Interaction of periplakin and envoplakin with intermediate filaments

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Summary

Periplakin is a component of desmosomes and the epidermal cornified envelope. Its N-terminal domain interacts with the plasma membrane; it heterodimerises with envoplakin via its rod domain; and its C-terminus interacts with intermediate filaments. Periplakin has the shortest C-terminus of the plakin family, comprising only the linker domain found in all conventional plakins. By transient transfection of COS7 cells and primary human epidermal keratinocytes with deletion mutants of the periplakin C-terminus we mapped sequences required for intermediate filament interaction to two regions of the linker motif that are most highly conserved amongst the plakins. The results were confirmed by overlay assays of the binding of in vitro translated periplakin constructs to keratins and vimentin. We found that envoplakin and periplakin could still associate with each other when parts of their rod domains were deleted and, surprisingly, that removal of the entire rod domain did not completely inhibit their interaction. Co-transfection of constructs containing the C-termini of envoplakin and periplakin suggested that the periplakin C-terminus may stabilise the interaction of the envoplakin C-terminus with intermediate filaments. We conclude that the periplakin C-terminus plays an important role in linking periplakin and envoplakin to intermediate filaments.

Key words: Envoplakin, Periplakin, Intermediate filaments, Cornified envelope, Keratinocytes

Introduction

Envoplakin and periplakin were originally identified as components of the cornified envelope, an insoluble layer of transglutaminase-crosslinked protein that is deposited beneath the plasma membrane of terminally differentiating epidermal keratinocytes (Simon and Green, 1984; Ruhrberg et al., 1996; Ruhrberg et al., 1997; Steinert and Marekov, 1999). They are also found in the desmosomes of keratinocytes and other epithelial cells (Ma and Sun, 1986; Ruhrberg et al., 1996; Ruhrberg et al., 1997); in addition, periplakin is highly expressed in the brain (Aho et al., 1998). Envoplakin and periplakin belong to the plakin family of cytolinker proteins, which also includes desmoplakin, plectin and BPAG1 (Ruhrberg and Watt, 1997; Fuchs and Yang, 1999; Herrmann and Aebi, 2000; Leung et al., 2001; Leung et al., 2002).

Like the other conventional plakins, envoplakin and periplakin have an N-terminal globular domain, a central rod domain comprising heptad repeats, and a C-terminal globular domain (Ruhrberg et al., 1996; Ruhrberg et al., 1997). When transfected into cells full length periplakin localises to desmosomes, the interdesmosomal plasma membrane and intermediate filaments, the N-terminal domain mediating the interaction with the plasma membrane and the C-terminus associating with intermediate filaments (DiColandrea et al., 2000). Full length envoplakin mainly accumulates in aggregates associated with intermediate filaments. The envoplakin rod domain is required for aggregation and the periplakin rod domain is necessary and sufficient to redistribute envoplakin to desmosomes (DiColandrea et al., 2000). The observations on envoplakin aggregates support the conclusion that envoplakin and periplakin can heterodimerise via their rod domains (Ruhrberg et al., 1997).

The C-termini of the plakins contain a variable number of tandem repeats (plakin repeats) that are predicted to fold into discrete subdomains, designated A, B and C, consisting of α helices separated by β turns (Sawamura et al., 1991; Wiche et al., 1991; Green et al., 1992; Ruhrberg and Watt, 1997; Kowalczyk et al., 1999). Desmoplakin has one A, one B and one C-type plakin repeat; plectin has five B repeats and one C; and different isoforms of BPAG1 have either no repeats, one A repeat or one B and one C repeat (reviewed by Leung et al., 2002). The C-termini of envoplakin and periplakin are considerably shorter than those of other plakins, with envoplakin having one C repeat, and periplakin no repeats (Ruhrberg et al., 1996; Ruhrberg et al., 1997; Leung et al., 2002).

The C-terminus of periplakin comprises only the L-subdomain (for 'linker'), which in the other plakins links the C repeat with the preceding subdomain or with the rod domain (Ruhrberg et al., 1997; Leung et al., 2001; Leung et al., 2002). The L-subdomain is the region of highest sequence conservation amongst different plakin family members and is also the most highly conserved between mouse and human (Mahoney et al., 1998; Määttä et al., 2000). It is lacking in the more distantly related proteins epiplakin (Fujiwara et al., 2001) and MACF/ACF-7, the mammalian homologue of *Drosophila* Kakapo (Gregory and Brown, 1998; Strumpf and Volk, 1998; Leung et al., 1999; Karakesisoglou et al., 2000; Leung et al.,

2001; Leung et al., 2002). In transient transfection experiments the C-terminus of periplakin associates with intermediate filaments, whereas the envoplakin C-terminal C box, lacking the linker motif, has a punctate distribution throughout the cytoplasm (DiColandrea et al., 2000). This led us to propose that the linker sequence mediates intermediate filament binding (DiColandrea et al., 2000).

The association of other plakins with intermediate filaments has already been examined in some detail, using a variety of experimental approaches. Yeast two-hybrid analysis of the C-terminus of desmoplakin is consistent with a role for the linker domain in the interaction of desmoplakin with vimentin; however, a sequence within the C subdomain is implicated in the interaction of desmoplakin with keratins, and peptide competition experiments suggest that the equivalent region of envoplakin and plectin has the same function (Meng et al., 1997). Recently the A, B and C subdomains of desmoplakin have been purified and tested for vimentin binding by co-sedimentation (Choi et al., 2002); these studies show that the linker region does not contribute significantly to binding affinity, but may provide the flexibility that allows the combination of B and C domains to bind more strongly than either domain individually. The intermediate filament binding region of plectin has been mapped to 50 amino acids linking the C-terminal repeat domains 5 and 6 [box B and C in the nomenclature of Green et al. (Green et al., 1992)]; the first 7 of these amino acids are within the B box, while the rest are within the linker domain (Nikolic et al., 1996; Steinböck et al., 2000).

The aim of our experiments was to carry out a more detailed study of the interaction of envoplakin and periplakin with intermediate filaments. We have mapped regions of the linker motif that are required for periplakin to associate with intermediate filaments; we show that periplakin stabilises the interaction of envoplakin with intermediate filaments; and we demonstrate that some envoplakin and periplakin interactions are not dependent on an intact rod domain.

Materials and Methods

Generation of cDNA constructs

All periplakin cDNA constructs were tagged at the C-terminus with influenza virus haemagglutinin (HA) and expressed in pCI-neo, as described previously (DiColandrea et al., 2000). To generate P-1/4R+L (Fig. 1) full-length periplakin (DiColandrea et al., 2000) was digested with *Eco*RI to yield a fragment consisting of amino acids 1588 and 1756 of periplakin. All periplakin C-terminal mutants were made by PCR deletion mutagenesis (Imai et al., 1991) using P-1/4R+L/pCI-neo as a template. Briefly, PCR of 25 cycles at 94°C for 2 minutes, 50-65°C (depending on the melting temperature of each primer) for 2 minutes, and 70°C for 4 minutes was performed in 50 μl of PCR reaction mixture containing 200 μM each of dNTPs, 2 ng plasmid DNA template, 2 μM each of the primers, 1 unit of *pfu* turbo DNA polymerase (Stratagene) and 5 μl of 10× buffer (Stratagene). 1 μl of 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) at 16°C for 1 hour. The resulting constructs were used to transform DH5α bacteria. Primers used were as follows. P-L (AA1646-1756): 5′-ATGAAGCGGGAGCAGCGGGAGAACCAC-CTGCGGCGCTCCATC-3′ and 5′-GTTGATTTCCGATTGGAGC-CTTCGGGTCTCCAGCTGCAGGTTTTG-3′; P-L-10AA(AA1588-1746): 5′-TACCCATACGACGTGCCAGACTACGCTTAGGCGACC-3′ and 5′-CTGGATGGACATATCCTTGTTGACATAGCGGTCATA-3′; P-L-20AA (AA1588-1736): 5′-TACCCATACGACGTGCCAGA-CTACGCTTAGGCGACC-3′ and 5′-GTCATACTGAGCAGGGGT-CAGCCTGCCA-3′; P-LΔDWEEI (P-L deletion mutant ΔAA1693-1699): 5′-TCAGTGAAGGGTCCCAATGGGGAGTCCTCAGTGA-TA-3′ and 5′-GCACTCCTGGCTTCTGAGTTTCACGAACATGT-TCCA-3′: P-LΔBox1 (P-L deletion mutant ΔAA1657-1699): 5′-TCAGTGAAGGGTCCCAATGGGGAGTCCTCAGTGATA-3′ and 5′-GGAGCGCCGCAGGTGGTTCTCCCGCTGCTCCCGCTT-3′; P-LΔBox2 (P-L deletion mutant Δ1711-1725): 5′-CTGCAGAGTGG-CAGGCTGACCCCTGCTCAG-3′ and 5′-GTGTATCACTGAGGA-CTCCCCATTGGGACCCTT-3′; P-LΔ1/2Box2 (P-L deletion mutant ΔAA1711-1719): 5′-TTCTCCATCGAAGAGGCCCTGCAGAGTG-GCAGG-3′ and 5′-GTGTATCACTGAGGACTCCCCATTGGGAC-CCTT-3′. The rod domain deleted periplakin construct (PΔRod) was made by PCR deletion mutagenesis using the full-length periplakin cDNA as a template and primers 5′-GGGATCCGGCACCTTCT-TGAGCACCTCCTTCC-3′ and 5′-AAGCGGGAGCAGCGGGAG-AACCACCTGCGGCGCTCCATCGT-3′.

