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

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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 "envoplakin."

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)[1] results in severe perturbation of epidermal differentiation and function (Huber et al., 1995).

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1. *Abbreviations used in this paper*: BPAG1, bullous pemphigoid antigen 1; DPI, desmoplakin I; FSG/PBS, fish skin gelatin in PBS; TGK, keratinocyte transglutaminase.

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 link 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-primed keratinocyte λgt11 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-primed λgt11 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 λgt11 library. A further cDNA clone, p210-23, was isolated from the second λgt11 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 (Sambrook et al., 1989). Hybridizations were performed at 65°C for 16 h in a hybridization buffer containing 0.5 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 λgt11 clones were subcloned into pBluescript II KS(+/−) (Stratagene Ltd., Cambridge, UK) for sequencing. The cDNA clones were sequenced by the dideoxy chain termination method using the Sequenase II kit (Amersham International plc., Bucks, UK) and oligonucleotides synthesized by Oligonucleotide Synthesis Services, ICRF.

Southern and Northern Blot Analyses

Genomic DNA was isolated from cultured human foreskin keratinocytes, digested to completion with restriction enzymes (New England Biolabs, Hitchin, UK), electrophoresed in a 1% agarose gel, and transferred to HybondN membrane (Amersham International plc.), according to the manufacturer's instructions. A DNA molecular weight standard (1-kbp ladder; GIBCO BRL, Paisley, UK) was 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% polyvinylpyrrolidine, 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.

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) were separated on 1% formaldehyde gels, as described (Sambrook et al., 1989). To facilitate the transfer of large RNA species, RNA was partially hydrolyzed by soaking the gel for 20 min in 50 mM NaOH, then neutralizing the gel for 30 min in 20 × SSC. The RNA was transferred to an HybondN membrane, as recommended by the manufacturer. Hybridizations were performed at 42°C for 16 h with probes, labeled as described above, in a hybridization buffer containing 50% formamide, 0.9 M NaCl, 50 mM sodium phosphate buffer, pH 7.2, 5 mM EDTA, pH 8.0, 0.1% BSA, 0.1% polyvinylpyrrolidine, 0.1% Ficoll (Pharmacia), and 100 μg/ml denatured and fragmented herring sperm DNA (Boehringer Mannheim). Washing was performed as described above. The involucrin probe was derived from plasmid pI-2 (Eckert and Green, 1986).

Computational Analysis of the Predicted Amino Acid Sequence

Secondary structure predictions were performed 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 with the program MacStripe (Knight and Kendrick-Jones, 1993), based on the algorithm described by Lupas et al. (1991). The predicted protein sequence was examined for potential transmembrane domains using MacVector 3.5, based on the method of Kyte and Doolittle (1982), and Top Pred II (Claros and von Heijne, 1994), based on the method of Argos and Rao (1986). The SwissProt and PIR protein databases were searched with the program BLAST at the National Center for Biotechnology Information (Bethesda, MD). Dotplot homology comparisons were performed using the programs COMPARE and DOTPLOT in the UWGCG (University of Wisconsin Genetics Computer Group) software suite.

Cell Culture

Normal human keratinocytes from newborn foreskins (strains kk, kp, and kq, passages 4–7) were cultured in the presence of mitomycin C–treated 3T3 feeder cells in a 3 + 1 mixture of DME and Ham's F12 medium supplemented with 10% FCS, 1.8×10^{-4} M adenine, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10^{-10} M cholera toxin, and 10 ng/ml epidermal growth factor, as previously described (Rheinwald and Green, 1975; Watt, 1994*a*). Keratinocytes were seeded at a concentration of 2×10^5/75-cm^2 flask, 10^5/25 cm^2-flask, or 2×10^4/35-mm dish.

Induction of Terminal Differentiation

To induce terminal differentiation, keratinocytes were disaggregated in trypsin/EDTA, washed, and resuspended at a final concentration of 5×10^5 cells/ml in growth medium containing 1.45% methylcellulose (Aldrich Chemical Co. Ltd., Milwaukee, WI), as described previously (Green, 1977; Watt, 1994*b*). The cell suspensions were cultured in bacterial culture plastic dishes coated with 0.4% polyHEMA (Aldrich Chemical Co. Ltd.) for 24 h and harvested by a fivefold dilution in ice-cold PBS followed by centrifugation.

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 limpit 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) (Arnemann et al., 1993). The mouse monoclonal DPI/II antibody used for immunofluorescence and immunogold EM was purchased from ICN (Thame, UK). The pan-desmoglein mouse mAb DG3.10 was purchased from Cymbus Bioscience Ltd. (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 (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). HRP-conjugated sheep anti-mouse or donkey anti-rabbit IgGs were purchased from Amersham International plc.

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 bromophenol 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 D_c 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 bromophenol blue was added, and the samples were heat de-

natured by boiling for 3 min. Samples and molecular weight standards (high range prestained protein molecular weight markers; GIBCO BRL) were resolved on a 6% polyacrylamide gel using the buffer system of Laemmli (1970) and transferred to nitrocellulose membrane (Schleicher & Schuell Inc., Dassel, Germany) in a transfer buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol (Towbin et al., 1979) at 30 V overnight. The membranes were stored dry at room temperature until use. For some experiments, samples were immunoprecipitated before blotting.

To inhibit nonspecific antibody binding to nitrocellulose, membranes were incubated for 1 h at room temperature in PBS containing 5% reconstituted skim milk powder (Marvel, Cadbury, UK) and 0.05% Tween 20. Each membrane was incubated for 1 h at room temperature with the primary antibody diluted in PBS containing 2.5% reconstituted skim milk powder and 0.05% Tween 20. After extensive washing in PBS containing 0.05% Tween 20, the membrane was incubated for 1 h at room temperature with an HRP-conjugated secondary antibody diluted 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 $MgCl_2$, and 0.5% Triton X-100; Fey et al., 1984) containing 250 mM $(NH_4)_2SO_4$, 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 transferrred 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 antiserum 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 antiserum 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 Axiophot microscope (Carl Zeiss Ltd., Oberkochen, Germany).

Keratinocyte colonies grown on coverslips in 35-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 antiserum CR-1, as described above. Double labeling was performed as described above with the rabbit antiserum 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.

After a 10-min incubation on a drop of PBS, the grids were blocked in 0.05% fish skin gelatin in PBS (FSG/PBS) for 30 min and then incubated with a 1:50 dilution of the antiserum CR-1 or the preimmune serum in FSG/PBS overnight at 4°C. The sections were washed three times with FSG/PBS and then incubated with a 1:35 dilution of protein A conjugated to 10 nm gold (Cell Biology Department, Utrecht, The Netherlands) in FSG/PBS for 30 min. After three washes each in PBS and distilled water, the sections were air-dried, contrasted with saturated aqueous uranyl acetate (12 min) and lead citrate (6 min), and examined with a Jeol 1200 electron microscope (JEOL UK Ltd., Herts, UK). For double labeling the sections were first labeled with CR-1, followed by protein A conjugated to 10 nm gold, and then with the mouse monoclonal DPI/II antibody described above, followed by a 1:35 dilution of goat anti–mouse IgG conjugated to 5 nm gold.

