IgG Autoantibodies from Bullous Pemphigoid (BP) Patients Bind Antigenic Sites on Both the Extracellular and the Intracellular Domains of the BP Antigen 180

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Bullous pemphigoid (BP) and gestational pemphigoid (PG) are subepidermal blistering disorders associated with autoantibodies directed against two components of hemidesmosomes: the BP antigen 180 (BP180) and the BP antigen 230 (BP230). Autoantibodies against the extracellular domain (ECD) of BP180 are thought to play an initiatory role in subepidermal blister formation. To characterize the targeted antigenic sites on BP180, we have assessed the reactivity of sera from BP and PG patients against eukaryotic recombinant proteins encompassing various portions of the ECD and the intracellular domain (ICD) of BP180. Twenty-two of 22 (100%) BP sera that immunoblotted BP180 in keratinocyte extracts, bound a mutant form consisting of the entire ECD of BP180, whereas only three of these 22 sera (14%) reacted against the ECD of BP180 lacking the NC16A membrane proximal region. Thirteen out of the 22 (59%) BP sera recognized the ICD of BP180. Circulating IgG from a representative BP patient that was affinity purified against the ECD of BP180 did not bind the ICD when reblotted, indicating that there was no antigenic cross-reactivity between the ECD and the ICD of BP180. Reactivity against the ICD of BP180 was further ascertained by immunofluorescence microscopy studies showing that nine of the 22 (41%) BP sera stained COS-7 cells expressing the ICD of BP180. Using deletion mutants of the ICD of BP180, the majority of the sera was found to recognize the central region of the ICD of BP180. Specifically, an immunodominant region was localized to an 87-amino acid segment located towards the NH_2-terminus of BP180. In contrast to BP sera, five of six (83%) PG sera contained IgG that recognized exclusively the NC16A region, whereas none bound to the ICD of BP180. Together, the results indicate that in BP, autoantibody reactivity to BP180 is not exclusively restricted to the NC16A region, but that additional antigenic determinants exist on the ICD of BP180. The observed heterogeneous immune response against BP180 might reflect intramolecular epitope spreading. Because the ICD of BP180 harbors functionally important regions, it is possible that autoantibodies against the ICD of BP180 have pathogenic significance for the progression of the disease. ***Key words: autoimmunity/hemidesmosome. J Invest Dermatol 112:141–147, 1999***

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease associated with *in situ* bound and circulating autoantibodies directed against the epidermal basement membrane zone (Jordon *et al*, 1967; Stanley, 1983). The typical features of the disease, which often affects the elderly, consist of large tense blisters arising on normal or erythematous skin (Lever, 1953). Patients' autoantibodies characteristically bind two components of hemidesmosomes (HD): the bullous pemphigoid antigen 230 (BP230, also termed bullous pemphigoid antigen 1) and the bullous pemphigoid antigen 180 (BP180, also termed bullous pemphigoid antigen 2, or type XVII collagen) (Stanley *et al*, 1981, 1984; Labib *et al*, 1986). HD are multiprotein junctional complexes, that mediate attachment of epithelial cells to the underlying basement membrane in stratified and some complex epithelia, and provide a linkage between elements of the cytoskeleton and the extracellular matrix (Borradori and Sonnenberg, 1996; Green and Jones, 1996). BP230, a cytoplasmic protein belonging to the plakin family (Green *et al*, 1992), is involved in the anchorage of keratin intermediate filaments to the plasma membrane (Guo *et al*, 1995; Yang *et al*, 1996). In BP230 null-mutant mice, HD lack the inner plaque and the connection of intermediate filaments with HD is severely impaired (Guo *et al*, 1995). In contrast, BP180 is a type II transmembrane protein with a large extracellular collagenous domain (ECD), which may act as a cell-surface receptor (Giudice *et al*, 1991, 1992; Hopkinson *et al*, 1992; Li *et al*, 1992). The idea that BP180 promotes cell-substrate adhesion is supported by the observation that mutations in the BP180 gene cause generalized atrophic benign epidermolysis bullosa, an inherited skin blistering disorder characterized by defective dermo–epidermal cohesion and impaired formation of HD (Jonkman *et al*, 1995; McGrath *et al*, 1995).

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Abbreviations: BP, bullous pemphigoid; ECD, extracellular domain; HD, hemidesmosomes; ICD, intracellular domain; PG, gestational pemphigoid.

Recent transfection studies have identified domains within BP180 that are important for the localization of the protein into HD (Hopkinson *et al*, 1995; Borradori *et al*, 1997). In addition, BP180 has been shown to interact with the α6β4 integrin (Hopkinson *et al*, 1995; Borradori *et al*, 1997; Aho and Uitto, 1998; Schaapveld *et al*, 1998), another transmembrane hemidesmosomal component, as well as to affect the recruitment of BP230 into HD (Borradori *et al*, 1998). These findings suggest that BP180 participates in the stabilization of HD by interacting, directly or indirectly, with various hemidesmosomal proteins.

