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# Interaction of plectin and intermediate filaments

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ABSTRACT

*Background:* Plectin, a member of the plakin family proteins, is a high molecular weight protein that is ubiquitously expressed. It acts as a cytolinker for the three major components of the cytoskeleton, namely actin microfilaments, microtubules and intermediate filaments.
*Objective:* The aim of our experiments was to identify new binding sites for intermediate filaments on plectin and to specify these sites.
*Methods:* We introduced truncated forms of plectin into several cell lines and observe interaction between plectin and intermediate filaments.
*Results:* We found that a linker region in the COOH-terminal end of plectin was required for the association of the protein with intermediate filaments. In addition, we also demonstrated that a serine residue at position 4645 of plectin may have a role on binding of plectin to intermediate filaments.
*Conclusion:* A linker region in the COOH-terminal end and serine residue at position 4645 may be important for the binding of plectin to intermediate filaments.


## 1. Introduction

Proteins of the plakin family are involved in the organization of the cytoskeleton [1,2]. Plakins have been shown to function as cytolinkers and/or scaffolding proteins; they connect intermediate filaments (IFs) to other cytoskeletal proteins and tether IFs to the plasma membrane [2]. The plakin family includes plectin, desmoplakin, BP230/BPAG1 envoplakin, periplakin, epiplakin and microtubule-actin crosslinking factor [2].

Plectin is a high molecular weight (500 kDa) protein that is ubiquitously expressed and acts as a cytolinker for the three major cyotoskeletal components, actin microfilaments, microtubules and IFs [1,3]. Plectin also plays an important role in maintaining the mechanical integrity of tissue [4]. The protein has a dimeric structure composed of a central coiled-coil, helical rod connected to large globular domain at each terminus [5,6]. The globular domains in plectin are responsible for the binding to cytoskeletal targets [7]. The COOH terminal domain is formed by 6 highly homologous repeating regions [8]. The five repeats adjacent to the central rod domain are termed B repeats and the sixth on the COOH terminal side is called C repeat [8]. These 5 B and 1 C repeats collectively form the plakin-repeat domain (PRD) [8]. Plectin is known to interact with IFs via the PRD that associates with vimentin and keratin 8/keratin 18 [9–11]. The $NH_2$-terminal globular domain is known to be responsible for binding to actin [12].

As mentioned above, plectin acts as a linker between cytoskeletal elements. In addition, it also connects the cytoskeleton to cytoskeleton-related proteins by its ability to bind to the myosin II motor proteins, α-spectrin and fodrin [13,14]. Moreover, plectin can mediate connection of keratin IFs to the major junction complex, hemidesmosomes, by its various binding site [2]. The importance of plectin in tissue integrity was confirmed by a study on mice with a targeted inactivation of the plectin gene: these mice showed defects in the integrity of the cytoskeleton in stratified epithelia, including skin and striated muscles, and died between 1 and 3 days after birth because of skin blistering and skeletal and cardiac defects [15].

Detailed analyses of the binding domain on plectin for IFs have been carried out and have identified a small stretch of basic residues in the fifth B repeat to the linker as important [9–11]. Here, we used deletion constructs to screen for new binding sites in plectin and also sought to further characterize the IF binding sites on the protein.

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## 2. Materials and methods

### 2.1. Generation of cDNA constructs (Fig. 1)

The following cDNA clones (GenBank accession number G02520) were constructed by PCR deletion/point mutagenesis according to the previously reported method using plectin C-ter-HA/pEGFP C2 as the template [16]: deletion of the last B repeat from plectin C-ter/pEGFP C2 (deletion mutant ΔAA 3971-4146; Plectin-C-ter ΔB repeat); deletion of the C repeat and tail from plectin C-ter/pEGFP C2 (deletion mutant ΔAA 4316-4574; Plectin-C-ter ΔCT); deletion of the B and C repeats from plectin C-ter/pEGFP (deletion mutant ΔAA 3971-4146 and 4316-4491; Plectin-C-ter ΔBC repeat); deletion of the tail from plectin C-ter/pEGFP (deletion mutant ΔAA 4492-4574; Plectin-C-ter Δtail); deletion of the B repeat, C repeat and the tail from plectin C-ter/pEGFP (deletion mutant ΔAA 3971-4146 and 4316-4574; Plectin-C-ter linker); a point mutation at the corresponding serine residue at S4645 (PLECTIN-C-ter$^{S4645G}$). The PCR primers used in this study is as follows: Plectin-C-ter B repeat forward: ACCATGAAG-GAACGGCTCTCGGTGTACCAG; Plectin-C-ter B repeat reverse: CAAGAGACACAGGCCCGTGGGGTCAG; Plectin-C-ter linker forward: CCGCTGAAGGAGAAGAAGCGGGA; Plectin-C-ter linker reverse: CGTCTCCCGTGTCCAGGATGCCAGCCAC; Plectin-C-ter C repeat forward: CTGGAGAAGGTGTCCATCAC; Plectin-C-ter C repeat reverse: CAGCAGCCGCCCCGTGCCCTC; Plectin-C-ter tail forward: GAGGCTGCCGCGCAGTCCACCAA; Plectin-C-ter S4645G forward; GGCTTTGACGCCACCGGCTCCGGCTTCTC. The following PCR conditions were used: 25 cycles at 94 °C for 2 min, 50–65 °C (depending on the melting temperature of the primer) for 2 min, and 70 °C for 4 min. The reactions were performed using a 50 μl PCR mixture containing 200 μM of each dNTP, 2 ng plasmid DNA template, 2 μM each of the primers, 1 unit of *pfu* turbo DNA polymerase (Stratagene, La Jolla, CA) and 5 μl of 10× buffer (Stratagene). One μl aliquot of the PCR product was self-ligated in 10 μl of 66 mM Tris–HCl, pH 7.4, 8 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 5 units of T4 polynucleotide kinase and 5 units of T4 DNA ligase (New England BioLabs, Hitchin, Hertfordshire, UK) at 16 °C for 1 h. The resulting constructs were used to transform DH5α bacteria. Some clones were inserted into a DsRed monomer vector (Invitrogen, Carlsbad, CA).