All envoplakin cDNA constructs were tagged at the C-terminus with the FLAG epitope and expressed in pCI-neo, as described previously (DiColandrea et al., 2000). Full-length envoplakin (DiColandrea et al., 2000) was used as a template to generate E-1/4R+L+C in pCI-neo by PCR. The primers used were 5′-AT-GCTCAACAGGGAGCGCACGGCCCGGCAGGC-3′ and 5′-GGTC-GTAAAGGCAAGTGCTGGCTCAGGCTGGGCTTGGC-3′. The C box was deleted from E-1/4R+L+C (deletion mutant ΔAA 1836-2011; E-1/4R+LΔC) by PCR deletion mutagenesis using these primers: 5′-CCAGCGGCACTGGAGGGGTACCGCTGCTA-3′ and 5′-CAGGA-GCAGCAGGCCGCTCAGGGGGTCTTT-3′. The rod domain deleted envoplakin construct (EΔRod) was made by PCR deletion mutagenesis using full-length envoplakin cDNA as a template and these primers: 5′-CTGGCTGAGCTCCTCCCGGCTCACCTTGG-CGTG-3′ and 5′-GACTACAAGGACGACGATGACAAGTGA-3′. Full-length envoplakin was digested with *Sac*II, removing two fragments that were self-ligated to yield E-Δ1/2N1/2R. All envoplakin and periplakin plasmid constructs were verified by dye-terminator cycle sequencing (Applied Biosystems).

Transient transfection of cells

COS7 cells (African green monkey kidney derived) and HeLa cells were maintained in DMEM supplemented with 10% FCS. The day before transfection, cells were seeded on glass coverslips in 24-well plates at the density of 5×10^4 cells/well. Cells were treated with transfection reaction mixture for 2-3 hours, washed with PBS, and transferred to DMEM containing 10% FCS. The transfection mixture consisted of Superfect reagent (Qiagen) combined with serum-free DMEM and plasmid DNA, according the manufacturer's protocol. Primary human keratinocytes were cultured and transfected as described previously (DiColandrea et al., 2000).

Immunofluorescence analysis

The following antibodies were used: LP34 [mouse monoclonal antibody to keratins 5, 6 and 18 (Lane et al., 1985)]; LL001 [mouse monoclonal antibody to keratin 14 (Lane, 1982)]; V9 (mouse monoclonal antibody to vimentin; Novocastra); TUB 1A2 (mouse monoclonal antibody to β-tubulin; Sigma-Aldrich); rabbit anti-HA (Y-11; Santa Cruz), rabbit anti-FLAG (Santa Cruz Biotechnology) and M2 (mouse monoclonal antibody to FLAG; Sigma). Alexa-488- or Alexa-594-conjugated goat anti-rabbit or -mouse IgG (Molecular Probes) was used as secondary antibody.

Cells on coverslips that were to be stained without prior saponin extraction were fixed in cold acetone/methanol 1:1 for 5 minutes on ice or in 4% paraformalehyde (Sigma)/PBS for 20 minutes at room temperature. Paraformaldehyde-fixed cells were subsequently

permeabilized with 0.2% Triton X-100 (Sigma) for 5 minutes at room temperature. After fixation cells were washed in PBS and blocked in a 1:500 dilution of normal goat serum (Sigma) in PBS for 10 minutes at room temperature. Cells were incubated with primary antibodies for 45 minutes at room temperature, washed in PBS, and then incubated for a further 45 minutes at room temperature with the appropriate Alexa-conjugated secondary antibodies. Polymerised actin was detected with Alexa-594-conjugated phalloidin (Molecular Probes). Nuclear counter staining was performed with TOTO-3 (Molecular Probes). After further washing in PBS and distilled water, coverslips were mounted in Gelvatol (Monsanto) and examined using a laser scanning confocal microscope (LSM 510; Carl Zeiss).

To remove detergent-labile cytoskeletal components, cells on coverslips were extracted with saponin prior to fixation. The methods of Svitkina et al. (Svitkina et al., 1995; Svitkina et al., 1996) and Herrmann and Wiche (Herrmann and Wiche, 1983) were used with some modifications. Briefly, cells were treated with 1% Triton X-100 or 0.6% saponin in imidazole buffer (50 mM imidazole, pH 6.8, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA) containing 4% polyethelene glycol (M_r 8000: PEG8000) (Sigma) for 30 minutes at room temperature. Extracted cells were washed gently in PBS, fixed in 4% paraformaldehyde/PBS at 4°C for 30 minutes or overnight and then processed as described above for immunofluorescence staining or as described below for scanning electron microscopy.

Scanning electron microscopy

Non-extracted cells and cells that had been extracted in saponin were gently washed in PBS and fixed in 4% paraformaldehyde in PBS overnight at 4°C. Fixed cells were dehydrated in graded ethanols up to 100%, then in two changes of acetone. Critical point drying was performed using a Polaron critical point dryer (Polaron, UK). Dried samples were rotary shadowed with platinum in a Polaron sputter coater and examined in a JEOL 5600 scanning electron microscope.

Overlay binding assay

Labelled periplakin protein probes used in the overlay assays were generated by in vitro transcription/translation using the TNT Quick Coupled Transcription/Translation System or TNT Coupled Wheat Germ Extract System (Promega, Madison, WI), in the presence of [^{35}S]-methionine (Amersham Pharmacia Biotech, Little Chalfont, UK). A partial desmoplakin I cDNA with an intact C-terminus [DPΔNwt; a kind gift of Kathy Green, Northwestern University Medical School (Stappenbeck and Green, 1992)] was subcloned into Bluescript KS+ (Stratagene) prior to in vitro translation. Human keratins 5 and 14 in pET vectors kindly provided by Elaine Fuchs (University of Chicago) (Coulombe and Fuchs, 1990) were also used for in vitro translation. Intermediate filament proteins were purified from cultured primary human keratinocytes and COS7 cells essentially as described (Steinert et al., 1982).

The overlay assays were performed as described (Merdes et al., 1991). Isolated intermediate filaments were resolved on 1-mm-thick SDS-polyacrylamide gels made using ultra-pure electrophoresis reagents (Bio-Rad Laboratories, Hercules, CA). Part of each gel was stained with Coomassie Brilliant Blue to visualise the proteins, and the remainder was transferred to nitocellulose membrane. [^{35}S]-methionine-labeled probes were diluted in gelatin buffer (0.9% NaCl, 20 mM Tris-HCl, pH 7.3, 1 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 20, 0.2% boiled gelatin, and 0.2 mM PMSF) such that all probes had the same cpm/unit volume. Blots were incubated with probes for 1 hour at room temperature in gelatin buffer then washed five times with gelatin buffer at room temperature. After the final wash, blots were incubated with Amplify Fluorographic Reagent (Amersham Pharmacia Biotech) and exposed to X-ray film (Bio Max MS, Kodak, NY).

