PLECTIN: GENERAL OVERVIEW AND APPRAISAL OF ITS POTENTIAL ROLE AS A SUBUNIT PROTEIN OF THE CYTOMATRIX

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I. INTRODUCTION

The cellular cytoplasm is viewed today as a highly structured cell compartment. It is believed that a great part of this structure is provided by the cytoskeleton, comprising microtubules, actin/myosin filaments, and intermediate filaments. An increasing body of evidence, much of it indirect in nature, however, suggests that the cytoskeleton is only part of a more complex structural entity, referred to as the cytoplasmic matrix or cytomatrix (for reviews see References 1 and 2). While cytoskeletal filaments have been well characterized both on the biochemical and ultrastructural level, relatively little is known to date about the molecular composition and spatial organization of other cytomatrix elements that are not typically filamentous. There is also very little understanding of how these cytomatrix elements interact with cytoskeletal filaments and whether they show preferences in binding to certain types of filaments.

The various filament systems of the cytoskeleton apparently have different functions. While there is little doubt that microtubules and actin filaments are both involved in various aspects of cellular dynamics and morphogenesis, including cell division and locomotion, the specific functions of intermediate filaments are still unknown. It is believed, however, that they play primarily a supportive (i.e., structural) role by providing a presumably flexible, though basically static, intracellular network that may serve as "mechanical integrator"[3] of the cytoplasm. This would not exclude the possibility that filament subunit proteins exchange dynamically along the filament backbone throughout the life cycle or at certain developmental stages of some cell types, as has recently been suggested for keratin filaments of simple epithelial cell lines.[4] Compatible with a chiefly skeletal function of intermediate filaments are both their cellular distribution, as visualized by immunofluorescence and electron microscopy, and their relatively high chemical stability. Furthermore, it has been proposed that intermediate filaments serve as a cellular anchoring device for the nucleus and the protein-synthesizing machinery (reviewed comprehensively in Reference 5).

Considering that intermediate filaments as a whole are less dynamic than other cytoskeletal filament systems and presumably are instrumental in positioning cellular organelles and in organizing cytoplasmic space, they seem to be better suited than microtubules or microfilaments to serve as a backbone of structural elements of the cytomatrix that are not typically filamentous. Within this conceptional framework, cytoplasmic proteins that bind to intermediate filaments would appear to be promising candidates for cytomatrix subunit components.

In an attempt to isolate microtubule-associated proteins (MAPs) by *in vitro* assembly of microtubules from soluble extracts of cultured cells (rat glioma C_6), a protein of M_r = 300,000, a weight similar to that of the major MAPs from mammalian brain, was found to

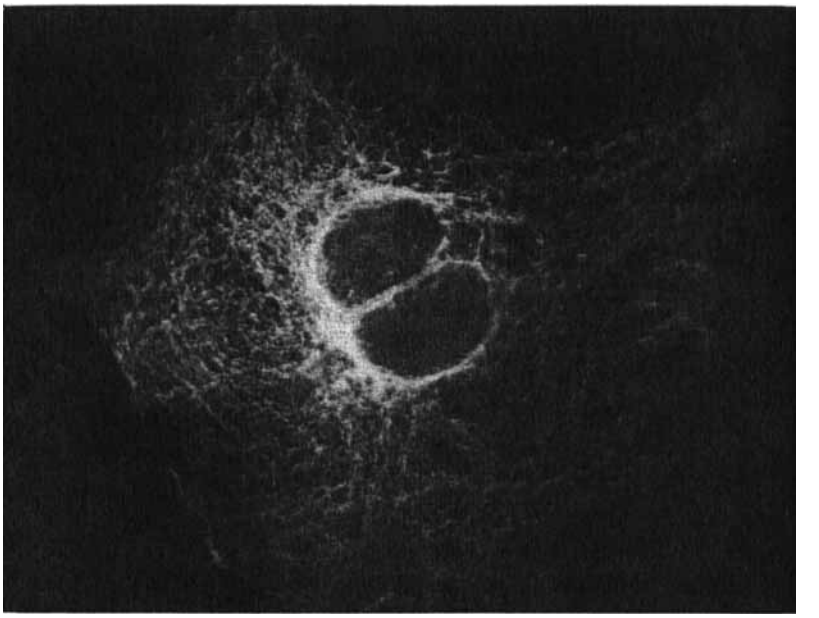

FIGURE 1. Immunofluorescence microscopy of a CHO cell using rabbit antibodies to plectin and fluoresceine-conjugated secondary antibodies. The cell was treated with a solution containing 0.15% Triton® X-100 prior to fixation and immunostaining. (Magnification × 1000.)

copurify with intermediate filaments rather than with microtubules.[6] Because of several properties, including M_r under denaturing conditions, extensive phosphorylation by endogenous kinases, and gross similarities in one-dimensional peptide maps, it was initially suggested that this protein was related to MAPs.[6,7] Although some additional data would have been consistent with this notion, such as partial copurification of the protein with microtubules in rounds of temperature-dependent *in vitro* assembly and disassembly and taxol-induced microtubule polymerization,[8] as well as similar isoelectric points as demonstrated by two-dimensional gel electrophoresis,[9] two lines of evidence clearly showed that this new protein was unrelated to MAPs. First, polyclonal antibodies raised against the protein purified from rat glioma C_6 cells in Western blot analysis showed no cross-reaction with mammalian brain MAPs and, vice versa, antibodies to hog brain MAPs were unreactive with the glioma C_6 protein.[8,10] Second, in a more refined peptide mapping analysis in which the protein was compared with individual MAP subcomponents separated on high-resolution gels, structural similarities were no longer detected.[11] Mainly because of its immunolocalization within dense cytoplasmic network arrays of cultured cells (see Figure 1) the new protein was given the name plectin, which is derived from πλεκτή, the Greek word for net- or meshwork.[12]

The aim of this article is to review the available data on plectin and to assess whether this protein exhibits properties that qualify it as a potential cytomatrix subunit protein. In our view, cytomatrix subunit proteins should not only be able to interact with intermediate filaments, but they should also meet several other criteria. First of all, the proteins should not be restricted in their distribution among different cell types and tissues, but rather should be of widespread occurrence. It would also seem advantageous if such proteins were capable of self-aggregation or co-aggregation with other putative cytomatrix proteins, so that they could convey structure to cytoplasmic areas not just in the immediate vicinity of the filaments, but also at more distant locations. In this way an intermediate filament-associated cytomatrix domain, rather than a mere surface complementation of filaments, could be established.

Such a feat would require that the proteins possess molecular sites specific for self-interaction or binding to other putative cytomatrix subunit components. Another favorable feature of cytomatrix subunit proteins would be if they were able to establish specific contacts with important structural elements of cells other than intermediate filaments, such as membranes and other types of cytoskeletal filaments. In this way, they could function as true connecting links of the cytoplasm. It would also be attractive if cytomatrix proteins were accessible to protein modifications, for instance, phosphorylation, or their functions would underly nucleotide- or Ca-ion-regulated control mechanisms. Therefore, all of these criteria are used, in evaluating plectin's potential role as a subunit protein of the cytomatrix.

II. OCCURRENCE AND IDENTIFICATION OF PLECTIN

Repeated extraction of cultured cells with a solution of 1% Triton® X-100 and 0.6 *M* potassium chloride yields insoluble cell residues that are enriched in intermediate filaments. The most prominent components of such residues, aside from the intermediate filament subunit proteins, were found to be high M_r proteins (~300,000) that comigrate with C_6 plectin upon electrophoresis on SDS-polyacrylamide gels. Up to now, preparations from more than a dozen different cell lines have been analyzed, all of which contained these proteins (Table 1). The amount of high M_r components relative to that of intermediate filament subunit proteins contained in the preparations varied, depending on the cell type. In general, it was higher in preparations from cells of mesenchymal origin compared to epithelial cell lines. For cultured fibroblast cell lines from rodents, plectin/vimentin mass ratios of up to 0.3 have been measured, equivalent to 1 plectin per 20 vimentin polypeptide chains.

When cell homogenates obtained from various tissues of rat and man were extracted in a similar way, polypeptides of the size of plectin were retained as part of the insoluble cell residues from all tissue types tested (Table 2). The relative amount of the high M_r material was, however, considerably lower than in corresponding preparations from cultured cells.

The M_r = 300,000 polypeptides found in Triton® X-100/high salt-insoluble cell fractions of various cultured cells and tissues were identified as analogs of C_6 cell plectin using immunoblotting and/or immunoprecipitation (Tables 1 and 2). These experiments were performed with highly specific antibody preparations obtained by immunization of rabbits with electrophoretically purified homogeneous preparations of C_6 cell plectin.[10]

Polypeptides similar in size to plectin were also detected in Triton® X-100/high salt-soluble cell fractions.[9,11] In fact, a quantitation of radiolabeled plectin in the soluble and insoluble fractions of cultured CHO cells yielded a ratio of 7:3 in favor of the soluble species. Thus, in marked contrast to the intermediate filament subunit proteins, major parts of plectin were extractable with Triton® X-100/high salt from fibroblast cells. However, whereas in the Triton® X-100/high salt-insoluble fraction plectin seemed to be the only high M_r species present, in the soluble fraction plectin apparently was only one of several.[11]

Quantitative estimates of the total cellular plectin made for cultured CHO cells and outer cell layers of bovine eye lens tissue revealed values near 1% of total cellular protein in both cases.[11,13] The widespread occurrence of plectin in mammalian cell cultures and tissues was also demonstrated by immunofluorescence microscopy using antisera or affinity-purified antibodies to plectin. So far, more than 20 different cell lines (Table 1) and a broad variety of tissues (Table 2) proved to be plectin-positive.