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 polyclonal antiserum (M) raised against the 210-kD cornified envelope precursor described by Simon and Green (1984).

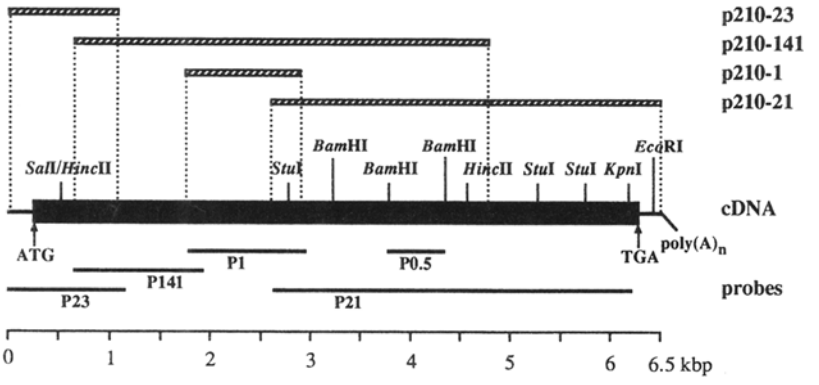

Figure 1. Isolated cDNA clones representing the mRNA for the 210-kD protein and partial restriction map of the composite cDNA. Clone names are shown on the right. The positions of the putative start codon (ATG) and stop codon (TGA) are indicated. The cDNA fragments used as probes are shown below the restriction map (P23, nucleotides 1–1082; P141, nucleotides 575–1976; P1, nucleotides 1775–2864; P21, nucleotides 2582–6153); P0.5, nucleotides 3205–3709.

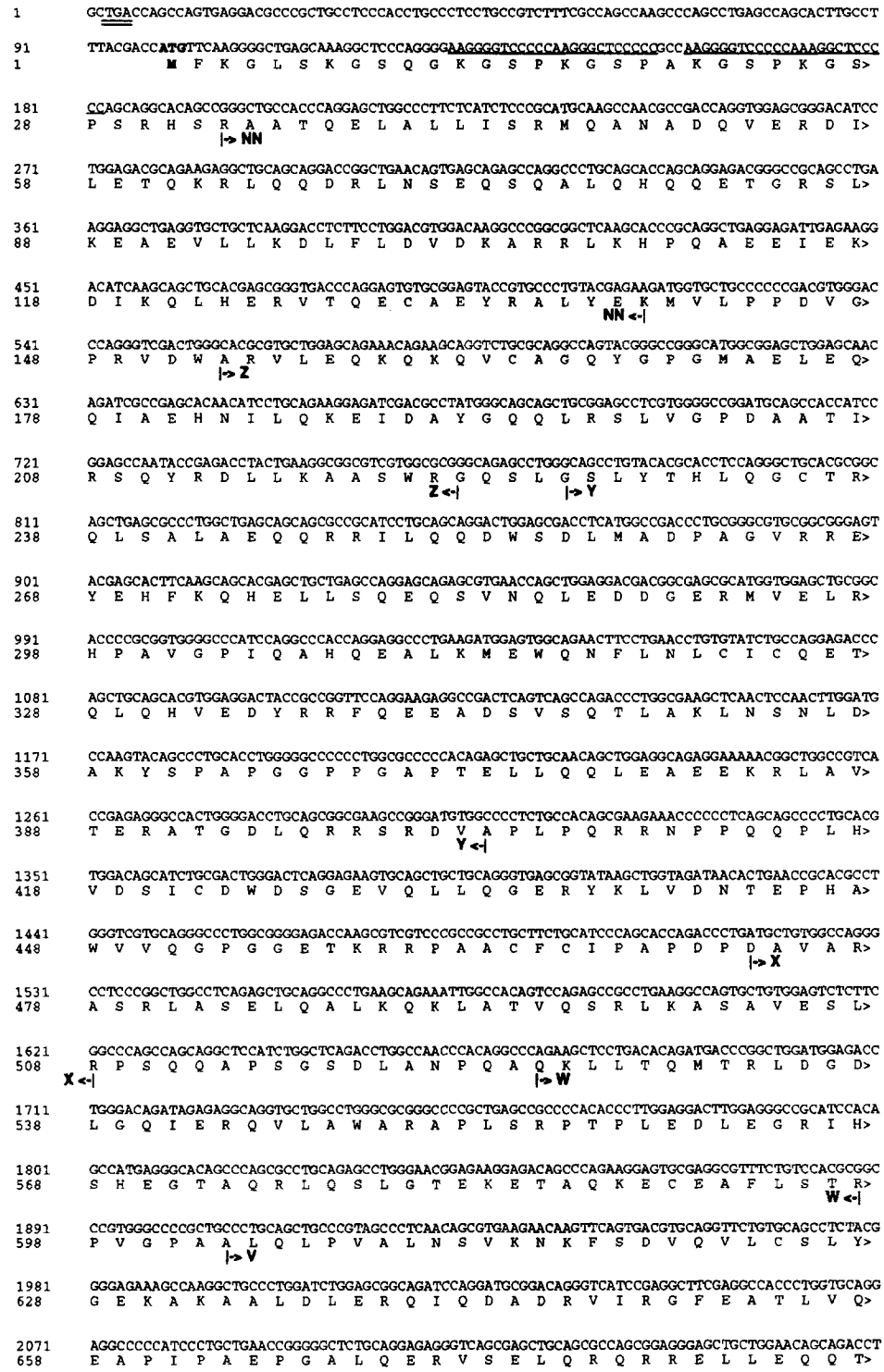

Figure 2. Nucleotide sequence of the composite cDNA encoding the 210-kD protein and predicted amino acid sequence. Numeration of the cDNA sequence begins with the first nucleotide of the putative translation initiation codon (ATG, ***bold***), which is preceded by an in-frame stop codon (TGA, *doubly underlined*) that is also present in genomic DNA (data not shown). Numeration of the deduced amino acid sequence begins with the first in-frame methionine of 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 NH_2-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.

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 encoded protein has a theoretical relative molecular mass of 231 kD, which is in good agreement with the apparent molecular mass of 210 kD in SDS-PAGE. The composite cDNA contains 6,457 bp, corresponding to the size of the 6.5-kb mRNA detected by Northern blot analysis (see below).

