

Expression of Plectin Mutant cDNA in Cultured Cells Indicates a Role of COOH-terminal Domain in Intermediate Filament Association

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Abstract. Plectin is an intermediate filament (IF) binding protein of exceptionally large size. Its molecular structure, revealed by EM and predicted by its sequence, indicates an NH₂-terminal globular domain, a long rodlike central domain, and a globular COOH-terminal domain containing six highly homologous repeat regions. To examine the role of the various domains in mediating plectin's interaction with IFs, we have constructed rat cDNAs encoding truncated plectin mutants under the control of the SV-40 promoter. Mutant proteins expressed in mammalian COS and PtK2 cells could be distinguished from endogenous wild type plectin by virtue of a short carboxy-terminal antigenic peptide (P tag). As shown by conventional and confocal immunofluorescence microscopy, the transient expression of plectin mutants containing all six or the last four of the repeat regions of the COOH-terminus, or the COOH-terminus and the rod, associated with IF networks of both the vimentin and

the cytokeratin type and eventually caused their collapse into perinuclear aggregates. Similar effects were observed upon expression of a protein encoded by a full length cDNA construct. Microtubules and microfilaments were unaffected. Unexpectedly, mutants containing the rod without any of the COOH-terminal repeats, accumulated almost exclusively within the nuclei of cells. When the rod was extended by the first one and a half of the COOH-terminal repeats, mutant proteins showed a partial cytoplasmic distribution, although association with intermediate filaments was not observed. Nuclear and diffuse cytoplasmic distribution was also observed upon expression of the NH₂-terminal domain without rod. These results indicate that sequences located roughly within the last two thirds of the globular COOH-terminus are indispensable for association of plectin with intermediate filaments in living cells.

PLECTIN is an abundant intermediate filament (IF)¹ binding protein found in a wide variety of different cell types. Based mainly on biochemical and immunolocalization studies we proposed that plectin plays a role in cross-linking of IF, the interlinking of IF with microtubules and microfilaments, and the anchoring of IF to the plasma membrane and the nuclear membrane (for reviews see Wiche, 1989; Foisner and Wiche, 1991). Furthermore, plectin's ability to form networks through self-association may convey structural stability to cytoplasmic areas which are devoid of cytoskeletal filaments.

Cloning and sequencing of rat plectin cDNA indicated a polypeptide of 4,140 amino acid residues (466,481 D) whose sequence was consistent with a three-domain structural model in which a long central rod domain, having mainly an

alpha-helical coiled coil conformation, is flanked by globular NH₂- and COOH-terminal domains (Wiche et al., 1991). The rod domain has five subregions ≈200-residues-long characterized by the presence of a 10.4-residue charge repeat that may be involved in the association between plectin molecules. The globular COOH-terminal domain has a prominent sixfold tandem repeat, with each repeat having a strongly conserved central region based on nine-tandem repeats of a 19-residue motif. The plectin sequence has several marked similarities to that of desmoplakin (Green et al., 1990; Virata et al., 1992) and bullous pemphigoid antigen (Sawamura et al., 1991). Similar to plectin, these proteins possess coiled coil rod domains with similar repeats of charged amino acids. However, the rod domains of desmoplakin and bullous pemphigoid antigen are shorter than that of plectin. Perhaps more significantly, desmoplakin and bullous pemphigoid antigen both have COOH-terminal globular domains with three and two, respectively, tandem repeats homologous to the six found in plectin.

1. *Abbreviations used in this paper:* IF, intermediate filament; MAP, microtubule-associated protein; NLS, nuclear localization signal.

Plectin's interaction partners identified on the molecular level include a nuclear (lamin B) and various cytoplasmic IF proteins (vimentin, desmin, the three neurofilament proteins, glial fibrillary acidic protein, and certain cytokeratins), microtubule-associated proteins (MAP 1 and MAP 2), spectrin-type proteins (α -spectrin and fodrin), and plectin itself. The *in vitro* interactions of plectin with the IF proteins vimentin and lamin B, have been shown to be differentially regulated by phosphorylation with protein kinases A and C (Foisner et al., 1991a). Moreover, it has been shown that the rearrangement of plectin structures observed during mitosis correlates with the phosphorylation of plectin by a p34^{cdc2}-related kinase and with a reduced binding affinity of the protein to vimentin (unpublished results). Binding inhibition assays using mAbs to plectin with epitopes residing on the rod domain of the molecule provided evidence for the involvement of this domain in plectin-vimentin and plectin-lamin B interactions, but the actual binding sites have not yet been characterized; neither has the possibility been ruled out that other molecular domains of plectin, such as the globular domains flanking the rod are involved in these interactions as well. Aside from some ultrastructural evidence pointing to an involvement of the globular end-terminal domains in the self-interaction of plectin, the location of binding sites of other known interaction partners of plectin, is largely unknown.

To study questions regarding the domain organization of plectin and to map functional epitopes more precisely, we have begun making cDNA constructs that enable the transient expression of plectin mutant proteins in cultured cells. The major early SV-40 promoter and enhancer were used to express mutant plectin cDNAs in two transfected cell lines, monkey COS and rat kangaroo PtK2, both of which are simple epithelial in origin and contain dense networks of vimentin filaments in addition to cytokeratin filaments. The studies reported here address the questions of which molecular domain of plectin is essential for IF association and whether IF organization is affected by the expression of plectin mutants. The data reported indicate that the COOH-terminal repeats, rather than the rod domain, are responsible for cytoplasmic localization. Unexpectedly, tailless plectin mutants localized in the nucleus.

Materials and Methods

Cell Culture

Monkey kidney COS cells and rat kangaroo PtK2 cells, obtained from the American Type Culture Collection (Rockville, MD), were cultivated in DME supplemented with 10% FCS.

Construction of Expression Plasmids

PCR mutagenesis was used to create a Sall site at the 5' end of the human keratin K14 cDNA contained in a modified version of the SV-40 promoter/enhancer-based mammalian expression vector pJK14-P (Albers and Fuchs, 1987), which lacks the EcoRI site downstream of the P tag (see Fig. 1 A). The new plasmid (pGW1) was digested with Sall to excise the keratin sequences and a synthetic Sall/EcoRI adaptor was then ligated to the vector, to generate plasmid pGW15 (Fig. 1 B). The adaptor-encoded EcoRI restriction site was used for the insertion of different EcoRI plectin constructs. The adaptor was designed so that it provided a start ATG in-frame with the coding sequence of clone C5 (see Fig. 1 in Wiche et al., 1991), and linked the P tag, also in-frame, to the 3' end of plectin.

Constructions of Plectin Mutants

The plectin coordinates given in the different constructs are based on the numbering used in Wiche et al. (1991).

pGW5. The EcoRI inserts of plectin clones C1 and C2 (see Fig. 1 in Wiche et al., 1991) were joined via a unique SacII site, to generate pBB1 (see Fig. 2). Subsequently, a Sall/BamHI adaptor designed in a way similar to the Sall/EcoRI adaptor described above was ligated to a 5.5-kb BamHI fragment derived from pBB1, spanning nucleotides 2431 to 8101. Excess adaptors were cleaved by digestion with Sall and removed by spin column chromatography on Sephacryl S-400 (Pharmacia, Uppsala, Sweden). The purified fragment was ligated to Sall-digested pGW1, yielding construct pGW5 (see Figs. 1 D and 2).

pGW10. A small part of the coding region of plectin clone C5 (see Fig. 1 in Wiche et al., 1991), which spans the last four repeats of plectin's carboxy-terminal domain and includes \approx 1 kb of untranslated sequences, was amplified by PCR using primers designed to remove the stop codon and create an EcoRI restriction site downstream from the coding sequence. The amplified sequence was ligated back into the original pUC/C5 plasmid and an EcoRI fragment of this plasmid was inserted into the EcoRI site of pGW15, to create plasmid pGW10 (see Figs. 1 C and 2). This plasmid contained the plectin coding sequence between bases 8396 and 12420.

pGW16. Because of the length of this construct and, in particular, the redundancy of the sequence in plectin's COOH-terminal repeat region, several PCR, and subcloning steps (not described) were necessary for the construction of this plasmid. In brief, an EcoRI restriction site, followed by an ATG translation start, was engineered by PCR in clone C1 (Wiche et al., 1991), at a position corresponding to the 5' end of plectin's carboxy-terminal domain. Another PCR was designed to reconstitute a truncated BstXI site at the 5' end of clone C5. These modified versions of clones 1 and 5 were joined in two sequential steps using conventional cloning techniques. The final plasmid (pGW16) extended from bases 6640 to 12420 (see Fig. 2).

pGW17. To construct this plasmid, pGW5 and pGW16 were joined via a unique BstBI site in their overlapping cDNA regions and a unique NdeI site in the vector. The resulting plasmid (pGW17) contained plectin's coding sequence between bases 2430 and 12420.

pAD11. This plasmid, containing the NH₂-terminal bases 1 to 1746 of plectin, was engineered by cancellation of the NsiI site at base 466, to render it unique, and elimination of the internal EcoRI site at base 484. Both sites were mutated by PCR without altering the amino acid sequence of plectin.

pAD14. Plasmid pAD11 was first combined with plasmid pWE88 (bases 483–8101; unpublished data) and then subcloned into pGW15. The resulting construct was finally combined with the COOH-terminal region of pGW17, to produce a full-length construct connected to P tag.

pAD15. The EcoRI site at the 3' end of pGW17 was exchanged with the natural stop codon from clone C5 (Wiche et al., 1991) via a unique SnaBI site. The sequence of this construct contains 848 bp of 3' untranslated sequence after the natural stop codon.

pAD17. This construct was made in two steps. First, the 4127-bp NcoI fragment of plectin extending from bases 2562–6687 was subcloned into a pUC derivative containing an NcoI restriction site in the polylinker. Then, the NheI-EcoRI fragment of this construct was subcloned into pGW17 resulting in a construct extending from bases 2430–6690 and encoding an extra sequence of nine amino acids preceding the P tag (see Fig. 1 E).

