

Development of an ELISA for the detection of autoantibodies to BP230

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Abstract

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease associated with autoantibodies against the transmembrane hemidesmosomal protein BP180/collagen type XVII and the intracellular plaque protein BP230. The aim of the present study was to develop an ELISA system for the detection of circulating autoantibodies to BP230. We generated five overlapping cDNA constructs covering the entire length of BP230 and expressed them in baculovirus-infected Sf21 insect cells. ELISA reactivity against BP230 was found in 63% of 56 BP patients' sera; the specificity of the ELISA was 93%. Epitope mapping studies showed that the fragment representing the C-terminal portion of BP230 was by far the most frequent target within the molecule. This ELISA provides a useful tool for the detection of autoantibodies to BP230 in BP and other diseases associated with an autoimmune response to this protein.

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Introduction

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease characterized by tissue-bound and circulating autoantibodies to the dermal–epidermal junction (DEJ) [1]. It primarily affects the elderly and is the most common autoimmune bullous disease in Europe [2,3]. Autoantibodies in BP are directed to two hemidesmosomal components, including a 230-kDa antigen (BP230), an intracellular constituent of the hemidesmosomal plaque [4,5], and the transmembrane protein BP180 [6,7]. In addition to BP, reactivity to BP230 may be found in patients with paraneoplastic pemphigus and less frequently in mucous membrane pemphigoid and pemphigoid gestationis [8–11].

BP230, also known as BPAg1, belongs to the plakin protein family [5,12–14]. It consists of a central α -helical coiled-coil region flanked by two globular end domains and several distinct subregions [14,15]. BP230 contributes to cytoskeletal organization by association of its carboxyl terminus with keratin filaments [16] and interaction of the N-terminal portion with both BP180 [17] and the β_4 chain of $\alpha_6\beta_4$ integrin [18].

Reactivity of BP sera with BP230, extracted from cultured keratinocytes or epidermis, was first reported by immunoprecipitation [4,19,20] and immunoblotting [12,19,21]. Subsequently, recombinant forms of BP230 have become available and it was demonstrated that immunoblot reactivity was strongest with the C-terminal portion of the molecule [9,12,22–25].

Reactivity of BP sera by indirect immunofluorescence (IF) microscopy on monkey esophagus or NaCl-split human skin reflects reactivity to various constituents of the dermal–epidermal junctions, including BP230 and BP180 [26]. Several specific and sensitive ELISA systems have been reported for the detection of autoantibodies to BP180 [27–31]. In contrast, the few reported ELISA-based detection systems for autoantibodies to BP230 were less sensitive and specific. These assays used either synthetic peptides [32] or bacterial fusion proteins [33] covering only small portions of the molecule or a fragment of murine BP230 [23], respectively.

In the present study, we developed an ELISA for the detection of autoantibodies to human BP230 utilizing recombinant forms covering the full-length of this autoantigen. Because of the low expression rate of full-length recombinant BP230, we generated five novel, overlapping fragments of BP230. The ELISA utilizing these fragments turned out to be as sensitive for the detection of antibodies to BP230 as immunoblotting using cellular BP230 derived

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from cultured keratinocytes. In addition, epitope mapping studies showed that BP sera preferentially react with the C-terminal portion of BP230.

Materials and methods

Patients and controls

Serum samples were obtained from 56 BP patients before initiation of treatment. The mean age of patients was 72 years (42–94 years); 25 were male and 31 were female. Direct IF microscopy of perilesional skin biopsies showed linear deposits of IgG and/or C3 at the DEJ in all patients. Indirect IF microscopy was performed on 1 M NaCl-split human skin as described [34]. All patients' sera showed circulating IgG binding to the epidermal site of the split with titers $\geq 1:10$. As controls, we used sera from healthy blood donors ($n = 56$). Additional controls included patients with pemphigus vulgaris ($n = 10$) and epidermolysis bullosa acquisita ($n = 10$) that showed an intercellular indirect IF staining and ELISA reactivity with desmoglein 3 or circulating IgG labeling the dermal site of NaCl-split skin and recombinant type VII collagen by immunoblotting, respectively [35,36].