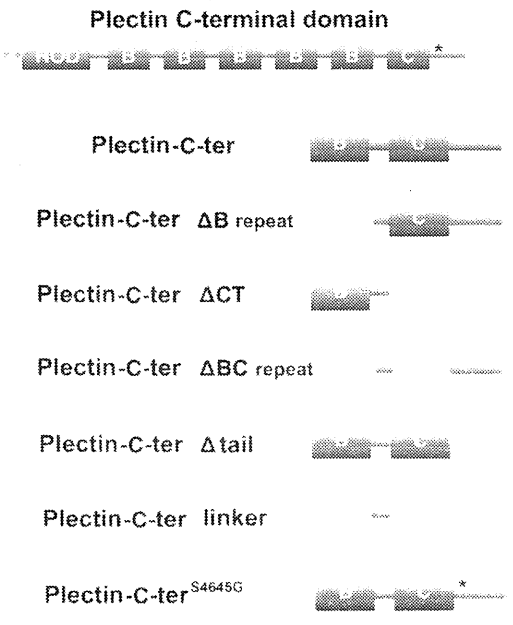


**Fig. 1.** Schematic diagrams of the constructs used in this study. ROD, rod domain; B, B repeat domains; C, C repeat domain; *serine 4645.

cDNA constructs of keratin (K) 5 and K14 were generated using standard cloning techniques. Briefly, random primers were used to synthesize cDNA from mRNA obtained from cultured normal human keratinocytes (NHKs) from normal human adult skin using ReverTra Ace Kit (Toyobo, Osaka, Japan). A pool of synthesized cDNA was used as the PCR template to generate human K5 and K14 constructs. Plasmid inserts were generated by PCR using the proofreading pfu turbo DNA polymerase (Stratagene, La Jolla, CA) and gene-specific sense and antisense primers. PCR products were subcloned into PCR Blunt 2.1 (Invitrogen, Carlsbad, CA) and inserted into pCI-neo (Promega, Madison, WI) in case of K5 and into pEDsRed-C2 monomer vector (Clontech, Mountain View, CA).

All plasmid constructs were verified using a dye-terminator cycle sequencing kit and an ABI PRISM 3000 (Applied Biosystems, Carlsbad, CA). The constructs of plectin short vectors used in this study are illustrated in Fig. 1.

### 2.2. Transient transfection of cells

COS7 cells (African green monkey kidney derived epithelial cell) were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS. NHKs were cultured first in defined K-SFM (Invitrogen) containing 0.15 mM $CaCl_2$ and Epilife Defined Growth Supplement. Then, we changed the media to K-SFM medium containing 1.8 mM $CaCl_2$ for 24 h before the experiment. The human adrenal adenocarcinoma cell line, SW-13, was obtained from Dainippon Pharma, Japan. SW-13 cells express vimentin in a mosaic pattern and the original population comprises vimentin-positive and vimentin-negative cells [17,18]. A vimentin-negative subclone of SW-13 cells, SW-13 VIM−/− cells, were selected after limiting dilution cloning. SW-13 VIM−/− cells were also maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS. The day before transfection, cells were seeded on 13 mm diameter glass coverslips in 24-well plates at a density of $5 \times 10^4$ cells/well. The cells were treated with the transfection reaction mixture for 2–3 h, washed with PBS, and transferred to DMEM containing 10% FCS (COS7 cells and SW-13 VIM−/− cells) or K-SFM (NHKs). The transfection mixture consisted of FuGene 6 transfection reagent (Roche, Indianapolis, IN) or ExGen 500 transfection reagent (Fermentas, Glen Burnie, MD) combined with DMEM (COS7 cells and SW-13 VIM−/− cells)/K-SFM (NHKs) and plasmid DNA, according to the manufacturer's protocol.

