Plectin and IFAP-300K Are Homologous Proteins Binding to Microtubule-associated Proteins 1 and 2 and to the 240-kilodalton Subunit of Spectrin*

Harald Herrmann‡ and Gerhard Wiche
From the Institute of Biochemistry, University of Vienna, Währinger Strasse 17, A-1090 Vienna, Austria

(Received for publication, September 4, 1986)

Structural and functional characteristics of plectin from intermediate filament preparations of rat glioma C6 cells were compared to those of the intermediate filament-associated protein of M, = 300,000 (IFAP-300K) of baby hamster kidney cells (Yang, H.-S., Lieska, N., Goldman, A. E., and Goldman, R. D. (1985) J. Cell Biol. 100, 620–631). After radiolabeling and proteolytic digestion under varied conditions, both proteins yielded nearly identical peptide maps. Immunological cross-reactivity, co-migration on one- and two-dimensional high-resolution gels, chromatofocusing, and amino acid analysis demonstrated structural homology as well. In vivo labeling with 32P showed that plectin was the target for cAMP-dependent protein kinases which phosphorylated 18-kDa domains at the end(s) of the molecule. Previously reported phosphorylation sites for cAMP-dependent and a newly identified site for Ca2+/calmodulin-dependent protein kinases were located on different domains. In solid-phase binding assays, plectin bound to vimentin, microtubule-associated proteins 1 and 2, the 240-kDa chain of brain fodrin, and α-spectrin from human erythrocytes. Similar characteristics were revealed for corresponding 300-kDa components of various other cell lines, supporting the concept that plectin is a general cytoskeletal cross-linking element, probably of multiple function.

Plectin, a phosphoprotein of M, = 300,000, originally was identified as a major component copurifying with intermediate filaments (IFs) after high salt/1% Triton X-100 extraction of C6 cells (1). It is a prominent substrate for cAMP-dependent as well as cAMP-independent protein kinases associated with the cytoskeleton (2). Plectin was first supposed to be related to MAP 2 (1), but was later shown to be a distinct protein based on peptide mapping and immunological criteria (2, 3). A 300-kDa band immunoreactive with antisera to C6 cell plectin has been shown to occur in IF preparations from a variety of cell types and tissues (2–5). In immunofluorescence microscopy studies using a polyclonal antibody preparation, this antigen was visualized throughout the cytoplasm of several tissue cell types (4, 5) and in dense cytoplasmic network arrays of cultured cell lines (2, 3, 5, 6). Moreover, in combination with immunoelectron microscopy, plectin was localized at junctional sites of various cell types such as hepatocytes and epithelial cells and at attachment sites of cytoplasmic filaments including Z discs and dense plaques of striated and smooth muscle (4, 5).

Recently, a high M, polypeptide, referred to as IF-associated protein of M, = 300,000 (IFAP-300K), has been identified in IF preparations of baby hamster kidney (BHK-21) cells (7, 8). This protein codistributed with BHK-21 IFs in situ as determined using a monoclonal antibody preparation, and cycled with the IF subunit proteins from BHK-21 cells in rounds of in vitro assembly/disassembly (7). However, because of its selective association with IFs of cultured cells, Goldman and co-workers concluded that IFAP-300K and plectin were distinct proteins (7, 8). Therefore, a direct comparison of both polypeptides by chemical as well as immunological methods became important. The data reported here suggest that both proteins, like those of various other fibroblast cell lines, indeed are closely related in primary structure. Furthermore, the identification of several new interaction partners of plectin by solid-phase binding assays supports the concept that this polypeptide is a general cytoplasmic cross-linker.

EXPERIMENTAL PROCEDURES

RESULTS

Chemical Comparison of Plectin and IFAP-300K—Extraction of cultured rat glioma C6 cell monolayers with buffers containing 0.77 M NaCl/1% Triton X-100 yielded an insoluble residue that consisted predominantly of a M, = 300,000 protein, plectin (6), and the IF subunit protein, vimentin (Fig. 1). A protein component of electrophoretic mobility identical to that of plectin was prominent also in parallel preparations from BHK-21 cells, together with vimentin and desmin (Fig. 1). The 300,000-Da proteins from both cell types were immunoprecipitated to a similar extent using antibodies to C6

* This work was supported in part by grants from the Austrian Research Fund (Österreichischer Fonds zur Förderung der Wissenschaftlichen Forschung). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a postdoctoral fellowship from Boehringer Ingelheim Funds. Made an oral presentation of the results contained in this paper at the Second European Congress on Cell Biology, Budapest, Hungary, July 7, 1986.

