

Cytoskeleton-associated Plectin: In Situ Localization, In Vitro Reconstitution, and Binding to Immobilized Intermediate Filament Proteins

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Abstract. The association and interaction of plectin (M_r 300,000) with intermediate filaments and filament subunit proteins were studied. Immunoelectron microscopy of whole mount cytoskeletons from various cultured cell lines (rat glioma C6, mouse BALB/c 3T3, and Chinese hamster ovary) and quick-frozen, deep-etched replicas of Triton X-100-extracted rat embryo fibroblast cells revealed that plectin was primarily located at junction sites and branching points of intermediate filaments. These results were corroborated by in vitro recombination studies using vimentin and plectin purified from C6 cells. Filaments assembled from mixtures of both proteins were extensively cross-linked by oligomeric plectin structures, as demonstrated by electron microscopy of negatively stained and rotary-shadowed specimens as well as by immunoelectron microscopy; the binding of plectin structures

on the surface of filaments and cross-link formation occurred without apparent periodicity. Plectin's cross-linking of reconstituted filaments was also shown by ultracentrifugation experiments. As revealed by the rotary-shadowing technique, filament-bound plectin structures were oligomeric and predominantly consisted of a central globular core region of 30–50 nm with extending filaments or filamentous loops. Solid-phase binding to proteolytically degraded vimentin fragments suggested that plectin interacts with the helical rod domain of vimentin, a highly conserved structural element of all intermediate filament proteins. Accordingly, plectin was found to bind to the glial fibrillar acidic protein, the three neurofilament polypeptides, and skin keratins. These results suggest that plectin is a cross-linker of vimentin filaments and possibly also of other intermediate filament types.

INTERMEDIATE filaments, one of the three major cytoskeletal filament systems, are biochemically well defined, but their cellular function is largely unknown. It is believed, however, that they play a role in the maintenance and generation of cell shape as well as in the structural organization of the cytoplasm, including the positioning of cell organelles (for a comprehensive review see Traub, 1985). In this context, polypeptides that are associated with or interact with intermediate filaments would seem to have a great potential as regulators of intermediate filament functions. Indeed, a number of polypeptides have been identified that co-distribute with intermediate filaments of various types. The most intensively studied of these intermediate filament-associated polypeptides are probably the neurofilament (NF)¹ components with apparent molecular weights of 160,000 (NF 160) and 200,000 (NF 200), which could actually be classified as true intermediate filament proteins, since they both

seem to have an extended structural domain in common with the M_r 68,000 neurofilament subunit protein (Weber et al., 1983; Geisler et al., 1985). Other intermediate filament-associated proteins are synemin of M_r 230,000 (Granger and Lazarides, 1980, 1984) and paranemin of M_r 280,000 (Breckler and Lazarides, 1982; Price and Lazarides, 1983); both proteins were originally found in muscle cells. To add to these are the low molecular weight filaggrins (Dale, 1977; Steinert et al., 1981; Harding and Scott, 1983) and their M_r 300,000 precursor, profilaggrin (Scott and Harding, 1981; Meek et al., 1983), identified in the epidermis, as well as epinemin of M_r 44,500 (Lawson, 1983, 1984) and an M_r 50,000 protein (Wang et al., 1983; Wang, 1985) found in association with vimentin filaments. Association with intermediate filaments has been also reported for the neuronal α -internexin (Pachter and Liem, 1985) and its nonneuronal counterpart β -internexin (Napolitano et al., 1985) as well as for neurofilament-associated protein, avian specific (Ciment et al., 1986), and for two antigens of 95 and 210 kD originally isolated from myofibrils of rat muscle (Lin, 1981; Lin and Feramisco, 1981). Regarding possible functions of these proteins, cross-linking of filaments has been demonstrated for synemin (Granger and Lazarides, 1982), filaggrins (Steinert

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1. *Abbreviations used in this paper:* GFA, glial fibrillar acidic protein; NF, neurofilament.

et al., 1981; Harding and Scott, 1983), and the 50-kD protein (Wang, 1985).

Aside from these proteins, most of which are considered to be genuinely intermediate filament-associated, only a few other polypeptides have been shown to interact specifically with intermediate filaments. High molecular weight proteins associated with microtubules were also found in preparations of neurofilaments (Aamodt and Williams, 1984; Letierrier et al., 1984), and bound to nitrocellulose-immobilized neurofilament protein subunits (Heimann et al., 1985; Furtner and Wiche, 1987). Association of spectrin, a major component of the membrane skeleton in erythrocytes, with vimentin was shown indirectly by microinjection of non-erythrocyte spectrin antibodies into fibroblasts (Mangeat and Burrige, 1984), and the association of spectrin with intermediate filaments of the desmin type was shown *in vitro* by co-sedimentation (Langley and Cohen, 1986). Furthermore, the interaction of vimentin filaments with the plasma membrane of erythrocytes was shown to occur via binding of ankyrin to the amino-terminal region of vimentin polypeptides (Georgatos and Marchesi, 1985; Georgatos et al., 1985).

Plectin (M_r 300,000) was initially identified as a major component of Triton X-100/high salt-resistant cell residues of various cultured cell lines (Pytela and Wiche, 1980; Wiche et al., 1982). Since these residues were highly enriched in intermediate filaments, plectin seemed to be another candidate for a genuine intermediate filament-associated protein. Accordingly IFAP-300 (Yang et al., 1985), a protein homologous to plectin by biochemical as well as immunological criteria (Herrmann and Wiche, 1987), co-localized with intermediate filaments of BHK-21 cells in immunofluorescence microscopy studies using a monoclonal antibody preparation. However, similar studies performed with a polyclonal antibody preparation on a series of different cell lines suggested that plectin was a constituent of dense cytoplasmic network arrays that were distinguishable from the more filamentous vimentin arrays (Wiche and Baker, 1982). Plectin and vimentin arrays became very similar, however, after extraction of cells with detergent, causing the release of a sizeable fraction of cellular plectin (Herrmann and Wiche, 1983). These observations suggested that only a part of cytoplasmic plectin was associated with intermediate filaments, while the rest was bound to either more soluble structures or formed a network on its own that was less resistant toward extraction. Plectin's presumptive role as an intermediate filament-interacting, rather than associated, protein is further supported by its immunolocalization at anchorage sites both of intermediate filaments and microfilaments, as revealed on frozen sections of various rat (Wiche et al., 1983; Zernig and Wiche, 1985) and human (Wiche et al., 1984) tissues. Moreover, the possible function of plectin as an interlinker between intermediate filaments and other cytoplasmic components was suggested on grounds of the protein's ability to interact with microtubules *in vitro* (Koszka et al., 1985) and to bind to nitrocellulose-transblotted high molecular weight microtubule-associated proteins MAP-1 and 2 as well as to α -spectrin-type proteins, constituents of the subplasma membrane skeleton (Herrmann and Wiche, 1987).

Here we focus on plectin's role in cross-linking intermediate filaments. We show the *in situ* localization of plectin at junctional sites of intermediate filaments, using immunoelectron microscopy of both whole-mount and deep-etched

replicas of fibroblast cytoskeletons. Furthermore, using different techniques, we demonstrate the interaction of chromatographically purified plectin with vimentin filaments assembled *in vitro*, and reveal the molecular arrangement of plectin/intermediate filament complexes on the ultrastructural level with the rotary shadowing technique. Furthermore, using solid-phase binding assays, we characterize the interacting subdomain of vimentin molecules and show that plectin binds to various other types of intermediate filament proteins.

