Enoplakin, a Novel Precursor of the Cornified Envelope That Has Homology to Desmoplakin

Christiana Ruhrberg,* M. A. Nasser Hajibagheri,† Marcia Simon,‡ Thomas P. Dooley,‖ and Fiona M. Watt*

*Keratinocyte Laboratory and †Electron Microscopy Unit, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom; ‡Department of Oral Biology and Pathology, School of Dental Medicine, Department of Dermatology, School of Medicine, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, New York 11794-8702; and ‖Department of Molecular Pharmacology, Southern Research Institute, Birmingham, Alabama 35205

Abstract. The cornified envelope is a layer of transglutaminase cross-linked protein that is deposited under the plasma membrane of keratinocytes in the outermost layers of the epidermis. We present the sequence of one of the cornified envelope precursors, a protein with an apparent molecular mass of 210 kD. The 210-kD protein is translated from a 6.5-kb mRNA that is transcribed from a single copy gene. The mRNA was upregulated during suspension-induced terminal differentiation of cultured human keratinocytes. Like other envelope precursors, the 210-kD protein became insoluble in SDS and β-mercaptoethanol on activation of transglutaminases in cultured keratinocytes. The protein was expressed in keratinizing and nonkeratinizing stratified squamous epithelia, but not in simple epithelia or nonepithelial cells. Immunofluorescence staining showed that in epidermal keratinocytes, both in vivo and in culture, the protein was upregulated during terminal differentiation and partially colocalized with desmosomal proteins. Immunogold EM confirmed the colocalization of the 210-kD protein and desmoplakin at desmosomes and on keratin filaments throughout the differentiated layers of the epidermis. Sequence analysis showed that the 210-kD protein is homologous to the keratin-binding proteins desmoplakin, bullous pemphigoid antigen 1, and plectin. These data suggest that the 210-kD protein may link the cornified envelope to desmosomes and keratin filaments. We propose that the 210-kD protein be named "enoplakin."

The cornified envelope is a layer of insoluble protein, ~15 nm thick, that is deposited under the plasma membrane of keratinocytes in the outermost layers of the epidermis (reviewed by Reichert et al., 1993; Simon, 1994). The cornified envelope provides a protective barrier between the environment and the living layers of the skin, and is believed to play an important role in maintaining the structural integrity of the epidermis. 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. In lamellar ichthyosis, an autosomal recessive disorder of the skin, reduced activity of the membrane-bound, keratinocyte-specific transglutaminase (TGK) results in severe perturbation of epidermal differentiation and function (Huber et al., 1995).

Current models propose that in the first step of cornified envelope assembly TGK catalyzes the cross-linking of involucrin at the plasma membrane, and that other, less abundant, envelope precursors such as cornifin, elafin, and the small proline-rich proteins are added subsequently (Eckert et al., 1993; Steinert and Marekov, 1995). The cytoplasmic surface of the envelope is believed to be composed of loricrin (Steinert and Marekov, 1995). All envelope precursors that have been characterized so far are soluble cytoplasmic proteins, with the exception of loricrin, which is a component of insoluble cytoplasmic aggregates (keratohyalin granules). It is not clear whether the cellular localization of TGK is sufficient to direct the assembly of the envelope to the inner face of the plasma membrane, or whether specific membrane-associated precursors are required for anchorage. A further unanswered question is how keratin filaments and desmosomes are linked to the cornified envelope (Haftek et al., 1991; Ming et al., 1994; Steinert and Marekov, 1995).

In 1984, Simon and Green identified two membrane-associated proteins with apparent molecular masses of 195 and 210 kD that become incorporated into the cornified envelope on transglutaminase activation. Antibodies to the two proteins were absorbed by isolated cornified enve-
lopes. Both proteins are expressed by epidermal keratinocytes, but not by dermal fibroblasts, and are upregulated during keratinocyte terminal differentiation. Simon and Green proposed that the two proteins might anchor other envelope proteins to the plasma membrane.

We now report the sequencing of overlapping cDNA clones encoding the 210-kD cornified envelope precursor. The predicted structure of the 210-kD protein, its homology with other known proteins, and its expression pattern strongly suggest that it is associated with the plasma membrane and may play keratin filaments and desmosomes to the cornified envelope.

**Materials and Methods**

**Screening of cDNA Libraries and cDNA Sequencing**

A mouse polyclonal antiserum (M) raised against the 210-kD protein (Simon and Green, 1984) was used to screen a random-primer keratinocyte kgt11 expression library, as described previously (Hudson et al., 1992), and a cDNA clone (p210-1) containing a 1-kbp insert was isolated. A probe (P1) derived from this clone was used to screen an oligo-dT−primed plasmid library (kindly provided by P. Jones, Imperial Cancer Research Fund, London) and a second random-primer kgt11 library (a gift from R. Buxton, National Institute for Medical Research, London), and two cDNA clones were isolated, p210-21 from the plasmid library and p210-141 from the kgt11 library. A further cDNA clone, p210-23, was isolated from the second kgt11 library using a probe (P141) containing the 5′ end of clone p210-141. For screening of libraries with DNA probes, cDNA fragments were radiolabeled by random priming (Sambo and Green, 1989). Hybridizations were performed at 65°C for 16 h in a hybridization buffer containing 5 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, pH 8.0, 1% BSA, 7% SDS, and 100 μg/ml denatured and fragmented herring sperm DNA (Boehringer Mannheim, Lewes, UK). Washing was performed at 65°C in 0.1 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate) and 0.1% SDS. The inserts of the kgt11 clones were subcloned into pBluescript II KS(−/+) (Stratagene Ltd., Cambridge, UK) for sequencing.