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2161    GCGTGCTGCGGCTACACCGCGCGCTGAAGGCCTCGGAGCACGCATGCGCTGCCCTGCAGAACAACTTCCAGGAGTTCTGCCAAGACCTGC
688     C  V  L  R  L  H  R  A  L  K  A  S  E  H  A  C  A  A  L  Q  N  N  F  Q  E  F  C  Q  D  L>

2251    CTCGCCAGCAGCGCCAGGTGCGAGCCCTCACCGACCGCTACCACGCCGTAGGGGACCAGCTGGACCTGCGGGAGAAGGTGGTGCAGGATG
718     P  R  Q  Q  R  Q  V  R  A  L  T  D  R  Y  H  A  V  G  D  Q  L  D  L  R  E  K  V  V  Q  D>

2341    CCGCCCTCACCTACCAGCAGTTCAAGAACTGCAAGGATAACCTGAGCTCCTGGCTGGAGCACCTGCCCCGCAGCCAGGTGCGGCCCAGCG
748     A  A  L  T  Y  Q  Q  F  K  N  C  K  D  N  L  S  S  W  L  E  H  L  P  R  S  Q  V  R  P  S>

2431    ACGGCCCCAGCCAGATCGCCTACAAGCTGCAGGCGCAGAAGAGGCTGACGCAGGAGATCCAGAGCCGAGAGCGGGACAGGGCCACAGCAT
778     D  G  P  S  Q  I  A  Y  K  L  Q  A  Q  K  R  L  T  Q  E  I  Q  S  R  E  R  D  R  A  T  A>

2521    CCCACCTCTCCCAGGCCCTGCAGGCAGCGCTCCAGGACTATGAGCTCCAGGCAGACACCTACCGCTGCTCTTTGGAGCCCACCCTGGCAG
808     S  H  L  S  Q  A  L  Q  A  A  L  Q  D  Y  E  L  Q  A  D  T  Y  R  C  S  L  E  P  T  L  A>

2611    TGTCAGCCCCCAAGAGACCCCGAGTGGCTCCCCTGCAAGAGAGCATCCAAGCCCAGGAGAAGAACCTTGCAAAGGCCTATACTGAGGTTG
838     V  S  A  P  K  R  P  R  V  A  P  L  Q  E  S  I  Q  A  Q  E  K  N  L  A  K  A  Y  T  E  V>

2701    CAGCAGCACAGCAGCAGCTGCTCCAGCAGCTGGAGTTTGCTAGAAAAATGCTGGAGAAGAAGGAGCTCAGTGAGGACATCCGAAGGACCC
868     A  A  A  Q  Q  Q  L  L  Q  Q  L  E  F  A  R  K  M  L  E  K  K  E  L  S  E  D  I  R  R  T>

2791    ATGATGCAAAGCAGGGCTCCGAGAGCCCTGCCCAAGCAGGGAGAGAGTCAGAGGCCCTGAAGGCCCAGCTGGAAGAGGAGAGGAAGCGGG
898     H  D  A  K  Q  G  S  E  S  P  A  Q  A  G  R  E  S  E  A  L  K  A  Q  L  E  E  E  R  K  R>
                                V <-|  |-> rod domain

2881    TGGCCCGGGTGCAGCATGAGCTGGAGGCGCAGAGGAGCCAACTGCTGCAGCTGAGGACCCAGCGGCCCTTGGAGAGGCTGGAGGAGAAGG
928     V  A  R  V  Q  H  E  L  E  A  Q  R  S  Q  L  L  Q  L  R  T  Q  R  P  L  E  R  L  E  E  K>

2971    AAGTGGTAGAGTTCTACCGGGACCCCCAGCTGGAGGGCAGCCTGTCCAGGGTGAAGGCCCAGGTGGAGGAGGAGGGCAAGCGGCGGGCTG
958     E  V  V  E  F  Y  R  D  P  Q  L  E  G  S  L  S  R  V  K  A  Q  V  E  E  E  G  K  R  R  A>

3061    GCCTGCAGGCAGACCTGGAAGTGGCAGCCCAGAAGGTCGTGCAGCTGGAAAGCAAGAGGAAGACCATGCAGCCTCATCTGCTGACCAAGG
988     G  L  Q  A  D  L  E  V  A  A  Q  K  V  V  Q  L  E  S  K  R  K  T  M  Q  P  H  L  L  T  K>

3151    AGGTCACCCAGGTGGAGAGGGACCCCGGCCTGGACAGCCAGGCGGCCCAGCTCAGGATCCAGATCCAGCAGCTCCGCGGGGAGGATGCCG
1018    E  V  T  Q  V  E  R  D  P  G  L  D  S  Q  A  A  Q  L  R  I  Q  I  Q  Q  L  R  G  E  D  A>

3241    TCATCTCGGCCCGGCTGGAAGGGCTGAAGAAGGAGCTACTGGCCCTTGAGAAGAGGGAGGTGGACGTGAAGGAGAAGGTCGTGGTGAAAG
1048    V  I  S  A  R  L  E  G  L  K  K  E  L  L  A  L  E  K  R  E  V  D  V  K  E  K  V  V  V  K>

3331    AGGTAGTCAAGGTGGAGAAGAATCTGGAAATGGTCAAGGCAGCCCAGGCTCTGAGGCTGCAGATGGAGGAGGATGCTGCGCGGAGGAAGC
1078    E  V  V  K  V  E  K  N  L  E  M  V  K  A  A  Q  A  L  R  L  Q  M  E  E  D  A  A  R  R  K>

3421    AGGCGGAGGAGGCTGTGGCCAAGCTACAGGCTCGCATCGAAGACCTGGAGCGGGCTATCAGCTCGGTGGAGCCCAAGGTCATCGTGAAGG
1108    Q  A  E  E  A  V  A  K  L  Q  A  R  I  E  D  L  E  R  A  I  S  S  V  E  P  K  V  I  V  K>

3511    AGGTGAAGAAGGTGGAGCAGGACCCAGGGCTCCTCCAGGAGTCCTCCAGGCTGAGGAGCCTCCTCGAGGAGGAGAGGACCAAGAACGCGA
1138    E  V  K  K  V  E  Q  D  P  G  L  L  Q  E  S  S  R  L  R  S  L  L  E  E  E  R  T  K  N  A>

3601    CGCTGGCCAGGGAGCTGAGCGACCTGCACAGCAAGTACAGCGTGGTGGAGAAGCAGAGGCCCAAAGTGCAGCTCCAGGAGCGCGTCCACG
1168    T  L  A  R  E  L  S  D  L  H  S  K  Y  S  V  V  E  K  Q  R  P  K  V  Q  L  Q  E  R  V  H>

3691    AGATCTTCCAGGTGGATCCGGAGACAGAGCAGGAGATCACTCGGCTCAAGGCCAAGCTGCAGGAGATGGCGGGCAAGAGGAGCGGTGTGG
1198    E  I  F  Q  V  D  P  E  T  E  Q  E  I  T  R  L  K  A  K  L  Q  E  M  A  G  K  R  S  G  V>

3781    AGAAGGAGGTGGAGAAGCTGCTGCCCGACCTGGAGGTCCTGCGGGCCCAGAAGCCCACGGTGGAGTACAAGGAGGTGACCCAGGAGGTGG
1228    E  K  E  V  E  K  L  L  P  D  L  E  V  L  R  A  Q  K  P  T  V  E  Y  K  E  V  T  Q  E  V>

3871    TGAGGCATGAGAGGAGCCCCGAGGTGCTGCGTGAGATTGACCGCCTGAAGGCTCAGCTCAACGAGCTCGTCAACAGCCACGGGCGCTCCC
1258    V  R  H  E  R  S  P  E  V  L  R  E  I  D  R  L  K  A  Q  L  N  E  L  V  N  S  H  G  R  S>