Results

Distribution of periplakin C-terminal deletion constructs in extracted and non-extracted cells

Fig. 1A shows the C-terminus of periplakin aligned with the conserved linker domains of other plakins. Identical residues are shown in light blue and conserved amino acids in dark blue (Mahoney et al., 1998; Määttä et al., 2000) in Fig. 1A, while in Fig. 1B both identical and conserved amino acids are shaded grey. We designated amino acids 1658-1698 as homologous box 1 and 1712-1724 as homologous box 2 (Fig. 1B). We previously reported that the C-terminus (amino acids 1639-1756) of periplakin had a filamentous cytoplasmic distribution when transiently transfected into keratinocytes and other cell types (DiColandrea et al., 2000). To examine this association in more detail we transfected cells with the full length C-terminus (P-L; amino acids 1646-1756) or a series of deletions that either truncated the C-terminus (P-L-10AA; P-L-20AA) or removed all or parts of the homologous boxes (P-LΔDWEEI, P-LΔBox2, P-LΔ1/2Box2, P-LΔBox1) (Fig. 1C). The constructs shown in Fig. 1C also contained part of the periplakin rod domain (amino acids 1588-1645; 1/4R). All of the constructs were tagged at the C-terminus with HA for ease of detection (DiColandrea et al., 2000).

As one method to evaluate the strength of association of the periplakin constructs with intermediate filaments we compared the localisation of the constructs in cells that had been fixed without prior extraction and cells that had been extracted in saponin prior to fixation. The method we used was based on those described previously that preserve the interaction between plectin and intermediate filaments (Herrmann and Wiche, 1983; Svitkina et al., 1995; Svitkina et al., 1996). Scanning electron microscopy revealed that after extraction with 0.6% saponin for 15 minutes there was significant retention of the plasma membrane (Fig. 2A), but when the extraction time was increased to 30 minutes (Fig. 2B,C) most of the plasma membrane was lost. This was confirmed by loss of the integral membrane protein HB-EGF after a 30 minute saponin treatment (as judged by immunofluorescence staining; data not shown). Cells extracted for 30 minutes no longer had an intact microtubule network (green in Fig. 2D,E), but polymerised actin (red in Fig. 2D-F) and intermediate filaments (green in Fig. 2F) were unaffected. The distribution of desmoplakin and plakoglobin was also unaffected by extraction (data not shown). When 1% Triton X-100 was substituted for saponin the effects on the cytoskeleton were similar (data not shown).

The results of transient transfection experiments in COS7 cells are summarised in Fig. 1C and illustrated in Fig. 3. In Fig. 1C, '+' refers to colocalisation that was sufficiently complete to make the distribution of periplakin almost indistinguishable from intermediate filaments (see, for example, Fig. 3A-C). '+/–' refers to partial colocalisation in which the filamentous distribution of the periplakin construct was evident without the need to overlay the intermediate filament staining pattern (Fig. 3J-L). '–' refers to constructs that had no obvious filamentous distribution even if some of the periplakin staining did overlap with intermediate filaments (see, for example, Fig. 3F).

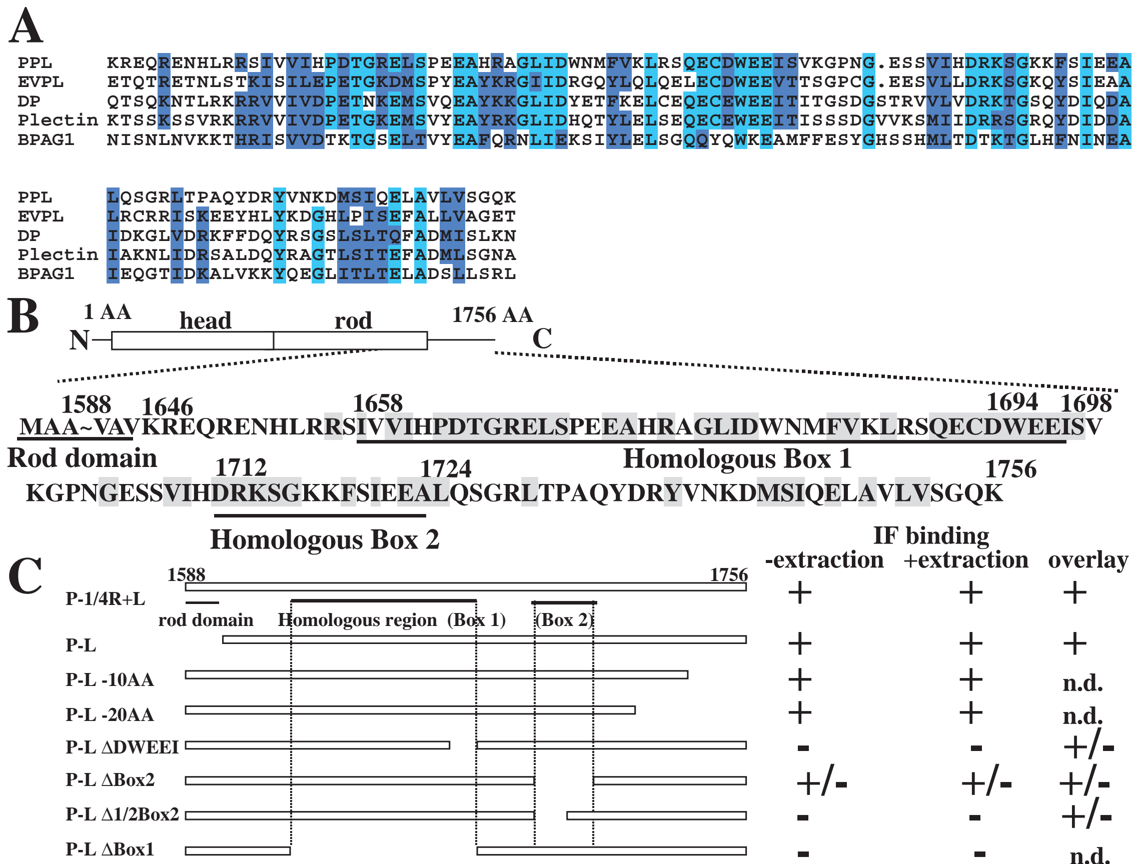

Fig. 1. The C-terminus of periplakin. (A) Alignment of the periplakin C-terminus with the linker sequences of other plakins. Light blue, identical amino acids; dark blue, conserved amino acids. Human periplakin (PPL) is shown from amino acid 1646; human envoplakin (EVPL) from amino acid 1675; human desmoplakin (DP) from amino acid 2454, rat plectin from amino acid 3722 and human BPAG1 from amino acid 2327 (Mahoney et al., 1998; Määttä et al., 2000). (B) Amino acid sequence of periplakin C-terminus, with residues that are most highly conserved between plakins (i.e. light or dark blue in A) shaded. The assignment of the boundary between the rod domain and linker domain is that described by Määttä et al. and DiColandrea et al. (Määttä et al., 2000; DiColandrea et al., 2000), except that the numbering has been corrected by one amino acid. (C) Periplakin C-terminal constructs tested in immunofluorescence and overlay assays. P, periplakin; R, rod domain; L, linker domain. The association of each construct with intermediate filaments (IF) was evaluated by immunofluorescence in transiently transfected COS7 cells stained with (+extraction) or without (–extraction) prior extraction and in overlay assays (overlay) with keratinocyte and COS7 cell intermediate filament preparations. +, colocalisation that was sufficiently strong to make the distribution of periplakin almost indistinguishable from intermediate filaments (strong binding in overlay assays); +/–, partial colocalisation in which the filamentous distribution of the periplakin constructs was evident without the need to overlay the intermediate filament staining pattern (weak binding in overlay assays); –, no obvious filamentous distribution; n.d., not determined.

