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Epiplakin, a Novel Member of the Plakin Family Originally Identified as a 450-kDa Human Epidermal Autoantigen

STRUCTURE AND TISSUE LOCALIZATION*

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A 450-kDa human epidermal autoantigen was originally identified as a protein that reacted with the serum from an individual with a subepidermal blistering disease. Molecular cloning of this protein has now shown that it contains 5065 amino acids and has a molecular mass of 552 kDa. As reported previously this protein, which we call epiplakin, belongs to the plakin family, but it has some very unusual features. Epiplakin has 13 domains that are homologous to the B domain in the COOH-terminal region of desmoplakin. The last five of these B domains, together with their associated linker regions, are particularly strongly conserved. However, epiplakin lacks a coiled-coil rod domain and an amino-terminal domain, both of which are found in all other known members of the plakin family. Furthermore, no dimerization motif was found in the sequence. Thus, it is likely that epiplakin exists *in vivo* as a single-chain structure. Epitope mapping experiments showed that the original patient's serum recognized a sequence unique to epiplakin, which was not found in plectin. Immunofluorescence staining revealed the presence of epiplakin in whole sheets of epidermis and esophagus, in glandular cells of eccrine sweat and parotid glands and in mucous epithelial cells in the stomach and colon.

Clarification of the basic structure of desmoplakin has been followed by the identification of many related proteins, such as BPAG1,[1] plectin, envoplakin, and periplakin (1–7). These proteins form a family known as the "plakin family" (8). Almost all members of this family have a common structure, with predicted globular amino-terminal and COOH-terminal domains that are separated by a central rod domain. Some homologous domain structures have been identified in both globular domains of many plakins, while the central domain is rich in heptad repeats and is believed to form a parallel α-helical coiled-coil structure with a dimerization partner (9). As suggested by this model, it has been demonstrated that desmoplakin I can form homodimers *in vitro* (10). Early investigations revealed that the COOH-terminal domains of plakins are involved in binding to intermediate filaments (11–13). The amino-terminal domains of desmoplakin and of BPAG1 are believed to bind to desmosomes or hemidesmosomes. Furthermore, some splicing variants of plectin and BPAG1 have actin- or microtubule-binding domains at their amino termini, and it has been proposed that these domains form cross-links between microfilaments and/or microtubules and intermediate filaments (14–16). Studies of a few inheritable diseases that appear to involve plectin or desmoplakin and of a BPAG1 null mouse have shown that each plakin plays a critical role in the tissue integrity in specific tissues (5, 17–19). Moreover, it seems likely that, in many autoimmune blistering diseases, plakins, located in the epidermis, might be target antigens, and these plakins are used for markers of specific diseases (2, 20–22). However, their pathological roles remain to be clarified.

Several years ago we described an individual with a subepidermal blistering disease that resembled bullous pemphigoid both clinically and pathologically (23). Immunoblot analysis revealed that the patient's serum did not react with the 230-kDa (BPAG1) and 180-kDa bullous pemphigoid antigens, whereas it did recognize a 450-kDa epidermal polypeptide. This polypeptide was expressed in human keratinocytes and in some transformed cell lines that included HeLa, KB, and A431 cells (24). In a preliminary study, to determine the molecular structure of this antigen, we screened a human keratinocyte cDNA library with the patient's serum. We isolated two kinds of cDNA: one encoding a protein that was strongly homologous to rat plectin and another encoding a protein, with partial homology to plectin, which appeared to be a novel and previously unidentified protein (25).

We report here the cloning of the cDNA and determination of the entire structure of the novel protein, which we compare with other members of the plakin family. We also demonstrate the tissue localization of this protein, as determined by Northern blotting and immunostaining. In our previous study, it was unclear whether the epitope that was recognized by our patient's serum was a sequence that is present within plectin itself. In this study, therefore, we also identified the major epitope of the protein.

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number AB051895.

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[1] The abbreviations used are: BPAG1, bullous pemphigoid antigen 1; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; ELISA, enzyme linked immunosorbent assay; GST, glutathione *S*-transferase; bp, base pair(s); BSA, bovine serum albumin; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline plus Tween 20.

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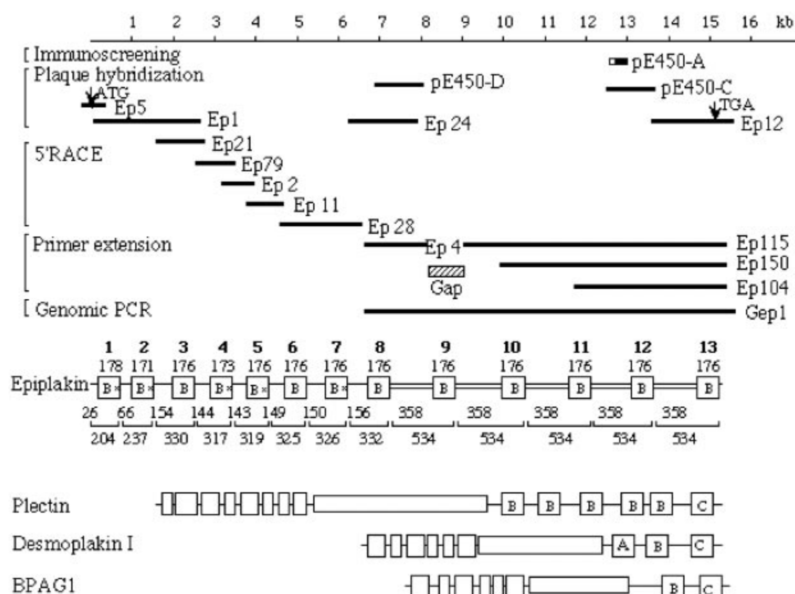

FIG. 1. **Cloning strategy for the isolation and sequencing of the human epiplakin cDNA and schematic representation of the deduced 552-kDa protein and comparisons with related members of the plakin family (9, 33).** The cDNAs isolated in this study allowed identification of a 15,195-bp ORF, which corresponds to contiguous cDNA sequences. Three previously isolated cDNAs, pE450-A (obtained by immunoscreening), pE450-C, and pE450-D (obtained by plaque hybridization), are also shown (25). The latter two clones overlapped correctly with part of the five repetitive region, but their position could not be determined. pE450-A was a hybrid clone that contained an irrelevant region of 78 bp (*box* adjacent to the *bold line*) at the 5′-terminal end. The gap of 760 bases in the message (*shaded box*) was covered by genomic sequence. The *numbers* at the *top* are domain numbers; *numbers* just above the *boxes* are the amino acid residue numbers of the domains; and *numbers* beneath the *lines* are the residue numbers of the linker regions; the *numbers* under the *line* below the *boxes* are the length of the separations between domains (in amino acid residues). The B domains in epiplakin are 70% homologous to the B domain in desmoplakin, and the B* domains are 46–49% homologous to the B domain in desmoplakin (see Table I). *Double-lined* linker regions and the five B domains (9, 10, 11, 12 and 13) to their right are almost perfectly identical (see Fig. 3*A*). A coiled-coil rod domain and an amino-terminal domains are shown as *open boxes*. Both are found in plectin, desmoplakin I, and BPAG1, but they are not found in epiplakin (33). The domain structures of the COOH-terminal ends of the three plakins are shown as *boxes* with *letters A*, *B*, or *C*.

EXPERIMENTAL PROCEDURES

Isolation of cDNA Clones and DNA Sequencing—A cDNA library prepared from HeLa cells (CLONTECH) was screened with the inserts of clones pE450-C and pE450-D, which had been isolated by extension cloning, using the same library, after the immunoscreening described in a previous report (25).

