Monoclonal Antibody Mapping of Structural and Functional Plectin Epitopes

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Abstract. To map structural and functional epitopes of the cytomatrix protein plectin, a set of mAbs was prepared by immunization of mice. Using immunoblot analysis of plectin fragments obtained after limited digestion with various proteases, two groups of mAbs were distinguished. The epitopes of one group (1) were located on a 130-kD terminal segment of the plectin 300-kD polypeptide chain, whereas those of the other group (2) bound within a 40kD segment confined to a central domain of the polypeptide chain. Domains containing the epitopes of group 2 mAbs were shown to include in vitro phosphorylation sites for kinase A, whereas kinase C phosphorylation sites were found on the same terminal segment that contained group 1 mAb epitopes. Rotary shadowing EM of mAb (Fab fragment) -decorated plectin molecules at various states of aggregation, ranging from characteristic dumbbell-shaped single molecules to highly complex multimeric structures, revealed that the epitopes of group 1 as well as those of group 2 mAbs were located on plectin's roughly 200-nm long rod domain interlinking its two globular end domains. Epitopes of group 1 mAbs were localized within a region near the center of the rod, those of group 2 in more peripheral sections near the globular end domains. Solid-phase binding assays carried out in the presence of Fab fragments of mAbs demonstrated an interference of certain group 1 mAbs in the interactions of plectin with vimentin and lamin B. On the other hand, plectin's self-interaction was inhibited mainly by Fab fragments with epitopes in the peripheral rod domain (group 2 mAbs). Together, these results suggested that the molecular binding sites of plectin for vimentin and lamin B, as well as the phosphorylation sites for kinase C, were confined to a defined central section of plectin's rod domain. In addition, they suggest an involvement of peripheral rod sections in plectin self-association.

PLECTIN (mol wt = 300,000) was originally identified as a major protein component of intermediate filament preparations obtained by high salt/1% Triton X-100 extraction of rat glioma C6 cells (Pytela and Wiche, 1980; Wiche et al., 1982). Immunofluorescence microscopy showed plectin to be present over a wide range of tissues and cell types (for review see Wiche, 1989). In line with its widespread distribution, plectin has been found to interact with a variety of proteins, including vimentin (Herrmann and Wiche, 1987; Weitzer and Wiche, 1987; Foisner et al., 1988*a*,*b*), microtubule-associated proteins 1 and 2 (Herrmann and Wiche, 1987), spectrin-type polypeptides (Herrmann and Wiche, 1987; Weitzer and Wiche, 1987), glial fibrillary acidic protein, all three neurofilament polypeptides, certain skin keratins (Foisner et al., 1988*a*) and lamin B (Foisner, R., P. Traub, and G. Wiche, manuscript submitted for publication). Plectin has also a strong tendency for self-association (Foisner and Wiche, 1987). Such a variety of binding partners suggests that plectin has a major role in cross-linking intermediate filaments (IFs)[1] with each other as well as in anchoring IFs at the plasma membrane and at the nuclear envelope. Furthermore, plectin may be part of the signal transduction mechanism involving kinases A and C, because in vitro as well as in vivo phosphorylation of the protein by those kinases differentially affected its binding affinities to vimentin and lamin B (Foisner, R., P. Traub, and G. Wiche, manuscript submitted for publication).

Molecular structure studies demonstrated that soluble plectin existed predominantly as a symmetrical dumbbell-shaped molecule, comprised of four identical 300-kD chains (Foisner and Wiche, 1987). The precise arrangement of the four constituent polypeptide chains within this structure, however, is still unknown. There is evidence suggesting that plectin's self-association occurs predominantly with its globular end domains, whereas its association with vimentin occurs with the rod domain (Foisner et al., 1988*b*).

To map structural as well as functional epitopes of plectin molecules with more precision, a set of mAbs to plectin was raised. It is shown here that five of these mAbs bind to dis-

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1. *Abbreviation used in this paper*: IF, intermediate filament.

tinct epitopes located along plectin's rod domain. Furthermore, based on inhibitory effects exerted by some of these mAbs in in vitro binding assays, the binding sites of two IF proteins, vimentin and lamin B, were assigned to a domain near the middle of plectin's rod.

Materials and Methods

Preparation of Proteins

Cell lysates and Triton X-100/high salt soluble and insoluble cell fractions were prepared from rat glioma C6 cells as described by Herrmann and Wiche (1983). Preparations of IFs, containing plectin, vimentin, and lamin B, and purified samples of plectin and vimentin were obtained from the same cell line essentially as reported previously (Foisner et al., 1988*a*). Limited proteolysis of plectin was done as by Foisner et al. (1988*b*), using elastase and *Staphylococcus aureus* protease V8 at plectin–protease mass ratios of 100:1 (the conditions of digestions are indicated in figures). For ^{125}I radiolabeling, proteins were desalted into 10 mM borate, pH 8.5, and incubated with [^{125}I]iodine and iodogen (Furtner and Wiche, 1987). For in vitro phosphorylation, plectin samples (0.1 mg/ml) were incubated in 20 mM Pipes, pH 7.4, 0.5 mM DTT, 10 mM $MgCl_2$ and 50 μM [^{32}P]ATP (100 mCi/mmol) containing either 30 μg/ml catalytic subunit of cAMP-dependent kinase (Sigma Chemie GmBH, Munich, FRG), or 5 μg/ml protein kinase C (prepared from porcine brain; 2.0 μmol P_i/mg·min sp act), 200 μg/ml L-α-phosphatidyl L-serine, 5 μg/ml diolein, and 500 μM Ca^{2+}, for 1 h at 37°C.

Isolation of mAbs and Fab Fragments

3-mo-old Balb/c mice were immunized with samples of purified plectin in 2 mM Tris-HCl, pH, 8.0 as will be described in detail elsewhere. Fusions of spleen cells with myelomas (P3-X63 Ag8.653, courtesy of Dr. Otto Majdic), cloning, and hybridoma growth were carried out following established procedures (Galfre and Milstein, 1981). Ascites production and purification of mAbs using a Mono Q column (Pharmacia, Uppsala, Sweden) was done as described by Zola (1988). Fab fragments were obtained by papain digestion of purified mAbs (Parham, 1986) and purified on a Mono Q column. Titers of antibody solutions were routinely measured by ELISA (Zola, 1988) using purified plectin as the antigen.