The majority of BP patients possess circulating IgG that bind BP230 and BP180 (Labib *et al*, 1986; Mueller *et al*, 1989; Giudice *et al*, 1994; Matsumura *et al*, 1996; Zillikens *et al*, 1997a). Specifically, recent studies have indicated that BP sera as well as gestational pemphigoid (PG) sera, a disease closely related to BP (Morrison *et al*, 1988), contain antibodies that predominantly react with an immunodominant region on the ECD of BP180 close to the transmembrane domain, the NC16A region (Giudice *et al*, 1993; Matsumura *et al*, 1996; Zillikens *et al*, 1997b). Because rabbit antibodies raised against the ECD of murine BP180, when injected into neonatal mice, induce a blistering disorder mimicking BP (Liu *et al*, 1993, 1995), it is thought that antibodies against the ECD of BP180 are pathogenetically important. Nevertheless, their exact contribution to the development of the disease in humans remains unclear. Furthermore, it is unknown whether BP180 harbors additional antigenic determinants. Recognition of distinct immunodominant epitopes on BP180 may be relevant for the initiation and perpetuation of the disease.

In this study, to further understand the pathophysiology of BP, we have sought to identify epitopes other that those located in the NC16A region by utilizing a series of recombinant forms of BP180 encompassing various portions of its ECD and its intracellular domain (ICD). The reactivity of 33 BP sera was assessed by immunoblotting analysis using extracts of transfected COS-7 cells as well as by immunofluorescence (IF) microscopy. The results indicate that BP180-specific IgG autoantibodies from BP patients recognize multiple epitopes in both the ECD and the ICD of BP180. It is likely that this heterogeneous response to BP180 reflects "intramolecular epitope spreading" (Vanderlugt and Miller, 1996; Chan *et al*, 1998) and may have pathogenic significance.

MATERIALS AND METHODS

Patients and controls Serum samples were obtained from adult patients with BP (n = 33), PG (n = 6), epidermolysis bullosa acquisita (EBA; n = 2), pemphigus vulgaris/pemphigus foliaceus (PV/PF; n = 8), and healthy volunteers (n = 8). The clinical diagnosis of BP was confirmed by histology (subepidermal blisters), direct IF microscopy (deposits of IgG and or C3 in epidermal basement membrane), and indirect IF microscopy (autoantibodies binding to the epidermal side of 1 M NaCl separated normal human skin at titers of ⩾1:20) (Gammon *et al*, 1984). In addition, by immunoblotting with keratinocyte extracts (Bernard *et al*, 1989), 22 BP sera had antibodies against BP180 (including four BP sera with antibodies against both BP180 and BP230), and 11 BP sera had antibodies uniquely against BP230 (sera uniformly diluted at 1:20). All PG patients were positive for anti-epidermal basement membrane zone antibodies by a complement fixation assay (Beutner *et al*, 1979), but bound neither BP180 nor BP230 in keratinocyte extracts by conventional immunoblotting. Serum samples from EBA patients immunoblotted type VII collagen in dermal extracts. All PV/PF patients showed typical histopathology, direct and indirect IF microscopy findings (autoantibodies reacting with the surface of epithelial cells of monkey esophagus).

Cells and antibodies The African monkey kidney cell line COS-7, which does not express endogenous BP180 and BP230 (Borradori *et al*, 1997; Niessen *et al*, 1997), was cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Paisley, U.K.) supplemented with 10% (vol/vol) bovine fetal calf serum, 100 U penicillin per ml, and 100 U streptomycin per ml. The cells were grown at 37°C in a humidified, 5% CO_2 atmosphere.

The following antibodies were used: mouse IgG1 monoclonal antibody (MoAb) anti-FLAG M2 against the FLAG peptide (DYKDDDDK) (IBI, Eastman Kodak, New Haven, CT); MoAb MYC 1–9E10.2 against a defined c-myc epitope (Evan *et al*, 1985); mouse IgG1 MoAb 1 A8c and the MoAb 233 directed against the ICD and ECD of BP180 (kindly donated by Dr. K. Owaribe, Nagoya University, Nagoya, Japan) (Nishizawa *et al*, 1993). Species-specific, affinity-isolated fluoroscein isothiocyanate-conjugated goat anti-mouse IgG (Nordic Immunochemicals Laboratory, Tilburg, The Netherlands), Texas red-conjugated goat anti-mouse IgG (Amersham, Bucks, U.K.), and fluoroscein isothiocyanate-conjugated goat anti-human IgG (Sigma, St. Louis, MO) were purchased.