For isolation of the 3′ end repeats, we constructed a cDNA library from the poly(A)$^+$ RNA of HeLa cells. cDNA was synthesized with a cDNA synthesis kit (Life Technologies, Inc.). We modified the procedure provided with the kit by adding trehalose to the reaction for first-strand synthesis (26) and, as specific primers for first-strand synthesis, we used 5′-CCAGACACAACAAGTATGCC-3′ for clone Ep115 and 5′-TAGCGCTTGACCGAGTCCATC-3′ for clone Ep 4. The cDNAs were ligated to an *Eco*RI adapter and then inserted into the *Eco*RI site of pUC 18 (Amersham Pharmacia Biotech) for construction of plasmid libraries. Each library was screened with the specific probe. To confirm the size of 3′ end repetitive region and cover a small gap of the message, genomic polymerase chain reaction (PCR) was performed using primers 5′-TCGAGAAGCAGGAAACCA-3′ and 5′-CCATATGACACATAGACGAC-3′.

To obtain some 5′-upstream cDNA sequences, we performed 5′-RACE (rapid amplification of cDNA ends) using the RACE System (Life Technologies) and total RNA from HeLa cells or the Marathon-Ready cDNAs System with adaptor-ligated double-stranded cDNA prepared from poly(A)$^+$ RNA from HeLa cells (CLONTECH). The products of RACE were cloned into the pGEM-T Easy vector (Promega).

Each cDNA clone and genomic clone of interest was sequenced by a double-strand strategy with an automatic DNA sequencer (Applied Biosystems). Internal primers were constructed for analysis of internal sequences.

Analysis of the Amino Acid Sequence—We searched various protein data bases, including Swiss-Prot and PIR, using the BLAST routine available from the National Center for Biotechnology Information (Bethesda, MD). Furthermore, we also made comparisons using the COMPARE and DOTPLOT programs available from the University of Wisconsin Genetics Computer Group (Madison, WI). We also used the MATCH routine to identify regions of sequence homology among proteins. Determinations of plausible secondary structure were made using several predictive techniques and results are presented here only in a preliminary form. Short regions of heptad substructure were delineated by hand rather than by direct computer analysis since the former method facilitated location of discontinuities in heptad phasing. Potential interchain ionic interactions between charged residues at positions 2d′-1g, 1g′-2e, 2a′-1g, 1g′-2a, 1e′-1d, and 1d′-1e were considered as a function of relative chain stagger and chain polarity (27, 28). The notation 2d′-1g means that the residue at position d of the second heptad of chain n′ interacts with that in position g of the first heptad of chain n.

Synthesis of Overlapping Peptides—Overlapping peptides were synthesized on derivatized cellulose membranes with Fmoc (9-fluorenylmethoxycarbonyl) amino acids according to the protocol from the manufacturer of the system (Auto spot robot ASP222; ABIMED Analysen-Technik GmbH, Langenfeld, Germany) (29, 30). The ASP222 software program was used to generate the amino acid sequences of decapeptides and the spotting schedule for each cycle of addition of an amino acid. Peptides spanning amino acid residues 2807–3337 of epiplakin were synthesized on cellulose membranes as a series of decapeptides with an overlap of eight amino acids. This region includes one of five highly conserved COOH-terminal repeats. The first cDNA clone that we obtained by immunoscreening encoded a part of this domain. To refine the definition of the epitope within the sequence LVPAKDQPGRQEKMSIYQAMWKGVLRPGT, we synthesized a series of decapeptides with an overlap of nine amino acids were synthesized on cellulose membranes. Enzyme immunoassays of the peptides on the cellulose membranes were performed with our patient's serum, alkaline phosphatase-conjugated antibodies against human IgG, and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. All reagents were purchased from Bio-Rad.

Chemical Synthesis of Peptides—The target peptides, namely peptide A (MSIYQAMWKGVLC), peptide B (TKGFFDPNTHENC), and peptide C (VKRYLEGTSCIAGVLVP), were synthesized by a solid-phase method (31). Peptide chains were elongated using an automated peptide synthesizer (model 9050; Millipore/Biosearch, Marlborough, MA) according to the standard operating programs. Peptides were purified by high-performance liquid chromatography on a reverse-phase column (Delta PakC18; Waters) with a Multisolvent Delivery System (600E; Waters). Peptides were eluted with a linear gradient from water to acetonitrile in 0.1% trifluoroacetic acid. The molecular mass and amino acid sequence of each purified peptide were confirmed by determinations of the matrix-assisted laser desorption ionization time of flight