Gels, Immunoblotting, and Overlays

SDS-PAGE (Laemmli, 1970) was used for both transblots (5.0, 7.5, or 10% acrylamide) and analysis of IgG and IgG fragments (8.75% acrylamide). For nonreducing gels, sample buffer without 2-mercaptoethanol was used. Transblotting of proteins to nitrocellulose was performed using the LKB (LKB-Pharmacia, Uppsala, Sweden) Novablot System according to instructions. Immunoblot analyses were done using the Protoblot Immunoscreening system (Promega Biotec, Madison, WI) with the following primary antibody dilutions: rabbit polyclonal antisera to plectin, 1:1,000; hybridoma supernatants (anti-plectin antibodies), 1:5; purified Fab fragments, 1:10. Overlays of transblots with ^{125}I-labeled plectin were done in 10 mM imidazole, pH 7.0, 170 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM 2-mercaptoethanol as described (Herrmann and Wiche, 1987), except that plectin was preincubated with or without Fab fragments (mass ratios of plectin to Fab fragments: 1:1) for 1 h at 37°C. Binding was measured by scanning of autoradiograms or by gamma counting of bands cut from nitrocellulose strips.

Binding Assays Using Dot Blotting

100-μl samples of purified plectin (0.1 mg/ml of 10 mM sodium borate, pH 8.5) and, as a control, BSA were spotted onto nitrocellulose using a dot-blot apparatus (Bio-Rad Laboratories, Richmond, CA. Stripes of nitrocellulose were then overlaid with radioactively labeled vimentin as described by Herrmann and Wiche (1987). For inhibition tests, strips were incubated in overlay buffer solution supplemented with comparable amounts of ascites fluid mAbs (final dilutions 1:20–1:100) for 1 h before the overlay and overlaid with ^{125}I-vimentin in the presence of mAbs. Binding was quantitated by excising spots and counting the radioactivity.

Electron Microscopy

Samples of purified plectin (50 μg/ml) were transferred into 10 mM borate, pH 8.0, using PD-10 desalting columns (Pharmacia, Uppsala, Sweden), and incubated with purified Fab fragments (5–10 μg/ml) for 2 h at 4°C. Samples were then prepared for rotary-shadowing EM according to the procedure of Tyler and Branton (1980) with slight modifications as described elsewhere (Foisner and Wiche, 1987).

Results

mAb Epitope Mapping by Immunoblot Analysis of Partially Digested Plectin

A series of mAbs was prepared using purified rat glioma C6 cell plectin as immunogen. When whole lysates (L) and Triton X-100/high salt-soluble (S) and -insoluble (P) cell fractions of C6 cells were analyzed by immunoblotting using supernatants of various hybridoma cultures, specific reactions with a 300-kD polypeptide present in these fractions were observed in all cases (Fig. 1). The same proteins were recognized by rabbit polyclonal antiserum to plectin (Fig. 1).

To examine whether these mAbs were directed against different epitopes, plectin samples were partially digested with two different proteases, transblotted to nitrocellulose, and incubated with each of five selected mAbs. The incubation of purified plectin samples (Fig. 2 *A*, lanes *1* and *2*) with elastase for 15 min at room temperature yielded as major fragments two pairs of closely spaced bands of apparent mol wts near 200,000 and 170,000, and a single band of mol wt = 130,000; two bands at 90,000 and 70,000 were minor components (Fig. 2 *B*, lane *1*). By comparing the staining patterns, two groups of mAbs were readily distinguished. One group, comprising mAbs 7A8, 1D8, and 10F6, stained all of the high mol wt fragments including those of mol wts 200,000, 170,000, and 130,000, but did not show an immunoreaction with the low-mol wt bands of 90,000 and 70,000 (Fig. 2 *B*, lanes *2–4*); however, one of them, 1D8, showed a relatively strong reaction with a fragment of mol wt <70,000 (Fig. 2 *B*, lane *3*). The second group (mAbs 6B8 and 1A2) recognized the high-mol wt bands at 200,000 and 170,000, both low-mol wt bands of 90,000 and 70,000, but not the major 130,000 fragment (Fig. 2 *B*, lanes *5* and *6*).

Digestion of purified plectin with protease V8 for 60 min at room temperature generated several well defined high-mol wt bands between 250,000 and 150,000 (Fig. 2 *C*, lane *1*). The most prominent immunoreactive bands were fragments of apparent mol wt 170,000 stained by both group 2 mAbs (6B8 and 1A2), and a fragment of 150kD that reacted only with mAb 1A2 (Fig. 2 *C*, lanes *5* and *6*). None of these fragments showed immunoreactivity with the group 1 mAbs (Fig. 2 *C*, lanes *2–4*).

Both experiments indicated that the epitopes of group 1 and group 2 mAbs were located within different domains of the plectin molecule. The epitopes of mAbs within each group were apparently situated close together, although subtle differences in the staining patterns and in the intensity of the bands suggested that all of the mAbs recognized distinct epitopes. With respect to group 1 mAbs, longer digestion of plectin with elastase and trypsin revealed that the epitopes of mAbs 7A8 and 1D8 were located closer to each other than they were to the mAb 10F6 epitope (data not shown).