3961    AGGAGCAGCTCATCCGCCTGCAGGGTGAGCGCGACGAGTGGAGGCGCGACGGGGCCAAGGTGGAGACCAAGACGGTGAGCAAGGAGGTGG
1288    Q  E  Q  L  I  R  L  Q  G  E  R  D  E  W  R  R  D  G  A  K  V  E  T  K  T  V  S  K  E  V>

4051    TGCGCCACGAGAAGGACCCGGTGCTGGAGAAAGAAGCAGAGTGGCTCCGCCAGGAGGTGCGGGAGGCGGCCCAGAAGAGGCGGGCCGCGG
1318    V  R  H  E  K  D  P  V  L  E  K  E  A  E  W  L  R  Q  E  V  R  E  A  A  Q  K  R  R  A  A>

4141    AGGACGCGGTGTACGAGCTGCAGAGCAAGCGCCTGCTGCTGGAGAGGAGGAAGCCCGAGGAGAAGGTGGTGGTGCAGGAGGTGGTGGTCA
1348    E  D  A  V  Y  E  L  Q  S  K  R  L  L  L  E  R  R  K  P  E  E  K  V  V  V  Q  E  V  V  V>
```

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 Garnier et al. (1978), predicted an NH_2-terminal domain characterized by the presence of short α-helical segments, a central do-

...continued

```
4231    CCCAGAAGGACCCGAAGCTGCGCGAGGAGCACAGCCGGCTGAGCGGGAGCCTGGATGAGGAGGTGGGCCGGCGGCGCCAGCTAGAGCTTG
1378    T  Q  K  D  P  K  L  R  E  E  H  S  R  L  S  G  S  L  D  E  E  V  G  R  R  R  Q  L  E  L>

4321    AGGTGCAGCAGCTGCGGGCCGGCGTGGAGGAGCAGGAGGGCCTGCTCAGCTTCCAGGAGGACCGCAGCAAGAAGCTGGCCGTGGAGAGGG
1408    E  V  Q  Q  L  R  A  G  V  E  E  Q  E  G  L  L  S  F  Q  E  D  R  S  K  K  L  A  V  E  R>

4411    AGCTGCGGCAGCTGACCTTGAGGATCCAGGAGCTCGAGAAGCGGCCTCCCACGGTTGCAGGAGAAGATCATCATGGAGGAAGTGGTCAAG
1438    E  L  R  Q  L  T  L  R  I  Q  E  L  E  K  R  P  P  T  V  A  G  E  D  H  H  G  G  S  G  Q>

4501    CTGGAGAAGGACCCGGACCTGGAGAAGTCCACGGAAGCCCTGCGTGGGACCTGGACCAGGAGAAGACCCAGGTAACCGAGCTGAATCGGG
1468    A  G  E  G  P  G  P  G  E  V  H  G  S  P  A  W  D  L  D  Q  E  K  T  Q  V  T  E  L  N  R>

4591    AGTGCAAGAACCTGCAGGTCCAGATTGACGTCCTCCAGAAAGCCAAATCGCAGGAGAAGACCATCTACAAGGAAGTGATCCGGGTGCAGA
1498    E  C  K  N  L  Q  V  Q  I  D  V  L  Q  K  A  K  S  Q  E  K  T  I  Y  K  E  V  I  R  V  Q>

4681    AGGACCGCGTCCTGGAAGATGAGCGGGCCCGCGTGTGGGAGATGCTCAACAGGGAGCGCACGGCCCGGCAGGCCCGGGAGGAGGAGGCAC
1528    K  D  R  V  L  E  D  E  R  A  R  V  W  E  M  L  N  R  E  R  T  A  R  Q  A  R  E  E  E  A>

4771    GGCGCCTGCGGGAGCGCATTGACCGGGCCGAGACGCTGGGGAGAACCTGGTCCCGGGAGGAGTCCGAGCTGCAGAGGGCCCGGGACCAGG
1558    R  R  L  R  E  R  I  D  R  A  E  T  L  G  R  T  W  S  R  E  E  S  E  L  Q  R  A  R  D  Q>

4861    CCGACCAGGAGTGTGGGCGGCTGCAGCAGGAGCTGCGGGCTCTGGAGAGGCAGAAGCAGCAGCAGACACTGCAGCTGCAGGAGGAGTCGA
1588    A  D  Q  E  C  G  R  L  Q  Q  E  L  R  A  L  E  R  Q  K  Q  Q  Q  T  L  Q  L  Q  E  E  S>

4951    AGCTGCTCAGCCAGAAGACGGAGAGCGAGCGACAGAAGGCGGCCCAGCGGGGCCAGGAGCTCTCGCGGCTGGAGGCGGCCATCCTCCGCG
1618    K  L  L  S  Q  K  T  E  S  E  R  Q  K  A  A  Q  R  G  Q  E  L  S  R  L  E  A  A  I  L  R>

5041    AGAAGGACCAGATCTACGAGAAGGAGCGGACGCTCCGGGACCTCCACGCCAAGGTGAGCCGGGAGGAGCTCAGCCAGGAGACCCAGACGC
1648    E  K  D  Q  I  Y  E  K  E  R  T  L  R  D  L  H  A  K  V  S  R  E  E  L  S  Q  E  T  Q  T>
                                                                                  rod domain <-|

5131    GAGAGACCAACCTTTCCACCAAGATCTCCATCCTGGAACCCGAGACGGGGAAGGACATGTCCCCATACGAGGCCTACAAGAGGGGCATCA
1678    R  E  T  N  L  S  T  K  I  S  I  L  E  P  E  T  G  K  D  M  S  P  Y  E  A  Y  K  R  G  I>

5221    TCGACAGGGGCCAGTACTTGCAGCTGCAGGAGCTCGAGTGTGACTGGGAGGAGGTCACCACCTCGGGGCCCTGTGGGGAGGAGTCTGTGC
1708    I  D  R  G  Q  Y  L  Q  L  Q  E  L  E  C  D  W  E  E  V  T  T  S  G  P  C  G  E  E  S  V>

5311    TCCTGGACCGCAAGAGCGGGAAGCAGTACTCCATCGAGGCCGCCCTCCGCTGCCGGCGCATCTCTAAGGAGGAGTACCATCTGTACAAGG
1738    L  L  D  R  K  S  G  K  Q  Y  S  I  E  A  A  L  R  C  R  R  I  S  K  E  E  Y  H  L  Y  K>

5401    ACGGCCACCTGCCCATCTCCGAGTTTGCGCTGCTTGTAGCTGGGGAGACCAAGCCAAGCTCCTCACTCTCCATCGGCTCTATCATCTCCA
1768    D  G  H  L  P  I  S  E  F  A  L  L  V  A  G  E  T  K  P  S  S  S  L  S  I  G  S  I  I  S>

5491    AGTCCCCGCTCGCCTCCCCGGCCCCCCAGAGCACCAGTTTCTTCTCTCCCAGCTTCTCTCTCGGGCTCGGTGATGACAGCTTCCCTATCG
1798    K  S  P  L  A  S  P  A  P  Q  S  T  S  F  F  S  P  S  F  S  L  G  L  G  D  D  S  F  P  I>