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MAATLGAGTP PRPQARSIAG VYVEASGQAQ SVYAAMEQGL LPAGLGQALL EAQAATGGLV DLARGQLLPV     70
                   ←1B*
SKALQQGLVG LELKEKLLAA ERATTGYPDP YGGEKLALFQ AIGKEVVDRA LGQSWLEVQL ATGGLVDPAQ    140
GVLVAPEPAC HQGLLDRETW HKLSELEPGT GDLRFLNPNT LERLTYHQLL ERCVRAPGSG LALLPLKITF    210
                                                                  1B*→
RSMGGAVSAA ELLEVGILDE QAVQGLREGR LAAVDVSARA EVRRYLEGTG SVAGVVLLPE GHKKSFFQAA    280
                                                                 ← 2B*
TEHLLPMGTA LPLLEAQAAT HTLVDPITGQ RLWVDEAVRA GLVSPELHEQ LLVAEQAVTG HHDPFSGSQI    350
PLFQAMKKGL VDRPLALRLL DAQLATGGLV CPARRLRLPL EAALRCGCLD EDTQRQLSQA GSFSDGTHGG    420
LRYEQLLALC VTDPETGLAF LPLSGGPRGG EPQGPPFIKY STRQALSTAT ATVSVGKFRG RPVSLWELLF    490
                 2B*→
SEAISSEQRA MLAQQYQEGT LSVEKLAAEL SATLEQAAAT ARVTFSGLRD TVTPGELLKA EIIDQDLYER    560
LEHGQATAKD VGSLASAQRY LQGTGCIAGL LLPGSQERLS IYEARCKGLL RPGTALILLE AQAATGFIID    630
                                  ←3B
PKANKGHSVE EALRAAVIGP DVFAKLLSAE RAVTGYTDPY TGQQISLFQA MQKGLIVREH GIRLLEAQIA    700
TGGVIDPVHS HRVPVDVAYR RGYFDQMLNL ILLDPSDDTK GFFDPNTHEN LTYLQLLERC VRDPETGLYL    770
                                                                         3B
LPLSSTQSPL VDSATQQAFQ NLLLSVKYGR FQGQRVSAWE LINSEYFSEG RRRQLLRRYR QREVTLGQVA    840
→
KLLEAETQRQ ADIMLPALRS RVTVHQLLEA GIIDQQLLDQ VLAGTISPEA LLLMDGVRRY LCGLGAVGGV    910
RLLPSGQRLS LYQAMRQKLL GPRVALALLE AQAATGTIMD PHSPESLSVD EAVRRGVVGP ELYGRLKRAE    980
    ←4B*
GAIAGFRDPF SGKQVSVFQA MKKGLIPWEQ AARLLEAQVA TGGIIDPTSH HHLPMPVAIQ RGYVDQEMET   1050
ALSSSSETFP TPDGQGRTSY AQLLEECPRD ETSGLHLLPL PESAPALPTE EQVQRSLQAV PGAKDGTSLW   1120
                                   4B*→
DLLSSCHFTE EQRRGLLEDV QEGRTTVPQL LASVQRWVQE TKLLAQARVM VPGPRGEVPA VWLLDAGIIT   1190
QETLEALAQG TQSPAQVAEQ PAVKACLWGT GCVAGVLLQP SGAKASIAQA VRDGLLPTGL GQRLLEAQVA   1260
                                             ←5B*
SGFLVDPLNN QRLSVEDAVK VGLVGRELSE QLGQAERAAA GYPDPYSRAS LSLWQAMEKG LVPQNEGLPL   1330
LQVQLATGGV VDPVHGVHLP QAAACRLGLL DTQTSQVLTA VDKDNKFFFD PSARDQVTYQ QLRERCVCDS   1400
ETGLLLLPLP SDTVLEVDDH TAVALRAMKV PVSTGRFKGC SVSLWDLLLS EYVGADKRRE LVALCRSGRA   1470
  5B*→
AALRQVVSAV TALVEAAERQ PLQATFRGLR KQVSARDLFR AQLISRKTLD ELSQGTTTVK EVAEMDSVKR   1540
SLEGGNFIAG VLIQGTQERM SIPEALRRHI LRPGTALVLL EAQAATGFII DPAENRKLTV EEAFKAGMFG   1610
                  ←6B
KETYVKLLSA ERAVTGYTDP YTGQQISLFQ AMQKDLIVRE HGIRLLEAQI ATGGIIDPVH SHRVPVDVAY   1680
RCGYFDEEMN RILADPSDDT KGFFDPNTHE NLTYLQLLER CVEDPETGLY LLQIIKKGEN YVYINEATRH   1750
                                                    6B→
VLQSRTAKMR VGRFADQVVS FWDLLSSPYF TEDRKRELIQ EYGAQSGGLE KLLEIITTTI EETETQNQGI   1820
KVAAIRGEVT AADLFNSRVI DQKTLHTLRV GRTGGQALST LECVKPYLEG SDCIAGVTVP STREVMSLHE   1890
                                                                   ←7B*
ASRKELIPAA FATWLLEAQA ATGFLLDPCT RQKLSVDEAV DVGLVNEELR ERLLKAERAA TGYRDPATGD   1960
TIPLFQAMQK QLIEKAEALR LLEVQVATGG VIDPQHHHRL PLETAYRRGC LHKDIYALIS DQKHMRKRFV   2030
DPNTQEKVSY RELQERCRPQ EDTGWVLFPV NKAARDSEHI DDETRRALEA EQVEITVGRF RGQKPTLWAL   2100
                        7B*→
LNSEYVTEEK KLQLVRMYRT HTRRALQTVA QLILELIEKQ ETSNKHLWFQ GIRRQITASE LLSSAIITEE   2170
MLQDLETGRS TTQELMEDDR VKRYLEGTSC IAGVLVPAKD QPGRQEKMSI YQAMWKGVLR PGTALVLLEA   2240
                                               ←8B
QAATGFVIDP VRNLRLSVEE PVPAGVVGSE IQEKLLSAER AVTGYTDPYT GQQISLFQAM QKDLIVREHG   2310
IRLLEAQIAT GGVIDPVHSH RVPVDVAYRR GYFDEEMNRV LADPSDDTKG FFDPNTHENL TYVQLLRRCV   2380
         ↓
PDPDTGLYML QLAGRGSAVH QLSEELRCAL RDARVTPGSG ALQGQSVSVW ELLFYREVSE DRRQDLLSRY   2450
    8B→
RAGTLTVEEL GATLTSLLAQ AQAQARAEAE AGSPRPDPRE ALRAATMEVK VGRLRGRAVP VWDVLASGYV   2520
SRAAREELLA EFGSGTLDLP ALTRRLTAII EEAEEAPGAR PQLQDARRGP REPGPAGRGD GDSGRSQREG   2590
QGEGETQEAA AAAAAARRQE QTLRDATMEV QRGQFQGRPV SVWDVLFSSY LSEARRDELL AQHAAGALGL   2660
PDLVAVLTRV IEETEERLSK VSFRGLRRQV SASELHTSGI LGPETLRDLA QGTKTLQEVT EMDSVKRYLE   2730
GTSCIAGVLV PAKDQPGRQE KMSIYQAMWK GVLRPGTALV LLEAQAATGF VIDPVRNLRL SVEEAVAAGV   2800
               ←9B
VGGEIQEKLL SAERAVTGYT DPYTGQQISL FQAMQKDLIV REHGIRLLEA QIATGGVIDP VHSHRVPVDV   2870
                                                          ↓
AYRRGYFDEE MNRVLADPSD DTKGFFDPNT HENLTYVQLL RRCVPDPDTG LYMLQLAGRG SAVHQLSEEL   2940
                                                          9B→
RCALRDARVT PGSGALQGQS VSVWELLFYR EVSEDRRQDL LSRYRAGTLT VEELGATLTS LLAQAQAQAR   3010
AEAEAGSPRP DPREALRAAT MEVKVGRLRG RAVPVWDVLA SGYVSGAARE ELLAEFGSGT LDLPALTRRL   3080
TAIIEEAEEA PGARPQLQDA WRGPREPGPA GRGDGDSGRS QREGQGEGET QEAAAAAAAA RRQEQTLRDA   3150
TMEVQRGQFQ GRPVSVWDVL FSSYLSEARR DELLAQHAAG ALGLPDLVAV LTRVIEETEE RLSKVSFRGL   3220
RRQVSASELH TSGILGPETL RDLAQGTKTL QEVTEMDSVK RYLEGTSCIA GVLVPAKDQP GRQEKMSIYQ   3290
                                                                   ←10B
AMWKGVLRPG TALVLLEAQA ATGFVIDPVR NLRLSVEEAV AAGVVGGEIQ EKLLSAERAV TGYTDPYTGQ   3360
QISLFQAMQK DLIVREHGIR LLEAQIATGG VIDPVHSHRV PVDVAYRRGY FDEEMNRVLA DPSDDTKGFF   3430
                              ↓
DPNTHENLTY VQLLRRCVPD PDTGLYMLQL AGRGSAVHQL SEELRCALRD ARVTPGSGAL QGQSVSVWEL   3500
                          10B→
LFYREVSEDR RQDLLSRYRA GTLTVEELGA TLTSLLAQAQ AQARAEAEAG SPRPDPREAL RAATMEVKVG   3570
RLRGRAVPVW DVLASGYVSG AAREELLAEF GSGTLDLPAL TRRLTAIIEE AEEAPGARPQ LQDAWRGPRE   3640
PGPAGRGDGD SGRSQREGQG EGETQEAAAA AAAARRQEQT LRDATMEVQR GQFQGRPVSV WDVLFSSYLS   3710
EARRDELLAQ HAAGALGLPD LVAVLTRVIE ETEERLSKVS FRGLRRQVSA SELHTSGILG PETLRDLAQG   3780
TKTLQEVTEM DSVKRYLEGT SCIAGVLVPA KDQPGRQEKM SIYQAMWKGV LRPGTALVLL EAQAATGFVI   3850
                                      ←11B
DPVRNLRLSV EEAVAAGVVG GEIQEKLLSA ERAVTGYTDP YTGQQISLFQ AMQKDLIVRE HGIRLLEAQI   3920
ATGGVIDPVH SHRVPVDVAY RRGYFDEEMN RVLADPSDDT KGFFDPNTHE NLTYVQLLRR CVPDPDTGLY   3990
 ↓                                                                      11B
MLQLAGRGSA VHQLSEELRC ALRDARVTPG SGALQGQSVS VWELLFYREV SEDRRQDLLS RYRASTLTVE   4060
→
ELGATLTSLL AQAQAQARAE AEAGSPRPDP REALRAATME VKVGRLRGRA VPVWDVLASG YVSRAAREEL   4130
LAEFGSGTLD LPALTRRLTA IIEEAEEAPG ARPQLQDAWR GPREPGPAGR GDGDSGRSQR EGQGEGETQE   4200
AAAATAAARR QEQTLRDATM EVQRGQFQGR PVSVWDVLFS SYLSEARRDE LLAQHAAGAL GLPDLVAVLT   4270
RVIEETEERL SKVSFRGLRR QVSASELHTS GILGPETLRD LAQGTKTLQE VTEMDSVKRY LEGTSCIAGV   4340
LVPAKDQPGR QEKMSIYQAM WKGVLRPGTA LVLLEAQAAT GFVIDPVRNL RLSVEEAVAA GVVGGEIQEK   4410
            ←12B
LLSAERAVTG YTDPYTGQQI SLFQAMQKDL IVREHGIRLL EAQIATGGVI DPVHSHRVPV DVAYRRGYFD   4480
                                                ↓
EEMNRVLADP SDDTKGFFDP NTHENLTYVQ LLRRCVPDPD TGLYMLQLAG RGSAVHQLSE ELRCALRDAR   4550
                                              12B→
VTPGSGALQG QSVSVWELLF YREVSEDRRQ DLLSRYRAGT LTVEELGATL TSLLAQAQAQ ARAEAEAGSP   4620
RPDPREALRA ATMEVKVGRL RGRAVPVWDV LASGYVSGAA REELLAEFGS GTLDLPALTR RLTAIIEEAE   4690
EAPGARPQLQ DAWRGPREPG PAGRGDGDSG RSQREGQGEG ETQEAAAAAA AARRQEQTLR DATMEVQRGQ   4760
FQGRPVSVWD VLFSSYLSEA RRDELLAQHA AGALGLPDLV AVLTRVIEET EERLSKVSFR GLRRQVSASE   4830
LHTSGILGPE TLRDLAQGTK TLQEVTEMDS VKRYLEGTSC IAGVLVPAKD QPGRQEKMSI YQAMWKGVLR   4900
                                                           ←13B
PGTALVLLEA QAATGFVIDP VRNLRLSVEE AVAAGVVGGE IQEKLLSAER AVTGYTDPYT GQQISLFQAM   4970
QKDLIVREHG IRLLEAQIAT GGVIDPVHSH RVPVDVAYRR GYFDEEMNRV LADPSDDTKG FFDPNTHENL   5040
                    ↓
TYLQLLQRAT LDPETGLLFL SLSLQ                                                   5065
              13B→
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FIG. 2. **The predicted sequence of 5065 amino acids of human epiplakin, as deduced from the single ORF within the 15.2-kilobase mRNA (DNA Data Bank of Japan, accession no. AB051895).** The calculated molecular mass of the protein is 55,2467 daltons. The positions of B and B* domain borders are indicated by *horizontal arrows* and *numbers* (*e.g.* 1B*, 2B*, . . . 13B.). The borders of the five COOH-terminal, strongly conserved, homologous repeats are also indicated by *vertical arrows*. The sequence on the basis of which we synthesized a series of eight amino acids overlap for epitope mapping, residues 2807–3337, is *shaded*. Peptide A (MSIYQAMWKGVL(C)) and peptide B (TKGFFDPNTHEN(C)) are indicated by *solid* and *dashed lines*, respectively. The sequence LVPAKDQPGRQEKMSIYQAMWKGVLRPGT, on the basis of which we synthesized a series of decapeptides with an overlap of nine amino acids to refine the identification of the epitope, is indicated in *bold italics*. The antigenic epitope (IYQAMWK) that was recognized by the patient's serum is shown in double-underlining. The sequence, residues 2443–2682, on the basis of which we produced the recombinant protein is indicated in *wavy underlining*.

