Identification of Two Collagen Domains within the Bullous Pemphigoid Autoantigen, BP180

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Abstract

Bullous pemphigoid (BP) is an autoimmune disease characterized by subepidermal vesicles and the presence of autoantibodies directed against the epidermal basement membrane zone. Previous studies have identified two protein components of the hemidesmosome, BP180 and BP230, as the primary antigenic targets of BP autoantibodies. We have recently reported the isolation of a 1.0-kb BP180 cDNA. Sequence analysis presented in this report reveals that this partial BP180 cDNA encodes two protein domains which have primary structures that are characteristic of the triple helical domains of collagens, i.e., glycine appears at every third position and over one-third of the remaining residues are proline. The two collagen domains have lengths of 242 and 30 amino acids and are separated by a noncollagen stretch of 12 amino acids. Collagenase digestion of the BP180 cDNA-encoded fusion protein generated a peptide fragment with a size that was consistent with the predicted locations of the collagenase digestion sites. A possible physiological function for the collagen domains of the BP180 hemidesmosomal protein may be to form stable interactions with constituents of the extracellular matrix of the cutaneous basement membrane zone. Such interactions may provide the molecular framework for the attachment of the basal keratinocyte and the basal lamina. (J. Clin. Invest. 1991, 87:734–738.)

Key words: autoimmunity • hemidesmosome • keratinocyte • basement membrane

Introduction

The hemidesmosome is a plasma membrane–associated organelle found in basal cells of stratified, squamous epithelia and is thought to be involved in the attachment of the epithelium to the underlying basal lamina. It appears that a disruption of the function of the hemidesmosome may play a role in the pathogenesis of the blistering skin disorder, bullous pemphigoid (BP).1 The hallmark of this disease is the appearance of subepidermal vesicles caused by the detachment of the epidermal basal cell layer from the basal lamina at the level of the lamina lucida. In the skin lesions of BP patients, hemidesmosomes cannot be visualized by electron microscopy (1, 2). Immunoelectron microscopic studies have demonstrated that circulating BP autoantibodies bind specifically to the hemidesmosomes of normal epidermis (3–7); however, it is important to note that direct evidence of the pathogenicity of these antihemidesmosomal autoantibodies is lacking.

Immunoblot and immunoprecipitation analyses have demonstrated that two large epidermal polypeptides, BP230 and BP180, are the major antigenic moieties recognized by BP autoantibodies (8–11). Recent studies dealing with the characterization of these autoantibodies have employed a molecular genetic approach involving the identification of BP antigen cDNA clones from a human keratinocyte cDNA expression library with the use of a serum from a BP patient (12, 13). Using this strategy, Stanley and co-workers (12) cloned and characterized a human epidermal cDNA which corresponds to a portion of the 230-kD BP antigen, BP230. Sequence analysis revealed a 1,992-bp open reading frame encoding a 76-kD peptide corresponding to the carboxy terminus of BP230. Northern analysis demonstrated that the BP230 probe hybridized to a 9-kb transcript.

More recently, our laboratory has reported the isolation of partial cDNA clones corresponding to both the BP230 (previously referred to as BP240) and BP180 human epidermal antigens (13). Northern blot analysis demonstrated that BP180 and BP230 are encoded by distinct RNA transcripts with lengths of 6.0 and 8.5 kb, respectively. A rabbit antiserum prepared against the BP180 cDNA-encoded fusion protein was shown to react specifically with BP180 on an immunoblot, stained the BMZ by indirect immunofluorescence, and labeled the epidermal hemidesmosome by immunoelectron microscopy. No cross-reactivity between BP180 and BP230 was detected. The sequence analysis of the BP180 cDNA is presented in the current report.