5581    CCGGGATCTATGACACAACCACAGACAACAAGTGCAGCATCAAGACGGCCGTGGCCAAGAACATGCTGGACCCCATCACTGGGCAGAAGC
1828    A  G  I  Y  D  T  T  T  D  N  K  C  S  I  K  T  A  V  A  K  N  M  L  D  P  I  T  G  Q  K>
                                |-> C

5671    TACTGGAGGCCCAGGCGGCCACAGGGGGCATCGTGGACCTGCTCAGCCGTGAGCGCTACTCTGTGCACAAGGCGATGGAGAGGGGCCTGA
1858    L  L  E  A  Q  A  A  T  G  G  I  V  D  L  L  S  R  E  R  Y  S  V  H  K  A  M  E  R  G  L>

5761    TCGAGAACACCTCCACACAGAGGCTGCTTAACGCCCAGAAGGCCTTCACCGGCATCGAGGACCCCGTCACCAAGAAGAGGCTCTCGGTGG
1888    I  E  N  T  S  T  Q  R  L  L  N  A  Q  K  A  F  T  G  I  E  D  P  V  T  K  K  R  L  S  V>

5851    GCGAGGCCGTCCAGAAGGGCTGGATGCCCCGGGAGAGCGTGCTCCCACACCTGCAGGTGCAGCACCTGACCGGGGGGCTCATCGACCCCA
1918    G  E  A  V  Q  K  G  W  M  P  R  E  S  V  L  P  H  L  Q  V  Q  H  L  T  G  G  L  I  D  P>

5941    AGAGGACAGGCCGCATCCCCATCCAGCAGGCCCTCCTCTCCGGGATGATCAGTGAAGAGCTGGCCCAGCTCCTGCAGGACGAGTCCAGCT
1948    K  R  T  G  R  I  P  I  Q  Q  A  L  L  S  G  M  I  S  E  E  L  A  Q  L  L  Q  D  E  S  S>

6031    ACGAGAAGGATTTGACAGACCCCATCTCCAAGGAACGGCTGAGCTACAAGGAGGCCATGGGCCGCTGCCGCAAAGACCCCCTGAGCGGCC
1978    Y  E  K  D  L  T  D  P  I  S  K  E  R  L  S  Y  K  E  A  M  G  R  C  R  K  D  P  L  S  G>

6121    TGCTGCTCCTGCCAGCGGCACTGGAGGGGTACCGCTGCTACCGCTCCGCCTCCCCCACCGTCCCGCGCTCCCTTCGCTGACACGGGCCAA
2008    L  L  L  L  P  A  A  L  E  G  Y  R  C  Y  R  S  A  S  P  T  V  P  R  S  L  R  *
              C <-|

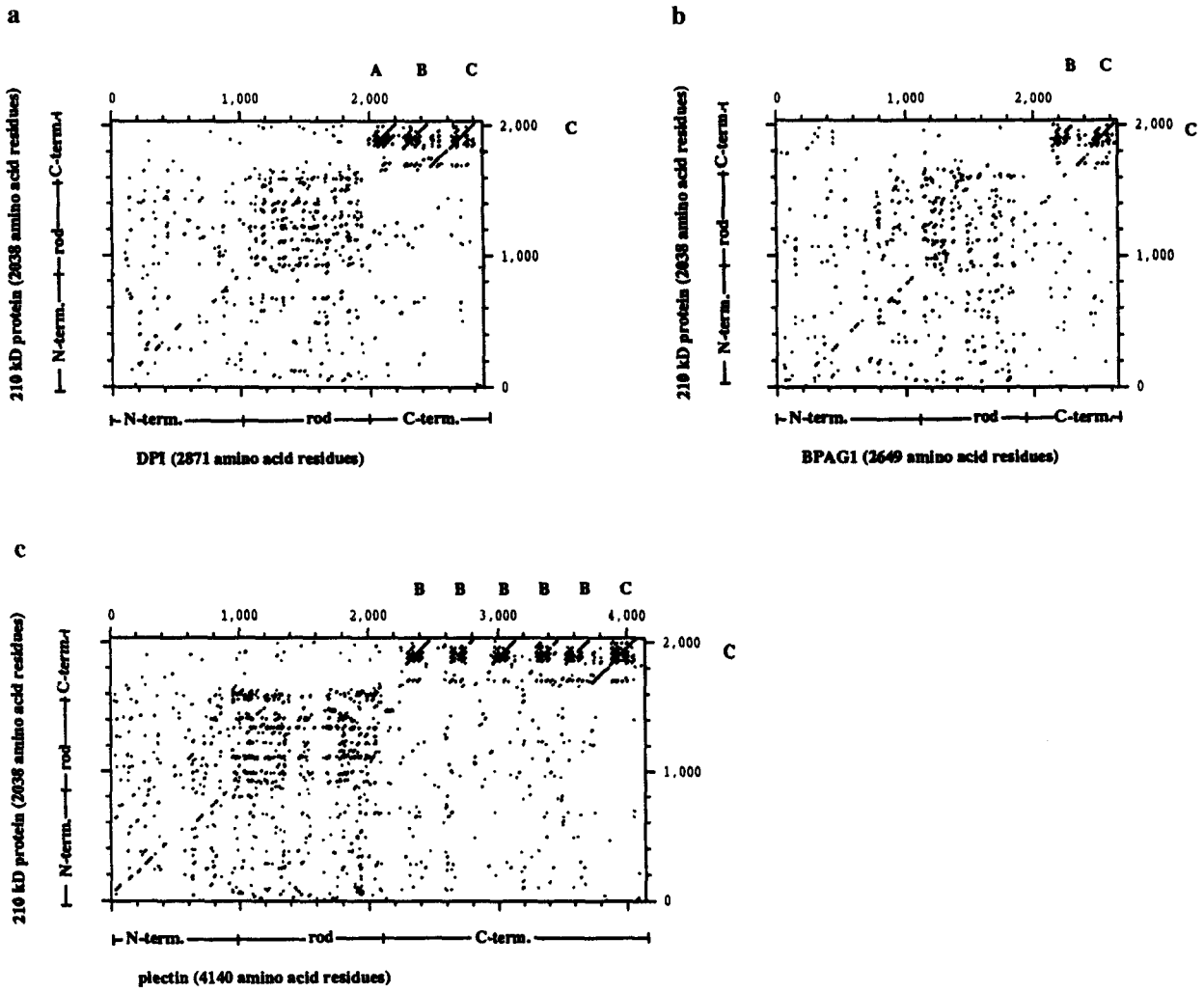
6211    GGAGCCAGTGGGGAAGTGCGTGTGTTGGGCCAGGTAGGATACGTACACCTCTTGCCTCAGAGCAGCCTCATCCCAGGCAGTGGGTCTTCC