Materials and Methods

Cell Culture

For preparative purposes, rat glioma cells, clone C6, were grown to confluence in roller bottles supplemented with DME and 10% newborn calf serum (Pytela and Wiche, 1980). For whole-mount immunoelectron microscopy, C6 cells were directly seeded onto electron microscopic grids. For quick-freezing, deep-etching electron microscopy, rat embryo fibroblasts were prepared from Wistar rats, cultured for 2–3 d in DME containing 10% heat-inactivated fetal calf serum, and then replated. These secondary cultures, used for all experiments after two to six passages, were adjusted to a concentration of 3×10^3 cells/ml, plated overnight in 1-ml aliquots onto 6-mm diam No. 3 coverslips in 24-well Linbro plates, and used the following day.

Preparation of Cytoskeletons and Immunocytochemistry

For whole-mount immunoelectron microscopy, cells grown overnight on collodion-coated UV-sterilized gold grids were incubated in extraction buffer containing 0.1% Triton X-100 (Sigma Chemical GmbH, Munich) as described by Small and Celis (1978), but with 2-(*N*-morpholino) ethanesulfonic acid (MES) instead of Pipes. A low salt-high salt-low salt treatment was performed according to Small and Sobieszek (1977) and Small and Celis (1978) to remove microtubules and actin. Extracted cells (whole-mount cytoskeletons) were then fixed in 3% paraformaldehyde in extraction buffer. Immunolabeling of cytoskeletons was performed according to De Mey et al. (1981) with the following modifications: (a) grids were first rinsed (3×10 min) in 20 mM Tris/HCl, pH 7.6, 155 mM NaCl, 2 mM EGTA, 2 mM MgCl₂, 0.001% streptomycin-sulfate (buffer A). (b) Preincubations were carried out in buffer A containing 5% normal goat serum and 0.1% BSA (Sigma Chemical GmbH) for 30 min. (c) Incubations with primary antibodies were performed for 60 min in buffer A (supplemented with 1% normal goat serum and 0.1% BSA) using rabbit antisera to plectin (Wiche and Baker, 1982) or vimentin (Wiche and Baker, 1982; Herrmann et al., 1985) or preimmune serum, all diluted 1:40 and centrifuged at 30,000 g for 2.5 min. (d) Incubations with prespun (4,800 g for 30 min) goat anti-rabbit IgG (IGS-reagent GAR G-5; Janssen Pharmaceutica, Beerse, Belgium), diluted 1:20 in buffer A containing 1% BSA were for 30 min. Samples were postfixed and negatively stained as described by Foisner and Wiche (1985).

For quick-freezing, deep-etching electron microscopy, rat embryo fibroblast cells, grown on glass coverslips, were rinsed in 8 mM Na₂HPO₄/1.5 mM KH₂PO₄, pH 7.2, 137 mM NaCl, 2.6 mM KCl (PBS) rinsed in 10 mM imidazole, pH 6.3, 100 mM KCl, 10 mM NaNO₃, 5 mM EGTA, 5 mM MgCl₂ (buffer B), and then extracted for 10–20 min at 0°C in buffer B plus 0.25 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% Triton X-100. After extraction with the detergent, cells were rinsed five times in cold buffer B, fixed for 25 min at 0°C in 1% stabilized formaldehyde (British Drug House) in buffer B, rinsed five times in PBS plus 20 mM lysine, and then incubated for 10 min in PBS containing 0.1% BSA (Pathocyte; Five Miles Research Products Division, Elkhart, IN) and 20 mM lysine. Coverslips were then incubated for 50–60 min at 20°C with either 20 μ l of a 1:50 dilution of rabbit antiserum to plectin, or a 1:50 dilution of normal rabbit serum, both in buffer B containing 0.1% BSA and 20 mM lysine and spun at 12,000 g for 5 min. Samples were then rinsed in buffer B containing 0.1% BSA and 20 mM lysine, and incubated as above in a 1:5 dilution of prespun (4,800 g, 10 min) goat anti-rabbit IgG, labeled with 10-nm colloidal gold particles. After repeated rinses in supplemented buffer B and PBS, the samples were fixed for 1 h at 20°C with either 1 or 2% glutaraldehyde in PBS and then stored at 4°C in PBS, before processing for quick-freezing, deep-etching, and electron microscopy, which were carried out as described previously (Lawson, 1984).

Table I. Co-assembly of Plectin with Vimentin in Rounds of Intermediate Filament Disassembly/Assembly

Fractions	Round	Mass ratio plectin/vimentin
S1		0.29
P1	1	0.28
S2		0.27
P2	2	0.23
S3		0.23

Triton X-100/high salt-insoluble cell residues from rat glioma C6 cells were solubilized in buffer C (S1), transferred to buffer D, incubated for 30 min at 30°C, and then centrifuged at 200,000 g for 1 h at 20°C. The pellet (P1) was homogenized in buffer C and centrifuged at 200,000 g for 1 h at 0°C. Supernatants (S2) were processed similar to S1. P2 and S3, fractions corresponding to P1 and S2.

Protein Purification

C6 cell residues insoluble in 1% Triton X-100/0.6 M KCl were prepared as described by Pytela and Wiche (1980) with slight modifications (Foisner and Wiche, 1987). Intermediate filament preparations were dissolved in 7 M urea (Serva, Heidelberg), 34 mM Pipes, pH 7.5, 1.4 mM EGTA, 1.4 mM MgCl₂, 5 mM 2-mercaptoethanol, 3 mM PMSF (buffer C) at 4°C, centrifuged at 200,000 g for 30 min at 4°C, and supernatants were freed of residual Triton X-100 by incubation with 0.3 mg Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) per milliliter of solution for 2 h at 4°C (Holloway, 1973). After centrifugation at 200,000 g for 60 min at 4°C, supernatants were run on Sepharose CL-4B (Pharmacia, Uppsala) columns in buffer C. Fractions containing plectin were immediately frozen at -70°C. Fractions containing vimentin were subjected to chromatography on CM-Sepharose CL-6B (Pharmacia) columns equilibrated in buffer C. Pure vimentin was eluted with buffer C containing 34 mM sodium citrate, pH 5.5, instead of Pipes.

Glial fibrillar acidic (GFA) protein and the neurofilament triplet proteins were isolated from hog spinal cord (Dahl et al., 1982). Proteins were partially separated on DEAE-Sepharose (Pharmacia) columns. Keratins were prepared by extraction of human skin with Triton X-100/high salt and gel permeation chromatography as above.

Protein concentrations were measured by the method of Lowry et al. (1951) and electrophoresis was on SDS 7, 10, or 12.5% polyacrylamide gels (Laemmli, 1970).

In Vitro Polymerization of Intermediate Filaments

For ultracentrifugation and electron microscopy, chromatographically purified vimentin and plectin samples were transferred to 2 mM Tris/HCl, pH 8-8.5, 5 mM 2-mercaptoethanol, 1 mM PMSF by chromatography on Bio-Gel P-10 (Bio-Rad Laboratories). Samples were centrifuged at 200,000 g for 60 min at 4°C, and filament assembly was initiated by adjusting supernatants to 100 mM imidazole/HCl, pH 6.8, 1 mM EGTA, 5 mM 2-mercaptoethanol, 1 mM PMSF (buffer D). Repeated rounds of polymerization and depolymerization of unfractionated intermediate filament preparations were performed as described in the legend to Table I.