**Southern and Northern Blot Analyses**

Genomic DNA was isolated from cultured human foreskin keratinocytes, digested to completion with restriction endonuclease (Biolabs, Hitchin, UK) electrophoresed in 1% agarose gel, and transferred to HybondN membrane (Amersham International plc., Bucks, UK) and oligonucleotides synthesized by Oligonucleotide Synthesis Services, ICRF. Total RNA was isolated from cultured human keratinocytes by extraction with guanidine thiocyanate (Sambrook et al., 1989). Poly(A) + RNA was purified from total RNA using oligo(dT)-cellulose spin columns (Pharmacia). 2 μg of poly(A) + RNA per lane and RNA molecular weight standards (RNA ladder; GIBCO BRL, Paisley, UK) were electrophoresed in the same gel. Hybridization was performed at 65°C for 16 h with a DNA probe (radiolabeled as described above) in a hybridization buffer containing 0.9 M NaCl, 50 mM sodium phosphate buffer, pH 7.2, 5 mM EDTA, pH 8.0, 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll (Pharmacia, St. Albans, UK), and 100 μg/ml denatured and fragmented herring sperm DNA (Boehringer Mannheim). Washing was performed as described above.

**Induction of Terminal Differentiation**

To induce terminal differentiation, keratinocytes were disaggregated in trypsin/EDTA, washed, and resuspended at a final concentration of 5 × 10⁶ cells/ml in growth medium containing 14.5% methylcellulose (Aldrich Chemical Co. Ltd., Milwaukee, WI) as described previously (Green, 1977; Watt, 1994). The cell suspensions were cultured in a 25-cm² flask, or 2 × 10⁴/35-mm dish.

**Antibodies**

A peptide corresponding to the COOH-terminal 14-amino acid residues of the protein encoded by the open reading frame of the isolated cDNA contig was synthesized and conjugated to keyhole limpet hemocyanin (Pierce Chemical Co., Rockford, IL) by Peptide Synthesis Services, ICRF. The conjugated peptide was injected into a rabbit to raise the antiserum CR-1. The polyclonal rabbit DPI/II antiserum used for immunoblotting was a kind gift from A. Magee (NIMR, London) (Arnemarm et al., 1993). The monoclonal antiserum DH1 (Dover and Watt, 1987) was purchased from Amersham International plc. (Chilworth, UK). Involucrin was detected with the polyclonal rabbit antiserum DH1 (Dover and Watt, 1987). FITC-conjugated goat anti-rabbit IgG was obtained from Vector Laboratories (Burlingame, CA). Goat anti-mouse IgG conjugated to 5-nm gold was purchased from Bio Cell International (Seattle, WA). Anti-rabbit IgGs were purchased from Amersham International plc. (Chilworth, UK). Goat anti-mouse IgG conjugated to 5-nm gold was purchased from Bio Cell International (Cardiff, UK).

**Immunoblotting**

Confluent keratinocyte cultures were washed in ice-cold PBS and lysed for 10 min in Laemmli SDS-PAGE sample buffer (Laemmli, 1970) containing 10% β-mercaptoethanol and 10 mM EDTA, but no bromphenol blue. Cell extracts were boiled for 5 min and then centrifuged at top speed in an Eppendorf microfuge for 5 min at 4°C to remove insoluble material. The protein content was determined using the Bio Rad D₅ assay system (Bio Rad). Extracts were stored at −70°C until further use. An equal volume of Laemmli SDS-PAGE sample buffer containing 10% β-mercaptoethanol and bromphenol blue was added, and the samples were heat de-
natured by boiling for 3 min. Samples and molecular weight standards (high purity proteins, Molecular Research Center, Inc.) were resolved on 6% polyacrylamide gel using the buffer system of Tris, 380 mM glycine, 0.1% SDS, and 20% methanol (Towbin et al., 1979) were resolved on a 6% polyacrylamide gel using the buffer system of 0.05% Tween 20, the membrane was incubated for 1 h at room temperature. After extensive washing in PBS containing 2.5% reconstituted skim milk powder and 0.05% Tween 20. The membrane was washed as before and then in PBS alone. The blot was developed using the ECL kit (Amersham International plc.) and exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY).

Immunoprecipitations

Protein was extracted from confluent keratinocyte cultures in ice-cold CSK buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl2, and 0.5% Triton X-100; Fey et al., 1984) containing 250 mM (NH4)2SO4, 2 mM PMSF, and 1 μg/ml leupeptin. Samples were precleared with protein A-Sepharose beads (Pharmacia) for 1 h on a rotating wheel at 4°C. The beads were pelleted, and the supernatants transferred to a clean tube. The supernatants were immunoprecipitated for 2 h with 10 μl antiserum, followed by incubation with protein A-Sepharose beads for 1 h on a rotating wheel at 4°C to collect the immune complexes. The protein A-Sepharose beads were washed in ice-cold CSK buffer five times and then boiled in Laemmli sample buffer containing 10% β-mercaptoethanol. The released proteins were separated on a 6% SDS-PAGE gel and transferred to nitrocellulose, as described above.

Induction of Cross-linking by Transglutaminases

Confluent keratinocyte cultures were washed twice in serum-free growth medium and incubated for 5 h in the same medium at 37°C in the presence of 0.04% Triton X-100 to induce formation of cornified envelopes (Rice and Green, 1979). To inhibit the activation of transglutaminases, control cultures were incubated for 30 min at 37°C in the presence of 20 mM cysteamine, pH 7.5, before the addition of Triton X-100. 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 an Eppendorf microfuge for 5 min at 4°C to remove insoluble material. Equal volumes of extracts were processed for immunoblotting as described above.