mass spectrometer (Kompact MALDI II; Kratos-Shimadzu, Tokyo, Japan) and a protein sequencer (491CLC; Applied Biosystems).

Each synthetic peptide (2 mg) was conjugated to bovine serum albumin (BSA; 10 mg; Sigma) using *N*-(4-maleimidobutyryloxy)succinimide (2 mg; Dojin, Kumamoto, Japan) according to the manufacturer's protocol.

Enzyme-linked Immunosorbent Assay (ELISA)—The ELISA was performed with microplates and BSA-conjugated peptides A, B, and C. Wells of microtiter plates (Nunc A/S, Kamstrupvej, Roskilde, Denmark) were coated with 50 ng of synthetic peptide that had been conjugated to BSA (0.5 μg/ml in PBS) with incubation at 4 °C overnight. After incubation with PBS that contained 0.05% Tween 20 (PBS-T) for 1 h at room temperature to block nonspecific binding, samples of serum (diluted 1:200 or serial 2-fold dilutions in PBS-T that contained 1% BSA) were added to the wells. After a 1-h incubation with shaking at room temperature, the wells were washed three times with PBS-T, and 100 μl of a solution of alkaline phosphatase-conjugated antibodies against human IgG antibody (Bio-Rad) that had been diluted to 1:1000 in PBS-T were added. After incubation for 1 h with shaking at room temperature, the wells were washed three times with PBS-T and twice with PBS. After a 1-h incubation with the substrate solution at room temperature, absorbance (405 nm) was determined with a SOFTMAX system (Molecular Devices Co., Sunnyvale, CA). The absorbance obtained with each synthetic peptide was corrected by subtraction of the value due to reaction of the antibodies with BSA by itself.

Immunoblotting—Epidermal extracts were obtained as described previously (23). After addition of dithiothreitol at a final concentration of 0.01 M, the solubilized proteins were separated by electrophoresis on an SDS-polyacrylamide gel (5% polyacrylamide) and transferred to an Immobilon™ polyvinylidene difluoride membrane (Millipore), which was probed with the original patient's serum or polyclonal antibodies against the synthetic peptide (see below) before or after incubation with the synthetic peptide.

Production of a Glutathione S-Transferase (GST) Fusion Protein—The cDNA that encoded a specific region of epiplakin was amplified by PCR with specific primers and subcloned in pGEX 4T-1 as an *Eco*RI fragment. The expression of the GST recombinant protein in *Escherichia coli* JM 109 was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside at 32 °C in LB medium supplemented with 50 μg/ml ampicillin. After culture for 3 h with isopropyl-1-thio-β-D-galactopyranoside, bacterial cells were harvested and lysed by sonication in PBS, with subsequent centrifugation at 15,000 × *g* for 10 min. Supernatants were incubated with 40 μl/ml glutathione-Sepharose (Amersham Pharmacia Biotech) for 30 min at 20 °C. The glutathione-Sepharose beads were washed with elution buffer that contained 15 mM glutathione to elute the GST recombinant proteins. The amino acid sequence of the protein was confirmed with the automated sequencer after Western blotting of proteins on Immobilon™ membranes.

Production of Antibodies against the Fusion Protein and a Synthetic Peptides—Antibodies against a recombinant protein were raised in rats. The purified GST recombinant protein was mixed with an equal volume of Freund's complete or incomplete adjuvant. Initial subcutaneous injections contained 100 μg of recombinant protein (plus complete Freund's adjuvant) per rat. Two booster injections, each consisting of the same amount of protein (plus Freund's incomplete adjuvant) were given 1 and 2 weeks after the initial injection. The rats were bled 3 weeks after the second booster injection. The inguinal lymph nodes were dissected out for production of monoclonal antibodies.

Purified peptide A (MSIYQAMWKGVLC) was conjugated with keyhole limpet hemocyanin using *N*-(4-maleimidobutyryloxy)succinimide and rabbits were immunized with the conjugate plus Freund's complete

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Table I
Comparison between segments of epiplakin and the COOH-terminal domains A, B and C of desmoplakin

All 13 domains, with the exception of domains 1, 2 and 4, consisted of 176 amino acid residues. These other three domains consisted of 178 (domain 1), 171 (domain 2), and 173 (domain 4) residues, respectively. An asterisk indicated B domains of epiplakin that are approximately 46–49% homologous to the B domain in desmoplakin.

Epiplakin		Desmoplakin					
		A		B		C	
Domain no.	Segment	Identities	%Ident	Identities	%Ident	Identities	%Ident
1	27–204	64	36	81	46*	52	30
2	271–441	66	39	79	46*	46	27
3	596–771	86	49	122	69	57	32
4	916–1088	78	45	83	48*	52	30
5	1232–1407	68	39	82	47*	52	30
6	1557–1732	83	47	123	70	57	32
7	1883–2058	77	44	87	49*	52	30
8	2215–2390	81	46	123	70	55	31
9	2749–2924	83	47	124	70	56	32
10	3283–3458	83	47	124	70	56	32
11	3817–3992	83	47	124	70	56	32
12	4351–4526	82	47	123	70	55	31
13	4885–5060	83	47	124	70	55	31

adjuvant. Antibodies (peptide A-specific antibodies) were purified by immunoaffinity gel chromatography on a peptide-immobilized column with absorption in 1 M NaCl (pH 7.5) and subsequent elution with 0.17 M Gly-HCl (pH 2.3) at room temperature.