Ultracentrifugation

For density gradient centrifugation, protein samples were loaded onto discontinuous sucrose gradients in 5 × 41-mm Ultra-Clear centrifuge tubes (Beckmann Instruments, Inc., Palo Alto, CA). The gradients consisted of 100-μl layers of 15, 25, 35 and 40% sucrose, all in buffer D. Centrifugation was carried out in a Beckman SW 50.1 rotor at 18,000 rpm for 30 min at 30°C. Fractions were analyzed by PAGE. Svedberg sedimentation velocity coefficients were calculated and normalized for aqueous solutions at 20°C ($S_{20,w}$) (McEwen, 1967).

Electron Microscopy of In Vitro Polymerized Filaments

Uranyl acetate staining and immunoelectron microscopy of intermediate filaments were performed as described by Foisner and Wiche (1985). In the latter case, rabbit antibodies to plectin or vimentin and goat anti-rabbit IgG, coupled to 5-nm gold particles (Janssen Pharmaceutica), were used. Rotary shadowing of samples sprayed onto mica was performed essentially as de-

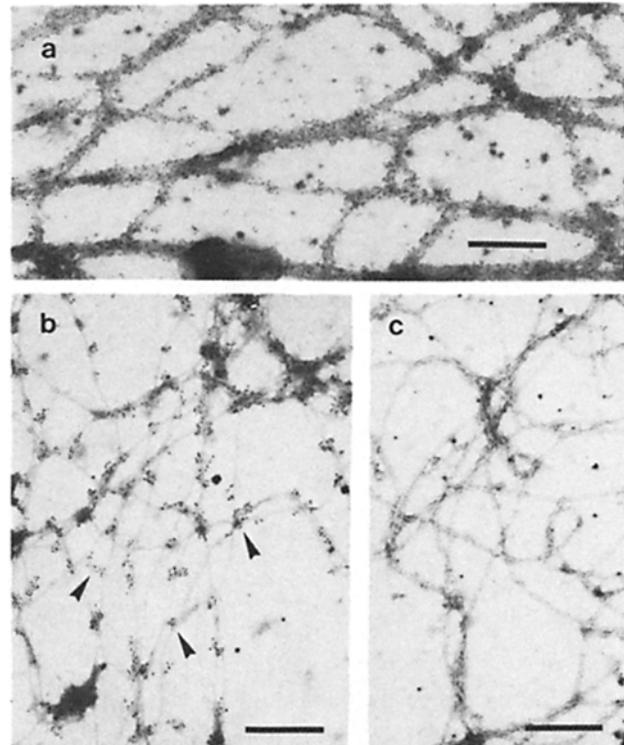


Figure 1. Whole-mount immunoelectron microscopy of plectin and vimentin in rat glioma C6 cytoskeletons. Glioma C6 cells grown on grids were prepared for immunoelectron microscopy as described in the text. (a) Antibodies to vimentin; (b) antibodies to plectin; (c) preimmune serum. Arrowheads in b, plectin located at cytoskeleton branching points. Bars, 0.25 μm.

scribed by Tyler and Branton (1980). All specimens were viewed in a JEOL 100C electron microscope operated at 80 kV.

Solid-Phase Binding Assay

Samples of purified vimentin, proteolytically cleaved with chymotrypsin (Sigma Chemical GmbH) in 5 mM borate, pH 8.5 (Herrmann et al., 1985), and all other preparations of intermediate filament proteins were subjected to electrophoresis on 7 or 10% polyacrylamide gels and transblotted to nitrocellulose sheets (Herrmann and Wiche, 1987). These sheets were overlaid with samples of purified plectin essentially as described by Herrmann and Wiche (1987). For detection of bound plectin, rabbit antibodies to plectin, either untreated or purified by preabsorption or by affinity chromatography, and secondary goat anti-rabbit antibodies coupled to alkaline phosphatase (Promega Biotec, Madison, WI) were used. For preabsorption, anti-plectin was incubated with keratins contained in gel slices. For affinity purification, anti-plectin was incubated with purified plectin linked to Affi-Gel 15 (Bio-Rad Laboratories), as described by the suppliers, and eluted with 0.2 M glycine, pH 2.5, and 150 mM NaCl.

Results

Immunoelectron Microscopy of Cytoskeleton-associated Plectin

To visualize cytoskeleton-associated plectin in situ, gold-immunoelectron microscopy was performed on microtubule- and actin-depleted cytoskeletons, which were prepared from C6 cells that had been grown directly on electron microscope grids. Incubation of fixed specimens with antibodies to vimentin resulted in a rather uniform and dense decoration of intermediate filaments with gold label (Fig. 1 a). In contrast,

