

Basic Amino Acid Residue Cluster within Nuclear Targeting Sequence Motif Is Essential for Cytoplasmic Plectin-Vimentin Network Junctions

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Abstract. We have generated a series of plectin deletion and mutagenized cDNA constructs to dissect the functional sequences that mediate plectin's interaction with intermediate filament (IF) networks, and scored their ability to coalign or disrupt intermediate filaments when ectopically expressed in rat kangaroo PtK2 cells. We show that a stretch of ~50 amino acid residues within plectin's carboxy-terminal repeat 5 domain serves as a unique binding site for both vimentin and cytokeratin IF networks of PtK2 cells. Part of the IF-binding domain was found to constitute a functional nuclear localization signal (NLS) motif, as demonstrated by nuclear import of cytoplasmic proteins linked to this sequence. Site directed mutagenesis revealed a specific cluster of four basic amino acid resi-

dues (arg⁴²⁷⁷-arg⁴²⁸⁰) residing within the NLS sequence motif to be essential for IF binding. When mutant proteins corresponding to those expressed in PtK2 cells were expressed in bacteria and purified proteins subjected to a sensitive quantitative overlay binding assay using Eu³⁺-labeled vimentin, the relative binding capacities of mutant proteins measured were fully consistent with the mutant's phenotypes observed in living cells. Using recombinant proteins we also show by negative staining and rotary shadowing electron microscopy that in vitro assembled vimentin intermediate filaments become packed into dense aggregates upon incubation with plectin repeat 5 domain, in contrast to repeat 4 domain or a mutated repeat 5 domain.

THE intermediate filament (IF)¹-binding protein plectin (Wiche et al., 1982) is abundantly expressed in many different tissues and cell types. It shows partial codistribution with different types of IFs and it is conspicuously arranged at plasma membrane attachment sites of IFs and microfilaments, including hemidesmosomes (Wiche et al., 1984), Z-line structures and dense plaques of striated and smooth muscle, and intercalated discs of cardiac muscle and focal contacts (Wiche et al., 1983; Zernig and Wiche, 1985; Seifert et al., 1992). Furthermore, in many tissues, among them kidney (Yaoita et al., 1996) and brain (Errante et al., 1994), plectin is prominently expressed in cells forming tissue layers at the interface of tissue and fluid filled cavities. These observations suggest a role of plectin in mechanically strengthening cells against pulling or shearing forces occurring along cell surfaces and at intracellular attachment sites of cytoskeletal filaments. Consistent with this idea is a recent report by Gache et al. (1996) showing that a defect in plectin expression is ob-

served in epidermolysis bullosa simplex (EBS)-MD, a skin blistering disease associated with muscular dystrophy.

As shown biochemically, apart from cytoplasmic as well as nuclear IF subunit proteins, such as vimentin, glial fibrillary acidic protein, certain cytokeratins, neurofilament proteins, and lamin B, plectin specifically interacts with the subplasma membrane protein fodrin and its erythroid counterpart α -spectrin, and it binds to high mol wt microtubule-associated proteins (Herrmann and Wiche, 1987; Foisner et al., 1988; 1991). Plectin is a prominent phosphoprotein and has been demonstrated to be an in vivo target for different types of kinases including Ca²⁺/calmodulin-dependent kinases, protein kinases A and C, and mitotic cyclin-dependent p34^{cdc2} kinase (Herrmann and Wiche, 1983, 1987; Foisner et al., 1991, 1996). Interestingly, in vitro assays showed that plectin's capacity to bind to vimentin and lamin B were differentially influenced by kinase A and kinase C phosphorylation (Foisner et al., 1991), and that phosphorylation of the molecule by p34^{cdc2} kinase during mitosis at a unique site correlated with its increased solubility and dissociation from cytoplasmic vimentin filament networks in CHO cells (Foisner et al., 1996; Malecz et al., 1996). Thus, phosphorylation involving distinct kinases probably is a major mechanism regulating the various molecular interactions of plectin that are of importance for its proposed function as a versatile cross-linking element of cytoarchitecture.

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1. *Abbreviations used in this paper:* EBS, epidermolysis bullosa simplex; IF, intermediate filament; NLS, nuclear localization signal; PK, pyruvate kinase.

Plectin (mol wt >530,000) is one of the largest polypeptides known. Ultrastructural studies (Foisner and Wiche, 1987), as well as secondary structure predictions based on the deduced amino acid sequences of both rat and human cDNAs and genomic clones (Wiche et al., 1991; Liu et al., 1996), revealed that the molecule is dumbbell-shaped, comprising a long central α -helical coiled coil rod structure flanked by two globular domains. The dominant structural feature of the carboxy-terminal domain, six highly homologous repeats exhibiting in their core a tandemly repeated 19-amino acid sequence motif, occur in lesser numbers also in other IF-associated proteins, such as the hemidesmosome-associated bullous pemphigoid antigen (BPA)-1 (Sawamura et al., 1991) and desmoplakin (Green et al., 1990), a constituent of desmosomes.

The cloning and sequencing of plectin cDNA opened new avenues to study questions related to the domain organization of the molecule and to a precise mapping of functional epitopes by expression of mutant forms of plectin in cell lines transiently transfected with cDNA constructs. Our initial studies, carried out with a rat kangaroo (PtK2) and a monkey (COS) cell line, both of which are of simple epithelial origin and contain dense networks of vimentin filaments in addition to cytokeratin filaments, indicated that plectin's carboxy-terminal globular domain, in particular sequences within the last two thirds (~1,500 amino acid residues) of the carboxy terminus, played a role in IF association (Wiche et al., 1993). NH₂-terminal globular or central rod domains, on the other hand, seemed to be dispensable for IF association. The study reported here was undertaken to localize and characterize plectin's carboxy-terminal IF-binding site(s) more precisely. In addition to studying the phenotype of plectin mutant proteins expressed in PtK2 cells in vivo, we performed quantitative and ultrastructural in vitro binding assays, using recombinant mutant proteins expressed in bacteria. We found that a sequence of ~50 amino acid residues within the fifth carboxy-terminal repeat domain, residing downstream of the tandem repeat core domain, is indispensable for binding to IFs. An essential part of this sequence has structural and functional characteristics typical of nuclear localization signals (NLS). Furthermore, we show that the in vitro decoration of vimentin filaments with the repeat 5 domain of plectin leads to filament network formation.

Materials and Methods

Construction of Expression Plasmids and Mutagenesis

Rat plectin cDNA fragments were amplified by PCR from previously defined plectin constructs using EcoRI-tailed primers, and cloned into pAD29, a modified version of pGW15 (Wiche et al., 1993), enabling the expression of carboxy-terminal *c-myc*-tagged proteins. Internal deletions and targeted mutagenesis of amino acid residues were generated by SOE-PCR (Horton et al., 1989). To prepare pAD29, sequences encoding the spacer amino acids and P epitope tag of pGW15 were excised as an EcoRI-BamHI fragment and replaced by the *c-myc* tag. One pair of synthetic oligonucleotides encoding the *c-myc* epitope, MEQKLISEEDLN, followed by a stop codon included within a XbaI recognition site and flanked by EcoRI and BamHI overhangs were ligated into pGW15, previously digested with EcoRI and BamHI. The sequence of the sense oligonucleotides was AATTCATGGAGCAAAAGCTCATTCTGAA-GAGGACTTGAATGTCTAGAGCG. In two cases (pBN16 and pBN18),

cDNA constructs subcloned into pAD29 were used directly for transfection into mammalian cells, in all others, HindIII-XbaI fragments excised from pAD29 constructs were subcloned into the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA). For bacterial expression, EcoRI fragments were subcloned into pBN120, a pET-15b (Novagen, Madison, WI) derivative in which the endogenous BglII and EcoRI sites were destroyed and a linker, containing an EcoRI cloning site and an in-frame stop codon, ligated between the NdeI and BamHI sites. Plasmid pBN88 encoding chicken pyruvate kinase (PK) (from leu 17 to met 52) fused to a *c-myc* tag at its 5' end and to plectin's NLS sequence at its 3' end was constructed by PCR amplification of the cDNA corresponding to plectin's 19-amino acid-long NLS domain, and subsequent in-frame ligation of the amplified sequences into plasmid BSmycPK Δ stp (a kind gift of Dr. M. Schmidt-Zachmann, DKFZ, Heidelberg, FRG) downstream of the PK coding sequence, as a HindIII-ClaI fragment. An XbaI-ApaI fragment containing the entire coding sequence for the fusion protein was then subcloned into a modified pRc/CMV expression vector, in which the sequences between the HindIII and ApaI in the vector cloning site were replaced by a linker containing XbaI and ClaI sites followed by a stop codon, without reconstitution of the HindIII restriction site. Control plasmid pBN86 (expressing myc-tagged PK without NLS) was obtained by subcloning an XbaI-ClaI fragment of plasmid BSmycPK Δ stp into this modified version of pRc/CMV. Plasmid constructs were verified by DNA sequencing.