Elastase seems to cleave plectin molecules sequentially

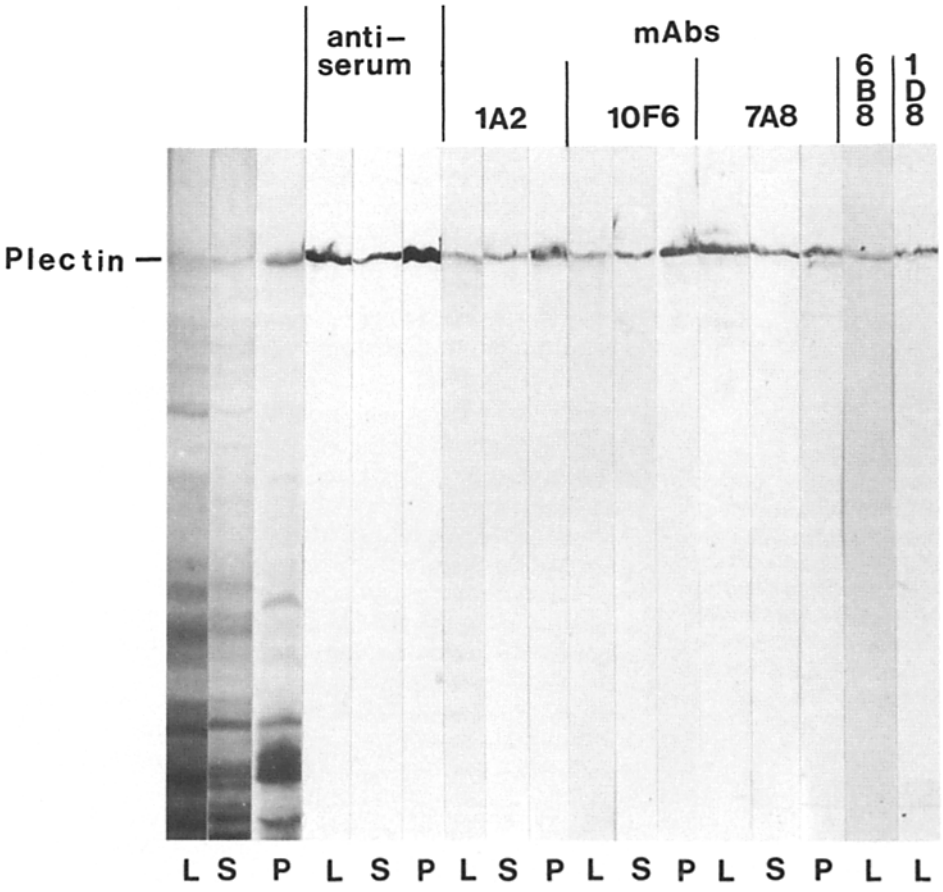

Figure 1. Specificity of mAbs demonstrated by immunoblotting. Total rat glioma C6 cell lysates (*L*) or Triton X-100/high salt soluble (*S*) and insoluble (*P*) cell fractions were subjected to immunoblotting using antiserum to plectin or hybridoma supernatants of mAbs, as indicated. Coomassie brilliant blue staining is shown in outermost three left lanes.

starting at the outermost globular ends (Foisner et al., 1988*b*). Thus, the 200-kD fragments which were immunoreactive with both group 1 and group 2 mAbs were probably end-terminal digestion intermediates of the 300-kD polypeptide chain, eventually yielding a 170-kD fragment, which was digested further to yield a 130-kD polypeptide (Fig. 3, *top*). Since the 170-kD fragment was still immunoreactive with all of the mAbs, whereas the 130-kD fragment reacted

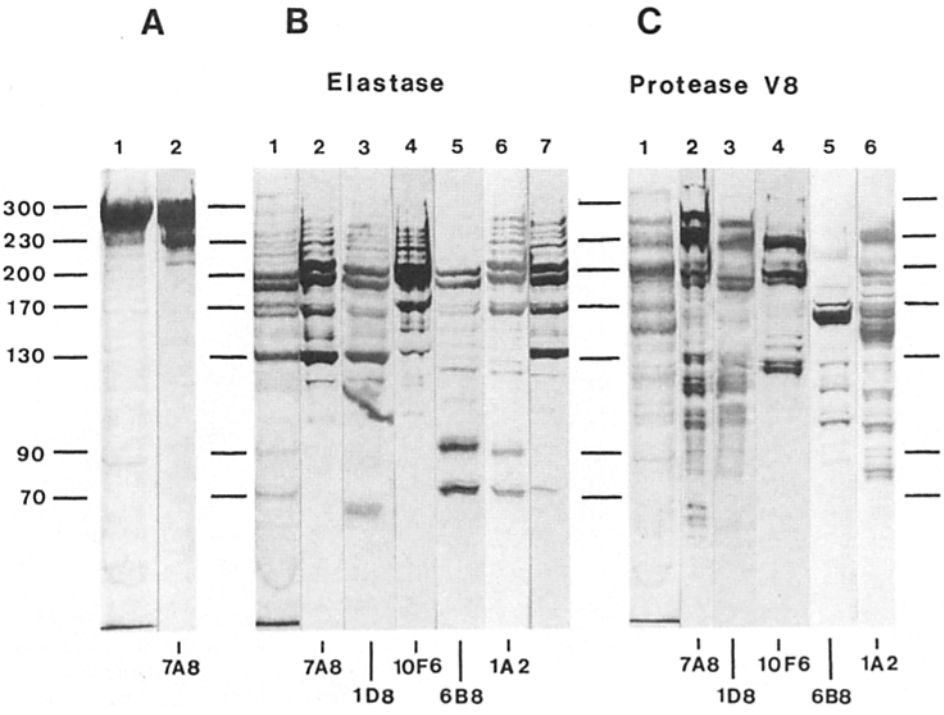

Figure 2. Epitope mapping by immunoblot analyses of proteolytic plectin fragments. Purified samples of plectin (*A*) were partially digested with elastase for 15 min at room temperature (*B*), or with *Staphylococcus aureus* protease V8 for 1 h (*C*). Lanes *1*, Coomassie brilliant blue staining; all other lanes, immunoblots using hybridoma supernatants of mAbs as indicated (lanes *2–6* in *B* and *C*) or polyclonal antiserum to plectin (lane *7* in *B*). Numbers, mol wt $\times 10^{-3}$.

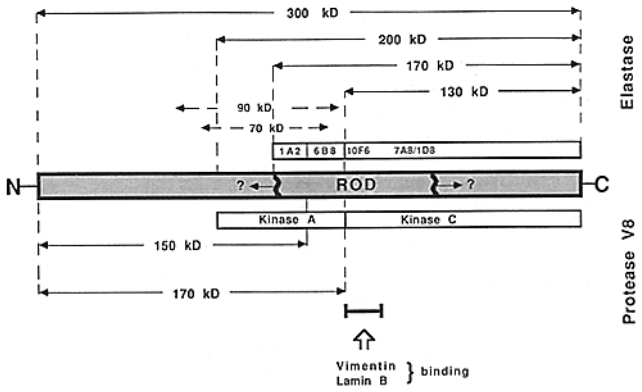

Figure 3. Map of epitopes and in vitro phosphorylation sites deduced from partial proteolysis of plectin. The plectin polypeptide chain is represented by the thick bar; amino (N)- and carboxy (C)-terminal ends as well as a portion of the rod forming sequences are indicated. The sizes and proposed positions of fragments derived by proteolysis with elastase (*top*) and protease V8 (*bottom*) are indicated by numbers and two-pointed arrows. Thin bars mark regions containing mAb epitopes (*top*) or kinase A and kinase C phosphorylation sites (*bottom*). Proposed vimentin and lamin B binding sites are indicated by a line bar. Note that the partial rod domain indicated is proportional in length to the epitope bearing polypeptide sequence; extentions (?) of the rod forming domain to either side are unknown.

only with group 1 mAbs (1D8, 7A8, 10F6), it seems likely that the epitopes of group 2 mAbs (1A2 and 6B8) resided within a 40-kD region of the 170-kD fragment, that did not overlap with the 130-kD fragment (Fig. 3). Furthermore, it was concluded that the group 2 mAb (1A2 and 6B8)-positive fragments of mol wt 90,000 and 70,000 included the immunoreactive parts of the 40-kD fragment without overlapping with the group 1 mAb epitopes contained in the 130-kD fragment.