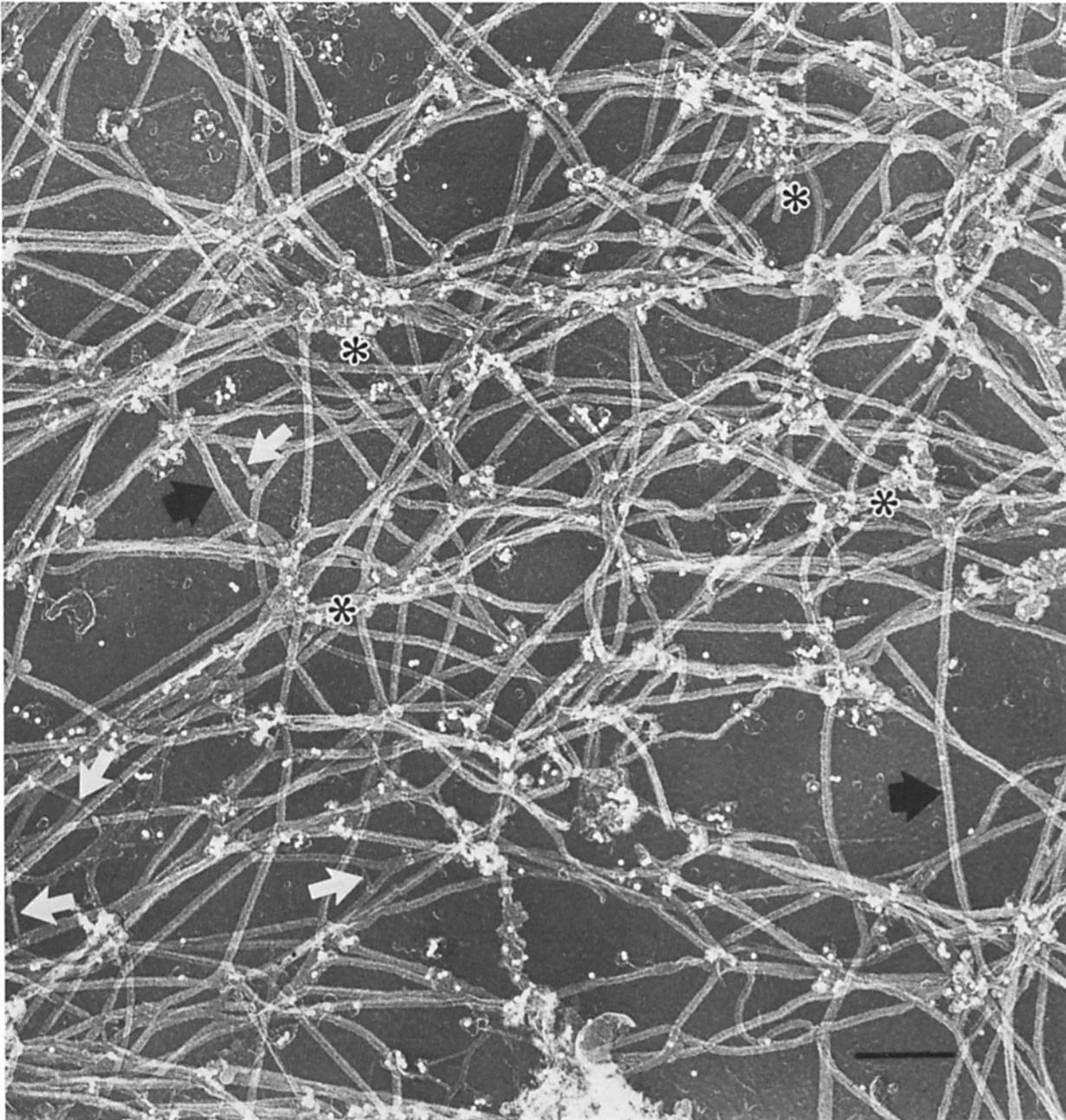


Figure 2. Deep-etched replica of rat embryo fibroblast cytoskeletons. Rat embryo fibroblast cells were grown on coverslips and prepared as described in the text. *Asterisks*, gold particles clustered over filament intersection points; *black arrows*, filament free of label; *white arrows*, small 2–3-nm unlabeled filaments. Bar, 150 nm.

when cytoskeletons were incubated with antibodies to plectin, the distribution of the gold label along the filaments was patchy (Fig. 1 *b*), concentrating mainly at branching points and junction sites of the filament network (*arrowheads*). When whole-mount cytoskeletons were incubated with preimmune serum, very little gold label was detected on the filaments (Fig. 1 *c*). Analogous results were obtained with mouse BALB/c 3T3, Chinese hamster ovary cells, and unfixed C6 cell cytoskeletons (data not shown).

Plectin's association with cytoskeletal filaments of cultured cells was also investigated by immunoelectron microscopy of quick-frozen and deep-etched rat embryo fibroblast cells ex-

tracted with 0.5% Triton X-100. In all instances, the antibodies to plectin were associated with intermediate filaments, which are easily recognized in deep-etched replicas by their smooth surface and characteristic wavy arrays. Anti-plectin was never found distributed along intermediate filaments (Fig. 2, *black arrows*) as has been described for other intermediate filament-associated proteins (see in Lawson, 1983), but was present only at crossover points (Fig. 2, *asterisks*). In these areas, up to 30 gold particles were found at every filament intersection, involving more than five intermediate filaments. No other filamentous structures were seen in association with anti-plectin. Small 2–3-nm filaments previ-

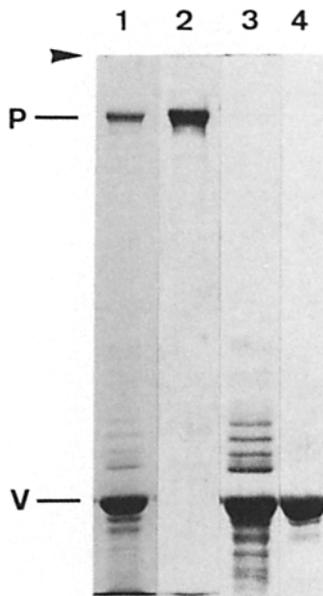


Figure 3. Electrophoresis of intermediate filament proteins from C6 cells. Lane 1, urea-solubilized intermediate filament preparation; lanes 2 and 3, plectin and vimentin fractions eluting from Sepharose CL-4B columns; lane 4, vimentin further purified on CM-Sepharose CL-6B columns. P, plectin; V, vimentin. Arrowhead, start of running gel.

ously observed in whole-mount (Schliwa and Van Blerkom, 1981) or embedded (Lawson, 1983) preparations of cytoskeletons were not labeled by anti-plectin (Fig. 2, white arrows) and neither were actin filaments (not shown). Control cells incubated with normal rabbit IgG and then with gold-labeled anti-rabbit IgG were unlabeled (data not shown). These results fully confirmed the *in situ* localization of plectin on whole-mount cytoskeletons.

In Vitro Interaction of Plectin with Intermediate Filaments

For *in vitro* recombination studies, plectin and the intermediate filament subunit protein vimentin were purified from 1% Triton X-100/0.6 M KCl-insoluble residues of rat glioma C6 cells. Both proteins were the major components of these residues (Fig. 3, lane 1), occurring at a mass ratio of 0.2 to 0.3 (plectin/vimentin) as determined by densitometric scanning of Coomassie Blue-stained polyacrylamide gels. Chromatography of the residues on molecular sieving columns after solubilization in 7 M urea yielded purified plectin preparations (Fig. 3, lane 2), which eluted shortly after the void volume. Fractions containing vimentin (Fig. 3, lane 3) were further purified on CM-Sepharose CL-6B columns, yielding the material shown in Fig. 3, lane 4.

Electron micrographs of uranyl acetate-stained filaments reconstituted from purified vimentin and plectin revealed a highly cross-linked network of 15–18-nm thick filaments (Fig. 4 a). In contrast to filaments polymerized from vimentin alone, which displayed smooth surfaces (Fig. 4 b), spherical particles of diameters ranging from 15 to 60 nm were found along the surface of the filaments (Fig. 4 a). A periodicity in the spatial arrangement of these globes along the filaments was not observed, but the particles were often located at crossing-over or branching sites of the filament network and appeared to form bridges and links between individual filaments (Fig. 4 a, arrowheads). The surface-attached structures were very similar in shape and size to those observed after negative staining of purified plectin alone (Fig. 4 c). Similar highly cross-linked networks were observed,

