17).

Based upon these discrepancies, the nature of the relationship between hamster IFAP300 and plectin have remained unclear. In this paper we resolve the question of the relationship between IFAP300 and plectin. Cloning and sequencing of most of the hamster plectin cDNA clearly demonstrates that plectin is found in hamster cells and that its sequence is highly conserved between species. Immunoblot analyses of recombinant fragments of hamster plectin show that the IFAP300 monoclonal antibody, 417D, recognizes hamster plectin. Moreover, we have been able to demonstrate that the IFAP300 epitope is located in the central part of the rod domain.

MATERIALS AND METHODS

Cloning and sequencing hamster plectin cDNA. Since the full-length rat (Accession No. X59601) and human (U53204) plectin cDNA sequences were known, our strategy was to clone the cDNA for BHK-21 (c13) cell plectin using reverse transcriptase-polymerase chain reactions (RT-PCR). Initially, the 3′-terminus of BHK plectin cDNA was amplified from a λgt22A cDNA expression library. This involved the use of a λgt11-2 primer and a primer corresponding to a region of plectin cDNA conserved in both the human and rat sequences. A 2.2 kbp fragment was identified and sequence analysis showed that it possessed ~85% identity to the 3′ end of the published rat plectin sequence. RT-PCR was used to amplify additional plectin sequence upstream of this partial cDNA sequence. BHK mRNA was isolated using the FastTrack 2.0 Kit for Isolation of mRNA (Invitrogen; Carlsbad, CA) and cDNA was synthesized from ~1 μg of mRNA using the SUPERSCRIPT II Reverse Transcriptase System (Life Technologies). Stepwise PCR amplification of plectin cDNA was performed using anti-sense primers complementary to a region of known BHK plectin cDNA and sense primers homologous to a region of plectin cDNA conserved in both the human and rat sequences. A total of six overlapping PCR fragments were characterized (see Fig. 1).

All PCR fragments were cloned using the TOPO TA Cloning kit (Invitrogen) and sequenced in both directions using BigDye Terminator Cycle Sequencing Ready Reaction mix (PE Applied Biosystems, Foster City, CA) and analyzed using an ABI 377 DNA Analyzer (PE Applied Biosystems).

Expression and immunoblotting of recombinant plectin fragments. Plectin cDNA fragments were subcloned into the prokaryotic expression vector pGEX-4T (Amersham Pharmacia Biotech; Piscataway, NJ). Individual plasmids were then expressed in BL21 (DE3) *E. coli* yielding plectin–glutathione-*S*-transferase fusion proteins. Total bacterial lysates were separated using standard SDS–PAGE gels for each of the fusion proteins and transferred onto nitrocellulose membranes. The membranes were blocked with 5% dry milk. The immune reaction was performed with either the monoclonal IFAP300 antibody 417D or guinea pig serum F3 which was raised against a peptide derived from a sequence identical in trout and human plectin (Pep2, Table 1). Trout plectin was isolated from high salt/NP-40 resistant fractions of RTG-2 cells by SDS–PAGE on 5% polyacrylamide gels and transferred by blotting to PVDF membranes (18). Fragments of the trypsin-digested polypeptides were isolated by HPLC and sequenced as described (19). The synthetic peptide, RLLFNDVQTLKDGRHC was elongated by five amino acids according to the human sequence and a C-terminal cysteine for coupling to hemocyanin.

Ultrastructural analysis. Immunogold labelling was carried out for electron microscopy using BHK-21 cytoskeleton preparations as previously described (20, 21), with the following exceptions: to optimize 417D antibody staining, cells were lysed in PEM buffer (100 mM PIPES, pH 6.9; 1 mM $MgCl_2$; 1 mM EGTA) containing 1% Triton X-100, 4% polyethylene glycol and taxol. Gelsolin treatment was carried out in the presence of taxol, and the cytoskeletal preparations were incubated with the primary antibody solution prior to glutaraldeyhde fixation.

RESULTS AND DISCUSSION

The IF-associated protein, IFAP300, originally isolated from BHK-21 cells (15, 16) has been defined almost exclusively by the monoclonal antibody 417D, which does not cross react with the originally identified

plectin of rat C6 glioma cells. Studies from our laboratory and others have previously shown that hamster IFAP300 and rat plectin, purified using identical protocols, have unique and shared epitopes and that peptide maps of ^{125}I-labeled proteins from C6-glioma and BHK-21 cells are similar (10, 17). These observations suggest that IFAP300 and plectin may be closely related or even orthologous proteins. Previous attempts to shed light on the nature of the relationship between these two proteins were focused on cloning the hamster cDNA which encodes the 417D monoclonal antibody epitope from λgt11 cDNA expression libraries. This initial effort was unsuccessful and likely reflected the limits of cloning large cDNAs using lambda expression libraries (17). The cDNA for rat plectin is ~15 kbp (8).