DNA Sequencing

The accuracy of all the constructs was confirmed by dideoxy sequencing of the double-stranded plasmid DNA (Sequenase kit, Version 2.0, United States Biochemical Corp., Cleveland, OH).

PCR

PCR was performed using standard conditions (Saiki et al., 1988). Products obtained after 30 cycles of amplification in a DNA Thermal Cycler (Perkin Elmer Corp., Norwalk, CT) were extracted in phenol, ethanol precipitated, and digested with the enzymes required for subcloning in the expression vectors.

DNA Transfections

DNA was transfected into COS and PtK2 cells attached to glass slides in 3-cm Petri dishes using the calcium phosphate precipitation method (Graham and Van der Erb, 1973). Cells were usually fixed 63 h posttransfection.

Immunofluorescence Microscopy

For immunofluorescent labeling, cells were usually fixed in freshly made 3.5% paraformaldehyde in 50 mM Pipes (pH 7.2), 1 mM EGTA, 1 mM MgCl₂ (PEM), supplemented with 0.1% Triton X-100, for 30–40 min at room temperature. In a few cases (visualization of endogenous plectin by mAb staining) cells were fixed in methanol at –20°C. After fixation, cells were washed in PBS and then incubated in 3% BSA (in PBS) at room temperature for 1 h to prevent nonspecific staining. Cells were then incubated with primary antibodies at room temperature for 40–60 min, extensively rinsed in PBS, and exposed to secondary antibodies at room temperature for 40 min. After repeated rinses in PBS and a dip into distilled water specimens were mounted in Moviol (Hoechst, Frankfurt, Germany). The following immunoreagents were used: rabbit antisera to substance P (Wako BioProducts, Richmond, VA), plectin (Wiche and Baker, 1982), lamin B (kindly provided by Dr. Traub, Max-Planck-Institute of Cell Biology Ladenburg/Heidelberg); mAbs to plectin (10F6; see Foisner et al., 1991b), human vimentin (Dacopatts, Denmark), human cytokeratins (AE1/AE3; Boehringer-Mannheim, Germany), β -tubulin (Amersham International, Buckinghamshire, England); Texas red–labeled goat anti–rabbit IgG and FITC-labeled goat anti–mouse IgG (Amersham International). Specimens were viewed in a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and photographs were taken using Ilford ASA 125 black and white film or Fuji DX 400 color slide film. Confocal microscopy was performed with a laser confocal system (MRC-600; Bio-Rad Laboratories, Cambridge, MA) equipped with an argon laser. Series of focal sections were combined in simple projection to obtain cell images. Transmitted light images were enhanced by subtraction of reference out-of-focus image and linear scaling.

EM

Cells grown on coverslips were extracted with 0.1% Triton X-100 in PEM for 30 s, and then fixed with a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde in PEM for 10–20 min. Specimens were then washed and processed for immunolabeling as described above. As secondary antibodies we used anti-rabbit IgG conjugated to 15-nm colloidal gold (Amersham International). Washed cells were postfixed in 2% glutaraldehyde, 50 mM phosphate, pH 6.8, followed by 0.1% OsO₄ in the same buffer. Embedding and sectioning was done following standard procedures.

SDS-PAGE and Immunoblotting

Transfected and control cells were collected from 3 cm Petri dishes and boiled in electrophoresis sample buffer (200 μ l/dish) for 5 min. Before loading on the gel, samples were centrifuged in a table top centrifuge for 5 min. Electrophoresis was performed using 5% polyacrylamide gels according to Laemmli (1970). Electrophoretically separated proteins were stained with Servablue-G (Serva, Heidelberg, Germany). For immunoblotting, separated proteins were transferred to nitrocellulose paper using a semi-dry transfer unit (Bio-Rad Laboratories), at 20 V for 1 h. After transfer, the membranes were washed in PBS and blocked by incubation in PBS containing 3% BSA for 40 min. Membranes then were incubated (1 h) in rabbit anti–substance P serum diluted 1:1000, or 10F6 hybridoma supernatant diluted 1:30, washed two times for 5 min with PBS and incubated with the appropriate secondary antibodies conjugated with alkaline phosphatase (Promega Biotec, Madison, WI). After final washes with four changes of PBS during 40–60 min, alkaline phosphatase activity was revealed using nitro blue tetrasolium as substrate.

Results

Epitope Tagging of Mutant Plectin

There were three major considerations in designing our vector to express mutant plectin in cultured cells. First, in light of the ubiquitous and abundant occurrence of plectin in cultured cells, it was desirable that the expression of mutant proteins took place at levels high enough for the proteins to compete with, and possibly override, the functions of the endogenous wild type plectin. Second, to visually follow the

expression of mutant plectin transfected into plectin-expressing cells by immunofluorescence microscopy, the constructs had to be tagged with sequences encoding an immunoreactive marker. Third, since a series of partial plectin clones encoding various domains of the molecule were already available with terminal EcoRI restriction sites (Wiche et al., 1991), the vector should preferentially have a corresponding cloning site.

Plasmid pJK14-P, an expression plasmid previously used for high level expression of wild type and mutant human keratin K14 in a variety of different cell lines (Albers and Fuchs, 1987, 1989; McCormick et al., 1991) fulfilled two of these requirements: (a) expression is driven by the SV-40 early promoter and enhancer, enabling high level expression particularly in T-antigen expressing cells, such as monkey kidney-derived COS cells; and (b) it contains a sequence (P tag), encoding a short COOH-terminal immunoreactive segment of neurosubstance P, which when ligated in reading frame to the COOH terminus of cDNA inserts, enables specific detection of expressed proteins due to the highly restrictive distribution of this peptide among cell types.

Plasmid pJK14-P was converted into a plectin mutant expression vector system by engineering a Sall site just 5' to the keratin 14 cDNA sequence (pGW1; Fig. 1 A), and replacement of the insert by an adaptor sequence containing an EcoRI cloning site and a preceding ATG initiation codon (pGW15; Fig. 1 B). Subcloning into pGW15 of a partial plectin cDNA construct, corresponding to the last four of the six repeat domains that form plectin's globular COOH-terminal domain, yielded plasmid pGW10 (Figs. 1 C and 2). Construct pGW5 was derived from plasmid pGW1 by replacing the keratin 14 gene with a 5.8-kb BamHI fragment of plectin, flanked by Sall/BamHI adaptors (Fig. 1 D). As shown in Fig. 2, this construct comprises the entire subdomain R2 of the rod, half of the subdomain R1, and roughly one and a half of the entailing six repeats forming the globular tail domain. All other plectin expression plasmids used in this study were constructed using plasmids pGW5 and pGW10 alone or in combination with other clones and constructs (see Materials and Methods for details). As illustrated in Fig. 2, plasmid pGW16 (\approx 4 kb) corresponded to all six COOH-terminal repeat domains of plectin, plasmid pGW17 (\approx 10 kb) comprised this COOH-terminal domain and the rod, plasmids pAD11 (\approx 1.7 kb) and pAD17 (\approx 4.2 kb) contained the NH₂-terminal domain without the rod, and the rod without the COOH-terminal globular domain, respectively, and plasmid pAD14 encoded the whole plectin molecule.

Mutant Plectin Proteins Are Expressed When Plectin Constructs Are Transfected into COS Cells

The kidney epithelial cell line COS, from monkey, was initially chosen for our transfection studies. This cell line expresses SV-40 large T-antigen, enabling high level expression of our SV-40 early promoter and enhancer-driven cDNA constructs (Gluzman, 1981). In addition, its cytoarchitecture comprises a number of potential interaction partners of plectin, including IF of the vimentin-type, microtubules, and nuclear lamins.