The 170- and 150-kD protease V8-derived fragments, which were immunoreactive only with group 2 mAbs, must have been located on a section of the chain that did not overlap with the elastase-derived 130-kD fragment (Fig. 3, *bottom*). The observation that 6B8 showed no reaction with the 150-kD fragment suggested that the binding site of this mAb was closer to the 130-kD elastase derived fragment than that of mAb 1A2.

From the combined results it may be concluded that the epitopes of all mAbs tested were located within an end-terminal 170-kD fragment of plectin's polypeptide chain, and that their sequence was 1D8/7A8, 10F6, 6B8, 1A2. Furthermore, immunoblot analysis of recombinant polypeptides derived from cDNA clones (data to be published elsewhere) revealed that this sequence was oriented such that 1D8/7A8 epitopes were proximal to plectin's COOH terminus.

Localization of In Vitro Phosphorylation Sites on Plectin Molecules

Plectin has been shown to be a major substrate of cAMP-dependent kinase A as well as of Ca^{2+}/phospholipid dependent kinase C in vitro and in vivo (Herrmann and Wiche, 1983, 1987; Foisner, R., B. Feldman, and G. Wiche, manuscript submitted for publication). However, the locations of the phosphorylation sites within the plectin molecule are still unknown. To assess which of the plectin fragments containing specific mAb epitopes included phosphorylation sites, purified plectin was radiolabeled by phosphorylation in vitro using either kinase A or kinase C. Subsequent proteolytic digestion yielded fragments of known immunoreactivity (Fig. 2). Autoradiography of kinase A phosphorylated samples revealed strong labeling of the major elastase-derived fragments of mol wts near 200,000 and 170,000, but no labeling of the 130-kD fragment (Fig. 4 *A*, lanes *2* and *2'*). In contrast, fragments of kinase C-phosphorylated samples showed labeling of all three high mol wt fragments (Fig. 4 *B*, lanes *2* and *2'*). Since those labeling patterns coincided with the staining patterns of group 2 and group 1 mAbs, respectively, the data suggested that phosphorylation sites of kinase A were located near the epitopes of mAbs 6B8 and 1A2, while those of kinase C were located within the 130-kD fragment immunoreactive with mAbs 10F6, 7A8, and 1D8 (Fig. 3). This was confirmed by autoradiography of protease V8-derived fragments, showing radiolabeling of group 2

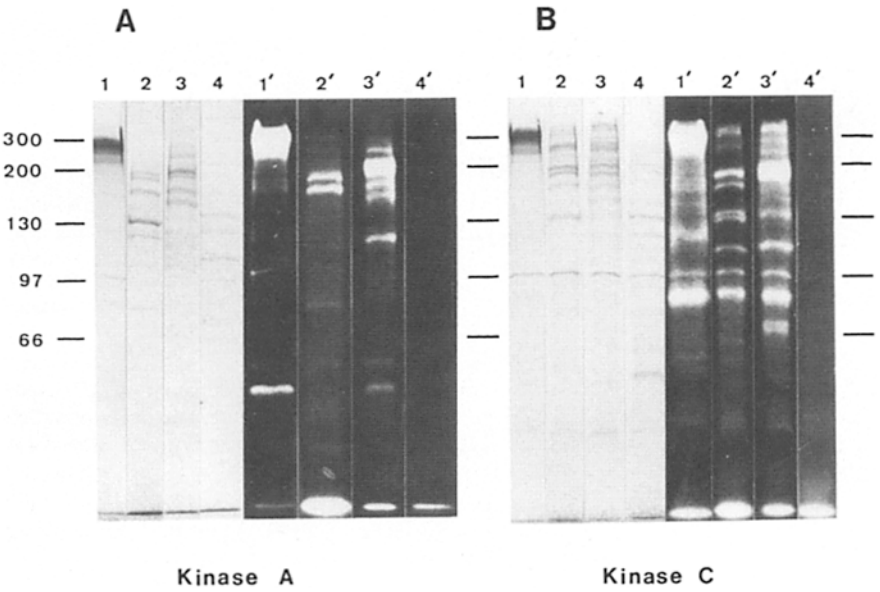

Figure 4. Localization of kinase A and kinase C phosphorylation sites on proteolytic plectin fragments. Purified samples of plectin were phosphorylated with kinase A (*A*) or kinase C (*B*) and afterwards digested with elastase for 15 min at room temperature (lanes *2* and *2'*), or with protease V8 for 60 min at room temperature (lanes *3* and *3'*), or with trypsin for 15 min at 37°C (lanes *4* and *4'*). Lanes *1–4*, Coomassie brilliant blue staining; lanes *1'–4'*, autoradiograms. Undigested samples are shown in lanes *1*. Numbers, mol wt × 10^{-3}.