The intact C-terminus of periplakin on its own (P-L) or connected to part of the rod domain (amino acids 1588-1645; P-1/4R+L) (green fluorescence) showed extensive colocalisation with vimentin intermediate filaments (red fluorescence) in both non-extracted (Fig. 3A,E) and saponin extracted (Fig. 3B-D and data not shown) cells. Deletion of the C-terminal 10 or 20 amino acids of periplakin had no effect on this distribution (P-L-10AA, P-L-20AA; Fig. 1C and data not shown). When all of homologous box 1 was deleted (P-LΔBox1) there was no association of the periplakin C-terminus with vimentin filaments and the construct was completely extractable with saponin (Fig. 1C and data not shown). Deletion of only 5 amino acids within box 1 (P-LΔDWEEI) had the same effect (Fig. 1C; green fluorescence in Fig. 3F-H).

Although homologous box 1 was required for the intermediate filament association of the periplakin C-terminus, it was not sufficient. Deletion of the first 8 amino acids of box 2 (P-LΔ1/2Box2) had the same effect as deletion of box 1 (Fig. 1C and data not shown). When the whole of box 2 was deleted (P-LΔBox2) most of the intermediate filament association was lost, but a small amount of vimentin-associated protein was retained after detergent extraction (Fig. 1C; green fluorescence in Fig. 3I,J,L). The distribution of several of the constructs (P-1/4+L, P-L, P-LΔDWEEI and P-LΔBox1) was examined in transiently transfected, non-extracted primary human keratinocytes (which have keratin filaments but no vimentin) and found to be the same as in COS7 cells (Fig. 3M-P and data not shown).

Direct binding of the periplakin C-terminus to keratins and vimentin

The immunofluorescence data suggested that the C-terminus of periplakin, corresponding to the linker domain in other plakin

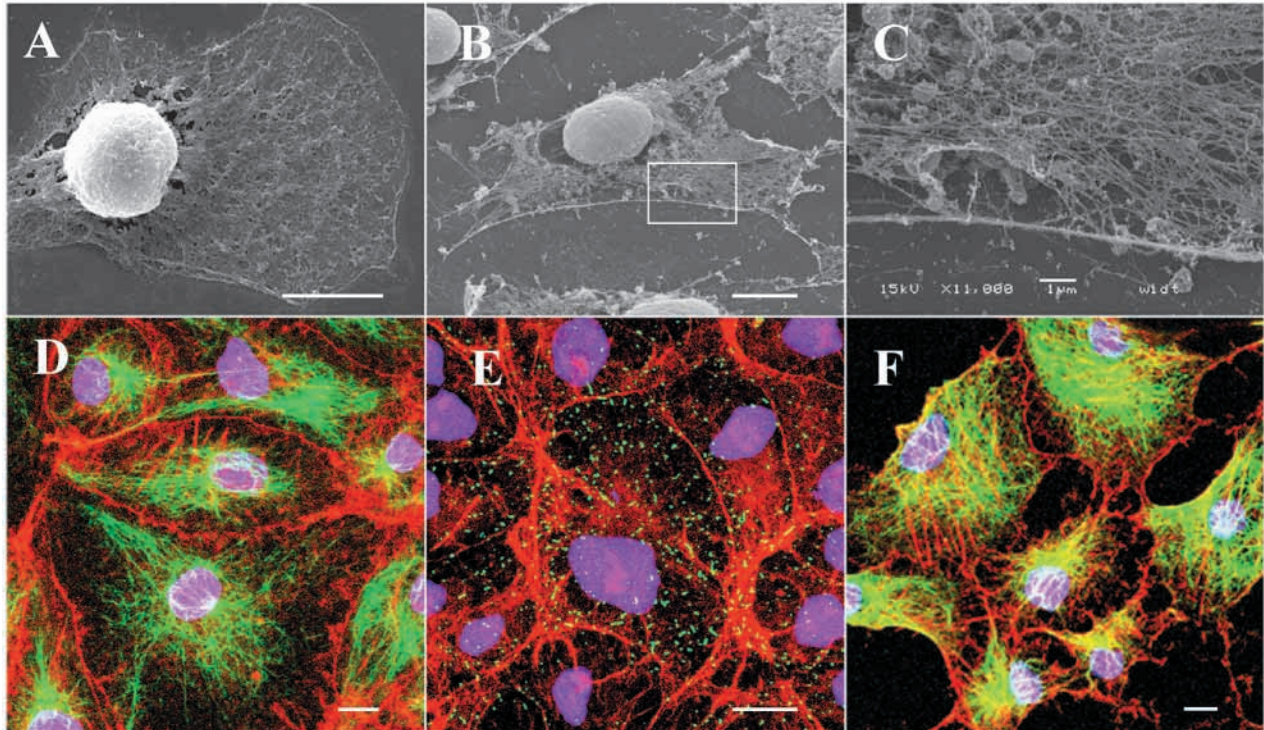

Fig. 2. Effects of detergent extraction on COS7 cells. (A-C) Scanning electron microscopy of cells extracted in 0.6% saponin for 15 minutes (A) or 30 minutes (B,C). Area boxed in B is shown at higher magnification in C. (D-F) Immunofluorescence staining of cells fixed without prior extraction (D) or cells extracted with saponin for 30 minutes (E,F). Green fluorescence: anti-β tubulin (D,E); anti-vimentin (F). Red fluorescence: phalloidin (D-F). Purple fluorescence: nuclei stained with TOTO-3. Bars, 10 μm (A,B,D-F), 1 μm (C).

family members, was sufficient to mediate the association of periplakin with intermediate filaments and that amino acids within the two regions that show highest homology with the other plakins were necessary for this association. To investigate whether the intermediate filament association was direct or indirect we carried out overlay assays with several of the constructs (Fig. 4; summarised in Fig. 1C).

[^{35}S]-methionine labelled recombinant periplakins were produced by in vitro translation (Fig. 4A). We prepared the intact C-terminus with part of the rod domain attached (P-1/4R+L) and three deletions within the C-terminus that interfered with intermediate filament colocalisation in the transfection experiments: P-LΔDWEEI, P-LΔ1/2Box2 and P-LΔBox2. As positive controls we expressed full length periplakin (P-full), a partial desmoplakin construct with an intact C-terminus that has previously been shown to associate with intermediate filament proteins [DP-C in Fig. 4B (see Stappenbeck and Green, 1992)], and keratins 5 and 14 (Coulombe and Fuchs, 1990; Smith and Fuchs, 1998). As a negative control we expressed the periplakin rod domain [P-R (see DiColandrea et al., 2000)] or used the empty expression vector, pCI-neo. All in vitro translated proteins migrated on SDS-PAGE gels with their predicted molecular masses, although there were additional, less abundant, lower molecular mass bands in the P-R and P-full preparations, probably reflecting degradation (Fig. 4A).

Intermediate filaments were isolated from cultured human keratinocytes and COS7 cells. Coomassie Brilliant Blue (CBB in Fig. 4B) staining of keratinocyte intermediate filament preparations resolved by SDS-PAGE revealed two doublets, corresponding to the major keratins expressed by keratinocytes in culture (K5, K14, K6 and K16; data not shown). The major intermediate filament proteins expressed by COS7 cells are vimentin, keratins 8 and 18 (data not shown); these bands migrated as a broad doublet, with vimentin and keratin 8 in the upper band and keratin 18 in the lower band (Fig. 4B).

The intermediate filament preparations were transferred to nitrocellulose membranes and incubated with equal amounts of radioactively labelled in vitro translated proteins, washed extensively and subjected to fluorography (Fig. 4B). Full length periplakin (P-full), K5 and K14 and the desmoplakin construct (DP-C) all bound to the keratinocyte and COS7 cell intermediate filament preparations, whereas P-R and pCI-neo did not. In keratinocyte and COS7 extracts the binding of P-1/4R+L was equal to P-full and stronger than that of the C-terminal deletion constructs, P-LΔDWEEI, P-LΔ1/2Box2, P-LΔBox2. The three deletion constructs were indistinguishable in the overlay assays, whereas in transfection experiments only P-LΔBox2 showed any colocalisation with intermediate filaments (see Fig. 1C, Fig. 3). All of the constructs tested bound preferentially to the upper doublet in the keratinocyte preparations, but bound equally to both bands in the COS7 cell preparations, possibly indicating different affinities for different intermediate filament proteins.

