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Periplakin, a Novel Component of Cornified Envelopes and Desmosomes That Belongs to the Plakin Family and Forms Complexes with Envoplakin

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Abstract. The cornified envelope is a layer of transglutaminase cross-linked protein that is assembled under the plasma membrane of keratinocytes in the outermost layers of the epidermis. We have determined the cDNA sequence of one of the proteins that becomes incorporated into the cornified envelope of cultured epidermal keratinocytes, a protein with an apparent molecular mass of 195 kD that is encoded by a mRNA with an estimated size of 6.3 kb. The protein is expressed in keratinizing and nonkeratinizing stratified squamous epithelia and in a number of other epithelia. Expression of the protein is upregulated during the terminal differentiation of epidermal keratinocytes in vivo and in culture. Immunogold electron microscopy was used to demonstrate an association of the 195-kD protein with the desmosomal plaque and with keratin filaments in the differentiated layers of the epidermis. Sequence analysis showed that the 195-kD protein is a member of the plakin family of proteins, to which envoplakin, desmoplakin, bullous pemphigoid antigen 1, and plectin belong. Envoplakin and the 195-kD protein coimmunoprecipitate. Analysis of their rod domain sequences suggests that the formation of both homodimers and heterodimers would be energetically favorable. Confocal immunofluorescent microscopy of cultured epidermal keratinocytes revealed that envoplakin and the 195-kD protein form a network radiating from desmosomes, and we speculate that the two proteins may provide a scaffolding onto which the cornified envelope is assembled. We propose to name the 195-kD protein periplakin.

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The cornified envelope is believed to play a major role in the function of the epidermis as a protective barrier between the body and the environment. The envelope is a layer of insoluble protein, ~15-nm thick, that is closely apposed to the cytoplasmic face of the plasma membrane of keratinocytes in the outermost layers of the epidermis (for review see Reichert et al., 1993; Simon, 1994). The envelope is made of several precursor proteins that are cross-linked by ϵ-(γ-glutamyl) lysine bonds in a calcium-dependent reaction that is catalyzed by epidermal transglutaminases. Mutation of the cornified envelope precursor loricrin or the membrane-bound, keratinocyte-specific transglutaminase results in severe perturbation of epidermal differentiation and function (Huber et al., 1995; Maestrini et al., 1996).

In 1984, Simon and Green (1984) identified two membrane-associated proteins with apparent molecular weights of 195 and 210 kD that are upregulated during terminal differentiation of cultured epidermal keratinocytes, and that are cross-linked on transglutaminase activation. We have recently described the sequence of the 210-kD cornified envelope precursor and named it envoplakin (Ruhrberg et al., 1996). Envoplakin is expressed in both keratinizing and nonkeratinizing, stratifed squamous epithelia and belongs to the plakin family, which includes the proteins desmoplakin, bullous pemphigoid antigen 1 (BPAG1),[1] and plectin (for review see Green et al., 1992; Ruhrberg and Watt, 1997). Envoplakin colocalizes with desmoplakin at desmosomal plaques and on keratin filaments throughout the differentiated layers of human epidermis (Ruhrberg et al., 1996), raising the possibility that envoplakin is involved in anchoring keratin filaments to desmosomes. The sequencing of peptides released on proteolytic digestion of isolated cornified envelopes has provided direct evidence that both desmoplakin and envoplakin are cross-linked into the cornified envelope (Robinson et al., 1997; Steinert and Marekov, 1997). In addition to their potential

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1. *Abbreviations used in this paper*: BPAG1, bullous pemphigoid antigen 1; CSK, cytoskeleton extraction.

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role in anchoring keratin filaments to desmosomes, the two proteins may therefore also anchor desmosomes and keratin filaments to the cornified envelope in terminally differentiated epidermal keratinocytes.

We have now sequenced overlapping cDNA clones encoding the 195-kD cornified envelope precursor and show that, like envoplakin, it belongs to the plakin family of proteins. Its expression pattern and subcellular localization suggest that the 195-kD protein, like envoplakin, is associated with desmosomes and with keratin filaments in human epidermis. We speculate that envoplakin and the 195-kD protein provide a scaffolding on which the cornified envelope is assembled.

Materials and Methods

Screening of cDNA Libraries and cDNA Sequencing

A mouse mAb, 3c, raised against the 195-kD protein of Simon and Green (1984) was used to screen a random primed keratinocyte λgt11 expression library (a gift from R. Buxton, National Institute of Medical Research [NIMR] London, UK) using the conditions described previously for immunoblotting (Ruhrberg et al., 1996), and the cDNA clone p195-1 was isolated. A probe (P195-1) derived from this clone was used to rescreen the λgt11 expression library and to screen an oligo dT–primed plasmid library (provided by P. Jones, Imperial Cancer Research Fund [ICRF], London, UK) as described previously (Ruhrberg et al., 1996), and two further cDNA clones were isolated, p195-111 from the λgt11 library, and p195-5 from the plasmid library. The inserts of the λgt11 clones were subcloned into pBluescript II KS (+/−) (Stratagene Ltd., Cambridge, UK) for sequencing. The cDNA clones were sequenced with oligonucleotides synthesized by Oligonucleotide Synthesis Services, ICRF, using the dideoxy chain termination method with the Sequenase II kit (Amersham International plc., Little Chalfont, UK) or the ABI PRISM™ Cycle Sequencing Ready Reaction kit with fluorescent dye terminators (Perkin-Elmer, Beaconsfield, UK).

Northern Blot Analysis

Northern blotting was performed as described previously, using mRNA isolated from human epidermal keratinocytes that were cultured on tissue culture plastic or suspended for 24 h in methylcellulose (Ruhrberg et al., 1996). Briefly, 2 μg of polyadenylated (poly[A]$^+$) RNA per lane were separated on 1% formaldehyde gels, transferred to HybondN membrane (Amersham International plc.) and hybridized at 42°C for 16 h with probes labeled by random priming. The probe P195-1 was used to investigate whether the mRNA for the 195-kD protein was upregulated during terminal differentiation of epidermal keratinocytes, using a Northern blot that had previously been hybridized with probes specific for envoplakin, involucrin, and glyceraldehyde-3-phosphate dehydrogenase (Ruhrberg et al., 1996).

Structure Predictions and Sequence Comparisons

Secondary structure predictions were obtained using the algorithms of Garnier et al. (1978) or Chou and Fasman (1978) as implemented by MacVector 3.5 (International Biotechnologies Inc., Cambridge, UK). Coiled-coil analyses were performed using the program MacStripe (Knight and Kendrick-Jones, 1993), based on the algorithm of Lupas et al. (1991), and the computer predictions were corrected by visual inspection. Interchain ionic interactions between the two strands of a coiled-coil polypeptide sequence were examined using the method described by Parry et al. (1977). The predicted protein sequence was examined for potential transmembrane domains using MacVector 3.5, based on the method of Kyte and Doolittle (1982), and TOPRED II (Claros and von Heijne, 1994), based on the method of Argos and Rao (1986). The Swiss-Prot and PIR protein databases were searched with the BLAST program at the National Center for Biotechnology Information (Bethesda, MD). Dotplot homology comparisons were made with the programs COMPARE and DOTPLOT in the University of Wisconsin Genetics Computer Group (UWGCG; Madison, WI) software suite.

Alignments of the NH_2- and COOH-terminal domains of the plakin proteins were performed using the UWGCG programs, BESTFIT for pairwise comparisons, and PILEUP for multiple sequence comparisons. In calculating the percentage amino acid identity between the 195-kD protein and other plakins we excluded the rod domain because of its high content of heptad repeats. To calculate identity in the NH_2- and COOH-terminal sequences gaps of greater than three amino acids were not included.

Antibodies

The mouse mAbs 3c and 1b, raised against the 195-kD cornified envelope precursor described by Simon and Green (1984), were a gift of M. Simon (State University of New York, Stony Brook, New York). The mAb AE11, specific for the 195-kD keratinocyte protein described by Ma and Sun (1986), was a generous gift of T.T. Sun (New York University School of Medicine, New York). The mouse antiserum to envoplakin, M, used for immunoblotting, and the rabbit antiserum to envoplakin, CR1, used for immunogold electron microscopy and immunoprecipitation, have been described previously (Simon and Green, 1984; Ruhrberg et al., 1996). The mouse mAb to desmoplakin, 11-5F, used for immunofluorescence microscopy and immunoblotting, was a gift of D. Garrod (University of Manchester, Manchester, UK) (Parrish et al., 1987). The mouse mAb to desmoplakin, 2.15, used for immunofluorescence microscopy, was purchased from ICN Pharmaceuticals Inc. (Thame, UK). The rabbit antiserum to desmoplakin, DP_{121}, used for immunofluorescence microscopy, was a gift of A. Magee (NIMR) (Arnemann et al., 1993). A rabbit antiserum raised against gel-purified bovine epidermal desmocollins that reacts with both desmocollins and desmoplakin was used for immunofluorescence microscopy and was provided by D. Garrod.

A peptide corresponding to 25 amino acid residues (RLTPAQYDRYVNKDMSIQELAVLVSG) in the predicted COOH terminus of the 195-kD protein was synthesized by Peptide Synthesis Services, ICRF, conjugated to keyhole limpit hemocyanin (Pierce Chemical Co., Rockford, IL), and then injected into a rabbit to generate the antiserum CR3. Antiserum CR5 was raised in a rabbit against a his-tagged recombinant polypeptide containing NH_2-terminal sequences of envoplakin (amino acid residues 222–793; Ruhrberg et al., 1996); the recombinant polypeptide was expressed from the isopropyl β-D-thiogalactoside–inducible expression vector pTrcHis (Invitrogen, San Diego, CA) in *Escherichia coli* XL1-blue (Stratagene Ltd.), and then was purified on nickel columns under denaturing conditions using the Xpress™ protein purification system (Invitrogen) as recommended by the manufacturer.

FITC-conjugated, goat anti–rabbit IgG and Texas red–conjugated, horse anti–mouse IgG were obtained from Vector Laboratories (Peterborough, UK). FITC-conjugated, goat anti–mouse IgG and rhodamine-conjugated, goat anti–rabbit IgG were purchased from Tago Inc. (Burlingame, CA). Goat anti–mouse IgG conjugated to 5 nm gold was purchased from Bio Cell International (Cardiff, UK). Horseradish peroxidase–conjugated sheep anti–mouse or donkey anti–rabbit IgGs were purchased from Amersham International plc.

Immunoblotting and Immunoprecipitation

The following primary human cultured cells were used for immunoblotting: keratinocytes from neonatal foreskin epidermis and adult esophagus (grown as described previously by Ruhrberg et al., 1996), and dermal fibroblasts from neonatal foreskin. Human cell lines used for immunoblotting were: MCF7 (breast carcinoma), MTSV-1 (SV40-transformed mammary epithelium), HCA-7 and SW1222 (both colon carcinoma), Chang liver, HPAF (pancreatic carcinoma), EJ/28 (bladder carcinoma), and HeLa (cervical carcinoma). Extracts of human colon, kidney, and heart muscle were purchased from CLONTECH (Palo Alto, CA).