Immunofluorescence

Human tissue from various body sites was obtained at biopsy or autopsy, embedded into OCT compound (Miles Inc., Stoke Poges, UK), and frozen in isopentane cooled in liquid nitrogen. Frozen tissue sections (6 μm) were air dried and incubated in PBS containing 0.2% fish skin gelatin for 30 min, followed by incubation for 1 h at room temperature in PBS containing 0.2% fish skin gelatin and a 100-fold dilution of the antisera CR-1 or the preimmune serum of rabbit CR-1. The sections were washed four times in PBS containing 0.2% fish skin gelatin and then incubated with FITC-conjugated goat anti-rabbit IgG for 1 h at room temperature, followed by three washes in PBS and one in distilled water. For double labeling, sections were first incubated with the rabbit antisera CR-1, followed by the mouse monoclonal DPI/II antibody, and then a mixture of rhodamine-conjugated anti–rabbit IgG and an FITC-conjugated anti–mouse IgG. The sections were mounted in Gelvatol (Monsanto, St. Louis, MO) and examined using an Axioskop microscope (Carl Zeiss Ltd., Oberkochen, Germany).

Keratinocyte colonies grown on coverslips in 25-mm dishes were washed three times in PBS and permeabilized with 0.1% Triton X-100 or with CSK immunoprecipitation buffer for 4 min at room temperature. After three additional washes in PBS, cells were stained with the antisera CR-1, as described above. Double labeling was performed as described above with the rabbit antisera CR-1 and the mouse monoclonal pan-desmoglein antibody DG3.10, or with CR-1 and a mouse monoclonal DPI/II antibody.

Immunogold EM

A piece of neonatal human foreskin was subjected to cryofixation using the HPM 010 high pressure freezing equipment (Leica) as described by Studer et al. (1989). The frozen sample was freeze-substituted in a Leica CS auto freeze substitution apparatus (Leica, Milton Keynes, UK), first in anhydrous acetone at ~70°C for 72 h, and then by warming to ~35°C for 48 h. The samples were washed with anhydrous acetone and embedded in Lowicryl K4M resin at ~35°C, followed by UV polymerization at ~35°C for 48 h. Thin sections were prepared with a diamond knife on a Reichert Ultracut E microtome (Leica) and mounted on carbon-coated grids. A piece of neonatal human foreskin was subjected to cryofixation using the HPM 010 high pressure freezing equipment (Leica) as described by Studer et al. (1989). The frozen sample was freeze-substituted in a Leica CS auto freeze substitution apparatus (Leica, Milton Keynes, UK), first in anhydrous acetone at ~70°C for 72 h, and then by warming to ~35°C for 48 h. The samples were washed with anhydrous acetone and embedded in Lowicryl K4M resin at ~35°C, followed by UV polymerization at ~35°C for 48 h. Thin sections were prepared with a diamond knife on a Reichert Ultracut E microtome (Leica) and mounted on carbon-coated grids.

Results

Isolation of Overlapping cDNA Clones Encoding the 210-kD Cornified Envelope Precursor

A random-primed keratinocyte λgt11 cDNA expression library was screened with a previously described mouse monoclonal antisera (M) raised against the 210-kD cornified envelope precursor described by Simon and Green (1984).
A probe (P1) derived from the isolated cDNA clone, p210-1, was used to isolate two overlapping cDNA clones, p210-141 and p210-21 (see Fig. 1). The clone p210-21 contained the polyadenylated 3' end of the cDNA. A probe (P141) derived from p210-141 was used to isolate a further cDNA clone, p210-23, which contained the putative translation start codon in a Kozak consensus sequence for translation initiation (Kozak, 1991), preceded by an in-frame stop codon (Fig. 2).

A map of the isolated cDNA clones and a partial restriction map of the composite cDNA are shown in Fig. 1. The cDNA sequence and the amino acid sequence encoded by the long open reading frame are shown in Fig. 2. The 6.5-kb mRNA detected by Northern blot analysis (see below). The molecular mass of 210 kD in SDS-PAGE. The composite cDNA contains 6,457 bp, corresponding to the size of the cDNA sequence and the amino acid sequence encoded by the long open reading frame (M, bold). A long direct repeat close to the beginning of the long open reading frame (underlined) precedes the region encoding the NN domain and encodes the amino acid sequence KGSP four times. The boundaries of the NH2-terminal domains (NN, Z, Y, X, W, and V), the central rod domain, and the COOH-terminal C domain are indicated by arrows (nomenclature of domains according to Green et al., 1992). The long open reading frame terminates in a stop codon (indicated by an asterisk), which is followed by a consensus polyadenylation signal (AATAAA) in the 3' untranslated region (doubly underlined). The sequence data are available from GenBank/EMBL/DDBJ under accession number U53786.
Analysis of the Predicted Amino Acid Sequence of the 210-kD Protein