Not much is known, however, about plectin species in cells of nonmammalian origin. Using immunofluorescence microscopy, anti-plectin-positive cytoplasmic structures have been observed in erythrophores from squirrel fish and in the parasitic hemoflagellate *Trypanosoma brucei;* cells from chicken and the unicellular *Paramecium* were nonreactive.[123]

Table 1
OCCURRENCE OF PLECTIN IN CULTURED CELLS

Species	Cell line	Cell type (origin)	Method[a]	Refs.
Man	HeLa	Epithelial (cervix carcinoma)	i,w	6,10,12
	A 431	Epithelial (skin carcinoma)	i,w	15 —, [b]
	SCC	Epithelial (tongue carcinoma)	i	15 —,[b]
	Chang	Epithelial (liver)	i	— [b], — [c]
	WI-38	Fibroblast (lung)	i	— [b], — [c]
	Primary culture	Fibroblast (skin)	i,w	15
	THP-1	Monocytes	i,p	15,120
Monkey	Vero	Fibroblast (kidney)	i	— [b], — [c]
Rat	MH_1C_1	Epithelial (hepatoma)	i	14
	Rat-1	Fibroblast	i,p	— [b],121
	C_6	Fibroblast (glial tumor)	e,i,p,w	6,8—10,12,29,32,44
Mouse	Ehrlich Lettre ascites	Epithelial (ascites carcinoma)	w	— [b], — [c]
	PAM 212	Epithelial (epidermis)	i	— [b],122
	L929	Fibroblast (connective tissue)	i,w	— [b], — [c]
	Balb/c 3T3	Fibroblast (mouse embryo)	e,i,w	10,12,44
	SV 101	Fibroblast (mouse embryo-SV40 transformed)	i,w	10
Hamster	CHO-K1	Epithelial (ovary)	e,i,p,w	6,9—11,44
	CHL	Fibroblast (lung)	g	6
	BHK-21	Fibroblast (kidney)	i,p,w	9,29
Cow	Primary culture	Epithelial (lens)	i	— [b]
Rat Kangaroo	PtK2	Epithelial (kidney)	i,w	10,12,14

[a] e, Electron microscopy; g, SDS polyacrylamide gel electrophoresis; i, immunofluorescence microscopy; p, immunoprecipitation; w, Western blotting.
[b] unpublished data.
[c] For specification of cell line see catalog of American Type culture collection, Rockville, Maryland.

Table 2
OCCURRENCE OF PLECTIN IN TISSUES, ISOLATED CELLS, AND SINGLE-CELL ORGANISM

Species	Tissue/isolated cells	Special structures/cell type and region	Method[a]	Refs
Man	Cornea	Basal membrane	i	15
	Skin (eyelid, foreskin)	Basal membrane	i	15
	Kidney	Mesangial cells of glomeruli, Bowman's capsule, tubular epithelium	i	—[b]
	Striated muscle	Z-lines	i	—[b]
	Connective tissue	Cytoplasm of fibroblasts and endothelial cells	i	—[b]
	Placenta		w	15
Rat	Cornea	Basal membrane	i	—[b]
	Lens	Lens fiber junctions	i	—[b]
	Tongue	Epithelial basal membrane	e,i,p	14
	Respiratory epithelium	Basal and apical region of epithelial cells	i	—[b]
	Stomach	Epithelial cells	i	14
	Small intestine/isolated epithelial cells	Basal and apical region epithelial cells	i	14
	Liver	Hepatocellular junctions, bile ducts	e,i,w	14
	Salivary gland	Basal and apical region of epithelial cells	i	14
	Kidney	Mesangial cells of glomeruli, Bowman's capsule, tubular epithelium	i	14
	Uterus	Basal and apical region of epithelial cells, endometrial stromal cells, smooth muscle	i	14
	Urinary bladder	Urothelial basal membrane, terminal bars, smooth muscle	e,i,p,w	14
	Testis	Basal membrane, Sertoli cells, spermatids	i	—[b]
	Epididymis	Basal and apical region of epithelial cells	i	—[b]
	Seminal vesicle	Smooth muscle	i	—[b]
	Pituitary gland	Rathke's pouch	i	—[b]
	Striated muscle	Z-Lines	e,i,p	14
	Cardiac muscle/isolated myocytes	Z-Lines, intercalated discs	e,i,p,w	14
	Smooth muscle	Lateral cytoplasmic densities	e,i,	14
	Connective tissue	Cytoplasm of fibroblasts and epithelial cells	i	14
	Spleen	Endothelial cells	i	—[b]
	Cerebellum	Cytoplasm of astrocytes, meningual fibroblasts and endothelial cells	i,	—[b]
Cow	Lens	Lens fiber junction	i	—[b]
	Lactating mammary gland	Basal membrane, glandular epithelium	i	—[b]
Squirrel fish	Isolated erythrophores	Cytoplasm	i	—[b]
Trypanosoma brucei		Cytoplasm, flagellum	i	—[b]

[a] e, Electron microscopy; g, SDS polyacrylamide gel electrophoresis; i, immunofluorescence microscopy; p, immunoprecipitation; w, Western blotting.

[b] Unpublished data.

III. CELLULAR LOCALIZATION OF PLECTIN

Immunofluorescence microscopy using antisera or affinity-purified antibodies to plectin revealed dense cytoplasmic network arrays in a series of cultured cell lines examined (Table 1). In fibroblast cells, these networks, consisting of seemingly short irregularly oriented fibers and lines of dots, were clearly distinguishable from microtubules and actin cables. The plectin networks were also distinguishable from intermediate filament arrays in that they were more delicate and appeared to be more densely packed than the latter. The overall spatial distribution of intermediate filaments and plectin networks within the cytoplasm of cells was very similar, however. This was also evident after treatment of 3T3 cells with high doses of colcemid when the plectin network was found massed up in the vicinity of vimentin filament loops and bundles typically formed under these conditions.[10] When fibroblast cells were extracted with solutions containing 0.15 to 0.5% Triton® X-100 prior to treatment with fixatives (Figure 1), the structures stained by anti-plectin appeared less dense and became almost superposable with vimentin filaments.[10,11]

In a series of cell lines derived from epithelial tissues of man, rat, mouse, hamster, cow, and rat kangaroo, antiplectin-stained cytoplasmic arrays resembled those revealed in fibroblast cells. Furthermore, in some of these cell lines, particularly in mouse PAM 212 cells, intercellular junctional complexes of unknown structure were conspicuously stained.

Immunofluorescence microscopy of frozen sections of various tissues from man, rat, and cow revealed the occurrence of plectin in a variety of cell types, including stratified and nonstratified epithelia, fibroblasts, endothelial cells, and astrocytes, as well as striated, smooth, and cardiac muscle. Prominent among plectin-negative cell types were neurons. The pattern of cellular staining varied among cell types. In fibroblasts, endothelial cells of vessels, and epithelia of bile duct, small intestine, uterus, urinary bladder, and stomach, staining was observed throughout the cytoplasm. Hepatocytes and smooth muscle cells, on the other hand, were stained primarily at their periphery. Epithelial cells of tongue and cardiac muscle cells showed both cytoplasmic and accentuated peripheral staining.

The nature of the structures stained in the interior and throughout the cytoplasm of fibroblastic and other cell types remains obscure. The generally dense staining pattern indicated a high concentration of relatively insoluble cytoplasmic plectin, but the distinction of individual substructures was hardly possible. This was still true when tissues were dissociated and cells viewed individually, such as in the case of absorptive epithelial cells from small intestine. Nevertheless, under these circumstances it became evident that plectin structures, unlike cytokeratin components, were not typically filamentous. Rather, they were similar to the dense network-type arrays consisting of short fibers and lines of dots characteristic of cultured cell lines.[14]

The immunolocalization at the periphery of various cell types suggested that plectin is associated with the subplasmamembrane protein skeleton, including various junctional complexes. In vertebrates, four major classes of junctions have been characterized: desmosomes, *adhaerens*-type junctions, tight junctions, and gap junctions. From immunoelectron microscopy using the peroxidase method, there is evidence that plectin is closely associated with desmosomal structures in liver, tongue, and cardiac muscle from rat,[14] as well as foreskin from man.[15] Although a precise ultrastructural localization within these junctional complexes was not possible using this technique, an enrichment of plectin near desmosomal dense plaques and its association with adjacent material extending into the cytoplasm, possibly filaments, was revealed. It should be noted that plectin, not being restricted to desmosomal structures, has to be distinguished, however, from proteins that are specific for such structures, such as desmoplakins[16,17] and band 5 protein.[18]

In liver, a conspicuous outlining of bile canaliculi was observed using immunofluorescence microscopy, suggesting that plectin is localized along hepatocyte junctions sealing the bile

canaliculi. It is possible, therefore, that plectin is also associated with tight junctions, which are known to form large parts of these areas.

A number of observations suggest that plectin is also associated with adhering junctions. Both immunofluorescence and immunoelectron microscopy revealed intercalated discs of heart muscle to be decorated by antiplectin in a seemingly uninterrupted pattern.[14,19] If plectin were exempt from *adhaerens*-type junctions, which are the major junctional complexes found in this region besides desmosomes, interrupted staining would be expected to occur. More convincingly, in cross-sections of smooth muscle cells, in particular, those from urinary bladder, dense patches spaced in regular intervals along the cellular periphery were immunoreactive with antibodies to plectin (Figure 2). In longitudinally sectioned cells, these patches became long, wavy lines running along the plasma membrane (Figure 2B; left-hand side). As demonstrated by double immunofluorescence microscopy, these patches (lines) also showed immunoreactivity with antibodies to vinculin, and thus represented microfilament anchorage sites, typically specified by *adhaerens*-type junctional complexes (for a review see Reference 20). The spatial arrangement within the junctional complex of plectin, vinculin, and of other proteins known to be part of *adhaerens*-type junctions, such as talin[21,22] and integral membrane glycoproteins,[20,23-26] remains to be established. A direct interaction of plectin with actin, the major constituent of microfilaments, is unlikely, however, at least on the grounds of negative *in vitro* binding experiments.[123] Similar considerations apply to plectin's molecular interaction partners at another microfilament anchorage site: the Z-lines of striated (skeletal and cardiac) muscle.