For immunoblotting, cultured cells were lysed in Laemmli SDS-PAGE sample buffer containing 10% β-mercaptoethanol and 10 mM EDTA as described previously (Ruhrberg et al., 1996). Tissue extracts were supplied in PAGE sample buffer, and 10% β-mercaptoethanol was added before analysis. Samples and molecular weight standards (high range, prestained protein molecular weight markers (GIBCO BRL, Paisley, UK), or rainbow molecular weight markers (Amersham International plc.) were resolved on 6% SDS-PAGE gels and immunoblotted as described previously (Ruhrberg et al., 1996).

In some experiments, subconfluent adherent keratinocyte cultures were incubated for various times with 50 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (sp act > 1,000 Ci/mmol; Trans^{35}S-label [ICN Pharmaceuticals

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Inc.]) before immunoprecipitation. For immunoprecipitation, ^{35}S-labeled or unlabeled confluent keratinocyte cultures were extracted in ice-cold cytoskeleton extraction (CSK) buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, and 0.5% Triton X-100; Fey et al., 1984) containing 250 mM $(NH_4)_2SO_4$, 10 mM EDTA, 2 mM PMSF, and 1 μg/ml leupeptin. Samples were immunoprecipitated as described previously (Ruhrberg et al., 1996), except that rabbit anti–mouse IgG (Sigma Chemical Co., Poole, UK) was added as a bridging antibody in immunoprecipitations with mouse mAbs. Immunoprecipitates were washed five times in ice-cold CSK buffer containing 300 mM NaCl, and then boiled in Laemmli sample buffer containing 10% β-mercaptoethanol. The released proteins were separated on 6% SDS-PAGE gels.

Induction of Cross-linking of Cornified Envelope Precursors by Transglutaminases

Confluent keratinocyte cultures were washed twice in serum-free FAD medium (Ruhrberg et al., 1996) and incubated for 5 h in the same medium at 37°C in the presence of 0.04% Triton X-100 or 100 μM of the calcium ionophore A23187 (Sigma Chemical Co.), dissolved in DMSO (final concentration 1%), to induce formation of cornified envelopes (Rice and Green, 1979). To inhibit transglutaminase activation, control cultures were incubated for 30 min at 37°C in the presence of 20 mM cystamine, pH 7.5, before addition of Triton X-100 or the calcium ionophore. For each incubation condition, protein was extracted from the cultures with equal volumes of Laemmli sample buffer containing 10% β-mercaptoethanol, but no bromophenol blue. Cell extracts were boiled for 10 min, and then centrifuged at top speed in a microfuge (Eppendorf, Madison, WI) for 5 min at 4°C to remove insoluble material. Equal volumes of extracts were processed for immunoblotting as described above.

Immunofluorescence Staining

Human tissue from various body sites was obtained at biopsy or autopsy, embedded in O.C.T. compound (Miles Inc., Stoke Poges, UK) and frozen in isopentane cooled in liquid nitrogen. Frozen tissue sections (3–6 μm) were air dried and either labeled immediately or fixed before labeling. Fixation conditions were either 5 min on dry ice in absolute methanol or 20 min in formaldehyde, which had been prepared by dissolving 3% (wt/vol) paraformaldehyde in PBS. Formaldehyde-fixed sections sections were then washed three times in PBS, followed by 10 min in 50 mM NH_4Cl in PBS, three washes in PBS and 5 min on dry ice in absolute methanol. Sections were stained with antibodies AE11, 3c, CR5, or 11-5F, followed by appropriate fluorescently conjugated secondary antibodies, as described previously (Ruhrberg et al., 1996). As controls, sections were labeled with preimmune serum or with secondary antibodies alone. Double labeling was performed with the rabbit antiserum CR5 and the mouse mAbs AE11 or 3c (envoplakin/p195), and with the rabbit antiserum DP_{121} and AE11 or 3c (desmoplakin/p195).

Keratinocyte colonies grown on glass coverslips were fixed for 20 min in formaldehyde as described above. Cells were permeabilized with CSK immunoprecipitation buffer for 10 min at room temperature, and after three additional washes in PBS stained with the antibodies as described above. For most double-labeling experiments, cells were extracted with CSK buffer for 4 min at room temperature before fixation to remove CSK-soluble protein. Double labeling was performed with the rabbit antisera CR1 or CR5 and the mouse mAbs 2.15 or 11-5F (envoplakin/desmoplakin), with CR5 and the mouse mAbs AE11 or 3c (envoplakin/p195), and with the rabbit antiserum DP_{121} or with a rabbit antiserum that recognizes desmocollins, desmoplakin, and AE11 or 3c (desmoplakin/p195).

Sections and coverslips were mounted in Gelvatol (Monsanto, St. Louis, MO) and examined using an Axiophot Microscope (Carl Zeiss Ltd., Oberkochen, Germany) or a laser scanning confocal microscope with a ×60 Plan-Apochromat 1.4 numerical aperture (NA) objective (model MRC-1000; Bio-Rad laboratories, Hercules, CA). For the analysis of the relative distributions of desmoplakin, envoplakin, and the 195-kD protein around desmosomes, Kalman-averaged (8 scans) confocal images were collected at 0.5-μm intervals in the z-axis using an iris aperture of 1–2.5-mm-diam. Images were captured within a linear range of fluorescent intensity based on the values of a standardized look-up table provided by the Comos confocal imaging software (Bio-Rad Laboratories). Images were processed with Photoshop 3.0 (Adobe Systems Inc., Mountain View, CA).

Immunogold Electron Microscopy

Thin sections of high pressure–frozen and freeze substituted–adult breast epidermis (a gift of E. Hunziker, University of Bern, Switzerland) and foreskin epidermis were immunogold-labeled as described before (Ruhrberg et al., 1996). For double labeling, the sections were first labeled with AE11, followed by a 1:35 dilution of goat anti–mouse IgG conjugated to 10 nm gold, and then with the rabbit antiserum CR1, followed by protein A conjugated to 5 nm gold (provided by the Cell Biology Department, University of Utrecht, The Netherlands). As a control, sections were labeled with secondary antibody or protein A–gold alone.

Results

Isolation of Overlapping cDNA Clones Encoding the 195-kD Cornified Envelope Precursor

A random-primed keratinocyte λgt11 cDNA expression library was screened with a mouse mAb (3c) raised against the 195-kD cornified envelope precursor described by Simon and Green (1984). A probe corresponding to an insert of the isolated cDNA clone, p195-1, was used to isolate two overlapping cDNA clones, p195-111 and p195-5 (Fig. 1). Clone p195-5 contained the polyadenylated 3′ end of the cDNA. The long open reading frame of clone p195-111 began with an ATG codon in a Kozak consensus sequence for translation initiation (Kozak, 1991); the position of this ATG codon corresponds to the start codon in the envoplakin gene, to which the 195-kD protein is closely related (see below).

A map of the isolated cDNA clones and a partial restriction map of the composite cDNA are shown in Fig. 1. Probes spanning the entire sequence of the composite cDNA detected only a single mRNA species of 6.3 kb in cultured human keratinocytes, suggesting that no abundant alternatively spliced mRNAs are expressed in this cell type (data not shown). The size of the composite cDNA (6.2 kbp) corresponds well to the estimated size of the mRNA detected by Northern blotting. The cDNA sequence and the amino acid sequence encoded by the long open reading frame are shown in Fig. 2. The encoded protein has a theoretical relative molecular weight of 205, which is in good agreement with the apparent molecular weight of 195 in SDS-PAGE.

The 195-kD Protein Is a Member of the Plakin Family

We searched the SwissProt and PIR protein databases with the predicted amino acid sequence of the 195-kD pro-

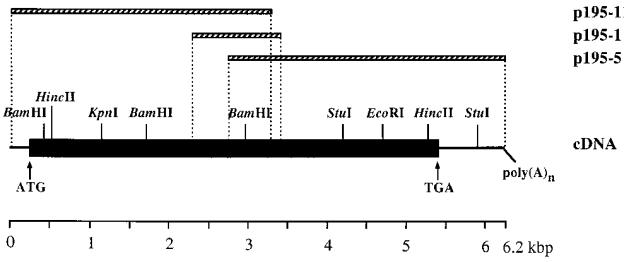

Figure 1. Isolated cDNA clones representing the mRNA of the 195-kD protein and partial restriction map of the composite cDNA. Clone names are shown on the right. The positions of the putative start codon (*ATG*) and stop codon (*TGA*) are indicated.

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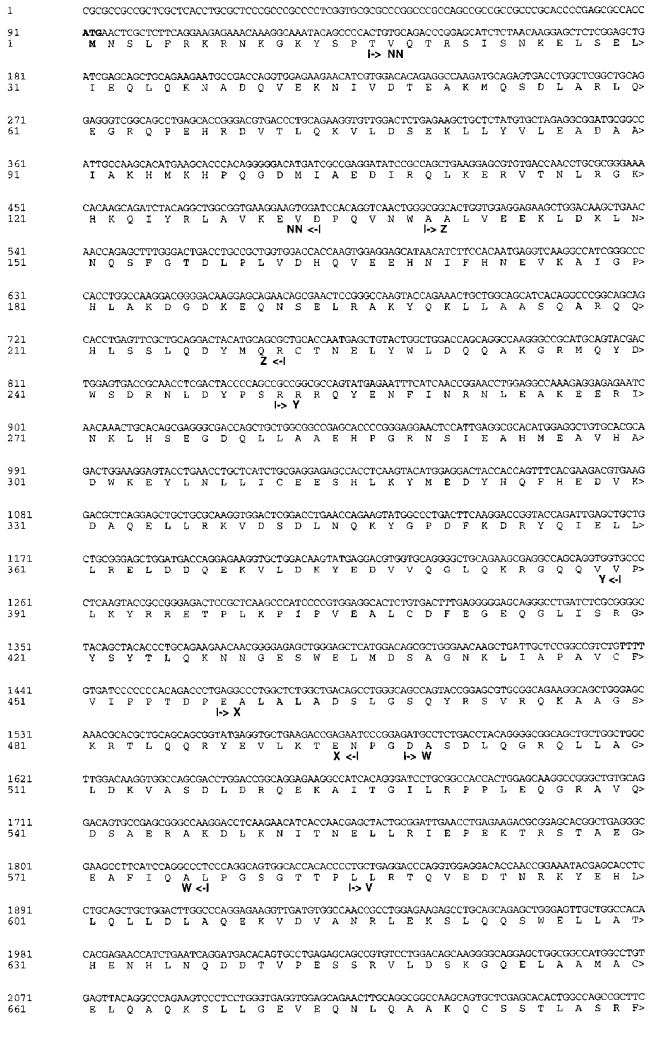