Methods

Isolation and characterization of the BP180 cDNA clone. As described in a previous report (13), a high titer BP serum containing autoantibodies against both the BP180 and BP230 epidermal antigens was used to screen an oligo dT-primed, human keratinocyte lambda gt11 cDNA library (Clontech Laboratories, Palo Alto, CA) after a modification of the protocol by Young and Davis (14). One immunoreactive clone, lambda-BP180, contained a 1.0-kb insert that was shown to encode a portion of the BP180 antigen. A second clone, lambda-BP230, contained a 1.6-kb insert corresponding to a portion of the BP230 coding region. This report focuses on the sequence analysis of the BP180 clone.
DNA sequence analysis. Nucleotide sequence analysis was performed as previously described (15). The Eco RI insert of the lambda-BP180 clone was subcloned into the Eco RI site of the plasmid pBlueScript IIISK* vector (Stratagene, La Jolla, CA). The cDNA was then analyzed according to the double-stranded DNA sequencing procedure of Chen and Seeburg (16), which is a modification of the dideoxy method of Sanger et al. (17). A series of oligonucleotide sequencing primers were synthesized in the Protein and Nucleic Acid Shared Facility at the Medical College of Wisconsin. A sequencing primer was allowed to anneal with the denatured recombinant plasmid DNA and was extended using Sequenase II, a derivative of T7 DNA polymerase (United States Biochemical Corp., Cleveland, OH). The reactions were carried out in the presence of appropriate mixtures of deoxy- and dideoxynucleotides. The termination of the nucleotide chain due to the incorporation of a dideoxynucleotide produced a sequencing ladder which was analyzed by urea/polyacrylamide gel electrophoresis. The sequence data was analyzed using the Genetics Computer Group sequence analysis software package (18). Homology searches were run against the GenBank and EMBL nucleotide sequence databases and the NBRF and Swiss protein sequence databases.

Production of cDNA-encoded BP180 fusion protein by lysogens. A lambda BP180 lysogen was produced by infecting Escherichia coli strain Y1089 with the lambda-BP180 bacteriophage after procedures described by Earnshaw et al. (19). When a liquid culture of the BP180 lysogen reached the late logarithmic phase of growth, fusion protein was induced by the addition of IPTG (10 mM) and lysis was induced by increasing the temperature to 42°C for 15 min. After an additional 2-h incubation at 37°C, the culture was centrifuged and the pellet was washed with Tris-buffered saline (TBS) at 4°C. The pellet was then treated with 1 mg/ml lysoyzyme, 0.1 mM PMSF in TBS, sonicated, and centrifuged. The pellet was washed with 1% triton X-100 in TBS and then solubilized in 6 M urea. The supernatant was dialyzed against H2O and stored as a lyophilized powder. The resin was resuspended in TBS and the insoluble fraction was washed with TBS until the supernatant showed no significant absorption at a wavelength of 280 nm. Aliquots of this insoluble material, containing the BP180 fusion protein, were treated with collagenase and analyzed as described below.

Collagenase digestion of the BP180 fusion protein. The insoluble fraction of the BP180 lysogen extract was treated with collagenase from Clostridium histolyticum (form III; Advance Biofactures Corp., Lynbrook, NY) in TBS containing 5 mM CaCl2 and 0.1 mM PMSF. Each 200-μl digestion reaction, which contained ~30 μg of BP180 fusion protein and 1–50 BTC units of collagenase, was incubated for various times at 37°C. The reactions were stopped by incubating at 95°C for 2 min in 1×SDS gel sample buffer. As a means of testing the specificity of the collagenase preparation, digestions were performed under the conditions described above on 30-μg aliquots of rat tail collagen type I (Collaborative Research, Inc., Bedford, MA) and BSA (Sigma Chemical Co., St. Louis, MO). Other controls included running the reactions after inactivation of the collagenase, either by preboiling the enzyme for 2 min or by adding 25 mM EDTA to the reaction mixture in place of the CaCl2.

SDS-PAGE and immunoblotting procedures. The SDS-PAGE and immunoblotting procedures performed in this study were minor modifications of the procedures described previously by Burnette (20), Labib et al. (10), and Morrison et al. (21). Briefly, the collagenase-digested BP180 lysogen extract and the control digestions were fractionated by SDS-PAGE according to the protocol of Laemmli (22). The fractionated proteins were electrophoretically transferred to nitrocellulose paper and then probed with a BP serum at a dilution of 1:400 in 4% PTX buffer (0.01 M phosphate, pH 7.5, 0.2% Triton X-100, 0.15 M NaCl, and 1 mM EGTA). Bound antibodies were detected using 125I-labeled Staphylococcus aureus protein A instead of the enzymatic method used by Labib et al. (10) and Morrison et al. (21). Immunoreactive protein bands recognized by the BP autoantibodies were visualized by autoradiography using Kodak XAR5 film with DuPont Lighting Plus intensifying screens.