Codistribution of vinculin and plectin was also observed on the light microscopical level in eye lens tissue from rat and cow. In this case, too, plectin was located in peripheral cell regions (Figure 3). However, junctional complexes of eye lens fiber cells seem more complex in composition than regular adhering junctions, because in addition to the proteins that are typical for this type of junction, they apparently contain proteins known from the nonjunctional plasma membrane skeleton, such as analogs of spectrin and protein 4.1.[27]

The occurrence of plectin at more than one type of junctional complex, as suggested in the original study on the protein's immunolocalization,[14] has recently found a parallel in the case of plakoglobin.[28] This protein, originally identified as a component of desmosomal structures,[18] was demonstrated to also occur at adhering junctions, including the vinculin-actin-associated intercellular junctions.[28] Interestingly, the cellular distribution of plakoglobin in lenses[27] is similar to that of plectin.

In mouse embryos, the only developmental system investigated thus far, plectin has been found in cells of all early embryonic stages, including the egg, two- to eight-cell morulae, and blastocysts.[123] Judged from immunofluorescence images, the level of plectin in early embryonic cells is high. Like in cultured cells, antiplectin-positive structures are found distributed all over the cytoplasm. After mild treatment with detergent, plectin was localized primarily in peripheral cell regions, creating a largely reticulate image (Figure 4).

IV. BIOCHEMICAL CHARACTERIZATION OF PLECTIN

A. Purification and Biochemical Properties

In attempts to directly extract plectin from cultured C_6 cells using borate- or phosphate-buffered solutions of various ionic strengths, it was observed that the amount of plectin solubilized increased with increasing pH, reaching a maximum at around 9.0. Under these conditions the ratio of solubilized plectin to solubilized vimentin was roughly comparable to that of intermediate filament-enriched Triton® X-100/high salt-resistant cell residues. However, attempts to purify plectin chromatographically from such extracts failed, mainly because of contaminants of similar high M_r. On the other hand, the purification of plectin was relatively simple if Triton® X-100/0.6 *M* KCl-insoluble cell residues were used as the

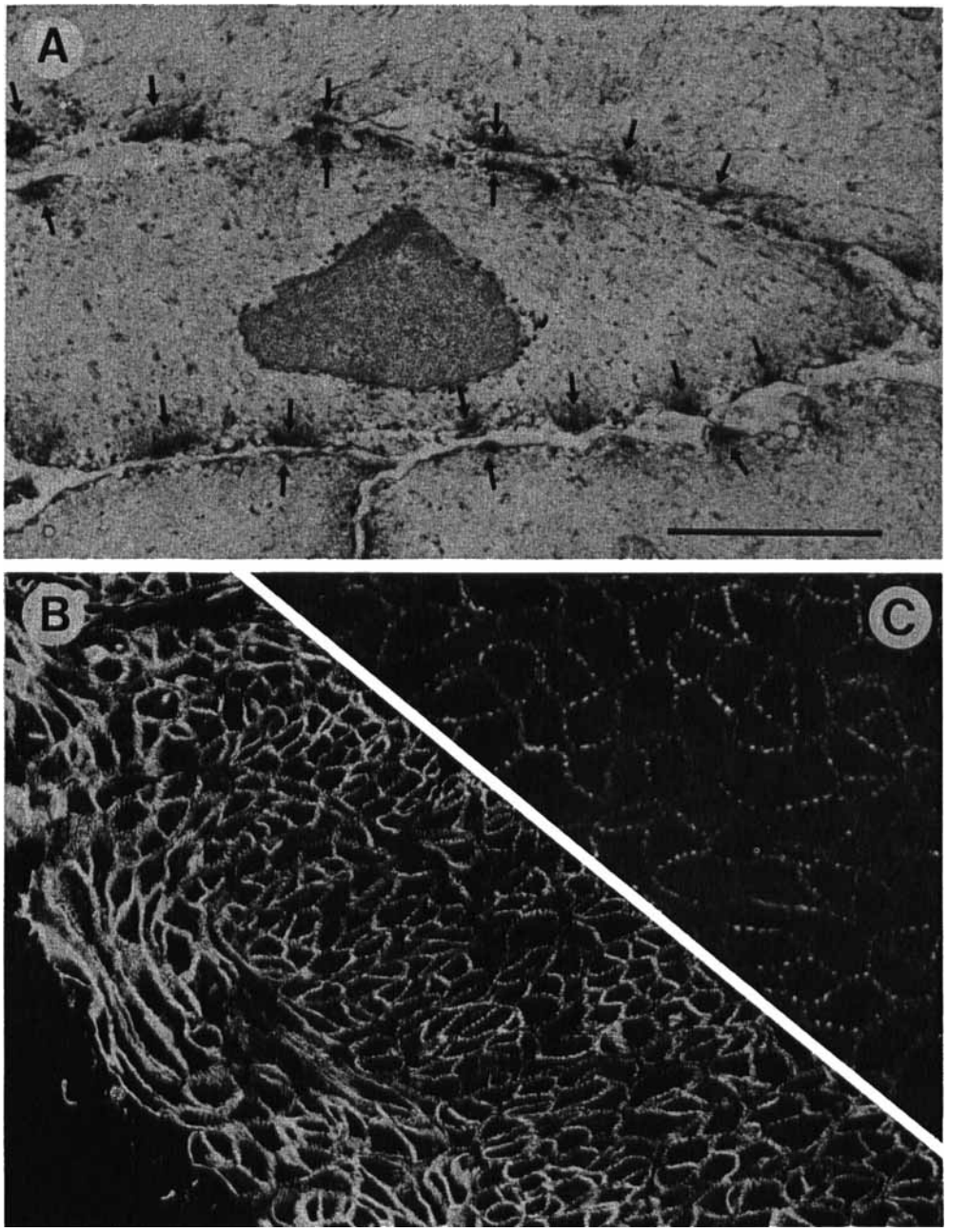

FIGURE 2. Immunolocalization of plectin in frozen sections of smooth muscle (rat urinary bladder). (A) Electron microscopy using the immunoperoxidase technique. Note preferential deposition of reaction product at regularly spaced regions (arrows) along the periphery of cells; bar 2 μm. (Magnification × 10,800.) (From Wiche, G., Krepler, R., Artlieb, U., Pytela, R., and Denk, H., *J. Cell Biol.*, 97, 887, 1983. With permission.) (B and C) Immunofluorescence microscopy using monoclonal (B), or rabbit antibodies (C) to plectin, and Texas red-conjugated secondary antibodies. In cells cross-sectioned perpendicular to their long axis dots and short lines are stained at the periphery in regular intervals (C and center part of B). In cells sectioned longitudinally, staining is observed at parallel lines running along the plasma membrane (B, left-hand side). [Magnification × 400 (B); Magnification × 950 (C).)] (Micrographs shown in B and C were kindly provided by U. Artlieb.)

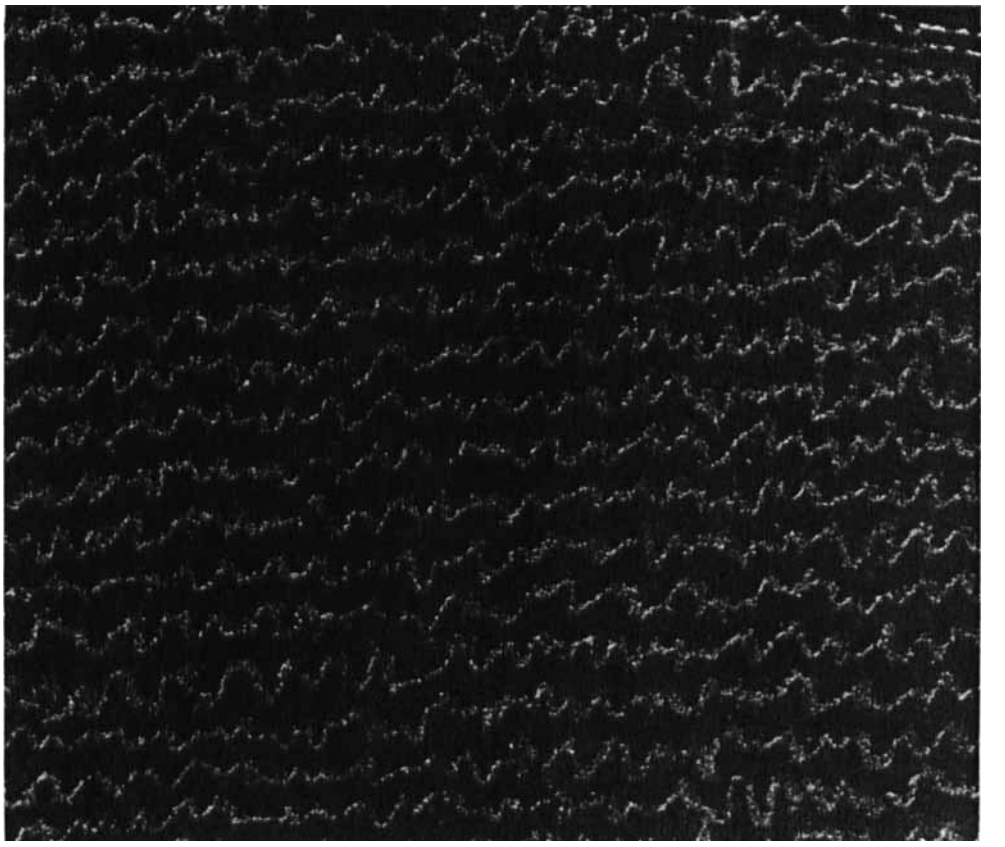

FIGURE 3. Immunofluorescence microscopy showing the localization of plectin on frozen sections of rat lens. Punctuate staining is seen along lens fiber junctions. (Magnification × 530.) (Data kindly provided by U. Artlieb.)

starting material, because plectin was the only high M_r protein present in these fractions in significant amounts. So far plectin has been successfully purified from the cell lines, C6 and BHK-21,[12,29] and from bovine lens tissue.[13]

In the procedure developed for cell cultures, roller bottle-grown cells are extracted with solutions containing Triton® X-100 and 0.6 *M* K/NaCl, and the insoluble cell residues are solubilized with 8 *M* urea. Fractions containing plectin of at least 90% purity can be obtained in one step by gel permeation chromatography in 8 *M* urea on Sepharose CL-4B columns. Although simple, this procedure permits only a relatively low yield (10% of the total cellular plectin), mainly because plectin is partially soluble in the extraction buffer.