The various mutant plectin constructs were transfected into COS cells, and cell extracts were prepared 63-h post-

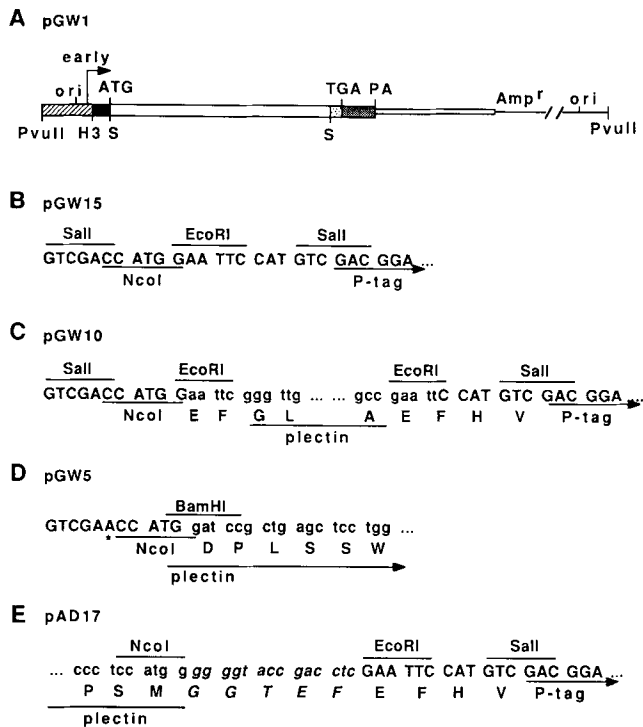


Figure 1. Plasmid construction for expression of plectin mutants. (A) Schematic drawing of pGW1. (SV-40) promoter; (H) HindIII/HincII polylinker of pSP64 (Promega Biotec) followed by the 5'-untranslated sequence of the K14 mRNA; (K) human keratin K14 gene; (L) sequence encoding a linker and 6 COOH-terminal amino acid residues of neurosubstance P followed by a stop codon; (S) 3'-untranslated sequences and polyadenylation signal (PA) of *Drosophila hsp70* gene; (A) pBR322; (H3) HindIII; and (S) Sall. For further details see Fig. 1 in Albers and Fuchs (1987). The start site and direction of transcription from the SV-40 promoter are indicated by an arrow. (B) Nucleotide sequences at the site of insertion (*EcoRI*) of plectin mutants into pGW15. (C, D, and E) Nucleotide sequences at relevant junctions between the expression vector and truncated plectin alleles in pGW10, pGW5, and pAD17, respectively. Note that the Sall/BamHI adaptor sequence did not regenerate the Sall site (*); expressed amino acid residues derived from the pUC polylinker are in italics.

transfection. When proteins were resolved by SDS-PAGE and subjected to staining with Coomassie blue, major discrete protein bands that had no counterpart in extracts from nontransfected cells were clearly detectable only in one case (Fig. 3 A, asterisk). The apparent size (280 kD) of the protein product encoded by pGW17 was close to that of intact plectin polypeptide chains which migrate at a position corresponding to 300 kD (Wiche, 1989); as mentioned previously (Wiche et al. 1991), plectin exhibits aberrant migration on gels (predicted molecular mass, 466 kD). In all the other cases, specific mutant protein bands were not detectable, probably because of insufficient electrophoretic resolution from endogenous proteins.

Immunoblot analysis of these cell extracts using antibodies specific for substance P revealed immunoreactive protein bands in all cases. The molecular masses of the major bands detected corresponded well with the sizes predicted on the basis of the cDNA sequence; minor bands of small sizes de-

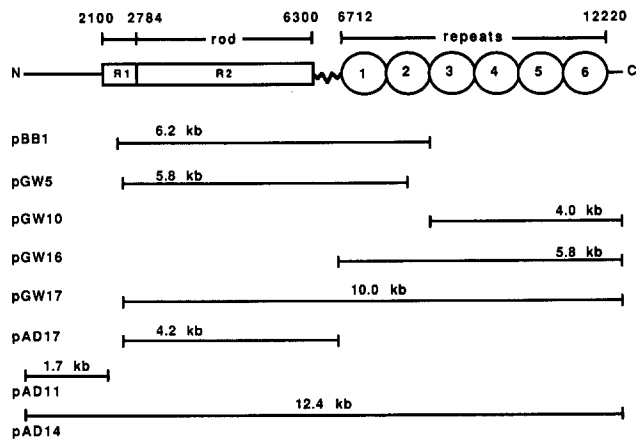


Figure 2. Schematic representation of the domain structure model of plectin and alignment of mutant cDNA constructs. Numbers in top row refer to plectin cDNA sequence as given in Wiche et al. (1991). Numbers within ellipsoids indicate repeat regions in COOH-terminal globular domain. R1 and R2, subdomains of plectin rod.

ected in a few cases most likely represented proteolytic degradation products of the major translation products.

In extracts from pGW17-, pAD17, and pGW5-transfected cells, the major immunoreactive bands detected by anti-P-tag staining (Fig. 3, lanes 1, 4 and 5, respectively) were also immunoreactive with mAbs to plectin (data not shown). In

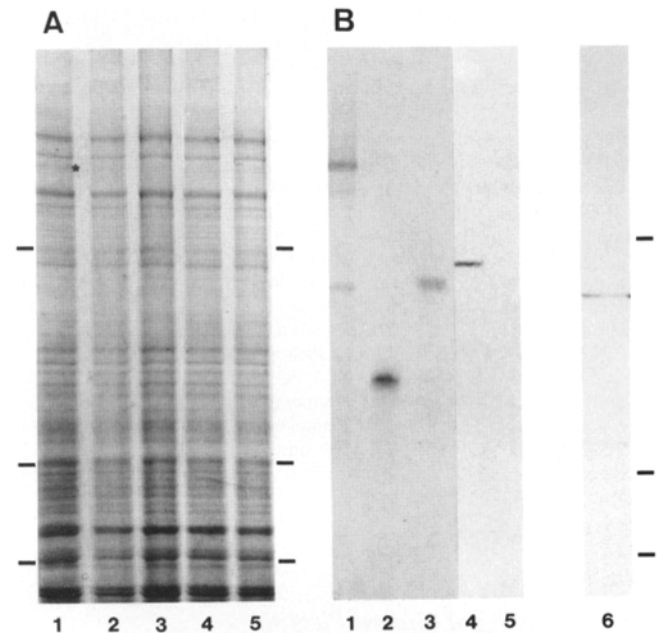


Figure 3. SDS-PAGE and immunoblot of plectin mutants. Lysates prepared from COS cells 63-h posttransfection were subjected to SDS-5% PAGE and immunoblotting as described in the text. (A) Servablau-G protein stain. (B) Immunoblotting using anti-P antibodies. (Lanes 1) pGW17; (lanes 2) pGW10; (lanes 3) pGW16; and (lanes 4) pGW5 transfected cells. (Lanes 5) untransfected cells; (lane 6) pAD17 transfected cells. Molecular weight ($\times 10^{-3}$) of markers is indicated. Asterisk in lane 1 denotes plectin mutant protein.