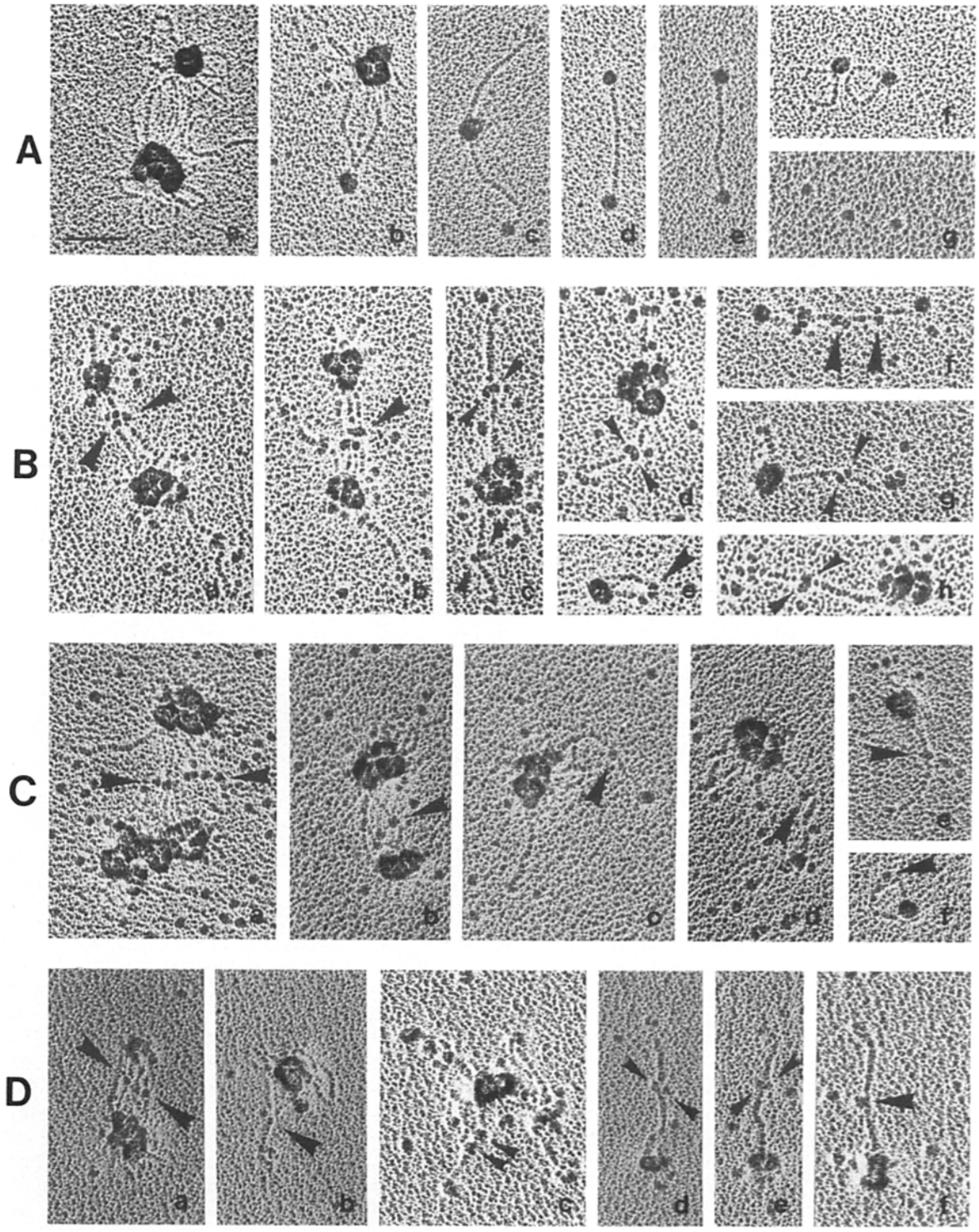

Figure 5. Rotary shadowing electron microscopy of various plectin structures. *A*, *a–f*, unlabeled plectin structures; *g*, purified Fab fragments (mAb 7A8). *B–D*, plectin structures decorated with Fab fragments of mAbs 1D8 (*B*), 7A8 (*C*), or 10F6 (*D*). Arrowheads denote Fab molecules bound to plectin's rod domain; for details see text. Bar, 100 nm.

mAb-positive fragments near 200,000, 170,000, and 150,000 in the case of kinase A phosphorylated samples (Fig. 4 *A*, lanes *3* and *3'*), but no labeling of the 170,000 and 150,000 group 2 mAb-positive fragments in the case of kinase C-treated samples (Fig. 4 *B*, lanes *3* and *3'*).

Ultrastructural Mapping of mAb Epitopes on Plectin Molecules

For the ultrastructural localization of mAb binding sites on plectin molecules, samples of purified plectin were incubated with intact mAbs or Fab fragments and viewed in the electron microscope after rotary shadowing. Initial experiments, using intact mAbs, indicated that the epitopes of the mAbs assayed were located on plectin's rod, rather than on its globular domains (Foisner et al., 1988*b*, and data not shown). However, due to extensive clustering of plectin molecules, an accurate mapping of epitopes along the rod domain was hardly possible. When Fab fragments rather than