### 2.3. Immunofluorescence analysis

Protein staining was performed using the following antibodies: mouse monoclonal anti-keratin 14 (LL002; Novocastra, Benton Lane, Newcastle, UK); mouse monoclonal anti-vimentin (V9; Novocastra), mouse monoclonal anti-β-tubulin (TUB 1A2; Sigma–Aldrich, Carlsbad, CA), rabbit anti-HA (Y-11; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Alexa 488- or Alexa 594-conjugated goat anti-mouse or anti-rabbit IgGs (Molecular Probes, Invitrogen, Carlsbad, CA) were used as secondary antibodies.

The cells were either fixed and stained without pretreatment or extracted with saponin before fixation. Cells in the former group were fixed in cold 1:1 acetone/methanol for 5 min on ice or in 4% paraformalehyde (Sigma–Aldrich)/PBS for 20 min at room temperature. Paraformaldehyde-fixed cells were subsequently permeabilized with 0.2% Triton X-100 (Sigma–Aldrich) for 5 min at room temperature. After fixation, cells were washed in PBS and blocked in a 1:500 dilution of normal goat serum (Sigma–Aldrich) in PBS for 10 min at room temperature. Cells were incubated with primary antibodies for 45 min at room temperature, washed in PBS, and then incubated for a further 45 min at room temperature with the appropriate Alexa-conjugated secondary antibodies.

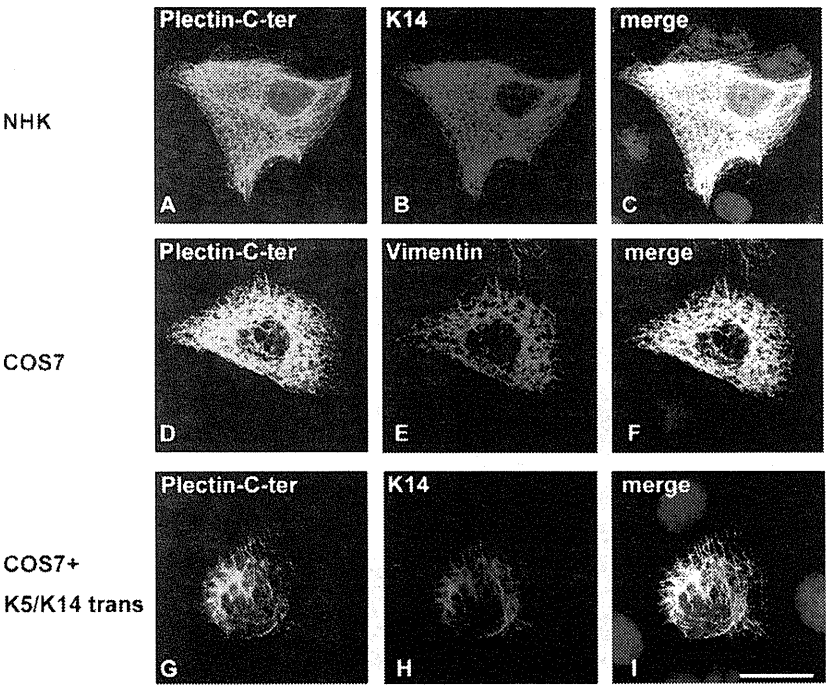


**Fig. 2.** Immunofluorescent staining of Plectin-C-ter and IF proteins. (Upper panel) Distribution of C-terminal plectin and K14 in NHKs: A, Plectin-C-ter; B, K14; C, merged image. Note the co-localization of the two proteins. (Middle panel) Distribution of C-terminal plectin and vimentin in COS7 cells: D, Plectin-C-ter; E, vimentin; F, merged image. Note the co-localization of the C-terminal plectin and vimentin. (Lower panel) Distribution of C-terminal plectin and K14 in K5/K14 vectors transfected-COS7 cells: G, Plectin-C-ter; H, K14; I, merged image. Note the co-localization of the C-terminal plectin and K14. The scale bar represents 20 μm.

Polymerized actin was detected with Alexa 594-conjugated phalloidin (Molecular Probes). Cell nuclei were counterstained with TOTO-3 (Molecular Probes). After further washing in PBS and distilled water, coverslips were mounted in Gelvatol (Monsanto, St. Louis, MO) and examined using a laser scanning confocal microscope (LSM 310 PASCAL; Carl Zeiss, Oberkochen, Germany).