Results

Sequence analysis. The entire length of both strands of the BP180 cDNA insert has been sequenced. The sense and antisense strands of the BP180 clone had previously been identified on the basis of Northern blot analyses using strand-specific cRNA probes (13). One open reading frame (ORF) in the sense orientation was found to extend the length of the BP180 clone. Using the 5’ terminal Eco RI site as a reference, it was determined that this BP180 ORF was in register with the lacZ reading frame of the original lambda gt11 BP180 cDNA clone, supporting the conclusion that this long ORF was the correct BP180 reading frame. This ORF encodes a peptide with a calculated molecular weight of 33,600 D or ~19% of the mass of the BP180 antigen isolated from human epidermis. The nucleotide sequence and the deduced amino acid sequence of the cloned portion of BP180 is shown in Fig. 1. A sequence comparison of the BP180 cDNA with that of the previously published BP230 cDNA (12) revealed no significant homology at either the nucleotide or amino acid levels.

A search of the GenBank (release 63) and EMBL (release 22) nucleotide sequence databases revealed that the BP180 cDNA exhibits a significant homology with the collagen gene family. Using the Genetics Computer Group program "FASTA", we obtained the best five matches with the following collagens: human alpha 1(II), human alpha 2(V), chicken chondrocyte-specific short collagen, chicken type X, and mouse alpha 1(IV). These sequences exhibited 53–54% identity with BP180 after introducing between 8 and 20 gaps over an average span of 860 bp.

An analysis of the deduced amino acid sequence of BP180 revealed several collagen-related features. Throughout most of the stretch from amino acid number 60 to 343, a glycine appears at every third position, a pattern which is characteristic of those regions of collagen polypeptides that are involved in the formation of a triple helix. Within this length of 284 amino acids, there occurs only one major break in the Gly-X-Y pattern: a noncollagen stretch of 12 amino acids which begins at position 302. The two major collagen domains, which have lengths of 242 and 30 amino acids, are indicated in Fig. 1 by the shaded boxes. A single amino acid interruption in the Gly-X-Y pattern of collagen domain I is located at amino acid position 132–133 (indicated in Fig. 1 by the nonshaded box). The proline content within the collagen regions was found to be quite high, (22% in collagen domain I and 40% in collagen domain II). These prolines are evenly distributed between the “X” and “Y” positions in the Gly-X-Y tripeptide repeat in both collagen domains.

Collagenase digestions. To further document the existence of a collagen structure within the cloned portion of BP180, the lysogen extract containing the BP180 cDNA-encoded fusion protein was digested with bacterial collagenase under various conditions and analyzed by SDS-PAGE (Fig. 2, lanes 1–5) and by immunoblotting with a BP serum (Fig. 2, lanes 6–10).

At time zero the lambda BP180 lysogen extract contained a major product of 135 kD and a minor one of 146 kD, both of which reacted on an immunoblot with a BP serum (Fig. 2, lanes 1 and 5) and with an anti-beta-galactosidase antiserum (data not shown). A (2-h) digestion of this extract with 1 BTC unit of collagenase at 37°C resulted in the disappearance of both the 146- and the 135-kD bands and the appearance of a band with an apparent molecular weight of 124 kD (lane 3).