Cell Culture, DNA Transfection, and Immunofluorescence Microscopy

Rat kangaroo PtK2 (CCL 56, Amer. Type Culture Collection, Rockville, MD) and transformed mouse epidermal keratinocytes Pam 212 (Yuspa et al., 1980) were grown attached to glass slides in plastic dishes in DMEM supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 10% of heat-inactivated FCS, at 37°C and 5% CO₂. Subconfluent cells were transiently transfected with 30 μ g plasmid DNA/10-cm plate using the calcium phosphate precipitation method (Graham and van der Erb, 1973). After fixation in methanol at -20°C, 40-60 h posttransfection cells were processed for immunofluorescence microscopy as previously described (Wiche et al., 1993). The following immunoreagents were used: affinity-purified goat anti-mouse vimentin (Giese and Traub, 1986; kindly provided by Dr. Peter Traub, MPI f. Zellbiologie, Ladenburg, Germany), guinea pig anti-mouse liver cytokeratin (Denk et al., 1981; kindly provided by Dr. H. Denk), and anti-myc mAb 1-9E10.2 (Amer. Type Culture Collection), as primary antibodies; and Texas red-conjugated AffiniPure donkey anti-mouse IgG (H+L); fluorescein (DTAF)-conjugated AffiniPure donkey anti-goat IgG (H+L); AMCATM fluorophore-conjugated AffiniPure donkey anti-guinea pig IgG (H+L) all with minimal species crossreactivity (Accurate, Westbury, NY), as secondary antibodies. Specimens were viewed in a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and photographs were taken using Ilford ASA 400 black and white film.

Expression of Plectin Mutants in Bacteria

His-tagged recombinant proteins encoded by pBN120 constructs were expressed in *E. coli* BL21(DE3) and purified from inclusion bodies by solubilization in 500 mM NaCl, 20 mM Tris, pH 7.9 (binding buffer) containing 5 mM imidazole and 6 M urea followed by affinity binding to His-Bind[®] metal chelation resin according to the manufacturer's (Novagen) protocol. Proteins bound were eluted from affinity columns with a gradient of 60 mM-1 M imidazole in binding buffer, and stored frozen at -20°C at protein concentrations of 0.5-1 mg/ml. The correct size of expressed proteins was verified by SDS 10% PAGE (see Fig. 5 A).

Preparation of Vimentin for In Vitro Assays

Vimentin was purified from rat glioma C6 cells using a protocol described in detail by Foisner et al. (1988). Stocks of vimentin in 7 M urea were stored frozen (-80°C) at a concentration of 1-2 mg/ml. For in vitro overlay binding assays, 100 μ l of vimentin stock were dialyzed against 50 mM NaHCO₃, pH 8.5, and then labeled with Eu³⁺ using 10 μ l Eu³⁺-labeling reagent following the instruction of the manufacturer (Wallac, Turku, Finland).

For electron microscopy, vimentin stocks were incubated with 1 mM DTT for 30 min on ice and subsequently spun at 200,000 g for 10 min at 4°C. Soluble fractions were desalted into 5 mM Tris, pH 8.5, 1 mM DTT, 1

mM EDTA using small columns of Sephadex PD-10 (Pharmacia, Uppsala, Sweden), and again spun at 200,000 g for 30 min. To assemble IFs in vitro, soluble fractions were diluted to a concentration of 150 µg/ml of protein, 1/10th volume of 1.6 M NaCl, 200 mM Tris, pH 7.0, was added, and mixtures incubated for 1 h at 37°C. Preassembled filaments, were mixed 1:1 (vol/vol) with solutions containing 150 µg/ml recombinant plectin mutant proteins in the same buffer, or with buffer alone, and subjected to a 1-h incubation at 37°C before processing for electron microscopy.

Protein concentrations were determined according to Bradford (1976).

Overlay Binding Assay

Microtiter plates were coated with 100 µl of rat plectin mutant proteins expressed in bacteria (100 nM in 100 mM sodium borate, pH 9.2) overnight at 4°C. Blocking was carried out with 4% BSA in PBS for 1 h and then binding with 100 nM Eu³⁺-labeled vimentin in 100 µl of overlay buffer (PBS, pH 7.5, containing 1 mM EGTA, 2 mM MgCl₂, 1 mM DTT, and 0.1% Tween 20) for 90 min at room temperature. After washing extensively, bound vimentin was determined by releasing complexed Eu³⁺ with Enhancement Solution and measuring the fluorescence with a Delphia time-resolved fluorometer (Wallac). The fluorescence values were converted to concentrations by comparison with a Eu³⁺ standard.

Cloning, PCR, and DNA Sequencing

Restriction enzymes and T4 DNA ligase used for cloning were purchased from Promega (Madison, WI); PCR was done with thermostable DNA polymerases Dynazyme (Finnzymes Oy, Espoo, Finland) or Taq (GIBCO BRL, Gaithersburg, MD) on a DNA Thermal Cycler (Perkin Elmer Corp., Norwalk, CT) using standard conditions (Saiki et al., 1988). Double-stranded plasmid DNA was sequenced using a Sequenase Version 2.0 sequencing kit (USB, Cleveland, OH).

Electron Microscopy

For uranyl acetate staining of specimens, drops (7 µl) of mixtures containing vimentin filaments polymerized in vitro and decorated with plectin mutant proteins (see above) were deposited onto Formvar/carbon-coated and glow-discharged 400 mesh Cu-grids for 30 s, and then stained with eight drops of 1% uranyl acetate for 20 s. For rotary shadowing, samples (50 µl) mixed with glycerol (1:1) were sprayed onto freshly cleaved mica and processed for electron microscopy essentially as described by Tyler and Branton (1980). The shadowing with platinum (thickness of film: 0.74 nm) occurred at an angle of 7°, shadowing with carbon (10 nm) at 90°. Specimens were visualized in a JEOL 1210 electron microscope operated at 80 kV.

Results

The Fifth Carboxy-Terminal Repeat Domain of Plectin Harbors a Unique Vimentin-binding Site

Previous transfection experiments using mutant forms of plectin indicated a role of plectin's carboxy-terminal globular domain in IF association (Wiche et al., 1993). To pinpoint the vimentin interaction site within this domain cDNA constructs encoding various parts of it (Fig. 1) were transiently expressed in PtK2 cells with a short immunoreactive sequence derived from the human *c-myc* protein fused to their 3' ends. A cDNA construct (pBN72; see Fig. 1), corresponding to roughly two thirds of the carboxy-terminal globular domain and encoding a mutant protein still capable of association with IFs, served as the basic template for all other mutants to be generated. The new constructs were scored positive for IF association if they showed any of the phenotypes characterized previously, including the alignment with filaments, or their bundling and collapse (Wiche et al., 1993). When a series of truncated versions of pBN72 (Fig. 1), lacking sequences from either its 3' or 5' ends, or from both ends, were ex-

