cDNA Cloning of the 210-kDa Paraneoplastic Pemphigus Antigen Reveals that Envoplakin Is a Component of the Antigen Complex

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Although the 210 and 190-kDa proteins are the most frequently detected antigens reacting with sera of patients with paraneoplastic pemphigus (PNP) in immunoblot analysis, there is still uncertainty as to the nature of these PNP antigens. To isolate and characterize a cDNA clone encoding the 210-kDa PNP antigen, we screened a human keratinocyte λ gt 11 cDNA expression library by the immunoperoxidase method with serum IgG from a PNP patient. The IgG used for the immunoscreening of a keratinocyte cDNA expression library recognized 210- and 190-kDa antigens by immunoblotting. A single clone, called here the PNP clone, producing a fusion protein that reacted strongly with the patient's IgG, was further characterized. Only the PNP patient's IgG, but not IgG from a normal control, pemphigus foliaceus, or pemphigus vulgaris patients, bound the plaques of this positive clone. Furthermore, PNP IgG affinity purified on plaques of this clone, but not unrelated clones, bound to keratinocyte cell surfaces by immunofluorescence and reacted with the 210-kDa PNP antigen by immunoblotting. *Eco*RI digestion of the clone's cDNA insert demonstrated a 1.4-kbp fragment. This cDNA insert was placed into a M13 mp 18 vector and sequenced. Sequence analysis revealed that the cDNA insert of the PNP clone encodes a part of the central rod domain and the COOH-terminal C domain of envoplakin, a newly defined precursor of the cornified envelope that is homologous to desmoplakin. This result demonstrates that the 210-kDa PNP antigen is envoplakin and PNP is an autoimmune disease that produces autoantibodies against intermediate filament–associated proteins in desmosomes and hemidesmosomes, desmoplakin, bullous pemphigoid antigen 1 (BPAG 1), and envoplakin. *Key words: keratinocyte λ gt 11 cDNA expression library/sequence analysis/intermediate filament-associated protein. J Invest Dermatol 109:365–369, 1997*

Paraneoplastic pemphigus (PNP) is an acquired autoimmune blistering disorder characterized by severe mucosal erosions and polymorphous skin lesions in association with underlying neoplasia (Anhalt *et al*, 1990; Horn and Anhalt, 1992; Oursler *et al*, 1992). Circulating autoantibodies bind not only to the cell surface of stratified squamous epithelia but also to simple, columnar, and transitional epithelia and immunoprecipitate antigen complex of five polypeptides with molecular masses of 250, 230, 210, 190, and 170 kDa (Anhalt *et al*, 1990; Oursler *et al*, 1992). These autoantibodies are pathogenic as demonstrated by passive transfer experiments (Anhalt *et al*, 1990).

By immunoprecipitation studies, it has been suggested that the 250- and 210-kDa proteins are desmoplakin (DP) I and II and that the 230-kDa protein is the bullous pemphigoid antigen 1 (BPAG 1), but the identity of the 190-kDa protein has not yet been established (Anhalt *et al*, 1990; Oursler *et al*, 1992). The 170-kDa protein that was thought to be an antigen degradation product of one of the higher molecular mass components is now regarded as a transmembrane molecule that may play an important role in the pathogenesis of PNP (Oursler *et al*, 1992).

Immunoblot analysis, however, demonstrated a slightly different reactivity from that of immunoprecipitation in that all the PNP sera reacted with a characteristic doublet of the 210- and 190-kDa proteins, but the 250-kDa DP 1 and 230-kDa BPAG 1 are less frequently detected, and the 170-kDa protein is not detected at all (Hashimoto *et al*, 1995). Further immunoblot analysis indicated that the 210-kDa protein is different from DP II. Upon immunoblotting of epidermal extracts, there was a slight but constant difference of mobility on the gel between the 210-kDa protein detected by PNP sera and DP II detected by the anti-DP monoclonal antibody (Hashimoto *et al*, 1995). A much more prominent difference between the 210-kDa PNP antigen and DP II was detected by immunoblotting of a bovine desmosome preparation (Hashimoto *et al*, 1995). In fact, the bands detected by anti-DP II monoclonal antibody are always located slightly higher than the 210-kDa protein. More confirmatory evidence of the dissimilarity between the 210-kDa protein and DP II comes from the finding that the 210-kDa PNP antigen and DP I are not always detected simultaneously by the same PNP serum, as in the previous immunoblotting studies (Rybojad *et al*, 1993; Hashimoto *et al*, 1995). DP I/II are known to be produced by alternative splicing from the same gene, and DP II is a part of DP I (Green *et al*, 1990). Therefore, if the 210-kDa PNP antigen is DP II, DP I and the 210-kDa PNP antigen