Cloning of BP230 cDNA fragments

Amplification and cloning procedures were performed as described previously for the generation of BP180 cDNA [37]. Briefly, primers for polymerase chain reactions (PCR) were synthesized by MWG Biotech (Ebersberg, Germany). DNA sequence data of BP230 were retrieved from GenBank DNA data bank using the most recent accession number M69225 as published by Sawamura et al. [13]. The full-length 7947 bp BP230 cDNA (nt 39–7985) was generated by polymerase chain reaction using the *Expand Long Template PCR System* (Roche, Penzberg, Germany) on a cDNA pool from cultured neonatal keratinocytes. The BP230 cDNA fragments encoding the overlapping proteins BP230-N1 (nt 39–2051), BP230-N2 (nt 1857–3257), BP230-N3 (nt 3078–5313), BP230-C1 (nt 5166–6604), and BP230-C2 (nt 6483–7985) were generated by applying PCR on the cloned full-length BP230 cDNA. Restriction sites for Kpn I/Sal I or Bam HI/Sal I (for BP230-C1) were introduced during the PCR procedure. Primer sequences for amplification of the BP230 fragments are depicted in Table 1. The full-length BP230 and the BP230 fragments were cloned into linearized pBHH2a and pBHH2b (for BP230-C1), respectively (Invitrogen, Carlsbad, CA), resulting in the recombinant transfer vectors pBBH-BP230-FL, pBBH-BP230-N1, pBBH-BP230-N2, pBBH-BP230-N3, pBBH-BP230-C1, and pBBH-BP230-C2. The presence of correct inserts was verified by DNA sequence analysis following the dideoxy method using a DyeDi-

Table 1

Primer sequences for PCR amplification of BP230 cDNA

Fragment	Size (bp)	Primer sequences (5'-3')
BP230	7968	F: <u>gacggtaccatgcacagtagtagttatgttacc</u> R: <u>ggatcgctgcacggaagaatagtagaggctagaattcctgtcatcagggg</u>
BP230-N1	2034	F: <u>gacggtaccatgcacagtagtagttatgttacc</u> R: <u>ggatcgctgcactattgaagccacattgctagctcga</u>
BP230-N2	1423	F: <u>gacggtaccatgtgccatctgtgtgcttcaccgttctccacc</u> R: <u>gacgtcgcacctcttgaataatgagatctgctgaa</u>
BP230-N3	2258	F: <u>gacggtaccgaaatagaaatgaaacagagcaaaatgg</u> R: <u>gacgtcgcacccatcttctccgaaactcctctgc</u>
BP230-C1	1461	F: <u>gacggtaccgaaagagttaaataacaggttgaa</u> R: <u>gacgtcgcactatgtctgacctgaaatgggagca</u>
BP230-C2	1525	F: <u>gacggtaccgaaagagttatcttgcctcagcgccgagag</u> R: <u>gacgtcgcacggaagaatagtaggctagaattcctgtcatcagggg</u>

Restriction sites for Bam HI (ggatcc), Kpn I (ggacc), and Sal I (gtcgact) are underlined. The upper sequence of each primer pair represents the forward primer (F), the lower the reverse primer (R).

deoxy Terminator kit and a 377 DNA sequence analyzer (Applied Biosystems, Foster City, CA).

Heterologous expression of overlapping BP230 fragments in Sf21 insect cells

Spodoptera frugiperda 21 (Sf21) insect cells (Invitrogen) grown in Grace's insect medium (Invitrogen) were co-transfected with one of the recombinant vectors pBBH-BP180-FL, pBBH230-N1, pBBH230-N2, pBBH230-N3, pBBH230-C1, or pBBH230-C2 and linearized baculovirus AcMNPV DNA (Invitrogen) using the liposome technique [37]. Recombinant baculovirus clones were identified and purified by plaque assay. Proteins were expressed by infecting the cells with recombinant baculovirus at a multiplicity of infection of 5.0. Three days post-infection, the corresponding recombinant hexa-histidine-tagged protein was purified from Sf21 insect cells by immobilized metal chelate affinity chromatography using Ni-NTA agarose (Qiagen, Hilden, Germany). Protein concentrations were measured by Bradford assay (Biorad, Hercules, CA). Recombinant forms of BP230, used in this study, are depicted in Fig. 1.