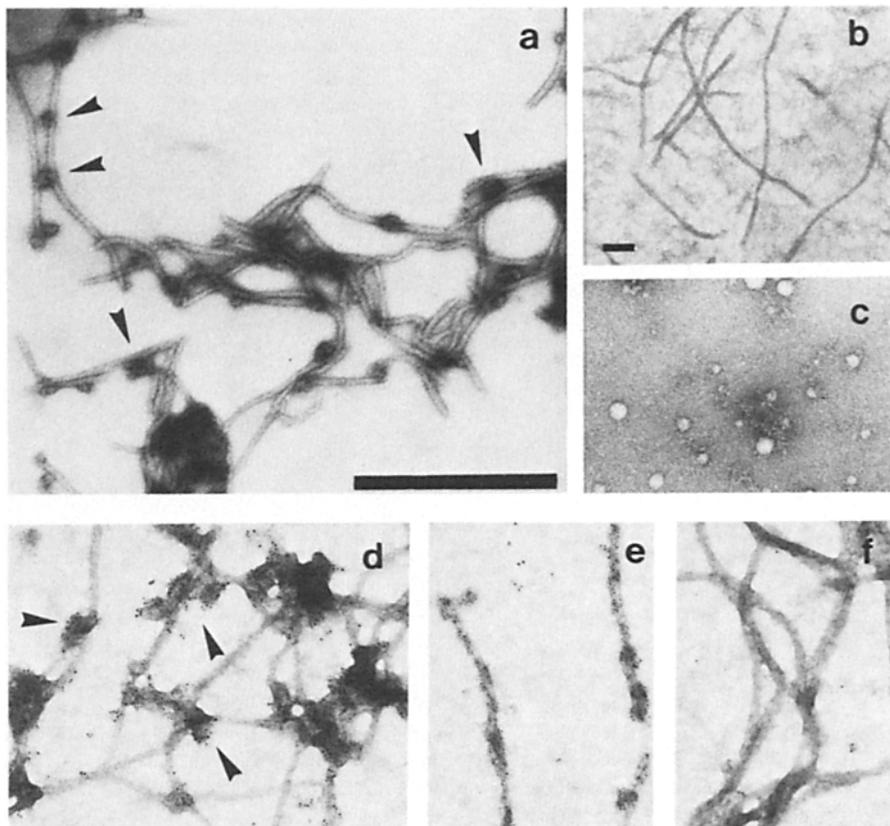


Figure 4. Uranyl acetate staining and immunoelectron microscopy of intermediate filaments reconstituted *in vitro* from purified vimentin and plectin. Samples of purified vimentin (0.15–0.17 mg/ml) (b, e, f) or plectin (0.08 mg/ml) (c), or mixtures of both (a, d) were incubated in buffer D for 45 min at 37°C and stained with uranyl acetate (a–c) or processed for immunoelectron microscopy (d–f) as described in the text. (d, f) Plectin antibodies; (e) vimentin antibodies. Arrowheads in a and d, plectin at junctions of filament network. Gold particles in d–f are better seen with the aid of a magnifying glass. Bars, 0.5 μm (a, d, e, f); 0.1 μm (b, c).

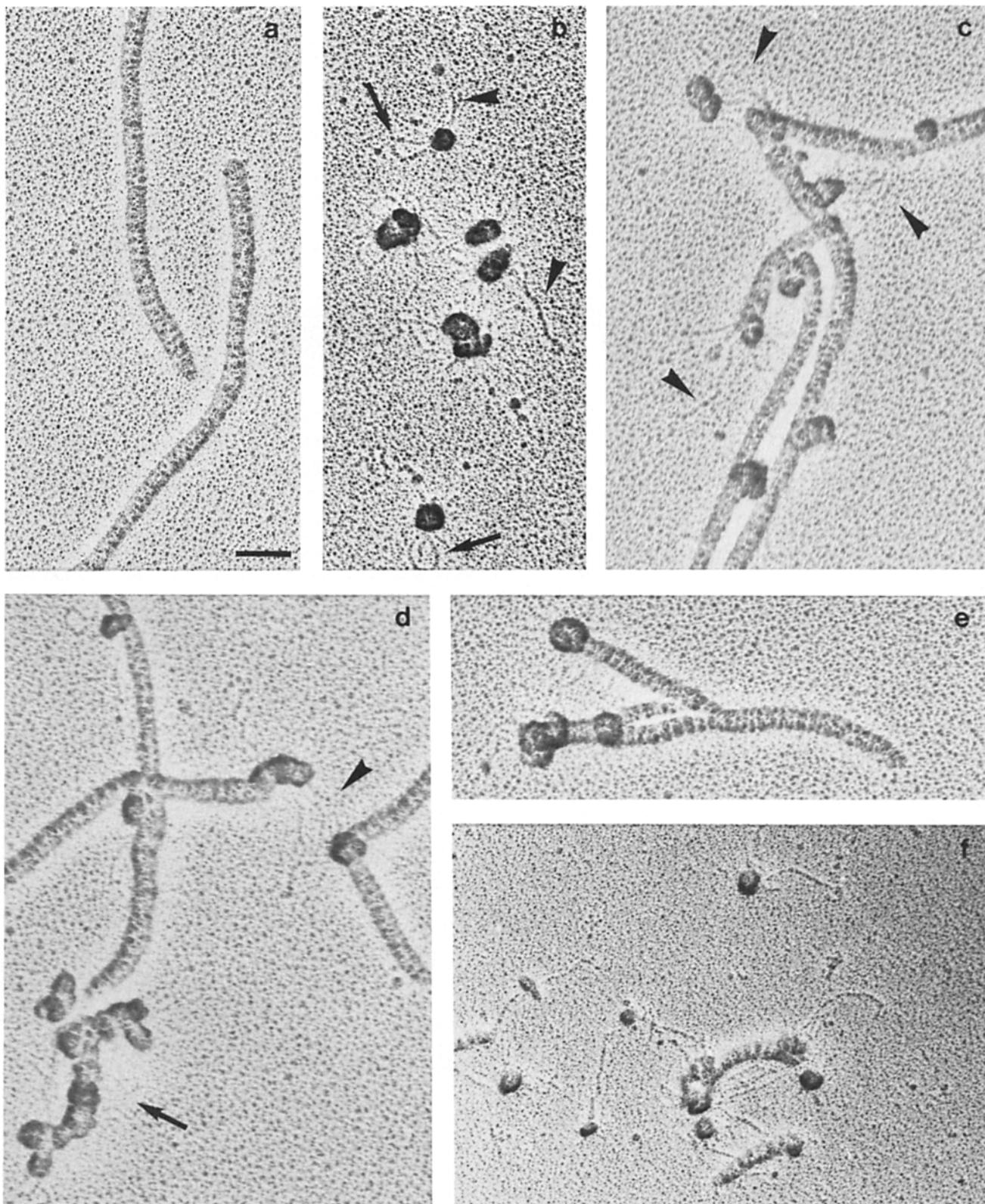


Figure 5. Rotary-shadowing electron microscopy of intermediate filaments reconstituted in vitro from purified vimentin and plectin. Purified vimentin (a), plectin (b), or mixtures of both (c-f) were incubated in buffer D for 45 min at 37°C and rotary shadowed as described in the text. Protein concentrations of vimentin were 0.15–0.20 mg/ml in a, c, and d or 0.05 mg/ml in e and f, those of plectin 0.05–0.08 mg/ml. Arrows in b, filamentous loops radiating from central core regions; arrowheads in b and c, filamentous protrusions; arrowhead in d, thin filaments cross-linking two intermediate filaments; arrowhead in d, aggregate of plectin oligomers attached to vimentin filament. Bar, 100 nm.

when preparations of unfractionated intermediate filaments (Fig. 3, lane 1) were solubilized and re-polymerized under the same conditions (data not shown).

To clearly demonstrate that the globular particles observed at the surface of reconstituted filaments consisted of plectin, immunoelectron microscopy was carried out using rabbit antibodies to plectin and gold-labeled secondary antibodies. On filaments reconstituted from vimentin and plectin, globular and amorphous structures, located mostly at branching points of the filament network, were densely decorated with gold particles (Fig. 4 *d*, *arrowheads*). As expected, filaments polymerized from vimentin alone were not decorated by antibodies to plectin (Fig. 4 *f*) but intensive labeling was observed using antibodies to vimentin (Fig. 4 *e*).