## 3. Results

### *3.1. Distribution of plectin and IFs in cells transfected with plectin deletion constructs*

We transfected normal human keratinocytes with a construct carrying the plectin C-terminus (Plectin-C-ter), which encom-

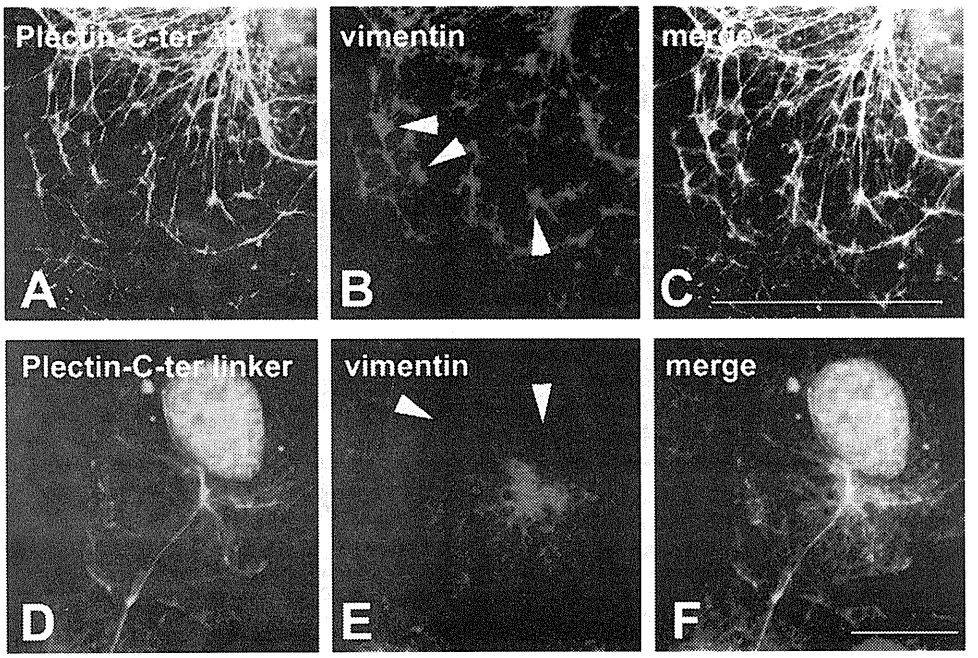


**Fig. 3.** Immunofluorescent staining of short constructs of Plectin-C-ter in COS7 cells. (Upper panel) A, Plectin-C-ter ΔB repeat; B, vimentin; C, merged image. (Lower panel) D, Plectin-C-ter linker; E, vimentin, F, merged image. Note the co-localization of plectin-C ΔB repeat protein and vimentin and the partial disruption of vimentin (arrowheads) in A–C. Note the dominant negative effect and disruption of vimentin (arrowheads) in transfection with the short C-terminal fragment (plectin-C-ter linker) on vimentin in D–F. The scale bar represents 20 μm.

passes B repeat, linker, C repeat and tail subdomains. Then, the patterns of expression of the Plectin-C-ter were compared with those of endogenous K14. Immunocytochemical staining showed expression of Plectin-C-ter as filamentous structures in the cells (Fig. 2A). These filaments were colocalized with the endogenous K14 in normal human keratinocytes (Fig. 2A–C). Next, we transfected COS7 cells with this construct, and compared expression of Plectin-C-ter with that of endogenous vimentin. The construct showed a filamentous staining pattern that was co-localized with vimentin IFs (Fig. 2D–F). Then, we transfected COS7 cells with this construct and full length K5 and full length K14 vectors (K5/K14 vectors), and compared expression of Plectin-C-ter with that of exogenous K14. The construct showed a filamentous staining pattern that was co-localized with K14 (Fig. 2G–I).