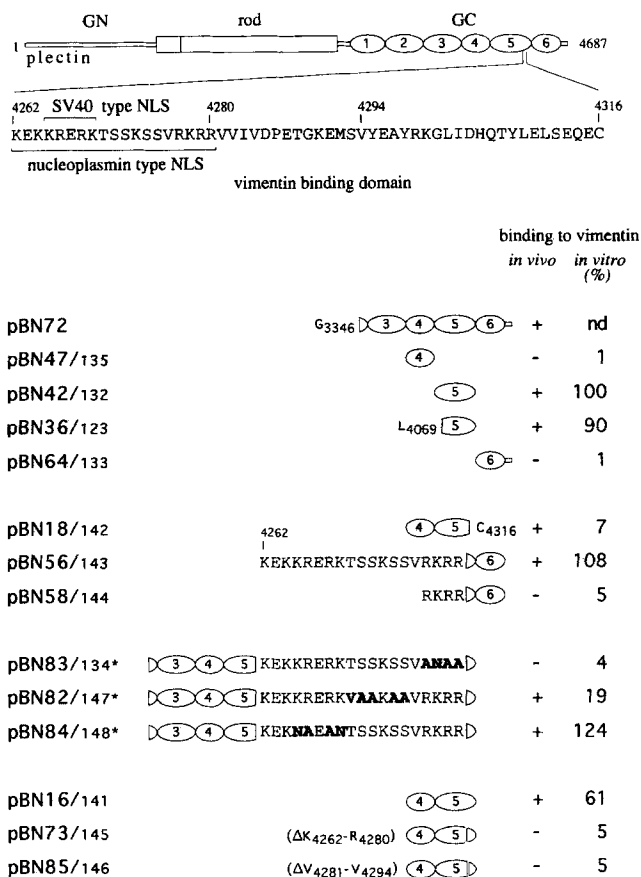


Figure 1. Overview of ectopically expressed plectin mutant proteins and summary of observed phenotypes. Schematic drawing on top represents the major molecular domains of plectin. GN and GC, NH₂- and COOH-terminal globular domains, respectively; numbered ellipsoids, repeat domains 1-6. Sequence below represents vimentin-binding domain with positions of relevant amino acid residues indicated. (The amino acid sequence extends to the published rat sequence (Wiche et al., 1991) at its amino terminus by 547 residues; the complete cDNA nucleotide sequence is available from the EMBL data library under accession number X59601.) Note that not all the amino acids shown (position 4281-4316) may be essential for vimentin binding (see text). Mutated amino acid residues are in bold. Consensus sequences of bipartite NLS and SV40 T antigen-like NLS motifs are indicated by brackets. pBN123, pBN132-135, and pBN141-148 represent bacterial expression vector constructs. Internal deletions in pBN73/145 and pBN85/146 are indicated. ±, positive or negative phenotypes observed *in vivo* after transient transfection of PtK2 cells. *In vitro* binding data (right hand column) are based on assays shown in Fig. 5 B, taking binding activity of repeat 5 as 100%. *nd*, not determined. *, pBN134, -147, and -148 were like pBN83, -82, and -84, respectively, except for starting at the 5' end of repeat 5.

amined, positive phenotypes were observed only with mutant proteins containing repeat 5 or certain parts of it (Figs. 2, A and B; 3, B and C; 4 A; and 8; see Fig. 1 for a survey). Two partially overlapping constructs, pBN18 (Fig. 2 A) and pBN56 (Fig. 2 B) showed a positive phenotype, in contrast to pBN58 (Fig. 2 C), a 5' truncated version of pBN56. Note, however, that the mutant protein encoded by pBN18, apart from being associated with distinct filaments, showed additional more diffuse distribution over

vimentin

myc-tag

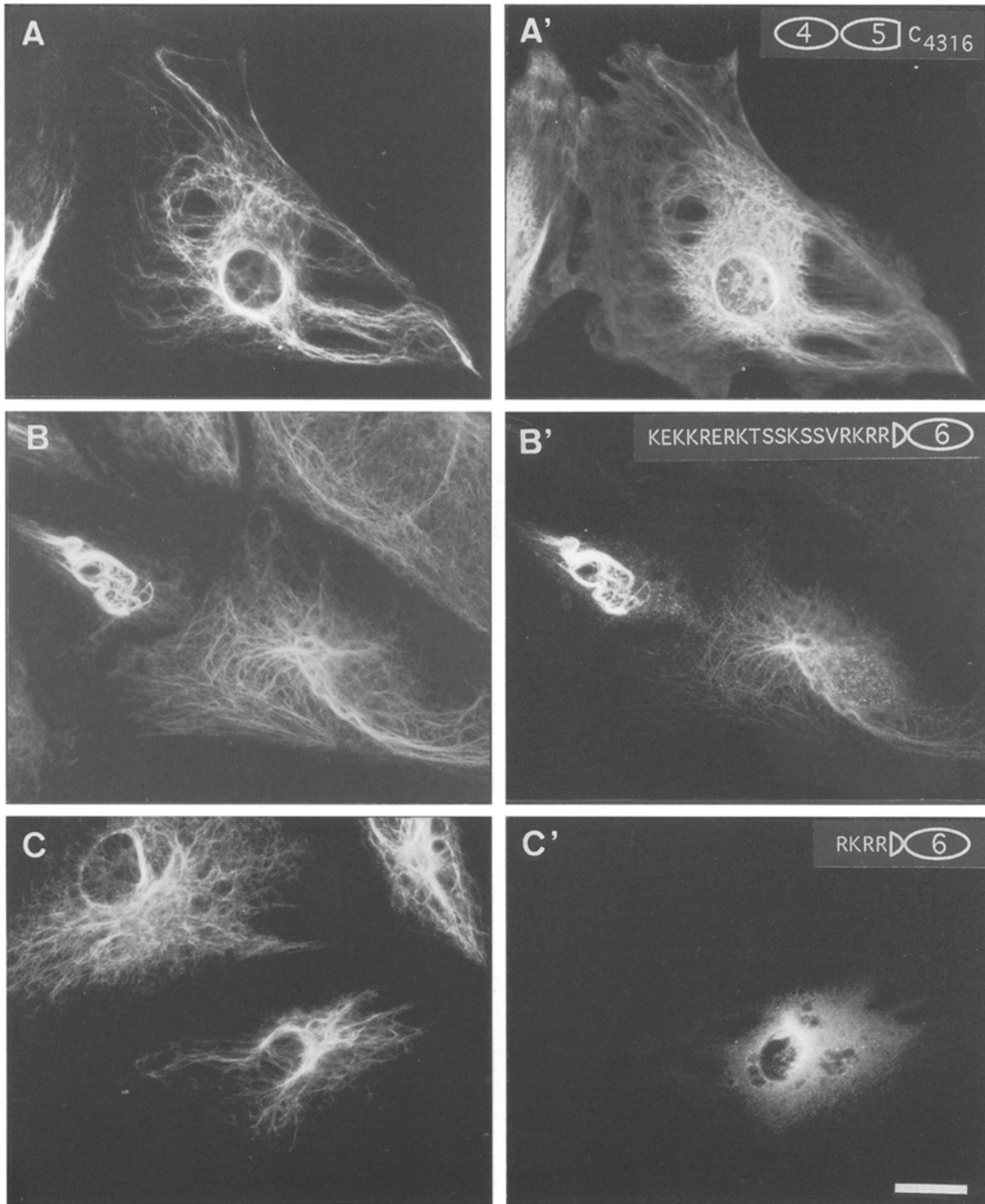


Figure 2. Expression of 3' and 5' truncated versions of plectin reveal vimentin-binding site in repeat 5. Double immunofluorescence microscopy is shown, primary antibodies are indicated. Expression plasmids: pBN18 (*A, A'*), pBN56 (*B, B'*), and pBN58 (*C, C'*). Note slightly diffuse (nonfilamentous) distribution in addition to IF-association of mutant protein in *A'*, prominent filament association in *B'*, and diffuse cytoplasmic distribution in *C'*. Bar, 15 μ m.

vimentin

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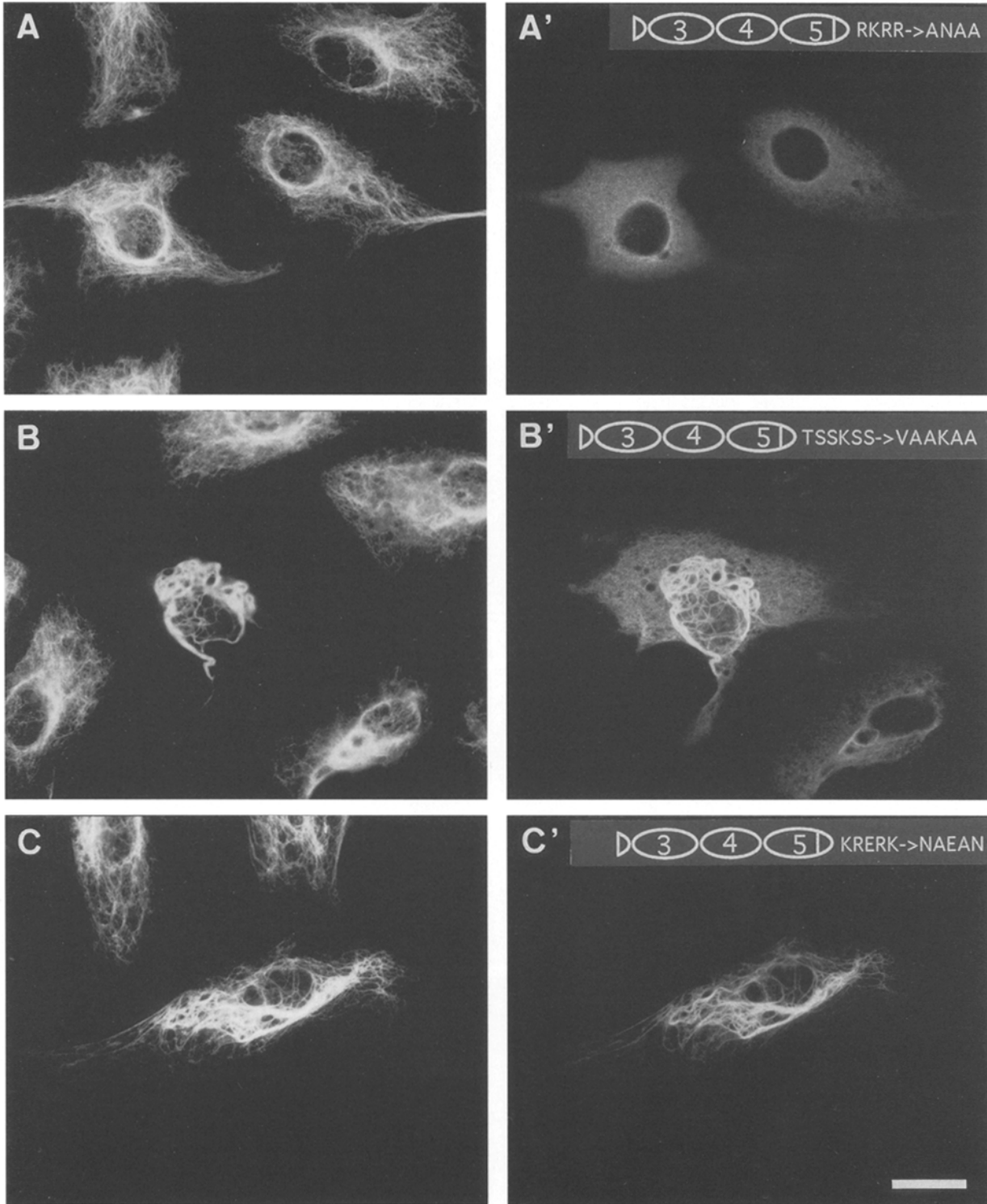