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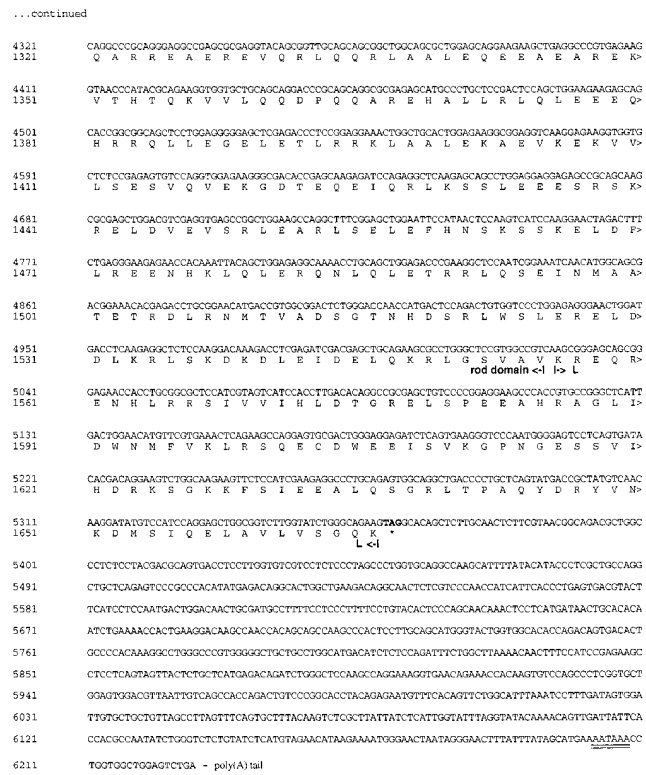

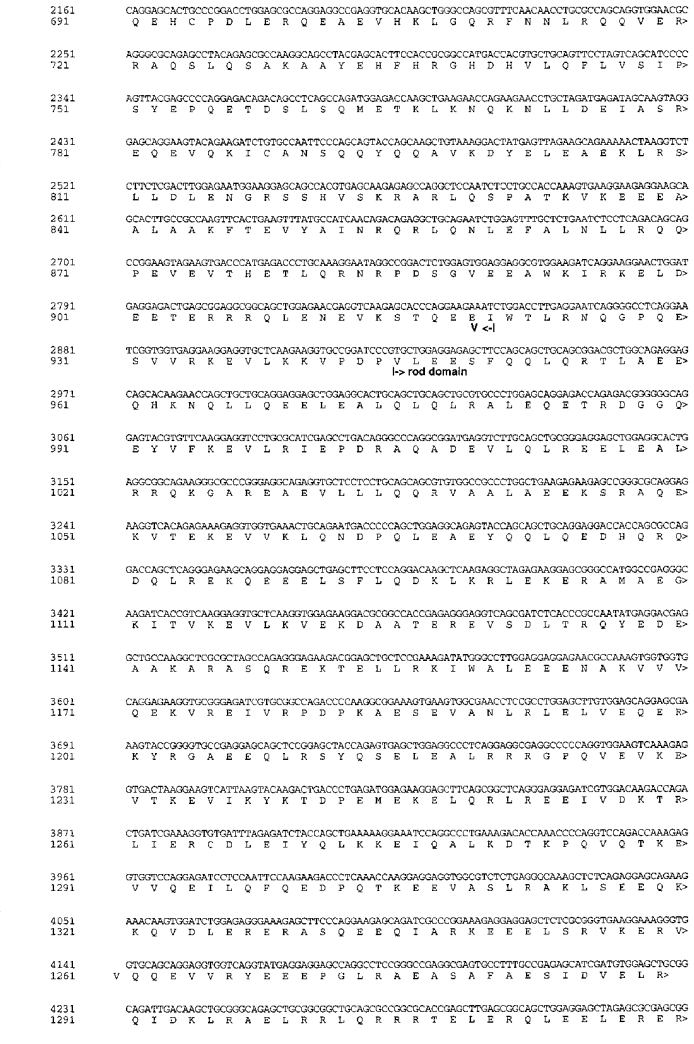

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Figure 2. Nucleotide sequence of the composite cDNA encoding the 195-kD protein and predicted amino acid sequence. Numeration of the cDNA sequence begins with the first nucleotide of the putative translation initiation codon (*ATG* [shown in bold face]). Numeration of the deduced amino acid sequence begins with the first methionine of the long open reading frame (*M* [shown in bold face]). The boundaries of the NH_2-terminal subdomains (*NN*, *V*, *W*, *X*, *Y*, and *Z*), the central rod domain, and the COOH-terminal L-subdomain are indicated by arrows (nomenclature of domains according to Green et al., 1992; except for *L*, linker region that precedes the C-subdomain in other family members). The long open reading frame terminates in a stop codon (*TAG*, *asterisk*), which is followed by a consensus polyadenylation signal (*AATAAA* [doubly underlined]) in the 3′ untranslated region. These sequence data are available from GenBank/EMBL/DDBJ under accession No. AF001691.

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tein and found extensive sequence homology to the proteins of the plakin family, which includes envoplakin, desmoplakin, BPAG1, plectin, and their alternative splice variants (Green et al., 1990; Sawamura et al., 1991*a*,*b*; Wiche et al., 1991; Virata et al., 1992; Liu et al., 1996; McLean et al., 1996; Ruhrberg et al., 1996; the sequence data of the extreme desmoplakin NH_2 terminus are available from GenBank/EMBL/DDBJ under accession No. M77830). The plakin proteins share a similar domain structure, characterized by the presence of globular end domains and a central rod domain that contains heptad repeats with a high probability of mediating coiled-coil formation with a dimerization partner (for review see Green et al., 1992; Ruhrberg and Watt, 1997).

As is the case for envoplakin, desmoplakin, BPAG1, and plectin, the NH_2 terminus of the 195-kD protein contains six putative α-helical subdomains, NN, Z, Y, X, W, and V, that are separated by non–α-helical regions and may fold into an antiparallel bundle (Figs. 2 and 3, *a*, *c*, *e*; see also Green et al., 1992; Ruhrberg et al., 1996). The COOH termini of desmoplakin, BPAG1, plectin, and envoplakin are predicted to be organized into discrete α-helical subdomains that are related by sequence; the most COOH-terminal of these α-helical regions, the C-subdomain, is present in all four proteins (Green et al., 1991; Ruhrberg et al., 1996). In contrast, the 195-kD protein does not contain a region with sequence homology to these subdomains; the only COOH-terminal region conserved between the 195-kD protein and the other plakins is the region that links the C-subdomain with the preceding domain in the other family members, and which we propose to name the L-subdomain (for "linker") (Figs. 2 and 3). The L-subdomain is predicted to adopt an α-helical conformation. As is the case for the other plakin proteins, the 195-kD protein is not predicted to contain a transmembrane domain. A schematic comparison of the predicted domain structure of envoplakin and the 195-kD protein is shown in Fig. 3 *a.*

A direct sequence comparison of the entire NH_2- and COOH-terminal amino acid sequences of the 195-kD protein with the respective domains of desmoplakin, BPAG1, plectin, and envoplakin showed that the 195-kD protein is most closely related to envoplakin. The sequence conservation in the NH_2- and COOH-terminal domains of the 195-kD protein and envoplakin is indicated by areas of lin-

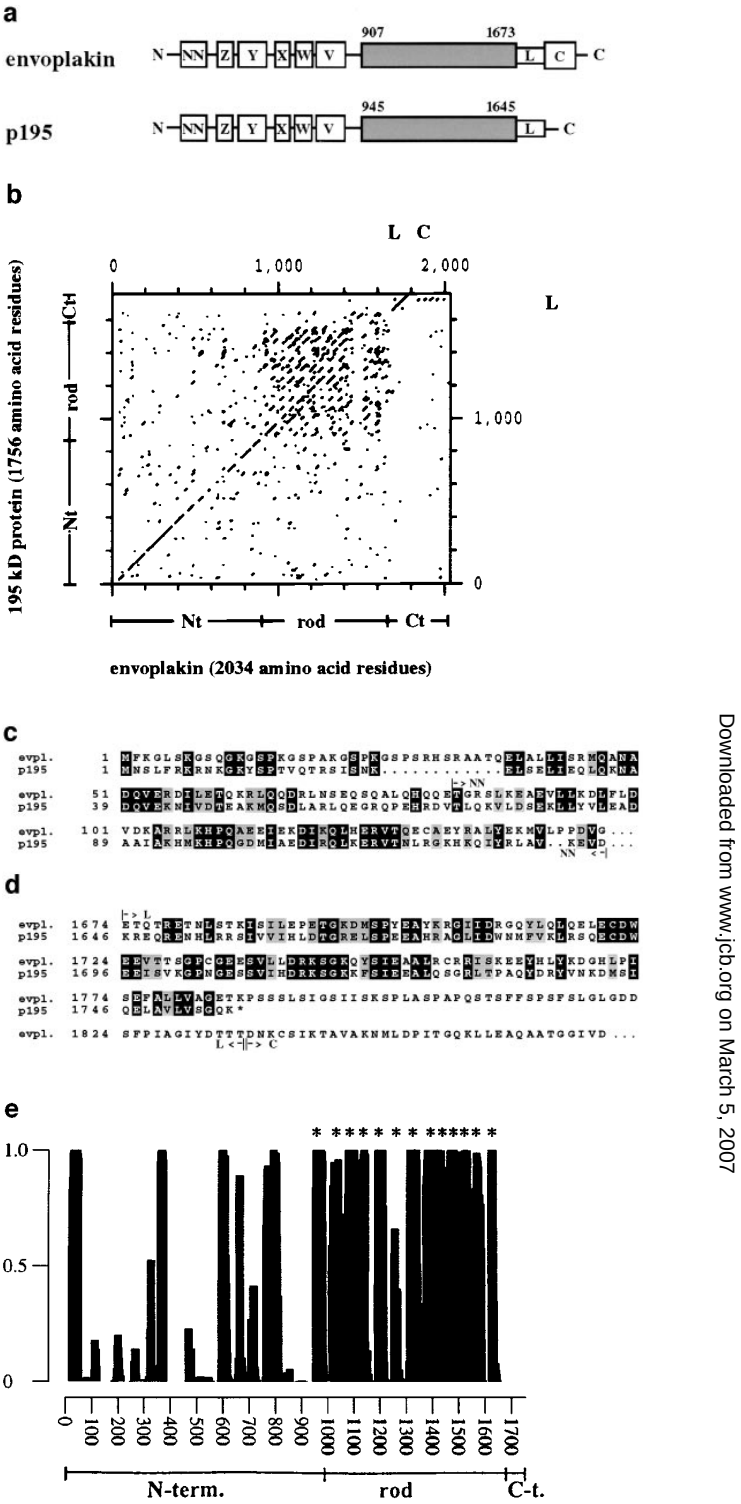

Figure 3. Similarity of the 195-kD protein to envoplakin. (*a*) Schematic representation of the domain structure of the 195-kD protein and envoplakin. The rod domains are represented by filled rectangles. For boundaries of the COOH-terminal linker region (L-subdomain) in envoplakin and the 195-kD protein see Figs. 2 and 3 *d*. (*b*) Dot matrix homology comparison of the 195-kD protein with envoplakin, performed with the software COMPARE and DOTPLOT; a dot was placed when 13 amino acids showed identity within a window of 20 amino acids. (*c* and *d*) Direct sequence comparison of the NH_2-terminal NN-subdomains (*c*) and the COOH-terminal L-domains (*d*) of the 195-kD protein and envoplakin. Amino acid residues in the sequence alignments are numbered according to their position in the protein sequences; the stop codon in the 195-kD protein is indicated with an asterisk; identical residues are shown on a black background, similar residues on a grey background. (*e*) Histogram of the probability of forming a coiled-coil (*y axis*), as predicted by the Lupas algorithm, versus the position in the amino acid sequence (*x axis*) for the 195-kD protein. Each division on the x axis scale corresponds to 100 amino acid residues. Regions with a value of $P > 0.9$ for >28 consecutive amino acid residues are predicted to adopt a coiled-coil conformation. The positions of the 13 heptad-rich subdomains (referred to in Tables I and II) in the rod domain are indicated above the histogram with asterisks. The boundaries of the NH_2- and COOH-terminal domains (*N-term.*, *C-t.*, respectively) and the rod domains are indicated below the histogram.