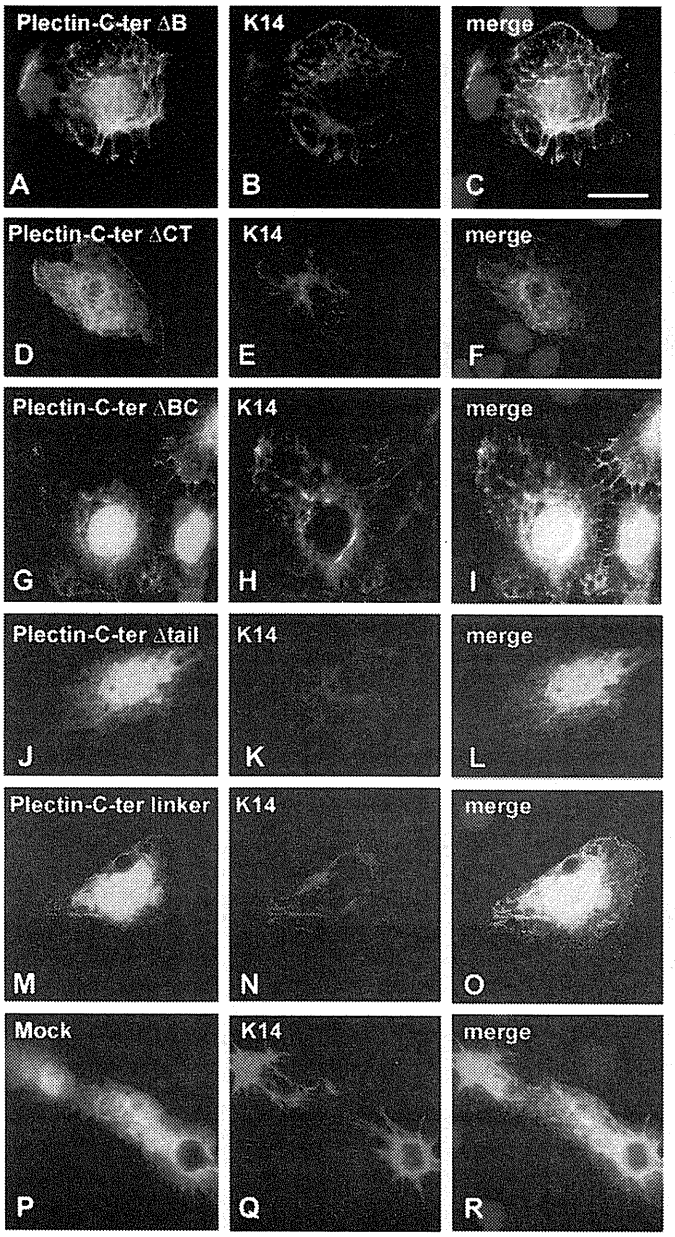


**Fig. 4.** Immunofluorescent staining of short constructs of Plectin-C-ter in K5/14 vectors-transfected COS7 cells. (A–C) Plectin-C-ter ΔB repeat, K5/14 vectors triple transfected cells: A, Plectin-C-ter ΔB repeat; B, K14, C, merged image. (D–F) Plectin-C-ter ΔCT, K5/14 vectors triple transfected cells: D, Plectin-C-ter ΔCT; B, K14, C, merged image. (G–I) Plectin-C-ter ΔBC repeat, K5/14 vectors triple transfected cells: G, Plectin-C-ter ΔBC repeat; H, K14, I, merged image. (J–L) Plectin-C-ter Δtail, K5/14 vectors triple transfected cells: J, Plectin-C-ter Δtail; K, K14, L, merged image. (M–O) Plectin-C-ter linker, K5/14 vectors triple transfected cells: M, Plectin-C-ter linker; N, K14, O, merged image. (P–R) Mock, K5/14 vectors triple transfected cells: P, mock, Q, K14, R, merged image. Note the co-localization of plectin-C-ter ΔB repeat, ΔBC repeat and Δtail protein and K14 and the partial disruption of keratin in A–C, G–I and J–L. The scale bar represents 20 μm.

### 3.2. Transfection of cells with short mutation constructs of Plectin-C-ter

Next, we transfected COS7 cells with constructs with shortened C-terminal domain in order to identify the region essential to IF binding. Five shortened constructs of the C-terminal domain were investigated: Plectin-C-ter ΔB repeat, Plectin-C-ter ΔCT, Plectin-C-ter ΔBC repeat, Plectin-C-ter Δtail, and Plectin-C-ter linker (Fig. 1). The Plectin-C-ter ΔB repeat fragment generally showed colocalization with vimentin IFs (Fig. 3A–C). However, the cells also showed disrupted distribution patterns of vimentin IFs; in particular, large accumulations of vimentin were occasionally