Figure 3. Targeted mutagenesis of NLS sequence domain within vimentin-binding site. Double immunofluorescence microscopy is shown, primary antibodies are indicated. Expression plasmids: pBN83 (*A, A'*), pBN82 (*B, B'*), and pBN84 (*C, C'*). Note nonfilamentous cytoplasmic staining in *A'*, filament association accompanied by diffuse cytoplasmic staining in *B'*, and exclusive filament association in *C'*. Bar, 15 μ m.

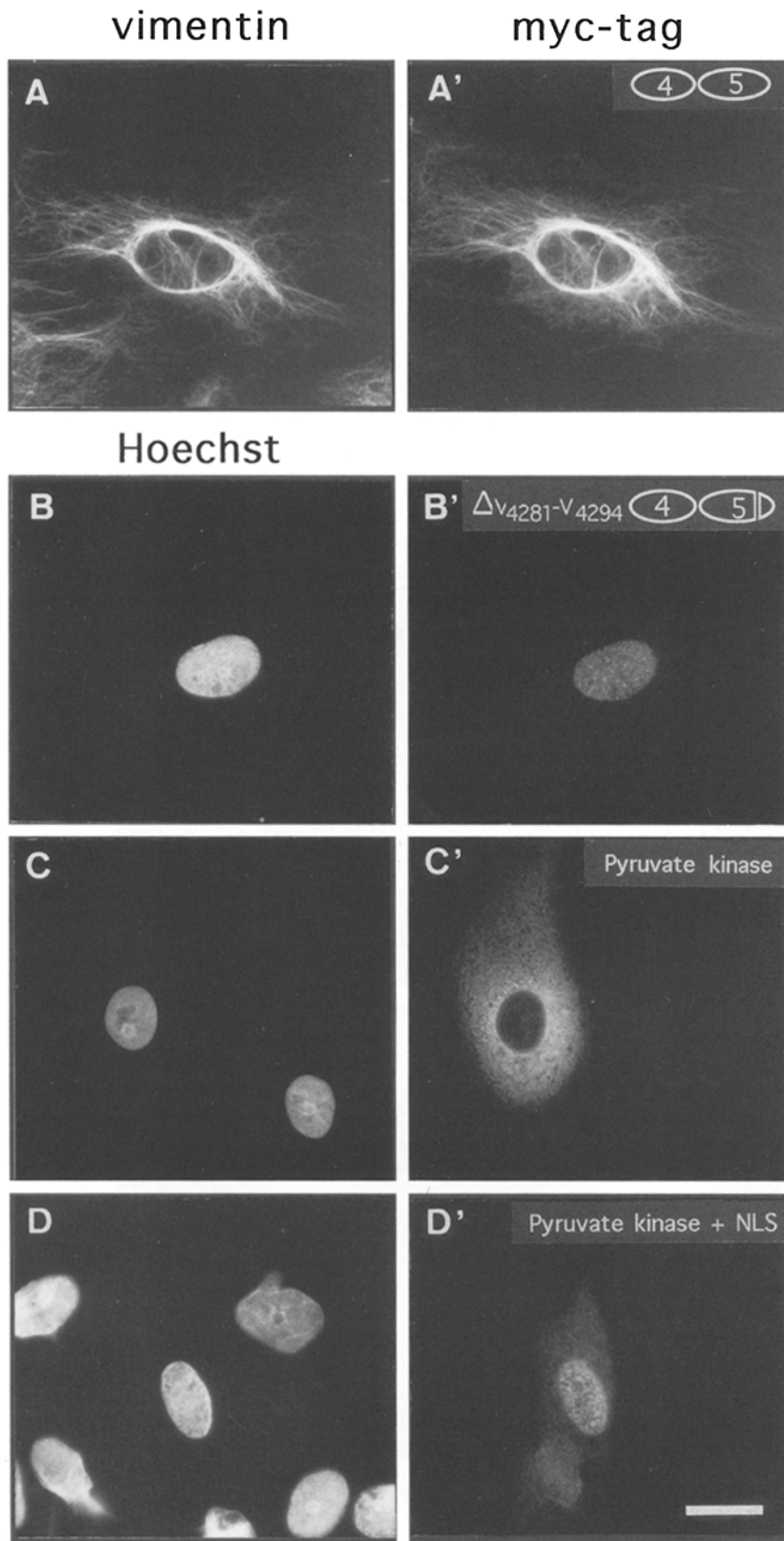


Figure 4. The NLS sequence domain within plectin's vimentin-binding site mediates nuclear import. Double immunofluorescence staining (*A, A'*) and immunostaining combined with nuclear (HOECHST dye) staining (*B, B'–D, D'*) of PtK2 cells are shown. Primary antibodies are indicated. Double panels show transfectants obtained using the following plasmids: *A, A'*, pBN16 (see Fig. 1); *B, B'*, pBN85 (see Fig. 1); *C, C'*, pBN86 (control myc-tagged pyruvate kinase); and *D, D'*, pBN88 (myc-tagged pyruvate kinase fused to plectin's 19-amino acid NLS sequence domain). Bar, 15 μ m.

cytoplasmic areas that apparently were free of filaments. Such a phenotype would have been consistent with a weaker binding of this mutant to the filamentous network, a notion supported by *in vitro* binding data (see below). Thus, apparently a sequence of ~50 amino acid residues within the carboxy-terminal one third of the repeat 5 domain (positions 4262-4316; see scheme on top of Fig. 1), was indispensable for filament association. As two other mutant proteins spanning over this domain but carrying deletions in the amino- and carboxy-terminal halves of it, pBN73 (data not shown) and pBN85 (Fig. 4 B), displayed negative phenotypes we concluded that most of this sequence, if not all of it, was required for colocalization with IFs in cells.

A Basic Cluster of Amino Acid Residues Located within a Functionally Intact NLS Sequence Motif Is Essential for Vimentin Binding

The NH₂-terminal part of the ~50-amino acid residue-long sequence required for filament association displayed structural characteristics typical of bipartite NLS sequence motifs, having two clusters of basic amino acids (residues 4262-4266; and 4277-4280; see Fig. 1) separated by a linker of 10 residues (Robbins et al., 1991). In addition, parts of this sequence, such as residues 4265-4269 (Fig. 1), qualified as typical SV40 T antigen-like NLS (Chelsky et al., 1989). The sequence connecting the basic clusters within this NLS motif contained one threonine and four serines, which could be targets for O-linked glycosylation and/or phosphorylation. In fact, several consensus sequence motifs for protein kinases, including kinases A and C, and cGMP-dependent kinase can be found within this sequence. To examine which structural elements of this sequence domain were essential for IF binding, several amino acid residues were alternatively mutated en bloc by site directed mutagenesis. Substitution of the basic cluster formed by arg⁴²⁷⁷-lys-arg-arg⁴²⁸⁰ (pBN83) by neutral amino acid residues led to a completely negative phenotype (Fig. 3 A), suggesting that this highly positively charged sequence was indispensable for IF binding. In contrast, neutralization of four more upstream SV40 T antigen-like basic residues by asparagine and alanine (pBN84) hardly had any detectable phenotypic effects (Fig. 3 C). Exchange of the threonine and four serines for valine and alanines in the segment connecting the two basic clusters, generated a mutant protein (pBN82) with a mixed phenotype, showing diffuse cytoplasmic distribution in addition to filament association (Fig. 3 B).