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ear homology in a dotplot matrix comparison (Fig. 3 *b*). The percentage amino acid identity between the 195-kD protein and envoplakin in the NH_2 terminus is 32%, compared with 25% for plectin, 23% for BPAG1, and 22% for desmoplakin. In the COOH terminus, the percentage amino acid identity with the 195-kD protein is 41% for envoplakin, 35% for plectin, 32% for desmoplakin, and 25% for BPAG1. Direct sequence comparisons of the amino acid sequences of the NH_2-terminal NN-subdomain and the COOH-terminal L-subdomain of the 195-kD protein and envoplakin are shown in Fig. 3, *c* and *d.*

Whereas the sequences of the NH_2- and COOH-terminal domains of all five plakins are well conserved, the central domain sequences are considerably diverged. However, a large number of heptad repeats with the potential of forming coiled-coils are present in the central domains (rod domains) of the 195-kD protein, envoplakin, desmoplakin, BPAG1, and plectin and appear as an area of "rectangular" homology in the dotplot matrices (Fig. 3, *b* and *e*; see also Ruhrberg et al., 1996). The rod domains of both the 195-kD protein and envoplakin are more closely related to each other than to other family members, with a similar overall length and a large number of heptad repeat–rich regions in different frames that are separated by stutter regions (Fig. 3 *e*; Ruhrberg et al., 1996).

Validation of the Identity of the 195-kD Protein as the Previously Described Cornified Envelope Precursor

To establish that the protein encoded by the cDNA we had isolated was indeed the 195-kD cornified envelope precursor described by Simon and Green (1984), we raised a rabbit antiserum (CR3) against a peptide corresponding to 25 amino acid residues at the COOH terminus of the protein encoded by the isolated cDNA. On immunoblots of keratinocyte extracts, CR3 detected a single protein with an apparent molecular mass of 195 kD that comigrated with the protein recognized by the 3c antibody that had been used to isolate cDNA clone p195-1 from a λgt11 expression library (Fig. 4 *a*). Furthermore, the protein detected by CR3 and 3c comigrated with the protein recognized by another mouse mAb, 1b, raised against the 195-kD cornified envelope protein described by Simon and Green (1984), and also with the 195 kD keratinocyte protein described by Ma and Sun (1986), and detected by antibody AE11 (data not shown). When we immunoprecipitated keratinocyte lysates with CR3, transferred the immunoprecipitates to nitrocellulose membranes, and then immunoblotted with 3c, 1b, or AE11, we found that all four antibodies recognized the same 195-kD protein (Fig. 4 *b*).

Confirmation That the 195-kD Protein Is Cross-linked by Transglutaminases and Is Upregulated during Keratinocyte Differentiation

When confluent keratinocyte cultures are treated with 0.04% Triton X-100 or the calcium ionophore A23187, the influx of calcium ions into the cells activates the cross-linking of cornified envelope precursors by transglutaminases (Rice and Green, 1979). Proteins that become cross-linked into the cornified envelope are no longer extractable by boiling the cell lysates in 1.6% SDS and 5 mM β-mercaptoethanol (Sun and Green, 1976; Simon and Green, 1984). Immunoblot analysis with antibodies CR3, 3c, and 1b showed that the 195-kD protein became nonextractable in SDS/β-mercaptoethanol after Triton X-100 (Fig. 5 *a*; and data not shown) or A23187 treatment (data not shown) of the cultures, suggesting that the 195-kD protein was cross-linked into cornified envelopes. When the cultures were treated with the transglutaminase inhibitor cystamine

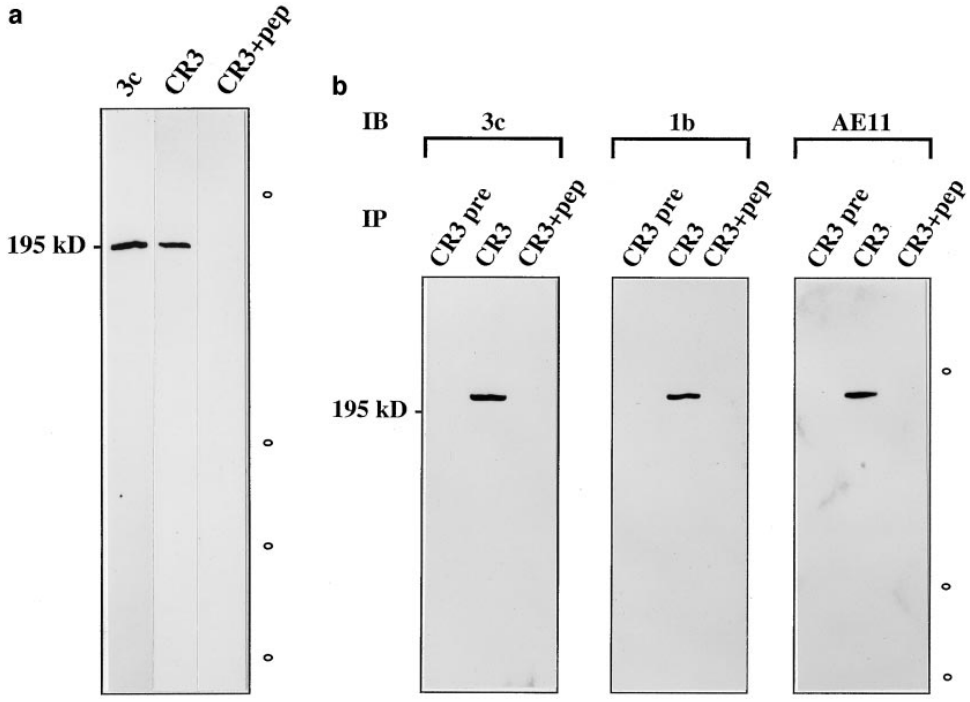

Figure 4. Confirmation that the isolated cDNA encodes the 195-kD cornified envelope precursor. (*a*) Immunoblot of keratinocyte extract probed with the antibody 3c (*3c*), raised against the 195-kD cornified envelope precursor described by Simon and Green (1984), with the antiserum CR3 (*CR3*), raised against a peptide corresponding to the COOH terminus encoded by the putative cDNA for the 195-kD protein, and with the antiserum CR3 in the presence of the peptide used for immunization (*CR3 + pep*). (*b*) Immunoprecipitation (*IP*) of keratinocyte extracts with CR3, CR3 in the presence of the peptide immunogen (*CR3 + pep*) or the preimmune serum (*CR3 pre*), followed by immunoblotting (*IB*) with antibodies 3c, 1b, or AE11. The mobility of the molecular weight standards is indicated: 200, 110, 75, and 43 kD.

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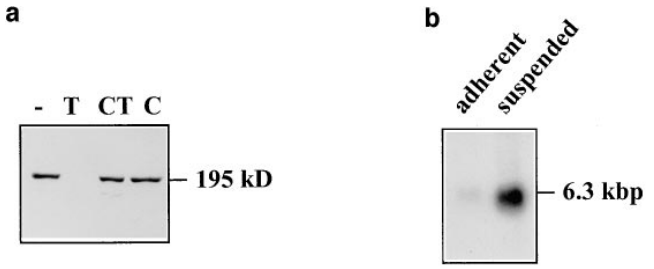

Figure 5. Confirmation that the 195-kD protein is a cornified envelope precursor, and upregulation of the mRNA encoding the 195-kD protein during terminal differentiation. (*a*) Adherent keratinocyte cultures were incubated for 5 h in serum-free medium in the absence (−) or presence of 0.04% Triton X-100 (*T*), 20 mM cystamine (*C*), or 20 mM cystamine and Triton X-100 (*CT*). A blot of the protein extracts was probed with the mouse antibody 1b to detect the 195-kD protein. (*b*) A Northern blot containing 2 μg poly(A)$^+$ RNA per lane from adherent or suspended keratinocyte cultures was hybridized with a radiolabeled DNA fragment derived from cDNA clone p195-1. The blot had been probed previously with an involucrin mRNA-specific probe as a control for the induction of terminal differentiation, with a glyceraldehyde-3-phosphate dehydrogenase-specific probe as a loading control, and with an envoplakin-specific probe (Ruhrberg et al., 1996).

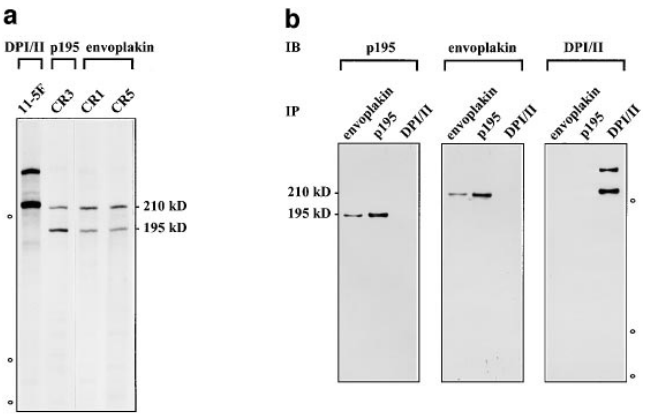

Figure 6. Coimmunoprecipitation of the 195-kD cornified envelope precursor and envoplakin. (*a*) Immunoprecipitation of Triton X-100 extracts of keratinocytes with antibodies specific for desmoplakin (*DPI/II*; lane *11-5F*), the 195-kD protein (lane *CR3*), and envoplakin (lanes *CR1* and *CR5*). (*b*) Immunoprecipitates (*IP*) with antibodies specific for desmoplakin (*DPI/II*; *11-5F*), the 195-kD protein (CR3), and envoplakin (CR1) were transferred to nitrocellulose and immunoblotted (*IB*) with antibodies specific for the 195-kD protein (3c), envoplakin (M), and desmoplakin (*DPI/II*; 11-5F). The mobility of the molecular weight markers is indicated: 200, 110, and 75 kD.