In this study, we reasoned that since plectin is a single copy gene in human and rat genomes, and we have found that the 417D antibody does not react with other types of plakins (data not shown), we next tried to determine whether the 417D mAb recognizes hamster plectin. This involved cloning BHK-21 plectin cDNAs using RT-PCR, expressing them in a prokaryotic expression system and then determining whether the 417D antibody recognized any of the expressed proteins. Using this approach, we have cloned a large partial cDNA that encodes hamster plectin. This cDNA is 14,618 base pairs long and starts at nucleotide 643 with respect to the rat C6 plectin cDNA sequence (Fig. 1A; Accession No. AF260753). Comparisons of this hamster sequence with the known full-length rat and human plectin cDNA sequences confirm that the hamster sequence is plectin and that its sequence is highly conserved between species. At the nucleotide level, hamster plectin cDNA is ~91% and ~87% identical to rat and human plectin, respectively (not shown). Analysis of the longest uninterrupted open reading frame in the hamster plectin cDNA shows that it encodes a protein at least 4474 amino acids long. Remarkably, comparisons of the hamster primary amino acid sequence shows ~96% and ~93% identity to rat and human plectin, respectively (not shown). These results demonstrate that plectin is highly conserved between species.

In order to determine specifically whether the 417D antibody recognizes plectin, fragments of hamster plectin were expressed as fusion proteins in prokaryotic cells. Immunoblot analyses of these plectin peptides were carried out to determine whether this antibody recognizes hamster plectin. The resulting data show that this antibody recognizes an epitope in the central α-helical domain of plectin (Fig. 1B and 1C). Alignment of the homologous hamster, human and rat plectin sequences shows that this epitope lies within the putative rod domain. Since the rod domain is highly conserved, this result surprised us because the monoclonal antibody 417D recognizes hamster IFAP300 and human plectin (not shown), but not rat plectin (17). However, the alignment of the specific amino acids containing the 417D epitope shows that differences in the primary sequences of hamster, human and rat plectin could explain the lack of cross-reactivity (Fig. 2). Even though this region shares a high degree of identity, 94% for human and 96% for rat, 5 out of the 178 residues within the epitope domain differ in rat plectin, but are conserved in hamster and human plectin. In addition, since 2 residues in this hamster sequence, positions 1 and 174, have been expressed in overlapping clones that were not recognized by 417D, the three residues remaining may be essential in determining the IFAP300 epitope. Indeed, it has been shown for a