We conclude that the association of the periplakin C-terminus with intermediate filaments observed by immunofluorescence (Fig. 3) involves direct binding to

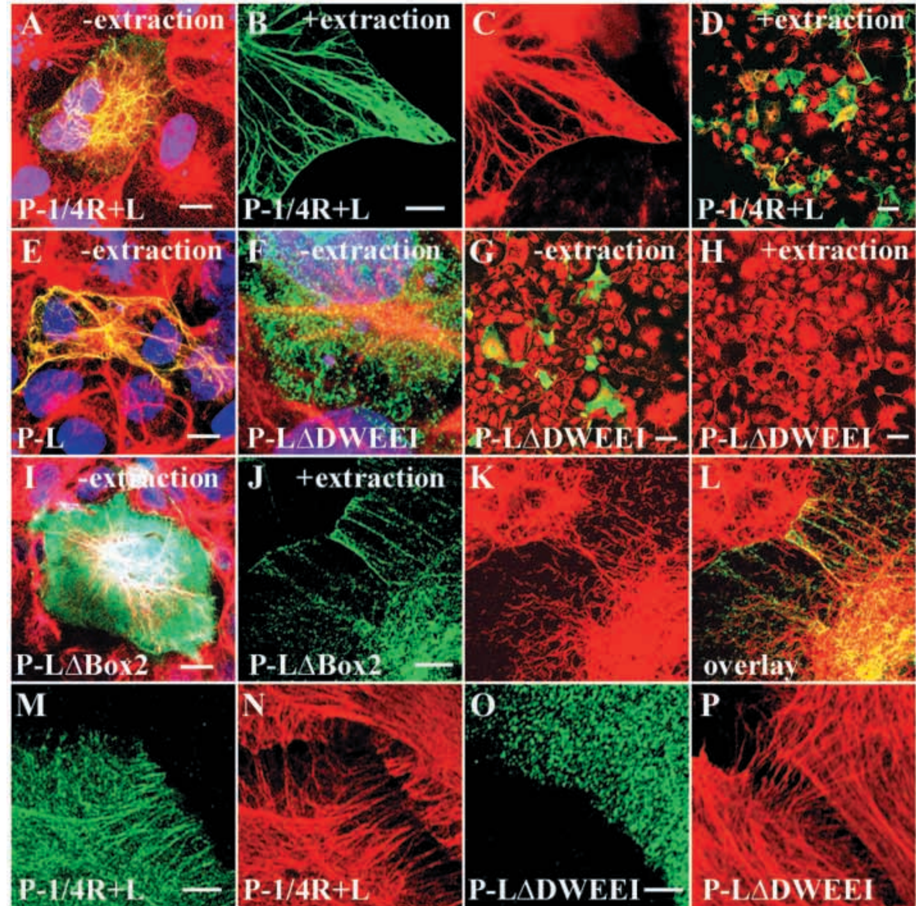

Fig. 3. Transfection of COS7 cells and keratinocytes with periplakin C-terminal constructs. (A-L) COS7 cells; (M-P) primary human epidermal keratinocytes. After transfection, the cells were fixed without prior extraction (A,E-G,I,M-P) or extracted with 0.6% saponin for 30 minutes before fixation (B-D,H,J-L). B and C are the same field; J-L are the same field; M and N are the same field; and O and P are the same field. See Fig. 1 for details of each construct. Green fluorescence: anti-HA (detects tagged periplakin constructs). Red fluorescence: anti-vimentin in COS7 cells; anti-keratin (LP34) in keratinocytes. Purple/blue fluorescence: nuclei stained with TOTO-3. Bars, 10 μm (A-C,E,F,I); 50 μm (D,G,H); 5 μm (J-P).

intermediate filament proteins and that amino acids within the two homology boxes are required for maximal binding.

Interactions of periplakin and envoplakin constructs containing rod domain deletions

Envoplakin and periplakin are known to associate via their rod domains (Ruhrberg et al., 1997; DiColandrea et al., 2000) and we wanted to investigate whether the two proteins could still interact when part or all of each rod domain was deleted. When full length envoplakin is transfected into cells it accumulates in aggregates with associated intermediate filaments, whether simple or complex keratins, vimentin or nuclear lamins (DiColandrea et al., 2000). Co-transfection with full length periplakin or its isolated rod domain prevents envoplakin aggregates from forming and targets both proteins to their correct subcellular locations, including desmosomes and intermediate filaments (DiColandrea et al., 2000). Removal of the rod domain of envoplakin or periplakin (EΔRod and PΔRod in Fig. 5) resulted in each protein having a diffuse, partially filamentous, distribution throughout the cytoplasm and nucleus (green in Fig. 6A,B). Each protein was completely lost from the cytoplasm of cells treated with saponin prior to fixation, although some protein remained in the nucleus (data not shown). Co-transfection of the two rod domain deleted constructs (EΔRod, PΔRod) resulted in the same distribution as when either construct was transfected individually (Fig. 6C). The constructs in the cytoplasm of doubly transfected cells were also completely extractable with saponin, although transfected cells could be detected by residual nuclear staining (Fig. 6D).

As expected, co-transfection of rod deleted periplakin (PΔRod) with full length envoplakin (E-full) failed to prevent envoplakin from forming detergent insoluble aggregates (Fig. 6E-G; E-full is shown in green in each panel and PΔRod in red). In most cells the rod deleted periplakin showed extensive colocalisation with envoplakin in aggregates (Fig. 6E,F). Since the aggregates have previously been shown to contain intermediate filament proteins (DiColandrea et al., 2000) periplakin could be binding via its C-terminus to intermediate filaments in the aggregates. However, in some doubly transfected cells the envoplakin aggregates were smaller (e.g. Fig. 6H; E-full is shown in green and PΔRod in red) than in singly transfected cells and these small aggregates were detergent sensitive (see Fig. 6I in which only large envoplakin aggregates, shown in green, remain in a detergent extracted cell; red indicates vimentin filaments). Thus PΔRod could partially alter the distribution of full length envoplakin by reducing the size and increasing the detergent solubility of envoplakin aggregates.

The effect of full length periplakin on the distribution of EΔRod was more pronounced. In the presence of P-full, EΔRod showed increased colocalisation with intermediate filaments (compare Fig. 6A with 6J-L; envoplakin signal is green; P-full is red in 6K,L). The intermediate filament association of EΔRod under those circumstances was, however, detergent sensitive (data not shown). These experiments demonstrate that full length periplakin could recruit EΔRod to intermediate filaments, suggesting that interactions between the two proteins can occur outwith the envoplakin rod domain.