Preparation of full-length BP230 from cultured human keratinocytes

Normal human keratinocytes were isolated from human neonatal foreskins and grown in KGM (Clonetics, La Jolla, CA) containing 0.15 mM Ca^{2+} at 37°C in a humidified atmosphere containing 5% CO_2 as described (JID, [26]). Confluent monolayers of third passage human keratinocytes were lysed in 2% sodium dodecylsulfate (SDS), 5% β -mercaptoethanol, 2 mM phenylmethyl-sulfonylfluoride (PMSF), and 2 mM EDTA in 62.5 mM Tris-HCl (pH 6.8) (Sigma, St. Louis, MO). Lysates were centrifuged at $20,000 \times g$ to remove cell debris, aliquoted, and stored at -80°C until use.

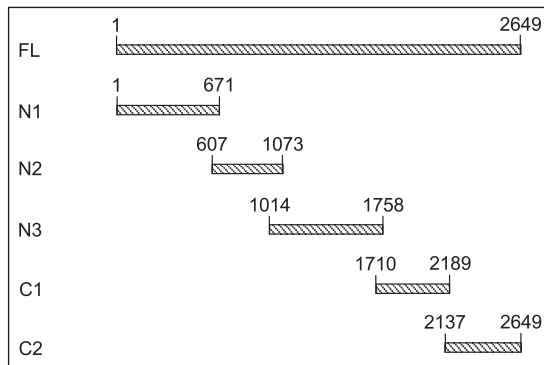


Fig. 1. Recombinant fragments of BP230 generated in this study. Full-length BP230 (FL) has been subdivided into five fragments (N1, N2, N3, C1, C2). Numbers refer to amino acid (aa) positions. At the N-terminus, recombinant proteins contain an RGS–hexahistidine moiety.

Immunoblot analysis

Recombinant proteins and lysates from cultured keratinocytes were fractionated by 12% and 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively. Proteins were transferred to nitrocellulose [38]. Blots were blocked for 1 h in 1% (v/v) Tween-20 in PBS buffer (154 mM NaCl, 20 mM KH_2PO_4 , pH 7.6) and subsequently labeled with patients' sera or monoclonal anti-RGSH₄-antibody (Qiagen) diluted 100-fold and 2000-fold in PBS, respectively. After a 12-h incubation at room temperature, blots were washed and incubated with rabbit anti-human or rabbit anti-mouse IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA). The NBT/BCIP staining system was used as a chromogenic substrate (Sigma, St. Louis, MO).

ELISA for detection of antibodies to BP230

The ELISA used five recombinant BP230 fragments; each was immobilized in a separate well of a 96-well microtiter plate (MaxiSorb™, Nunc, Roskilde, Denmark) and subsequently probed with a series of BP and control sera using a horseradish-peroxidase-conjugated secondary antibody.

Defining the optimal working conditions of the assay followed a previously established procedure [27]. Chess board titrations were performed with serial dilutions of recombinant BP230-N1, BP230-N2, BP230-N3, BP230-C1, and BP230-C2, respectively (1–50 µg/ml in PBS, pH 7.6), serial dilutions of secondary antibody (1:10,000 through 1:30,000 dilutions), and a constant 50-fold dilution of primary antibody (BP-sera with medium range immunoblot reactivity to BP230). Analysis of the curves generated by plotting antigen concentration versus OD₄₉₀ revealed that a constant 12,500-fold dilution of the secondary antibody produced optimal discrimination in this assay.