The abbreviations used are: IF, intermediate filament; IFAP-300K, IF-associated protein of M, = 300,000; MAP, microtubule-associated protein; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; 1-d, one-dimensional; DTI, dithiothreitol; EGTA, ethylenediamine(oxethylendentriol)tetraacetic acid; NP-40, Nonidet P-40.

‡ Portions of this paper (including "Experimental Procedures" and Figs. 5 and 7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3044, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
were prepared by procedure B (see "Experimental Procedures") and boiled after addition of electrophoresis sample buffer. Immunoprecipitation from boiled samples was done with antibodies to boiled plectin, vimentin, and desmin. The positions of plectin, vimentin, and desmin are indicated by numbers (M, \times 10^9).

**FIG. 1. Identification of plectin in high salt/Triton X-100-insoluble cell fractions.** Rat glioma C6 (lanes 1 and 2) and BHK-21 (lanes 3 and 4) cell monolayers were labeled with [3H]-leucine. IFs were prepared by procedure B (see "Experimental Procedures") and boiled after addition of electrophoresis sample buffer. Immunoprecipitates prepared by procedure B were labeled by reductive methylation, and 300,000-Da protein bands were excised from 5% gels after autoradiographic detection and digested during re-electrophoresis with 0.5 \( \mu \)g of V8 protease, and fragments were separated on 12.5% gels. M, \times 10^9.

**FIG. 2. Peptide mapping of plectin and IFAP-300K.** IF residues prepared by procedure B were labeled by reductive methylation, and 300,000-Da protein bands were excised from 5% gels after autodigestion by re-electrophoresis with 0.5 \( \mu \)g of V8 protease, and fragments were separated on 12.5% gels. M, \times 10^9.

plectin (Fig. 1, lanes 2 and 4). This indicated that the two proteins were immunologically related. We then labeled IF preparations from both cell lines in vitro with tritium by reductive methylation and isolated the bands of apparent M, = 300,000 corresponding to plectin and IFAP-300K from 5% polyacrylamide gels. One-dimensional peptide mapping by the limited proteolysis technique (9) using V8 protease produced nearly identical peptide patterns from both proteins (Fig. 2). Analogous results were obtained using chymotrypsin and subtilisin (data not shown). This indicated a high degree of similarity in primary structure and confirmed the immunological relationship of both proteins. Upon analysis of IF preparations on high-resolution gels, polypeptides of M, between 300,000 and 280,000 became apparent in addition to the co-migrating 300,000-Da bands (Fig. 3). These bands, too, reacted with antibodies to plectin (data not shown). In accordance with data reported for IFAP-300K (7), one-dimensional peptide mapping (data not shown) was similar to trypsin fingerprinting (Fig. 3) revealed similar patterns indicating that these lower bands most likely were derived by endogenous proteolysis of the 300,000-Da polypeptide. Analogous results were obtained for the high M, polypeptides in IF preparations from other cell lines (data not shown).

Lieska et al. (8) reported that IFAP-300K could not be focused under conventional isoelectric focusing conditions because most of the material did not enter the focusing gel. However, following the method of Garrels (10), we found that a considerable part of the 300,000-Da polypeptides present in C6 and BHK-21 IF preparations entered the first-dimension gel and focused at a pH of 4.7-5.0 (Fig. 4). These results were corroborated by determining the pI of C6 plectin on chromatofocusing columns. Like IFAP-300K (8), plectin eluted in pH fractions of 5.5-4.9 (Fig. 5). Upon washes with polybuffer of pH 2.4, the remaining plectin came off (last three lanes in Fig. 5). To verify the structural relationship of the 300-kDa protein species eluting with different pH value, 300-kDa bands from fraction at pH 5.3, 4.0, and 3.6 as well as from the sample loaded (Fig. 5) were iodinated and subjected to limited digestion with V8 protease. All cleavage patterns obtained were virtually identical (data not shown). Furthermore, the amino acid analysis of C6 plectin and BHK-21 IFAP-300K revealed no difference.\(^3\) Taking these data together, we conclude that the 300-kDa proteins present in IF preparations from BHK-21 and various other cultured cell lines are homologous to C6 cell plectin.