The deduced amino acid sequence of the 210-kD protein (Fig. 2) contains 36% hydrophobic residues (alanine, leucine, isoleucine, phenylalanine, tryptophan, and valine) and 32% charged residues (aspartate, glutamate, lysine, and arginine). A hydrophilicity plot based on the method of Kyte and Doolittle (1982) revealed that the protein was generally very hydrophilic and did not contain any regions with a high probability of forming a transmembrane domain (data not shown). The absence of potential transmembrane domains was confirmed using the algorithm of Argos and Rao (1986). Secondary structure analyses, based on the methods of Chou and Fasman (1978) or Gamier et al. (1978), predicted an NH₂-terminal domain characterized by the presence of short α-helical segments, a central do-
Figure 2. Homology of the 210-kD protein to DPI, BPAG1, and plectin. Dot matrix homology comparisons of the 210-kD protein with DPI (a), BPAG1 (b), and plectin (c) were performed with the software COMPARE and DOTPLOT; a dot was placed when 13 amino acids showed identity within a window of 20 amino acids. Secondary structure predictions (see text and Fig. 4) suggest that the domain structure of the 210-kD protein is similar to that of DPI, BPAG1, and plectin (d). The designation of the NH$_2$-terminal domains NN, Z,
Y, X, W, and V, and the COOH-terminal repeat domains A, B, and C for DPI, BPAG1, and plectin is according to Green et al. (1992); for domain boundaries of the 210-kD protein, see Fig. 2. The rod domains are represented by filled rectangles. (GSRX)₆ and (GSRX)₄ refer to repeat motifs at the COOH terminus of DPI and plectin, respectively.
Figure 4. Direct sequence comparison and coiled coil analysis of the 210-kD protein, DPI, BPAG1, and plectin. Sequence comparison of the NH₂ termini (a) and the COOH termini (b) of the four homologues (domain boundaries of DPI, BPAG1, and plectin according to Green et al., 1992). Amino acid residues in the sequence alignments are numbered according to their position in the protein sequence; stop codons are indicated with asterisks. (c-f) Histograms of the probability of forming a coiled coil (y axis), as predicted by the Lupaš algorithm, versus the position in the amino acid sequence (x axis) for the 210-kD protein (e), plectin (d), DPI (e), and BPAG1 (f). Each division on the x axis scale corresponds to 100 amino acid residues. Regions with a value of P > 0.9 for more than 28 consecutive amino acid residues are predicted to adopt a coiled coil conformation.
main containing long stretches of α-helical regions interrupted by short nonhelical spacers, and a COOH-terminal globular domain containing short α-helical regions separated by many turns (data not shown).

We searched the SwissProt and PIR protein databases with the predicted amino acid sequence of the 210-kD protein and found homology to the proteins human desmoplakin I (DPI), human bullous pemphigoid antigen 1 (BPAG1), and rat plectin (Green et al., 1990, 1992; Sawamura et al., 1991; Virata et al., 1991; Wiche et al., 1991; the sequence of the extreme DPI NH2-terminus is available under GenBank/EMBL/DDBJ accession no. M77830; for sequence of human plectin see Note Added in Proof). Fig. 3, a–c shows dot-plot homology comparisons of the 210-kD protein with DPI, BPAG1, and plectin. Each of the four proteins consists of an NH2-terminal domain, a central domain, and a COOH-terminal domain containing a variable number of repeats (Fig. 3, a–c; Green et al., 1992). The sequence of the NH2- and COOH-terminal domains appears to be well conserved between the four proteins, indicated by areas of linear homology in the dot-plot matrices. The central domain sequences are considerably diverged, although a large number of heptad repeats with the potential of forming coiled coils are present in the central domains (rod domains) of all four proteins (see below), and they appear as an area of “rectangular” homology in the dot-plot matrices (Fig. 3, a–c).

A schematic comparison of the domain structure of DPI, BPAG1, plectin, and the 210-kD protein is shown in Fig. 3 d. The NH2 termini of the four homologous proteins each contain six putative α-helical subdomains, NN, Z, Y, X, W, and V, which are separated by nonhelical regions (Green et al., 1992; data not shown). Unlike the other proteins, the 210-kD protein has two pairs of KGSP tandem repeats preceding the NN domain (Fig. 2), each containing a potential protein kinase C (PKC) phosphorylation site (SPK). The COOH-terminal repeats of DPI, plectin, and BPAG1 are predicted to fold into discrete α-helical subdomains that were first described for DPI (Green et al., 1990). DPI contains three such subdomains (termed A, B, and C), BPAG1 contains two (B and C), plectin contains six (five B and one C) (Green et al., 1990, 1992; Sawamura et al., 1991; Wiche et al., 1991), and the 210-kD protein contains one (C) (Fig. 3, a–d).

A comparison of the predicted amino acid sequences of the entire NH2 terminus and the COOH-terminal C domain of the 210-kD protein with the respective domains of DPI, BPAG1, and plectin suggested that the 210-kD protein is most closely related to plectin (conservation is highest in the COOH-terminal C domain with preceding linker region: 36% identity), even though plectin is almost twice as large as the 210-kD protein and contains a larger rod domain as well as additional COOH-terminal repeat domains. Fig. 4, a–b, shows a direct sequence comparison of the extreme NH2 and COOH termini of the 210-kD protein, DPI, BPAG1, and plectin. For DPI, BPAG1, and plectin, the start of the NN-domain corresponds to the predicted start of an α-helical region (Green et al., 1992).

**Figure 5.** Analysis of the gene and mRNA encoding the 210-kD protein. (a) A Southern blot containing human genomic DNA (10 μg/ lane) digested with the indicated restriction enzymes was probed with the radiolabeled BamHI fragment P0.5. A single hybridizing fragment was present in each restriction digest. DNA molecular weight standard sizes are indicated (lane MW). (b) Northern blots containing 2 μg poly(A)⁺ RNA from cultured human keratinocytes were probed with radiolabeled cDNA fragments P23, P141, P1, and P21, corresponding to the entire coding region of the mRNA encoding the 210-kD protein. A single hybridizing fragment was detected by all probes. RNA molecular weight standard sizes are indicated on the left. (c) A Northern blot containing 2 μg poly(A)⁺ RNA per lane from adherent or suspended keratinocyte cultures was hybridized with a probe (P141) specific for the mRNA encoding the 210-kD protein, stripped, and reprobed with an involucrin mRNA-specific probe as a control for the induction of terminal differentiation and with a GAPDH-specific probe as a loading control.
In the 210-kD protein, however, the start of the α-helical region begins with arginine 33, and so although the NN domains of the four homologues shown in Fig. 4a are aligned optimally to reveal primary sequence conservation, secondary structure predictions suggest an NH2-terminal shift of the NN domain of the 210-kD protein relative to the other homologues.