Since plectin is highly sensitive to proteolysis, it is necessary to add protease inhibitors to all buffer solutions used for isolating and purifying plectin from cells. Among a series of protease inhibitors tested, 1 to 3 m*M* PMSF was very effective in reducing proteolytic breakdown. The degree of proteolysis is further dependent on the procedure used for cell lysis and the preparation of intermediate filament-enriched cell residues. Intermediate filaments conventionally prepared by Triton® X-100/high salt extraction of cells that were in suspension after having been dislodged from vessel walls, usually contained, in addition to the major plectin band, several minor bands of higher electrophoretic mobility, which were clearly identified as plectin breakdown species by immunoblotting and peptide mapping.[9,29] When, as in the case of BHK-21 cells, the procedure was sped up by extracting adhering cells on ice without prior disloding of the cells, only one major band without degradation

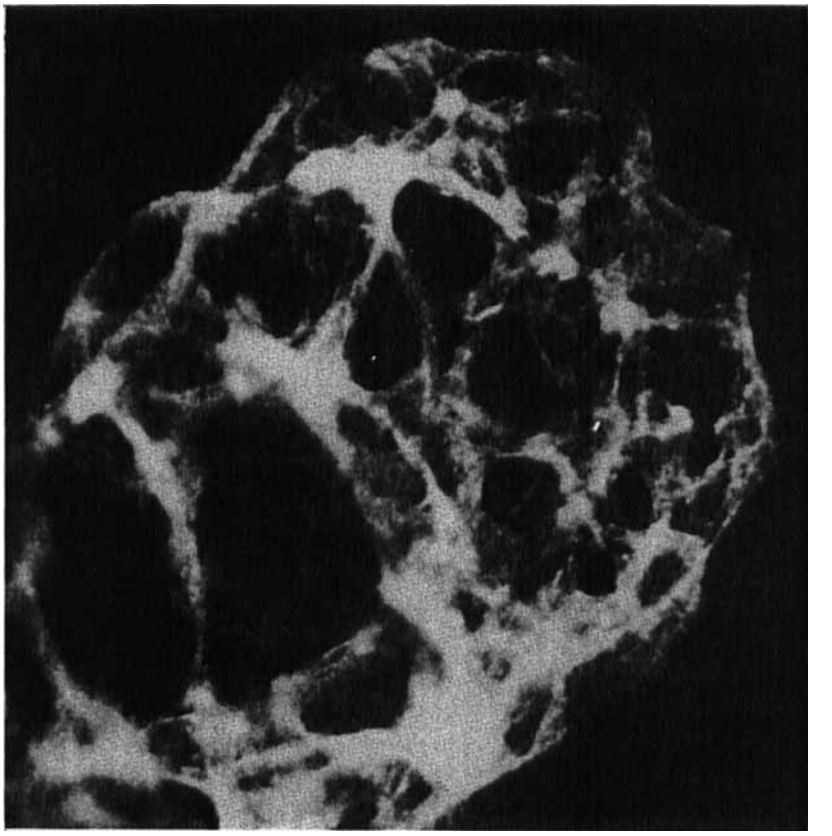

FIGURE 4. Immunofluorescence microscopy of a 3-d-old mouse embryo (blastocyst) using rabbit antibodies to plectin and rhoadmine-conjugated anti-IgG. Methanol/aceton-treated mouse embryos were extracted with solutions containing 0.1% sodium dodecyl sulfate and 0.1% NP40 prior to immunostaining. (The experiment was performed by the author in collaboration with H. Condamine at the Institut Pasteur, Paris.) (Magnification × 1400.)

was observed. A reduction in the generation of breakdown products was also observed when monolayer cultures were incubated first with solutions containing 1% Triton® X-100 and then were brought to 0.7 to 0.8 *M* K/NaCl by stepwise addition of salt. Once purified, solubilized plectin samples are quite resistant toward proteolytic degradation, even if they are repeatedly frozen and thawed.

A number of things were critically important for the efficient purification of plectin from eye lens tissue. First, only the outer epithelial and cortical cell layers were utilized, since it was found that they were much richer in plectin than the nuclear cell region. Second, grinding of the tissue to a fine powder under submersion in liquid nitrogen greatly facilitated subsequent cell extractions, and, third, the solubilizing effects of Triton® X-100/high salt on plectin were effectively counteracted through the addition of 0.1 *M* $MgCl_2$ to the tissue extraction buffer. This modification increased the amount of Triton® X-100/high salt-insoluble lens cell plectin from 30 to over 95% of the total cellular plectin. Similar to plectin from glioma C_6 cells, lens cell plectin could be extensively purified chromatographically in one step after solubilization of the extraction-resistant cell residues in solutions containing either 8 *M* urea or 1% sodium lauroylsarcosinate. Solubilization of plectin in 1% sodium lauroylsarcosinate was slightly less effective than in 8 *M* urea, but, since it appeared to have less influence on the native structure of lens cell plectin, as indicated by circular dichroism

studies, it became the method of choice. Sodium lauroylsarcosinate-solubilized plectin could be separated from other proteins by gel permeation chromatography on Sephacryl S-500 columns. The overall yield achieved was about 300 mg of purified plectin per 100 lenses (= 200 g).

The Triton® X-100/high salt-insoluble plectin from lens tissue was also partially soluble in phosphate- and borate-buffered solutions, pH 8.7, but only after relatively long periods of homogenization and dialysis. Therefore, even in the presence of protease inhibitors, proteolytic degradation of plectin was increased, compared to the quicker solubilization in solutions containing 8 *M* urea or 1% sodium lauroylsarcosinate. Interestingly, Mg ions seemed to make plectin, as well as the intermediate filament subunit proteins, more resistant toward Triton® X-100/high salt extraction. Without removal of Mg ions with EDTA, neither plectin nor vimentin were solubilizable in phosphate-buffered solutions, although under these conditions a selective solubilization of plectin was achieved in borate buffers. If Mg ions were removed, however, 60 to 80% of both plectin and vimentin could be solubilized in borate- and in phosphate-buffered solutions. Thus, it seems that the solubility properties of plectin and vimentin are similar, albeit distinguishable. With respect to detergents, plectin is readily solubilized in phosphate-buffered 0.5 to 1% sodium lauroylsarcosinate, but not in nonionic detergents such as Tween 40. Also, in some ionic detergents, such as desoxycholate, plectin is only moderately soluble. It is likely that plectin's relatively high content of hydrophobic amino acid residues[13] is a major factor in determining its solubility.

Under denaturing conditions plectin has an apparent M_r of 300,000, as shown by SDS-polyacrylamide gel electrophoresis. It comigrates with the microtubule-associated protein MAP-2,[30] provided the conditions of electrophoresis are exactly according to Laemmli.[31] Deviations from these conditions, however, can have effects on the migratory behavior of plectin. For instance, if the pH of the running buffer is increased from the commonly used 8.3 to 8.9, the plectin band is shifted to a position above MAP-2. Reducing agents did not influence plectin's mobility in gel electrophoresis, indicating that plectin polypeptide chains are not disulfide linked. In gel-permeation chromatography of samples solubilized in 1% sodium lauroylsarcosinate or 8 *M* urea, plectin eluted near or with the void volume, indicating a M_r of over 1×10^6. Thus, native plectin molecules are apparently multimers of M_r = 300,000 polypeptide chains that are not, or only partially, dissociated by 1% sodium lauroylsarcosinate or 8 *M* urea. Since detergent-solubilized plectin eluted from gel permeation columns within a single sharp peak, it is likely that the eluted species represented native plectin molecules. When dissolved in urea, however, plectin showed a different chromatographic behavior inasmuch as it eluted free of contaminants only in early fractions, whereas the rest, "smearing" all over the elution profile, coeluted with other proteins. This indicated a more heterogeneous size distribution of plectin in urea compared to the other solvents and its partial dissociation into smaller molecular entities. A similar behavior of urea-solubilized samples was observed after chromatography on DEAE-5PW columns. Without urea, plectin bound irreversibly to all ion-exchange resins tested.

The M_r of plectin in solution was estimated as 1.15×10^6 by gel permeation chromatography, using a combination of four HPLC columns and rod-shaped molecular weight standards.[13] In an alternative approach, plectin's M_r under nondenaturing conditions was determined by molecular cross-linking of plectin in solution with dimethyl suberimidate and electrophoretic analysis of cross-linked products on size-calibrated agarose gels.[32] When samples of purified plectin were incubated with increasing amounts of cross-linking reagent, a transition was observed from the monomeric (M_r = 300,000) to an oligomeric form of plectin ($M_r = 1.2 \times 10^6$), which, according to its M_r, apparently consisted of four 300-kDa polypeptide chains. This was confirmed in experiments performed under conditions where the chemical cross-linking reaction was less favored; in this case dimeric and trimeric intermediates were picked up.