**Localization of Phosphorylation Sites**—As previously observed for Chinese hamster ovary cells (2), the major phosphorylated V8 fragments derived from C6 plectin and BHK-21 IFAP-300K were of M, = 14,000-18,000 as revealed by analysis on high percentage (15%) polyacrylamide gels (Fig. 6). Hardly any labeled fragments were seen between this M, range and 300,000, a fact strongly corroborated by analysis on low percentage (6.25%) gels. In contrast, parallel experi-

\(^3\) G. Weitzer, unpublished data.
Interaction of Plectin with MAPs and Spectrin

The removal of low Mr, phosphopeptides by V8 eliminated all of the label from the molecule and left behind unlabeled fragments of high Mr. These fragmentation patterns were observed over a wide concentration range of protease (1 ng to 10 µg). These data strongly suggest, therefore, that plectin is phosphorylated at the end(s) of the polypeptide chain.

In addition to this bulk label, we observed labeled V8 fragments of ~30 kDa that were only generated at medium protease concentration. By incubating permeabilized cells with [γ-32P]ATP in the presence or absence of Ca2+/calmodulin, these fragments were shown to be derived from a domain of plectin phosphorylated exclusively by a Ca2+/calmodulin-dependent protein kinase (Fig. 7). Thus, the phosphorylation sites affected by cAMP-independent and Ca2+/calmodulin-dependent kinases clearly were located on different molecular domains.

**Solid-phase Binding Assays**—In order to detect possible binding partners of plectin, blots of proteins to be assayed were overlaid with 32P- or 125I-labeled C6 plectin. 32P-labeled plectin bound to vimentin from IF preparations as well as to chromatographically purified vimentin (Fig. 8, lane 1), confirming previous copolymerization experiments (6). Furthermore, 125I-plectin bound to MAP 1 and MAP 2 of two-times-cycled hog brain microtubule preparations, but not to tubulin (lane 2). In agreement with plectin's binding to a prominent 240-kDa protein of brain membrane preparations (data not shown), 125I-plectin bound to the 240-kDa subunit of fodrin purified from brain (lane 3). Moreover, 125I-plectin bound to

---

**Fig. 4.** Two-dimensional gel electrophoresis of plectin. C6 or BHK-21 cells were labeled in vivo with 32P, and IFs prepared by procedure B were taken up in sample solution as described by Garrels (10). Isoelectric focusing (IEF) was performed on 14-cm-long 3.5% polyacrylamide gels containing 2% Serva pH 3-10 and 2% Serva pH 4-6 ampholytes. The basic end (2 cm) of the focusing gel was discarded in order to prevent contamination with nonfocused proteins during pre-equilibration with electrophoresis sample buffer. Probably because less was loaded, C6 plectin focused somewhat sharper than BHK-21 plectin. For measuring the pH gradient, gels focused in parallel were cut into 5-mm slices, each slice was incubated with 1 ml of 9.2 M urea in degassed H2O, and the pH was determined on a pH meter (30). P, plectin; V, vimentin; D, desmin. PAGE, polyacrylamide gel electrophoresis.

**Fig. 6.** Proteolytic fragmentation patterns of 32P-labeled plectin. Rat glioma C6 and BHK-21 cells were labeled in the logarithmic growth phase with 32P, using 0.5-0.1 mCi/ml for 3–12 h as described (2) except that no depletion was performed. Labeled samples were then run on 5% gels, and plectin bands detected by autoradiography were excised. Plectin contained in these bands was digested during re-electrophoresis with 0.5 µg of V8 on 6.25 or 15% gels, as indicated. Mr, × 10^9.

**Fig. 8.** Binding of purified plectin to proteins transblotted to nitrocellulose. A, overlays with 32P (lane 1) or 125I (lanes 2–4) labeled pectin. 32P-labeled plectin (1.2–1.6 × 10^6 cpm/µg) was purified (see text) from cells radiolabeled in vivo with 32P; (2) and used at a concentration of 0.1–1 µg/ml. 125I-labeled plectin (0.2–2.0 × 10^6 cpm/µg) was prepared by labeling chromatographically purified plectin with 125I-labeled Bolton-Hunter reagent in 100 mM borate (pH 8.5) for 30 min on ice and used at 0.05–1.0 µg/ml. Gel blots of the following purified samples (see “Experimental Procedures”) are shown: lane 1, C6 cell vimentin; lane 2, hog brain microtubule proteins; lane 3, hog brain fodrin; lane 4, human erythrocyte spectrin. Blots from full-length gels are shown. Lane 1 was from a 6.25% gel, and lanes 2–4 were from 5% gels. Autoradiography was for 16 h to 4 days. B, overlays with unlabeled plectin. Protein blots of chromatographically purified hog brain fodrin preparations, separated on 5% gels (lane 1, Coomassie Brilliant Blue stain), were overlaid with 0.4 µg of chromatographically purified plectin/ml of PBS (lane 2) or with PBS alone (lane 3), followed by antiserum to C6 plectin. Bound immunoglobulins in lanes 2 and 3 were detected by the Proto-Blot color reaction (see text).