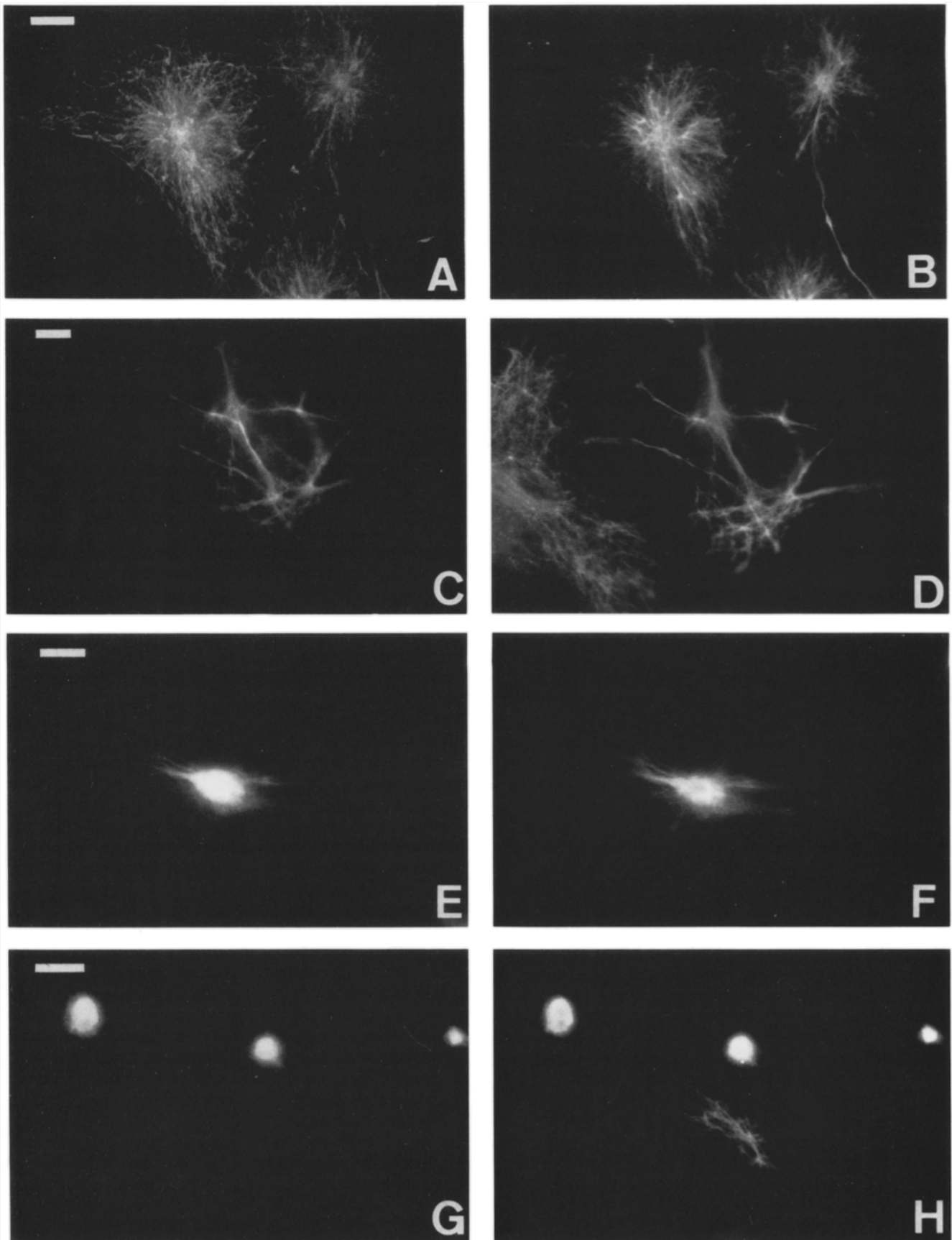


Figure 4. Association of plectin mutants containing four or more COOH-terminal repeat domains with vimentin filaments in COS cells and formation of aggregates. Double immunofluorescence microscopy is shown; see text for detailed protocol. (A) Anti-plectin; (C, E, and G) anti-P tag, Texas red optics; (B, D, F, and H) anti-vimentin, FITC optics. (A and B) Control (untransfected cells); (C and D) pGW10-transfected cells; (E and F) pGW16-transfected cells; and (G and H) pGW17-transfected cells. Bars, 10 μ m.

contrast, the mutant products from pGW10 and pGW16 were unreactive with mAbs. This was expected since all of the epitopes of a series of mAbs available reside within the rod domain of plectin molecules (Foisner et al., 1991b; Wiche et al., 1991), which is out of the coding region of these constructs (see Fig. 2).

Plectin Mutants Containing Multiple COOH-terminal Repeat Domains Associate with the Vimentin Network of COS Cells and Cause Its Collapse

Fig. 4 (A and B) illustrates the organization of plectin and vimentin networks in normal, untransfected COS cells as visualized by double immunofluorescence microscopy. Similar to various other cell lines studied previously, plectin exhibited codistribution with vimentin filaments throughout the cytoplasm. As observed in general, however, plectin structures appeared somewhat more dense and slightly more delicate than the vimentin network.

When COS cells were transfected with constructs pGW10, pGW16, or pGW17, which encode the last four (pGW10) or all six COOH-terminal repeat domains (pGW16 and pGW17), the newly synthesized mutant proteins colocalized with vimentin filaments. In a few cells, where extended filament networks existed, mutant proteins distributed all along the endogenous filaments (Fig. 4, C and D). However, the

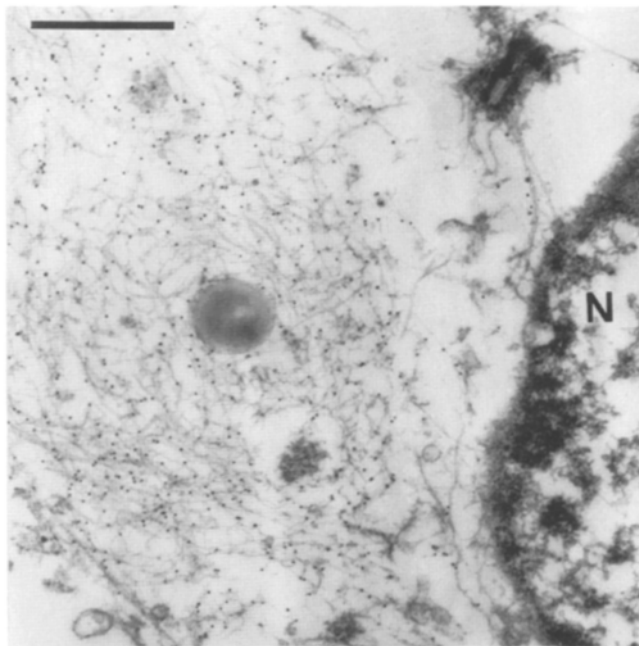


Figure 5. Immunoelectron microscopy (anti-P tag) of filamentous perinuclear aggregates. pGW17-transfected cells were processed for immunoelectron microscopy as described in the text. Diameter of filaments: ~10 nm. Bar, 10 μ m.

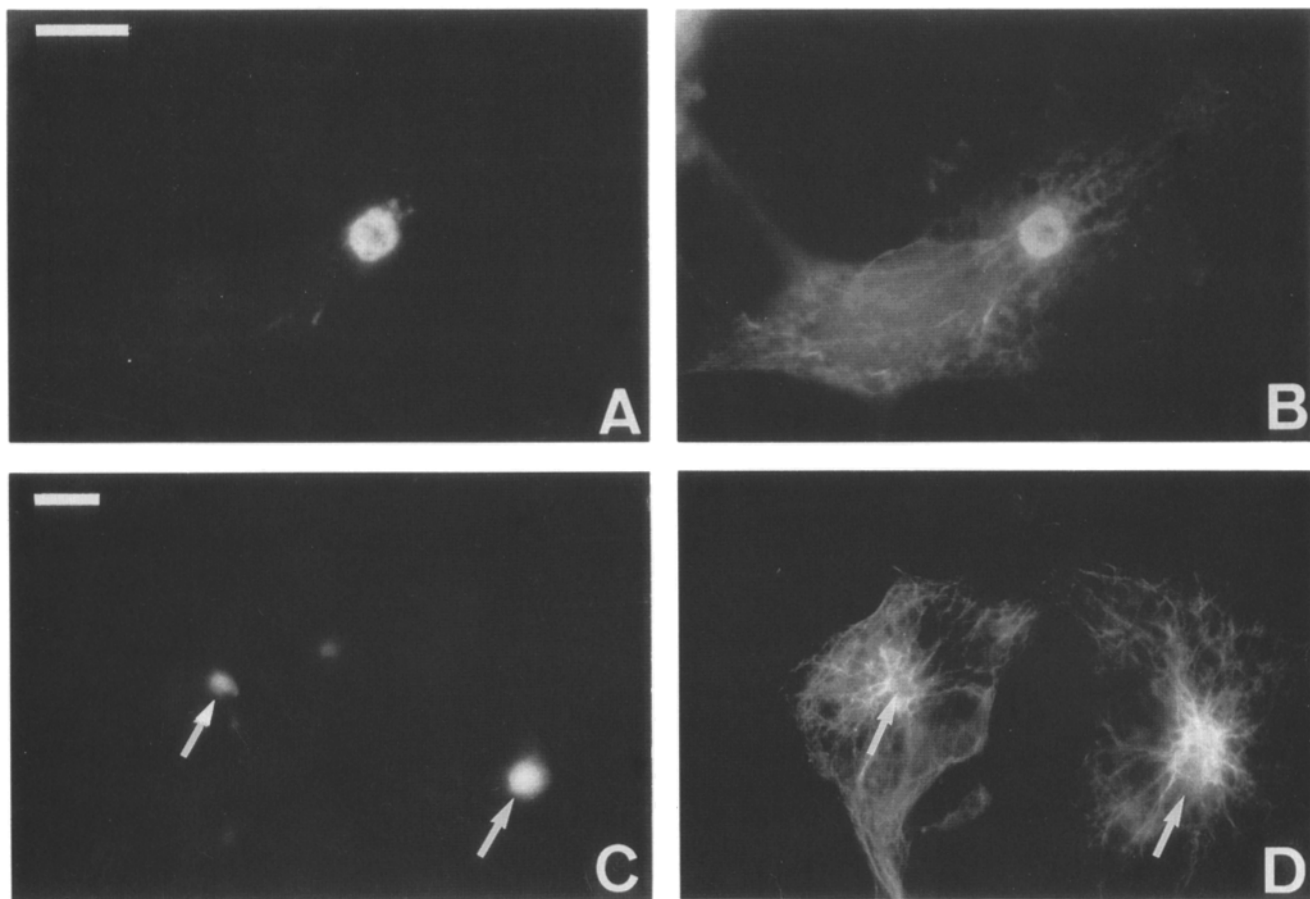


Figure 6. Distribution of endogenous plectin and microtubule networks after collapse of vimentin filaments in transfected COS cells. Double immunofluorescence microscopy of pGW17-transfected cells is shown. (A and C) Anti-P tag, Texas red optics; (B and D) anti-plectin (mAb 10F6) and anti-tubulin, respectively, FITC optics. (Arrows) Positions of perinuclear aggregates. Note that endogenous (not vimentin-associated) plectin visualized in cytoplasm (B) represented only part of the endogenous plectin (see text). Bar, 10 μ m.