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Abbreviations: BPAG 1, bullous pemphigoid antigen 1; DP, desmoplakin; PNP, paraneoplastic pemphigus.

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should always be detected simultaneously with the same PNP serum. Furthermore, the 210- and 190-kDa antigens are recognized by all PNP sera in immunoblotting analyses, indicating that these antigens may play an important role in pathogenesis of PNP (Hashimoto *et al*, 1995). These findings have prompted us to investigate these antigens more precisely.

The purpose of this study was to isolate and characterize a cDNA clone with coding sequences for the 210-kDa PNP antigen and to clarify the relationship between the 210-kDa antigen and DP II. We have used the autoantibodies from a PNP patient to identify and clone a cDNA that encodes the 210-kDa antigen. Sequence analysis of the cDNA clone encoding the 210-kDa PNP antigen revealed that the 210-kDa PNP antigen is identical to human envoplakin, a recently reported new member of the desmoplakin gene family.

MATERIALS AND METHODS

Sera One PNP serum that gave particularly clean immunoblotting of the 210-kDa and 190-kDa PNP antigen was orginally used to screen the cDNA expression library. Immunoprecipitation was performed using published techniques (Stanley *et al*, 1984; Oursler *et al*, 1992), and the findings were also characteristic of paraneoplastic pemphigus, with strong reactivity to 210-kDa, 190-kDa, and 170-kDa proteins but faint binding to the 250-kDa and 230-kDa proteins. IgG was isolated from the serum by diethylaminoethyl column (Bio-Rad, Hercules, CA) and concentrated using Centricon-30 concentrators (Amicon Co. Danvers, MA). Control sera were obtained from patients with pemphigus vulgaris and pemphigus foliaceus. We also used normal human sera as controls.

Screening of λ gt 11 Expression Library Human keratinocyte λ gt 11 expression library was purchased from Clontech (Palo Alto, CA). Screening of clones expressing the fusion protein of the 210-kDa PNP antigen was as previously described with a few modifications (Stanley *et al*, 1988; Amagai *et al*, 1991). Recombinant phages were screened at a density of 50,000 plaque-forming units per 150-mm plate of *Escherichia coli* Y 1090. The plates were incubated at 42°C for 4 h, overlaid with nitrocellulose membranes (Amersham, Buckinghamshire, U.K.), saturated with isopropyl thiogalactopyranoside (GIBCO, Grand Island, NY), and further incubated for 3 h at 37°C. Membranes were washed in 3% skim milk in Tris(hydroxymethyl) aminomethane-HCl–buffered saline (pH 8.0) for 1 h at room temperature and incubated at 4°C overnight with the primary antibody solution containing 2% bovine serum albumin and preabsorbed with antigen lysate of *E. coli*. Incubation was done at room temperature for 2 h with biotinylated anti-human IgG (DAKO, Copenhagen, Denmark), and another incubation for 1 h with streptavidin-biotinylated horseradish peroxidase complex (DAKO) was followed by staining with 4-chloro-1-naphthol (Sigma, St. Louis, MO).