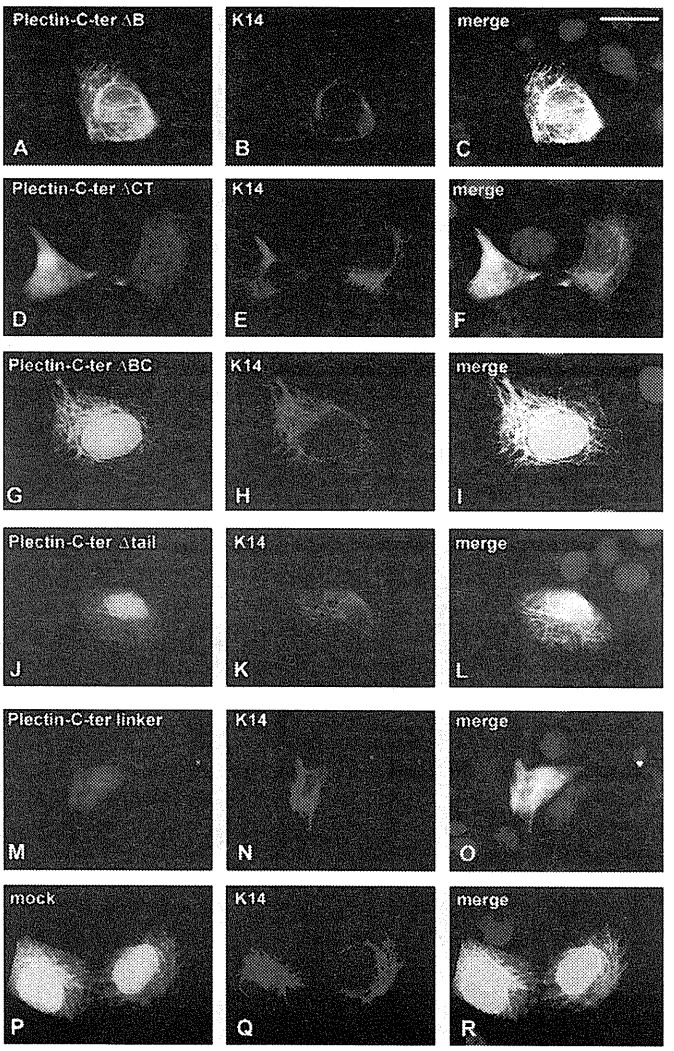


**Fig. 5.** Immunofluorescent staining of short constructs of Plectin-C-ter in NHKs. (A–C) Plectin-C-ter ΔB repeat transfected cells: A, Plectin-C-ter ΔB repeat; B, K14, C, merged image. (D–F) Plectin-C-ter ΔCT transfected cells: D, Plectin-C-ter ΔCT; B, K14, F, merged image. (G–I) Plectin-C-ter ΔBC transfected cells: G, Plectin-C-ter ΔBC repeat; H, K14, I, merged image. (J–L) Plectin-C-ter Δtail transfected cells: J, Plectin-C-ter Δtail; K, K14, L, merged image. (M–O) Plectin-C-ter linker transfected cells: M, Plectin-C-ter linker; N, K14, O, merged image. (P–R) Mock transfected cells: P, mock, Q, K14, R, merged image. Note the co-localization of plectin-C-ter ΔB repeat, ΔBC repeat and Δtail protein and K14 and the partial disruption of keratin in A–C, G–I and J–L. The scale bar represents 20 μm.

present (Fig. 3B, arrowheads). In sharp contrast, the plectin-C-ter linker fragment did not show co-localization with vimentin (Fig. 3D–F). Expression of vimentin was also disrupted, with large accumulations and formation of short filaments (Fig. 3E, arrowheads). No vimentin under this condition showed intact filamentous pattern. These results suggest that the transfection with the Plectin-C-ter linker construct showed dominant negative effect on vimentin IFs. Similar results were obtained in cells transfected with the Plectin-C-ter ΔBC repeat construct, which is composed of linker domain and C-terminal tail just adjacent to C subdomain (data not shown). Next, we co-transfected plectin-C-ter short fragment vectors, including ΔB repeat, ΔCT, ΔBC, Δtail and linker construct, and K5/K14 vectors into COS7 cells. The results revealed co-localization of plectin proteins and K14 and the partial disruption of K14 in plectin-C-ter ΔB, ΔBC and Δtail-transfected cells, while no co-localization was observed in plectin-C-ter ΔCT and linker-transfected cells (Fig. 4A–R). Finally, we co-transfected plectin-C-ter short fragment vectors, including ΔB repeat, ΔCT, ΔBC, Δtail and linker construct into NHKs. The results revealed co-localization of plectin proteins and K14 and the partial disruption of K14 in plectin-C-ter ΔB, ΔBC and Δtail-transfected cells, while no co-localization was observed in plectin-C-ter ΔCT and linker-transfected cells (Fig. 5A–R).

### 3.3. The role of S4645 of Plectin-C-ter

The previous results suggested the possible role of the tail domain for the binding of plectin to IFs. Previously, Fontao et al. reported that the 2849 serine residue of the COOH-terminal extremity of desmoplakin could weakly bind IFs [19]. Since desmoplakin is also a member of the plakin family, we induced a point mutation in the C-terminal region of plectin at the corresponding serine residue at S4645 (Plectin-C-ter$^{S4645G}$). We transfected COS7 cells and NHKs with the PLECTIN-C-ter$^{S4645G}$ construct. The results revealed that in the cell periphery, reduced colocalization was observed in COS7 cells (Fig. 6A–D). Intriguingly, the plectin was associated with large bundles of vimentin in some part (arrowheads) (Fig. 6A–D). Moreover, aggregates of vimentin co-localized with mutated plectin C-terminus was observed in NHKs (Fig. 6E–H). These results revealed that S4645G mutation in plectin may impact the binding of plectin to IFs.