Northern Dot Blot Analysis—An RNA Master Blot (PT3004-1) was purchased from CLONTECH and Northern dot blotting was performed according to the protocol from CLONTECH. The product of PCR obtained with 5′-GGCGCCAGGACCTGCTGA-3′ and 5′-CACCTTGCTGAGCCGCTCCT-3′ as primers and Ep12 as template was labeled with ^{32}P and used as the specific probe for epiplakin mRNA. The product of PCR obtained with 5′-CCACACCACGGTGGACGA-3′ and 5′-ACTCAGCAGCTGCCTCTG-3′ as primers and pE 450-B as template was used as the specific probe for plectin mRNA (25). Ubiquitin cDNA was used as a control probe.

Immunofluorescence Staining—Human tissues were obtained at biopsy or autopsy, embedded into OCT compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan) and frozen in liquid nitrogen. Frozen sections (5 μm) were air-dried and incubated in PBS for 5 min and then for 1 h at room temperature in PBS that contained 100-fold diluted specific antiserum or antiserum against GST. The sections were washed three times in PBS and then incubated with fluorescein isothiocyanate-conjugated (FITC-conjugated) goat antibodies against rat (for peptide-specific antiserum) or against goat (for GST-specific antiserum) IgG for 1 h at room temperature, with three subsequent washes in PBS. Sections were mounted in immersion oil (Olympus) and examined under a fluorescence microscope (UFX-DX; Nikon).

RESULTS

Isolation of Overlapping cDNA Clones That Encoded a Human Epidermal Autoantigen—We first screened the HeLa cell λgt11 library of random-primed cDNA using the 5′ end of clone pE 450-D as probe (Fig. 1). The longest clone, designated clone Ep24, was isolated, and primers from the 5′ end of clone Ep24 were used for 5′-RACE to identify five overlapping clones (clones Ep28, Ep11, Ep2, Ep79, and Ep21). The original HeLa cell cDNA library was then screened again, and clones Ep1 and Ep5 were obtained. In clone Ep5, the putative initiation codon ATG was preceded by an in-frame termination codon.

For downstream cloning, we screened the HeLa cell cDNA library using the 3′ end of clone pE 450-C (Fig. 1). We isolated several almost identical clones and a clone that included TGA (clone Ep12). From these results, we postulated that the 3′ side of the message had highly homologous repeats of 1602 bp. Clones pE 450-C and pE 450-D both overlapped with parts of the repetitive region. However, the corresponding position could not be identified in this region because of the presence of five strongly homologous repeats. We constructed a cDNA library using a specific primer from the 3′- terminal region of clone Ep12. The longest clone, Ep115, was 6.3 kilobases long and it was isolated together with several shorter clones, Ep150 and Ep104. We constructed another cDNA library using a specific primer from the repetitive region of the 1.6-kilobase pair repeat and isolated a single clone, designated Ep 4.

We performed genomic PCR to amplify the repetitive region and obtained a clone that was slightly more than 9 kilobase pairs in length and contained no introns. The gap of 760 bases in the message that had resulted in a gap in the cDNA was, thus, covered by genomic sequence.

The Human Epidermal Autoantigen Is a Member of the Plakin Family but Has Unusual Features—It appeared that an open reading frame began at the second ATG triplet, which was preceded by an in-frame TGA triplet, because the first ATG triplet was not preceded by a consensus sequence that would favor the initiation of translation (32). The total length of the ORF was 15,195 bp, and the predicted amino acid sequence (5065 amino acids) is shown in Fig. 2. The theoretical relative molecular mass was calculated to be 552,467 Da and was in reasonable agreement with the molecular mass of 450 kDa determined previously by SDS-polyacrylamide gel electrophoresis. The key feature of the protein was the presence of 13 B domains of the type first identified in the COOH-terminal domain of desmoplakin (1). This feature alone clearly identified epiplakin as a member of the plakin family, which includes desmoplakin, BPAG1, and plectin. However, several unusual features were also found, as follows. The B domains themselves could be divided into two groups: one group with ~70% identity to the B domain in desmoplakin (domains 3, 6, and 8–13; indicated by *B* in Fig. 1), and another group with ~45–50% identity to the B domain in desmoplakin (domains 1, 2, 4, 5, and 7; indicated by *B** in Fig. 1). However, all 13 repeats were more similar to the B domain rather than to the A or C domain that were also first recognized in the COOH-terminal domain of desmoplakin (Table I and Ref. 1). We found, moreover, that the linker regions (358 residues in length) that preceded domains 9–13 and followed these five B domains were almost perfectly identical (Fig. 3*A*). In addition, we identified three homologous segments within each of five strongly conserved linker regions (Fig. 3*B*). For example, in the linker region (residues 2391–2748), two of the segments (residues 2391–2472 and 2473–2554) were 30% identical, with particularly high identity over the last 63 residues. After a glycine-proline-rich segment, a third homologous region (residues 2613–2675), which was shorter than the other two, similarly showed homology over the same region. We predicted that these quasi-repeats should contain two or three heptad-containing segments that are separated from one another by β-turns and/or short β-strands. The total potential number of heptad-containing segments was nine, and we postulated that these segments

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A

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2391 QLAGRGSAVH QLSEELRCAL RDARVTPGSG ALQGQSVSVW ELLFYREVSE 2440
2925 QLAGRGSAVH QLSEELRCAL RDARVTPGSG ALQGQSVSVW ELLFYREVSE 2974
3459 QLAGRGSAVH QLSEELRCAL RDARVTPGSG ALQGQSVSVW ELLFYREVSE 3508
3993 QLAGRGSAVH QLSEELRCAL RDARVTPGSG ALQGQSVSVW ELLFYREVSE 4042
4527 QLAGRGSAVH QLSEELRCAL RDARVTPGSG ALQGQSVSVW ELLFYREVSE 4576

                  *
2441 DRRQDLLSRY RAGTLTVEEL GATLTSLLAQ AQAQARAEAE AGSPRPDPRE 2490
2975 DRRQDLLSRY RAGTLTVEEL GATLTSLLAQ AQAQARAEAE AGSPRPDPRE 3024
3509 DRRQDLLSRY RAGTLTVEEL GATLTSLLAQ AQAQARAEAE AGSPRPDPRE 3558
4043 DRRQDLLSRY RASTLTVEEL GATLTSLLAQ AQAQARAEAE AGSPRPDPRE 4092
4577 DRRQDLLSRY RAGTLTVEEL GATLTSLLAQ AQAQARAEAE AGSPRPDPRE 4626

                                      *
2491 ALRAATMEVK VGRLRGRAVP VWDVLASGYV SRAAREELLA EFGSGTLDLP 2540
3025 ALRAATMEVK VGRLRGRAVP VWDVLASGYV SGAAREELLA EFGSGTLDLP 3074
3559 ALRAATMEVK VGRLRGRAVP VWDVLASGYV SGAAREELLA EFGSGTLDLP 3608
4093 ALRAATMEVK VGRLRGRAVP VWDVLASGYV SRAAREELLA EFGSGTLDLP 4142
4627 ALRAATMEVK VGRLRGRAVP VWDVLASGYV SGAAREELLA EFGSGTLDLP 4676

                                *
2541 ALTRRLTAII EEAEEAPGAR PQLQDARRGP REPGPAGRGD GDSGRSQREG 2590
3075 ALTRRLTAII EEAEEAPGAR PQLQDAWRGP REPGPAGRGD GDSGRSQREG 3124
3609 ALTRRLTAII EEAEEAPGAR PQLQDAWRGP REPGPAGRGD GDSGRSQREG 3658
4143 ALTRRLTAII EEAEEAPGAR PQLQDAWRGP REPGPAGRGD GDSGRSQREG 4192
4677 ALTRRLTAII EEAEEAPGAR PQLQDAWRGP REPGPAGRGD GDSGRSQREG 4726