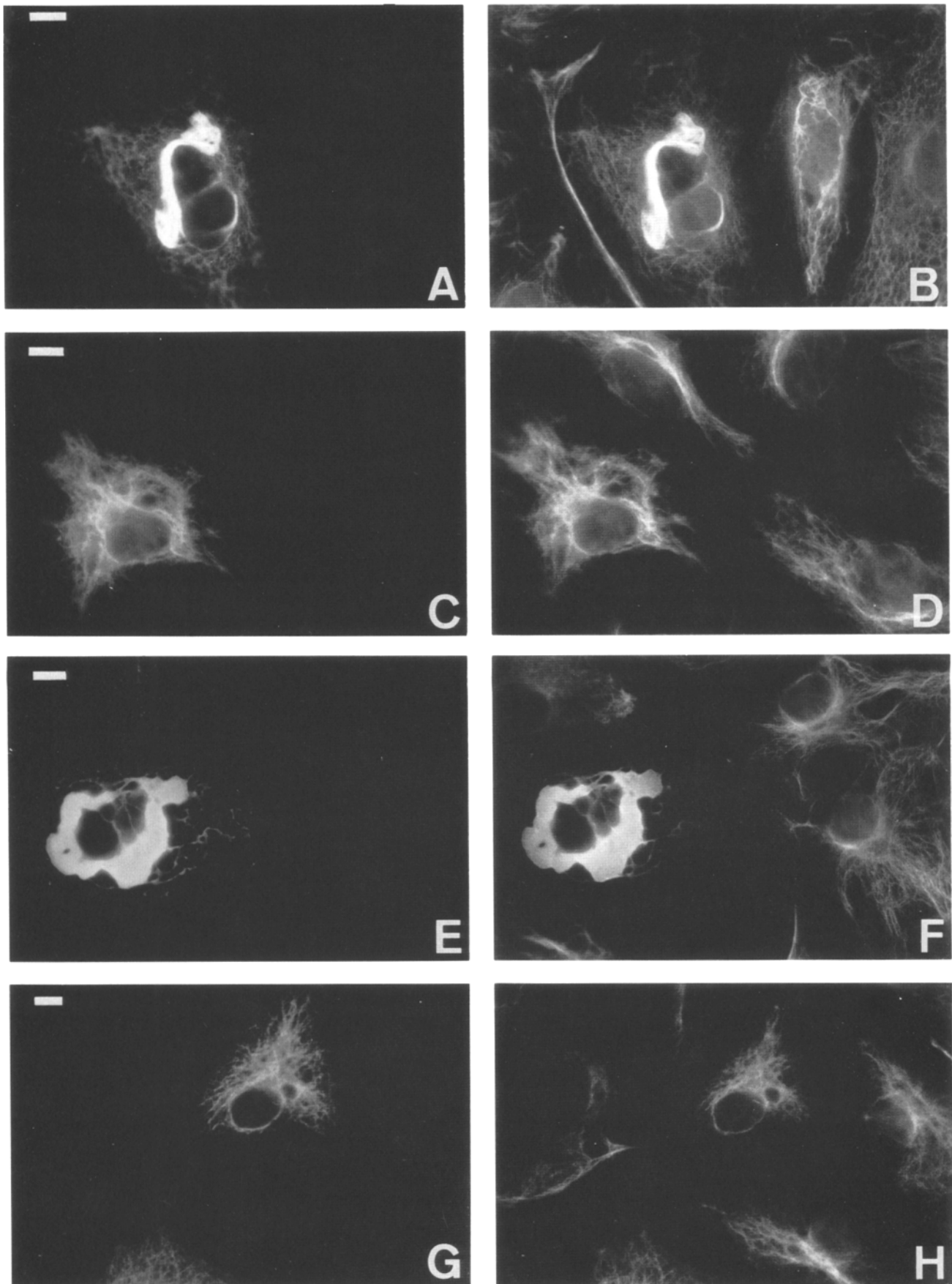


Figure 7. Plectin constructs containing four or more COOH-terminal repeat domains associate with IF networks of PtK2 cells and cause their collapse. Double immunofluorescence microscopy of PtK2 cells transfected with plasmid constructs pGW10 (*A* and *B*); pGW16 (*C* and *D*); pGW17 (*E* and *F*); and pAD14 (*G* and *H*) is shown. (*A*, *C*, *E*, and *G*) Anti-P tag, Texas red optics; (*B* and *H*) anti-vimentin, FITC optics; and (*D* and *F*) anti-cytokeratin, FITC optics. Bars, 10 μm .

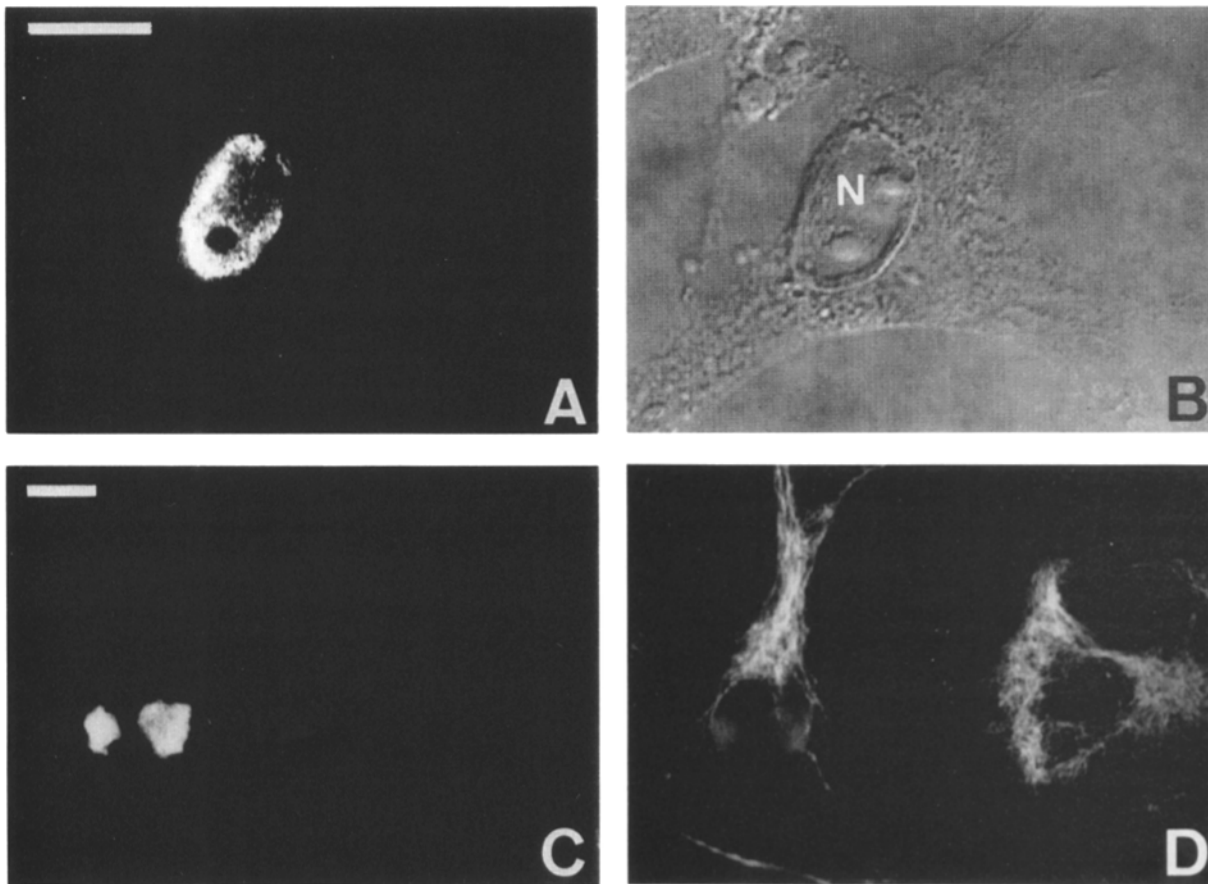


Figure 8. Distribution of plectin mutants lacking COOH-terminal repeat domains. (A and B) Immunofluorescence and transmission light microscopy, respectively, of PtK2 cells transfected with plasmid pAD17. (A) Anti-P tag, Texas red optics. (N) nucleus. (C and D) Double immunofluorescence microscopy of plasmid pAD17-transfected PtK2 cells. (C) Anti-P tag, Texas red optics; (D) anti-vimentin, FITC optics. Note that in A, C, and D, optical sections obtained by confocal laser microscopy are shown, resulting in a slight optical overspill of C into D. Bars, 10 μ m.