### 3.4. Co-distribution of the plectin-C-ter and actin microfilament in vimentin-null cells

Plectin acts as a cross-linker for actin microfilaments, IFs and microtubules. In order to determine further the interaction of plectin with cytoskeletal components, we transfected vimentin-null (SW-13 VIM−/−) cells with the plectin-C-ter construct and mutants. The results revealed that some plectin-C-ter protein distributed precisely along actin microfilaments without the existence of vimentin IFs, although the others did not (Fig. 7A–C: lower magnification, Fig. 7D–F: higher magnification). The pattern of the expression of plectin and vimentin was same as endogenous plectin and actin in normal SW-13 cells (data not shown). In sharp contrast, all the plectin-C-ter mutants, including Plectin-C-ter ΔB repeat, ΔCT, ΔBC repeat, Δtail and linker, did not co-localize with and possibly disrupted actin microfilaments (Fig. 7G–O).

## 4. Discussion

The C-terminal B repeat and their linker regions on plakins are known to have a critical role in binding to IFs [19]. In this study, we confirmed that the last linker region on plectin is required for binding with IFs. Additionally, we found that Ser 4645, the residue that corresponds to a known minor binding site on desmoplakin, may have a role in binding of plectin to IFs.

The results presented here provide further insights into the IF binding sites in plectin that were first identified in four previous studies. Steinböck et al., Reipert et al. and Nikolic et al. reported that the IF binding site on plectin was located in an approximately 50 amino acid residue region linking the fifth B subdomain and the linker region [9–11]. They concluded that plectin showed greater binding with vimentin IFs compared to K5/14 IFs. Moreover, Favre et al. reported that the association of plectin with both desmin and vimentin predominantly dependent on its fifth B subdomain and downstream linker region [20]. In a review, Wiche predicted that the minimal binding domain of plectin must be the linker region, which shows less evolutionary conservation compared to other plakins [6]. Our results confirmed this prediction; the linker between the fifth B repeat and the C repeat plays a crucial role in the binding of plectins to IFs. The precise role of the linker region has yet to be elucidated; however, it is most likely to form loop-like

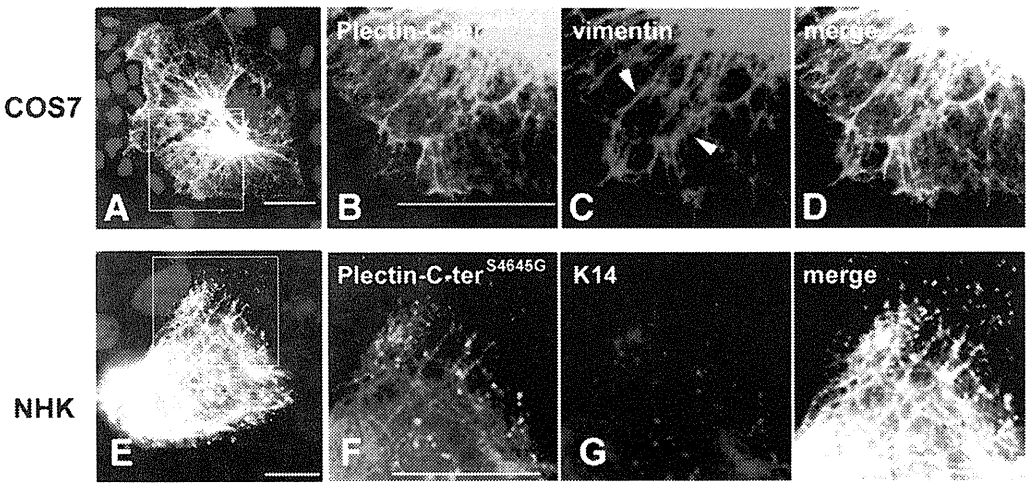


**Fig. 6.** Immunofluorescent staining of COS7 cells and NHKs transformed with the Plectin-C-ter$^{S4645G}$ construct. A, low magnification merged image of transformed COS7 cell. B, Plectin-C-ter$^{S4645G}$; C, vimentin; D, merged image. (B–D) Higher magnification images of the boxed area in A. E, low magnification merged image of transformed NHK; F, Plectin-C-ter$^{S4645G}$; G, K14; D, merged image. (F–H) Higher magnification images of the boxed area in E. Arrowheads in C represent large bundles of vimentin. The scale bar represents 20 μm