Although the NLS sequence domain of plectin's vimentin-binding region was insufficient without its carboxy-terminal flanking sequence to form a functional vimentin-binding domain, its NLS function seemed to be intact, as indicated by the exclusively nuclear localization of pBN85-encoded mutant protein, which contained the NLS domain but lacked its carboxy-terminal flanking sequence (Fig. 4 B). A similar mutant protein lacking the NLS domain, but containing its carboxy-terminal flanking sequence (pBN73; see Fig. 1) did not show this phenotype (data not shown). Moreover, when the 19-amino acid residue motif comprising plectin's bipartite NLS structure was linked by genetic engineering to pyruvate kinase, a typical cytoplasmic pro-

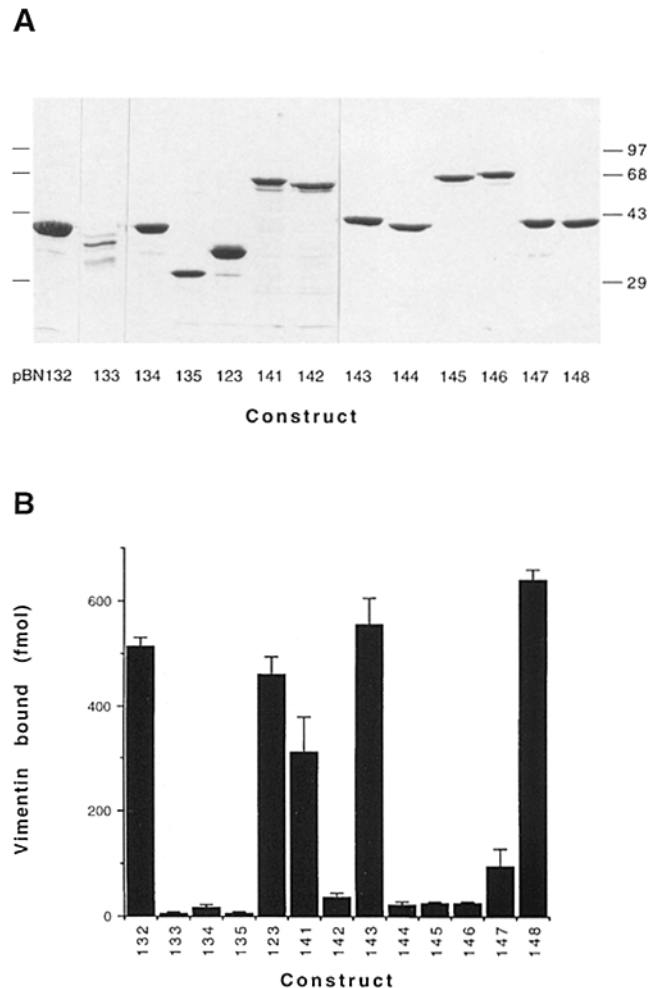


Figure 5. SDS-PAGE (A) and binding to Eu³⁺-labeled vimentin (B) of plectin mutant proteins expressed in bacteria. (A) Plectin mutants expressed in bacteria were purified as described in the text. Purified proteins were analyzed by SDS 10% PAGE. Gels were then stained with Coomassie blue. (B) Microtiter plates were coated with purified mutant proteins at equivalent concentrations (100 nM) and binding of 100 nM Eu³⁺-vimentin carried out as described. Data are presented as the mean ± SD of triplicate determinations. The experiment is typical of three.

tein, a myc-tagged version of the enzyme was found predominantly within the nuclear compartment, after ectopic expression in PtK2 cells (Fig. 4 D). In contrast, myc-tagged pyruvate kinase without the plectin NLS sequence retained its cytoplasmic localization (Fig. 4 C).

Mutant Proteins Expressed in Bacteria Display Binding Properties Similar to Those Expressed in Mammalian Cells

In vitro binding experiments were performed with corresponding plectin mutant proteins expressed in bacteria as histidine-tagged versions to enable their purification via affinity chromatography (Fig. 5 A). In a first series of experiments purified mutant proteins were coated onto microtiter plates, overlaid with Eu³⁺-labeled vimentin, and bound vimentin quantitatively determined after release of complexed Eu³⁺ and detection by time-resolved fluorome-

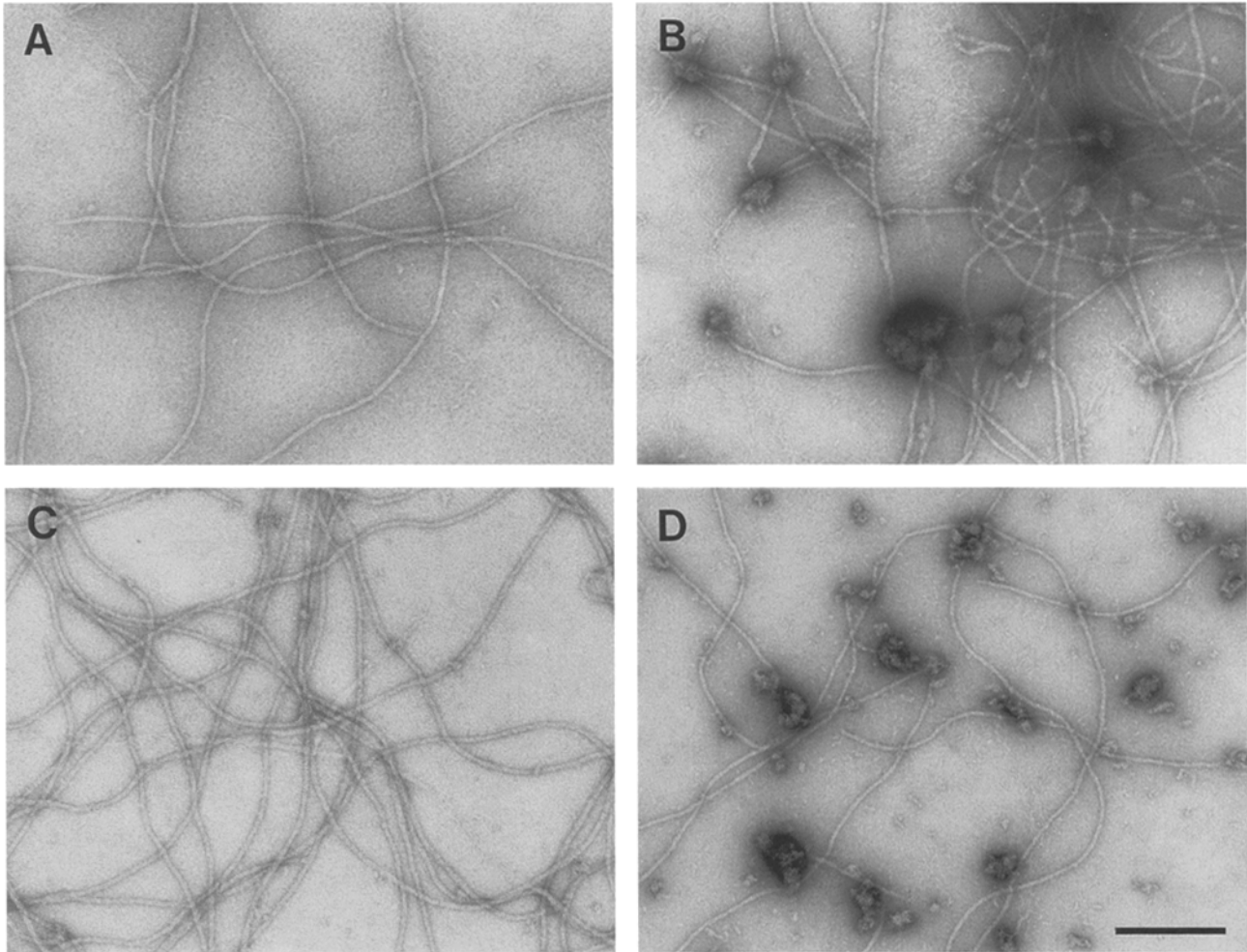


Figure 6. Electron microscopy of uranyl acetate-stained vimentin IFs decorated with plectin mutant proteins expressed in bacteria. IFs were reconstituted from purified C6 cell vimentin stocks and incubated with affinity-purified recombinant mutant proteins as described in the text. Negatively stained specimens of the following mixtures are shown: *A*, IFs alone (control); *B*, IFs and plectin repeat 5 (pBN132); *C*, IFs and plectin repeat 4 (pBN135); and *D*, IFs and mutagenized plectin repeat 5 (pBN134). Note loose tangles of filaments visualized in *A*, *C*, and *D*, but dense filamentous aggregates in *B*. Bar, 300 nm.