As shown by a number of techniques, the net charge of plectin molecules is negative. In two-dimensional gel electrophoresis plectin focuses between pH 4.7 and 5.0, slightly toward the acidic side of microtubule-associated proteins MAP-1 and MAP-2, as demonstrated by coelectrophoresis of the proteins.[9,29] From chromatofocusing columns the major part of plectin elutes in fractions of pH 4.7 to 5.0; the remaining part, which probably represents plectin aggregates, elutes in fractions of even lower pH.[29] Finally, the amino acid analysis of plectin from three different sources[13] showed a predominance of potentially acidic vs. basic residues (an average of 24 and 15.6%, respectively).

Plectin is a phosphoprotein serving as a target for cAMP-independent, cAMP-dependent, and Ca/calmodulin-dependent kinases. Phosphorylation takes place primarily on serin, moderately on threonin, but not on tyrosine residues. As a target of cAMP-dependent kinases plectin seems amenable to hormonal regulation, but a hormone-dependent phosphorylation of the protein so far has not been demonstrated. In an attempt to associate phosphorylation sites with molecular domains of plectin, ^{32}P-plectin species from C_6 and BHK-21 cells were subjected to proteolytic digestion by V8 protease, and the fragments were analyzed electrophoretically. The major phosphorylated fragments obtained were of M_r 14,000 to 18,000; between this M_r range and 300,000 M_r, hardly any labeled fragments were seen. In contrast, ^{3}H- and ^{125}I-labeled plectin species analyzed in parallel showed a multitude of fragments in this M_r range.[29] Since the removal of low M_r phosphopeptides by the protease eliminated nearly all of the label from the molecule, leaving behind unlabeled fragments of high M_r, these data strongly suggested that plectin is phosphorylated at the ends of the polypeptide chain. However, only the bulk label, i.e., the cAMP-independent phosphorylation, was located at these sites; cAMP-dependent and Ca/calmodulin-dependent phosphorylation occurred at different sites, apparently located on more central molecular domains.

Plectin was not stained by the fuchsine reagent, nor was it labeled when cell cultures were incubated with radioactive carbohydrate precursors.[123] Therefore, plectin is not a glycoprotein. Furthermore, enzymatic activities intrinsic to plectin have not been found yet.

All plectins of mammalian origin examined to date show immunoreactivity with antibodies to C_6 cell plectin, indicating a certain degree of structural homology. Quantitative immunological studies aimed at determining the extent of homology have not been conducted; based on combined results from a series of immunoprecipitation, immunoblotting, and immunofluorescence microscopy experiments, it seems that plectins from mammalian cells have a rather conserved primary structure. This notion is supported by comparative one-dimensional peptide mapping of plectins from different cell lines using protein species radiolabeled in various ways.[9,29] Furthermore, plectins from a number of sources tested exhibited a pI of 4.8 to 5.0, and thus were indistinguishable in chromatofocusing and two-dimensional gel electrophoresis.[29] Extensive similariaties in the primary structure of different plectin species were also demonstrated by amino acid analyses.[13]

B. Hydrodynamic Parameters, Conformation, and Molecular Structure

The sedimentation velocity coefficient of C_6 cell plectin determined by ultracentrifugation under nondenaturing conditions was near 10 S. This analysis was done in low ionic strength buffers, such as 2 m*M* Tris/HCl, pH 8.0, at low protein concentration, and at low temperatures (5 to 10°C); otherwise, due to plectin's tendency to aggregate, sedimentation boundaries were too broad. Sedimentation velocity centrifugation of plectin dissolved in 7 *M* urea yielded broad sedimentation boundaries corresponding to values of 12 to 15 S. This indicated that the molecular structure of plectin in urea was altered compared to low ionic strength buffers, probably due to unfolding of the polypeptide chains. Based on the M_r, the sedimentation coefficient and the partial specific volume derived from the amino acid composition, a frictional coefficient of 5.3×10^{-7} g/sec^{-1} was estimated for plectin molecules in solutions of low ionic strength. This indicated a very elongated shape of plectin molecules.

The Stokes radius of C_6 plectin derived from the hydrodynamic data was 28 nm. The frictional coefficient and Stokes radius calculated for plectin solubilized in 7 *M* urea were considerably lower than those of plectin in urea-free solutions, again indicating alterations in the molecular structure of plectin caused by urea.

The Stokes radius of plectin dissolved in solutions of low ionic strength was also determined by HPLC, using a combination of different gel permeation columns and spherical, as well as elongated, proteins as standards. For plectin isolated from glioma C_6 cells a value of 27 nm was obtained, whereas values of 13 and 26 nm were obtained for plectin purified from lens cell tissue. The 13- and 26-nm plectin species from lens cells were apparently in equilibrium, with a predominance of the 13-nm form. The plectin species with the smaller Stokes radius probably corresponded to an intramolecularly folded plectin tetramer (see below) or, less likely, to dimeric plectin molecules.

The circular dichroism spectrum of plectin purified in solutions containing 7 *M* urea and then desalted into low ionic strength buffer, best conformed to 30% α-helix, 9% β-structure, and 62% aperiodic structure. Samples purified under less denaturing conditions (0.5% sodium *N*-lauroylsarcosinate instead of urea), yielded similar spectra with a slightly higher α-helical content of 35%. Thus, most of plectin's secondary structure was apparently recovered after removal of urea. As indicated by spectra of samples at various concentrations of urea, plectin seemed totally unfolded in 7 *M* urea, but only partially in 3.5 *M* urea.

Electron microscopy of purified and rotary-shadowed C_6 or lens cell plectin dissolved in low ionic strength buffer revealed globular as well as filamentous structures. The only particles of clearly defined structure and size were filaments terminated at each end by globes of equal size. It was assumed, therefore, that these dumbbell-like structures represented the predominant form of plectin molecules in solution. Additional structures observed, like single globes of various sizes and globes with filamentous protrusions of different lengths, probably represented parts of the major dumbbell-like structures that had been disrupted by shearing forces during spraying of the samples onto the electron microscope grids. Also, many of the dumbbell-like structures displayed breaks close to the middle of the rod section, and a few rods seemed to be longitudinally split into two polypeptide strands. Electron microscopy of negatively stained plectin samples revealed structures similar to those seen in shadowed specimens. The average contour length between the centers of the terminal globes of plectin molecules was 193 nm, the diameter of the rod 2 nm, and the diameter of the globular end domains 9 nm.

Based on the microscopic dimensions of dumbbell-like structures their molar mass was calculated as 1.12 to 1.16 $\times$ 10^6. This value was in reasonable agreement with the value of 1.2 $\times$ 10^6 deduced from chemical cross-linking experiments. The center-to-center distance of plectin's globular end domains in average was 171 nm, only slightly lower than the contour length of 193 nm. A stiffness parameter of 0.041, calculated on this basis, indicated that plectin molecules were more rigid than, for example, myosin rods, laminin, and fibronectin.

For a number of reasons, it is likely that the rod domain of dumbbell-shaped plectin molecules consist mainly of a double-stranded coiled coil α-helix. First, its diameter was very similar to that of other coiled coil α-helical structures, such as myosin rods. Second, plectin's secondary structure unfolded sharply at a midpoint temperature and with a vant Hoff transition enthalpy, typical for α-helical coiled coil structures. Third, the M_r of the rod estimated from its ultrastructural dimensions (480 000) accounted for about 40% of plectin's total M_r (1200 000). This would be consistent with the 30% α-helical structure revealed by circular dichorism spectroscopy, assuming that most of the rod domain is in fact α-helical. Having coiled coil α-helical domains, plectin molecules seem to follow structural principles found in a variety of other rod-like proteins, such as tropomyosin,[33-35] myosin,[36] laminin,[37] and most intermediate filament subunit proteins.[38,39]

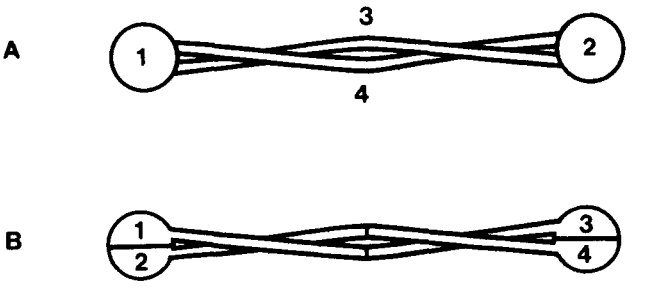

FIGURE 5. Models of polypeptide chain arrangement in tetrameric plectin molecules. (A) Polypeptide chains 1 and 2 each form one globular end domain, polypeptide chains 3 and 4 form the coiled coil rod section. Structurally different polypeptide chains are required for rod and globular domains. (B) Polypeptide chains 1 to 4 are each part of the globular as well as of the rod domains. All four polypeptide chains are structurally equivalent. Models are not drawn to scale.