BP180 Contains Two Collagen Domains
This collagenase-resistant polypeptide reacted with BP autoan-tibodies by immunoblotting (lane 8), indicating that it arose as a breakdown of the BP180 fusion protein. Comparison of the Coomassie blue–stained gel profiles of the 0- and 2-h time points reveals that none of the bacterial proteins were degraded by the collagenase preparation. This demonstrates that, under the conditions of this assay, contaminating proteolytic activity was not detected. A 5-min digestion of the BP180 lysogen ex-

Figure 1. Nucleotide and deduced amino acid sequence of the BP180 cDNA clone. Shaded boxes denote the two collagen domains. Collagen domain I extends from amino acid number 60 to 301, and collagen domain II extends from amino acid 314 to 343. Nonshaded box located within collagen domain I denotes a single amino acid interruption in the Gly-X-Y pattern.

Figure 2. Collagenase digestion of the BP180 fusion protein. A BP180 lysogen extract containing the BP180 fusion protein was subjected to collagenase digestion and the products were fractionated by SDS-PAGE. Lanes 1–5 were stained with Coomassie blue (CB), and lanes 6–10 are immunoblots (IB) labeled with a BP serum. A 200-μl aliquot of the extract was incubated with 1 BTC unit of bacterial collagenase in TBS-Ca++ at 37°C for the indicated times: zero time (lanes 1 and 6), 5 min (lanes 2 and 7), and 2 h (lanes 3 and 8). The 2-h collagenase digestion generated a 124-kD collagenase-resistant peptide which reacted with the BP serum (lane 8). Longer incubation times, up to 24 h, or increased amounts of enzyme, up to 50 U, showed no further degradation of the 124-kD band. The 5-min time point shows incomplete digestion of the BP180 fusion protein (lane 7). The digestion of the BP180 fusion protein was inhibited by the addition of EDTA to the reaction mixture in place of the CaCl₂ (lanes 4 and 9) or by heat inactivation of the enzyme (lanes 3 and 10).
extract with 1 BTC unit of collagenase showed incomplete digestion, with both the 135- and the 124-kd bands visible (lane 2), both of which were labeled with the BP serum (lane 7).

The digestion of the BP180 fusion protein with collagenase was completely inhibited by either including EDTA in the reaction mixture (Fig. 2, lanes 4 and 9), or by boiling the enzyme for 2 min before adding it to the lysogen extract (lanes 5 and 10). The PAGE and immunoblot analyses of the products of these control reactions gave patterns which, as expected, were identical to those of the zero time point. Digestions of rat collagen type I and BSA were used to test the specificity of the collagenase. A 2-h incubation at 37°C with 1 BTC unit of collagenase resulted in complete digestion of 30 µg of rat collagen, whereas a 30-µg aliquot of BSA showed no detectable degradation after collagenase treatments lasting up to 24 h (data not shown).

Discussion

The sera of BP patients contain autoantibodies which bind hemidesmosomal antigens of squamous epithelia (3–7) and react by immunoblotting and immunoprecipitation with two large epidermal proteins, i.e., the BP230 and the BP180 antigens (8–11). Stanley et al. (12) were the first to clone a cDNA corresponding to one of the BP antigens, a clone which was shown to encode the COOH-terminal one-third of the BP230 antigen. Sequence analysis has revealed that BP230 shares a significant homology with desmoplakins I and II (23), components of the desmosomal plaque, but does not share homology with other known matrix or structural proteins.

In the present paper we report the sequence of a 1,047-bp human epidermal cDNA which, by immunological criteria, had previously been shown to encode a portion of BP180 (13). This clone corresponds to approximately one-sixth of the size of the BP180 transcript previously reported by our laboratory (6 kb; reference 13). No information is available concerning the position of this cloned sequence relative to the 5′ or 3′ terminus of the BP180 RNA transcript; however, because this clone was isolated from an oligo-dt-primed library, it is unlikely that it corresponds to a region near the 5′ end of this long transcript.

Based on sequence analysis, the fusion protein encoded by the lambda BP180 recombinant clone has a predicted size of 148 kD; however, two protein product were detected in the BP180 lysogen extract (see Fig. 2, lanes I and 6). A 146-kD immunoreactive polypeptide, which may correspond to the intact BP180 fusion protein, was found in low amounts, whereas the major immunoreactive product of the BP180 clone had an apparent molecular weight of 135 kD. Both bands showed reactivity with a BP serum and with a rabbit anti-beta-galactosidase antisera. Efforts to eliminate proteolysis during the extraction procedure had no effect on the relative amounts of these two polypeptides, suggesting that both the 146- and the 135-kD polypeptides may be generated by the lysogen in culture.