Affinity Purification of Antibodies on λ gt 11 Plaque Protein To obtain the affinity-purified IgG that bound fusion protein produced by the PNPgt11 clone, we plated the isolated clone at a density of 5,000–8,000 plaques/150-mm plate. Fusion protein synthesis was induced as described above with isopropyl thiogalactopyranoside-saturated nitrocellulose membranes. These membranes were then incubated with the PNP IgG (diluted 1:20 in 2% bovine serum albumin in 10 mM Tris(hydroxymethyl)aminomethane-HCl, pH 7.4) at 4°C overnight. After the membranes were washed five times with 3% bovine serum albumin in Tris(hydroxymethyl)-aminomethane-HCl–buffered saline, the bound antibodies were eluted twice with 10 ml of 20 mM sodium citrate buffer (pH 3.2) at 37°C for 15 min each on a orbital shaker. The eluates were immediately neutralized with 2 M Tris(hydroxymethyl)aminomethane (pH 7.5). The eluted, neutralized IgG was dialyzed against Tris(hydroxymethyl)aminomethane-HCl–buffered saline at 4°C, then concentrated on a Centricon-30 (Amicon Co.) to a final volume of 100 μl and used for indirect immunofluorescence and immunoblot analysis.

Immunoblot Analysis Immunoblotting was performed as previously described (Hashimoto *et al*, 1990). In brief, cultured human keratinocytes were placed on ice, scraped with a scraper into sodium dodecyl sulfate sample buffer [1.5% sodium dodecyl sulfate, 10 mM Tris(hydroxymethyl)-aminomethane-HCl, pH 6.8, 2 mM ethylenediamine tetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 5% mercaptoethanol, 5 mg leupeptin, antipain, chymostain, and pepstain per ml (Sigma)], and homogenized in a glass homogenizer. The sample was boiled for 5 min and centrifuged at 15,000 × *g* for 30 min. The supernatant was frozen at −70°C until used for electrophoresis.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970) and electrophoretically transferred to a nitrocellulose membrane (Towbin *et al*, 1979). The membrane was cut into strips and incubated with patient's IgG and affinity-purified IgG and then with peroxidase-conjugated rabbit anti-human IgG (DAKO). The reaction was visualized with 4-chloro-1-naphthol (Sigma) in the presence of 0.025% H_2O_2.

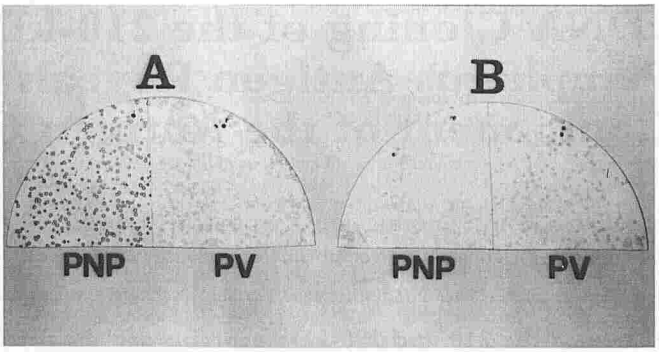

Figure 1. Immunoperoxidase staining of plaques of the purified λ gt 11 paraneoplastic pemphigus (PNP) clone *(A)* and keratinocyte cDNA library plaques *(B)* on nitrocellulose membranes. The PNP IgG react specifically with the PNP clone plaques but pemphigus vulgaris (PV) IgG does not react with the PNP clone plaques. Neither the PNP IgG nor the PV IgG react with the keratinocyte cDNA library plaques.

Purification of Positive Clone DNA Phage DNA was purified as decribed previously (Sambrook *et al*, 1989), and digested with *Eco*RI. The 1.4-kbp insert DNA was isolated from the agarose gel with Geneclean II kit (Bio 101, La Jolla, CA).

DNA Sequencing and Analysis cDNA inserts were subcloned into M13 mp 18 vectors. Single-stranded DNA was prepared and sequenced by a dye termination method (Smith *et al*, 1986) using ABI PRISM 377 DNA sequencer (Perkin-Elmer, Branchburg, NJ). The sequence data were analyzed using the PC Gene sequence analysis software package (Intelligenetics, Mountain View, CA). Homology searches were run against the GenBank.

RESULTS

Immunoscreening of a cDNA λ gt 11 Library Recombinant phages were plated with *E. coli* Y1090, and plaques were immunoscreened with IgG purified from a PNP patient's serum. Of 5×10^5 recombinant clones, one positive clone, called here the PNP clone, was further characterized.