cDNA constructs The BP180 nucleotide and protein sequences are numbered according to Giudice *et al* (1992) and Hopkinson *et al* (1995), respectively. Full-length BP180 was obtained from human keratinocyte RNA by reverse transcriptase-polymerase chain reaction with primers based on the published sequence of human BP180 (GenBank accession number M91669) as reported elsewhere (Borradori *et al*, 1997). The chimeric cDNA-constructs encoding the membrane localization sequence of K-Ras (KMSKDGKKKKKKSKTKCVIM) (GenBank accession number M54968 and M38506) fused with cDNA encoding either the entire ICD of BP180 or the ICD with increasing internal truncations have been previously described (Borradori *et al*, 1997). The various constructs were cloned using the X*ba* I and/or a N*ot* I restriction site in the eukaryotic expression vector pCI-neo (Promega, Madison, WI). The clone coding the entire ECD of BP180 including the last COOH-terminal 4 amino acids (IALA) of the transmembrane domain, was derived from a novel cDNA-construct BV13 kindly provided by Dr. K. Yancey (National Cancer Institute, Bethesda, MD) (Haase *et al*, 1998). This cDNA BV13, the correctness of which was verified by sequence analysis, was first cleaved with *X*baI and *N*ot I. The *X*ba I and *N*ot I blunt-ended fragment from this clone was then implanted in frame into the blunt-ended *N*ot I site of the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA), which at the 5′ end of its multiple cloning site contained a sequence encoding the myc-epitope (MEQKLISQQDL) (kindly provided by Dr. E. Sander, The Netherlands Cancer Institute, Amsterdam). To generate a cDNA clone coding for the ECD of BP180 lacking the NC16A region, wild-type BP180 was used as a template in polymerase chain reactions with recombinant *Pfu*-polymerase (Stratagene, La Jolla, CA) using a 5′ primer that contained a *N*ot I site (underlined) and the nucleotides corresponding to the sequence 1777 and 1800 of BP180 (italic) (CT-CTCGCGGCCGC*TATGGAACAGGAAAATGGAAATCTC*), whereas the 3′ end primer contained a *N*ot I and a *X*ba I site, as well as nucleotides corresponding to the sequence 4579–4599 of BP180 (CGAT-GCGGCCGCTCTAGA*TCACGGCTTGACAGCAATACT*). The *N*ot 1 cleaved polymerase chain reaction product was cloned into the *N*ot I digested pcDNA3 vector encoding the myc-epitope.

Transfection experiments COS-7 cells were transfected using the DEAE-dextran method (Cullen, 1987) and assayed for gene expression after 48 h.

Immunofluorescence microscopy Cells grown on glass coverslips in 6 well tissue culture plates were fixed with 1% formaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.5% Triton X-100 for 5 min. After rinsing with PBS and blocking with 2% (wt/vol) bovine serum albumin in PBS for 30 min at 37°C, the cells were incubated with primary antibody for 30 min at 37°C, then washed three times with PBS. Sera from BP patients and controls were uniformly diluted at 1:20 in PBS. The cells were then incubated with fluoroscein isothiocyanate- or Texas-Red conjugated anti-mouse IgG or anti-human IgG for 30 min at 37°C. The coverslips were subsequently washed, mounted with Vectashield (Vector, Burlinghame, CA), and viewed under a confocal laser scanning microscope (LSM-410, Carl Zeiss AG, Zürich, Switzerland).

Western blot analysis Cells were lyzed with 1% sodium dodecyl sulfate in 25 mM Tris-HCl, pH 7.5, 4 mM ethylenediamine tetraacetic acid, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg leupeptin per ml, and 10 μg soybean trypsin inhibitor per ml. Protein concentration in the cell lysates was determined with the BCA protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein were loaded on a 7.5% or 12% sodium dodecyl sulfate-polyacrylamide gel, separated, and electrophoretically transferred to nitrocellulose sheets as previously described (Niessen *et al*, 1997; Borradori *et al*, 1998). The filters were incubated in TBST (10 mM Tris-HCl, pH 7.6, and 150 mM NaCl, 0.1% Tween-20) containing 2% (wt/vol) bovine serum albumin and 2% (wt/vol) milk powder for 12 h at 4°C. The nitrocellulose paper was then cut into strips and incubated with a 1:20 dilution of each tested serum in TBS-0.5% Nonidet P40 for 2 h at room temperature. After washing four times for 5 min in TBS-0.5% Nonidet P40, the strips were then incubated with peroxidase-conjugated goat anti-human IgG (H + L) antibody (Institut

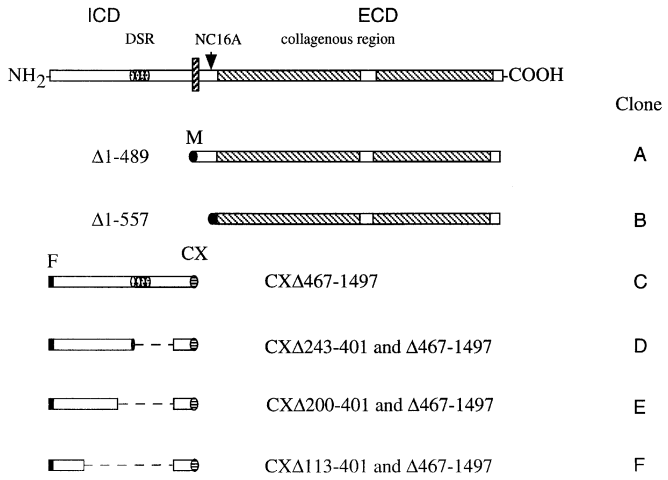

Figure 1. Schematic representation of wild-type and mutant forms of BP180. The upper two cDNA constructs encode either the extracellular domain of BP180 (clone A) or the ECD lacking the NC16A region (clone B). The lower four cDNA constructs (clones C–F) represent chimeric cDNA constructs encoding the membrane localization sequence of K-Ras fused to the intracellular domain (ICD) of BP180 with increasing internal truncations. ICD, intracellular domain; ECD, extracellular domain; M, c-myc tag; F, FLAG tag; DSR, degenerate set of four 24–26 residue tandem repeats; CX, membrane localization sequences of K-Ras. Truncations introduced by cloning procedures are indicated by the segment of amino acids that were deleted. The protein sequence of BP180 is numbered according to Hopkinson *et al* (1995).