To further examine the role of the rod domain in periplakin/envoplakin interactions we transfected COS7 cells with E-Δ1/2N1/2R. This construct consists of the first half of the envoplakin-N terminal domain, the C-terminal half of the rod domain and the complete C-terminus (Fig. 5). E-Δ1/2N1/2R formed cytoplasmic aggregates (Fig. 7A-D; envoplakin shown in green, vimentin in red) that were largely

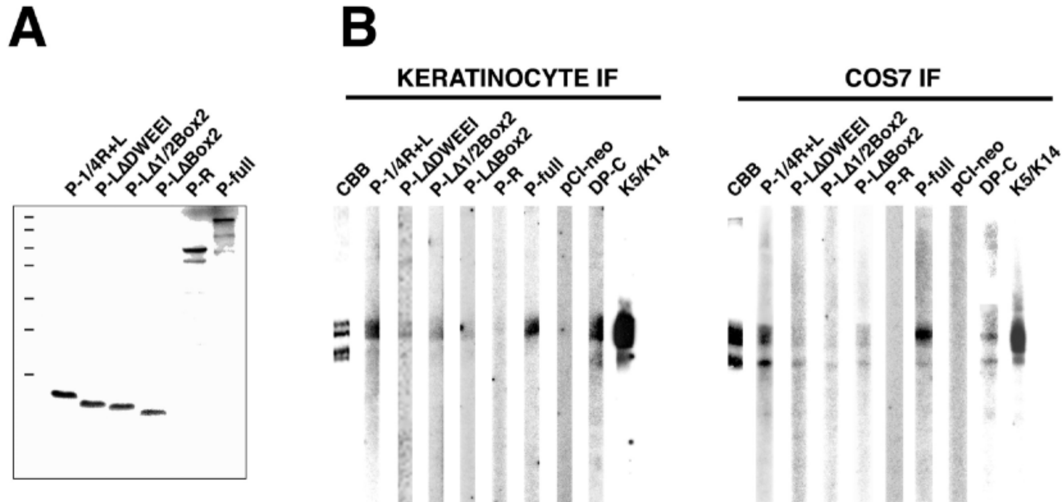

Fig. 4. Overlay assays of the interaction of periplakin constructs with intermediate filaments. (A) Autoradiography of [^{35}S]-methionine-labeled in vitro translated probes. Mobility of molecular mass standards of 200, 150, 100, 75, 50, 37 and 25 kDa are indicated. (B) Binding of [^{35}S]-methionine-labeled periplakin recombinant proteins to unlabelled intermediate filaments (IF) extracted from cultured epidermal keratinocytes and COS7 cells. CBB: Coomassie Brilliant Blue staining of intermediate filament preparations prior to transfer to nitrocellulose. Probes are described in Figs 1 and 5 or as follows. P-R, periplakin rod domain; pCI-neo, empty vector control; DP-C, desmoplakin construct with intact C-terminus [DPΔNwt (Stappenbeck and Green, 1992)]; K5/K14, keratin 5 and 14.

removed by saponin extraction (Fig. 7E-H; envoplakin in green, vimentin in red), in contrast to the aggregates formed by full length envoplakin [(DiColandrea et al., 2000) and data not shown]. Co-transfection with full length periplakin resulted in the envoplakin construct colocalising with intermediate filaments and becoming non extractable in saponin (Fig. 7K,L; envoplakin is green in each panel). Some bundling of intermediate filaments was observed in doubly transfected cells (Fig. 7K,L) that was not seen in cells transfected with full length periplakin alone. Fig. 7I,J shows full length periplakin (green) colocalising with vimentin (red, panel I) or keratins (red, panel J).

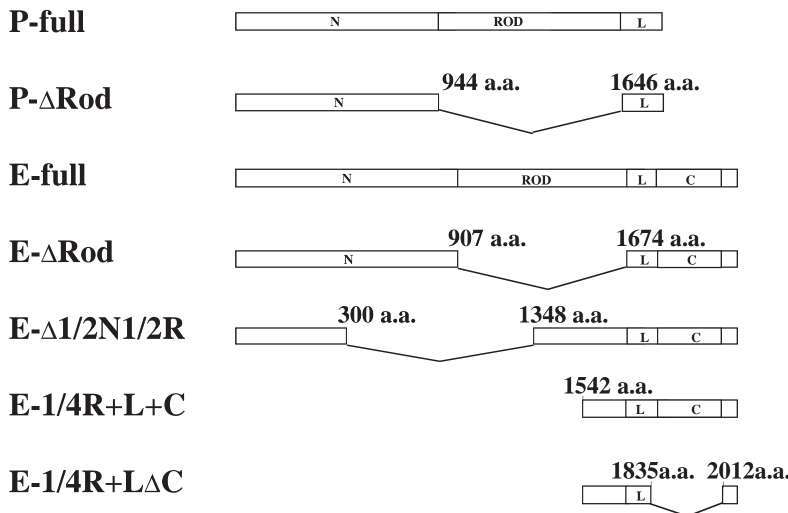

Fig. 5. Summary of constructs with rod domain deletions. P, periplakin; E, envoplakin; full, full length protein; N, N-terminus; R, rod domain; L, linker domain; C, C box subdomain of C terminus; a.a., amino acid.

We conclude that removal of part of the N-terminus and rod domain does not prevent envoplakin from aggregating, nor from being rescued by full length periplakin. All of the observations made in non-extracted COS7 cells using the constructs shown in Fig. 5 were confirmed in HeLa cells and primary human keratinocytes (data not shown).

Periplakin stabilises the association of envoplakin with intermediate filaments

The effects of P-full on EΔRod (Fig. 6) and E-Δ1/2N1/2R (Fig. 7) suggested that periplakin could affect the association of envoplakin with intermediate filaments. In addition to containing the linker motif, the C-terminus of envoplakin contains one plakin repeat, designated a C subdomain (Ruhrberg et al., 1996; Ruhrberg and Watt, 1997). We have previously reported that when transiently transfected into keratinocytes the C subdomain of envoplakin is distributed in a punctate pattern throughout the cytoplasm and does not show any colocalisation with the cytoskeleton (DiColandrea et al., 2000). When the entire envoplakin C-terminus, comprising the linker and C box, coupled to part of the rod domain, was transfected into COS7 cells (E-1/4R+L+C in Fig. 5; see Fig. 8A-C) its distribution was primarily punctate in the cytoplasm but showed partial colocalisation with intermediate filaments (envoplakin is green in panels A,B; vimentin is red in A,C). This protein was completely extracted by saponin treatment (Fig. 8D; note that there is no residual green fluorescence attributable to the envoplakin construct). Deletion of the C box had no effect on the distribution (Fig. 8E-G) or detergent solubility (Fig. 8H) of the envoplakin C-terminus (green in panels E,F,H; vimentin is red). However,

when E-1/4R+L+C was co-transfected with P-1/4R+L the envoplakin protein showed extensive colocalisation with intermediate filaments (Fig. 8I; green fluorescence denotes envoplakin construct; red fluorescence denotes periplakin construct) and could not be extracted with saponin (Fig. 8J-L; green: envoplakin; red: periplakin).

These experiments confirm that envoplakin and periplakin can still interact when most of their rod domains have been

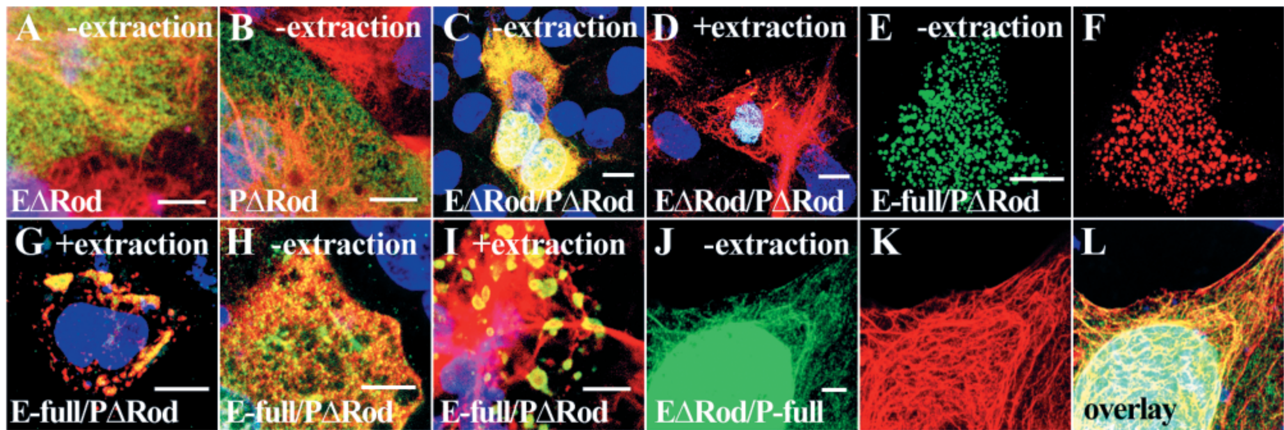

Fig. 6. Immunofluorescence analysis of COS7 cells transfected with full length envoplakin (E-full) or periplakin (P-full) or constructs lacking the rod domains (envoplakin, EΔRod; peiplakin, PΔRod). The cells were fixed without prior extraction (A-C,E,F,H,J-L) or extracted before fixation (D,G,I). Green fluorescence: anti-FLAG (detects tagged envoplakin; A,C-E,G-J,L); anti-HA (detects tagged periplakin; B). Red fluorescence: anti-HA (C,F,G,H,K,L); anti-vimentin (A,B,D,I). Blue fluorescence: nuclei stained with TOTO-3. In D note residual nuclear fluorescence attributable to EΔRod. Bars, 20 μm (C-H); 5 μm (A,B,I-L).