To verify that the fusion protein produced by this PNP clone specifically bound antibodies in PNP sera, we purified the clone so that the immunoperoxidase staining of this clone could be compared with the background staining of other clones. Immunoblotting revealed that this fusion protein reacted specifically with the patient's IgG. No reaction occurred with IgG from pemphigus vulgaris and pemphigus foliaceus patients and normal individuals **(Fig 1)**. These results suggest that this PNP clone encodes a major antigenic site of the PNP antigen.

To further confirm that the antibodies binding to the products of this clone were typical PNP antibodies, in that they were capable of binding to the keratinocyte cell surfaces and could bind the 210-kDa PNP antigen on immunoblots, we affinity-purified the IgG that bound the purified PNP clone. The purified PNP clone on a nitrocellulose membrane was incubated with the PNP IgG. After extensive washing, bound PNP IgG was eluted with sodium citrate buffer **(Fig 2*A*)**. Indirect immunofluorescent staining showed that this affinity-purified IgG bound to the keratinocyte cell surface of epidermis **(Fig 2*Ba*)** but did not bind to other tissues that express DP such as urinary bladder or myocardium (data not shown), suggesting that the tissue distribution of the PNP antigen and DP is not identical. The IgG from the same PNP IgG similarly eluted from plagues of control λ gt 11 clones did not show staining in the epidermis **(Fig 2*Bb*)**. The staining of the affinity-purified IgG was more prominent in the upper spinous and granular layers **(Fig 2*Ba*)**.