Pasteur, Marnes la Coquette, France) or goat anti-mouse Ig (Amersham Life Sciences, Zürich, Switzerland). After washing, the strips were developed with 3,3′-diaminobenzidine-4 HCl (0.5 mg per ml) in 100 mM Tris-HCl pH 7.4 containing 0.01% H_2O_2.

Affinity purification studies IgG from a representative BP patient was affinity purified against the ECD of BP180 immobilized on a nitrocellulose paper. Extracts of transfected COS-7 cells were resolved by electrophoresis and transferred to nitrocellulose paper as described above. Horizontal strips of nitrocellulose paper containing the ECD of BP180 (or control peptides) were incubated with patient serum (diluted 1:5) overnight at 4°C. Bound antibodies were eluted from experimental (and control) nitrocellulose strips with 100 mM glycine, pH 2.5, for 10 min, then immediately neutralized with one-tenth volume of 1 M Tris, pH 8.0, and finally tested against the ICD and the ECD (control) of BP180 by immunoblotting analyses as described above.

RESULTS

Circulating IgG from BP patients bind multiple epitopes of the ECD of BP180 Most sera from BP patients contain IgG that bind the NC16A region of the ECD of BP180 (Giudice *et al*, 1993; Liu *et al*, 1993; Zillikens *et al*, 1997a). To further characterize the antigenic sites recognized on BP180, we assayed by immunoblotting the reactivity of BP sera against a series of mutant forms of BP180, which were expressed in COS-7 cells after transfection with the corresponding cDNA (**Fig 1**). To identify the recombinant proteins, the constructs were tagged at their 5′ end with either the myc-epitope or the FLAG peptide. Twenty-two of 22 (100%) BP sera that immunoblotted BP180 in keratinocyte extracts, reacted against a BP180 mutant encompassing the entire ECD of BP180 (clone A). (**Fig 2**, **Table I**). The bound protein had an apparent molecular weight of 100 kDa, as predicted on the basis of the corresponding cDNA sequence, and was recognized by both the MoAb 9E10 anti-myc-epitope and the MoAb 233 against the ECD of BP180 (not shown). Only three of these 22 (14%) BP sera contained IgG that recognized clone B-encoded BP180 recombinant consisting of the ECD of BP180 devoided of the NC16A region (**Fig 2**). The bound protein was close to its predicted molecular weight of 93 kDa. In addition, one of 11 BP patients (9%) exhibiting exclusively BP230-reactivity with keratinocyte extracts recognized clone A-encoded protein (not shown). In contrast, serum samples from normal volunteers (n = 8), patients with EBA (n = 2), and patients with PV/PF (n = 8) did not recognize clone A- or clone B-encoded recombinant proteins (not shown). The results indicate that, although BP180-specific autoantibodies predominantly recognize epitopes of the NC16A region, additional epitopes of the ECD of BP180 exist.

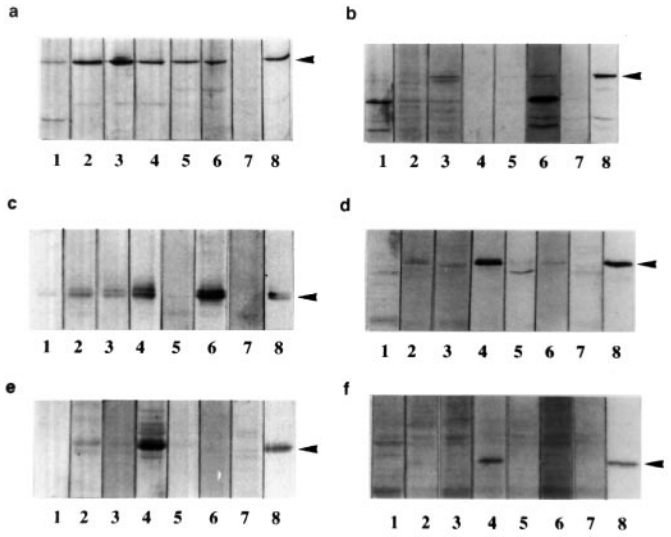

Figure 2. IgG autoantibodies from BP patients bind multiple antigenic regions on BP180. Reactivity of BP sera was assessed by immunoblotting as described in *Materials and Methods* against BP180 mutants (listed in **Fig 1**), which were expressed in transfected COS-7 cells. Reactivity against (*a*) clone A-, (*b*) clone B-, (*c*) clone C-, (*d*) clone D-, (*e*) clone E-, (*f*) clone F-encoded BP180 mutants. *Lanes 1–8* correspond to sera from patients BP2, BP6, BP8, BP12, BP16, BP21, a normal human volunteer, MoAb 9E10 anti-myc-epitope (A and B) or MoAb anti-FLAG peptide (*c*, *d*), respectively. Proteins closed to their predicted mass of 101, 93, 51.7, 35.3, 30.9, 21.4 kDa for clones A, B, C, D, E, and F, respectively, are recognized (*arrow*) by MoAb 9E10 (*a*, *b*) or MoAb anti-FLAG peptide (*c–f*). Clone C-encoded mutant appeared as a doublet (*c*), most likely due to proteolytic degradation. Each of the six BP sera exhibits a different pattern of immunoreactivity and is representative of the reactivity patterns observed with other BP sera. Note that some additional reactive bands were occasionally found with both experimental and control sera and most likely represented either unspecific background or reactivity against proteins of unknown identity in COS-7 cell extracts. Samples were separated by 7.5% (*a*, *b*) or 12.5% (in *c–f*) sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions.