(Siefring et al., 1978) before addition of Triton X-100 or A23187, cross-linking of the 195-kD protein was inhibited (Fig. 5 *a*; and data not shown).

The level of the 195-kD protein is increased in differentiating keratinocytes in culture (Simon and Green, 1984). We used Nothern blotting to examine whether the mRNA for the 195-kD protein was also upregulated during terminal differentiation. Cultured human keratinocytes can be induced to undergo terminal differentiation when they are disaggregated and placed in suspension for 24 h. The level of the mRNA for the 195-kD protein was upregulated in suspension (Fig. 5 *b*), as are the mRNAs for envoplakin and involucrin, known cornified envelope precursors (Nicholson and Watt, 1991; Ruhrberg et al., 1996).

Coimmunoprecipitation of the 195-kD Protein and Envoplakin

On immunoblots, CR3 and AE11 recognized a single band corresponding to the 195-kD cornified envelope precursor, whereas the antienvoplakin antibodies, CR1 and CR5, recognized a single band corresponding to the 210-kD envoplakin (Fig. 4; and data not shown; Ma and Sun, 1986; Ruhrberg et al., 1996). In contrast, when each antibody was used to immunoprecipitate the Triton X-100–soluble protein fraction of keratinocyte cultures labeled overnight with [^{35}S]cysteine/[^{35}S]methionine, all four antibodies coimmunoprecipitated a 210- and a 195-kD protein (Fig. 6 *a*; and data not shown). The identity of the 210- and 195-kD proteins as envoplakin and the 195-kD cornified envelope precursor, respectively, was established by immunoblotting of the immunoprecipitates (Fig. 6 *b*; and data not shown). Envoplakin and the 195-kD protein were coimmunoprecipitated in keratinocytes labeled with [^{35}S]cysteine/[^{35}S]methionine for only 15 min, suggesting that the proteins complexed soon after translation (data not shown). The complex was dissociated by boiling in 1% SDS and 10% β-mercaptoethanol, but reformed after 10-fold dilution in immunoprecipitation buffer (data not shown). While envoplakin and the 195-kD cornified envelope precursor were coimmunoprecipitated, neither protein immunoprecipitated with the desmoplakin antibody 11-5F (Fig. 6, *a* and *b*), suggesting that in the Triton X-100–soluble pool envoplakin and the 195-kD protein can associate with each other, but not with desmoplakin.

Calculation of Potential Ionic Interactions between the Rod Domains of the 195-kD Protein and Envoplakin

The coimmunoprecipitation of the 195-kD protein and envoplakin raised the possibility that the two proteins might form heterodimers, and we therefore examined the predicted amino acid sequence of the rod domain of each protein. By visual inspection of the rod domain sequences of envoplakin and the 195-kD protein, we could assign the boundaries of individual heptad-containing subdomains and their frames more accurately than the computer algorithm had done. The frame of a heptad was assigned according to the position of the first amino acid in a continuous, heptad-containing region, where amino acid residues in the heptad are designated a through g (nomenclature according to McLachlan and Stewart, 1975) and amino acid residues in positions a and d are responsible for hydrophobic interactions at the core of the coiled-coil. The envoplakin and 195-kD protein rod domains both contained 13 heptad-rich subdomains of similar length and in the same frame, with an additional short heptad–containing region (designated 10-A) present in envoplakin, but not the 195-kD protein (Fig. 3 *e*; and Table I).

The relative polarity of coiled-coil chains, and their relative axial displacement, are both specified primarily by

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Table I. Heptad-containing Subdomains in the Rod Domains of the 195-kD Protein and Envoplakin

	p195		Envoplakin	
Subdomain	Amino acid No.	Frame	Amino acid No.	Frame
1	945–984	c	909–948	c
2	1,005–1,058	c	967–1,020	c
3	1,066–1,118	d	1,028–1,080	d
4	1,124–1,169	b	1,086–1,131	b
5	1,184–1,222	d	1,148–1,186	d
6	1,225–1,239	e	1,189–1,203	e
7	1,243–1,280	c	1,205–1,242	c
8	1,303–1,356	c	1,265–1,318	c
9	1,364–1,402	d	1,326–1,364	d
10	1,408–1,450	c	1,383–1,425	c
10-A	–	–	1,431–1,452	g
11	1,465–1,503	g	1,484–1,522	g
12	1,511–1,600	c	1,531–1,620	c
13	1,611–1,645	g	1,637–1,671	g

Amino acid residues are numbered according to their position in the predicted protein sequences, beginning from the NH_2 terminus. The frame of each heptad subdomain refers to the position of the first amino acid residue in its first heptad, where the heptad positions are named *a* to *g* in accordance with the nomenclature of McLachlan and Stewart (1975).

Table II. Predicted Interchain Ionic Interactions between the Rod Domains of the 195-kD Protein and Envoplakin

A

Stagger	1	2	3	4	5	6	7	8	9	10	11	12	13	Sum
±2	0	2	1	1	−5	0	−2	2	1	2	3	0	4	9
±1	−2	−2	−4	−5	−2	1	0	−4	3	−3	−5	3	−5	−25
0	0	12	4	4	4	2	6	10	0	4	6	8	6	66

B

Stagger	1	2	3	4	5	6	7	8	9	10	10-A	11	12	13	Sum
±2	0	1	−2	0	1	1	−1	−4	−2	3	1	3	1	1	3
±1	1	2	6	1	0	0	−1	2	0	−2	0	0	−5	−2	2
0	6	0	−2	2	6	−2	4	8	2	2	2	6	6	2	42

C

Stagger	1	2	3	4	5	6	7	8	9	10	11	12	13	Sum
−2	−1	4	4	−1	−1	0	−1	−1	0	3	3	0	3	12
−1	−1	−2	−1	−2	1	−1	0	0	0	−3	−3	1	−5	−16
0	2	6	7	2	1	0	6	3	0	4	4	8	5	48
1	0	−2	−2	−1	0	0	0	3	1	−2	−3	−4	−3	−13
2	2	1	2	0	−3	1	−3	−5	0	1	3	−2	2	−1

Ionic interactions in the rod domains of parallel 195-kD protein homodimers (*A*), envoplakin homodimers (*B*), and 195-kD protein/envoplakin heterodimers (*C*), are shown. The sum of possible ionic interactions for a parallel in register (*stagger 0*) arrangement and with relative axial displacements of the heptads by one or two heptads (*stagger ±1 and ±2*) are shown separately for each heptad-rich subdomain in columns *1–13*, and the sum of the ionic interactions involving all heptad subdomains is shown in the final column. The subdomain *10-A*, present only in envoplakin, has not been included in (*C*). We looked at interactions between amino acid pairs at positions 1g′–2e, 2e′–1g, 2a′–1g, 1g′–2a, 1e′–1d, and 1d′–1e (see main text). Ionic interactions were scored as follows: interactions of the type ± were scored as +1, whereas interactions of the type +/+ or −/− were scored as −1; all other interactions were scored as 0 (Parry et al., 1977).

ionic interactions between amino acid residues in positions e and g of the two chains (McLachlan and Stewart, 1975). To assess the extent to which homodimers or heterodimers of the 195-kD protein and envoplakin could be stabilized by ionic interaction, we used the scoring method described by Parry et al. (1977), who used this method to correctly predict the heterodimerization of type I and type II keratins. We looked at interactions between amino acid pairs at positions 1g′–2e, 2e′–1g, 2a′–1g, 1g′–2a, 1e′–1d, and 1d′–1e, where "1" is the heptad closer to the NH_2 terminus and "2" the following heptad; a to g and a′ to g′ represent the heptad positions in the 195-kD protein and envoplakin, respectively (see Parry et al., 1977). Ionic interactions were scored as follows: interactions of the type +/− were considered stabilizing and scored as +1, whereas interactions of the type +/+ or −/− were considered destabilizing and scored as −1. Using this analysis, we could confirm that the boundaries of each heptad-containing subdomain had been assigned correctly for both proteins; we identified many potential stabilizing ionic interactions when the boundaries of the heptad-containing subdomains were as described in Table I (Table II, *Stagger 0*), but there were few stabilizing ionic interactions when the heptad-containing subdomains were shifted relative to each other by one or two heptads (Table II, *Stagger ±1* or *±2*).

The values obtained when the interchain ionic interactions in the rod domains of the 195-kD protein and envoplakin are summed are consistent with the possibility that both can form homodimers (Table II, *A* and *B*). Parallel 195-kD protein homodimers would be stabilized by an average of 0.79 ionic interactions per pair of heptads in a two-stranded, coiled-coil rope. This value was obtained by dividing the number of amino acid residues in heptads (i.e., 585) (Table I) by 7, and using the resultant number (i.e., 83.6) to divide the sum of the predicted interchain ionic interactions (i.e., 66) (Table II *A*). Parallel envoplakin homodimers would be stabilized by an average of 0.48 ionic interactions per heptad in a two-stranded, coiled-coil rope (42 ionic interactions per 607 amino acid residues in heptads; Table II *B*). This analysis also showed that parallel 195-kD protein/envoplakin heterodimers would be stabilized by an average of 0.57 ionic interactions per common heptad pair (48 ionic interactions per 585 amino acid residues in heptads; Table II *C*) and therefore appear energetically as favorable as envoplakin homodimers. The corresponding value for a known parallel homodimer, α-tropomyosin, has been calculated as 0.74, whereas the corresponding value for a known parallel heterodimer, the type I and type II keratin heterodimer, has been calculated as 0.48 ionic interactions per heptad in the rod domains (Parry et al., 1977).

In summary, the analysis of the rod domain sequences of envoplakin and the 195-kD protein predicts that they could form two-stranded, in register parallel homo- or heterodimers. 195-kD protein homodimers were predicted to be the energetically most stable conformation, but 195-kD protein/envoplakin heterodimers were predicted to be as stable as envoplakin homodimers.