H. Herrmaan and G. Wiche, unpublished data.
α-spectrin from human erythrocytes (lane 4). Overlays with heat-denatured 125I-plectin showed no binding to any of the blotted proteins (data not shown), indicating that a native conformation of plectin is needed for binding. In further studies, the binding of plectin to fodrin was shown to depend on the plectin concentration, and unblotted plectin was found to compete with the labeled protein for binding (data not shown). The binding of plectin to the 240-kDa polypeptide chain of fodrin, α-spectrin, MAP 1, and MAP 2 was further demonstrated by similar overlays using unblotted plectin whose binding was detected by incubation with antibodies to plectin followed by secondary antibody coupled to alkaline phosphatase, as shown for fodrin in Fig. 8B. Control blots that were not incubated with plectin showed no reaction with plectin antibodies, eliminating the possibility of fodrin cross-reacting with antibodies to plectin (Fig. 8B, lane 3). The various binding patterns of plectin were not influenced by 1 mM ATP or by Ca2+/calmodulin. Binding of plectin to rabbit muscle actin or myosin was not observed (data not shown). Comparable results were obtained in parallel experiments carried out with plectin from Chinese hamster ovary and BHK-21 cells.

DISCUSSION

In this report, we show that plectin, originally identified in high salt/detergent-resistant cytoskeletal residues from rat glioma C6 cells, and IFAP-300K from BHK-21 cells are homologous proteins. Homology was demonstrated by immunological cross-reactivity, peptide maps, co-migration on high-resolution gels, identical PI on two-dimensional gels and chromatofocusing columns, similar amino acid composition, and comparable characteristics of phosphorylation by endogenous protein kinases. The two proteins also show an analogous distribution to various cell fractions (2). Moreover, 300,000-Da proteins found in all other cell lines tested (Chinese hamster ovary, 3T3, HeLa, A-431, and WI-38) were immunologically related and hardly distinguishable from plectin by peptide mapping. Thus, plectin’s primary structure seems to have been highly conserved in evolution.

Yang et al. (7) pointed out that BHK-21 cell IFAP-300K did not show cross-reactivity with MAPs; and therefore the protein seemed to be distinct from plectin, which originally we had claimed to be structurally related to MAPs (1). However, in later studies with well-defined antibody preparations, we had ruled out any immunological relationship between plectin and MAPs; furthermore, peptide mapping showed that plectin and MAP 2 are not homologous (2, 3). Differences exist in cellular associations reported for IFAP-300K and plectin. Whereas IFAP-300K was reported to be selectively associated with IFs of BHK-21 cells (7, 8), plectin has been shown to be widespread both with regard to cell types and organelles (2-6, 11). Caution is necessary, however, in comparing both sets of data because a single monoclonal antibody preparation was used in the studies on IFAP-300K, whereas all studies on plectin antigens were carried out with conventional affinity-purified rabbit antibodies. The likelihood that accessibility of epitopes for antibodies becomes a limiting factor that ultimately may lead to an apparent restricted distribution of a particular antigen is much higher in studies with a single monoclonal antibody compared to those with conventional rabbit antibodies which recognize several different epitopes along one polypeptide chain. In fact, the following observations suggest the association of the 300-kDa protein from BHK-21 cells with cellular components other than IFs. First, like C6 plectin, the protein specifically bound to several polypeptides aside from vimentin in solid-phase bindings assays. Second, analogous to C6 plectin, the major part of the protein was found in the high salt/Triton X-100-soluble fractions in contrast to IF subunit proteins.

In an extension of previous studies, the bulk of plectin’s phosphorylation sites, acted on by a cAMP-independent kinase, have been located on domains less than 20 kDa from the end(s) of the molecule. The phosphorylation of plectin at the molecular end domains is similar to that of other cytoskeletal proteins of high Mr, e.g. β-spectrin (12), myosin (13), the neurofilament protein H (14), fibronectin (15), and, to a certain degree, MAP 2 (16). In this study, plectin has been identified additionally as a substrate for Ca2+/calmodulin-independent kinases. The site(s) of this phosphorylation resides on a V8-generated 30-kDa fragment of unknown molecular location. Furthermore, cAMP-dependent phosphorylation occurs on a distinct 25-kDa fragment whose location is also unknown (2). Therefore, these three kinases seem to phosphorylate plectin on distinct domains of the molecule. The finding that three independently regulated kinase systems operate on the plectin molecule opens up ample possibilities for sophisticated regulation at the molecular level.