IgG antibodies from BP patients immunoblot a recombinant protein encompassing the ICD of BP180 Prompted by the observation that a cDNA clone coding for a portion of the cytoplasmic domain of BP180 was isolated using a BP serum sample (Hopkinson *et al*, 1992), we examined the reactivity of BP sera using extracts of COS-7 cells transfected with a cDNA construct coding for a chimeric protein consisting of the ICD of BP180 fused to the membrane localization sequence of K-Ras (clone C). Thirteen of 22 (59%) BP patients with BP180-reactivity contained IgG autoantibodies that reacted with clone C-encoded BP180 mutant, a protein of ≈52 kDa (**Fig 2**, **Table I**). The electrophoretic mobility of this protein was the same as that of the protein recognized by the MoAb against the FLAG peptide and the MoAb 1A8c against the ICD of BP180 (not shown). When BP sera showing uniquely BP230-reactivity were tested, two of 11 (18%) sera immunoblotted clone C-encoded protein. In contrast, serum samples from normal volunteers (n = 8), patients with EBA (n = 2), and patients with PV/PV (n = 8) were negative. Finally, to exclude the possibility that autoantibodies directed against the ECD of BP180 cross-reacted with its ICD, IgG from a representative BP patient (BP7) with circulating autoantibodies binding both the ECD and the ICD of BP180 was affinity-purified against the ECD of BP180 and tested against the ICD by immunoblotting. Patient's

Table I. Clinical status of bullous pemphigoid (BP) patients and reactivity of their sera in immunoblot analyses against clone A-, clone B-, or clone C-encoded BP180 mutants and in immunofluorescence microscopy studies of COS-7 cells expressing clone C-encoded protein. All patients sera immunoblotted BP180 in keratinocyte extracts

					Immunoblotting[b]			Immunofluorescence[b]
Patients	Age	Duration	Skin lesions	Treatment[a]	A	B	C	C
BP1	77	10 y	back, limbs	CB 4 mg, PDN 30 mg	++	–	–	–
BP2	81	1 mo	generalized	Aza 100 mg, Pred 60 mg	+	–	+	+
BP3	83	1 mo	limbs	none	+	+	++	+
BP4	92	1 mo	back, mouth	CB 4 mg, PDN 25 mg	++	–	++	+
BP5	75	2 mo	trunk	CB 4 mg, PDN 15 mg	+	–	+	+
BP6	76	6 mo	generalized	CB 6 mg, PDN 30 mg	++	–	++	–
BP7	74	2 mo	limbs, face	CTX 100 mg, PDN 20 mg	++	–	++	+
BP8	86	1 mo	limbs, trunk	CB 6 mg, PDN 40 mg	++	+	++	–
BP9	75	5 mo	generalized	CB 4 mg, PDN 40 mg	++	–	++	–
BP10	85	4 mo	limbs, trunk	CB 6 mg, PDN 40 mg	+	–	–	–
BP11	53	2 mo	trunk	PDN 40 mg	+	–	+	–
BP12	87	5 wk	trunk	Aza 100 mg, PDN 40 mg	++	–	++	+
BP13	70	4 y	none	none	++	–	–	–
BP14	98	2 wk	limbs	PDN 40 mg	++	–	–	–
BP15	61	6 y	none	none	+	–	–	–
BP16	55	8 wk	trunk, arms	Aza 50 mg, PDN 16 mg	++	–	–	–
BP17	92	2 wk	trunk	Aza 100 mg, PDN 40 mg	++	–	++	+
BP18	91	3 mo	generalized	Aza 100 mg, PDN 100 mg	++	–	++	+
BP19	96	1 mo	trunk	none	++	–	–	+
BP20	88	1 y	generalized	Aza 50 mg, PDN 30 mg	++	–	–	–
BP21	86	3 mo	limbs, face	Aza 150 mg, PDN 30 mg	++	+	++	–
BP22	90	1 mo	limbs, trunk	PDN 50 mg	++	–	–	–
Positive sera					22/22 (100%)	3/22 (14%)	13/22 (59%)	9/22 (41%)

[a]CB, chlorambucil; CTX, cyclophosphamide; PDN, prednisone.
[b]Intensity of the labeling: ++, strong positive; +, positive; –, negative.

