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A Monoclonal Antibody against the Paraneoplastic Pemphigus (PNP) Antigen, Envoplakin: cDNA Sequences Encoding the Variable Regions of Heavy and Light Chains

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Paraneoplastic pemphigus (PNP) is an acquired autoimmune disorder characterized by severe mucosal erosion, and polymorphous cutaneous lesions associated with neoplasia. PNP patients have circulating autoantibodies that bind to stratified and nonstratified epithelia. Previously, we showed that envoplakin was a component of the antigen complex recognized by PNP sera. In the present study we generated a monoclonal antibody, EVP-YS, against human envoplakin. The antibody bound to keratinocyte cell surfaces and reacted with the 210-kDa PNP antigen, confirming its specificity for envoplakin. The variable regions of the heavy (H) and light (L) chain genes were cloned from the hybridoma and shown to belong to mouse H chain subgroup III and κ light chain subgroup V, respectively. The L chain of EVP-YS was 98% identical to the κ chains of some autoantibodies and anti-nucleic acid antibodies, and had an identical amino acid sequence in all three complementary determining regions, suggesting that the H chains determine the specificity of the EVP-YS-envoplakin interaction. The EVP-YS antibody can be used to evaluate the sensitivity and specificity of clinical, histological, and immunological criteria for diagnosing PNP.

Keywords: Envoplakin; Monoclonal Antibody; Paraneoplastic Pemphigus (PNP).

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Introduction

Paraneoplastic pemphigus (PNP) is an acquired autoimmune blistering disorder characterized by severe mucosal erosion, and polymorphous skin lesions in association with underlying neoplasia (Anhalt *et al.*, 1990; Horn and Anhalt, 1992; Oursler *et al.*, 1992). Circulating autoantibodies in PNP patients bind not only to the cell surface of stratified squamous epithelia but also to simple, columnar and transitional epithelia, and immunoprecipitate antigen complexes consisting of five polypeptides with molecular masses of 250, 230, 210, 190, and 170 kDa (Anhalt *et al.*, 1990). These autoantibodies are pathogenic, as demonstrated by passive transfer experiments (Anhalt *et al.*, 1990). We have previously shown that the 210 kDa PNP antigen is envoplakin, a member of the plakin family (Kim *et al.*, 1997). It is now clear that PNP autoantibodies recognize a number of plakin family proteins including plectin, desmoplakin, BP230, envoplakin and periplakin (Aho *et al.*, 1999; Mshoney *et al.*, 1998).

Plakin family proteins are expressed in tissues that experience mechanical stress, such as epithelia and muscle, where they play a vital role in maintaining tissue integrity by cross-linking cytoskeletal filaments and anchoring them to membrane complexes. Hence, both autoimmune and inherited diseases that affect plakins can lead to disorders characterized by tissue fragility and skin blistering (Leung *et al.*, 2002). Envoplakin is a component of the epidermal cornified envelope that is localized in the desmosomes of stratified epithelial cells. Its expression increases along with the differentiation of keratinizing stratified squamous epithelia (Simon and Green, 1984). In the present study, we developed a monoclonal antibody against human recombinant envoplakin and, using the hybridoma cells, we sequenced the envoplakin-specific variable regions of the heavy and light chain cDNAs. This monoclonal antibody can be used to characterize the envoplakin associated with PNP.

Materials and Methods

Preparation of glutathione (GST)-fused human envoplakin To produce antigen for generating monoclonal antibody against human envoplakin, a 1.4 kb *Eco*RI fragment of λ gt11 PNP DNA which contains coding sequences for part of the rod domain, the linker and the COOH-terminal C-domain of 210-kDa envoplakin (Kim *et al*., 1997) was inserted downstream of a cDNA encoding GST in pGEX4T-1 (Pharmacia). The resulting plasmid was transformed into *E. coli* DH5α, and the expressed GST-envoplakin was purified with GST Sepharose beads (Pharmacia). The recombinant envoplakin was digested with thrombin and extracted from SDS/PAGE by electro-elution followed by dialysis in Tris-buffered saline (pH 7.2) at 4°C overnight (Fig. 1).