The BP180 sequence showed no significant homology, at either the nucleotide or the amino acid level, with the previously published sequence of BP230 (12). This information is consistent with the results of a previous report from our laboratory (13) which provided evidence that these two BP antigens are not closely related. A Northern blot analysis demonstrated that BP230 and BP180 are encoded by distinct RNA transcripts showing no cross-hybridization. Immunological analyses which involved the use of BP autoantibodies affinity-purified against either the lambda-BP180 or the lambda-BP230 fusion protein showed no cross-reactivity between these two epidermal autoantigens. The above experiments failed to detect any homology between BP230 and BP180; however, because all of these analyses utilized partial cDNA clones for both BP230 and BP180, a definitive statement concerning the relatedness of these two antigens cannot yet be made. It is still possible that the segments of these two BP antigens that remain to be cloned may exhibit some homology.

Comparison to the BP180 sequence with sequences stored in both nucleotide and amino acid sequence databases revealed striking homologies with the collagen gene family. Analysis of the deduced amino acid sequence of the BP180 clone identified two protein domains in which a glycine residue appears at every third position. The Gly-X-Y tandem repeat is a primary structure characteristic of those regions of collagen chains which are involved in the formation of a triple helix (24). The strict conservation of the Gly-X-Y pattern in collagens is due to the fact that the repeating glycine residues are positioned within the interior of the collagen triple helix and, because of size restrictions, no other amino acid can be accommodated at these positions.

As a means of independently demonstrating the presence of collagen sequences within the cloned segment of BP180, the lysogen extract containing the BP180 cDNA-encoded fusion protein was assayed for digestion after treatment with collagenase from Clostridium histolyticum. The substrate specificities of the collagenases from this bacterium have been analyzed extensively with the use of synthetic peptides (25–28). Based on this information, we predicted that complete collagenase digestion of the beta-galactosidase-BP180 fusion protein would generate one major collagenase-resistant peptide of 124 kD and a variety of smaller peptides ranging in size from 3 to 39 amino acids. The predicted 124-kD collagenase-resistant fragment consists of a 114-kD portion of the beta-galactosidase protein and a 95 amino acid stretch (residues 1–95 from Fig. 1) encoded by the BP180 cDNA. Analysis of the products of the BP180 lysogen digestions demonstrated that both the 146- and 135-kD forms of the BP180 fusion protein are substrates for the bacterial collagenase. Furthermore, the observed size of the major collagenase digestion product, 124 kD, corresponded very well with the size predicted from the sequence analysis.

Immunoblot analysis of the collagenase digestion products also revealed information concerning the general location of an epitope recognized by BP autoantibodies. The 124-kD collagenase digestion product of the BP180 fusion protein reacted, by immunoblot, with a BP serum. This indicated that one or more epitopes recognized by BP autoantibodies against the BP180 antigen are present within the first stretch of 95 amino acids encoded by the 5′ end of the BP180 cDNA insert. This 95-amino acid region consists of a 59–amino acid noncollagenous NH2-terminal domain and a 36–amino acid segment of collagen domain I. This observation does not exclude the possibility that BP epitopes are also present on other portions of the BP180 antigen. A more precise mapping of the BP epitopes encoded by the BP180 cDNA is currently under way.

In summary, the BP180 cDNA encodes a 33-kD segment of the BP180 antigen containing two collagen domains. The physiological role of these collagen domains may be to interact with one or more of the various matrix proteins present in the epi-
dermal basement membrane zone, e.g., laminin, fibronectin, collagen (type IV or VII), a proteoglycan, or an integrin. Such molecular interactions could possibly play an essential role in the adherence of epidermal basal cells to the dermis. Moreover, it is possible that a disruption of such adhesive interactions (autoantibody-mediated or otherwise) may be the trigger for epidermal cell detachment in BP patients. Testing of these hypotheses will be the focus of future investigations.

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References