The optimized ELISA was run under the following conditions. Wells were coated for 2 h at 37°C with 100 µl PBS, pH 7.6 containing 2.4 µg BP230-N1, 0.9 µg BP230-N2, 0.8

µg BP230-N3, 0.5 µg BP230-C1, or 1.2 µg BP230-C2. Wells were washed three times with PBS, pH 7.6, using a 96-well washer (Tecan, Switzerland) and blocked with PBS containing 0.5% BSA, 0.5% casein, and 0.5% Tween-20 at room temperature for 2 h. Subsequently, wells were incubated for 1 h with 100 µl serum diluted 50-fold in blocking buffer. After washing as above, each well was incubated for 1 h at room temperature with rabbit anti-human IgG (H + L) (Jackson ImmunoResearch Laboratories) diluted 12,500-fold in PBS, pH 7.6. Following another series of washes, 100 µl of freshly dissolved substrate (*o*-phenylenediamine in 0.1% H_2O_2) was added to each well. Ten minutes after substrate addition, the reaction was stopped by the addition of 100 µl 1 M H_2SO_4 and the OD₄₉₀ was measured using a microplate reader (Dynex MRX, Chantilly, VA). Each serum was assayed in duplicates.

Results

Cloning of five overlapping BP230 cDNA fragments

Total RNA was extracted from human neonatal keratinocytes and reversed transcribed. The 7968-bp BP230 cDNA encoding the full-length BP230 was obtained by PCR using the primers shown in Table 1. This cDNA was cloned into the transfer vector pBBH2a resulting in pBBH-BP230. In addition, cDNA fragments encoding five overlapping fragments of BP230 were subcloned into pBBH2a and pBBH2b resulting in the recombinant transfer vectors pBBH-BP230-N1, pBBH-BP230-N2, pBBH-BP230-N3, pBBH-BP230-C1, and pBBH-BP230-C2, respectively. Restriction analysis and DNA sequence analysis revealed the correct insertion and orientation of the cDNA fragments (Fig. 2).

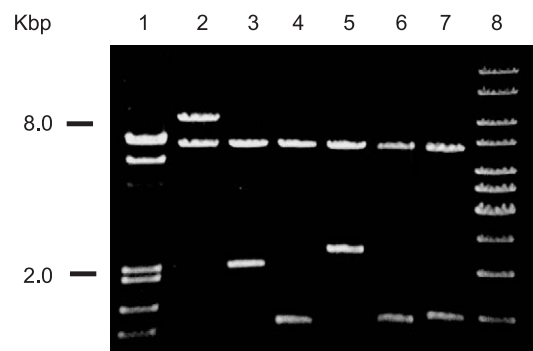


Fig. 2. Agarose gel electrophoresis of cloned BP230 cDNA fragments. To confirm the presence of BP230 cDNA inserts, recombinant baculovirus transfer vectors were digested with BamHI/Sal I or KpnI/Sal I (pBBH-BP230-C1). DNA fragments were separated by 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining. Lane 1, molecular weight marker (MWM III, Roche); lane 2, full-length BP230 cDNA; lane 3, BP230-N1 cDNA; lane 4, BP230-N2 cDNA; lane 5, BP230-N3 cDNA; lane 6, BP230-C1 cDNA; lane 7, BP230-C2 cDNA; lane 8, molecular weight marker (1 kbp ladder, BRL). Numbers to the left refer to cDNA sizes of 8.0 and 2.0 kbp of the 1 kbp ladder.

Expression of recombinant forms of BP230 in infected Sf21 insect cells

The five overlapping BP230 fragments were expressed in baculovirus-infected Sf21 insect cells and were isolated by immobilized metal chelate affinity chromatography. Due both to low expression rates and rapid degradation, recombinant full-length BP230 could not be obtained in sufficient amounts to be used for ELISA experiments. Migration of the different BP230 fragments as detected by immunoblotting using MoAB RGSH₄ antibody is depicted in Fig. 3, panel A. Immunoreactivity of a representative BP serum with the recombinant BP230 fragments is shown in Fig. 3, panel B.