The algorithm of Lupas et al. (1991) identified heptad repeats with a high probability of forming intermolecular coiled coils in the central regions of all four proteins (Fig. 4, c–f). In contrast to DPI, BPAG1, and plectin, the rod domain of the 210-kD protein contains a large number of stutter regions (Fig. 4, c–f). The NH2-terminal regions of all four proteins also contain heptad repeats, but they are very short (<4 heptads; Fig. 4, c–f) and may cause each NH2 terminus to fold into an antiparallel bundle rather than forming an extensive coiled coil (Green et al., 1992).

**The 210-kD Protein Is Encoded by a Single-copy Gene, and the Corresponding mRNA Is Upregulated during Keratinocyte Terminal Differentiation**

A Southern blot containing human genomic DNA digested with various restriction enzymes was hybridized to a probe, P0.5, derived from the cDNA clone p210-21 (see Fig. 1). The probe detected a single restriction fragment in each digest, suggesting that the 210-kD protein is encoded by a single-copy gene (Fig. 5a). Probes spanning the entire sequence of the composite cDNA detected only a single mRNA species of 6.5 kb in cultured human keratinocytes, suggesting that no abundant alternatively spliced mRNAs are expressed in this cell type (Fig. 5b).

Cultured human keratinocytes can be induced to undergo terminal differentiation when they are disaggregated and placed into suspension (Green, 1977). The level of the mRNA for the 210-kD protein was upregulated in suspension culture, as was the mRNA for involucrin, a known cornified envelope precursor (Fig. 5c; Nicholson and Watt, 1991).

**Validation of the Identity of the 210-kD Protein as a Cornified Envelope Precursor**

To establish that the protein encoded by the cDNA we had isolated was indeed the 210-kD cornified envelope precursor described by Simon and Green (1984), and to obtain a reagent for further studies, we raised a rabbit antiserum, CR-1, against a peptide corresponding to the COOH-terminal 14-amino acid residues encoded by the isolated cDNA. On immunoblots of keratinocyte extracts, CR-1 detected a single protein with an apparent molecular mass of 210 kD that comigrated with the protein recognized by the mouse polyclonal antiserum M, raised against the 210-kD protein by Simon and Green (1984), and used to isolate cDNA clone p210-1 from a Agt11 expression library (Fig. 6a, left-hand side). When CR-1 immunoprecipitates were transferred to nitrocellulose and probed with antiserum M, the 210-kD cornified envelope precursor was detected (Fig. 6a, right-hand side), establishing that CR-1 and M recognize the same protein.

To further confirm that the 210-kD protein was incorporated into the cornified envelope, we treated confluent keratinocyte cultures with 0.04% Triton X-100. This treatment causes an influx of calcium ions into the cells and 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 cells lysates in 1.6% SDS and 5% β-mercaptoethanol (Sun and Green, 1976; Simon and Green, 1984). Both the 210-kD protein and involucrin became nonextractable in SDS/β-mercaptoethanol after Triton X-100 treatment of the cultures for 5 h (Fig. 6b) or when cornified envelope formation was induced with 0.8 M sodium chloride (data not shown), the method used by Simon and Green (1984). When the cultures were treated with the transglutaminase inhibitor cysteamine (Siefring et al., 1978) before the addition of Triton X-100, cross-linking of the 210-kD protein and involucrin was inhibited (Fig. 6b). In contrast, ~50% of desmoplakin remained soluble in SDS/β-mercaptoethanol after induction of cross-linking (Fig. 6b).

**Expression and Cellular Localization of the 210-kD Protein**

Indirect immunofluorescent staining of unfixed frozen sections of human skin showed that the antiserum CR-1 detected an epitope in the epidermis, but not the dermis (Fig. 7, a and b). Staining was most prominent in the cell periphery (Fig. 7b), suggesting that the 210-kD protein was asso-
Figure 7. The tissue distribution and cellular localization of the 210-kD protein, as determined by indirect immunofluorescence with the CR-1 antiserum. (a–c) Epidermis. (a) Preimmune serum from rabbit CR-1, (b and c) double labeling with CR-1 (b) and an antibody to DPII (c), (d) cervical mucosa labeled with CR-1; (e–g) cultured keratinocytes; (e) single labeling with CR-1; (f and g) double labeling with CR-1 (f), and an antibody to DPII (g). Bars: (a–c) 50 μm; (d and e) 100 μm; (f and g) 20 μm. Arrows in b and c show position of the dermal/epidermal boundary.

Ruhrberg et al. Cornified Envelope Protein with Homology to Desmoplakin
associated with the plasma membrane. Nonspecific labeling of the stratum corneum with preimmune serum was variable and precluded any conclusions regarding CR-1 labeling of this layer. CR-1 staining was strongest in the upper spinous and granular layers (Fig. 7 b). In contrast, an antibody specific for DPI/II showed strong labeling of the basal layer, as well as the suprabasal layers (Fig. 7, b vs. c).

To examine the distribution of the 210-kD protein, we stained frozen sections of various tissues with CR-1 (Fig. 7, b and d; data not shown). All stratified squamous epithelia examined (epidermis from neonatal foreskin and adult breast, keratinized and nonkeratinized oral mucosa, oesophageal, and cervical mucosa) were positively stained. CR-1 did not stain the simple epithelium of endocervical glands. The antiserum also failed to stain fibroblasts or endothelial cells in the dermis and did not stain brain tissue. These results are consistent with the earlier conclusion that the 210-kD protein is keratinocyte-specific (Simon and Green, 1984).