Solid-phase binding assays, commonly used to demonstrate specific protein interactions (17-20), led to the identification of several interaction partners of plectin including vimentin, MAP 1, and MAP 2. The in vitro interaction of plectin with C6 microtubules, assembled by temperature-dependent or taxol-induced polymerization, has previously been shown using immunoblotting and immunoelectron microscopy (21). Copolymerization experiments also indicated plectin’s interaction with vimentin filaments (6, 8). Plectin’s ability to bind to vimentin and high Mr, MAPs, as demonstrated here, suggests that one of its possible cellular functions is the cross-linking of cytoplasmic microtubules with IFs. The physical connection of these two systems has been proposed based on their cellular codistribution (22) and the collapse of IFs into perinuclear coils after disruption of microtubules with colcemid (23, 24). Other binding partners of plectin were the 240,000-Da chain of hog brain fodrin and the α-spectrin chain of human erythrocytes. Both proteins have been shown to possess similar primary structure, whereas β-spectrin and the 235,000-Da chain of fodrin are each unique (25). Thus, plectin probably binds to a common domain of these 240,000-Da spectrins.

If the binding partners identified in vitro are also interact-
ing with plectin in vivo, plectin may cross-link IFs with microtubules and connect these filament systems via spectrin to the plasma membrane as described in Fig. 3. Furthermore, plectin may cross-link IFs with microfilaments via MAP 2 (26) and/or spectrin (27).

In conclusion, our data demonstrate that plectin has a conserved primary structure and is ubiquitously distributed in cultured fibroblasts as well as other cell types. Its structural conservation, including strategically located phosphorylation sites, suggests that this protein is involved in important cellular functions. As a potential multifunctional cross-linking element of the cytoskeleton, plectin molecules are expected to possess several specific binding domains whose characterization is in progress.

Acknowledgments—We thank John Wyatt and J. Mitchell Dalton for technical assistance and Benjamin Feldman for comments on the manuscript.

REFERENCES

Polyacrylamide gel electrophoresis and peptide mapping - 1-d SDS-polyacrylamide gel electrophoresis was done according to Laemmli (33) as previously described (3). In several experiments the pH of the running buffer was adjusted to pH 8.9. 2-d gel electrophoresis was done essentially as described by Guerra (16) with 24 ampholytes pH 4 - 6 plus 24 ampholytes pH 3 - 10. 1- and 2-d peptide mapping was performed as previously described (9,29,32).

Protein blotting and overlay experiments - For overlay experiments proteins of various samples were separated on 15% polyacrylamide gels and blotted to nitrocellulose for 12 h. 300 nA) in 25 mM borate, pH 8.8, 2 mM EDTA, 1 mM 2-mercaptoethanol, 20% methanol. The papers were then blocked (18, 37°C in PBS containing 5% defatted milk powder, 1 mM EGTA, 1 mM 2-mercaptoethanol, 0.005% NP-40 and 0.5 mM MgCl₂) and briefly washed with PBS. Overlays with samples of plectin were performed for 3 h at 37°C with constant agitation in the same solution used for blocking but without milk powder. The concentration of plectin in the overlay solution was between 0.05 and 1 μg/ml. Radiolabeled plectin was homogeneous as judged from autoradiographs of electrophoresis gels. Incubations were stopped by washing 8 times with PBS (5 to 10 min each). When radiolabeled plectin samples were used, the nitrocellulose papers were blotted dry and autoradiographed. Unlabeled plectin was detected by conjugation to alkaline phosphatase and color development (Probe-Blot Immunoblotting system) according to instructions provided by the manufacturer.

Other procedures - Microtubule proteins from hog brain and brain membranes were prepared as described (31, 34 and 17). DEAE-column purified hog brain fodrin was prepared essentially as described by Davies and Bennett (17); the identity of fodrin was confirmed by Ca²⁺/CaM-dependent binding of radiolabeled calmodulin (unpublished data). Spectrin was prepared by extraction of human red cell ghosts at 37°C in low ionic strength buffers (13). Vimentin was purified from C6 glial preparations by molecular sieve chromatography followed by chromatofocusing on DEAE-Sepharose CL-6B. Immunoprecipitation of SDS-boiled samples was done by the method of Blobe and Melter (37) as described (2). Protein was quantitated using the method of Lowry et al. (38) with bovine serum albumin as standard. Quantitation of stained protein profiles was done in a Beckman DU 88 spectrophotometer.