Expression and Cellular Localization of the 195-kD Protein in Tissues

Indirect immunofluorescent staining of unfixed frozen sections of human skin with the mAb 3c and AE11 to the 195-kD protein confirmed that the protein was expressed in the epidermis, but not the dermis (Fig. 7, *a* and *c*; and data not

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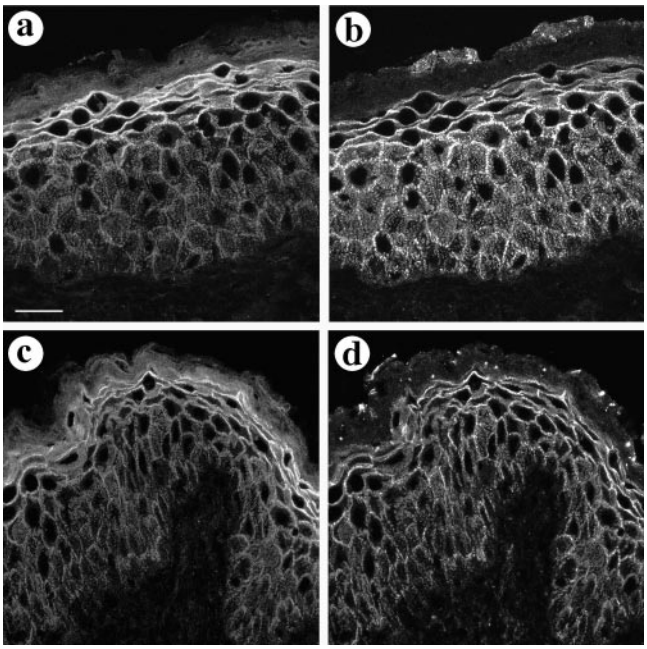

Figure 7. Expression of the 195-kD protein in human epidermis. Double-label immunofluorescence of human epidermis with an antibody specific for the 195-kD protein (3c) (*a* and *c*) and desmoplakin (DP_{121}) (*b*), or envoplakin (CR5) (*d*). Bar, 25 μm.

shown) (Ma and Sun, 1986). In contrast to desmoplakin staining, which is intense in all living layers of the epidermis, staining for the 195-kD protein was increased in the upper spinous and granular layers relative to the lower spinous and basal layers (compare Fig. 7, *a* and *c* with *b*). Staining was most prominent at the cell periphery and in the lower half of the epidermis appeared punctate. Double-label immunofluorescent staining with antibodies to the 195-kD protein and desmoplakin (Fig 7, *a* and *b*) or envoplakin (Fig. 7, *c* and *d*) showed colocalization of these proteins in the cell periphery in all living layers of the epidermis, suggesting that the 195-kD protein associated with desmosomes. None of the antibodies to the 195-kD protein stained the stratum corneum, suggesting that the epitope is masked in the cornified layers, as observed for involucrin (Ishida-Yamamoto et al., 1996).

To examine the distribution of the 195-kD protein in other tissues, we stained frozen sections with AE11 or 3c. All stratified squamous epithelia examined (epidermis from neonatal foreskin and adult breast, keratinized and nonkeratinized oral mucosa, esophageal, and cervical mucosa) were positively stained (Fig. 8, *a* and *c*; and data not shown) (Ma and Sun, 1986). In contrast to the other stratified squamous epithelia examined, in esophagus there was a striking difference between the labeling obtained with antibodies to the 195-kD protein and envoplakin. Whereas there was strong staining of all the suprabasal layers with an antibody to the 195-kD protein (Fig. 8 *c*), envoplakin labeling was strongest in the outermost layers (Fig. 8 *d*).

Expression of the two proteins was not restricted to keratinocytes (Fig. 8; and data not shown). There was clear, positive staining of the pseudo-stratified epithelium of human mammary glands (Fig. 8 *b*), the transitional epithelium of the urinary bladder (Fig. 8 *g*) and the simple epithelial gastric mucosa (Fig. 8 *f*) with antibodies to the 195-kD protein and envoplakin. In contrast to desmoplakin antibodies, AE11 and CR5 staining in thymus was confined to the concentric rings of Hassall's bodies (Fig. 8 *e*). We did not see convincing staining above background in the simple epithelial lining of the glomerular capsule or tubules in human kidney, nor the simple epithelium of endocervical glands, nor in pancreas, duodenum and colon, all of which stained positive for desmoplakin. We did not see staining of nonepithelial cells in the dermis, including fibroblasts and endothelial cells, nor did antibodies to the 195-kD protein and envoplakin stain brain tissue, skeletal muscle, cardiac muscle (which was desmoplakin-positive), or the nonstriated muscle of esophagus, bladder, and stomach. Immunoblotting of tissue extracts with antibodies to the 195-kD protein and envoplakin was negative for heart (desmoplakin-positive), but there was faint immunoreactivity for envoplakin in colon and for the 195-kD protein in kidney (data not shown).

Positive staining for envoplakin and the 195-kD protein was concentrated at the cell periphery in the tissues we examined. In mammary gland epithelium, staining was more restricted than observed with desmoplakin antibodies and was confined to the luminal cell layer (Fig. 8 *b*). In stomach mucosa, staining was concentrated in a ring at the upper lateral membranes (Fig. 8 *f*, *arrow*), and the apical cell surface appeared unlabeled. In bladder, the apical plasma membrane of the most superficial cells (umbrella cells) was intensely stained (Fig. 8 *g*, *arrow*).

Using immunoblotting, we readily detected the 195-kD protein in cultured human cells derived from a number of epithelia, but not in primary human foreskin fibroblasts (Fig. 9; and data not shown). Cells that were positive included primary esophageal keratinocytes and two mammary gland–derived lines, MCF7 and MTSV-1. These data are consistent with the observations of Ma and Sun (1986), who detected the AE11 epitope by immunoblotting in cultured mammary epithelial cells, but not in fibroblasts or capillary endothelial cells. The 195-kD protein was also expressed, albeit at lower levels, in two colonic mucosa–derived cell lines, HCA-7 and SW1222, and in the bladder line, EJ/28, but not in the Chang liver cell line. Although frozen sections of human pancreas did not stain positive for the 195-kD protein as described above, the protein was detected by immunoblotting in the pancreatic carcinoma line HPAF. Similar results were obtained when cell extracts were immunoblotted for envoplakin, with the exception that envoplakin was weakly detectable in the Chang liver line (data not shown). Blots were reprobed with an antitubulin antibody as a positive control (data not shown).

To examine the distribution of the 195-kD protein at the ultrastructural level, we labeled thin sections of epidermis, prepared by high pressure freezing/freeze substitution, with the antibody AE11. Like antibodies to envoplakin, AE11 labeled the cytoplasmic surface of the desmosomal plaque (Fig. 10, *b*, *d*, and *e*). As observed for envoplakin staining (Ruhrberg et al., 1996), AE11 labeled keratin filaments associated with desmosomes and keratin filaments throughout the cytoplasm in the lower cornified layers (Fig. 10 *a*). There was no specific labeling of keratohyalin

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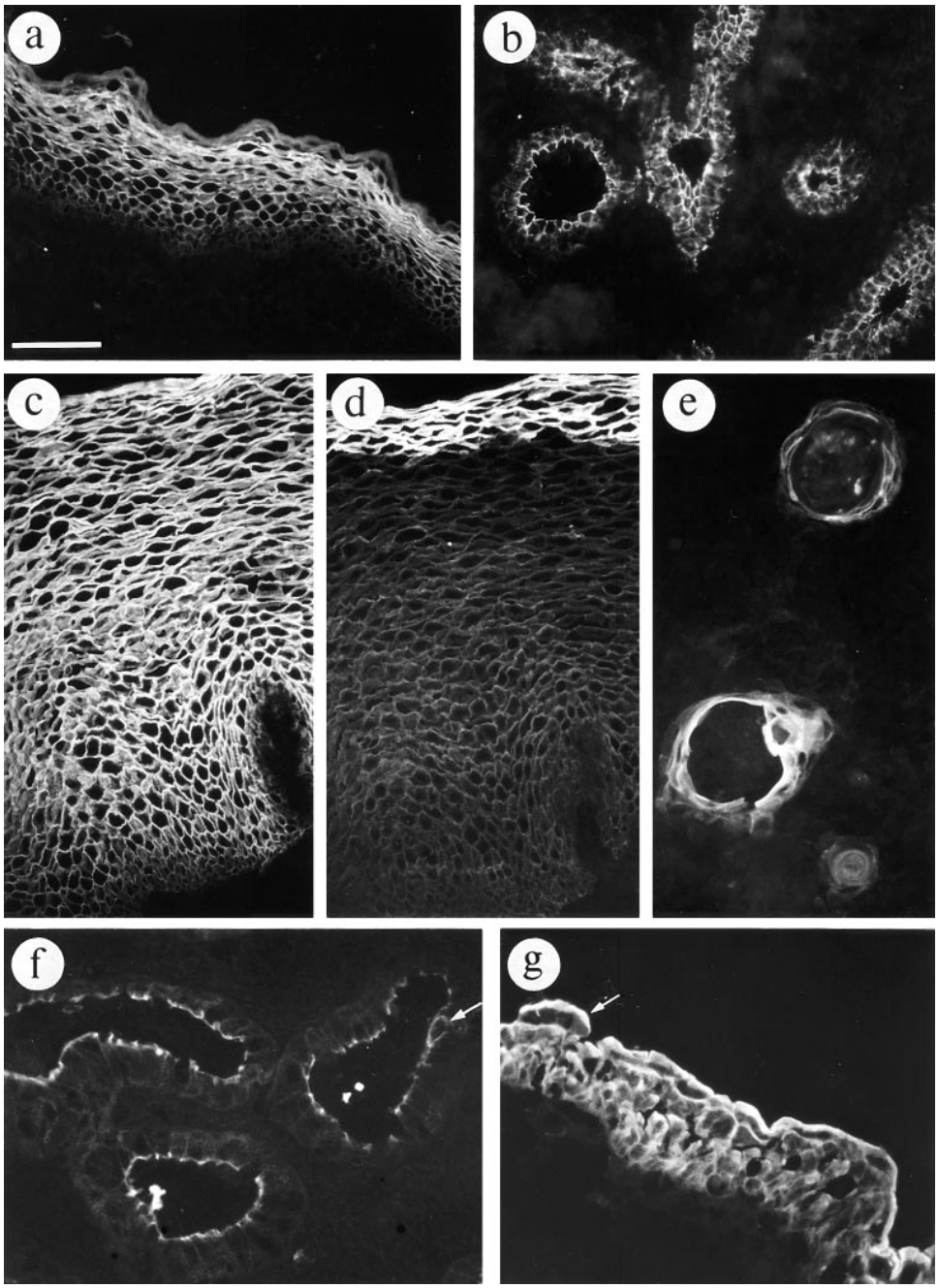

Figure 8. Tissue distribution of the 195-kD protein. Immunofluorescent staining of human cervical mucosa (*a*), mammary epithelium (*b*), esophageal mucosa (*c* and *d*), thymus (*e*), stomach mucosa (*f*), and bladder epithelium (*g*) with AE11, specific for the 195-kD protein (*a–c*, and *e–g*), and CR5, specific for envoplakin (*d*). The Hassall's bodies in *e* are positively stained. Arrow in *f* indicates cell with labeling of upper lateral membrane; apical membrane is not labeled. Arrow in *g* indicates labeling of apical plasma membrane. Bar: (*a*, *b*, and *e–g*) 100 μm; and (*c* and *d*) 200 μm.

granules (Fig. 10 *e*) or hemidesmosomes (Fig. 10 *c*), and in basal cells the desmosomal labeling was very weak (Fig. 10 *c*). The level of staining in the nucleus and cytoplasm of basal cells (Fig. 10 *c*) was the same as the background level found in sections incubated with protein A–gold or gold-conjugated secondary antibodies alone (data not shown).