Regarding the molecular arrangement of the four M_r = 300,000 polypeptide chains that constitute the dumbbell-shaped plectin molecule, two models can be considered (Figure 5). In one, two polypeptide chains form the coiled coil filamentous domain and, each of the other two, the two globular domains (Figure 5A). This arrangement would require at least two components of different primary structure and conformation for rod and globe structures. Since there is no evidence for such heterogeneity of plectin preparations, this model is considered unlikely. In the other model (Figure 5B), two plectin polypeptide chains associate laterally to form one globular domain and one half of the rod. Two such structures, joining at the tips of their half rods, would then yield the symmetric tetramer. This model is favored, not only because of the striking symmetry of the dumbbell-like structures, but also due to the observation of dumbbell-like structures that were broken characteristically near the midpoint of the rod section, where mechanical disruption would be expected to most likely occur. This model is also supported by the chemical cross-linking data that revealed predominantly dimeric and tetrameric structures, but relatively few trimers. Moreover, the cross-linking experiments showed that dimer formation was kinetically favored over tetramer formation, indicating that two each of the four polypeptide chains constituting the tetramer were in closer, maybe also tighter, contact with each other than with the other chains. The data on plectin's molecular and hydrodynamic properties, as well as those on its conformation and ultrastructure, are summarized in Table 3.

V. INTERACTION PARTNERS OF PLECTIN

A. Self-Association

While preparations of plectin dissolved in low ionic strength buffers and kept at 4°C were visualized in the electron microscope predominently as single dumbbell-like structures of defined size, globes of mainly varying sizes with radiating thin filaments were observed at 37°C. Apparently these structures were formed through the associating globular head domains of tetrameric plectin molecules. This head-to-head association seemed to occur intramolecularly through backfolding of the rod, thereby forming an extended loop, or intermolecularly by association of several single plectin molecules into a larger globular core region, with the rod domains and the free globular domains radiating outward. Linear associations of several plectin molecules were observed, too. Still larger aggregates of plectin were observed in buffer solutions of higher ionic strength. Such conditions yielded star-like structures that displayed a globular core region of about 50 nm in diameter and surface-

Table 3
MOLECULAR CHARACTERISTICS OF PLECTIN

Molecular weight	
SDS-electrophoresis	300,000
SDS-electrophoresis (cross-linked)	1,200,000
HPLC	1,150,000
Isoelectric point	
Isoelectric focusing	4.7—5.0
Chromatofocusing	4.7—5.5
Amino acid composition	
Acidic residues	24%
Basic residues	15%
Sedimentation velocity coefficient	
Low ionic strength	10 S
7 *M* urea	12—15 S
Frictional coefficient	
Low ionic strength	5.3×10^{-7} g × sec^{-1}
7 *M* urea	2.5—3.1 × 10^{-7} g × sec^{-1}
Stokes radius	
Sedimentation	28 nm
HPLC	13 nm, 27 nm
Conformation	
α-helix	30—35%
β-sheet	9%
Aperiodic	62%
Midpoint temp (therm. unfold.)	45°C
Dimensions	
Length of rod	184 nm
Diameter of rod	2 nm
Diameter of globular ends	9 nm

attached filamentous loops up to 100 nm long. Several star-like plectin assemblies were often interconnected via their radiating filaments. In addition, star-like structures interconnected by rather straight filamentous bridges were observed, suggesting that the two globular head domains of individual plectin molecules bound to the cores of two different star-like assemblies. Since the interaction of plectin's globular head domains seemed to be specific, it is possible that star-like plectin oligomers are structural elements of cells. Specific head-to-head association has been reported for other multidomain proteins consisting of rod-like and globular substructures, such as procollagen IV,[40] vinculin,[41] laminin,[42] and nidogen.[43]

B. Intermediate Filaments and Subunit Proteins

Plectin's interaction with intermediate filaments was studied using a number of different techniques. The *in situ* association of plectin with intermediate filaments of cultured cells was shown by gold-immunoelectron microscopy of microtubule- and actin-depleted cytoskeletons, as well as of quick-frozen and deep-etched fibroblast cells mildly extracted with detergent.[44] In both cases filament-associated plectin was visualized primarily at junction and crossover sites of the filament network. It was not found distributed along the surface of the filaments, as has been reported for other intermediate filament-associated proteins, such as epinemin.[45,46] Antiplectin-positive structures connecting adjacent intermediate filaments were seen as well.

The interaction of plectin with intermediate filaments was also shown *in vitro* using chromatographically purified fractions of both vimentin and plectin.[13,44] As revealed by electron microscopy of negatively stained specimens, intermediate filaments reconstituted from vimentin alone displayed a smooth surface and no interlinking. In contrast, filaments reconstituted from mixtures of purified fractions of vimentin and plectin showed spherical

particles attached to their surfaces and, as a whole, resembled a highly cross-linked network. No periodicity in the attachment of globes along the filaments was apparent, but they were preferentially located at crossing-over or branching sites of the filament network. Similar highly cross-linked networks were observed when preparations of unfractionated intermediate filaments were solubilized and repolymerized. The filament-attached globes were clearly identified as plectin structures by gold-immunoelectron microscopy. Using the rotary shadowing technique, numerous thin filaments up to 200 nm in length protruding from the surface-attached globular structures were visualized. Thus, these structures represented associations of single dumbbell-shaped plectin molecules consisting of a globular core region with filamentous protrusions, as seen after incubation of purified plectin alone, at high concentrations of protein or salt. Clusters of two or more oligomeric cores interconnected by their filamentous protrusions were also found associated with vimentin filaments. They were attached, often densely packed, either to individual filaments or to two filaments running in parallel. The ability of plectin to interlink vimentin filaments was also demonstrated by sedimentation experiments in the ultracentrifuge. Aggregates of plectin and intermediate filaments on the average were approximately ten times larger than those of plectin or vimentin samples alone. The mass ratio of plectin to vimentin in aggregates formed *in vitro* was in the range of 1 plectin molecule per 20 vimentin molecules, very similar to the proportion found in crude filament preparations. This proportion was roughly maintained, even after repeated rounds of solubilization and *in vitro* assembly of unfractionated intermediate filaments, strongly indicating that the interaction of plectin with vimentin filaments was specific.[13] Regarding other intermediate filament types, copurification of plectin was also observed with preparations of neurofilaments.[44]

A multidomain structure is characteristic for all intermediate filament subunit proteins. A central rod domain consisting of coiled coil polypeptide helices interrupted by nonhelical portions is generally flanked by less ordered head and tail domains. Plectin overlays of proteolytic vimentin fragments, which had been electrophoretically separated and immobilized on nitrocellulose sheets, revealed that plectin bound to all fragments containing the intact central rod domain of the intermediate filament protein.

Because of the conservative structure of the central rod domain of intermediate filament proteins, it was not unexpected to find that plectin bound to intermediate filament subunit proteins other than vimentin, including the glial fibrillary acidic protein (GFA), all three of the neurofilament proteins (NF 200, NF 160, and NF 68), and cytokeratins.[44] Regarding the latter, it was interesting to find that of the five keratins contained in preparations of intermediate filaments from human skin, only keratins 10 and 11, characteristic for keratinizing fully differentiated epidermal cells,[47] showed binding to plectin.

Could domains of intermediate filament subunit proteins other than the rod be involved in the interaction of plectin with intermediate filaments? In the assembled filament, the central rod domains of the subunit proteins are presumably associated laterally, forming the filament backbone. The amino-terminal head domains, being essential for filament polymerization,[48-50] supposedly are tightly incorporated into the filament structure, while the carboxy-terminal tail domain of vimentin, and most likely that of other intermediate filament proteins, having no influence on polymerization, is probably located at the surface of the filament. Therefore, the tail domain, though not directly binding to plectin, could still be involved in regulating the interaction of plectin with intermediate filaments. A negative control mechanism of this domain seems not unlikely, considering the lack of plectin binding along most of the filament's surface, as observed using a variety of techniques.[44]

C. Microtubules and Microtubule Proteins

Using immunoblotting techniques, plectin has been shown to copurify with microtubules assembled *in vitro* from extracts of cultured rat glioma C_6 cells.[8] Codistribution was observed

in repeated rounds of temperature-dependent *in vitro* polymerization, as well as after taxol-induced microtubule assembly. Furthermore, gold-immunoelectron microscopy of *in vitro* assembled microtubules revealed a patchy arrangement of plectin molecules along the microtubule surface.[8]

Overlays of hog brain microtubule proteins, which had been electrophoretically separated on SDS-polyacrylamide gels and transferred to nitrocellulose sheets with purified samples of plectin, showed that both MAP-1 and MAP-2, but not tubulin, possessed binding sites for plectin.[29]

D. Proteins of the Subplasma Membrane Skeleton

In solid phase-binding assays of electrophoretically separated and nitrocellulose-transblotted proteins with plectin purified from glioma C_6 cells, the binding of plectin to the 240-kDa subunit of fodrin from mammalian brain and to the α-polypeptide chain (240 kDa) of erythrocyte spectrin was demonstrated.[29] These structurally related polypeptides,[51] together with their more diverse complementary chains, the 235-kDa fodrin subunit and β-spectrin (220 kDa), respectively, constitute the major components of the subplasma membrane skeleton. Evidence for complex formation between plectin and fodrin also came from experiments carried out with material from lens cells.[13] Fodrin codistributed with plectin and vimentin in repeated rounds of *in vitro* filament polymerization in a nearly constant ratio. Moreover, when high-salt/Triton® X-100 insoluble lens cell proteins were partially solubilized and subjected to molecular sieving column chromatography, a soluble complex of proteins eluting in the exclusion volume showed a composition similar to that of intermediate filaments prepared by rounds of *in vitro* polymerization/depolymerization. A complex of similar composition could be reconstituted after solubilization in urea, separation of the proteins by column chromatography, and subsequent combination in the absence of urea. Several other proteins, most notably one of M_r 130,000, and some of M_r below 50,000, presumably including actin, were also part of these complexes. However, not all of these proteins were necessarily plectin-binding species. For example, in view of plectin's inability to directly interact with actin *in vitro*, the most likely interaction partner of the presumptive actin component would be fodrin.