                  *
2591 QGEGETQEAA AAAAAARRQE QTLRDATMEV QRGQFQGRPV SVWDVLFSSY 2640
3125 QGEGETQEAA AAAAAARRQE QTLRDATMEV QRGQFQGRPV SVWDVLFSSY 3174
3659 QGEGETQEAA AAAAAARRQE QTLRDATMEV QRGQFQGRPV SVWDVLFSSY 3708
4193 QGEGETQEAA AATAAARRQE QTLRDATMEV QRGQFQGRPV SVWDVLFSSY 4242
4727 QGEGETQEAA AAAAAARRQE QTLRDATMEV QRGQFQGRPV SVWDVLFSSY 4776

2641 LSEARRDELL AQHAAGALGL PDLVAVLTRV IEETEERLSK VSFRGLRRQV 2690
3175 LSEARRDELL AQHAAGALGL PDLVAVLTRV IEETEERLSK VSFRGLRRQV 3224
3709 LSEARRDELL AQHAAGALGL PDLVAVLTRV IEETEERLSK VSFRGLRRQV 3758
4243 LSEARRDELL AQHAAGALGL PDLVAVLTRV IEETEERLSK VSFRGLRRQV 4292
4777 LSEARRDELL AQHAAGALGL PDLVAVLTRV IEETEERLSK VSFRGLRRQV 4826

2691 SASELHTSGI LGPETLRDLA QGTKTLQEVT EMDSVKRYLE GTSCIAGVLV 2740
3225 SASELHTSGI LGPETLRDLA QGTKTLQEVT EMDSVKRYLE GTSCIAGVLV 3274
3759 SASELHTSGI LGPETLRDLA QGTKTLQEVT EMDSVKRYLE GTSCIAGVLV 3808
4293 SASELHTSGI LGPETLRDLA QGTKTLQEVT EMDSVKRYLE GTSCIAGVLV 4342
4827 SASELHTSGI LGPETLRDLA QGTKTLQEVT EMDSVKRYLE GTSCIAGVLV 4876

2741 PAKDQPGRQE KMSIYQAMWK GVLRPGTALV LLEAQAATGF VIDPVRNLRL 2790
3275 PAKDQPGRQE KMSIYQAMWK GVLRPGTALV LLEAQAATGF VIDPVRNLRL 3324
3809 PAKDQPGRQE KMSIYQAMWK GVLRPGTALV LLEAQAATGF VIDPVRNLRL 3858
4343 PAKDQPGRQE KMSIYQAMWK GVLRPGTALV LLEAQAATGF VIDPVRNLRL 4392
4877 PAKDQPGRQE KMSIYQAMWK GVLRPGTALV LLEAQAATGF VIDPVRNLRL 4926

2791 SVEEAVAAGV VGGEIQEKLL SAERAVTGYT DPYTGQQISL FQAMQKDLIV 2840
3225 SVEEAVAAGV VGGEIQEKLL SAERAVTGYT DPYTGQQISL FQAMQKDLIV 3374
3859 SVEEAVAAGV VGGEIQEKLL SAERAVTGYT DPYTGQQISL FQAMQKDLIV 3908
4393 SVEEAVAAGV VGGEIQEKLL SAERAVTGYT DPYTGQQISL FQAMQKDLIV 4442
4927 SVEEAVAAGV VGGEIQEKLL SAERAVTGYT DPYTGQQISL FQAMQKDLIV 4976

2841 REHGIRLLEA QIATGGVIDP VHSHRVPVDV AYRRGYFDEE MNRVLADPSD 2890
3375 REHGIRLLEA QIATGGVIDP VHSHRVPVDV AYRRGYFDEE MNRVLADPSD 3424
3909 REHGIRLLEA QIATGGVIDP VHSHRVPVDV AYRRGYFDEE MNRVLADPSD 3958
4443 REHGIRLLEA QIATGGVIDP VHSHRVPVDV AYRRGYFDEE MNRVLADPSD 4492
4977 REHGIRLLEA QIATGGVIDP VHSHRVPVDV AYRRGYFDEE MNRVLADPSD 5026

                       *      * ***  *     **
2891 DTKGFFDPNT HENLTYVQLL RRCVPDPDTG LYML 2924
2425 DTKGFFDPNT HENLTYVQLL RRCVPDPDTG LYML 3458
3959 DTKGFFDPNT HENLTYVQLL RRCVPDPDTG LYML 3992
4393 DTKGFFDPNT HENLTYVQLL RRCVPDPDTG LYML 4526
5027 DTKGFFDPNT HENLTYLQLL QRATLDPETG LLFL 5060
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B

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2391 QLAGRGSAVH QLSEELRCAL RDARVTPGSG ALQGQSVSVW ELLFYREVSE 2440
2473 AQARAEAEAG SPRPDPREAL RAATMEVKVG RLRGRAVPVW DVLASGYVSG 2552
2613 ---------- ---------- RDATMEVQRG QFQGRPVSVW DVLFSSYLSE 2643