Distribution of the 195-kD Protein, Envoplakin, and Desmoplakin in Cultured Keratinocytes

To examine the expression of the 195-kD protein in cultured keratinocytes, stratified colonies were stained with antibodies 3c or AE11. Cells were either fixed in paraformaldehyde dissolved in PBS, and then permeabilized with CSK buffer, or extracted with CSK buffer before fixation. In cultures fixed before extraction, we observed weak staining throughout the cytoplasm in all layers and in association with the plasma membrane in the suprabasal layers (data not shown). When the colonies were extracted with CSK buffer before fixation, staining was strong and concentrated in punctate regions at the plasma membrane in the suprabasal layers (Fig. 11, *a* and *c*). Similar results were obtained when stratified keratinocyte cultures were stained with an envoplakin antiserum (data not shown; see also Ruhrberg et al., 1996). Double-label immunofluorescence with antibodies 3c or AE11 and antibodies specific for desmoplakin (Fig. 11, *a* and *b*) or envoplakin (Fig. 11, *c* and *d*) showed partial colocalization of the 195-kD protein

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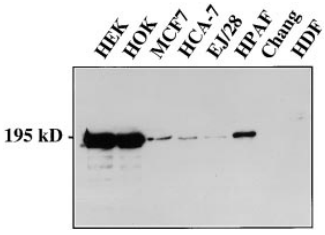

Figure 9. Immunoblot of cultured cells with the 1b antibody to the 195-kD protein. *HDF*, human dermal fibroblasts; *HEK*, human epidermal keratinocytes; *HOK*, human esophageal keratinocytes. 70 μg total protein was loaded per lane, except in the *HEK* and *HOK* lanes, where 15 μg was loaded.

with these proteins in the suprabasal layers. However, the staining for the 195-kD protein and envoplakin appeared more diffuse than the desmoplakin staining (compare Fig. 11, *a–d*). Occasionally, we observed colocalization of 195-kD protein and envoplakin with desmoplakin staining at cell borders in the basal layer in areas where cells appeared larger than neighboring basal cells, indicating that they had begun to terminally differentiate.

To gain a clearer view of the relative spatial organization of the 195-kD protein, envoplakin, and desmoplakin, we double labeled stratified keratinocyte cultures with antibodies specific for these proteins and examined their staining patterns in single 0.5-μm z sections acquired by laser scanning confocal microscopy. When areas where suprabasal cells made adhesive contact with the layer below were viewed "en face" at high magnification (Fig. 12), a distinct staining pattern was observed for all three proteins. Antidesmoplakin antibodies stained discrete areas (Fig. 12, *a*, *b*, *d*, and *f*), corresponding to individual desmo-

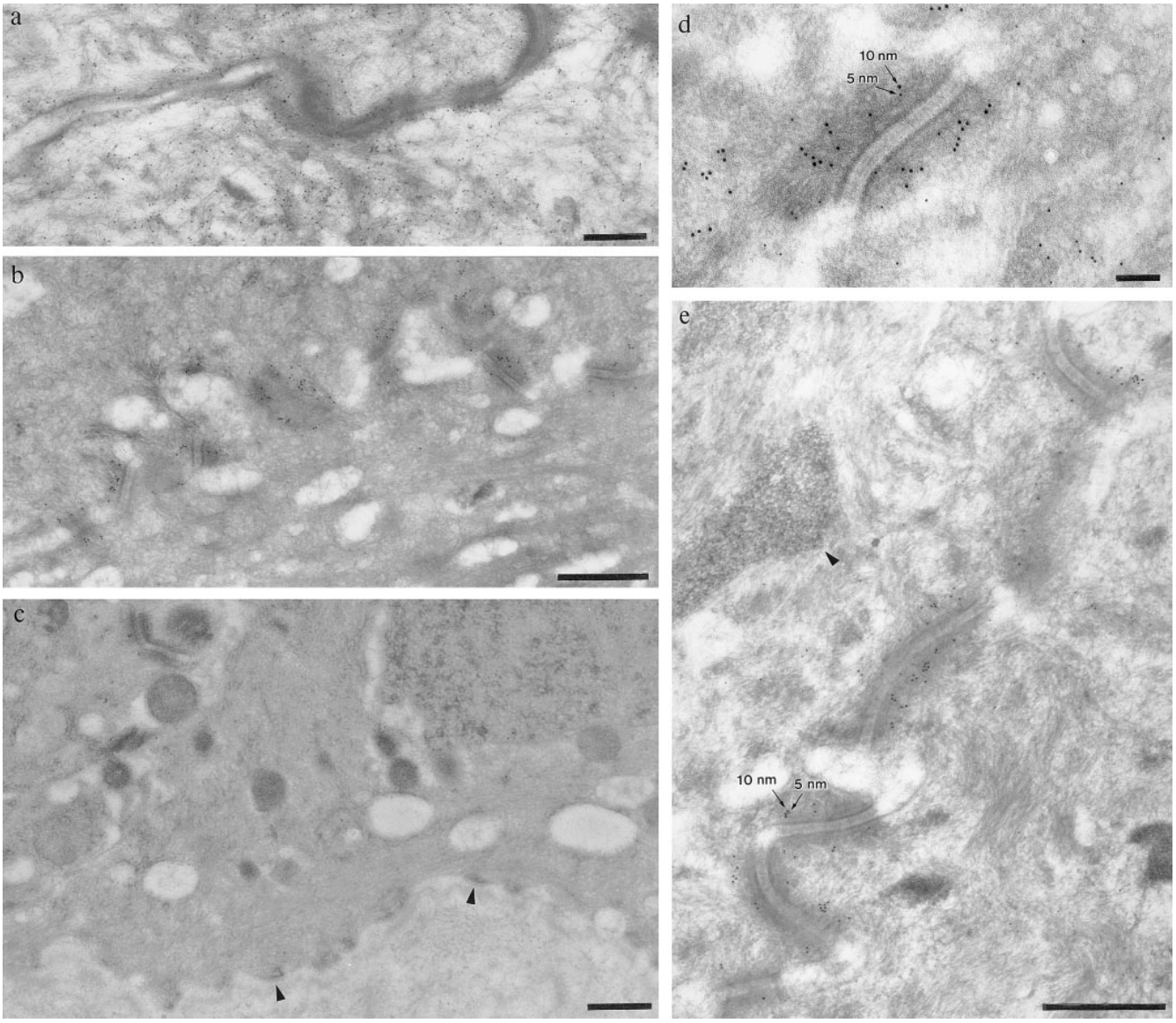

Figure 10. Localization of the 195-kD protein in epidermis, as determined by immunogold electron microscopy with the AE11 antibody. (*a*) Labeling with AE11; (*b–e*) double labeling with AE11 (10 nm gold) and an antibody specific for envoplakin (CR1, 5 nm gold). Breast epidermis (*a–d*); foreskin epidermis (*e*). Lowest cornified layers (*a*); upper spinous layers (*b* and *d*); basal layer (*c*); granular layer (*e*). Arrowheads in *c* indicate the basement membrane zone; arrowhead in *e* indicates a keratohyalin granule. Bars: (*d*) 100 nm; and (*a–c*, and *e*) 500 nm.

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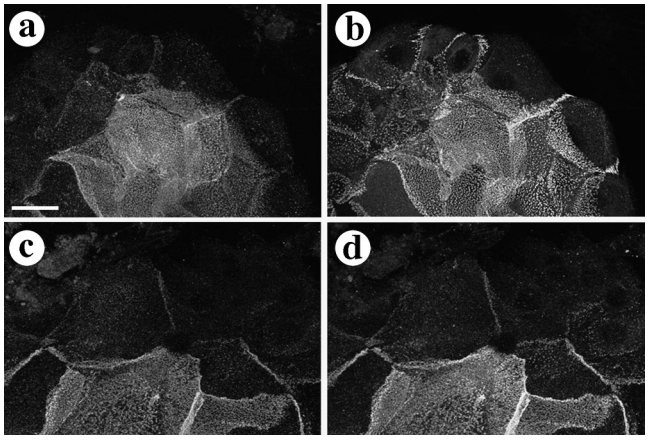

Figure 11. Localization of the 195-kD protein in stratified colonies of cultured keratinocytes. Double-label immunofluorescence of stratified epidermal keratinocytes with an antibody specific for the 195-kD protein (AE11) (*a* and *c*), and desmoplakin (DP_{121}) (*b*), or envoplakin (CR5) (*d*). Bar, 25 μm.

somal plaques, as previously reported (Duden and Franke, 1988). The 195-kD protein and envoplakin showed partial colocalization with desmoplakin; but in addition, staining extended beyond the areas defined by desmoplakin antibodies (Fig. 12, *c–f*). There appeared to be less overlap between 195-kD protein and desmoplakin staining than between envoplakin and desmoplakin staining (compare Fig. 12, *c* and *d* with *e* and *f*). As a control, we performed double-label immunofluorescence with two different desmoplakin antibodies, and they gave good colocalization (Fig. 12, *a* and *b*). We further confirmed the relative distribution of the 195-kD protein, envoplakin, and other desmosome proteins using different combinations of antibodies (Fig. 13).

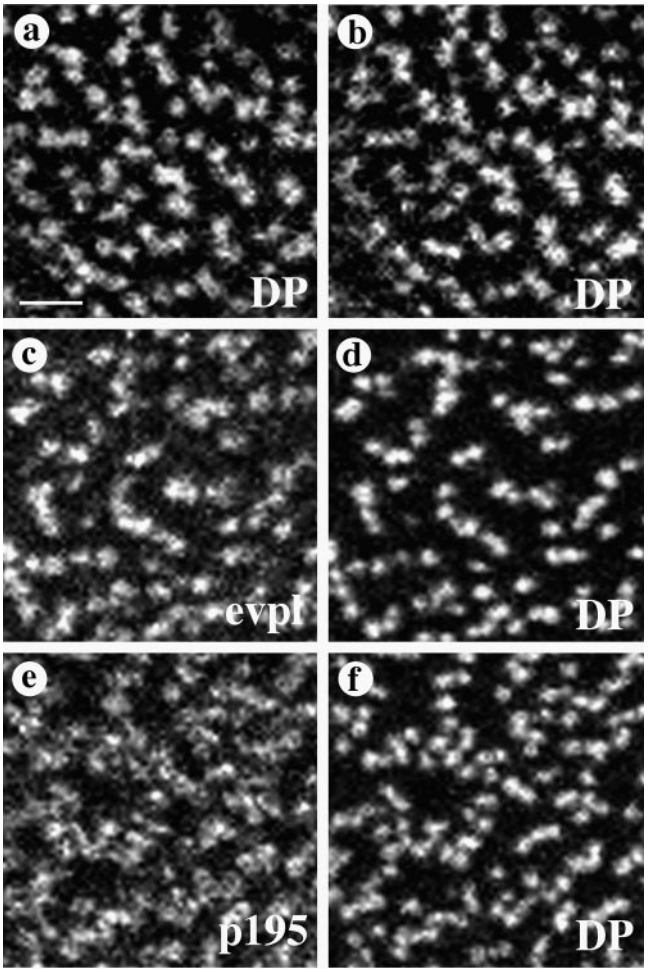

Figure 12. Relative organization of the 195-kD protein, envoplakin, and desmoplakin. "En face" view of desmosomes in 0.5-μm z sections, taken in the suprabasal layers of stratifed colonies of cultured keratinocytes that were fluorescently double labeled with antibodies specific for desmoplakin (11-5F: *a* and *f*; DP_{121}: *b* and *d*) and envoplakin (CR5: *c*), or the 195-kD protein (AE11: *e*). Bar, 2 μm.