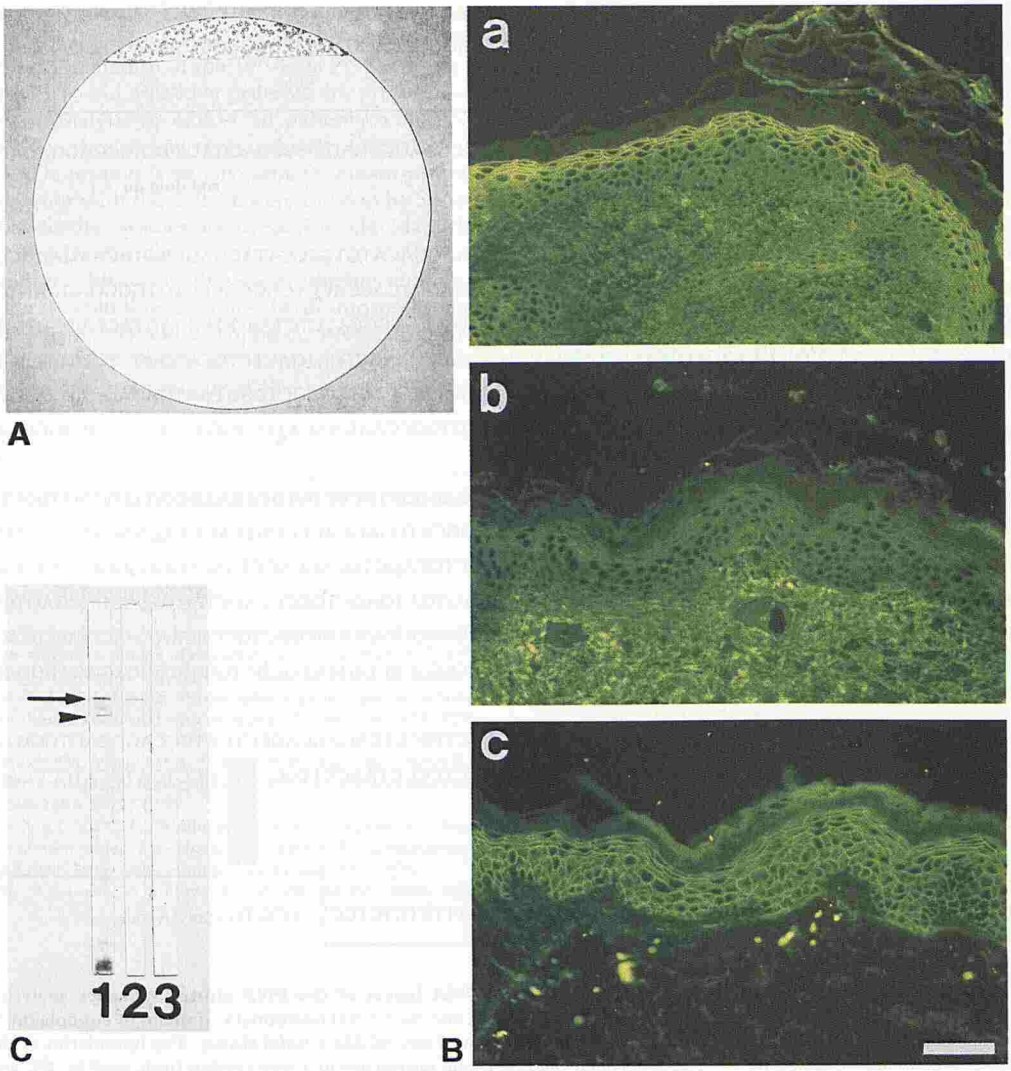

Figure 2. PNP IgG affinity-purified on plaques of the λ gt 11 PNP clone stains the keratinocyte cell surfaces by immunofluorescence and react with the 210-kDa PNP antigen. *(A)* Immunoperoxidase staining of plaques of the purified PNP clone on a nitrocellulose membrane. The membrane was incubated with PNP IgG and washed. A small piece of the top of the membrane was cut off and stained. The bound PNP IgG on the rest of the membrane was eluted with acid sodium citrate, then similarly stained. Much of the affinity-purified IgG was eluted, accounting for the less intense staining after elution. *(B)* Indirect immunofluorescence on normal skin with the PNP IgG affinity-purified on the plaques of the PNP clone shows staining of the keratinocyte cell surfaces *(a)*. IgG from the same PNP IgG similarly eluted from plaques of control λ gt 11 clones did not demonstrate this staining *(b)*. The staining of affinity-purified IgG is more prominent in the upper spinous and granular layers. In contrast, monoclonal antibody to DPI/II shows even staining throughout the epidermis *(c)*. *Scale bar*, 50 μm. *(C)* Immunoblot analysis of cultured keratinocytes extract demonstrates the PNP IgG *(lane 1)* react with both the 210 (→) and 190-kDa antigen (➤), and PNP IgG affinity purified on plaques of the PNP clone (lane 2) react with only the 210-kDa antigen, but PNP IgG affinity purified on plaques of control clones (lane 3) do not react with PNP antigen.

In contrast, an antibody specific for DPI/II (Boehringer Mannheim, Mannheim, Germany) showed even staining throughout the epidermis **(Fig 2*Bc*)**. These results suggest that expression of the PNP antigen increased during terminal differentiation of epidermis and that the staining pattern of PNP antigen and DP is different in epidermis. In addition, the affinity-purified IgG bound the 210-kDa PNP antigen extracted from cultured keratinocytes **(Fig 2*C*)**. These results confirm that the cDNA insert of the PNP λ gt 11 clone contains coding sequences for a major antigenic site of 210-kDa PNP antigen. *Eco*RI digestion of the λ gt 11 PNP clone revealed a cDNA insert of 1.4-kbp, as determined by agarose gel electrophoresis.

DNA Sequencing of the PNP cDNA Coding Region Sequence analysis revealed that the cDNA insert of the PNP clone encodes a part of the central rod domain and the COOH-terminal C domain of envoplakin (nucleotides 5028–6452). Of the 1425 nucleotides of the PNP cDNA, only four nucleotides are mismatched with the published sequence of envoplakin **(Fig 3)**. This result confirmed that the 210-kDa PNP antigen is identical to envoplakin, a novel precursor protein of the cornified envelope that is homologous to desmoplakin.

DISCUSSION

In order to further characterize the 210-kDa PNP antigen, we screened the human keratinocyte cDNA library with IgG from a PNP patient and isolated a cDNA clone that encodes a portion of the 210-kDa PNP antigen. Sequence analysis revealed that the isolated cDNA encodes a part of the central rod domain and the COOH-terminal C domain of envoplakin, which is a newly defined precursor protein of cornified envelope (Ruhrberg *et al*, 1996). This result also suggests that a major antigenic site of the envoplakin resides in the C domain of the COOH terminus.