VI. FUNCTIONS OF PLECTIN

The evidence discussed suggests that plectin is a cross-linking element of the cytomatrix with several functions (Figure 6). One of these functions seems well established: the cross-linking of intermediate filaments in cultured cells. The immunoelectron microscopic location of plectin at crossover and junction sites of filaments reconstituted *in vitro* and of cytoskeletons studied *in situ*, as well as a number of other observations (see above), strongly favor this idea. Based on the *in vitro* reconstitution experiments showing that under conditions near physiological, plectin structures interlinking intermediate filaments were oligomeric (individual or clustered star-like arrangements), it is likely that in the cell, too, bridging elements consisting of plectin are made up of several molecules.

Besides interlinking intermediate filaments, plectin may also be involved in anchoring intermediate filaments into the plasma membrane. Its ability to interact with the well-conserved and ubiquitously distributed α-chain of the spectrin dimer suggests that plectin could be the direct link between intermediate filaments and the membrane protein skeleton. This does not exclude the possibility that the interaction between intermediate filaments and cellular membranes can also occur through the direct binding of intermediate filament subunit proteins to membrane components, as suggested on the basis of *in vitro* experiments.[52-55]

The interaction of plectin with the high M_r MAPs 1 and 2 suggests another function of plectin, namely, the interlinking of intermediate filaments and microtubules. It has long

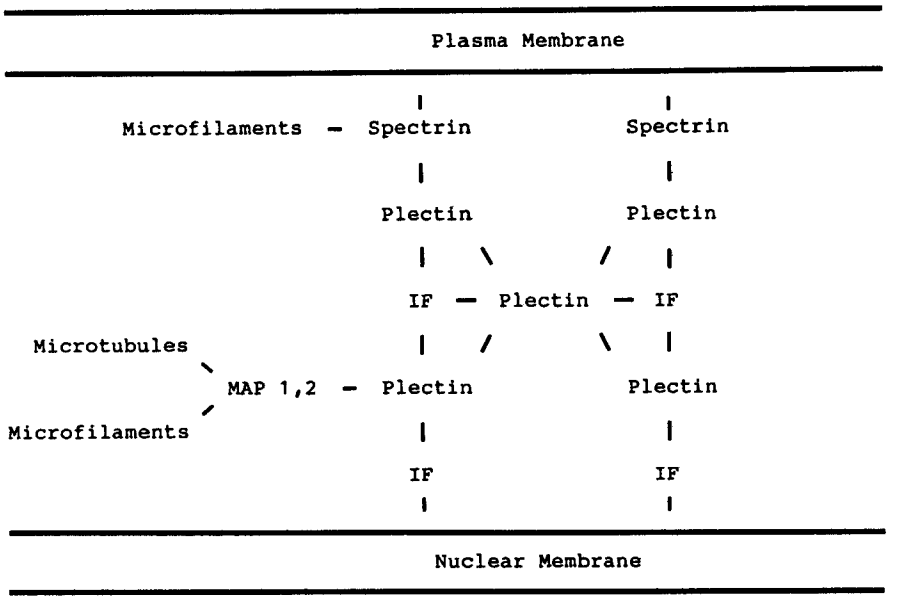

FIGURE 6. Schematic representation of plectin's proposed integration into the cytoplasmic space. The scheme takes into account the demonstrated ability of plectin molecules to interact with themselves, with intermediate filaments, and with microtubular proteins, as well as with components of the subplasma membrane skeleton.

been recognized that these two cytoskeletal systems in the cell are interconnected in some ways. Their spatial codistribution and apparent interconnection by filamentous cross-bridges has been observed in cells of different types, including neurons,[56-58] cultured fibroblasts,[59-61] embryonic chicken gizzard cells,[62] and epithelia-derived cells.[63] A functional linkage between the two systems is suggested also by experiments showing that the organization of the cellular intermediate filament network is profoundly affected after drug-induced disruption[64-68] or relocation[69,70] of microtubular arrays. Furthermore, the observation that microtubules and neurofilaments move together as one component of slow axonal transport in neurons[71,72] provides additional evidence for both systems forming a coherent structural unit. However, the proposed function of plectin as a linking element of microtubules and intermediate filaments may be relevant only in cell types where these two filament systems are not as closely associated, as apparently they are in neurons. In fibroblasts, for instance, plectin may be instrumental in establishing bridges between the two filamentous systems that span over much larger distances than in the case of neurons. In neurons, on the other hand, component proteins of microtubules and neurofilaments may be directly involved in the formation of contacts. Consistent with this view is the ability of high M_r MAPs to bind to neurofilament proteins, as demonstrated using solid phase binding assays,[73,74] as well as the absence of plectin from neurons as revealed by immunocytochemistry (see above).

Considering that high-M_r MAPs have been shown previously to bind to filamentous actin,[75-77] the binding of plectin to MAPs may also play a role in establishing contacts between intermediate filaments and actin filaments. Another, more indirect, mechanism of intermediate filament/microfilament interaction may proceed via spectrin-type molecules.

Several observations would be consistent with the idea that plectin is capable of forming a cytoplasmic network-type array of its own that is independent of the other well-characterized cytoskeletal filament systems. One is the ability of plectin molecules to interact specifically with themselves, yielding complex star-like assemblies. These structures, occurring indi-

vidually as well as in clusters, in turn, appear capable of self-interaction. Thus, it seems possible that in the cell there exists a whole network of star-like plectin aggregates. These aggregates could interact with various partners via the filamentous extensions frequently observed at their periphery. In the absence of potential interaction partners, these extensions might bend backward to generate loop-like structures, as seen *in vitro*. Plectin networks could thus be envisaged as a kind of anastomizing structure with extended or backfolded arms, depending on the availability of interaction partners in the surrounding environment. In this way plectin may function as an important structural element of the cytomatrix.

VII. RELATIONSHIP TO OTHER PROTEINS

A. IFAP-300K

A high-M_r polypeptide, referred to as intermediate filament-associated protein of M_r = 300,000 (IFAP-300K) has been identified in intermediate filament preparations of baby hamster kidney (BHK-21) cells.[78] This protein codistributed with BHK-21 intermediate filaments *in situ*, as determined using a monoclonal antibody preparation, and cocycled with intermediate filament subunit proteins from BHK-21 cells in repeated rounds of *in vitro* polymerization and depolymerization. Furthermore, networks of intermediate filaments showing the location of IFAP-300K protein at points of contact and intersections between intermediate filaments were reconstituted *in vitro* from purified protein components.[79] Because of its selective association with intermediate filaments of cultured cells, Goldman and co-workers concluded that IFAP-300K and plectin were distinct proteins.[78,79] However, homology of both proteins was later revealed in a study by Herrmann and Wiche.[29] Homology was demonstrated by immunological cross-reactivity, peptide maps, comigration on high-resolution gels, identical pI on two-dimensional gels and chromatofocusing columns, similar amino acid composition, and comparable characteristics of phosphorylation by endogenous kinases. The two proteins also showed an analogous distribution to various cell fractions. Like IFAP-300K, M_r = 300,000 proteins found in several lines, including Chinese hamster ovary, mouse Balb/c3T3, and human HeLa, A-431, and WI-38 cells, were immunologically related and hardly distinguishable from plectin by peptide mapping.[9,29] Therefore, the differences in cellular associations reported for IFAP-300K and plectin most likely are due to the immunoreagents used. IFAP-300K's immunolocalization exclusively on intermediate filaments of BHK-21 cells is based on data obtained with a single monoclonal antibody preparation, whereas the widespread occurrence of plectin both with regard to cell types and tissues was deduced from studies with polyclonal rabbit antisera and affinity-purified 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 that recognize several different epitopes on a particular antigen. In fact, a series of monoclonal antibody preparations to plectin that were more recently obtained confirm the widespread occurrence of the protein, previously revealed only with the conventional antibodies.[123]

B. Paranemin

A protein potentially related to plectin is paranemin,[80,81] which was originally identified in chick embryonic skeletal muscle. This protein is very similar to plectin with respect to M_r (280,000) and isoelectric point (4.0 to 4.5). It has been found to codistribute with vimentin and desmin in cultured myogenic cells from chicken embryos. In early myotubes it was colocalized with cytoplasmic intermediate filaments; in late myotubes with Z-lines. Its expression seems developmentally regulated, because it was not detectable, at least not by immunofluorescence microscopy, in fast and slow skeletal muscle after hatching. It is found

in mature myocardiac cell and in smooth muscle cells of elastic arteries, but not in smooth muscle of muscular arteries. Endothelial cells of muscular vessels were paranemin-positive; those of elastic vessels negative.[82] Similarly, only subpopulations of fibroblast cells were identified as paranemin-positive. Neurons, epithelial, and glial cells were negative, too. Cross-reaction of paranemin antibodies with proteins of species other than chicken was not reported. Thus, paranemin seems to be much more restricted in its occurrence compared to plectin. However, proteins related to paranemin may be expressed and present in a wider spectrum of cells than hitherto revealed, but they may not be detectable with the available antisera. Whether paranemin, like plectin, can cross-link intermediate filaments or is capable of self-interaction or interaction with other elements of the cytoskeleton is not known.

C. Synemin

Synemin (M_r = 230,000), first described by Granger and Lazarides,[83] might be related to plectin in function. This protein, originally isolated from chicken smooth muscle,[83,84] where it occurs in an amount that is 50 to 100 times lower than that of the muscle-specific intermediate filament protein desmin, is also expressed in adult and embryonic skeletal muscle, as well as in cardiac muscle.[81] Besides in muscle tissues, synemin has been found in relative abundance only in avian erythrocytes,[85] in lens cells,[86] and in ependymal tanycytes.[86] Thus, in contrast to plectin, synemin seems to exist in a very limited number of cell types. Notably, however, synemin from avian lens cells copurifies as a complex with vimentin-, actin-, and spectrin-type proteins, strikingly similar to plectin from mammalian lens tissue.