Discussion

In this study we describe the cDNA sequence of the membrane-associated, 195-kD cornified envelope precursor first identified by Simon and Green (1984), and we show that it is identical to the 195-kD protein identified by Ma and Sun (1986) as an antigen in keratinocyte protein lysates that is recognized by the AE11 antibody. The level of the 195-kD protein is increased during keratinocyte terminal differentiation in culture and in vivo, and the protein localizes to keratin filaments and desmosomes in differentiated keratinocytes.

The 195-kD cornified envelope precursor is a member of the plakin family, other members of which are of crucial importance in maintaining the integrity of the skin and other tissues (Guo et al., 1995; McLean et al., 1996; Uitto et al., 1996; Yang et al., 1996; for review see Ruhrberg and Watt, 1997). Like the other plakins, the 195-kD protein is predicted to contain a globular NH_2-terminal domain, consisting of a bundle of antiparallel α helices (NN, Z, Y, X, W, and V), and a central coiled-coil rod domain. The COOH terminus of the 195-kD protein differs from those of the other plakins in that it lacks any of the sequence-related subdomains, designated A, B, or C, which consist of α helices separated by β turns. The COOH terminus of the 195-kD protein does however contain the α-helical linker region that connects the C-subdomain with the preceding COOH-terminal subdomain in the other plakins. The primary sequences of the NH_2 and COOH termini of the 195-kD protein show >20% sequence identity to the corresponding regions of BPAG1, plectin, desmoplakin, and envoplakin, the sequence similarity being greatest in the L-subdomain; the 195-kD protein is most closely related to envoplakin. Interestingly, all the plakins show some sequence similarity to the protein ACF7, encoded by a partial cDNA from a brain library (Bernier et al., 1996); however, until its complete cDNA sequence and domain structure have been determined, it is not clear how ACF7 is related to other family members.

The COOH-terminal domains of desmoplakin, BPAG1, and plectin mediate the association of each protein with intermediate filaments (Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Wiche et al., 1993; Kouklis et al., 1994; for review see Ruhrberg and Watt, 1997). In the case of plectin, a 50–amino acid region in the L-subdomain, in

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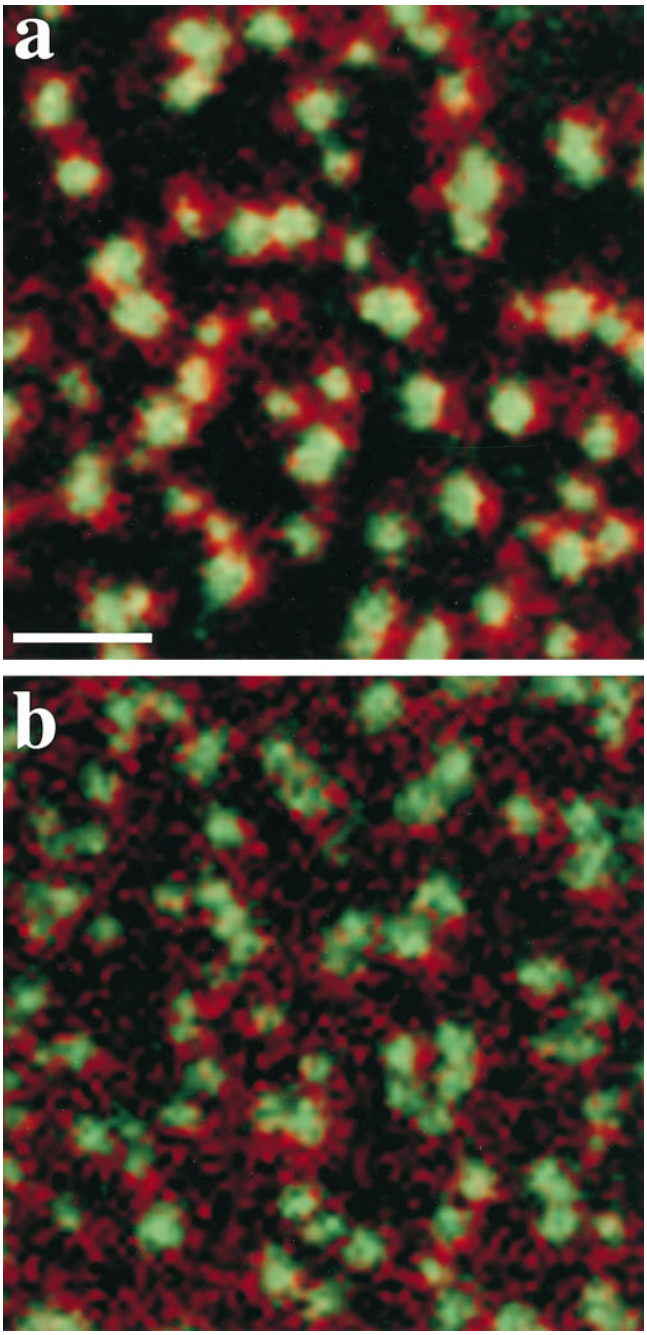

Figure 13. Relative organization of the 195-kD protein and envoplakin at desmosomes. Overlay of en face views of desmosomes in 0.5-μm z sections, taken in the suprabasal layers of stratified keratinocyte colonies that had been fluorescently labeled (*a*) with CR1, specific for envoplakin (*red*), and 2.15, specific for desmoplakin (*green*), or (*b*) with AE11, specific for the 195-kD protein (*red*), and an antiserum specific for desmocollins and desmoplakin (*green*). Bar, 2 μm.

the context of a B- or a C-subdomain, is required for the association of plectin with vimentin intermediate filaments in intact cells and in cell extracts (Nikolic et al., 1996). In agreement with this, recombinant desmoplakin polypeptides containing the entire COOH terminus, including the L-subdomain, localize to vimentin and keratin filaments, whereas polypeptides containing the C-subdomain, but not the L-subdomain, do not (Stappenbeck et al., 1993; Green, K., personal communication). It is therefore possible that envoplakin, which contains an L-subdomain and a C-subdomain, is able to directly bind intermediate filaments, whereas the 195-kD protein could not, although further experiments are required to test these ideas. Coimmunoprecipitation experiments show that the 195-kD protein can form complexes with envoplakin, raising the possibility that it is recruited to keratin filaments via its interaction with envoplakin.

As observed for desmoplakin (Pasdar and Nelson, 1988 *a*,*b*), envoplakin and the 195-kD protein are localized in two pools that can be distinguished on the basis of their solubility in CSK buffer: the CSK-soluble pool, which is distributed throughout the cytoplasm, and the CSK-insoluble pool, which is stably associated with desmosomes. Our immunoprecipitation experiments suggest that neither protein interacts with desmoplakin in the CSK-soluble pool. The mobility of the 195-kD protein is reduced in the CSK-insoluble pool (Ma and Sun, 1986), and it is known that the association of desmoplakin with keratin filaments and its incorporation into desmosomes are regulated by phosphorylation (Sheu et al., 1989; Stappenbeck et al., 1994). Further experiments are required to determine if the 195-kD protein and envoplakin undergo posttranslational modifications, and whether this might regulate their cellular distribution or their interactions with each other and with other proteins such as desmoplakin.

A detailed sequence analysis of the 195-kD protein and envoplakin showed that their rod domains are more closely related to each other than to those of the other plakins, and suggested that the two proteins could form two-stranded, in register, parallel homodimers or heterodimers, which would be stabilized by extensive interchain ion pairing. Whereas there is no evidence that other members of the plakin family can heterodimerize (O'Keefe et al., 1989; Tang et al., 1996; see also Ruhrberg and Watt, 1997), there is evidence, in the case of plectin, that homodimers might assemble into higher order complexes (Foisner et al., 1988; Wiche et al., 1991; Svitkina et al., 1996). It is also possible that higher order complexes consisting of more than one type of plakin can form; plectin and BPAG1 are both required for the anchorage of keratin filaments to hemidesmosomes, and loss of plectin can affect the localization of BPAG1 to the hemidesmosomal plaque (Guo et al., 1995; Yang et al., 1996; see also Uitto et al., 1996). Further experiments are required to determine whether the coimmunoprecipitates of the 195-kD protein and envoplakin represent heterodimers or consist of homodimers assembled into higher order complexes, and whether the envoplakin and 195-kD protein networks observed by immunofluorescence are built of homo- or heterodimers. It is clear from our localization studies in tissues and in cultured keratinocytes that the 195-kD protein and envoplakin do not completely codistribute and thus, even if heterodimers can form, homodimers must exist.

Although the 195-kD protein and envoplakin were originally identified as precursors of the cornified envelope in cultured keratinocytes, their expression in a variety of other epithelia suggests that they might have additional functions. The sequence homology of the 195-kD protein

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and envoplakin to desmoplakin, and their localization to desmosomes and keratin filaments raise the possibility that they, together with desmoplakin, are involved in anchoring keratin filaments to desmosomes. The 195-kD protein and envoplakin are not constitutive components of all desmosomes, and their presence may alter the properties of these junctions, as has been proposed for pinin and may be the case for plakophilin 1, two other components of the cytoplasmic plaque of desmosomes with cell type–specific expression patterns (Heid et al., 1994; Schmidt et al., 1994; Ouyang and Sugrue, 1996). One possibility is that envoplakin and the 195-kD protein influence the mechanical integrity of desmosomes, by analogy with the role of BPAG1 in hemidesmosomes (Guo et al., 1995). Additional functions of the proteins may be inferred from the staining we observed in bladder; the 195-kD protein and envoplakin were concentrated at the exposed apical membrane of the superficial epithelial cells (umbrella cells), a membrane that is devoid of desmosomes but contains rigid plaques known as the asymmetrical unit membrane (Surya et al., 1990).

Confocal analysis of cultured epidermal keratinocytes revealed an overlapping but distinct distribution for desmoplakin, envoplakin, and the 195-kD protein, with envoplakin and the 195-kD protein being organized in an extensive network surrounding desmoplakin. Steinert and Marekov (1995, 1997) have shown, by the controlled proteolysis of intact cornified envelopes, that involucrin is one of the first proteins to be incorporated into the cornified envelope, and that it is cross-linked to desmoplakin and envoplakin. Whereas further experiments are needed to test our hypothesis, we propose that a network of plakins, organized around the desmosomal core, could provide a scaffolding onto which involucrin and other cytoplasmic precursors of the cornified envelope could be cross-linked by transglutaminases. Furthermore, the cross-linking of desmoplakin, envoplakin, and the 195-kD protein into the cornified envelope could explain how the cornified envelope is linked to desmosomes and keratin filaments (Haftek et al., 1991; Ming et al., 1994; Steinert and Marekov, 1995, 1997). Because it is a new member of the plakin family that is organized around desmosomes in differentiated keratinocytes, we propose to name the 195-kD protein periplakin.

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