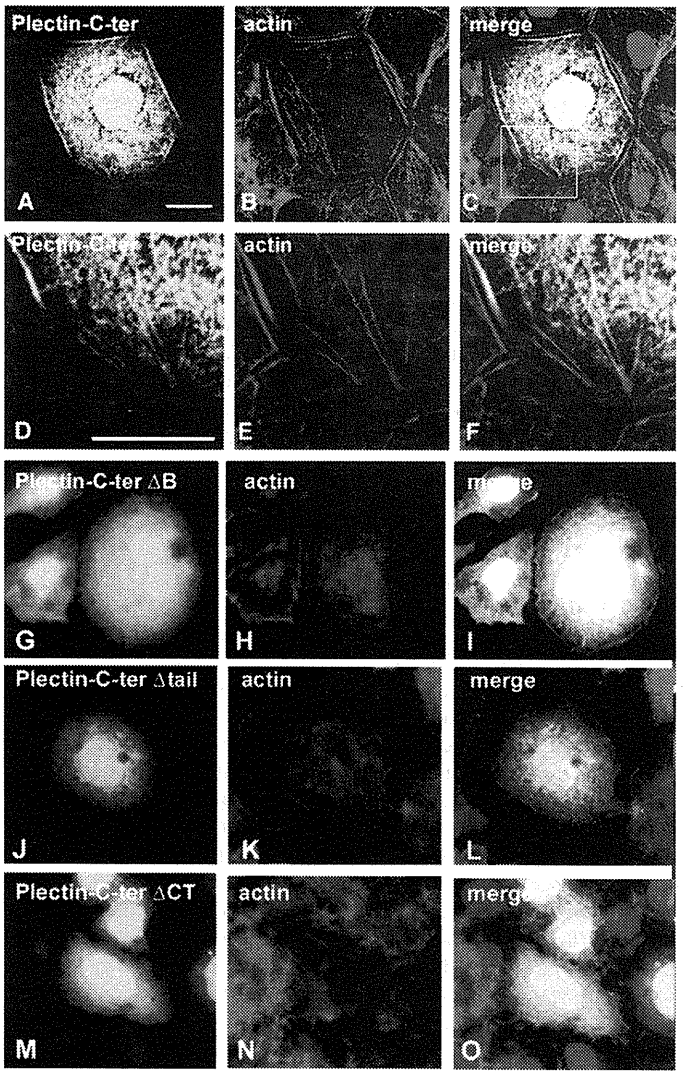


**Fig. 7.** Immunofluorescent staining of Plectin-C-ter, its short fragments and actin microfilaments in SW-13 VIM−/− cells. A, D, Plectin-C-ter; B, E, actin microfilaments; C, F, merged images. (A–C) Low magnification image; (D–F), higher magnification image of box area indicated in C. Note the identical expression of the Plectin-C-ter and actin filaments in some parts. (G–I) G, Plectin-C-ter ΔB repeat; H, actin microfilaments; I, merged images. (J–L) J, Plectin-C-ter Δtail; K, actin microfilaments; L, merged images. (M–O) M, Plectin-C-ter ΔCT; N, actin microfilaments; O, merged images. The scale bar represents 20 μm.

structures on the surface of the C-terminal globular domain of plectin that can bind to other proteins [6]. Fontao et al. reported similar roles of the linker regions for other members of the plakin family, namely, desmoplakins and bullous pemphigoid antigen-1e (BPAG1e) [19].

Intriguingly, the transfection of plectin-C-ter ΔCT, Δtail and ΔBC repeat construct caused at least partial inhibition of plectin to IF binding in COS7 cells and NHKs. We do not know the discrepancy between previous 4 reports and ours in light of the role of the binding of plectin C repeat and tail to IFs. However, it may be due to the difference of the experimental design.

There is increasing evidence to indicate that plectin–IF interactions are regulated by protein kinase-dependent phosphorylation. For example, the interaction between plectin and lamin B is significantly decreased by phosphorylation of plectin by cAMP-dependent protein kinase (PKA) or protein kinase C (PKC). By contrast, binding between plectin and vimentin is increased after PKA-phosphorylation but is decreased after PKC-phosphorylation [21]. We also found here that the serine residue at position 4645 in the tail domain of the C-terminus may have a role in the binding of plectin to vimentin IFs. Currently, the candidate protein kinase of this residue is not known. In desmoplakin, the corresponding serine residue in the COOH-terminal domain is believed to be phosphorylated in a protein kinase A-dependent manner [19]. Interestingly, although the corresponded mutation in desmoplakin caused stronger binding of desmoplakin to IF [22], the same mutation in plectin reported here caused weaker binding of plectin to IF at the periphery of COS7 cells but normal binding of the other portion of cells. Moreover, aggregated co-localization of plectin and K14 was observed in NHKs. We speculated that phosphorylation of this site plays some roles of plectin–IF binding. The phosphorylation of this site may stabilize the binding of plectin to IF at the cell periphery and may regulate the binding of plectin to IF in the other cell area. Further study is required to elucidate this.

Other intriguing issue to be discussed is that in vimentin-null SW-13 VIM−/− cells transfected with plectin-C-ter mutants, all the mutant proteins did not co-localize with and possibly disrupted actin microfilaments. The ability of each plectin-C-ter mutant to disrupt actin microfilaments did not correspond with that of each construct to IFs. As these results were still preliminary, further study is required to fully elucidate this.

In conclusion, we have shown that the linker region of the plectin C-terminus mediates the association of plectin with IFs. We also have demonstrated that the plectin C-terminus tail fragment may also play some roles on the association of plectin with IFs.

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