try (Soini and Lövgren, 1987). In agreement with the results obtained by cDNA transfection into cultured cells, strong binding was observed only with those mutant proteins which contained a fully intact vimentin-binding domain comprising the NLS sequence domain in repeat 5 and its carboxy-terminal flanking structure (Fig. 5 *B*). Scatchard analysis of binding data obtained with pBN132- and pBN123-encoded mutant proteins, corresponding to the entire repeat 5 domain and its amino-terminal truncated version, respectively, revealed dissociation constants in the range of $K_d = 0.5\text{--}0.9 \times 10^{-7}$ M, similar to purified native plectin (data not shown). On a scale in which the binding efficiency of the polypeptide corresponding to wild-type repeat 5 (pBN132) was arbitrarily taken as 100% (Fig. 1, last column), only two mutant proteins (pBN143 and pBN148) showed stronger binding (108% and 124%, respectively) maybe due to better accessibility of their vimentin-binding site. Repeat 4 and repeat 6 including plectin's tail showed no binding. In fact, since the mutant protein representing repeats 4 and 5 combined (pBN141) exhibited significantly reduced binding to vi-

mentin (61%) compared to repeat 5 protein alone, it seemed that the repeat 4 domain exerted an inhibitory effect. However, alternative explanations, including the possibility that repeat 5 being not in its usual protein sequence context, in particular without the preceding repeat 4 sequence, may have an artificially increased affinity for vimentin, cannot be ruled out. The carboxy-terminal truncated version (pBN142) of the mutant protein encoded by pBN141, which displayed a weak but still positive phenotype upon expression in cultured cells (pBN18; see Fig. 2 *A*), showed also rather little binding *in vitro*. Very little insignificant binding was observed with all other mutant proteins displaying negative phenotypes in transfection experiments, such as the deletion mutants pBN145 and pBN146, the amino-terminal truncated version of pBN143, pBN144, and mutant pBN134 carrying mutations in the essential basic amino acid residue cluster within the NLS sequence motif. Mutant pBN147 in which the cluster of hydroxy-amino acid residues had been mutagenized, showed only moderate binding activity (19%) compared to wild-type repeat 5 (pBN132). Again, this was consistent

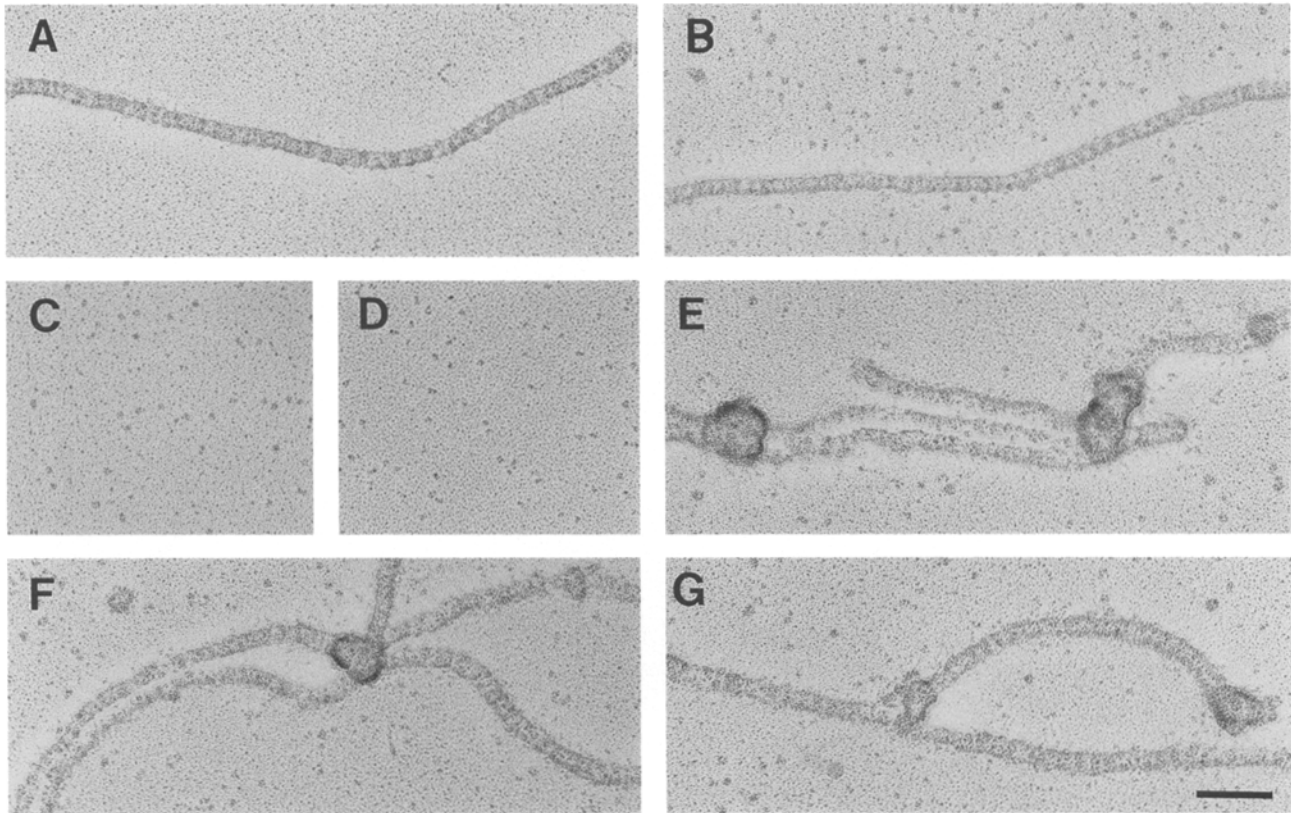


Figure 7. Plectin mutant protein-vimentin IF complexes visualized by rotary shadowing electron microscopy. Vimentin IFs were preassembled *in vitro*, incubated with or without purified recombinant plectin mutant proteins, and processed for rotary shadowing electron microscopy as described in the text. *A*, IFs alone; *B*, IFs and plectin repeat 4 (pBN135); *C*, plectin repeat 4 alone; *D*, plectin repeat 5 alone (pBN132); and *E–G*, IFs and plectin repeat 5. Note large filament-associated protein aggregates in *E–G*, not observed in the absence of filaments (*D*). Bar, 100 nm.

with our results obtained by cDNA transfection into cultured cells, considering that the corresponding mutant expressed in PtK2 cells in addition to filament association showed a somewhat diffuse distribution throughout the cytoplasm (Fig. 3 *B*). Thus, in general the *in vitro* binding data were in full agreement with the immunolocalization of mutant proteins expressed in cultured cells.

Decoration of Vimentin Filaments with Plectin Repeat 5 Domain Leads to Network Formation In Vitro

To assess effects on ultrastructure, filaments assembled *in vitro* from purified rat glioma C6 cell vimentin were incubated with selected plectin mutant proteins and examined by electron microscopy. After staining with uranyl acetate, filaments assembled from vimentin alone were observed predominantly in the form of loose filamentous meshworks (Fig. 6 *A*), whereas filaments incubated at a roughly equimolar ratio with plectin repeat 5 formed dense aggregates. In general these aggregates were too dense to enable resolution of substructures, except for areas at their periphery, where filaments were spread more evenly upon the grid. In such areas the filaments were heavily decorated with globular structures of various sizes, presumably consisting of clustered repeat 5 protein domains (Fig. 6 *B*). These structures were associated predominantly with junctional sites of the filament network, but occasionally they

were also observed at the ends of single individual filaments. Notably, some of the filaments appeared to be shorter, compared to those visualized in the control sample without mutant protein (compare Fig. 6, *A* and *B*). Globular structures not associated with filaments were rarely observed. No distinct structures were observed in negatively stained samples containing repeat 5 protein without IFs (data not shown). Statistics of several micrographs indicated that of the ~400 filament-bound protein complexes evaluated, 91% appeared to be junctional, i.e., they were associated with more than a single continuous filament, 2.5% were situated at the ends of single filaments, and 6.5% appeared to be laterally associated with filaments.

When similar filament preparations were incubated with recombinant plectin repeat 4 domain, lacking a vimentin-binding site (Fig. 6 *C*), the ultrastructural appearance of filament networks closely resembled that of the control sample (compare Fig. 6 *C* with 6 *A*). Finally, filaments were incubated with the basic cluster-defective pBN134-encoded mutant protein exhibiting only very low vimentin-binding activity in solid phase binding assays (Fig. 5 *B*). In this case the vimentin filament network, although decorated with numerous globular aggregates, appeared much less dense and more spread out (Fig. 6 *D*) than networks decorated with wild-type repeat 5 domain (Fig. 6 *B*). In addition, a relatively high background of globular material not associated with filaments was noticeable.