When stratified colonies of cultured foreskin keratinocytes were permeabilized and stained with CR-1, staining was concentrated at the plasma membrane in the first suprabasal layers, but became more uniform in the uppermost layers (Fig. 7 e), confirming that the 210-kD protein is upregulated during differentiation of epidermal keratinocytes. At higher magnification, the staining appeared punctate, reminiscent of desmosomal junctions (Fig. 7 f; see Watt et al., 1984). Double-label immunofluorescence with CR-1 and antibodies specific for desmogleins (data not shown) or DPI/II (Fig. 7, f and g) showed colocalization of the 210-kD protein with these desmosomal proteins in differentiating cells.

The 210-kD protein could not be detected with the CR-1 antiserum in cultured keratinocytes or epidermis treated with aldehyde fixatives. We could, however, carry out immunogold EM when epidermal sections were prepared by high pressure freezing and freeze substitution. In these sections, there was very little labeling with the preimmune serum of any of the cell layers, including the stratum corneum (Fig. 8 d). CR-1 showed extensive colocalization with a DPI/II antibody in the satellite region of desmosomes (Fig. 8, a and b) and along keratin filaments throughout the cytoplasm of differentiated keratinocytes (Fig. 8 c). We saw no evidence of specific labeling of keratohyalin granules with either CR-1 or anti-DPI/II (Fig. 8 c). The CR-1 antibody labeled cornified cells strongly, and the labeling was not confined to the cell periphery (Fig. 8 e).

**Discussion**

In this study, we describe the cloning and sequencing of overlapping cDNA clones that encode the membrane-associated 210-kD cornified envelope precursor first described by Simon and Green (1984). The authenticity of the isolated cDNA was established with an antiserum (CR-1) raised against a peptide corresponding to the predicted COOH-terminal 14-amino acid residues of the protein encoded by the isolated cDNA. On immunoblots, the CR-1 antiserum detected a single protein with an apparent molecular mass of 210 kD, which was also recognized by an antiserum raised against the 210-kD cornified envelope precursor by Simon and Green (1984). Furthermore, the protein detected by the CR-1 antiserum was cross-linked into the cornified envelope on transglutaminase activation in cultured keratinocytes, as reported by Simon and Green (1984).

The 210-kD protein appears to be keratinocyte-specific, but, as observed for involucrin (Banks-Schlegel and Green, 1981), its expression is not restricted to keratinizing stratified squamous epithelia. Expression of the 210-kD protein increased during terminal differentiation of epidermal keratinocytes both in vivo and in culture. When cultured epidermal keratinocytes were disaggregated and placed in suspension to induce terminal differentiation, the mRNA for the 210-kD protein was upregulated, suggesting that the increase in 210-kD protein during epidermal differentiation is at least partly caused by increased transcription or increased mRNA stability.

The 210-kD cornified envelope precursor is homologous to the intermediate filament–associated proteins desmoplakin I (DPI), bullous pemphigoid antigen 1 (BPAG1), and plectin. Sequence analyses predict that the four homologous proteins have a similar domain structure with an NH2-terminal globular domain, a central rod domain, and a COOH-terminal globular domain (Green et al., 1990; Sawamura et al., 1991a; Virata et al., 1992; Wiche et al., 1991). The NH2 termini of BPAG1, plectin, and DPI are predicted to each contain a bundle of antiparallel α-helices (NN, Z, Y, X, W, and V; Green et al., 1992), which are also conserved in the 210-kD protein. The COOH termini of the four homologues contain a variable number of tandem repeats that are predicted to be organized into discrete subdomains that consist of α helices separated by β turns, and which were first described for DPI (Green et al., 1990). DPI contains three such subdomains, BPAG1 two, plectin six (Green et al., 1990; Sawamura et al., 1991a; Virata et al., 1992; Wiche et al., 1991), and the 210-kD protein one. The C-domain, with its preceding linker region, is the only COOH-terminal subdomain conserved in all four proteins. A comparison of the predicted amino acid sequences of the NH2- and COOH-terminal domains of the 210-kD protein with the respective domains of human DPI, human BPAG1, and rat plectin shows that the primary sequence of the 210-kD protein is most closely related to plectin, even though plectin is almost twice as large as the 210-kD protein, with a larger rod and additional COOH-terminal repeats.

Although the central domain sequences of the four proteins are highly diverged, they all have a large number of heptad repeats with the potential of forming coiled coils with a dimerisation partner. There is direct evidence for homodimerization of DPI (O’Keefe et al., 1989), and ro-
tary shadowing suggests that the same is true for plectin (Foisner and Wiche, 1987). In contrast to DPI, BPAG1, and plectin, the rod domain of the 210-kD protein contains a large number of stutter regions, and this may have implications for its structure and its interaction with other proteins.

A single mRNA species of 6.5 kb encoding the 210-kD protein can be detected on Northern blots of human keratinocytes. In contrast, two alternatively spliced desmoplakin mRNAs, encoding DPI and DPII, are transcribed from the desmoplakin gene, and DPII is believed to contain an abbreviated rod domain (Virata et al., 1992). BPAG1 expression is confined to basal keratinocytes (Sawamura et al., 1991b; Tamai et al., 1993), but alternative splice products containing BPAG1 exons, BFG in the pancreatic carcinoma cell line FG (Hopkinson and Jones, 1994) and dystonin in the brain (Brown et al., 1995), have been described.