After rotary shadowing (Tyler and Branton, 1980), filaments polymerized from purified vimentin alone typically displayed a longitudinal periodicity of ~ 21 nm (Henderson et al., 1982; Milam and Erickson, 1982) (Fig. 5 *a*). Filaments reconstituted from mixtures of vimentin and plectin at concentrations of 0.15–0.20 mg/ml and 0.05–0.08 mg/ml, respectively, were irregularly decorated with globular structures of 30–60 nm in diameter (Fig. 5, *c* and *d*). In agreement with uranyl acetate-stained specimens (Fig. 4), many of the filament-attached structures were located at branching and crossover points, creating a highly cross-linked filament network (Fig. 5, *c* and *d*). Numerous thin filaments up to 200-nm long protruded from the globular structures (Fig. 5, *c* and *d*, *arrowheads*). When samples of purified plectin were incubated without vimentin under comparable conditions (Fig. 5 *b*), structures similar to the ones visualized at the surface of assembled filaments (Fig. 5 *c*) were observed. As reported previously (Foisner and Wiche, 1987), these structures represented oligomeric states of plectin molecules that are formed by head-to-head association of single dumbbell-shaped molecules in conditions of physiological ionic strength. The interaction between the globular head domains of plectin molecules within these oligomers occurred both intramolecularly, giving rise to loops (Fig. 5 *b*, *arrows*), and intermolecularly, generating globular core regions with filamentous protrusions, up to 200-nm long and terminated by small globes (Fig. 5 *b*, *arrowheads*). Clusters consisting of two or more oligomeric core regions interconnected by their filamentous protrusions were also observed. These structures were found associated with vimentin filaments as well. They were attached, often densely packed, to either individual filaments (Fig. 5 *d*, *arrow*), or as cross-bridges between two filaments (Fig. 5 *d*, *arrowhead*). Extensively cross-linked networks were also observed when plectin was added to preassembled vimentin filaments (data not shown), indicating that plectin bound to the surface of the filaments. Filaments assembled from mixtures of plectin and vimentin containing considerably lower concentrations of vimentin (0.05 mg/ml) were relatively short and often had plectin oligomers attached to their ends (Fig. 5, *e* and *f*). In addition, numerous plectin oligomers not attached to filaments, but mostly interconnected by head-to-head self-association, were observed (Fig. 5 *f*).

If plectin can indeed interlink vimentin filaments, as indicated by electron microscopy of uranyl acetate-stained as well as rotary-shadowed specimens, intermediate filaments assembled in the presence of plectin should sediment in the ultracentrifuge considerably faster than filaments assembled

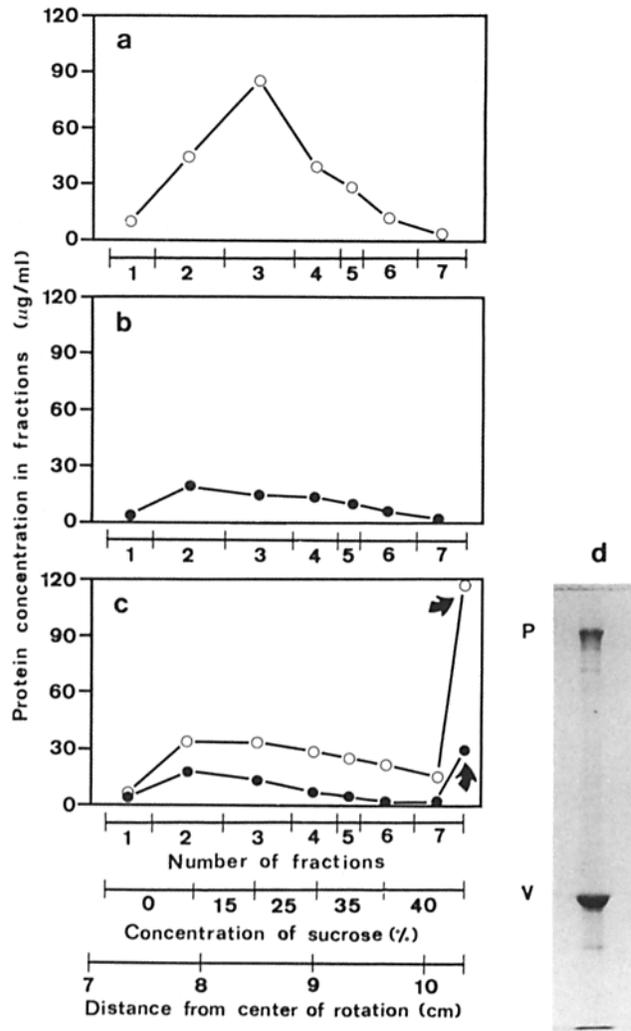


Figure 6. Cross-linking of vimentin filaments by plectin demonstrated by sucrose gradient centrifugation. Purified fractions of vimentin (*a*), plectin (*b*), and a mixture of both (*c*) were incubated as described in Fig. 4. 120 μ l of each sample were then centrifuged on discontinuous sucrose gradients (see text) and seven fractions collected from each gradient. Volumes of fractions were: 70 μ l (fractions 1 and 4), 100 μ l (2 and 3), 80 μ l (6), 30 μ l (5), and 50 μ l (7). Pellets were dissolved in 50- μ l electrophoresis sample buffer containing 8 M urea. Three times concentrated sample buffer was added to aliquots of fractions, and samples were analyzed by electrophoresis and densitometry of stained bands. The amount of protein in gel bands was estimated by running standards of plectin and vimentin of known concentration in parallel. Bars along abscissae indicate relative size of fractions (1, uppermost; 7, lowest fraction taken). (○) Vimentin; (●) plectin. (*d*) Electrophoresis of material in pellet of *c*. Arrows in *c* denote pellet fractions.

without plectin. To test this, three samples—vimentin alone, plectin alone, and a mixture of both—were incubated under assembly conditions and centrifuged through discontinuous sucrose gradients (Fig. 6). In these experiments, neither the vimentin nor the plectin polymers formed by themselves were large enough to yield pellets. Vimentin filaments displayed a rather symmetric size distribution with 46% of the total protein sedimenting with an approximate sedimentation coefficient, $s_{20,w}$, of 250S around the interface of the 15 and

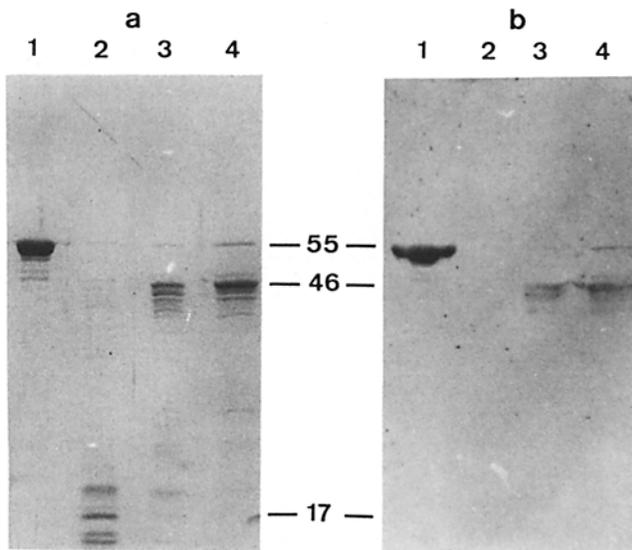


Figure 7. Solid-phase binding of plectin to vimentin fragments. Purified vimentin was incubated without (lanes 1) or with chymotrypsin in 5 mM borate, pH 8.5, at protease/vimentin mass ratios of 1:100 (lanes 2), 1:250 (lanes 3), and 1:500 (lanes 4) for 10 min at room temperature and fragments separated on SDS 12.5% polyacrylamide gels. (a) Coomassie Blue-stained gel; (b) Western blots incubated in sequence with plectin, affinity-purified antibodies to plectin and secondary antibodies linked to alkaline phosphatase as described in the text. Numbers, $M_r \cdot 10^{-3}$.