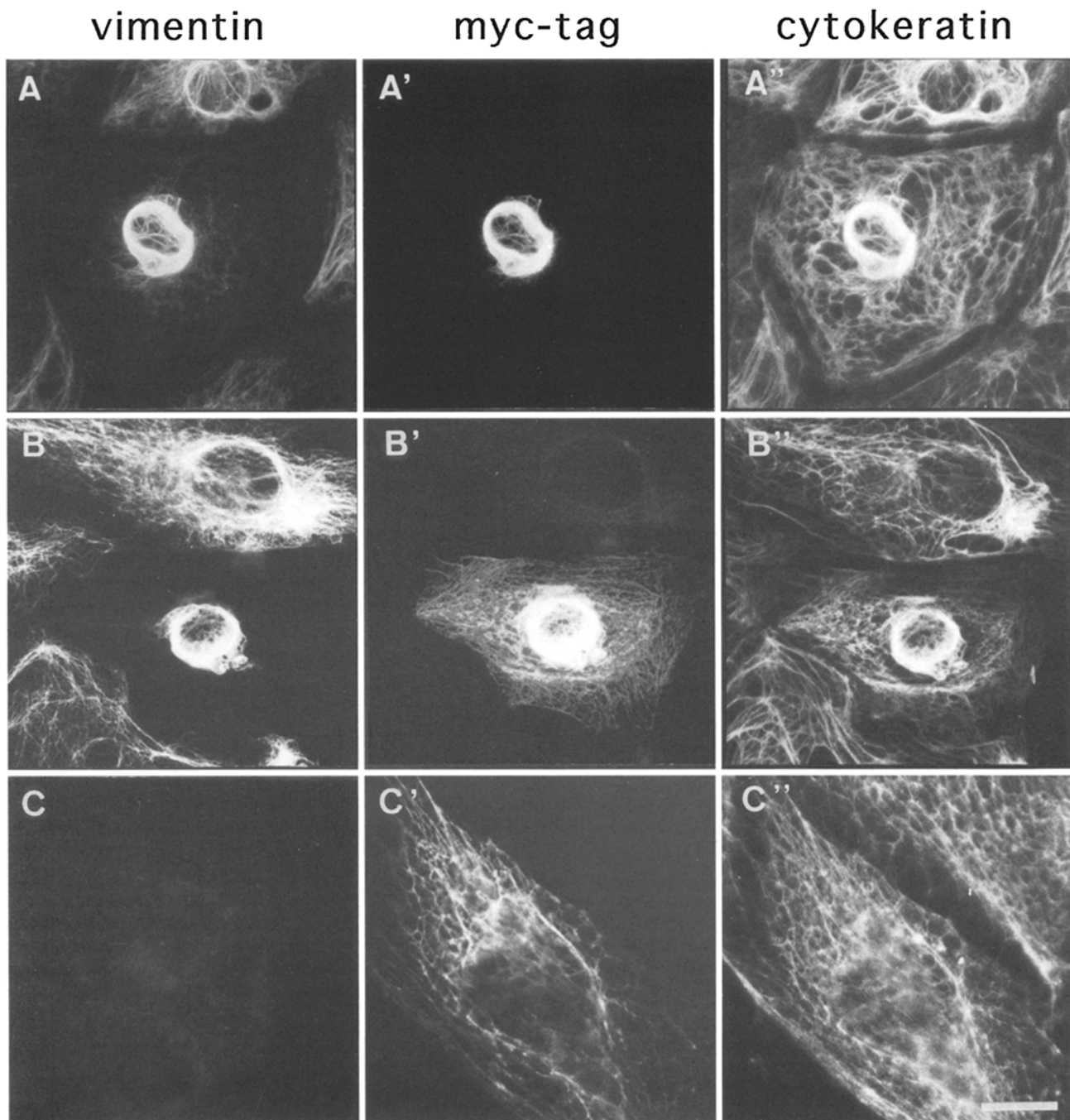


Figure 8. Association of plectin repeat 5 domain with cytokeratin IFs in PtK2 (A, A', A'' and B, B', B'') and Pam 212 cells (C, C', C'') demonstrated by triple immunofluorescence microscopy. Primary antibodies used are indicated. Plasmid pBN36 was used for transfection. Note codistribution of mutant protein with collapsed vimentin IFs in both A' and B', but additional association with extended cytokeratin networks only in B'. No staining of Pam 212 cells was observed with antibodies to vimentin (C). Bar, 15 μ m.

Additional structural detail was revealed when similar specimens were visualized after rotary shadowing with heavy metals (Tyler and Branton, 1980). In this case, in samples of both repeat 4 alone (Fig. 7 C) and 5 alone (Fig. 7 D), we observed particles of fairly homogenous sizes with diameters between ~ 5 and ~ 10 nm. Although hardly any such particles were detected in preparations of *in vitro* assembled filaments that were processed in the absence of recombinant plectin mutant proteins (Fig. 7 A), they were

clearly seen spread over the entire grid in preparations of filaments incubated with either plectin repeat 4 or 5. However, in contrast to plectin repeat 4 proteins, which, as expected, showed no preferential association with filaments (Fig. 7 B), in the case of repeat 5 proteins large filament-associated protein aggregates were observed apart from the unbound smaller particles (Fig. 7, E–G). Similar to uranyl acetate-stained specimens, the aggregated proteins were predominantly found at junctional sites of the fila-

ment network (Fig. 7, *E* and *F*) and occasionally also at the ends of relatively short filaments (Fig. 7 *G*), generating cap-like structures. It is likely that capped rather than continuous filaments constituted at least part of some junctional complexes, considering the often uneven number of protruding filaments (for example Fig. 7 *F*). Since large globular structures resembling those observed at filament junctions were not detected in the control sample containing repeat 5 alone (Fig. 7 *D*), it is likely that they formed only upon binding of repeat 5 to filaments. This may occur due to the clustering of free-binding sites for plectin in certain areas of the filament, such as at their ends, or cooperative interactions between bound repeat 5 domains, or both. Studies to distinguish between these and other possible mechanisms have been initiated.

Ectopically Expressed Plectin Repeat 5 Domain Binds to Both Vimentin and Cytokeratin Filaments

To investigate whether the IF-binding site within repeat 5 was vimentin-specific the effects of its overexpression (pBN36) on both the vimentin and the cytokeratin IF networks were studied in PtK2 cells using triple fluorescence microscopy. In transiently transfected cells expressing comparatively low levels of the mutant protein, codistribution with vimentin but not cytokeratin IFs was observed; these included cells in which the vimentin filament system had collapsed into perinuclear aggregates, while the cytokeratin filament system was still intact (Fig. 8 *A*). However, in cells with high level expression (as judged by fluorescence intensities) the mutant protein was found associated with both collapsed vimentin filaments and an intact (Fig. 8 *B*), or similarly collapsed, cytoplasmic cytokeratin network (data not shown). These observations were consistent with the notion that repeat 5 in fact contained binding sites for both vimentin and cytokeratin IFs. Apparently, however, the mutant protein bound to filaments of the vimentin type with preference over those of the cytokeratin type. The ability of plectin repeat 5 protein to associate with cytokeratin filaments was not restricted to cells derived from simple epithelia, such as PtK2, but applied also to epidermal, mostly vimentin-free keratinocytes, such as mouse Pam 212 cells (Fig. 8 *C*) and human A431 cells (not shown).

Discussion

Our initial study on plectin-IF interactions using transient transfection of plectin mutant cDNAs into cultured cells (Wiche et al., 1993) indicated that the COOH-terminal domain of plectin, which contains several repeats of homologous structure, plays an important role in this association. Using a similar approach in conjunction with *in vitro* binding and electron microscopic assays involving plectin mutant polypeptides expressed in bacteria, we have now narrowed down the vimentin-binding site of plectin to a relatively short stretch of ~50 amino acid residues residing at the end of its COOH-terminal repeat 5 domain. In addition, we show evidence that plectin's repeat 5 domain contains also a binding site for cytokeratins.