Detection of autoantibodies to BP230 by ELISA

Fifty-six BP and 76 control sera (from 56 healthy controls, 10 pemphigus vulgaris patients, and 10 epider-

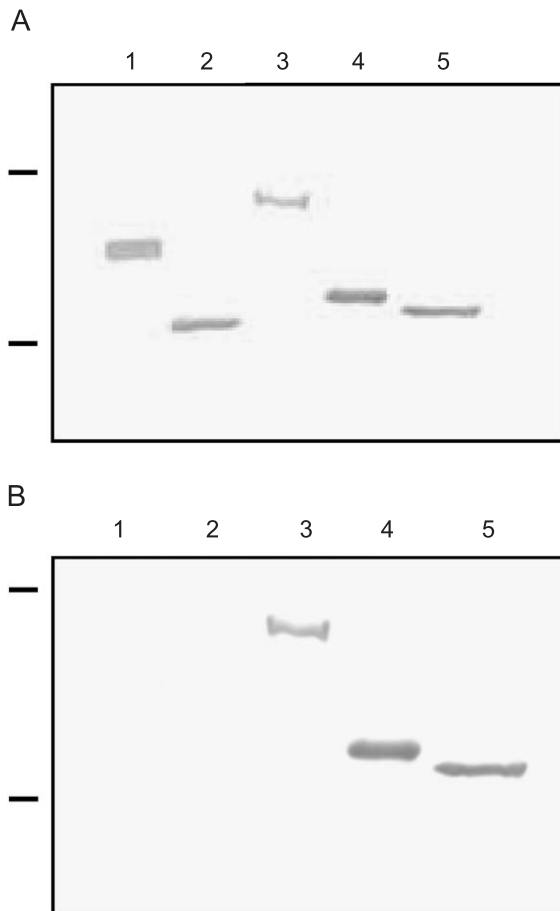


Fig. 3. Immunoblot analysis of recombinant forms of BP230. Fractionated proteins were transferred to nitrocellulose membranes and labeled with MoAB anti-RGSH₄ (panel A) and a representative BP serum (panel B). Bound antibodies were visualized by alkaline phosphatase-conjugated anti-mouse/human IgG. Lane 1, BP230-N1; lane 2, BP230-N2; lane 3, BP230-N3; lane 4, BP230-C1; lane 5, BP230-C2. Scale bars to the left of each panel refer to molecular weight markers of 94 and 45 kD, respectively.

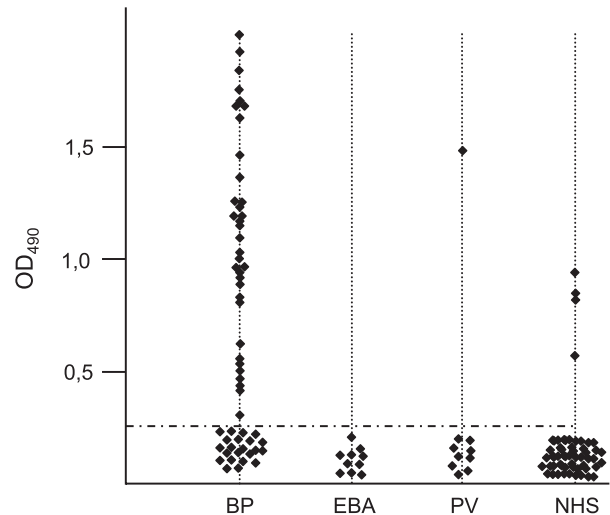


Fig. 4. ELISA analysis of BP sera using 5 overlapping recombinant forms of BP230. Thirty-five of 56 BP sera recognized at least one of the recombinant BP230 fragments. Samples were analyzed in duplicates and results are expressed as optical densities (OD) measured at 490 nm. The cut-off of the assay is shown as dotted line and was calculated by the mean $OD_{490} + 3SD$ of sera from healthy controls ($n = 56$). For each patient and control, reactivity with the specific BP230 fragment is depicted that showed the highest immunoreactivity.

molysis bullosa acquisita patients) were tested for reactivity with the five recombinant BP230 fragments using the above-described ELISA protocol. Sera with an OD_{490} greater than the mean value plus three standard deviations of the healthy control sera were considered positive. The cut-off for each antigen was set at an OD_{490} of 0.3. As shown in Fig. 4, 35 of 56 (63%) BP sera recognized at