2441 DRRQDLLSRY RAGTLTVEEL GATLTSLLAQ AQ 2472
2523 AAREELLAEF GSGTLDLPAL TRRLTAIIEE AE 2554
2644 ARRDELLAQH AAGALGLPDL VAVLTRVIEE TE 2675
```

FIG. 3. **The structure of the COOH-terminal domains of epiplakin.** *A*, comparison of the sequences of five COOH-terminal, strongly conserved, homologous repeats. Each repeat consisted of the linker regions (358 residues in length) followed by B domains 9, 10, 11, 12, and 13 (176 residues in length). In total, 534 amino acids were almost perfectly identical. Amino acids that are identical in five repeats are *shaded*. *Asterisk* (*) indicates the different amino acids. *B*, comparison of the sequences of three homologous segments within one of five strongly conserved linker regions (residues 2391–2748). The sequences of three homologous segments are compared with each other. Two of these segments (residues 2391–2472 and 2473–2554) are 30% identical, with particularly homology over the last 63 residues. The third homologous region (residues 2613–2675) is shorter than the former two but, similarly, exhibits the homology over the same region. Amino acids that are identical in three segments are *shaded*.

should be grouped in two or three bundles that contain antiparallel α-helices, a motif found in the structures of many other globular proteins. We failed to identify a coiled-coil rod domain and an amino-terminal domain, both of which are characteristic features of all other known members of the plakin family. Furthermore, no dimerization motif was found in the sequence, suggesting that epiplakin probably exists *in vivo* as a single-chain structure. A search for other functional protein motifs did not reveal any transmembrane sequences or any vimentin- or actin-binding domains (13, 16).

The Antigenic Epitope in Epiplakin—To map the linear epitope in epiplakin accurately, we tested the original patient's serum, which had been used for cDNA screening, for reactivity against 264 consecutive cellulose-bound linear peptides of 10 amino acids in length with an 8-amino acid overlap in the region between amino acids residues 2807 and 3337 of epiplakin (Fig. 2). This region covers one of five strongly conserved COOH-terminal repeats and includes the sequence that was recognized by the patient's serum (25). The patient's serum reacted strongly with the amino acids sequence MSIYQAMWKGVL and weakly with amino acids TKGFFDPNTH. The former sequence is unique to epiplakin, but the latter is also found in plectin and is highly homologous, with the exception of glutamic acid at the last position instead of histidine (TKGFFDPNTE), to a peptide in desmoplakin. To confirm the reactivity of the serum, we performed an ELISA and found that the patient's serum reacted only with the former peptide (Fig. 4*A*). To refine the identification of the epitope, we exposed a series of decapeptides with nine overlapping amino acids derived from the sequence LVPAKDQPGRQEKMSIYQAMWKGVLRPGT (residues 2739–2767) to the patient's serum in another dot blot test. We detected four strongly positive spots, corresponding to sequences from KMSIYQAMWK to IYQAMWKGVL (Fig. 4*B*) and, thus, the major epitope recognized by

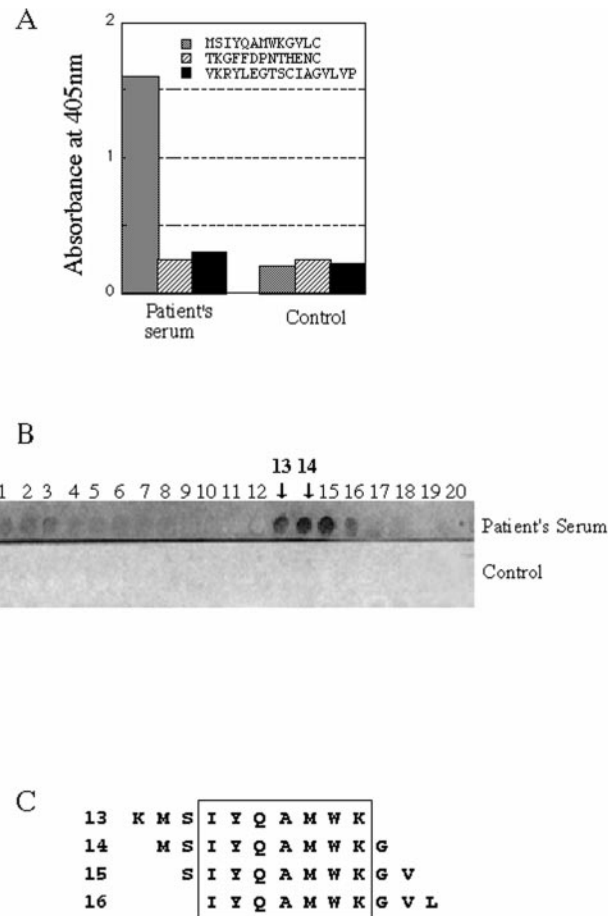

FIG. 4. **The Antigenic epitope in epiplakin.** *A*, enzyme-linked immunosorbent assays using MSIYQAMWKGVLC, TKGFFDPNTHENC, and VKRYLEGTSCIAGVLVP (negative control). The patient's serum reacted with the first peptide but not with the second and the control peptide. Serum from a control patient did not react with any of these peptides. *B*, a series of decapeptides with nine overlapping amino acids from within the sequence LVPAKDQPGRQEKMSIYQAMWKGVLRPGT (residues 2739–2767) was synthesized on a membrane and exposed to the patient's serum. Four strongly positive spots, corresponding to regions from KMSIYQAMWK (no. 13) to IYQAMWKGVL (no. 16), were observed. *C*, the major epitope that was recognized by the patient's serum was determined to be IYQAMWK. The common sequence from KMSIYQAMWK (no. 13) to IYQAMWKGVL (no. 16) was determined to be an antigenic epitope.

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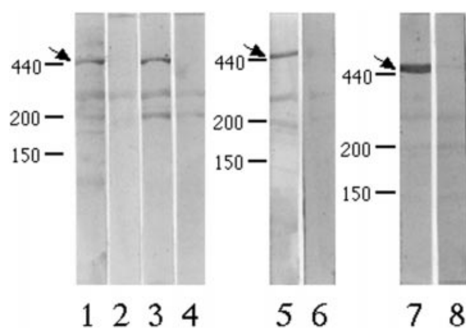

FIG. 5. **Western blotting using several antisera against the 450-kDa epidermal antigen.** An extract of human epidermis was subjected to immunoblotting analysis with the patient's serum before (*lane 1*) and after (*lane 2*) incubation with the synthetic peptide MSIYQAMWKGVLC, and with polyclonal antibodies against the synthetic peptide before (*lane 3*) and after (*lane 4*) incubation with the same synthetic peptide. Polyclonal antiserum (*lane 5*) and monoclonal antibodies (*lane 7*) against the GST-epiplakin fusion protein, but not the control polyclonal antiserum (*lane 6*) and monoclonal antibodies (*lane 8*) against GST itself, recognized the 450-kDa epidermal antigen. Two smaller weakly stained bands in almost all lanes are artifacts due to large amount of desmoplakin I and II in the epidermal samples. *Arrowheads* indicate the position of the 450-kDa epidermal antigen. The position of molecular size standards (in kilodaltons) are shown on the *left*.

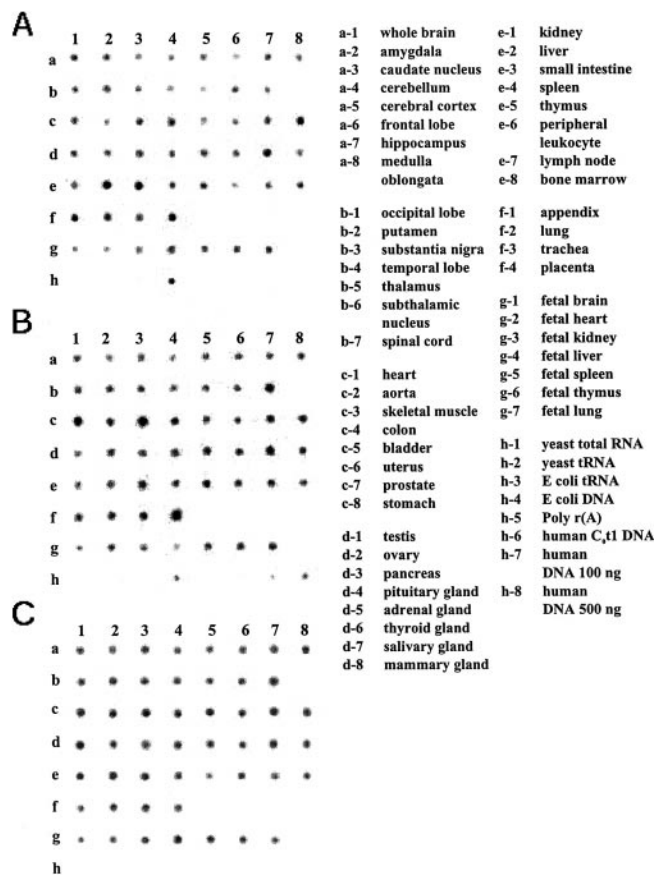

FIG. 6. **Northern dot blots demonstrating the expression of epiplakin in various organs.** Northen analysis of poly(A)$^+$ RNA from various human tissues with probes that corresponded to a specific sequence of epiplakin cDNA (*A*), plectin cDNA (*B*), or ubiquitin cDNA (*C*) as controls. In contrast to plectin, transcripts for epiplakin were strongly expressed in liver, salivary glands, placenta, and digestive organs, and to a lesser extent in lung, bone marrow, and brain. The type and position of poly(A)$^+$ RNAs and controls dotted on the membranes are shown in the *right side*.

the patient's serum was determined to be IYQAMWK (Fig. 4*C*). Western blotting showed that the patient's serum, after absorption with the synthetic peptide MSIYQAMWKGVLC, no longer reacted with the 450-kDa epidermal autoantigen (Fig. 5, *lanes 1* and *2*). These data indicated that antibodies in the patient's serum recognized mainly the unique epitope in epiplakin.

Distribution of Epiplakin as Revealed by Northern Dot Blots of Transcripts—We used a specific cDNA probe that encoded linker regions for Northern hybridization on the RNA Master Blot. The results are shown in Fig. 6*A*. The dot blots indicated that epiplakin was widely distributed in a variety of tissues as was plectin (Fig. 6*B*), but the dominant tissues were different. The signal due to plectin transcripts was strong in the case of muscle, heart, placenta, and spinal cord. By contrast, the epiplakin probe reacted strongly with RNA from liver, small intestine, colon, salivary glands, stomach, and appendix and somewhat less strongly with RNA derived from the placenta, lung, brain, and bone marrow.

Localization of Epiplakin in the Entire Stratified Epithelium and Also in Simple Epithelium—Polyclonal antibodies against the synthetic peptide MSIYQAMWKGVLC reacted with the 450-kDa epidermal antigen before but not after absorption with the synthetic peptide (Fig. 5, *lanes 3* and *4*). Both polyclonal and monoclonal antibodies against the recombinant protein composed of epiplakin (residues 2443–2682) and GST also reacted with the 450-kDa epidermal antigen (Fig. 5, *lanes 5* and *7*). This sequence is within one of five conserved linker regions that is not homologous to human plectin (Fig. 2).