In 1984, Simon and Green identified two membrane-associated proteins with apparent molecular masses of 195 and 210 kDa that

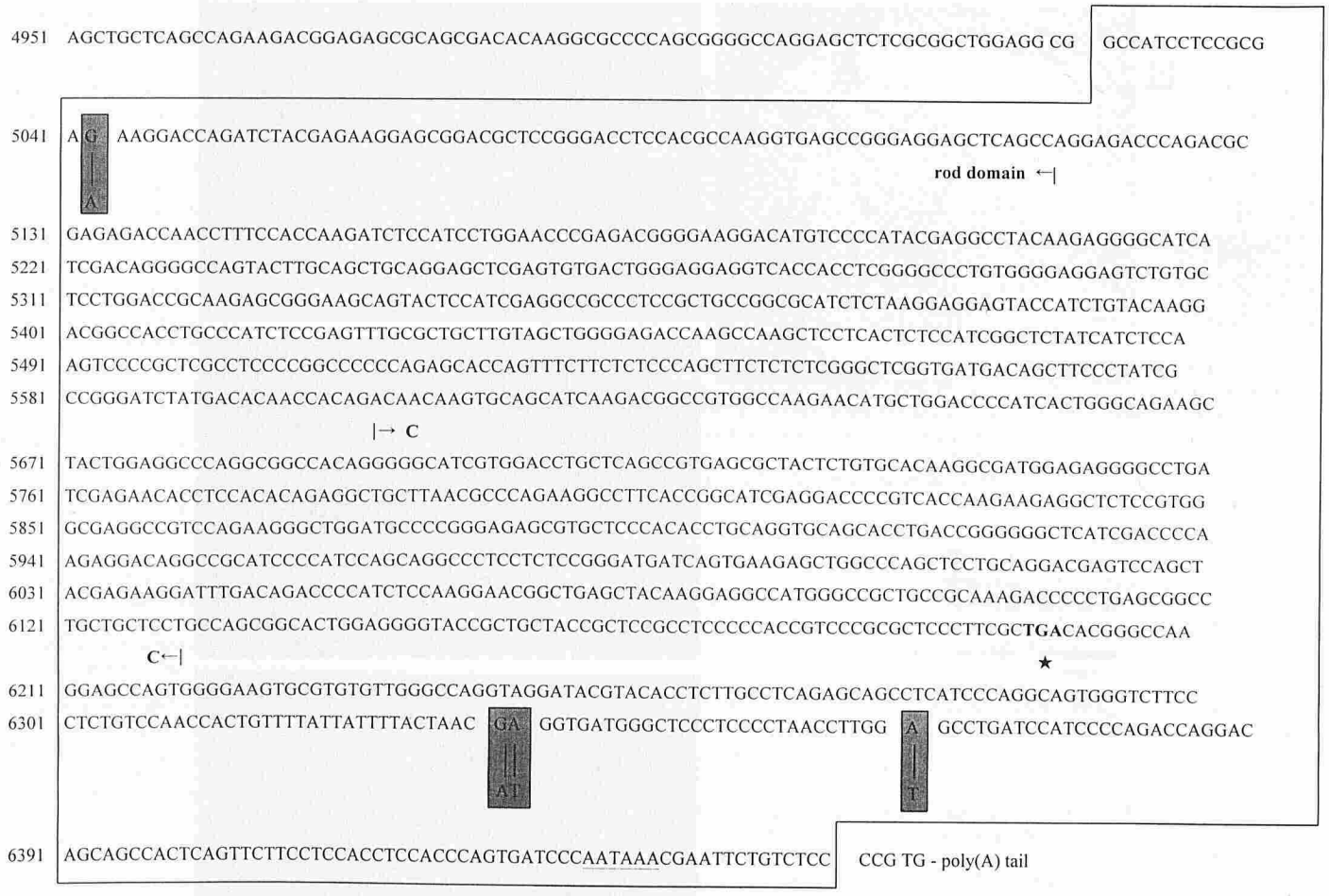

Figure 3. Nucleotide sequence of the envoplakin compared with the cDNA insert of the PNP clone. Sequence analysis has revealed that the cDNA insert of the PNP clone (*boxed area*) encodes a part of the central rod domain and the COOH-terminal C domain of envoplakin. Only four nucleotides are mismatched between the cDNA insert of the PNP clone and the published data of envoplakin (*shaded blocks*). The boundaries of the central rod domain, and the COOH-terminal C domain are indicated by →. The long open reading frame terminates in a stop cordon (indicated by ★), and the polyadenylation signal, AATAAA, is *underlined*. The sequence data of envoplakin are available from GenBank/EMBL/DDBJ under accession number U53786.