6301    CTCTGTCCAACCACTGTTTTATTATTTTACTAACGAGGTGATGGGCTCCCTCCCCTAACCTTGGAGCCTGATCCATCCCCAGACCAGGAC

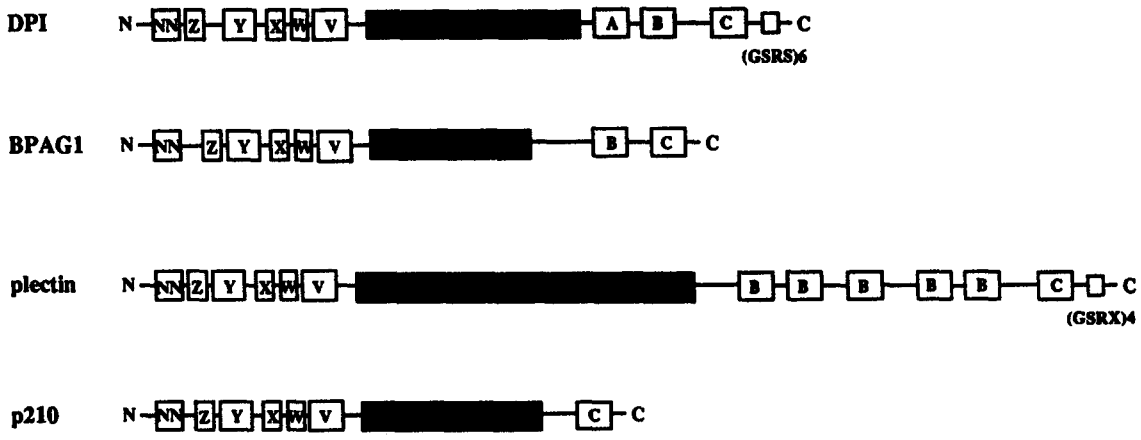
6391    AGCAGCCACTCAGTTCTTCCTCCACCTCCACCCAGTGATCCCAATAAACGAATTCTGTCTCCCCGTG - poly(A) tail
```

Figure 2.

Figure 3. 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,



d



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. (GSRS)6 and (GSRX)4 refer to repeat motifs at the COOH terminus of DPI and plectin, respectively.

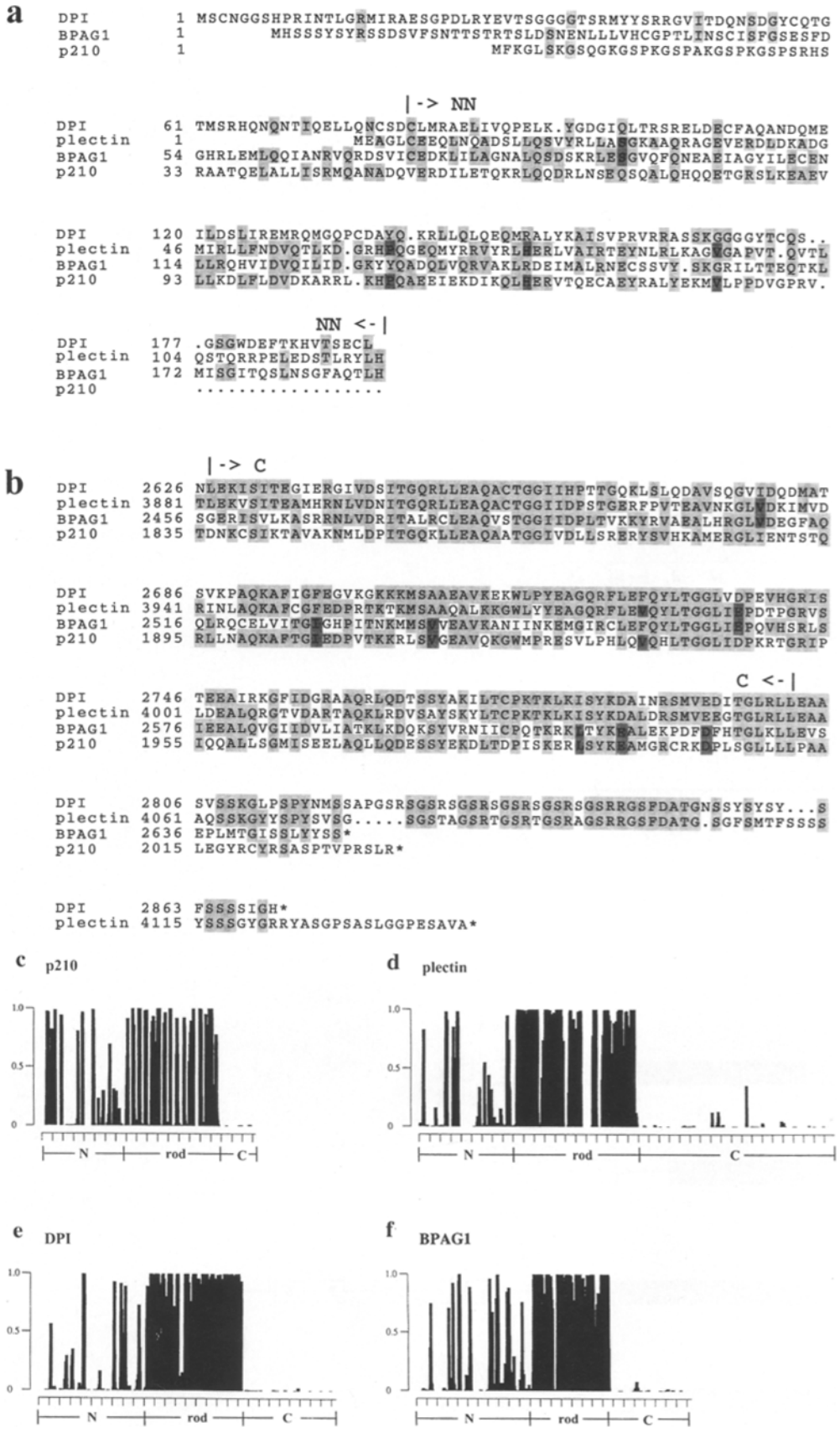


Figure 4. Direct sequence comparison and coiled coil analysis of the 210-kD protein, DPI, BPAG1, and plectin. Sequence comparison of the NH_2 termini (*a*) and the COOH termini (*b*) of the four homologues (domain boundaries of DPI, BPAG1, and plectin according to Green et al., 1992). Amino acid residues 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 Lupas algorithm, versus the position in the amino acid sequence (*x* axis) for the 210-kD protein (*c*), 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*a*; Virata et al., 1991; Wiche et al., 1991; the sequence of the extreme DPI NH_2-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 NH_2-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 NH_2- 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 NH_2 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) phophorylation 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*a*; 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 NH_2 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 regon: 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 NH_2 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).

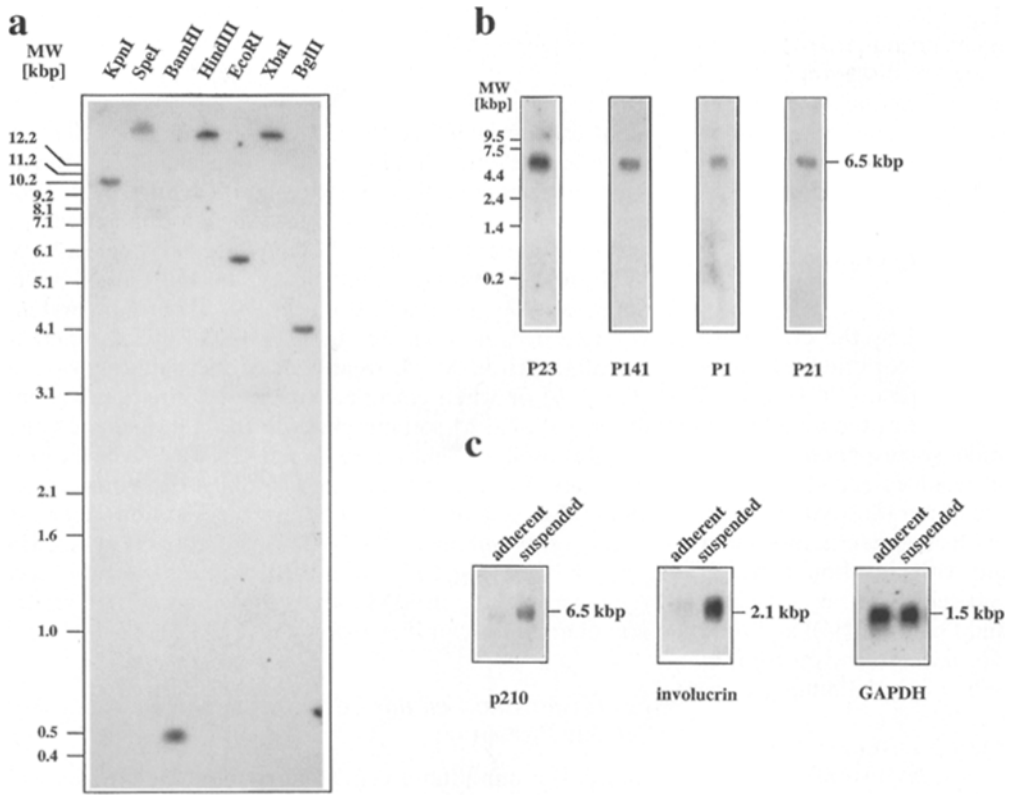


Figure 5. Analysis of the gene and mRNA encoding the 210-kD protein. (*a*) A Southern blot containing human genomic DNA (10 μg/lane) digested with the indicated restriction enzymes was probed with the radiolabeled BamHI fragment P0.5. A single hybridizing fragment was present in each restriction digest. 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. 4 *a* are aligned optimally to reveal primary sequence conservation, secondary structure predictions suggest an NH_2-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 NH_2-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 NH_2 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. 5 *a*). 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. 5 *b*).