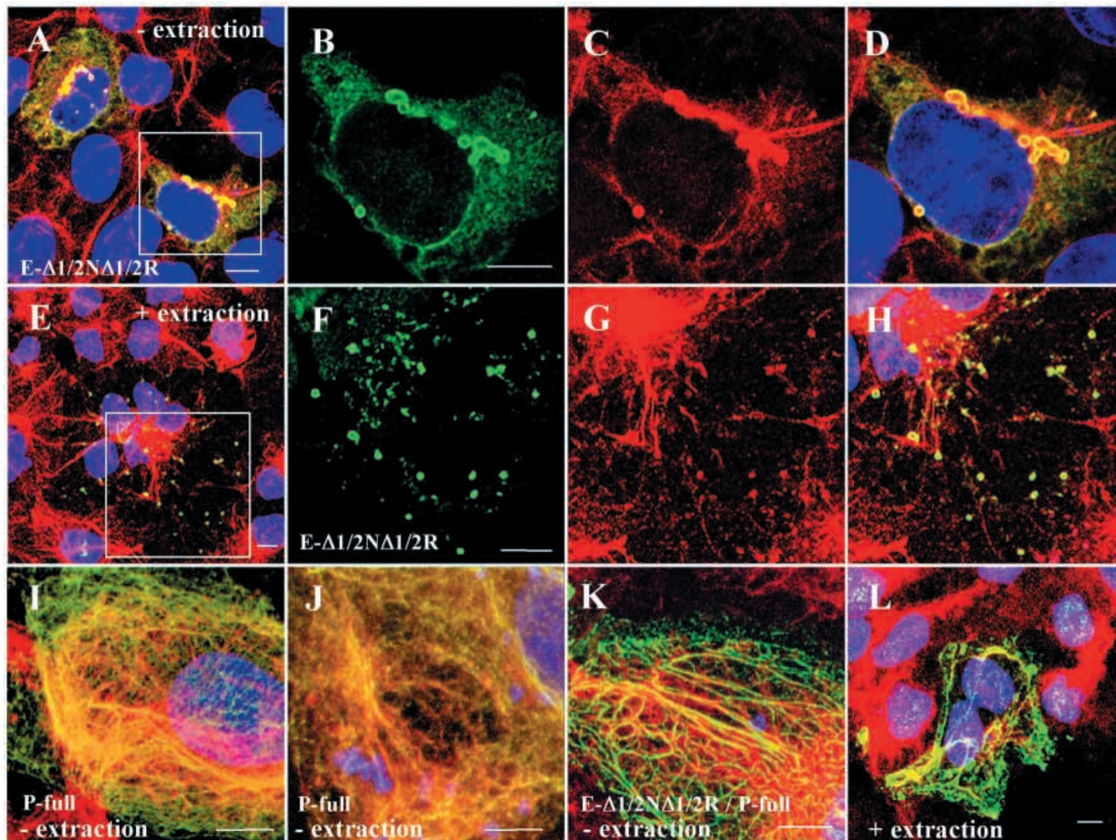

Fig. 7. Immunofluorescence analysis of COS7 cells transfected with E-Δ1/2NΔ1/2R alone (A-H) or in combination with P-full (K,L) or with P-full alone (I,J). Cells were fixed without prior extraction (A-D, I-K) or extracted with 0.6% saponin before fixation (E-H,L). Green fluorescence: anti-FLAG (to detect tagged envoplakin; A,B,D-F,H,K,L); anti-HA (to detect tagged periplakin; I,J). Red fluorescence: anti-vimentin (A,C-E,G-I,K,L), anti-keratin (J). Boxed areas in A,E are shown at higher magnification in B-D and F-H, respectively. Bars, 10 μm (A-H,L); 5 μm (I-K).

deleted and suggest that periplakin enhances and stabilises the interaction of envoplakin with intermediate filaments.

Discussion

We have shown that the periplakin C-terminus, the shortest of all the plakin C-terminal domains, is able to associate with keratin and vimentin intermediate filaments on the basis of colocalisation in transiently transfected cells and direct binding in overlay assays. We have shown that deletions within the two most highly conserved regions of the linker domain decrease the interaction. Constructs that showed extensive or partial colocalisation with intermediate filaments in non-extracted cells survived detergent extraction, whereas constructs that did not colocalise were completely extracted. The results of the overlay assays were consistent with the immunofluorescence data, except that two of the deletion constructs (P-LΔDWEEI and P-LΔ1/2Box2) that did not colocalise in cells showed weak binding in the overlays. The overlay assays suggest that periplakin may bind more effectively to some intermediate filament proteins than others, although this remains to be examined in detail.

We have previously shown that the C subdomain of envoplakin does not colocalise with intermediate filaments in transiently transfected cells (DiColandrea et al., 2000); the same is true of the isolated C box of desmoplakin (Stappenbeck et al., 1993). Different regions of the desmoplakin C-terminus interact with keratins and vimentin (Stappenbeck et al., 1993; Meng et al., 1997) and in assays with purified recombinant proteins, the desmoplakin C-terminus is reported to interact directly with keratin 5, but not with simple type II keratins, vimentin or type I keratins (Kouklis et al., 1994). Our overlay data support a preferred interaction of desmoplakin with keratin 5 rather than 14; however, we also saw some binding to COS7 cell intermediate filaments (predominantly vimentin and keratins 8 and 18).

Our understanding of the interaction of desmoplakin with intermediate filaments has been greatly increased by the recent studies of Choi et al. (Choi et al., 2002). They compared the binding of the C-terminal A, B and C subdomains of desmoplakin to vimentin in a co-sedimentation assay and also obtained the crystal structures of the B and C subdomains. All three subdomains bound vimentin when added in molar excess. A construct spanning domains A and B did not bind more