In general, the cellular distribution of synemin is indistinguishable from desmin and vimentin. In skeletal muscle, for example, it colocalizes with desmin and vimentin at the periphery of the Z-line structures. Synemin from avian smooth muscle, erythrocytes, and lens cells seem to be closely related immunologically, but there is little if any immunological relationship between avian and mammalian synemins.

Like plectin, synemin cocycles with intermediate filament subunit proteins in rounds of filament assembly and disassembly without having a noticeable effect on the rate and extent of filament assembly. However, contrary to plectin, it binds to the core filament with a periodicity (180 nm).[85,86] Solubilization of intermediate filaments from chicken gizzard smooth muscle in urea yielded a soluble form of synemin (M_r = 980), which, like plectin, appears to be a tetramer consisting of four equal polypeptide chains. Contrary to plectin, however, these tetramers are globular in shape. Synemin is slightly less acidic (pI = 5.34) than plectin, and its amino acid composition is distinct, so are its Stokes radius (78 Å) and S value (22.4). However, similar to plectin, synemin is a phosphoprotein with a high capacity to incorporate ^{32}P-phosphate and a susceptibility for cAMP-dependent phosphorylation. In both cases, serin residues (95%) are the major phosphate acceptors and threonine residues (5%) are the minor phosphate acceptors.

Synemin has been postulated to be a mediator of filament/filament interaction and a cross-linker between intermediate filaments and the plasma membrane in certain cell types. Furthermore, it has been suggested that synemin self-interaction plays a role in the cross-linking of filaments.[85] Within this concept, synemin and plectin seem functionally related.

D. Neurofilament Proteins

A functional relationship of plectin may also exist with neurofilament proteins (NFs) 200 and 160. NFs polymerized *in vitro* from mammalian brain tissue consist mainly of three polypeptides with M_rs of 200,000 (NF 200), 160,000 (NF 160), and 68,000 (NF 68). According to available sequence data, all three polypeptides possess extensively conserved α-helical rod domains, allowing (in principle) for proper self-association in each case. However, only NF 68 easily forms long intermediate filaments *in vitro*.[87,88] NF 160 is able

to form intermediate filaments, too, but only under restricted experimental conditions.[89] Self-assembly of NF 200 yield short curly structures of smaller diameter than those formed from NF 68 and NF 160.[89] The reduced self-assembling ability of the NFs of higher M_r is probably due to interferences by their long-tail piece domains, which, in contrast to the central rod domains, seem to be of nonconserved structure. While the helical, well-conserved rod domain of high-M_r NFs most likely is involved in the construction of the filament backbone, the tail regions presumably form protrusions extending from the filament surface. These protrusions could be instrumental in mediating filament interactions with other components. Thin polypeptide strands interconnecting neurofilaments were indeed decorated by antibodies to NF 200, as visualized by the quick-freezing/deep-etching replica technique.[90] Whether NF 200 is the sole constituent of these bridges, or whether other proteins are part of these structures as well, is not known. Thus, like plectin, NF 200 and possibly NF 160 may be responsible for intermediate filament cross-linking.

Similar to plectin, high-M_r NFs may also serve as bridging components between intermediate filaments (neurofilaments) and microtubules. This interaction is very likely to take place via the high-M_r microtubule-associated proteins MAP-1 and MAP-2 that decorate the surface of microtubules with thin projections[91] and have been shown to bind to and cross-link neurofilaments *in vitro*.[92,93] Moreover, both MAP-1 and MAP-2 have been shown to interact with NFs on the molecular level using solid phase-binding assays.[73,74] Thus, NF 200 and NF 160 apparently possess two binding sites: one for neurofilaments and one for MAPs. In this respect, too, they resemble plectin, which also interacts with both MAPs as well as with intermediate filament proteins (including NFs). In view of plectin's absence from neurons and the fact that neurofilaments are specific for neurons, NF 200 and NF 160 might represent variants of cytomatrix proteins that are specifically tailored to the requirements of neuronal cell morphology and function. NF 200 also resembles plectin insofar as it is capable of self-association, forming polymeric structures that are clearly different from *bona fide* intermediate filaments.

E. High-M_r Microtubule-Associated Proteins

As potential cytomatrix proteins, the microtubule-associated proteins MAP-1 and MAP-2 might also be related to plectin in function, although regarding structure, they are clearly different from plectin. Like plectin, MAPs possess binding sites for a number of different interaction partners[74] (for a review see Reference 94). They copurify with microtubules in rounds of *in vitro* assembly and disassembly, and their binding to tubulin subunits, or fragments thereof, has been demonstrated on the molecular level.[74,95] Furthermore, they bind to and cross-link NFs *in vitro*, as shown by different techniques, including solid phase binding assays.[73,74,92,93] Interestingly, both MAP-1 and MAP-2 possess binding sites for MAP-2, providing the possibility of cytomatrix network formation through self-interacting MAPs.[74] This self-interaction *in vitro* can be promoted by taxol,[96] a drug known to stabilize microtubules polymerized *in vitro* and *in vivo*.[97,98] MAP polymers formed with taxol resemble either large network-type aggregates with little substructure or more regular structures consisting of 5-nm thick filaments arrayed in parallel.[96] Although proteins structurally related to MAP-1 and to MAP-2 seem to be of widespread occurrence in cultured cells and tissues[99,100] (reviewed in References 94 and 101), apparently this type of MAPs is by far more abundant in brain than in other tissues. Therefore, like NFs, MAP-1 and MAP-2 could be of particular importance for cytomatrix features typical of neuronal cells.

F. Filaggrin

Another protein potentially related to plectin in function is filaggrin,[102] which was originally isolated from mammalian epidermis.[103,104] Filaggrin comprises a family of basic, histidine-rich proteins, whose size and amino acid composition vary among species.[105]

However, all filaggrins seem to be related in function. They bind to keratin, desmin, and vimentin filaments *in vitro* and cause the formation of macrofibers, tight, highly organized filament bundles. Because of their ability to interact with and bundle intermediate filaments of different types, it is assumed that filaggrin molecules, analogous to plectin, bind to the conserved α-helical rod domains of intermediate filaments.[102] Because of its filament-bundling ability, filaggrin has been suggested to form the interfilamentous matrix of the stratum corneum.[104] Thus, filaggrins could be the keratinocyte-specific pendent of high-M_r NFs characteristic of neurons. Filaggrin is synthesized as a high-M_r precursor form, profilaggrin[106-111] (M_r in rat over 500,000), that resembles plectin with respect to its high content in phosphoamino acids, which alters the basic net charge, typical of filaggrins, to acidic or neutral.[106,107,112] Unlike plectin, however, in this form, it shows no affinity to intermediate filament proteins.

A number of other proteins found in association with intermediate filaments have not been characterized sufficiently to allow an evaluation of whether, like plectin, they can be considered as potential subunit components of the cytomatrix. These include epinemin[45,46] of M_r 44,500, a M_r 50,000 protein found associated with vimentin filaments,[113,114] α-internexin,[115] a neuronal protein, and β-internexin,[116] its nonneuronal counterpart, as well as NAPA,[117] a neurofilament-binding protein, and two antigens of 95 kDa and 210 kDa originally isolated from myofibrils of rat muscle.[118,119]

VIII. CONCLUSIONS AND PERSPECTIVES

Considering the data available, plectin indeed seems well qualified as a potential subunit component of the cytomatrix. Possessing a binding site for the well-conserved α-helical rod domain of intermediate filament subunit proteins, it is, in principle, equipped for interactions with intermediate filaments of all types. Thus, plectin's presumptive function as a cross-linking and anchorage element of intermediate filaments, *a priori*, is not restricted to certain cell types. However, in highly specialized cells, such as epidermal cells and neurons, filaggrins, as well as neurofilament proteins and MAPs of high M_r, may take over functions similar to those of plectin. A difference in function between plectin and these other presumptive cytomatrix proteins may be that only plectin, because of its high tendency for self-interaction, is capable of forming cytomatrix connecting structures that span over relatively long distances. This may not be a requirement for neuron-, or epidermal cell-specific cytomatrix proteins, whose task presumably is the bridging or bundling of juxtaposed intermediate filaments.

Consistent with the idea of plectin being a general intermediate filament cross-linking element is its widespread occurrence both with respect to tissues and cell types. For none of the other proteins that have been found to associate with, or to interact with, intermediate filaments has such a similarly wide distribution been reported.

Multifaceted binding characteristics of the kind observed with plectin molecules are probably features to be expected for genuine cytomatrix subunit components. Being provided with binding sites for a number of cytoplasmic polypeptides that are almost certainly of strategic importance for the organization of the cytoskeleton, such as MAPs and spectrin-type molecules, plectin appears to be capable of not only interlinking different cytoplasmic filament systems, but also of anchoring these systems into the subplasma membrane skeleton. In fact, the multiplicity of its demonstrated interaction partners makes this protein the seemingly most versatile cytoplasmic cross-linking element reported to date. The existence of binding sites on the plectin molecule that are specific for a number of different interaction partners does not imply, however, that in a given cell all of them would be simultaneously occupied. It is more likely that plectin's molecular interactions depend on the availability of cellular binding partners in a particular cell type or cell compartment. Moreover, post-

translational protein modifications, such as phosphorylation, may alter its binding specificities during tissue development and differentiation of cells.

In future studies it should be possible to ascribe the various binding sites of plectin to specific molecular domains, in particular to the rod of the globular end pieces. This task is likely to be achieved most effectively using a combination of protein chemical and recombinant DNA techniques. Furthermore, microinjection of domain-specific antibodies and transfection of cells with manipulated-plectin genes could be helpful in unraveling the cellular functions of plectin in a more comprehensive way.

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