Production of a monoclonal antibody to human envoplakin Ten mg of thrombin-treated envoplakin was mixed 1:1 with complete Freund's adjuvant (Sigma) in phosphate-buffered saline (PBS), and five mice were immunized intraperitoneally with this antigen. The immunized splenocytes were harvested and incubated with Sp2/0-Ag-14 myeloma cells in 50% PEG 4000 for 2 weeks. The supernatants of the hybridoma cell cultures were tested for anti-envoplakin antibody by enzyme-linked immunosorbant assay (ELISA). Cells giving positive signals were cloned through three rounds of selection by limiting dilution.

Competition ELISA The affinity of hybridoma-derived EVP-YS was evaluated by competition ELISA (Kim *et al*., 2001; Orfanoudakis *et al*., 1993). The amount of EVP-YS antibody giving half-maximal binding to envoplakin-coated plates was determined by ELISA by serial dilution of the hybridoma supernatant. For this purpose, recombinant envoplakin (10 µg) was dispensed in 96-well plates and incubated at 4°C for overnight. The plates were washed, and the wells blocked with 1% BSA in PBS containing 0.05% Tween (PBS-T) for 2 h at room temperature. After washing, 100 µl aliquots of serial dilutions of the hybridoma culture supernatant were incubated for 2 h at room temperature. Following washing with PBS-T, rabbit anti-mouse IgG-peroxidase conjugate (DAKO) was added for 1 h. After washing, 100 µl of ABTS (KPL, MD, USA) was added to each well, and the O.D. was measured at 405 nm. For competition ELISA, serially diluted envoplakin was mixed with an equal volume of twice the concentration of EVP-YS antibody giving half-maximal binding, and incubated for 2 h at room temperature. The envoplakin-bound EVP-YS antibody solution was added the envoplakin- or periplakin-coated plates, and the rest of the assay was as described above. The envoplakin concentration giving 50% inhibition of maximum binding represents the inhibition constant, *Ki*.

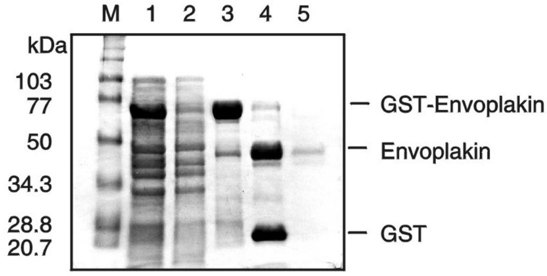

Fig. 1. Purification of recombinant envoplakin by Glutathione-Sepharose column chromatography. Lane 1, total *E. coli* lysate; lane 2, flow-through following binding of the lysate to Glutathione-Sepharose; lane 3, GST-Envoplakin following elution with reduced glutathione; lane 4, thrombin-digested GST-Envoplakin; lane 5, the electro-eluted envoplakin used to immunize mice.

Immunofluorescence microscopy Immunofluorescence microscopy was performed essentially as described (Bahn *et al.*, 2002). Frozen sections (5 µm) of neonatal foreskin were air-dried and incubated in PBS for 5 min, and then for 2 h at room temperature in PBS containing 10-fold diluted monoclonal anti-envoplakin EVP-YS antibody. The sections were washed three times in PBS and incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (DAKO) for 1 h at room temperature, with three subsequent washes in PBS. Sections were mounted in glycerol/PBS solution and examined with a fluorescence microscope.

Immunoblot analysis Immunoblotting was performed as described (Hashimoto *et al.*, 1990). In brief, cultured human keratinocytes were lysed in SDS sample buffer [1.5% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 6.8, 2 mM ethylenediamine tetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 5% mercaptoethanol, 5 mg leupeptin, antipain, chymostain, and pepstain (Sigma) per ml], and homogenized with a glass homogenizer. Samples were boiled for 5 min and centrifuged at 15,000 × *g* for 30 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes that were cut into strips and incubated with patients' IgG or monoclonal anti-envoplakin antibody and then with peroxidase-conjugated rabbit anti-human or anti-mouse IgG (DAKO). Reactions were visualized with 4-chloro-1-naphthol (Sigma) in the presence of 0.025% H_2O_2.