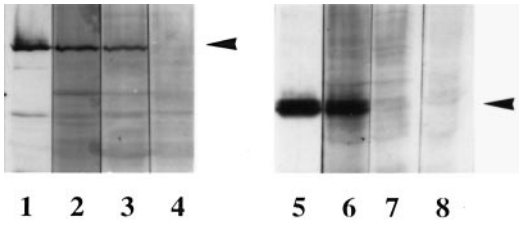

Figure 3. Affinity-purified IgG antibodies against the ECD of BP180 do not bind its ICD when reblotted. IgG autoantibodies from a representative BP patient (BP7) were affinity purified against the ECD of BP180 (or 200 kDa control polypeptides) immobilized on nitrocellulose paper and assessed against the ICD of BP180 (and its ECD, control) by immunoblotting as described in *Materials and Methods*. Reactivity against clone A- (*lanes 1–4*) and clone C- (*lanes 5–8*) encoded mutants corresponding to the ECD and ICD of BP180, respectively. *Lane 1*, MoAb 9E10 anti-myc-epitope; *lanes 2* and *6*, BP7 serum; *lanes 3* and 7, BP7 serum affinity-purified IgG against the ECD of BP180; *lanes 4* and *8*, affinity-purified IgG against 200 kDa control peptides; *lane 5*, MoAb anti-FLAG peptide. The migration position of the two BP180 mutants is indicated (*arrow*). Samples were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions.

IgG affinity purified against the ECD of BP180 did not immunoblot the ICD (**Fig 3**), but was reactive against the ECD in the same manner as total serum IgG from this patient. In contrast, eluates from control proteins showed no reactivity with either the ECD or the ICD of BP180. The results indicate that the ICD of BP180 harbors antigenic sites that are specifically recognized by IgG autoantibodies from BP patients.

IgG autoantibodies from BP patients stain transfected COS-7 cells expressing the ICD of BP180 To further ascertain that patients' sera bind the ICD of BP180, confocal laser IF microscopy studies of transfected COS-7 cells expressing the ICD of 180 were performed. As previously described (Borradori *et al*, 1997), the mutant protein encoded by clone C was properly expressed at the plasma membrane in transfected cells as assessed using the MoAb against the FLAG peptide tag (**Fig 4**). When cells were simultaneously processed with BP sera, nine of 22 (41%) BP sera with BP180-reactivity using keratinocyte extracts stained the transfected cells in a pattern indistinguishable from that obtained with the MoAb against the FLAG peptide (**Fig 4**, **Table I**); however, only eight of the 13 (62%) BP sera that immunoblotted clone C-encoded protein were positive in IF analysis, whereas the remaining five immunoblot-positive BP sera (38%) did not show any obvious staining of transfected cells. Only one of nine (11%) IF-positive BP sera was negative on immunoblot analysis. Finally, serum samples from BP patients with BP230 reactivity (n = 11), EBA

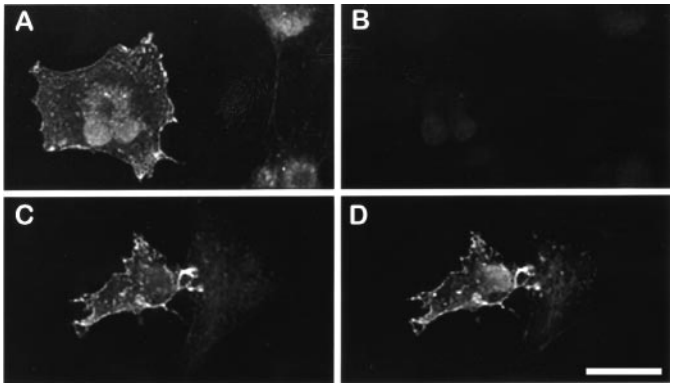

Figure 4. Sera from BP patients stain transfected COS-7 cells expressing the ICD of BP180. Confocal double immunofluorescence microscopy. Cells grown on glass coverslips were transfected with clone C encoding a chimeric protein composed of the ICD of BP180 combined with the membrane targeting sequence of K-Ras. After 36 h, cells were fixed with 1% formaldehyde, permeabilized with 0.5% Triton X-100, and subjected to double immunofluorescence using the MoAb anti-FLAG M2 peptide (*A*, *C*) and a serum from a normal volunteer (*B*), or BP12 serum (*D*). Species-specific, Texas red-conjugated goat anti-mouse IgG (*A*, *C*) and fluoroscein isothiocyanate-conjugated goat anti-human IgG (*B*, *D*). *Scale bar*: 25 μm.

Table II. Reactivity in immunoblot analyses of BP sera against clone D-, clone E-, or clone F-encoded BP180 mutants. These patients sera contained IgG immunoblotting clone C encoded BP180 recombinant

BP sera	D	E	F
BP2	–[a]	–	–
BP3	+	+	–
BP4	+	+	–
BP5	+	+	–
BP6	+	+	–
BP7	+	+	–
BP8	–	–	–
BP9	–	–	–
BP11	+	+	–
BP12	++	++	++
BP17	++	+	–
BP18	+	+	+
BP21	+	–	–
Positive sera	10/13 (77%)	9/13 (69%)	2/13 (15%)

[a]Interpretation of the results: ++, strong positive; +, positive; –, negative.

(n = 2), PV/PV (n = 8), and normal volunteers (n = 8) did not react with clone C-encoded protein in these IF microscopy studies. These results confirm that the ICD of BP180 contains additional antigenic sites and further suggest that immunoblot analysis is more sensitive than IF microscopy studies in detecting antibodies to the ICD of BP180.