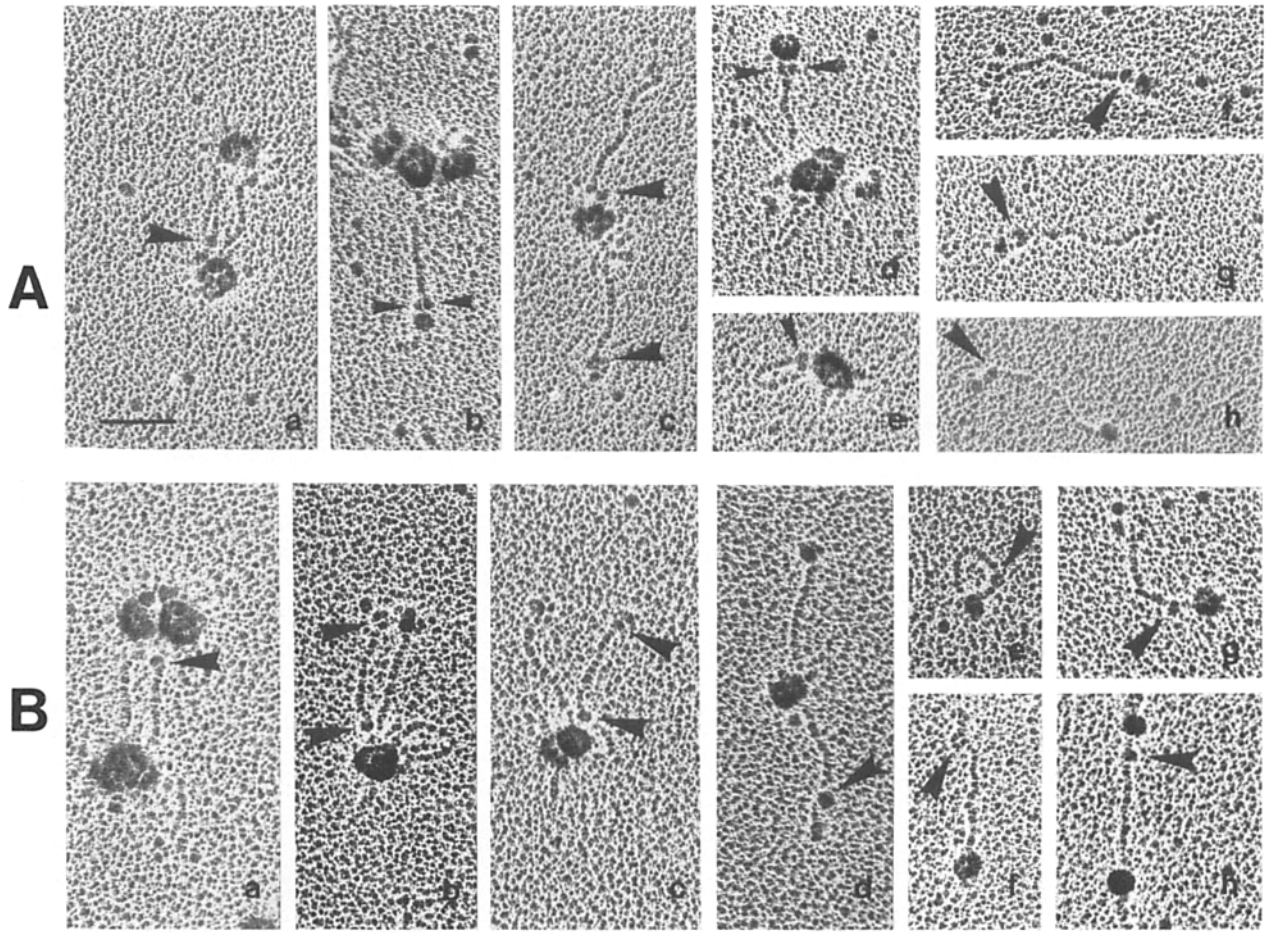

Figure 6. Rotary shadowing EM of plectin structures labeled with Fab fragments of mAbs 1A2 (*A*) and 6B8 (*B*). Arrowheads denote Fab molecules bound to plectin's rod domain; for details see text. Bar, 100 nm.

intact mAbs were used, antibody-decorated plectin structures were better defined allowing a more precise localization of epitopes. Fig. 5 *A*, *a–f*, depicts unlabeled plectin structures representative of those observed after incubation with Fab fragments. Because of plectin's strong tendency to self-associate under the conditions of antibody incubation, the most frequently observed plectin structures consisted of several plectin molecules clustered via plectin's globular domains and interlinked by their filamentous rod domains (Fig. 5 *A*, *a* and *b*). In addition, oligomeric plectin structures with a single core region of clustered globes (Fig. 5 *A*, *c*) or single dumbbell-shaped (Fig. 5 *A*, *d* and *e*) or loop-shaped (Fig. 5 *A*, *f*) molecules were observed, although less frequently. Fab fragments, visualized as small globes, 10–15 nm in diameter (Fig. 5 *A*, *g*), were easily detected on the rod domains of labeled plectin structures (Fig. 5, *B–D* and Fig. 6, *A* and *B*). The binding of Fab fragments to plectin's globular domains, which would be seen less clearly on plectin polymers, but should be visible on single plectin structures due to an enlargement of plectin's globes, was not detected. Confirming the data of immunoblot analyses, two major groups of mAbs could be distinguished: those of one group (1D8, 7A8, and 10F6) bound near the middle of plectin's rod domain (Fig. 5, *B–D*), whereas those of the other (1A2, 6B8) bound to the rod closer to the globular domains (Fig. 6, *A–B*). These locations were visualized on rods interlinking clustered globular domains of complex plectin structures (Figs. 5, *B–D*, and 6, *A* and *B*; *arrowheads*, in *a* and *b*) or more clearly on plectin structures freely extending from clusters (*arrowheads* in *c* and *d*) or on single elongated and looped plectin molecules (*arrowheads* in *e–h*). Frequently, two Fab molecules were observed that were bound to the rod domain at laterally opposite sites (*arrowheads* in Figs. 5 *B*, *c*, *d*, and *f–h*; 5 *D*, *d* and *e*; and 6 *A*, *b* and *d*), or were labeling the rod at two sites of equal distances from its middle (arrowheads in Figs. 5 *B*, *f*; *D*, *c*; and 6 *A*, *c*; *B*, *b* and *c*). Such symmetrical labeling patterns were in agreement with a twofold symmetry proposed for the molecular arrangement of the four 300-kD polypeptide chains constituting the dumbbell-shaped plectin molecule (Foisner and Wiche, 1987). However, a simultaneous symmetric labeling of plectin molecules by four Fab molecules (Fig. 5 *B*, *f*), as may be expected for a tetrameric molecule, was observed only rarely, probably due to shearing forces disrupting such structures during sample preparation.

Measurements of the center to center distances between Fab molecules and the globular ends of plectin structures on micrographs of at least 50 specimens per antibody yielded histograms showing the relative amount of a given Fab fragment that bound within 5 nm long sections of plectin's rod (Fig. 7). Fab fragments of mAbs 1D8, 7A8, and 10F6 bound within 20–25-nm-long stretches of plectin's rod positioned at distances of between 65 and 90 nm from the center of the