Cloning and sequencing of the envoplakin-specific variable regions of heavy (V_H) and light (V_L) chain cDNAs Total RNA was extracted from 1 × 10^7 hybridoma cells using Tri-reagent (Molecular, Cincinnati) and first-strand cDNA was made in 20 µl reaction mixtures in reverse transcriptase buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT], with 5 µg total RNA and Moloney murine leukemia virus reverse transcriptase, 250 ng random primers and Rnasin (Promega). The cDNA of the V_H gene was PCR-amplified with a reverse Cγ primer complementary to the sequence of the constant region of the γ (Cγ) chain gene, and forward H1-3 primers complementary

Table 1. Primers used for cloning EVP-YS V_L and V_H genes.

Name	Sequence (5′ → 3′)	Groups
Cκ	CCAGGGGCCAGTGGATAGACAAGCTTGGGTGTCGTTT	IgG L-chain constant
Cγ	AAGATGGATCCAGTTGGTGCAGCATCAGC	IgG H-chain constant
H1	(C/G)CAGCTGCAG(C/G)AGTC(A/T)GG	IgG H-chain (IB, IIA)*
H2	(C/G)(A/C)AACTGCAG(C/G)AGTC(A/T)GG	IgG H-chain (IIA, B, C, VA)*
H3	(C/G)(A/C)AGCTGCAG(C/G)AGTC(A/T)GG	IgG H-chain (IIIA, C, D)*
L1	GACATTGTGATG(A/T)C(A/T)CAGTCTCCA	IgG L-chain (I)*
L2	GA(G/C)AGGTGCAGCT(T/G)(C/A)AGGAGTCAGGA	IgG L-chain (III)*
L3	(A/G)A(A/C)ATTGTGCTGAC(A/C)CA(A/G)TCTCC(A/T)	IgG L-chain (V)*

* Indicates mouse H- and L-chain groups as described by Kabat *et al.* (1991).

to the DNA sequences of the N-termini of heavy γ chain genes (Table 1). The cDNA of the V_L gene was amplified with a Cκ reverse primer and L1-3 forward primers complementary to the DNA sequences of the N-termini of light κ chains under the same conditions as used for the V_H genes. The PCR products were cloned into pBluescript KS(+) (Stratagene) and sequenced by the dideoxy chain termination method.

Results and Discussion

To produce monoclonal antibodies specific for human envoplakin, we expressed a GST-fusion of envoplakin in bacteria, and electro-eluted it from SDS-PAGE. The renatured recombinant protein was used as antigen to immunize BALB/c mice (Fig. 1), and the splenocytes obtained from an envoplakin-immune mouse were fused with myeloma SP2/0 cells. Of the monoclonal antibodies produced, EVP-YS bound to purified envoplakin with high affinity (data not shown). Figure 2A shows that a 1:20 dilution of the culture supernatant of the EVP-YS hybridoma recognized recombinant envoplakin but not periplakin. Competition ELISA showed that the envoplakin concentration giving 50% inhibition of maximum binding was 2.2×10^{-8} M (Fig. 2B). Periplakin did not compete with envoplakin for binding. Because the EVP-YS hybridoma was generated using a non-natural form of the antigen, we assessed whether the antibody recognized endogenous envoplakin expressed on human keratinocytes. Indirect immunofluorescence assays showed that the antibody bound to the cell surface of the keratinocytes of human skin. Staining with the antibody was more prominent in the upper spinous and granular layers, just as it is with affinity-purified IgG from PNP patients (Kim *et al.*, 1997) (Fig. 3A). Immunoblot analysis of proteins extracted from cultured keratinocytes revealed that EVP-YS bound to the 210-kDa PNP antigen (Fig. 3B, lane indicated by *EVP-YS*). We also showed that monoclonal antibody against periplakin (PRP-YS) recognized a major 190-kDa molecular weight species. Both envoplakin and periplakin are PNP antigens that react strongly with PNP sera (Borradori *et al.*, 1998). Indeed, PNP patients' serum detected a characteristic doublet of 210-kDa and 190-kDa (Fig. 3B, lane indicated by *PNP serum*), corresponding to the envoplakin and periplakin bands detected by their respective monoclonal antibodies. These results indicate that EVP-YS is capable of binding to keratinocyte cell surfaces where it presumably binds to envoplakin.