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. 5 *c*; 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 λgt11 expression library (Fig. 6 *a*, *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. 6 *a*, *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 cell 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. 6 *b*) 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. 6 *b*). In contrast, ~50% of desmoplakin remained soluble in SDS/β-mercaptoethanol after induction of cross-linking (Fig. 6 *b*).

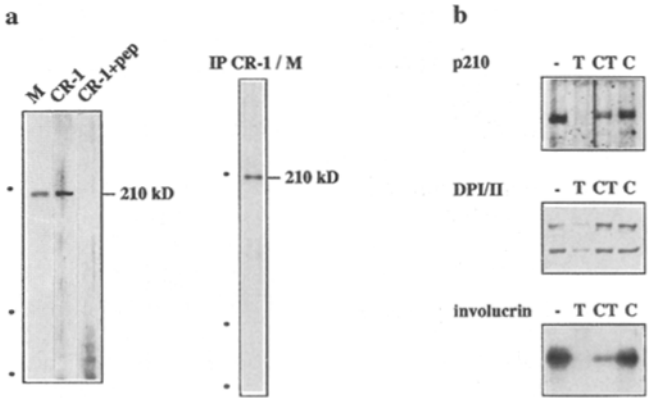


Figure 6. Confirmation that the isolated cDNA encodes the 210-kD cornified envelope precursor. (*a*) (*Left-hand side*): Immunoblot of keratinocyte extract probed with an antiserum raised against the 210-kD cornified envelope precursor described by Simon and Green (1984) (lane *M*), with an antiserum raised against a peptide corresponding to the COOH terminus encoded by the putative cDNA for the 210-kD protein (lane *CR-1*), and with the antiserum CR-1 in the presence of the peptide used for immunization (lane *CR-1 + pep*). (*Right-hand side*): Immunoprecipitation of keratinocyte extract with CR-1, followed by immunoblotting with antiserum M (lane *IP CR-1/ M*). The mobility of the molecular mass standards is indicated: 220, 110, and 75 kD. (*b*) 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 cysteamine (*C*), or 20 mM cysteamine and Triton X-100 (*CT*). Immunoblots of the protein extracts were probed with CR-1 to detect the 210-kD protein, with a DPI/II antiserum to detect DPI and DPII (apparent molecular masses 250 and 215 kD, respectively), or with DH1 to detect involucrin (apparent molecular mass 140 kD).

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. 7 *b*), suggesting that the 210-kD protein was asso-

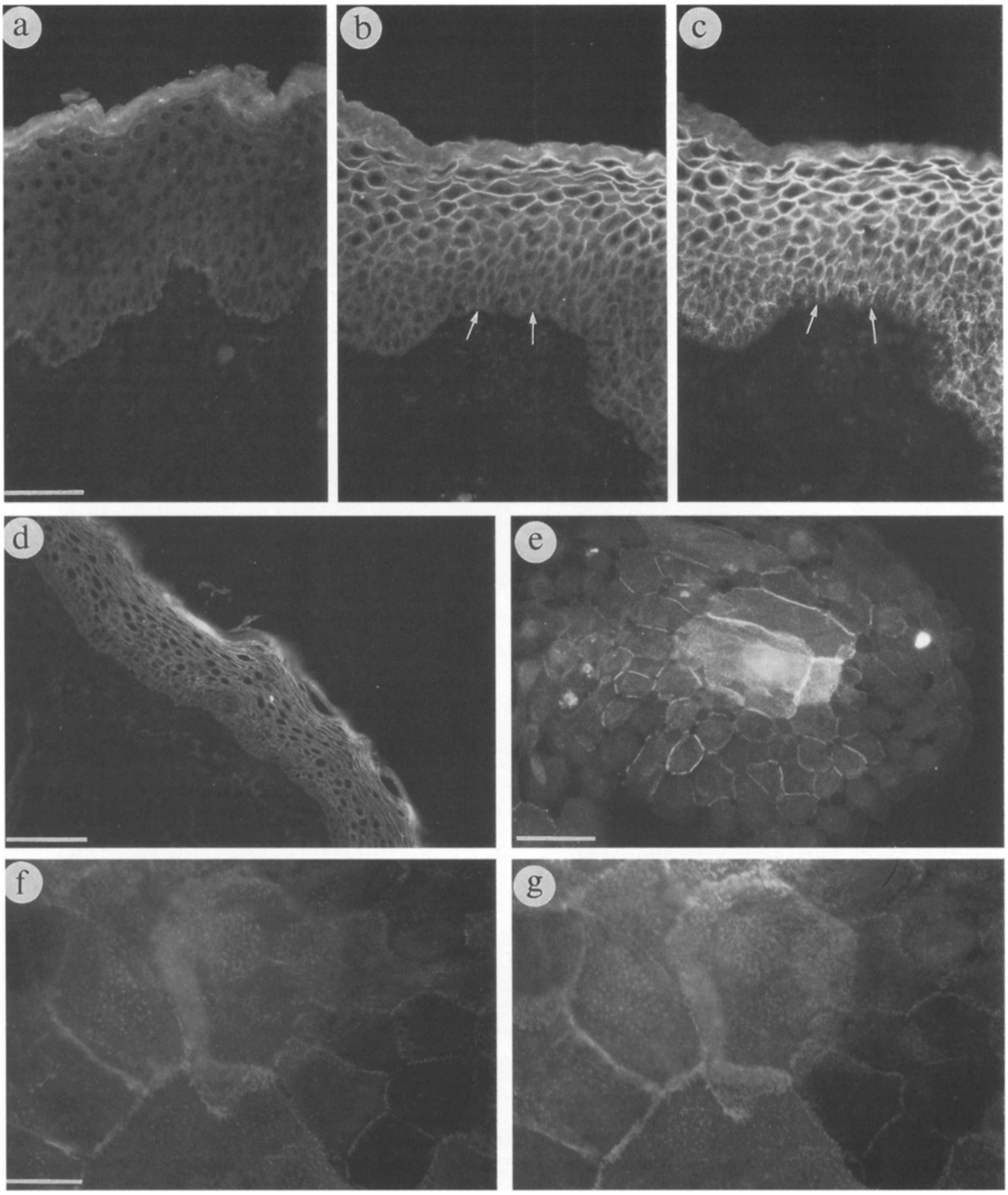