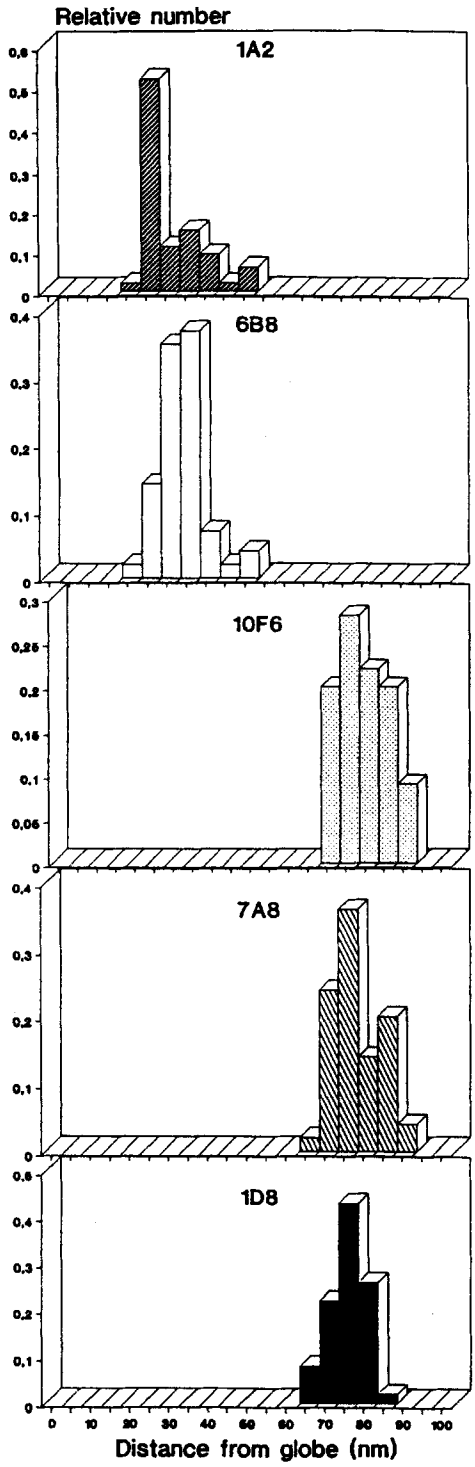

Figure 7. Histograms showing the localization of mAb epitopes along plectin's rod domain. Bars indicate the relative amount of a given Fab fragment localized within 5-nm-long stretches of plectin's rod domain. Center to center distances between Fab fragments and peripheral globes of plectin molecules were measured. Values are the result of at least 50 measurements in each case.

globes and 10–35 nm from the middle of the rod. The binding sites of mAbs 1A2 and 6B8 were located in regions separated 20–40 nm from each globe.

Since all group 1 and group 2 mAbs were localized on the rod section of plectin molecules, the polypeptide sequence specifying these epitopes must be part of this domain. Given that plectin's rod domain, which is most likely a double-stranded α-helical structure, has a length of ~180 nm and a proposed mol wt of 480,000 (Foisner and Wiche, 1987), the roughly 65 nm distance measured between the outermost binding sites of group 1 and group 2 mAbs would correspond to a mol wt of ~80,000–90,000 per polypeptide chain, or 160,000–180,000 of double-stranded rod. According to the immunoblot analysis (Fig. 2), this sequence should be confined to an internal stretch of the polypeptide chain, as indicated in Fig. 3. The exact position with regard to the entire rod forming domain remains to be established.

Plectin Interactions Inhibited by mAbs

Plectin has been shown to bind to several different interaction partners, such as a number of intermediate filament subunit proteins, fodrin, MAPs 1 and 2, and plectin itself. It is unknown whether plectin possesses unique binding sites for these proteins or whether common binding sites exist. In an attempt to map plectin's binding sites for the two intermediate filament proteins vimentin and lamin B and its self-interaction site(s), ^{125}I-radiolabeled plectin was incubated with Fab fragments of one of several mAbs before its overlay onto transblotted, nitrocellulose-immobilized crude IF preparations known to contain the proteins to be tested (data not shown). Antibodies that bound to plectin domains involved in any of these interactions were expected to exhibit inhibitory effects. Since intact mAbs, due to their bivalency, enhanced rather than inhibited plectin–plectin interactions, only Fab fragments were used in these experiments.

Both the plectin–vimentin and the plectin–lamin B interaction were strongly inhibited (>50%) by mAb 10F6, as quantitatively analyzed by scanning autoradiograms or by counting excised bands (Table I). This suggested that the binding sites of plectin for vimentin and lamin B were similar, if not identical and located close to the binding site of 10F6 (Fig. 3). Fab fragments of mAb 6B8 inhibited plectin–plectin interactions strongly but had no detectable effect on plectin–vimentin and plectin–lamin B binding, suggesting that these two types of interactions take place on distinct binding sites of the plectin molecule. Moreover, considering the locations of mAb 10F6 and 6B8 epitopes on plectin molecules (Fig. 7), these data suggested that plectin-vimentin as well as plectin-lamin B interactions involved more central parts

Table I. Inhibition of Plectin Interactions by Fab Fragments of Various mAbs

Binding partner	mAbs				
	1A2	6B8	10F6	7A8	1D8
Vimentin	19	9	67	28	31
Lamin B	8	1	63	47	22
Plectin	10	70	11	12	30

Transblotted samples of crude IF preparations containing plectin, lamin B, and vimentin were overlaid with ^{125}I-labeled plectin (preincubated with or without Fab fragments) as described in the text. Percent inhibition of binding was calculated by comparing amounts of bound plectin in the absence (100%) and presence of Fab fragments. Numbers (%) represent means of three experiments.

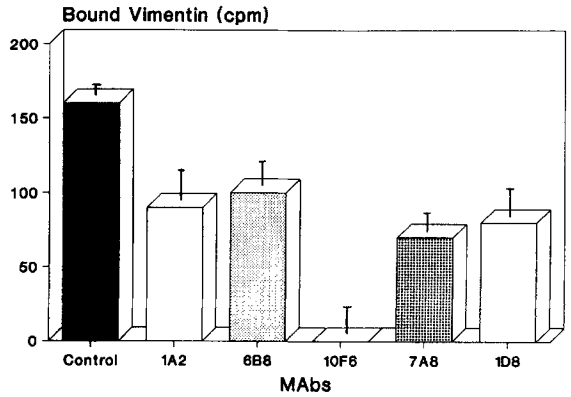

Figure 8. Inhibition of plectin-vimentin binding by mAbs. Native samples of purified plectin spotted onto nitrocellulose were incubated with various ascites fluids and then overlaid with ^{125}I-labeled vimentin as described in the text. Binding values were obtained by counting the radioactivity of excised dots and subtraction of blanks where BSA was substituted for plectin. Bars represent mean values of three experiments; maximal deviations were as indicated.

of plectin's rod domain (Fig. 3, *bottom*) than plectin-plectin interactions.

To avoid possible pitfalls of solid phase binding assays due to partial or complete denaturation of transblotted samples, dot-blot analyses using native protein samples were performed as an alternative approach to study plectin–vimentin interactions. Native samples of plectin spotted onto nitrocellulose were blocked with mAbs and then overlaid with radiolabeled vimentin in the presence of mAbs. Alternatively, native vimentin spotted to nitrocellulose was overlaid with radiolabeled plectin in the presence of Fab fragments. Both types of experiments again revealed strongest inhibition of plectin–vimentin interaction by mAb 10F6 (Fig. 8; and data not shown). Thus, these results were fully consistent with the data obtained by solid phase overlays using partly denatured binding partners.