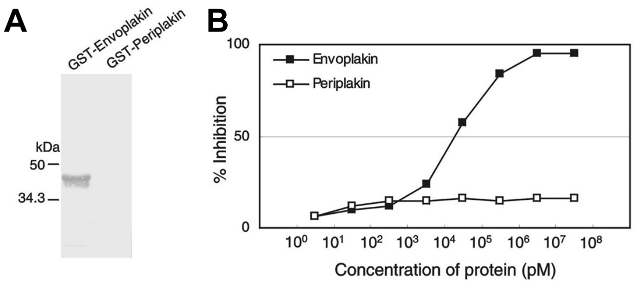

Fig. 2. The specificity of EVP-YS monoclonal antibody. **A.** Immunoblot analysis using a 1:20 dilution of the culture supernatant of EVP-YS hybridoma cells with GST-fused envoplakin and periplakin. **B.** Competition ELISA for determining the affinity of EVP-YS. See **Materials and Methods** for details. Competition data for envoplakin and periplakin against envoplakin-bound EVP-YS antibody are presented.

The V_H cDNA of monoclonal antibody EVP-YS was prepared from total RNA of EVP-YS hybridoma cells and cloned by RT-PCR with a reverse primer that hybridizes to the constant region (Cγ primer) and a forward primer H3 (Table 1). The PCR product, a DNA fragment of approximately 380 bp, was cloned into pBluescript. The nucleotide sequences and deduced amino acid sequences of the V_H region are shown in Fig. 4A. Comparison of the amino acid sequence of the EVP-YS V_H region with the sequence of 10 murine V_H groups indicates that the V_H segment of EVP-YS belongs to the subgroup III, according to Kabat's classification (Kabat *et al.*, 1991). PCR using a forward L3 primer and reverse Cκ primer was performed to clone the V_L cDNA of EVP-YS. The deduced amino acid sequence revealed a typical mouse variable region κ light chain (Fig. 4B), with 98% homology to

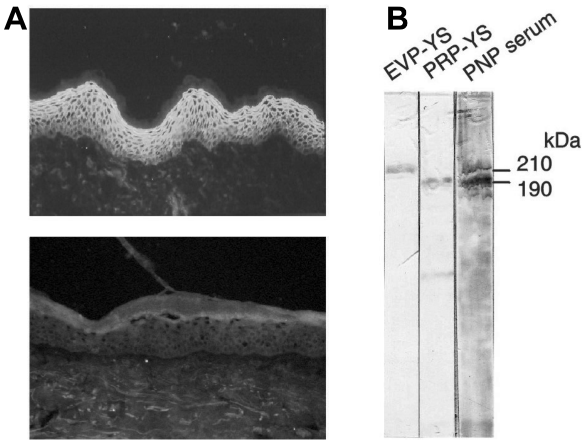

Fig. 3. EVP-YS monoclonal antibody stains the keratinocyte cell surface and reacts with the 210-kDa PNP antigen. **A.** Upper, indirect immunofluorescence analysis of normal skin shows staining of the keratinocyte surface. Staining is more prominent in the upper spinous and granular layer. Lower, background staining with secondary antibody conjugated with FITC. **B.** Immunoblot analysis of cultured keratinocyte extract. *EVP-YS*, monoclonal antibody against human envoplakin; *PRP-YS*, monoclonal antibody against periplakin; and *PNP serum*, PNP patients' serum.