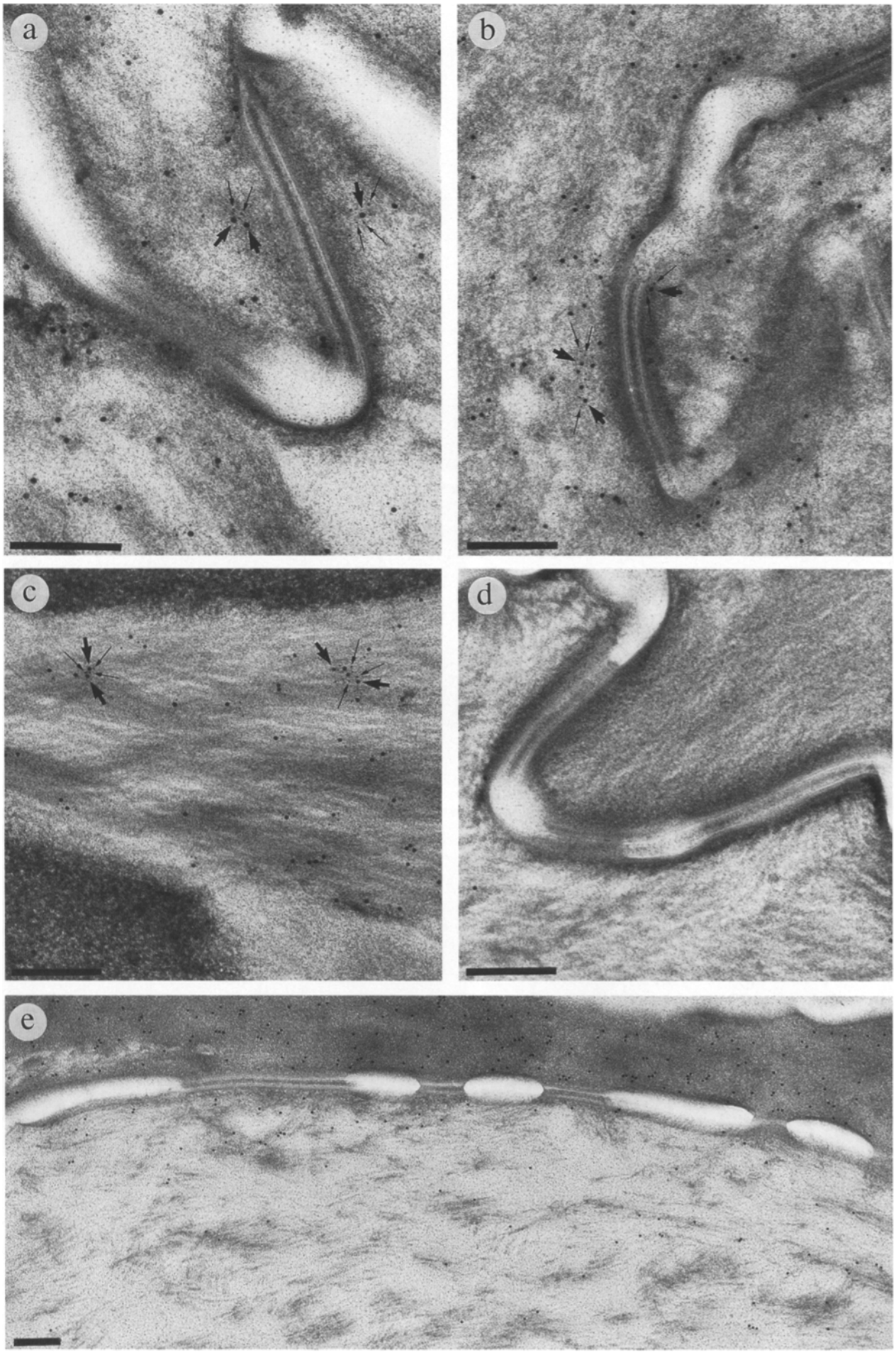


Figure 7. The tissue distribution and cellular localization of the 210-kD protein, as determined by indirect immunofluorescence with the CR-1 antiserum. (*a–c*) Epidermis. (*a*) Preimmune serum from rabbit CR-1, (*b* and *c*) double labeling with CR-1 (*b*) and an antibody to DPI/II (*c*), (*d*) cervical 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 DPI/II (*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.


a
b
c
d
e

ciated 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 NH_2-terminal globular domain, a central rod domain, and a COOH-terminal globular domain (Green et al., 1990; Sawamura et al., 1991*a*; Virata et al., 1992; Wiche et al., 1991). The NH_2 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., 1991*a*; 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 NH_2- 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-

Figure 8. Localization of the 210-kD protein in epidermis, as determined by immunogold EM with the CR-1 antiserum. (*a–c*) Double labeling with CR-1 (10 nm gold) and an antibody to DPI/II (5 nm gold); some of the areas showing colocalization of CR-1 (*large arrows*) and DPI/II (*small arrows*) are indicated by arrows. (*d*) Labeling with the preimmune serum of rabbit CR-1. (*e*) Single labeling with CR-1. The upper cell is a cornified cell. Bar, 200 nm.

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., 1991*b*; Tamai et al., 1993), but alternative splice products containing BPAG1 exons, BPFG 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 labeleled 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 detergents and one that is easily extracted with low detergent concentrations (Duden and Franke, 1988; Pasdar and Nelson, 1988*a*,*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 NH_2-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 NH_2-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 NH_2-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 NH_2-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."

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

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