25% sucrose layers (Fig. 6 a). Plectin polymers were more heterogeneous in size with a relative peak at the interphase between the 0 and 15% sucrose layers corresponding to $\sim s_{20,w} = 150S$ (Fig. 6 b). In the pellet fraction of the mixture, 32 and 27% of the total vimentin and plectin were recovered, respectively (Fig. 6, c and d). The sedimented plectin/intermediate filament aggregates had Svedberg coefficients of at least $s_{20,w} = 2,300S$ and, thus, were approximately ten times larger than the bulk of structures formed after incubation of plectin and vimentin samples alone. The mass ratio of plectin to vimentin in the aggregates sedimenting to the bottom of the tube was 0.25, a proportion very similar to that usually found in crude (unfractionated) filament preparations (Fig. 3, lane 1). Similar proportions of both proteins were also determined in pellets obtained after solubilization of unfractionated filament preparations in urea followed by re-assembly and sucrose gradient centrifugation (data not shown). Moreover, nearly constant plectin/vimentin proportions were measured even after repeated rounds of solubilization and *in vitro* assembly of unfractionated intermediate filaments (Table I), indicating specificity of the plectin/vimentin interaction.

Interaction of Plectin with Solid Phase-bound Intermediate Filament Proteins

The subunit proteins of intermediate filaments characteristically contain rod domains consisting of coiled-coil polypeptide helices interrupted by nonhelical portions, and less ordered head and tail domains (Geisler et al., 1982; Steinert et al., 1985). To determine which of these domains were involved in the interaction with plectin, vimentin was subjected to limited chymotryptic digestion, yielding fragments

of known size and domain structure (Geisler et al., 1982; Nelson and Traub, 1983) (Fig. 7 a). Separated fragments transblotted to nitrocellulose sheets were overlaid with preparations of purified plectin and then incubated with affinity-purified anti-plectin and secondary antibodies linked to alkaline phosphatase. As shown in Fig. 7 b, plectin bound to undigested vimentin (lane 1) as well as to vimentin fragments between 38 and 45 kD (Fig. 7, a and b, lanes 3 and 4) containing vimentin's central rod domain (Geisler et al., 1982; Perides et al., 1987). It did not bind to groups of fragments of M_r 15,000–20,000 and 25,000–28,000 (Fig. 7, a and b, lanes 3), or to vimentin fragments that were obtained after digestion with relatively high concentrations of chymotrypsin and presumably lacked the rod domains (Fig. 7, a and b, lanes 2). Controls with antibodies to plectin without prior incubation of the blots with plectin samples revealed no stain. A specific interaction of plectin with vimentin was demonstrated also by overlays of transblotted and partially renatured plectin with ^{125}I -labeled C6 cell vimentin (data not shown).

Since the rod domain is a well-preserved structural element common to all intermediate filament proteins, it was of interest to examine whether plectin bound to intermediate filament subunit proteins other than vimentin. When crude neurofilament protein fractions from hog spinal cord (Fig. 8 a, lane 1) transblotted to nitrocellulose sheets were overlaid with purified plectin and the bound plectin was visualized with antibodies, four bands of M_r 300,000, 200,000, 68,000, and 50,000 were strongly stained; minor reactions occurred with components of apparent M_r 160,000 and 42,000 (Fig. 8 a, lane 2). Blots that were not overlaid with plectin, but only incubated with antibodies to plectin followed by detection with secondary antibody coupled to alkaline phosphatase, showed a strong reaction only at 300 kD (Fig. 8 a, lane 3), suggesting that plectin was present in these preparations. Thus, exogenous plectin apparently was reactive with all three of the neurofilament proteins, and with co-purifying glial fibrillary acidic protein (GFA). To confirm this, the binding of plectin to chromatographically fractionated GFA (Fig. 8 b) and neurofilament subunit proteins (Fig. 8, c and d) was assayed. The preparation of GFA proteins used (Fig. 8 b, lane 1) contained two protein bands, one of 50 kD and the other of 42 kD, that were immunoreactive with antibodies to GFA, as established by Western blotting experiments (data not shown). One of these bands (50 kD) represented intact GFA; the other was probably a degradation product containing the rod domain of GFA. As expected, plectin bound to both of these protein species (Fig. 8 b, lane 2). Strong binding was also observed (Fig. 8 c, lane 2) when fractions highly enriched in NF 200 (Fig. 8 c, lane 1) were probed. In fractions enriched in NF 68 and NF 160 (Fig. 8 d, lanes 1 and 1'), both proteins showed high affinity to plectin (Fig. 8 d, lanes 2 and 2'). Binding of plectin to any of the other proteins contained in these fractions, including tubulin, was not observed. When increasing amounts of the proteins were loaded on the gel, the amount of plectin bound to the neurofilament subunit proteins increased correspondingly (shown for two samples in Fig. 8 d). Finally, plectin's binding to human skin keratins was tested. In a keratin preparation consisting of cytokeratins 1, 5, 10, 11, and 14 (Moll et al., 1982) (Fig. 8 e, lane 1), plectin bound exclusively to keratins 10 and 11 (Fig. 8 e, lane 2). In a control experiment,