Discussion

In this study, the epitopes of a set of mAbs were mapped on plectin molecules using Western blot analyses of partial digests of the protein and EM of rotary shadowed plectin structures after decoration with mAbs. This analysis, in combination with Western blot type and dot-blot solid-phase binding assays performed in the presence of various mAbs, led to the assignment of vimentin and lamin B binding sites to a central segment of the rod domain of the plectin molecule.

A number of criteria indicated that all mAbs described here recognized different epitopes. According to their immunoreactivity with proteolytic fragments of plectin, two groups of mAbs were distinguished, with members of each group showing similar, though not identical staining patterns. Furthermore, based on the fragmentation patterns observed upon digestion of intact plectin samples with elastase and protease V8, as well as of a recombinant plectin polypeptide with endogenous bacterial proteases (data to be published elsewhere), the sequence of these mAb epitopes was determined as 7A8/1D8-10F6-6B8-1A2, starting from the carboxy terminus. Group 2 mAbs 1A2 and 6B8 showed very similar staining patterns of proteolytic fragments implying that their epitopes were in close vicinity. This was confirmed by the assignment of both epitopes to a relatively short portion of the 300 kD polypeptide chain. In the case of group 1 mAbs, the epitope localization was somewhat less accurate, as the smallest well defined fragment identified to contain all epitopes still had a size of 130 kD. However, taking into account the distance between group 1 and group 2 mAb epitopes as revealed at the ultrastructural level, it is quite likely that the binding sites of group 1 mAbs were located within the NH_2-terminal half of the 130-kD fragment (Fig. 3). In addition, there was evidence that the epitope of mAb 10F6 was located closer to the amino terminal end of the 130-kD fragment than the epitopes of the other members of this group (7A8 and 1D8). Regarding mAbs 7A8 and 1D8, differences in their staining patterns were not pronounced enough to establish the relative orientation of their epitopes.

The ultrastructural epitope mapping using rotary shadowed specimens showed that all of the mAbs tested bound to plectin's rod domain. For a more precise localization of the mAb binding sites, it was crucial to use monovalent Fab fragments rather than intact mAbs to avoid antibody-dependent clustering of plectin structures. However, even then the precision of single epitope mapping along the rod was limited by plectin's tendency to form complex structures through self-association under the conditions of Fab decoration. Due to this, precise length measurements along the rods of complex aggregates were rather difficult. However, based on statistical values obtained from significant numbers of measured specimens in various states of aggregation, it was possible to clearly define epitope bearing stretches of the rod. By this type of analysis, it was possible to distinguish the same two groups of mAbs that had been previously found by immunostaining of proteolytically fragmented plectin. The members of one group (mAbs 10F6, 1D8, and 7A8) each bound within relatively short regions (20–25 nm) over one stretch situated close to the middle of the rod. Well separated from this site were partially overlapping epitopes of the other group (mAbs 1A2 and 6B8), which were found within a 25-nm-long stretch near the globular end domains of plectin molecules. The relative positions of epitopes within both stretches, however, could not be determined unambiguously, partly due to the limited resolution of the rotary shadowing technique.

Based on chemical cross-linking and hydrodynamic data, plectin molecules have been suggested to be formed by a symmetrical association of four identical polypeptide chains (Foisner and Wiche, 1987). Binding of two mAbs at laterally opposite sides of plectin's rod, as observed in a significant number of cases, confirmed the proposed double-stranded organization of plectin's rod, as well as the lateral symmetry of the molecule. The longitudinal symmetry of plectin molecules, on the other hand, was indicated by the following observations: (*a*) occasionally two, or in a rare number of cases two pairs of Fab fragments, were observed that bound symmetrically to the center of the rod. (*b*) Oligomeric plectin structures consisting of large clustered core regions and of single radiating plectin molecules were labeled at the extending rod domains either near to the core structure, or close to the peripheral globes. Therefore, the rod sections of com-

plex plectin molecules, like those of single ones, apparently had two equivalent mAb binding sites, located symmetrically to the rod center.

Since it was found that plectin's interactions with vimentin and lamin B were both strongly inhibited by mAb 10F6, using two types of assays, we consider it likely that the rod domain containing the epitope of this mAb included also the binding sites for these proteins, or at least is involved in some other way in these interactions. Consistent with this is that mAb 7A8, whose epitope was found to overlap with that of mAb 10F6, was also significantly inhibiting both interactions. Thus, plectin's interactions with vimentin, lamin B, and possibly other IF proteins probably involve a central portion of plectin's rod domain. The plectin–plectin interaction on the other hand, which was primarily inhibited by mAb 6B8, whose epitope was mapped closely to plectin's globular domains, may involve the rod section adjacent to the globes.

Regarding the mechanism of mAb interference in the in vitro interactions of plectin with various proteins, a number of possibilities exist. We consider it likely that the inhibition of plectin–vimentin and plectin–lamin B interactions by mAbs binding to more central portions of plectin's rod domain is due to a direct competition between antibodies and binding partners for a similar binding site, or to the steric hindrance of a protein binding site by an antibody bound to a closely associated epitope. In this context it is of interest that the binding affinities of plectin to vimentin and to itself have been found to be on the order of those reported for antibody–antigen interactions (unpublished data). Thus, the complete blockage of plectin's interactions with these proteins could hardly be expected except at a high excess of mAbs compared to binding proteins, which was not the case under the conditions of the experiments reported here. Contrary to plectin–IF protein interaction, the plectin–plectin interaction appears to occur mainly via plectin's globular domains, as was suggested previously by results obtained mainly by rotary shadowing EM (Foisner and Wiche, 1987; Foisner et al., 1988*b*). Thus, the inhibition of plectin–plectin interaction by an mAb (6B8) that binds to the rod section proximal to the globes may be due to indirect effects, such as conformational alterations of protein binding sites located on the globular domains. A direct involvement of this rod section in plectin–plectin interaction cannot be ruled out, however, because of the limited resolution inherent in rotary shadowing EM.

The isolation of a repertoire of mAbs specific for different functional epitopes of plectin opens up several possibilities for future research. In particular, these mAbs should be useful for the purification and further biochemical characterization of proteolytically derived molecular domains of plectin of defined functions. Furthermore, they should be of great value for studies of the structure–function relationship of plectin molecules on the nucleic acid level. Finally, this new set of anti-plectin mAbs provides a more reproducible, and hence more reliable, tool for histological studies, compared to the originally prepared polyclonal antisera.

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