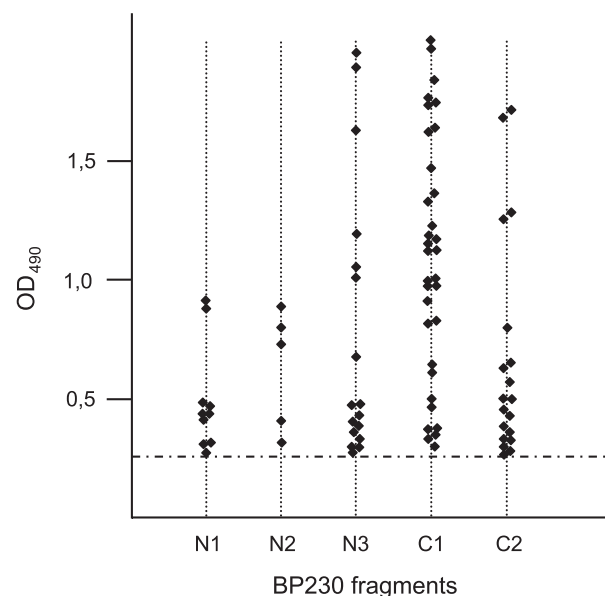


Fig. 5. Epitope mapping of BP sera using five overlapping recombinant forms of BP230. The C-terminal fragment BP230-C1 was the most frequent autoantigenic target within the BP230 molecule. Thirty-three of 56 sera (63%) reacted with this fragment. Dotted line indicates the cut-off of the assay.

Table 2

ELISA reactivity of BP sera with recombinant BP230. Only sera with reactivity to cell-derived BP230 were included ($n = 32$)

	BP230 fragments					Total
	N1	N2	N3	C1	C2	
<i>N</i>	9	5	13	22	12	23
	28%	16%	41%	69%	38%	72%

N1, recombinant BP230-N1; N2, recombinant BP230-N2; N3, recombinant BP230-N3; C1, recombinant BP230-C1; C2, recombinant BP230-C2.

least one of the recombinant BP230 fragments by ELISA. Control sera showed no reactivity with the BP230 fragments, except for BP230-N3 that was recognized by 1 of the 10 PV (10%) and 4 of the 56 (7%) healthy control sera. Taken together, 5 of the 76 control sera reacted with BP230-N3 resulting in an overall specificity of the ELISA of 93%.

Epitope mapping of autoantibodies to BP230

Thirty-three of our 56 BP sera (59%) contained antibodies against recombinant BP230-C1 (Fig. 5). These 33 sera represent 94% of the 35 BP sera, which reacted with at least one recombinant form of BP230. In 7 of these 35 BP sera (20%) BP230-C1 was the only recombinant target by ELISA. Only 10 of 56 (18%) and 5 of 56 (9%) recognized BP230-N1 and BP230-N2, respectively. BP230-N3, located adjacent to the N-terminus of the C1 fragment, was targeted by 17 of 56 BP sera (30%) and BP230-C2, next to the C-terminal end of the C1 fragment, by 19 sera (34%). All BP sera that reacted with BP230-N1 or BP230-N2 also recognized BP230-C1. All but two sera reactive with BP230-C2 also reacted with BP230-C1.

Immunoblot reactivity of BP patients with cell-derived BP230

Thirty-two of our 56 BP sera (57%) recognized BP230 derived from cellular extracts of cultured keratinocytes. Twenty-three of these 32 sera (72%) reacted with at least one of the recombinant BP230 fragments by ELISA; all but one of these 32 cases labeled BP230-C1. Further reactivity of these sera is shown in Table 2.

Discussion

In this study, we cloned the full-length BP230 cDNA and expressed overlapping recombinant fragments, covering the entire molecule, in baculovirus-infected insect cells. Utilizing these fragments, we developed an ELISA for the detection of circulating autoantibodies to BP230. In addition, like previous reports, we show that the major epitopes targeted by antibodies to BP230 are located at the C-terminal portion of the protein.