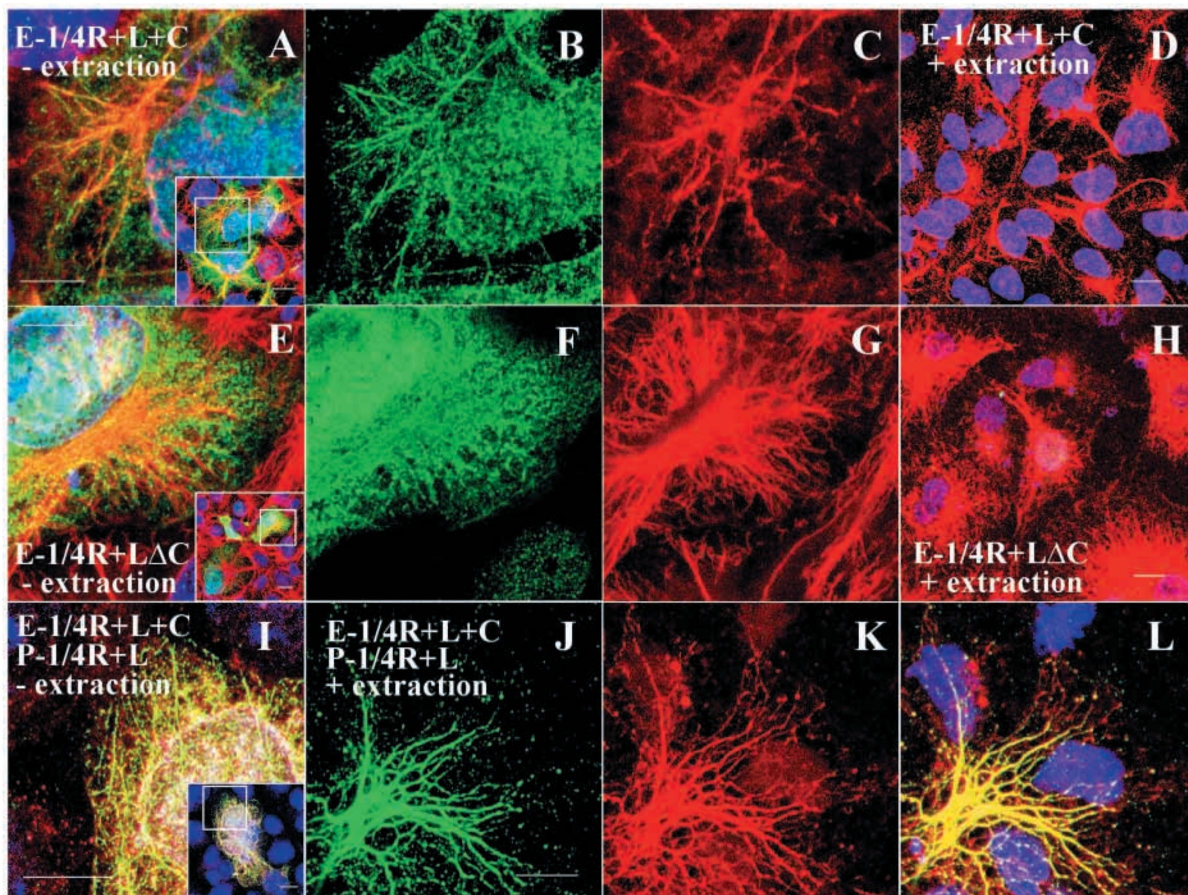

Fig. 8. Immunofluorescence analysis of COS7 cells transfected with envoplakin C-terminal constructs, alone (A-H) or in combination with the periplakin P-1/4R+L construct (I-L). Cells were fixed without prior extraction (A-C,E-G,I) or extracted before fixation (D,H,J-L). Green fluorescence: anti-FLAG (to detect tagged envoplakin). Red fluorescence: anti-vimentin (A,C,D,E,G,H); anti-HA (to detect tagged periplakin; I,K,L). Blue/purple fluorescence: nuclei stained with TOTO-3. Boxed inserts in A,E,I are shown at higher magnification in A-C, E-G and I, respectively. J-L are the same field. In D there is no residual green fluorescence and therefore no evidence that the cells shown were actually transfected. In H the central cell has weak residual green fluorescence. Bars, 10 μm.

strongly than either subdomain alone, whereas the combination of B and C did show enhanced binding. The B and C subdomains are connected by the linker sequence; since the combination of B plus linker did not increase vimentin binding the linker may contribute indirectly by providing the flexibility to allow more than one subdomain to bind simultaneously (Choi et al., 2002). The crystal structures of subdomains B and C suggest that a basic groove within the desmoplakin C-terminal domain may form the intermediate filament binding site (Choi et al., 2002).

The intermediate filament binding domain of plectin binds to and crosslinks all types of intermediate filament, but shows a higher affinity for vimentin than keratins 5 and 14 (Steinböck et al., 2000). One role of the C-terminal plakin repeats may be to modulate the affinity of the linker for particular classes of intermediate filament (Meng et al., 1997; Steinböck et al., 2000). It will be particularly interesting to know whether epiplakin, which consists of 13 B subdomains, but lacks the conserved linker motif, has any intermediate filament association (Fujiwara et al., 2001). Phosphorylation of plectin and desmoplakin regulates their interaction with intermediate filaments (Foisner et al., 1991; Stappenbeck et al., 1994; Wiche, 1998; Kowalczyk et al., 1999), but it remains to be determined whether this is also the case for envoplakin and periplakin.

The ability of the envoplakin C-terminus to colocalise with intermediate filaments was enhanced in the presence of the C-terminus of periplakin (Fig. 8), suggesting that periplakin not only mediates the association of envoplakin with the interdesmosomal plasma membrane (DiColandrea et al., 2000) but also regulates its association with intermediate filaments. As overlay assays were not performed with envoplakin constructs it is possible that rather than periplakin enhancing direct binding of envoplakin to intermediate filaments envoplakin binds to periplakin and only periplakin binds intermediate filaments. This seems, however, unlikely, given that envoplakin aggregates associate with intermediate filaments in the absence of periplakin (DiColandrea et al., 2000) and that all of the other conventional plakins bind intermediate filament proteins directly (reviewed by Leung et al., 2000). The envoplakin linker construct (E-1/4R+LΔC in Fig. 8) did show some colocalisation with vimentin filaments; however, it is surprising that it was not as strong as that of the periplakin linker (Fig. 3), given that the sequence conservation of the periplakin linker is highest with envoplakin (Ruhrberg et al., 1997; Mahoney et al., 1998; Määttä et al., 2000). One way to investigate this further would be by generating point mutations in the periplakin linker to systematically convert the sequence to that of envoplakin and test the effects on intermediate filament binding.

The envoplakin and periplakin C-terminal constructs tested for interaction (E-1/4R+L+C and P-1/4R+L) each contained a partial rod domain and this may have been sufficient for heterodimerisation. Alternatively the two C-termini may interact directly, by analogy with the ability of the plectin intermediate filament binding domain to self-associate (Wiche et al., 1993; Steinböck et al., 2000). We did not observe any collapse of the intermediate filament network in the presence of the periplakin C-terminus, in contrast to the effects of the plectin intermediate filament binding domain (Steinböck et al., 2000). However, the bundling of filaments in cells transfected with E-Δ1/2NΔ1/2R and full length periplakin (Fig. 7K,L) would suggest that envoplakin and periplakin can, like plectin, influence filament dynamics and crosslinking (Steinböck et al., 2000).

While the rod domains are undoubtedly important for the interaction of periplakin and envoplakin, most likely mediating heterodimer formation (Ruhrberg et al., 1997; DiColandrea et al., 2000), our data suggest that the proteins may be able to interact even when there are deletions within each rod domain. Thus full length periplakin could prevent an envoplakin construct lacking the N-terminal half of the rod domain from forming aggregates and facilitated its association with intermediate filaments (Fig. 7), and P-1/4R+L influenced the distribution and extractability of E-1/4R+L+C (Fig. 8). Perhaps most surprising was the finding that the two proteins appeared able to interact even when one was completely lacking the rod domain; this was most striking in cells co-transfected with EΔRod and P-full, where the presence of P-full allowed recruitment of EΔRod to intermediate filaments (Fig. 6). There is good evidence that plakins can form higher order complexes than dimers, although the nature of the interactions involved is currently unclear (reviewed by Ruhrberg and Watt, 1997; Wiche, 1998; Kowalzcyk et al., 1999). For example, plectin can self-associate in vitro and forms filamentous side arms that emerge from vimentin filaments (Foisner et al., 1988; Svitkina et al., 1996; Wiche, 1998). Acidic and basic residues are distributed periodically along the rod domains of plakins and ionic interactions could play a role in formation of higher order filamentous structures (Green et al., 1992). In the case of envoplakin and periplakin constructs lacking the rod domains other types of association must be involved and it is attractive to suggest a role for the linker domains, by analogy with the ability of the plectin intermediate filament binding domain to self-associate (Wiche et al., 1993; Steinböck et al., 2000).

In conclusion, we have shown that conserved sequences within the periplakin C-terminus mediate the association of periplakin with intermediate filaments, demonstrated that periplakin regulates the association of envoplakin with intermediate filaments, and obtained evidence to suggest that the interactions between envoplakin and periplakin extend to regions outwith their rod domains. The challenge now is to uncover the role of these interactions in the context of intact tissues, in particular the epidermis, where envoplakin and periplakin are early precursors in the assembly of the cornified envelope (Kalinin et al., 2001).

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Note added in proof

A recent study demonstrated a specific interaction of the periplakin linker domain with keratin 8 and vimentin (Kazerounian et al., 2002).

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