The central region of the ICD of BP180 contains immunodominant epitopes To map the antigenic sites recognized by BP autoantibodies in the ICD of BP180, 13 BP sera that immunoblotted the ICD of BP180 were assayed against a series of mutant proteins, which consisted of the membrane localization sequence of K-Ras fused to the ICD of BP180 with increasing internal deletions (clones D, E, and F) (**Fig 2**, **Table II**). Ten of these 13 (77%) BP sera were reactive against clone D-encoded BP180 mutant showing a deletion of a 159-amino acids stretch in the central portion of the ICD of BP180. In addition, nine of the 13 (69%) BP sera also reacted with clone E-encoded protein with a deletion of a 202-amino acids stretch. In contrast, the protein encoded by clone F, which exhibited an additional deletion of 87 amino acids towards the NH_2-terminus, was recognized by only two of the 13 (15%) BP sera. The results indicate that BP sera recognize multiple epitopes located in the ICD of BP180. The observation that the mutant BP180 protein consisting of a 265-amino acids segment (clone E) was still recognized by the majority of these BP sera, whereas a BP180 mutant with an additional 87-amino acids deletion towards the NH_2-terminus was not (clone F), suggested that major antigenic determinants reside within this 87-amino acids stretch. It is noteworthy that this region contains two stretches of seven amino acids (residues 130–136 and 175–181) showing high antigenicity as predicted by the James and Wolf algorithm (Jameson and Wolf, 1988). In addition, MoAb 1A8c against the ICD of BP180 is reactive against this 87-amino acids segment (Borradori *et al*, 1997), suggesting that this region is also immunogenic in mice.

IgG antibodies from PG patients bind the NC16A region of BP180 Previous studies have suggested that sera from PG patients recognize the NC16A region of the ECD of BP180 (Giudice *et al*, 1993). To further assess the reactivity of PG sera, six PG sera were tested using clone A-, clone B-, and clone C-encoded proteins. Five of six (83%) PG sera immunoblotted the ECD of BP180 (clone A) (**Fig 5**), whereas none (0%) of the sera recognized a recombinant consisting of the ECD of BP180 devoid of the NC16A region (clone B). Finally, no reactivity was observed against the ICD of BP180 (clone C) in both immunoblotting (**Fig 5**) and IF microscopy studies of transfected COS-7 cells (not shown). Thus, the immune response to BP180 in PG patients appears to be largely restricted to the NC16A region.

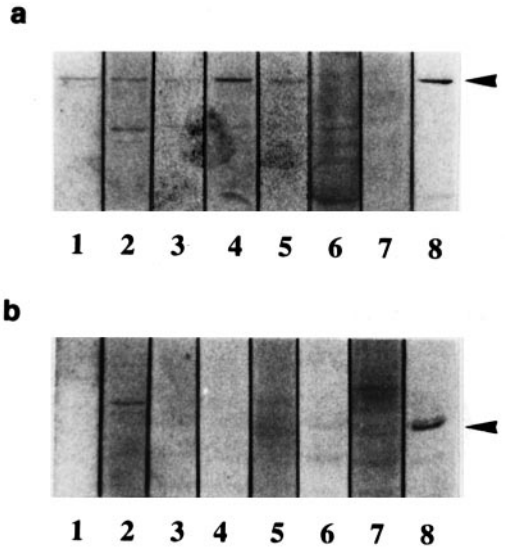

Figure 5. IgG autoantibodies from PG patients bind the ECD of BP180. Reactivity of PG sera was assessed by immunoblotting as described in *Materials and Methods* against clone A- and clone C-encoded mutant forms of BP180 (*a* and *b*, respectively). *Lanes 1–8* correspond to sera from patients PG1, PG2, PG3, PG4, PG5, PG6, a normal human volunteer, MoAb 9E10 anti-myc epitope (in *a*) or MoAb anti-FLAG peptide (*b*), respectively. The migration position of the two BP180 mutants is indicated by an *arrow*. Samples were separated by 7.5% (*a*) or 12.5% (*b*) sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions.

DISCUSSION

This study demonstrates that BP autoantibodies recognize antigenic sites spread over the entire BP180 molecule. BP sera were found to contain IgG autoantibodies that predominantly recognize not only the NC16A region on the ECD of BP180, but also the central portion of its ICD.

Previous studies using bacterial fusion proteins have indicated that the reactivity to BP180 in BP patients is largely restricted to the NC16A region, a 76-amino acid stretch located between the transmembrane domain and the large extracellular collagenous portion of BP180 (Giudice *et al*, 1993, 1994; Matsumura *et al*, 1996; Zillikens *et al*, 1997b). Our study confirms that BP180-reactive autoantibodies recognize the NC16A region; however, the observation that at least 13% of the BP180-reactive sera have antibodies against a BP180 mutant encompassing the ECD without the NC16A region (clone B) indicates that epitopes other than the NC16A region exist on the ECD of BP180. Support for this idea derives from recent findings demonstrating that some BP sera bind the distal COOH-terminal region of BP180 (Balding *et al*, 1996). These results provide an explanation for the observation that preadsorption of BP sera with the NC16A region does not always completely abolish their reactivity against the ECD of BP180 (Zillikens *et al*, 1997b).