Since our attempts to purify sufficient amounts of the recombinant full-length BP230 were not successful, we generated five overlapping fragments covering the entire BP230 molecule. These were expressed in insect cells because the production in bacteria resulted in a much lower yield, mainly due increased degradation of the proteins. Previous studies using recombinant or synthetic BP230 fragments did not cover the entire molecule [12,22–25,32,33,39,40], except for the recent work by Hamada et al. [9].

Utilizing the five BP230 fragments in an ELISA, we detected autoantibodies to at least one fragment in 63% of BP sera. BP230-C1 was found to be the most frequent target; 94% of the BP sera that were positive by ELISA reacted with this C-terminal portion of BP230. Sera from a large number of controls, including healthy volunteers and patients with pemphigus vulgaris or epidermolysis bullosa acquisita, did not label any of the BP230 fragments, except for fragment N3. The overall specificity of our ELISA was 93%.

By immunoblotting, using BP230 extracted from epidermis or cultured keratinocytes, a large number of studies reported sensitivities ranging between 42% and 68% [21,22,33,39,41–43]. Some studies reported higher sensitivities; these, however, only included sera with indirect IF titer of 1:40 and greater [20,39,44] or did not specify the criteria for selecting the sera [23]. By immunoblotting of BP230 extracted from cultured keratinocytes, we found reactivity in 57% of our sera. This well compares with our ELISA sensitivity (63%). Because we used fragments, but not the full-length form of BP230, reactivity of autoantibodies with conformational epitopes could not be detected in our ELISA. Similarly, by immunoblot analysis, only reactivity with linear epitopes can be demonstrated due to the denaturing conditions before SDS-PAGE. The inability of our ELISA to detect conformational epitopes may explain the lower sensitivity of immunoblotting and our ELISA compared with immunoprecipitation using cellular BP230 from cultured keratinocytes that detected autoantibodies to BP230 in up to 97% of BP sera [19,20,41,42]. In addition, one may speculate that reactivity to the intracellularly located BP230 increases with the duration of the disease. BP sera, obtained in the initial phase of the disease, may therefore be less reactive with BP230 than sera from patients with a longer disease history. Sera included in the present study were all from patients in the initial phase of the disease, before treatment was initiated. Possibly, some of the previous studies also considered patients with longer standing disease.

Only a few reports described the use of synthetic or recombinant BP230 fragments for detection of patients' autoantibodies. In a first attempt, to replace epidermal or cell-derived BP230, six non-overlapping synthetic peptides (19 mers) were used in an ELISA that detected reactivity to BP230 in 58% of patients [32]. Subsequently, a recombinant C-terminal fragment of murine BP230 was recognized by

63% [12] and 52% [23] of BP sera by immunoblotting and ELISA, respectively. When using recombinant GST-BP230 fusion proteins by immunoblotting or ELISA, sensitivities for the detection of reactivity to BP230 varied markedly, depending on the specificity of the assay [24,32]. In addition, a recombinant 55-kDa fragment representing a C-terminal fragment of human BP230 was generated. This was targeted by 62% of BP sera by immunoblotting [22,39,40]. More recently, a recombinant 703 amino acids stretch of the C-terminal portion of BP230 was generated. When this fragment was used for immunoblotting, 84% of BP sera that were selected on the basis to react with cell-derived BP230, labeled the recombinant fragment [25]. In addition, this previous study identified an immunodominant region within the N-terminus of this fragment. In fact, this portion of BP230 corresponds to the C-terminal portion of fragment BP230-C1 which was the most frequent target among our BP230 recombinants. Hamada et al. [9], utilizing three BP230 fragments, found anti-BP230 reactivity in 80% of their BP sera, which were selected by an indirect IF titer of 1:40 and greater; the fragment that was most frequently targeted also represents the C-terminal portion BP230 [9].

In conclusion, we developed an ELISA for the detection of autoantibodies to BP230 utilizing five overlapping recombinant fragments covering the entire antigen. This ELISA should be a useful tool for the diagnosis of BP and other autoimmune diseases associated with autoantibodies to BP230. Ultimately, ELISA systems for the detection of reactivity to both BP180 and BP230 may help to replace indirect IF microscopy on organ sections in these diseases.

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