majority of cells transfected with these constructs displayed gross perturbations in their vimentin filament networks. In these cells, vimentin staining was often confined to dense perinuclear aggregates with none or only a few extending filament bundles (Fig. 4, F and H). Most of these aggregates exhibited a filamentous substructure, as demonstrated by immunogold EM of transfected COS cells (Fig. 5); occasionally, we observed also dense aggregates of amorphous structure (data not shown). A few cells exhibited cytoskeletal structures resembling transition states between extended networks and fully collapsed aggregates (data not shown). In all cases, the location of plectin mutant proteins, as revealed by P tag staining, was virtually indistinguishable from that of vimentin (Fig. 4, E-H). In contrast, at least part of the endogenous COS cell plectin did not codistribute with vimentin, but retained its cytoplasmic distribution, as demonstrated by mAb staining (Fig. 6, A and B). In contrast to the major alterations in the plectin-vimentin network, the microtubule network and actin stress fibers of COS cells appeared unaffected by expression of COOH-terminal plectin segments (Fig. 6, C and D; anti-P staining in frame C and anti-tubulin staining in frame D; actin data not shown).

Plectin Mutants Containing Multiple Repeat Domains Affect Both Vimentin and Cytokeratin Filament Networks in PtK2 Cells

To distinguish between the effects of mutant plectin proteins on vimentin versus cytokeratin filament networks, we transfected various constructs into PtK2 cells. Like COS cells, PtK2 cells have both vimentin and cytokeratin networks. However, the unusually flat morphology of PtK2 cells enables a clear distinction between the two IF networks.

In general, the effects of pGW10-, pGW16-, and pGW17-encoded plectin mutants on the vimentin network of transfected PtK2 cells were similar to those observed in the case of COS cells, although the morphologies of P tag-positive structures were more varied. In cases of transfected cells with partially collapsed or seemingly intact (rare) IF systems, plectin mutants were colocalized with filaments of both types (Figs. 7, A-D). However, in the majority of cells transfected with constructs containing repeat domains, a near complete collapse of IF organization was observed (Figs. 7, E and F; vimentin data not shown). Whenever a partial or complete collapse of these structures had occurred,

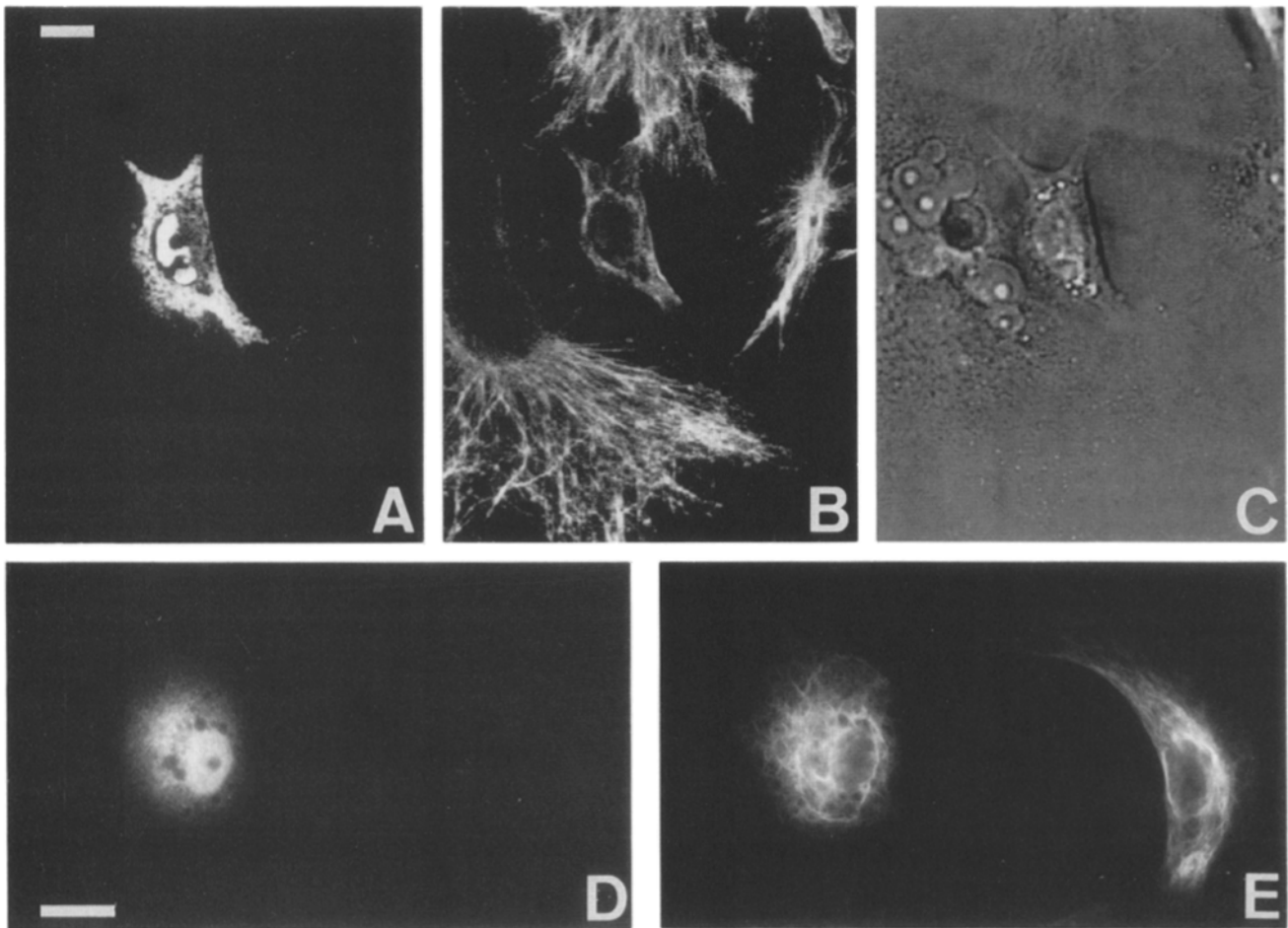


Figure 9. Plectin mutants encoded by plasmids pGW5 and pAD11 accumulate in both the nuclear and the cytoplasmic cell compartments. See Fig. 2 for plasmid alignment with plectin domains. Confocal double immunofluorescence (*A* and *B*) and transmission light microscopy (*C*) of COS cells, and conventional double immunofluorescence microscopy of PtK2 cells are shown. (*A* and *D*) Anti-P tag, Texas red optics; (*B* and *E*) anti-vimentin, FITC optics. Bars: (*A–C*) 10 μm ; (*D* and *E*) 20 μm .

dense P tag-positive aggregates were found in close proximity to the nucleus. However, unlike the globular aggregates observed in COS cells, the structures in PtK2 cells were found surrounding the nucleus in ring- or horse shoe-like fashion. Aside from plectin mutant proteins, these structures contained both vimentin and cytokeratins, as indicated by bright immunostaining with both of the corresponding antibodies. It has not yet been clarified, whether plectin mutants perturbed each IF network independently, or whether the collapse of one (vimentin?) caused the collapse of the other.

In control experiments carried out with full-length construct pAD14 similar effects were observed. P tag-positive staining was associated with extended (Fig. 7, *G* and *H*) as well as partially or fully collapsed (data not shown) vimentin networks; in addition, P tag-positive structures were observed in more peripheral cell regions, which were largely vimentin-negative. Overall, this distribution was highly reminiscent of that of plectin in untransfected cells (see Fig. 4 *A*). Results obtained with a construct whose coding sequence corresponded to that of pGW17, except that it terminated at the natural stop codon of plectin, and contained no P tag, were indistinguishable from those obtained with pGW17 (data not shown). This indicated that P tagging had no effect on the behavior of plectin mutants.

Plectin Mutants Lacking COOH-terminal Repeat Domains Accumulate in the Nucleus

To determine whether the removal of the COOH-terminal part of the molecule containing the six repeat domains, had any effect on the cellular distribution of plectin mutants, transfections of COS and PtK2 cells were carried out, using construct pAD17, which encodes the rod but not the repeats (see Fig. 2). Unexpectedly, in both cell lines the pAD17 mutant protein was found predominantly in the nuclei of transfected cells (Fig. 8). In COS cells the mutant protein was only exceptionally located in both nuclear and cytoplasmic compartments, while in the case of PtK2 cells, the proportion of cells displaying both nuclear and cytoplasmic staining versus those displaying solely nuclear staining was slightly higher. Focal sections obtained by confocal microscopy (Fig. 8, *A* and *C*) revealed that the mutant protein was uniformly distributed throughout the nucleoplasm with the exception of nucleolar areas. Furthermore, the nuclear lamina appeared unaffected by the nuclear presence of the mutant protein, as shown by double immunofluorescence confocal microscopy using anti-lamin B antibodies (data not shown).