Immunofluorescence analysis suggested that the 210-kD protein is associated with the plasma membrane and shows partial colocalization with desmosomal proteins. Immunogold EM showed colocalization of the 210-kD protein and DPI/II at desmosomes and along keratin filaments in differentiating keratinocytes. At the EM level, the antiserum to the 210-kD protein labeled cornified cells strongly, but labeling was not concentrated at the cell periphery; it is possible that the epitope becomes inaccessible in mature envelopes (Steinert and Marekov, 1995; Ishida-Yamamoto et al., 1996).

The extensive association of DPI/II and the 210-kD protein with keratin filaments has not been observed previously by immunofluorescence or immunogold EM. Epithelial cells are known to contain two different pools of DPI/II, one that cannot be extracted by nonionic detergent and one that is easily extracted with low detergent concentrations (Dudan and Franke, 1988; Pasdar and Nelson, 1988a,b). Our evidence suggests that there are also two pools of the 210-kD protein: Triton X-100-soluble protein is readily immunoprecipitated by the CR-1 antiserum (Fig. 6 a), yet immunofluorescence staining of Triton X-100-extracted keratinocytes shows that they also contain insoluble 210-kD protein (Fig. 7, e and f). We suggest that the detergent-soluble pools of DPI/II and the 210-kD protein may be weakly associated with keratin filaments, and that this association is not preserved by conventional chemical fixation and permeabilization procedures. However, it is preserved by the high pressure freezing/freeze substitution technique that we used for our EM analysis.

There is evidence that desmosomal components (Haftek et al., 1991; Steinert and Marekov, 1995) and keratin filaments (Haftek et al., 1991; Ming et al., 1994; see also Fig. 5 b) become incorporated into the cornified envelope. We speculate that the 210-kD protein may anchor them to the envelope. In addition to our EM evidence, the sequence homology between the 210-kD protein, plectin, DPI/II, and BPAG1 strongly suggests such a role. Plectin is found in a wide variety of different cell types and is thought to play a role in the cross-linking of intermediate filaments to each other, as well as to microtubules and microfilaments, and may anchor these networks to the cell membrane (reviewed by Foisner and Wiche, 1991). Association of plectin with vimentin and keratins is dependent on its COOH-terminal domain (Wiche et al., 1993). DPI and DPII are the most abundant constituents of desmosomes (reviewed by Schwarz et al., 1990), and they anchor keratin filaments to desmosomes: the DPI/II COOH terminus associates with the keratin network when overexpressed in cultured cells (Stappenbeck et al., 1992, 1993), and it binds a subset of keratins in vitro (Kouklis et al., 1995). BPAG1 is involved in anchoring the keratin network to hemidesmosomes in keratinocytes (Guo et al., 1995, and references therein).

We have not determined whether the 210-kD protein is a direct substrate for transglutaminases, or whether it becomes cross-linked into the envelope in association with other envelope precursors, but it is interesting that the NH2-terminal domain, but not the rod or the COOH-terminal domain of the 210-kD protein, contains a larger proportion of glutamine residues (11.6%) than DPI/II (8.1%), plectin (8.1%), or BPAG1 (7%). Like its homologues, the 210-kD protein lacks a transmembrane domain, but the NH2-terminal domain may mediate association with desmosomes, as observed for DPI/II (Stappenbeck et al., 1992), and may be in close proximity to membrane-bound transglutaminase (TGK) (Chakravarty and Rice, 1989). It is conceivable that its relatively high NH2-terminal glutamine content could make the 210-kD protein a better substrate for cross-linking by transglutaminases than DPI/II. Alternatively, or additionally, free carboxyl groups of NH2-terminal glutamine residues that are not cross-linked by transglutaminase could serve as attachment sites for hydroxyceramides, the major component of the lipid monolayer that replaces the plasma membrane in fully differentiated corneocytes (Reichert et al., 1993).

In conclusion, the 210-kD protein is a cornified envelope precursor that is likely to link the envelope to both keratin filaments and desmosomes. Because the 210-kD protein is an envelope precursor that belongs to the desmoplakin family and because of deficits in our classical education, we propose that the 210-kD protein be named "envoplakin."

We thank everyone who provided us with reagents; Michael P. Mitchell and Paul S. Freemont for advice on protein sequence analysis; Kenneth J. Blight and Stephen Gschmeissner for their help with EM; George Elia for preparation of frozen sections; and Stella Keeble and R. Dawn Obermoeller for their contributions to the project.

Received for publication 22 December 1995 and in revised form 26 April 1996.

Note Added in Proof: The human plectin gene has now been sequenced. See Liu, C.-G., C. Maercker, M.J. Castaño, R. Hauptman, and G. Wiche. 1996. Proc. Natl. Acad. Sci. USA. 93:4278–4283.

References

Argos, P., and J.K. Rao. 1986. Prediction of protein structure. Methods Enzymol. 130:185–207.

Arnemann, J., K.H. Sullivan, A.I. Magee, I.A. King, and R.S. Buxton. 1993. Stratification-related expression of isoforms of the desmosomal cadherins in human epidermis. J. Cell Sci. 104:741–750.

Banks-Schlegel, S., and H. Green. 1981. Involucrin synthesis and tissue assembly by keratinocytes in natural and cultured human epithelia. J. Cell Biol. 90:722–727.

Brown, A., G. Bernier, M. Mathieu, J. Rossant, and R. Kothary. 1995. The mouse dystonia musculorum gene is a neural isoform of bulbous pempigoid antigen 1. Nature Genetics. 10:301–306.

Chakravarty, R., and R.H. Rice. 1989. Acylation of keratinocyte transglutaminase by palmitic and myristic acids in the membrane anchorage region. J. Biol. Chem. 264:625–629.