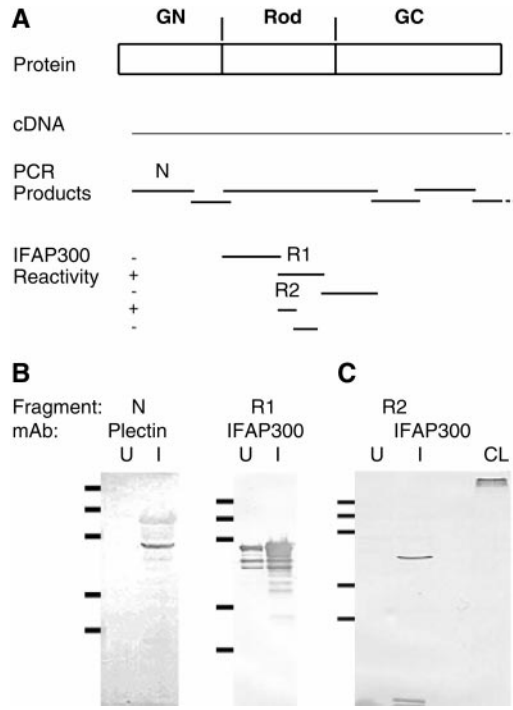

FIG. 1. Monoclonal IFAP300 antibody 417D recognizes the plectin rod domain. (A) Diagrammatic representation of plectin protein structure and the extent to which the hamster cDNA has been cloned. Hamster plectin cDNA was assembled from five RT-PCR products containing protein coding regions, while the 3′-untranslated region was PCR amplified from a λgt22A cDNA expression library. PCR products labeled N, R1, and R2 represent regions encoding plectin recognized by plectin or IFAP300 antibodies. (B) Immunoblot analysis of plectin fragments expressed in *E. coli* showing that a guinea pig serum raised against a highly conserved 15-amino-acid-long plectin peptide in the amino-terminal domain also recognizes a recombinant amino-terminal hamster plectin fragment. The IFAP300 mAb 417D recognizes an epitope in the recombinant plectin fragment labeled R1. (C) Expression of smaller pieces of the rod domain narrowed the 417D epitope to a region of 178 amino acids in the center of the α-helical rod domain labeled R2. GN, globular amino-terminal domain; Rod, α-helical coiled-coil structure; GC, globular carboxyl-terminal domain. (+) and (−) indicate whether 417D reacts or does not react with the plectin fragment encoded by the respective PCR product. U, uninduced protein expression; I, protein expression induced with 1 μM IPTG. CL, whole BHK-21 cell lysate. Molecular weight standards for blots, from top to bottom, 208, 127, 85, 45, and 32.8 kDa.

```
Hamster: 1    RVLTEKLAAISEATRLKTEAEIALKEKEAENERLRRLAEDEAFQRRRLEEQAALHKADIEE 61
Human:   1970 ...A......G.....................................Q..... 2030
Rat:     1973 G.....................................................Q..... 2033


Hamster: 62   RLAQLRKASESELERQKGLVEDTLRQRRQVEEEILALKVSFEKAAAGKAELELELGRIRSS 122
Human:   2031 .........D.............................A.....................N 2091
Rat:     2034 .....................................M...A.....................N 2094


Hamster: 123  AEDTMRSKEQAEQEAARQRQLAAEEEQRRREAEERVQKSLAAEEEAARQRKAALEE 178
Human:   2092 ....L.......L.............R.............................. 2147
Rat:     2095 .........L.............................R.............V.... 2150
```

FIG. 2. Alignment of the smallest hamster plectin fragment (R2), recognized by the monoclonal antibody 417D, with human and rat plectin shows that the primary structure of the rod domain is highly conserved. Single letters in the human and rat sequences denote amino acid differences with respect to the hamster sequence. Only five amino acids out of 178 are identical in hamster and human, and different in the rat plectin sequence. These differences in the primary amino acid sequence may be essential in determining why 417D recognizes hamster and human, but not rat C6 glioma cell plectin.

monoclonal antibody (3B4) raised against bovine vimentin, that a single amino acid difference in the primary sequence of mouse and human protein, within the epitope, caused the non-reactivity of that antibody with mouse vimentin (22).

Immunoblot analysis of recombinant hamster plectin fragments with a peptide antibody directed against a highly conserved sequence found in the amino-terminus of trout plectin also recognized the amino-terminal domain of hamster plectin (Fig. 1B). Moreover, immunoprecipitation of plectin from BHK-21 IF preparations with 417D revealed a protein of an apparent molecular weight of 300,000 that was recognized with a guinea pig serum generated against recombinant C-terminal repeat six of human plectin (data not shown; for antibodies see (23)). This confirms that the hamster expresses plectin and that the recombinant proteins expressed were plectin, and not a closely related protein. Together, the demonstration that BHK-21 cells have mRNA encoding plectin and that the 417D antibody recognizes an expressed domain of plectin, confirms that IFAP300 is plectin.

In order to lend further support to this conclusion, an ultrastructural analysis was carried out to determine whether the 417D monoclonal antibody recognizes typical plectin-type structures in BHK cell cytoskeletal preparations. Ultrastructural studies of cells treated to reduce their actin content demonstrate that vimentin IF have a millipede-like appearance with the "legs" appearing to interconnect IF with microtubules, the actin-based cytoskeleton and possibly membrane components (21). These legs or projections contain plectin as determined by immunogold labeling with monoclonal antibodies (21). Similarly, labeling actin-depleted BHK cell cytoskeletal preparations shows that the 417D monoclonal antibody also recognizes the legs projecting from IF (Fig. 3). The position of the gold particles, at the middle of these projections, provides further proof that the epitope for this antibody lies in the central region of the projection. These observations confirm that the antigen recognized by the 417D antibody is plectin and that the epitope recognized by the antibody lies in the central portion of the molecule. This localization of the 417D epitope in the central portion of the plectin crossbridges corresponds closely to the location of another plectin rod-specific monoclonal antibody (21).

In summary, this study demonstrates by immunological crossreactivity, cloning and sequencing of the cDNA and immunoelectron microscopy denotes that IFAP300 is plectin.

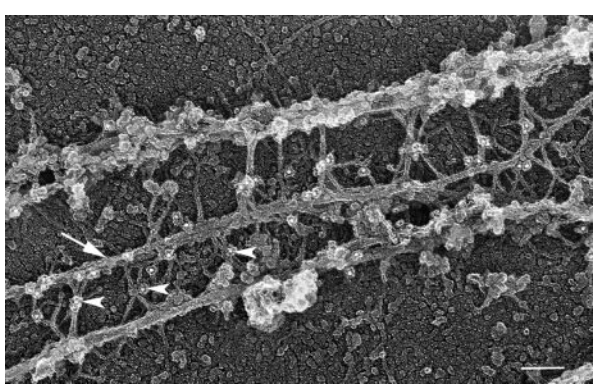

FIG. 3. Plectin side arms on intermediate filaments. Electron microscopy of actin-depleted BHK-21 cytoskeletons shows that IF have a millipede-like appearance. Immunogold-labeling (arrowheads) shows that the monoclonal antibody 417D recognizes the side arms that project away from an IF (arrow). Scale bar, 100 nm.