Cells transfected with pAD17 displayed normal morphologies, and their cytoplasmic vimentin network appeared unaffected (Fig. 8, *C* and *D*). The distribution of endogenous

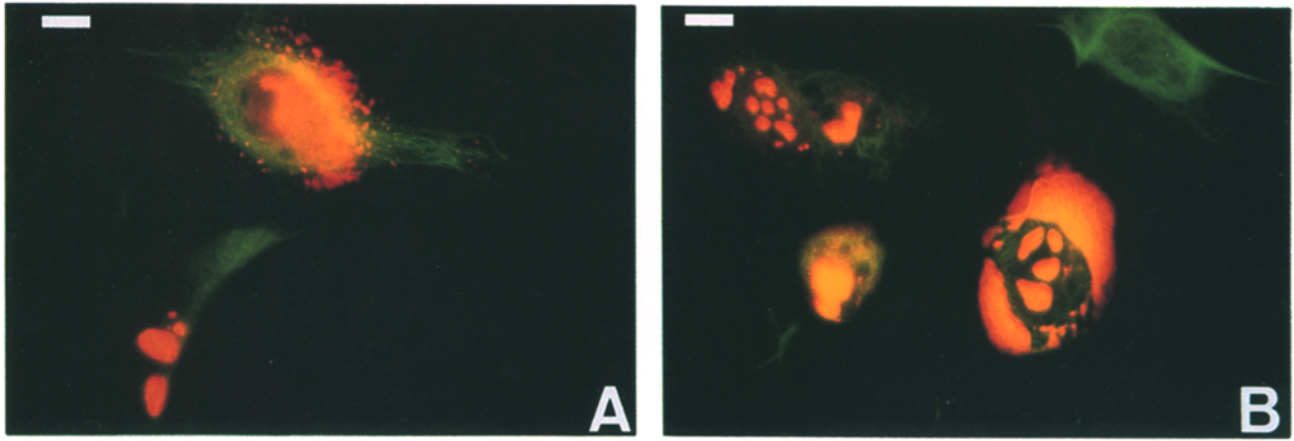


Figure 10. Cytoplasmic pGW5-encoded plectin mutants do not codistribute with IF networks in transfected PtK2 cells. (A) Double immunofluorescence microscopy using anti-P tag (Texas red optics) and anti-vimentin (FITC optics). (B) Double immunofluorescence microscopy using anti-P tag (Texas red optics) and anti-cytokeratin (FITC optics). Note nuclear as well as cytoplasmic localization of mutant proteins in both cases. Bars, 10 μm .

(cytoplasmic) plectin could not be monitored independently of P-tagged mutant protein because all of the available anti-plectin antibody preparations are immunoreactive with the mutant (rat) species, and attempts to raise antibodies to domains of plectin other than the rod have failed thus far. However, when COS cells were double-labeled using anti-P tag and anti-plectin antisera, we always observed cytoplasmic staining of filamentous structures in addition to bright nuclear staining. Since this was only occasionally observed when cells were stained with anti-P tag alone (see above), it appears that endogenous cytoplasmic plectin was largely unaffected by nuclear import of rod mutants.

The difference in localization between the plectin rod mutant (encoded by pAD17) and the mutant containing the rod and the COOH-terminal repeat domain (encoded by pGW17) suggested that the COOH-terminal repeats might be involved in appropriately localizing plectin to the cytoplasm. To determine whether a smaller number of repeats were sufficient to maintain plectin in the cytoplasm, we transfected COS and PtK2 cells with pGW5, consisting of the rod and roughly one and a half of the first six COOH-terminal repeats. In this case, the encoded mutant proteins were found in nuclear as well as in cytoplasmic compartments in both cell lines (Figs. 9 and 10). However, an exclusive occurrence of mutant proteins within the nuclear compartment was never observed, indicating a difference between the localization behavior of pGW5- and pAD17-encoded products. Interestingly, the mutant protein encoded by pGW5 did not codistribute with vimentin or cytokeratin filaments in the cytoplasm. Instead, it was diffusely distributed over the cytoplasm and/or was associated with spot-like structures (Fig. 10, A and B). In this regard, the distribution of pGW5 mutant proteins containing only one and a half repeat domains was clearly different from that of mutant proteins representing plectin's tail and containing either all six (pGW16 and pGW17) or the last four (pGW10) of the repeat domains. Instead of being associated with vimentin filaments, pGW5 mutant proteins in fact seemed to accumulate in cytoplasmic areas that were complementary to those with high filament densities. Together, these data suggested that: (a) either more than one carboxy-terminal repeat region or COOH-terminal sequences other

than the repeats are needed for efficient interaction between plectin and IF networks; and (b) interactions between plectin and IF networks may be necessary to keep plectin from localizing in the nucleus. A behavior similar to that of the pGW5-encoded mutant, diffuse distribution over nucleus and cytoplasm, was displayed by a mutant protein (pAD11-encoded) consisting of the NH₂-terminal domain of plectin (Fig. 9, D and E).

Discussion

Plectin has all the features of a versatile cytoplasmic cross-linking protein. Its interaction partners range from constituent proteins of the subplasma membrane skeleton and the nuclear lamina to IF proteins and microtubule-associated proteins. In addition, the protein self-associates giving rise to network-like arrays. Using recombinant DNA technology we have begun a comprehensive investigation of plectin's cellular function(s), with emphasis on the molecular mapping of structural epitopes involved in its diverse interactions. The evidence presented here, which is based on the transient transfection of plectin mutant cDNAs into COS and PtK2 cells, indicates that the COOH-terminal domain of plectin, consisting of six repeats of homologous structure, plays an important role in plectin's association with cytoplasmic IF. Truncated plectin mutants comprising the NH₂-terminal domain or the rod, but lacking the COOH-terminal repeat domains were shown to have lost their ability to associate with IF.

Our cDNA constructs were all designed such that the 3' ends of the mutant proteins were linked via short sequences (13–18 amino acid residues) to the COOH-terminal last six amino acid residues of the neuropeptide substance P (Fig. 1). This enabled the expression of mutant plectin alleles with immunoreactive P tags in all cases. Control experiments carried out with a construct encoding a P-tagless mutant protein (corresponding to pGW17) showed that the addition of P tag sequences did not affect the behavior of the mutant protein.

The phenotype of cytoplasmic IF structures in cells overexpressing plectin mutants with multiple COOH-terminal repeat domains (pGW10, pGW16, and pGW17) often varied

considerably. Between the two extremes of seemingly unperturbed IF network arrays and perinuclear dense aggregates without extending single or bundled filaments, were a variety of structures which could be considered transition states between the two forms. Rough estimates based on fluorescence intensities indicated that the relative amounts of mutant proteins expressed in transfectants with seemingly unperturbed IF network arrays were low compared to transfected cells with partially or fully collapsed networks. Thus, whether plectin mutant proteins associated with IF network arrays without disrupting them, or whether a collapse occurred, most likely was dependent on the level of expression of the mutant proteins.

We do not yet know the molecular mechanism(s) by which plectin mutants disrupt the endogenous IF network. EM data indicated that the perinuclear aggregates observed consisted of filamentous structures with a diameter characteristic of IF. Thus, plectin mutants may induce a gradual aggregation of filaments, eventually leading to a collapse of the IF network into perinuclear structures. Coupled with the knowledge that plectin itself can self-associate to form network-like arrays (Foisner and Wiche, 1987), it seems most likely that plectin mutants act by perturbing their own cytoskeletal structures, which in turn exerts perturbations on the IF network. The initial effect of mutant plectin overexpression could, therefore, be the formation of filamentous aggregates. Amorphous aggregates, which were occasionally observed, may result by disruption of filaments, succeeding their aggregation when highly over-expressed plectin mutant proteins "mask" self-interaction sites of IF subunit proteins. Further work is needed to clarify this point.

Perinuclear aggregates formed after overexpression of mutant proteins, containing multiple COOH-terminal repeat domains and lacking the rod (pGW10 and pGW16), were immunoreactive not only with mutant-specific anti-P tag antibodies, but also with a number of mAbs to plectin (data not shown). Since the epitopes of these mAbs are known to reside on the rod domain of plectin (Foisner et al., 1991a; Wiche et al., 1991), these aggregates must have contained endogenous plectin, aside from mutant proteins. However, a considerable fraction of endogenous plectin did not colocalize with mutant proteins and collapsed IF networks, but was still distributed all over the cytoplasm (Fig. 6, *A* and *B*). A plausible explanation for this phenomenon is that only a fraction of endogenous plectin is tightly associated with IFs, whereas the remainder is associated with other cytoplasmic (plasma membrane-associated?) structures. This notion is consistent with biochemical data showing the existence of insoluble (IF-associated) and more soluble (not IF-associated) pools of plectin in cultured cells and tissues (Wiche, 1989).