A

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CTGCAGGAGTCAGGGGGAGGGTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCA  60
L  Q  E  S  G  G  G  L  V  K  P  G  G  S  L  K  L  S  C  A   20

GCCTCTGGATTCGCTTTCAGTAGCTATGACATGTCTTGGGTTCGCCAGACTCCGGAGGAG  120
A  S  G  F  A  F  S  S  Y  D  M  S  W  V  R  Q  T  P  E  E   40
                    CDR1
AGGCTGGAGTGGGTCGCATACATTAGTAGTGGTGGTGGTAGCACCTACTATCCAGACACT  180
R  L  E  W  V  A  Y  I  S  S  G  G  G  S  T  Y  Y  P  D  T   60
                                  CDR2
GTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTACCTGCAAATG  240
V  K  G  R  F  T  I  S  R  D  N  A  K  N  T  L  Y  L  Q  M   80

AGCAGTCTGAAGTCTGAGGACACAGCCATGTATTACTGTGCAAGACAGGGGAGGTACGAC  300
S  S  L  K  S  E  D  T  A  M  Y  Y  C  A  R  Q  G  R  Y  D   100

GGGGTTTACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCT  360
G  V  Y  Y  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S  A   120
  CDR3
AAAACGCACCCAAGCTTA
K  T  H  P  S  L
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B

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TCTAGAAACATTGTGCTGACCCAGTCTCCTGCTTCCTTAGCTGTATCTCTGGGGCAGAGG  60
S  R  N  I  V  L  T  Q  S  P  A  S  L  A  V  S  L  G  Q  R   20

GCCACCATCTCATACAGGGCCAGCAAAAGTGTCAGTACATCTGGCTATAGTTATATGCAC  120
A  T  I  S  Y  R  A  S  K  S  V  S  T  S  G  Y  S  Y  M  H   40
                         CDR1
TTGAACCAACAGAAACCAGGACAGCCACCCAGACTCCTCATCTATCTTGTATCCAACCTA  180
L  N  Q  Q  K  P  G  Q  P  P  R  L  L  I  Y  L  V  S  N  L   60
                                              CDR2
GAATCTGGGGTCCCTGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACCCTCAAC  240
E  S  G  V  P  A  R  F  S  G  S  G  S  G  T  D  F  T  L  N   80

ATCCATCCTGTGGAGGAGGAGGATGCTGCAACCTATTACTGTCAGCACATTAGGGAGCTT  300
I  H  P  V  E  E  E  D  A  A  T  Y  Y  C  Q  H  I  R  E  L   100
                                             CDR3
ACACGTTCGGAGGGGAACCAAGCTGGAAATAAAACGAGC
T  R  S  E  G  D  Q  A  G  N  K  T  S
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Fig. 4. Nucleotide sequence of the EVP-YS variable region gene and the deduced amino acid sequence. Complementary determining regions (CDRs) are underlined. (A) V_H gene and (B) V_L gene.

the light chains of autoantibodies and anti-nucleic acid antibodies, and with identical amino acid sequences in complementary determining region (CDR)-1, -2 and -3 (Brown *et al.*, 1998). Other light chains of antibodies such as anti-keratin, anti-p53 and anti-estrogen receptor, have also shown homology to sequences in GenBank. Please explain--editor. Since the EVP-YS light chain does not have a unique specificity for envoplakin, its specificity is probably determined by its heavy chains.

PNP is an autoimmune blistering disease characterized by the production of autoantibodies mainly directed against proteins of the plakin family, which include envoplakin and periplakin. Although autoantibody specificities overlap between the different types of pemphigus such as PNP, pemphigus vulgaris (PV) and pemphigus foliaceus (PF), the detection of envoplakin by immunoblotting is both a sensitive and specific feature of PNP (Joly *et al.*, 2000). Thus, we believe that monoclonal antibody EVP-YS will be useful for evaluating the sensitivity and specificity of clinical, histological, and immunological criteria for the diagnosis of PNP.

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