The six COOH-terminal repeat domains of plectin share a strongly conserved ~200 residue long central region that

is constructed from tandem repeats of a 19-residue motif. The IF-binding domain characterized in this study resides right after the repeat 5 core region and comprises ~50 residues. Overall, this region shares hardly any homology with the corresponding regions of the other five repeats constituting the COOH-terminal globular domain of plectin. (Note that the regions between the core domains of each repeat and the beginning of the next repeat downstream including the COOH terminus vary in length between 7 [repeat 4] and 109 [repeat 5] amino acid residues.) While the amino-terminal boundary of the IF-binding site could be defined rather precisely (lys⁴²⁶²), using both transfection and *in vitro* binding assays (Figs. 2 and 5), its carboxy-terminal extension was less evident. A deletion mutant protein (pBN85) which lacks 14 flanking amino acid residues downstream of the essential NLS-like sequence domain displayed clearly a negative phenotype, while a carboxy-terminal truncated mutant protein (pBN18) which extends 36 amino acid residues beyond the NLS-like sequence domain produced a positive/negative phenotype (Figs. 2 and 5), indicating that binding occurred but only to a limited extent. Since the phenotype of a similar mutant (pBN17) with a 35 residue longer flanking sequence was unambiguously positive (data not shown), we conclude that a sequence starting at the amino terminus of the bipartite NLS domain and containing between 55 and 90 downstream amino acid residues is required for optimal binding. The flanking domain upstream of the NLS sequence, on the other hand, seems dispensable, if not inhibitory for binding, considering that an amino-terminal truncated mutant protein (pBN56) starting just with the first basic cluster of the NLS sequence domain showed higher binding activity than mutants containing upstream flanking sequences, including one corresponding to repeats 4 and 5 combined (pBN16).

In vitro mutagenesis of different structural elements of the NLS sequence domain within plectin's IF-binding site indicated that the arg-lys-arg-arg cluster at position 4277-4280 at the carboxy-terminal boundary of the NLS domain was crucial for binding. Unimpaired function of the IF-binding site seemed to require also one or more residues of the hydroxyamino acid cluster (thr⁴²⁷⁰-ser⁴²⁷⁵) preceding this basic cluster, since its mutagenesis effected a substantial decrease in binding. Interestingly, neutralization of a more 5' basic cluster (residues 4265-4269) had a slightly positive rather than negative effect on binding. It remains to be shown whether constituents of these clusters serve as targets for secondary protein modifications, such as phosphorylation or acetylation.

It might be fortuitous that the same sequence motif that enables a protein to enter the nucleus serves as an essential structural element of the plectin-IF network complex, a cytoskeletal junction thought to provide cells with mechanical strength. On the other hand, the observation that plectin binds to IFs via a domain comprising an NLS sequence motif may suggest that IFs, in particular those of the vimentin type, may serve as cytoplasmic docking sites for proteins containing such sequences. Such docking could be important during various cell cycle phases, particularly M-phase, when nuclear proteins are no longer spatially restricted by a nuclear envelope. In this context it is of interest that plectin and vimentin structures, which co-

distribute in cultured cells throughout interphase, dissociate during M-phase in conjunction with plectin phosphorylation by p34^{cdc2} protein kinase (Foisner et al., 1996). A possible scenario could thus involve the same site on vimentin molecules in plectin-vimentin network formation leading to mechanical strengthening of cells during interphase, and cytoplasmic docking of nuclear proteins during M-phase. In postmitotic cells, reestablished plectin-vimentin interactions could lead to the release of NLS-bearing proteins and their re-entry into the nucleus. Consistent with this hypothesis, vimentin filaments have been shown during mitosis to serve as a transient docking site for inner nuclear membrane vesicles carrying on their surface proteins with NLS sequences, such as lamin B and nuclear membrane protein p58 (Maison et al., 1993). Plectin may also play a role in cellular functions involving its direct interaction with cytoplasmic NLS-binding proteins of the nuclear transport carrier machinery, such as Srp1-like proteins (Adam, 1995). The potential ability of plectin to interact with such proteins was indicated by efficient nuclear import of a plectin mutant protein (pBN85) having an intact NLS sequence, but lacking the remaining part of the vimentin-binding domain. However, any of the above possibilities related to a potential role of plectin's NLS signal motif apart from IF binding, as well as to mechanisms mediating nuclear import of plectin mutants, remain speculative and require further investigation.

Sharing extensive structural homology, particularly in their COOH-terminal repeat domains, plectin, desmoplakin, and BPA-1, are considered to comprise a novel gene family involved in IF organization (Wiche et al., 1991; Green et al., 1992; Liu et al., 1996). In fact, in desmoplakin, but not BPA-1, the region corresponding to plectin's vimentin-binding domain, including its NLS sequence motif, is highly conserved. The COOH-terminal globular domain of desmoplakin comprises three repeat domains (Green et al., 1990) compared to six found in plectin, with the highest degree of homology found in the last two domains of both molecules. Upon examination of the sequence alignment of plectin's repeat 5 and desmoplakin's repeat B domains, an equivalent NLS sequence motif was found in desmoplakin at positions 1328-1346. Human and rat plectin are 100% identical in this region (Liu et al., 1996). Consistent with our analysis of plectin, based on truncation mutagenesis, Stappenbeck et al. (1993) suggested that the molecular domain of desmoplakin necessary for binding to vimentin resided within the COOH-terminal globular domain somewhere upstream of repeat C. Whether the region identified in plectin also serves as a vimentin-binding site in desmoplakin remains to be shown, especially as a couple of nonconserved cysteine residues found in these regions may alter the conformation and function of the corresponding domains in both proteins.

The appearances of IF networks reconstituted from *in vitro* assembled vimentin filaments and plectin repeat 5 domain were similar to those reported for filaments decorated with whole plectin molecules purified from glioma C6 cells (Foisner et al., 1988). Intact plectin molecules in solution resemble dumbbell-shaped rod structures that have a high tendency to aggregate via their globular end domains flanking the rod (Foisner and Wiche, 1987; Weitzer and Wiche, 1987). Under the conditions we used

for visualizing IF networks in the electron microscope, both whole plectin molecules (Foisner et al., 1988) and recombinant repeat 5 protein (this study) appear as globular filament-attached aggregates of ill-defined structure and size. In both cases one can assume that at least some of the highly hydrophilic IF-binding sites of plectin molecules are exposed on the surface of these aggregates leading to the bridging of filaments and eventually to their packaging into dense networks. This may also be the mechanism underlying the collapse of IF networks in cultured cells upon transfection of cDNA constructs encoding mutant proteins containing plectin's IF-binding site.

It may be argued that recombinant proteins expressed in bacteria might possess different properties, including binding specificities, compared to their counterparts expressed in mammalian cells. This seemed to pose little problem, however, in this study because in general the results obtained by transfection of plectin mutant cDNA into cultured cells were fully consistent with those based on *in vitro* binding assays using proteins expressed in bacteria. The quantitative *in vitro* assay we applied in this study measures the binding of Eu³⁺-labeled vimentin in solution to plectin proteins immobilized on plastic dishes. One of the advantages of this assay was that due to its high sensitivity the concentration of soluble vimentin could be kept low enough (nM range) to minimize distorting effects due to aggregation of the IF protein. In fact, we observed a concentration-dependent binding of vimentin to immobilized plectin repeat 5 in the range of 1–500 nM, enabling the determination of a $K_d = 90$ nM, similar to that of purified whole plectin, by Scatchard plot analysis. A similar, more detailed study of the binding behavior of recombinant plectin mutant proteins corresponding to different domains of the molecule and including other previously identified binding partners of plectin is currently being carried out (to be published elsewhere).

Transient transfection of cDNAs into simple epithelia-derived PtK2 cells, which expressed both a vimentin and a cytokeratin IF network, and into the epidermal keratinocyte cell lines, Pam 212 and A431, which expressed only cytokeratins, indicated that repeat 5 contained a cytokeratin-binding site in addition to its vimentin-binding site. Since in PtK2 cells none of the mutant proteins deficient in binding to vimentin showed binding to the cytokeratin network, it is likely that plectin interacts with both filament systems of this cell type via the same molecular domain. However, association of the mutant protein with the cytokeratin network of PtK2 cells seemed to be less favored than that with vimentin, indicating differential binding affinities of the repeat 5 domain for these networks. Whether this might apply also to cytokeratin filaments of different subunit composition, such as those from simple epithelia vs those from stratified epithelia, or basal vs suprabasal epidermal keratinocytes, remains an interesting question to be studied. Furthermore, the possibility that molecular domains other than, or in addition to, repeat 5 are involved in plectin's interactions with various cytokeratin IFs cannot be ruled out. In fact, preliminary *in vitro* binding data suggest that plectin has more than one binding site for keratins isolated from human skin (unpublished results).

In line with plectin's proposed role as a cytoskeletal

cross-linking element that provides cells with mechanical strength, studies by Gache et al. (1996) indicate that defects in plectin play a role in epidermolysis bullosa simplex (EBS) with muscular dystrophy, an autosomal recessive skin blistering disease associated with a dystrophic muscle condition. Moreover, genetic linkage analysis indicates that defects in plectin may also be involved in EBS-Ogna, a severe skin blistering disease associated with a dominant mutation (Gedde-Dahl, 1990; and unpublished observations). It will be a challenging task for future research to investigate whether the mutations of the plectin gene involved in these and other diseases affect the IF-binding site of plectin characterized in this study.

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