become incorporated into the cornified envelope upon transglutaminase activation. Antibodies to the two proteins were absorbed by isolated cornified envelopes. 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. In 1996, Ruhrberg *et al* reported the sequencing of overlapping cDNA clones encoding the 210-kDa cornified envelope precursor, which was identified previously by Simon and Green, and they proposed that the 210-kDa protein be named "envoplakin." The envoplakin was expressed in keratinizing and nonkeratinizing stratified squamous epithelia, but not in simple epithelia or nonepithelial cells. Immunolocalization analysis at the electron microscopic level indicates that both DP and envoplakin are found in the region of intermediate filament attachment in desmosomes (Ruhrberg *et al*, 1996). Sequence analysis showed that envoplakin is homologus to the keratin-binding proteins DP, BPAG 1, and plectin. The predicted structure of envoplakin, its homology with other known proteins, and its expression pattern strongly suggest that it is associated with plasma membrane and may link keratin filaments and desmosomes to the cornified envelope. Currently, DP, BPAG 1, plection, and envoplakin are defined as a new gene family of cell adhesion junction plaque proteins. These proteins are thought to share similar functions in that they are involved in the anchorage of cytoskeletons to plasma membrane.

Recently, a relationship between the diseases and the defects of these keratin-binding proteins has been described. A knockout mouse for BPAG 1 produced a phenotype of skin fragility and neuromuscular disease (Guo *et al*, 1995). Very recently, McLean *et al* (1996) reported that a mutation of the plectin gene led to the muscular dystrophy–epidermolysis bullosa simplex phenotype through loss of plectin protein expression. The role of the antibodies against these keratin-binding proteins in the pathogenesis of PNP, however, remains unknown at present. Autoantibodies of pemphigus can easily reach pemphigus antigen and cause acantholysis, since they are located in the desmosomal core spaces. In contrast, envoplakin and DP are located in the cytoplasmic plaques in desmosomes, and therefore it is less likely that autoantibodies against these proteins directly induce acantholytic blisters. It seems reasonable to suppose that instead of DP and envoplakin, other transmembrane proteins, possibly the 170-kDa protein and the pemphigus vulgaris antigen, have pathogenic roles in PNP. Oursler *et al* (1992) demonstrated that the 170-kDa protein is a transmembrane glycoprotein and suggested that it may play an important role in the pathogenesis of PNP. Recent immunoblot analysis demonstrated that some, if not all, PNP sera reacted with pemphigus vulgaris antigen, indicating that pemphigus vulgaris antigen may be involved in the pathogenesis of PNP (Joly *et al*, 1994; Hashimoto *et al*, 1995).

From previous immunoblot studies, it seems likely that there are two groups of PNP patients with different immune responses, one

group showing strong reactivity to DP I/II and the other showing strong reactivity to 210-kDa envoplakin and a 190-kDa protein (Hashimoto *et al*, 1995). The nature of the 190-kDa PNP antigen is still unknown. As the 210- and 190-kDa proteins are present as a doublet in immunoblot analysis in almost all patients with PNP (Hashimoto *et al*, 1995), they seem to be closely related. It is possible that the 190-kDa protein is an envelope precursor protein that was identified together with the 210-kDa envoplakin by Simon and Green (1984), but further studies are needed to elucidate the exact nature of the 190-kDa protein.

PNP is the autoimmune blistering disease producing autoantibodies against the keratin-binding proteins in desmosomes and hemidesmosomes, DP, BPAG 1, and envoplakin. PNP is the first human autoimmune disease in which autoantibodies against the envoplakin are a prominent component of the humoral autoimmune response.

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