Chou, P.Y., and G.D. Fasman. 1987. Prediction of the secondary structure of

The Journal of Cell Biology, Volume 134, 1996

728
proteins from their amino acid sequence. *Adv. Enzymol. Relat. Areas Mol. Biol.* 47:45-148.

Claro, M.G., and G. von Heijne. 1994. TopPred II: an improved software for membrane structure predictions. *CABIOS* 10:685-686.

Dover, R., and F.M. Watt. 1987. Measurement of the rate of epidermal terminal differentiation: expression of involucrin by S-phase keratinocytes in culture and in psoriatic plaques. *J. Invest. Dermatol.* 89:349-352.

Duden, R., and W.W. Franke. 1988. Organization of desmoplasmal plaque proteins in cells growing at low calcium concentrations. *J. Cell Biol.* 107:1049-1063.

Eckert, R.L., and H. Green. 1986. Structure and evolution of the human involucrin gene. *Cell* 46:583-589.

Eckert, R.L., M.B. Yaffe, J.F. Crish, S. Murthy, E.A. Rorke, and J.F. Welter. 1993. Invovlucrin — structure and role in envelope assembly. *J. Invest. Dermatol.* 100:613-617.

Fey, E.G., K.M. Wan, and S. Penman. 1984. Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three-dimensional organization and protein composition. *J. Cell Biol.* 98:1973-1984.

Foisner, R., and G. Wiche. 1987. Structure and hydrodynamic properties of plectin molecules. *J. Mol. Biol.* 198:513-531.

Foisner, R., and G. Wiche. 1991. Intermediate filament-associated proteins. *Curr. Opin. Cell Biol.* 3:75-81.

Garnier, D.J., D.J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* 120:97-120.

Green, H. 1977. Terminal differentiation of cultured human epidermal cells. *Cell* 11:405-416.

Green, K.J., D.A. Parry, P.M. Steiner, M.L. Virata, R.M. Wagner, B.D. Angst, and L.A.Nilles. 1990. Structure of the human desmoplakin. Implications for Ca2+—induced cross-linking of membrane proteins in intact human erythrocytes. *Biochemistry.* 27:2598-2604.

Simon, M., and H. Green. 1984. Participation of membrane-associated proteins in the formation of the cross-linked envelope of the keratinocyte. *Cell.* 36: 733-741.

Sawamura, D., K.H. Li, M.L. Chu, and J. Uitto. 1991a. Human bullous pemphigoid antigen (BPAG1). Amino acid sequences deduced from cloned cDNA predict biologically important peptide segments and protein domains. *J. Biol. Chem.* 266:17874-17879.

Simon, M. 1994. The epidermal cornified envelope and its precursors. In *The Keratinocyte Handbook.* I.M. Leigh, E.B. Lane, and F.M. Watt, editors. Cambridge University Press, Cambridge. 275-292.

Stappenbeck, T.-S., and K.J. Green. 1992. The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells. *J. Cell Biol.* 116:1197-1209.

Stappenbeck, T.-S., E.A. Bornscheuer, C.M. Corcoran, H.H. Luo, M.L. Virata, and K.J. Green. 1993. Functional analysis of desmoplakin domains: specification of the interaction with keratin versus vimentin intermediate filament networks. *J. Cell Biol.* 123:691-705.

Steiner, F.M., and L.N. Marekova. 1995. The proteins elafin, filagerin, keratin intermediate filament, keratin, and small proline-rich proteins 1 and 2 are isopeptide cross-linked components of the human epidermal cornified cell envelope. *J. Biol. Chem.* 270:17702-17711.

Studes, D., M. Michel, and M. Mueller. 1989. High pressure freezing comes of age. *Scanning Microscopy Supplement.* 3:253-269.

Sun, T.T., and H. Green. 1976. Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. *Cell.* 9:511-521.

Tama, K., D. Sawamura, H.C. Do, Y. Tama, K.Li, and J. Uitto. 1993. The human 250-kD bullous pemphigoid antigen gene (BPAG1). Exon-intron organization and identification of regulatory tissue specific elements in the promoter region. *J. Clin. Invest.* 92:814-822.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.

Virata, M.L., R.M. Wagner, D.A. Parry, and K.J. Green. 1992. Molecular structure of the human desmoplakin I and II amino terminus. *Proc. Natl. Acad. Sci. USA.* 93:544-548.

Watt, F.M., D.L. Mattey, and D.R. Garrod. 1984. Calcium-induced reorganization of desmosomal components in cultured human keratinocytes. *J. Cell Biol.* 99:2211-2215.

Watt, F.M. 1994a. Cultivation of human epidermal keratinocytes with a 3T3 feeder layer. In *Cell Biology: A Laboratory Handbook.* J.E. Celis, editor. Academic Press, Inc., San Diego. 83-89.

Watt, F.M. 1994b. Suspension-induced terminal differentiation of keratinocytes. In *Keratinocyte Methods.* I. L. Leigh, and F. M. Watt, editors. Cambridge University Press, Cambridge. 113.

Wiche, G., B. Becker, K. Luber, G. Weitzer, M.J. Castanon, R. Hauptmann, C. Striatsos, and M. Stewart. 1991. Cloning and sequencing of rat plectin indicate s a 466-kD polypeptide chain with a three-domain structure based on a central alpha-helical coiled coil. *J. Cell Biol.* 114:83-99.

Wiche, G., D. Gronov, A. Donovan, M.J. Castanon, and E. Fuchs. 1993. Expression of plectin mutant cDNA in cultured cells indicates a role of COOH-terminal domain in intermediate filament association. *J. Cell Biol.* 121:607-619.