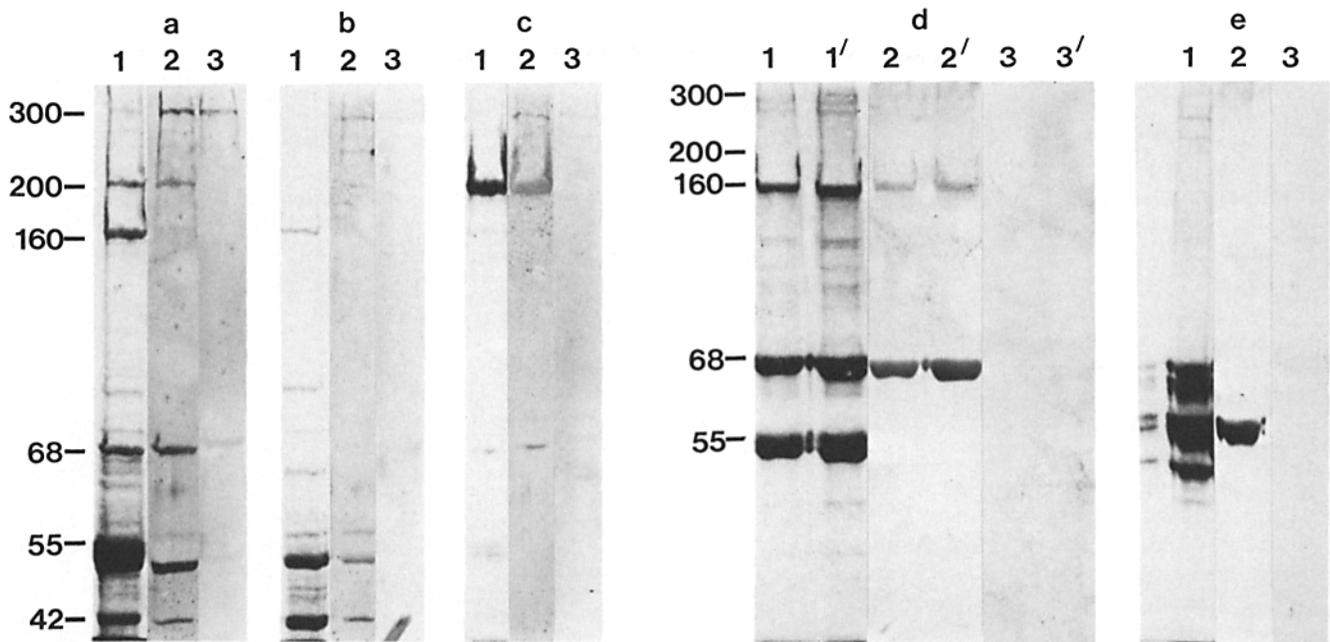


Figure 8. Solid-phase binding of plectin to intermediate filament subunit proteins. Intermediate filament proteins were prepared as described in the text. Coomassie Blue-stained gels (lanes 1) and Western blots (lanes 2 and 3) of various protein fractions are shown. Blots were overlaid with purified plectin (lanes 2) or buffer alone (lanes 3), followed by plectin antibodies and secondary antibodies linked to alkaline phosphatase; in *e* plectin antibodies preabsorbed with keratins were used. Lanes 1'-3' in *d*, amounts of proteins loaded on gels was about 2× higher compared to lanes 1-3. 7 and 12% polyacrylamide gels were used in *a-c*, and *d* and *e*, respectively. (*a*) Preparations of crude neurofilaments; (*b*) GFA proteins; (*c*) NF 200; (*d*) NF 68 and NF 160; (*e*) skin keratins. Numbers, $M_r \cdot 10^{-3}$.

the keratin-preabsorbed anti-plectin used in these experiments showed no reaction (Fig. 8 *e*, lane 3).

Discussion

Here we demonstrate the preferential localization of plectin at junction sites of intermediate filaments using electron microscopy of whole-mount and of quick-frozen, deep-etched cytoskeletons from cultured fibroblast cells. The implication from these data, namely that plectin could be responsible for intermediate filament network formation by cross-linking vimentin filaments, was supported by ultracentrifugation and electron microscope analysis of intermediate filaments reconstituted *in vitro* from purified protein components. Rotary shadowing experiments of *in vitro* reconstituted filaments revealed that the plectin bridges consisted of complex structures assembled by self-association of dumbbell-shaped plectin molecules at their globular end domains. Furthermore, on the basis of solid-phase binding assays, it was concluded that plectin structures interacted with the structurally conserved rod domain of vimentin and bound to several other intermediate filament proteins.

It could be argued that the *in situ* immunolocalization of plectin structures primarily at intermediate filament cross-over sites, rather than along the surface of the filaments, is a procedural artifact. For several reasons, however, we believe that this is unlikely. First, electron microscopy of intermediate filaments, polymerized *in vitro* from mixtures of purified vimentin and plectin, and whole-mount cytoskeletons showed similar ultrastructures; a periodic arrangement of plectin structures along the surfaces of filaments, as has been reported for true filament-associated proteins, such as

synemin (Granger and Lazarides, 1982), was not observed even when polymerization was performed with excess plectin. Second, plectin/vimentin mass ratios remained nearly constant even after repeated assembly and disassembly of filaments, indicating a defined number of binding sites. Third, the mass ratio of both proteins was similar in preparations of intermediate filaments polymerized *in vitro* and in unfractionated cytoskeletons. Finally, it has been shown previously by immunofluorescence microscopy and biochemical analysis of detergent-extracted and non-extracted cells that only a fraction of the total cellular plectin is intermediate filament-associated, while the rest seems to be either associated with other more soluble structures or involved in a network of its own.

In vitro reconstitution experiments, filamentous networks looked alike morphologically, regardless of whether plectin and vimentin were mixed before filament polymerization or plectin was added to pre-formed filaments. We conclude, therefore, that plectin interacts with sites exposed on the surface of filaments without affecting the structure of the filaments themselves. How a plectin binding site is specified on the filament surface remains unclear. One possibility is that binding of plectin to vimentin occurs at filament branching or interacting sites which are somehow defined within the filament structure. Alternatively, plectin's binding to intermediate filaments could be induced or stabilized by contact with more than one filament. Both models would explain a role of plectin in the formation of cross-bridges and generation of networks.

Filament-associated plectin structures, visualized after rotary shadowing of *in vitro* reconstituted intermediate filaments, possessed a well defined structure consisting of a

globular core region of 30–60-nm diam with numerous filamentous loops and spike-like protrusions. Since similar structures were recently shown to represent oligomers of tetrameric plectin molecules (Foisner and Wiche, 1987), it can be concluded that oligomers of plectin, rather than single molecules, are forming the bridges that interlink intermediate filaments in vitro.

Solid-phase binding assays of plectin to proteolytically cleaved vimentin suggested that plectin binds to the rod domain of vimentin. This α -helical domain, well conserved among intermediate filament proteins, is generally flanked and terminated by less structured and more variable head and tail domains (Geisler et al., 1982, 1985b; Geisler and Weber, 1982; Steinert et al., 1985). In the assembled filament, the central rod domains are presumably associated laterally (Ip et al., 1985), in units of four (Geisler et al., 1985a), forming the filament backbone. The amino-terminal head domain, being essential for filament polymerization (Traub and Vorgias, 1983; Kaufmann et al., 1985; Perides et al., 1987), is tightly incorporated into the filament structure, while the carboxy-terminal tail domain with no influence on polymerization is probably located at the surface of the filaments. Thus, the carboxy-terminal tail domain of vimentin, and possible of other intermediate filament proteins, may not bind to plectin, but could still be involved in regulating the interaction of plectin with intermediate filaments; a negative control mechanism would not be unlikely, considering the lack of plectin binding along most of the filament's surface.

Since the central rod domain of intermediate filament subunits is structurally highly preserved, plectin's binding to intermediate filament proteins other than vimentin was expected and presumably also occurs at the helical, and not the variable, molecular domains of these proteins. Hence the interaction of proteins or other cellular components with intermediate filaments via the specific head and tail regions of subunit proteins, as suggested by Kaufmann et al. (1985), is probably restricted to genuine intermediate filament-associated proteins that presumably bind to one subtype of intermediate filaments only.

In vitro cross-linking of BHK-21 intermediate filaments by the protein IFAP 300 has previously been suggested by Lieska et al. (1985). In their studies, immuno- and negative staining electron microscopy were used, both giving relatively low resolution on the ultrastructural level compared to the rotary-shadowing technique. Our studies confirm and significantly extend the initial analysis of IFAP 300, since we have shown that plectin and IFAP 300 are homologous in structure (Herrmann and Wiche, 1987). Furthermore, the establishment of plectin's function as a cross-linking element of vimentin filaments extends this homology to the functional level.

Plectin seems to fulfill a number of criteria expected for a cytoplasmic cross-linking element of mammalian cells. It is ubiquitous as well as abundant and occurs at cellular sites that are of importance for the organization of the cytoskeleton, including anchorage sites of intermediate filaments and microfilaments. It interlinks vimentin filaments and possibly other types of intermediate filaments. It has been found in association with microtubules polymerized in vitro from cultured cells (Koszka et al., 1985), and microtubule-associated proteins of high molecular weight have been identified as interacting components (Herrmann and Wiche, 1987). Also,

spectrin-like molecules, major components of the sub-plasma membrane protein skeleton, have been characterized as in vitro interaction partners of plectin (Herrmann and Wiche, 1987; Weitzer and Wiche, 1987). Furthermore, the oligomeric and polymeric aggregates of self-assembled plectin in solution (Foisner and Wiche, 1987) could be part of cytoplasmic network arrays that spatially stabilize and cross-link various cellular components. It will be of interest, therefore, to further characterize the various binding domains of the plectin molecule and to study the mechanisms that control the various interactions of this protein.

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