The novel finding of our study is that a substantial number of BP180-reactive sera contain IgG autoantibodies that bind the ICD of BP180. Immunoblot analysis demonstrated that 59% of BP180-reactive sera contained IgG that recognized a mutant protein encompassing the ICD of BP180. In addition, IF microscopy studies disclosed that 41% of BP180-reactive sera stained COS-7 cells expressing the ICD of BP180. Finally, affinity purification studies using circulating IgG autoantibodies of a representative BP patient indicated that there was no antigenic cross-reactivity between the ECD and the ICD of BP180. These results firmly establish that anti-BP180 reactivity is not confined to the ECD of BP180, but that additional antigenic sites exist on the ICD. Specifically, by using a series of mutants with increasing internal deletions of the ICD of BP180, many BP sera were found to recognize a segment of 87 amino acids towards the NH_2-terminus of BP180. This segment, which is also targeted by MoAb 1A8c

against BP180 (Borradori *et al*, 1997), thus appears to encompass a major antigenic determinant.

The failure to characterize the antigenicity of the ICD of BP180 in previous studies may be explained by the fact that the reactivity of BP sera was exclusively assessed utilizing bacterial fusion proteins encompassing either the NC16A region or the distal COOH-terminus of the ECD of BP180, whereas the ICD was not tested. Furthermore, the use of recombinant BP180 forms expressed in an eukaryotic system, which yields proteins with potential proper post-translational modifications, might have facilitated the identification of additional autoantibody specificities in this study.

The identification of at least two regions of autoantibody-reactivity on BP180 is not surprising. Ample evidence exists indicating that during the course of an autoimmune disease B and T cell responses are not restricted to a unique "immunodominant" epitope, but that additional "secondary" epitopes within the same proteins are recognized (Vanderlugt and Miller, 1996; Chan *et al*, 1998). This phenomenom termed "epitope spreading" appears to be important for the perpetuation and progression of the disease. The observation that in BP two distinct molecules (i.e., BP180 and BP230) are targeted on which multiples epitopes are recognized (Rico *et al*, 1990; Tanaka *et al*, 1991; Giudice *et al*, 1993; Balding *et al*, 1996; Zillikens *et al*, 1997b), suggests that epitope spreading also occurs in this disorder; however, although it has been conjectured that autoantibodies to the ECD of BP180 have an initiatory role in the development of BP, whereas antibodies directed against cytoplasmic antigenic determinants such as BP230 arise as a secondary event (Giudice *et al*, 1993; Liu *et al*, 1993), experimental data supporting this hypothesis are missing. Although in this study no obvious correlation could be established between clinical stage or extent of the disease and the fine specificities of anti-BP180 autoantibodes, prospective studies aimed at characterizing the epitopes on BP180 and BP230, which are recognized by B cells, are required to verify this hypothesis. In addition, there is evidence that autoreactive T cell responses to BP180 are important in inducing B cells reactivity to BP180 (Büdinger *et al*, 1998). The nature of the antigenic determinants recognized by T cells, which provide help to B cells to produce autoantibodies, is likely to be critical for the initiation of the events leading to overt pathology.

Because recent studies have shown that autoantibodies can penetrate into living cells, reach their intracellular targets, and affect their function (Alarcón-Segovia *et al*, 1996), it is conceivable that autoantibodies against the ICD of BP180 have pathogenic significance. Thus, it is tempting to correlate our epitope mapping results with the finding that the ICD of BP180 is functionally important. First, it has been shown that the ICD of BP180 contains sequences critical for the incorporation of the protein into HD (Hopkinson *et al*, 1995; Borradori *et al*, 1997). Specifically, the 87-amino acid segment recognized by many sera reactive against the ICD is required for the recruitment of the protein into HD (Borradori *et al*, 1997). Second, the central region of the ICD of BP180 harbors binding site(s) critical for the efficient interaction of BP180 with the β4 subunit of the α6β4 integrin (Borradori *et al*, 1997; Aho and Uitto, 1998; Schaapveld *et al*, 1998). Based on these findings, it is possible that autoantibodies to the ICD of BP180 contribute to dermo–epidermal separation by impairing the interaction of BP180 with other elements of HD and thus their stabilization.

This report strengthens the idea that PG sera predominantly recognize epitopes localized within the NC16A region (Giudice *et al*, 1993, 1994), as five of six (83%) PG sera bound exclusively this region. In apparent contrast to what has been found with BP sera, none of the PG sera showed reactivity against the ICD of BP180; however, because of the small number of PG sera analyzed, further studies are needed to assess whether this lack of reactivity against the ICD is a distinctive feature of PG, which may explain differences in certain clinical and immunopathologic features observed between BP and PG.

Finally, immunoblot analyses using mutant forms of BP180 expressed in transfected COS-7 cells appear to represent a rapid, efficient, and specific technique to detect circulating autoantibodies against BP180. In all patients showing reactivity against BP180 by conventional immunoblotting using keratinocyte extracts, anti-BP180 autoantibodies were detected by this approach. In addition, the method appeared to increase the sensitivity of the assay for the detection of anti-BP180 antibodies, because three of 11 (27%) BP sera and five of six (83%) PG sera, which were otherwise BP180-negative by immunoblotting studies, showed reactivity against either the ECD or the ICD of BP180.

In conclusion, our study demonstrates that multiple antigenic sites are recognized on BP180. Two major immunodominant regions exist, which are localized to the extracellular, membrane-proximal NC16A region and to the central portion of the ICD of BP180. It is likely that this heterogeneous immune response to BP180 reflects intramolecular epitope spreading and may have pathogenic significance.

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