We performed indirect immunofluorescence staining of human skin using the monoclonal antibodies against the recombinant protein of epiplakin. The antibodies reacted with the entire epidermis and immunostaining was especially prominent over basal keratinocytes (Fig. 7*A*). When we used polyclonal antibodies against the synthetic peptide, which is the same epitope recognized by the patient's serum, we obtained the similar patterns (Fig. 7*C*). Using the polyclonal antiserum against the recombinant protein of epiplakin, the epithelium of eccrine sweat glands and hair follicles were also positive for immunostaining (Fig. 7, *E* and *G*). In the esophagus, the same antibodies reacted with all the sheets of the stratified epithelium, including the basal layer (Fig. 7*I*). Immunofluorescence was apparent in acinous cells in the parotid gland and in mucous epithelial cells of the stomach and colon (Fig. 7, *K*, *M*, and *O*).

DISCUSSION

In this report, we have described the cloning and sequencing of overlapping cDNA clones and a genomic clone for a small gap region that together correspond to the entire transcript for a novel member of the plakin family. Epiplakin appears to be a 450-kDa human epidermal autoantigen that is homologous to plectin and other members of the plakin family (1–7, 25). The COOH-terminal region of plectin contains five homologous segments known as the B domain and one other homologous (but different) segment, known as the C domain. Both the B and C domains contain a 39-residue quasi-repeat, which is believed to form a stretch of α-helix that is followed by β-turns (9). Epiplakin, by contrast, contains only B domains (13 in total), and these domains are distributed along the sequence with relatively uniform spacing. Furthermore, epiplakin does not appear to contain either a coiled-coil rod domain or an amino-terminal domain. These features alone indicate that epiplakin belongs to a new category of plakins (33, 34).

The COOH-terminal domain of epiplakin includes five strongly conserved repeats of 534 amino acid residues, each of which includes linker regions and a single B domain. Moreover, each linker domain includes three homologous subdomains. We identified a number of heptad-containing regions in the linker

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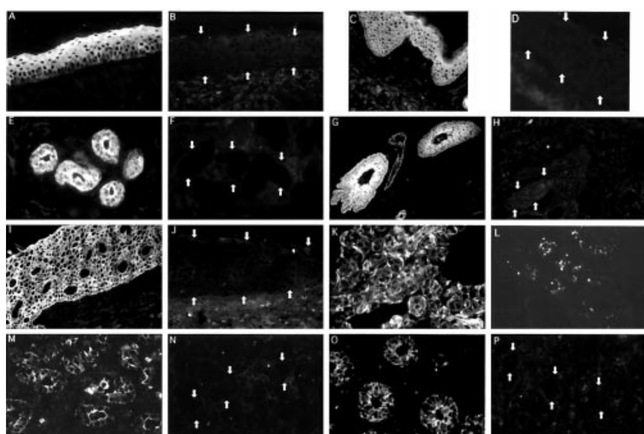

FIG. 7. **Presence of epiplakin in the entire epidermis as revealed by immunofluorescence staining.** Frozen sections of human skin (*A–H*), of esophagus (*I* and *J*), of salivary gland (*K* and *L*), of stomach (*M* and *N*), and of colon (*O* and *P*) were immunostained with monoclonal antibodies (*A*), polyclonal antiserum against recombinant protein of epiplakin (*E*, *G*, *I*, *K*, *M*, and *O*), polyclonal antibodies against the synthetic peptide MSIYQAMWKGVLC(C). And monoclonal antibodies against the GST protein (*B*), polyclonal antiserum against GST protein (*F*, *H*, *J*, *L*, *N*, and *P*) or normal rabbit serum (*D*) were used as negative controls. Epiplakin was detected in the entire epidermis, in particular in suprabasal keratinocytes. The epithelium of eccrine sweat glands and hair follicles were also immunostained. In the esophagus, the same antibodies reacted with all the sheets of stratified epithelium including the basal layer. Immunofluorescence was apparent in acinous cells in the parotid gland and in mucous epithelial cells of the stomach and colon. *White arrows* indicate the positions of epidermis (*B* and *D*), sweat glands (*F*), hair follicles (*H*), stratified epithelium of the esophagus (*J*), mucous epithelial cells of the stomach (*N*), and colon (*P*).

regions, but it is likely that they form parts of α-helical bundles rather than an extended coiled-coil rod. The reasons for this hypothesis are as follows. Two-stranded coiled-coil structures extend, on average, over about 11 heptads between breaks in heptad continuity, and such structures display characteristic patterns of charged residues, in particular at positions e and g (1). By contrast, α-helices that form part of a bundle are typically about three or four heptads long, and the e and g positions are commonly occupied by apolar or noncharged residues. In the amino acid sequence of epiplakin, the characteristics of the heptad-containing regions in the linkers are rather similar to those of a bundle of α-helices of the type commonly seen in globular proteins. In the same context, these heptad-containing sequences in epiplakin do not have the characteristics of a classical dimerization motif and, thus, the probable single-chain nature of epiplakin represents another unusual feature of this protein.

The unique features of the repeated structures in epiplakin undoubtedly contribute to the protein's function *in vivo*. It has been suggested that the COOH-terminal domains of desmoplakin, plectin, and BPAG1 all interact with intermediate filaments (11–14). Epiplakin, an apparently novel human epidermal autoantigen, might also be expected to bind to such filaments. However, no vimentin-binding domain, of the type found in rat plectin (13), was detected within epiplakin; thus, the types of intermediate filament that might act as partners might be limited.

Northern dot blots revealed that epiplakin is expressed at relatively high levels in the brain, in particular, in the amygdala, putamen, subthalamic nucleus, hippocampus, and occipital lobe. By contrast, plectin was detected most prominently in spinal cord and to a lesser extent in other neuronal tissue. Thus, epiplakin might interact with intermediate filaments other than keratins.

In a previous report, we postulated that epiplakin might be distributed in other organs in addition to the epidermis (25). Northern blotting and immunofluorescence staining revealed that the stratified or simple epithelium of the digestive system and several types of glandular epithelium, including that of sweat and salivary glands, expressed this protein. In our immunofluorescence study of skin, it appeared that the protein was expressed in the entire sheet and, in some cases, more strongly in the suprabasal layer of the epidermis. These data are inconsistent with those in our previous study, in which we detected immunofluorescence exclusively in basal keratinocytes (25). In the previous study, we used polyclonal antibodies against a fusion protein derived from a hybrid clone (pE 450-A) and encoded by a segment of 276 bp. We tried to identify the epitope recognized by these antibodies. At maximum dilution, they recognized the sequence SGDQYNDDEIDA, which is encoded by the 5′ region of 78 bp (designated X) of the hybrid clone and irrelevant part of the antigen.

The protein encoded by the full-length cDNA had no signal peptide at its amino terminus and no transmembrane sequence, so it seems likely that it is localized in the cytoplasm. The results of our immunofluorescence study support this hypothesis. As is the case for BPAG1, the pathological role of the autoantibodies against the epiplakin is not known.

The presence of autoantibodies against desmoplakin, envoplakin, periplakin and plectin has been reported in the serum of patients with paraneoplastic pemphigus, an infrequently occurring autoimmune blistering disease (20–22). The serum of the patient whom we discussed in a previous report recognized a sequence in epiplakin that is not found in plectin.

In conclusion, we have clarified the structure and tissue distribution of a novel human epidermal autoantigen, which is an unusual member of the plakin family that was originally found as an autoantigen in a patient with a subepidermal blistering disease. The functions of this protein and the genetic diseases in which it is involved should be studied in further detail.

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