In a recent study on transient transfection of COS-7 and NIH-3T3 cells with desmoplakin mutants, Stappenbeck and Green (1992) observed effects that were in part similar to those we report here. They show that the expression of desmoplakin mutants comprising three COOH-terminal repeat regions each of which is highly homologous to the six repeat regions of plectin (Wiche et al., 1991), leads to the coalignment of the mutant protein with IF networks and eventually to the disruption of IF, followed by the accumulation of perinuclear aggregates consisting of mutant protein and IF proteins. Thus the COOH-terminal domains of plectin and

desmoplakin exhibit similar properties when overexpressed in cultured cells. This may indicate that the COOH-terminal repeat domains of both proteins share a common function, which could be essential for IF association. In the case of plectin, whose ability to directly interact with IF has been shown on the protein level in a number of ways (Foisner et al., 1988, 1991a,b), it would be tempting to speculate that the 19 amino acid sequence motifs tandemly occurring over nine times within the core of each repeat domain (Wiche et al., 1991) represent IF interaction sites. If so, it is likely that many of these motifs are required for efficient IF binding, because pGW5-encoded mutant plectin comprising the rod and one and a half COOH-terminal repeat domains (containing in total ≈ 15 19-amino acid sequence motifs) did not seem to associate appreciably with cytoplasmic IF, although its distribution seemed partially cytoplasmic. On the other hand, mutants comprising the last four or all six repeat regions, containing near 40 and 60 19-amino acid sequence motifs, respectively, associated and eventually disrupted IF networks, independent of whether (pGW17), or not (pGW16) they also contained the rod domain.

The situation is more complex in the case of desmoplakin, because previous attempts using biochemical techniques have been unable to demonstrate an interaction between desmoplakin and IF (for a more comprehensive discussion see Stappenbeck and Green, 1992). Thus, the multiplicity of the 19-amino acid sequence motif alone may not suffice for IF binding. Certain secondary structural features, yet to be characterized, may be equally important. Alternatively, COOH-terminal sequences other than the 19-amino acid repeat motifs, such as the short COOH-terminal sequences entailing the repeat domains, may be involved in binding to IF.

We were surprised that plectin mutants comprising the rod (pAD17) or the NH₂-terminal (pAD11) domains, but deficient in COOH-terminal repeats, were localized within the nuclear compartment. In the case of mutants containing the rod, there was a clear correlation between nuclear versus cytoplasmic distribution and the mutants' content of COOH-terminal repeats. While mutants lacking all six of plectin's repeat regions (pAD17) were exclusively found in the nuclear compartment, those comprising the rod plus the entailing one and a half repeat domains (pGW5) were distributed roughly equally between nucleus and cytoplasm, and those with all six COOH-terminal repeats were nearly exclusively cytoplasmic. Thus, it seems that the signal for nuclear accumulation of plectin was counteracted by structural features of the repeat regions in a dose-dependent way. The simplest explanation for these results is that the cytoplasmic occurrence of plectin mutants is dictated by their IF binding activity. However, this does not explain why pGW5-encoded mutants were diffusely distributed throughout the cytoplasm, rather than being filament associated. Future experiments will be necessary to elucidate precisely how cytoplasmic localization is determined.

The unexpected appearance of plectin rod or NH₂-terminal segments within the nuclear compartment could be explained in several ways. It is possible that these mutants contain nuclear localization signals that are masked in the native form of the protein but become exposed when the protein lacks the COOH-terminal domain. Intramolecular masking of a nuclear localization signal (NLS) by the COOH-terminal half of the 110-kD polypeptide precursor of the

mammalian transcription factor NF- κ B has been described recently (Blank et al., 1991; Henkel et al., 1992). In this case, proteolytic processing of the precursor renders the NLS accessible to NLS-binding proteins and nuclear uptake ensues. Unmasking of the NLS by phospho- or dephosphorylation of amino acid residues within or near the NLS has also been described (Moll and Nasmyth, 1991; Rihs et al., 1991). A putative NLS consensus sequence (Chelsky et al., 1989) was indeed found within the pAD17-encoded rod construct. It resides within the RI subdomain of the rod (amino acid residues 895–898) close to the beginning of the R2 domain (see Fig. 10 in Wiche et al., 1991). It remains to be tested, however, whether this signal is functional, because such minimal basic sequences are not always sufficient to ensure nuclear import (for a review see Silver, 1991). In the NH₂-terminal repeat, however, no such signals were found.

An alternative mode of transporting these plectin segments into the nucleus might be via complex formation with a protein that does have an NLS. Such "piggy-back" mechanisms may preferentially involve α -helical elements of secondary structure. It is noteworthy that two recently described nuclear proteins, NuMA (Yang et al., 1992; Compton et al., 1992) and NUF1 (Mirzayan et al., 1992), have central coiled coil domains, similar to plectin's rod in length and secondary structure. As components of the nuclear skeleton, both NuMA and NUF1 are imported into the nucleus upon synthesis in the cytoplasm. In this regard, it may be relevant that Bader et al. (1991) recently reported the accumulation of tailless cytokeratins in the nucleus of transfected fibroblast cells, since the tailless cytokeratins were also composed predominantly of α -helical coiled coil structures.

Among the most intriguing questions remaining is whether the ability to import plectin to the nucleus is functionally significant. Considering that the bulk of plectin clearly is localized in the cytoplasm, the answer is probably no. However, posttranslational processing of plectin may occur naturally at low levels. This may give rise to subunits that are able to enter the nucleus, but due to their relatively low amount have previously escaped detection. Given the recent discovery of nuclear proteins with some properties similar to plectin (see above), this possibility seems attractive.

The rod domains of plectin and desmoplakin (and of BP antigen) share extensive heptad repeat substructure, express the same periodic distribution of charged residues, and each are predicted to form two stranded α -helical coiled coil structures. Despite lacking significant homologies in the overall primary structure, the rod domains of these proteins could therefore serve similar functions. However, when cells were transfected with mutants of plectin or desmoplakin encoding only the rod section of the molecules results obtained were clearly different in both cases. Whereas the plectin mutants were found exclusively in the nuclear compartment of cells, corresponding desmoplakin mutants were diffusely distributed over the cytoplasm (Stappenbeck and Green, 1992). Although this does not exclude that both molecular domains have certain functions in common, it suggests others are different.

Based on mAb mapping of plectin epitopes and binding inhibition assays, we suggested previously that a central part of the rod domain of plectin molecules is involved in plectin–vimentin and plectin–lamin B binding (Foisner et al., 1991b). However, our transfection experiments suggest that

not the rod but the COOH-terminal globular domain is indispensable for IF binding. These results are not necessarily mutually exclusive. As discussed in our original report (Foisner et al., 1991b), the region of the rod harboring the epitope for mAb 10F6, which inhibits the binding of plectin to both vimentin and lamin B with a relatively high efficiency (63–67%), may not provide the actual binding sites, but may be indirectly involved in binding. Alternatively, there may exist several different binding sites, only one or a number of which reside within the rod. Binding sites residing in certain domains of the molecule may be differentially accessible under the conditions of the various binding assays employed. Thus, binding sites residing within the COOH-terminal repeat region of plectin may be dominant over rod-based sites within the intracellular environment, but masked or less accessible under *in vitro* conditions. One approach that we are currently taking to solve these questions is to express recombinant plectin mutants in bacteria, purify them, and study their biochemical properties, in particular their binding to the various cytoplasmic interaction partners of plectin previously identified.

In conclusion, the transient expression in COS and PtK2 cells of truncated plectin mutants containing an immunologically detectable tag has enabled us to gain insights into the dynamic behavior of this protein in living cells, and to investigate the function(s) of different molecular domains of plectin in their natural environment. The observation that certain plectin mutants associate with, and eventually cause the collapse of cytoplasmic IF, is fully consistent with previous biochemical and histochemical data, suggesting that plectin binds to, and acts as a crosslinking element of IF. The transfection data have provided the first evidence that plectin's tail domain, containing a series of six homologous sequence regions, is largely indispensable for IF association. The finding that tailless rod or NH₂-terminal domains of plectin, on the other hand, accumulate in the nuclear compartment is intriguing, but further work is needed to evaluate its biological significance. The mutant cDNA described should also be useful in studies aimed at expressing plectin wild type and mutant proteins in bacteria for detailed biochemical analyses of the recombinant proteins. Combined with transfection experiments such studies are likely to enable the pinpointing of contact sites between plectin and its various interaction partners to the actual amino acids. Furthermore, experiments are being started to produce transgenic mice, in order to investigate the role of plectin in development and cell differentiation.

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