ACKNOWLEDGMENTS

We thank Dr. Hans Heid for peptide sequence analysis of trout plectin. Bryan H. Clubb was supported by a postdoctoral fellowship from the Medical Research Council of Canada. This work was supported by a National Institute of Dental Research Grant (NIDR 1 PO1 DE12328) to R. D. Goldman.

REFERENCES

1. Pytela, R., and Wiche, G. (1980) *PNAS* **77,** 4808–4812.
2. Wiche, G., Krepler, R., Artlieb, U., Pytela, R., and Denk, H. (1983) *J. Cell Biol.* **97,** 887–901.
3. Wiche, G., Herrmann, H., Leichtfried, F., and Pytela, R. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **46,** 475–482.
4. McMillan, J. R., McGrath, J. A., Tidman, M. J., and Eady, R. A. (1998) *J. Invest. Dermat.* **110,** 132–137.
5. McLean, W. H., Pulkkinen, L., Smith, F. J., 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., Leigh, I. M., Christiano, A. M., and Uitto, J. (1996) *Genes Dev.* **10,** 1724–1735.
6. Schaapveld, R. Q., Borradori, L., Geerts, D., van Leusden, M. R., Kuikman, I., Nievers, M. G., Niessen, C. M., Steenbergen, R. D., Snijders, P. J., and Sonnenberg, A. (1998) *J. Cell Biol.* **142,** 271–284.
7. Liu, C. G., Maercker, C., Castanon, M. J., Hauptmann, R., and Wiche, G. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4278–4283.
8. Wiche, G., Becker, B., Luber, K., Weitzer, G., Castanon, M. J., Hauptmann, R., Stratowa, C., and Stewart, M. (1991) *J. Cell Biol.* **114,** 83–99.
9. Geerts, D., Fontao, L., Nievers, M. G., Schaapveld, R. Q., Purkis, P. E., Wheeler, G. N., Lane, E. B., Leigh, I. M., and Sonnenberg, A. (1999) *J. Cell Biol.* **147,** 417–434.
10. Herrmann, H., and Wiche, G. (1987) *J. Biol. Chem.* **262,** 1320–1325.
11. Nikolic, B., Mac Nulty, E., Mir, B., and Wiche, G. (1996) *J. Cell Biol.* **134,** 1455–1467.
12. Koss-Harnes, D., Jahnsen, F. L., Wiche, G., Soyland, E., Brandtzaeg, P., and Gedde-Dahl, T., Jr. (1997) *Exp. Dermatol.* **6,** 41–48.
13. Smith, F. J., Eady, R. A., 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 Bono, A. G., Owaribe, K., Wiche, G., Pulkkinen, L., Uitto, J., McLean, W. H., and Lane, E. B. (1996) *Nature Genetics* **13,** 450–457.
14. Gache, Y., Chavanas, S., Lacour, J. P., Wiche, G., Owaribe, K., Meneguzzi, G., and Ortonne, J. P. (1996) *J. Clin. Invest.* **97,** 2289–2298.
15. Yang, H. Y., Lieska, N., Goldman, A. E., and Goldman, R. D. (1985) *J. Cell Biol.* **100,** 620–631.
16. Lieska, N., Yang, H. Y., and Goldman, R. D. (1985) *J. Cell Biol.* **101,** 802–813.
17. Skalli, O., Jones, J. C., Gagescu, R., and Goldman, R. D. (1994) *J. Cell Biol.* **125,** 159–170.
18. Herrmann, H., Muenick, M. D., Brettel, M., Fouquet, B., and Markl, J. (1996) *J. Cell Sci.* **109,** 569–578.
19. Hess, H., Heid, H., and Franke, W. W. (1993) *J. Cell Biol.* **122,** 1043–1052.
20. Svitkina, T. M., and Borisy, G. G. (1998) *Methods Enzymol.* **298,** 570–592.
21. Svitkina, T. M., Verkhovsky, A. B., and Borisy, G. G. (1996) *J. Cell Biol.* **135,** 991–1007.
22. Bohn, W., Wiegers, W., Beuttenmuller , M., and Traub, P. (1992) *Exp. Cell Res.* **201,** 1–7.
23. 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) *Eur. J. Cell Biol.* **78,** 288–295.