Cytoplasmic Domain of the 180-kD Bullous Pemphigoid Antigen, a Hemidesmosomal Component: Molecular and Cell Biologic Characterization

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Using a serum sample of a bullous pemphigoid (BP) patient we have isolated a cDNA clone encoding a portion of a 180-kD polypeptide component of the hemidesmosome, the "BP180 autoantigen." The identity of the clone was confirmed by the generation of a fusion protein antibody that recognizes BP180 in both a basal epithelial cell extract of bovine tongue and extracts of human epidermal cells. Immunoelectron microscopy indicates that the 588-bp cDNA encodes a cytoplasmic fragment of BP180. Furthermore, the wide species reactivity of the fusion protein suggests that this portion of BP180 is highly conserved. In cultured human epidermal cells processed for confocal immunofluorescence microscopy, the fusion protein antibody generates a punctate cell substrate-associated staining pattern that is similar to that seen using BP230 antibodies. Using the original BP180 cDNA we have now isolated additional cDNA clones encoding approximately 1800bp of BP180 the 3′ sequence of which overlaps with the sequence detailed in Giudice et al (J Clin Invest 87:734–738, 1991). Secondary structural analyses have been undertaken on the predicted amino acids encoded by the 1800bp. These suggest that the collagen-like sequences of BP180 described by Giudice et al (ibid.) are separated by a putative transmembrane region from the domain of BP180 recognized by our fusion protein antibody. Indeed, BP180 appears to belong to a relatively rare group of proteins in which the N-terminus is located in the cytoplasm and the C-terminus is extracellular. We detail some preliminary biochemical experiments in support of this hypothesis. We discuss possible functions of BP180 and BP230 in the hemidesmosome. *J Invest Dermatol 99:264–270, 1992*

The sera of patients with bullous pemphigoid (BP) contain autoantibodies against components of an adherens junction termed the hemidesmosome [1–5]. This junction is located along the basal surface of basal epithelial cells and is involved in the interaction of the epithelial cell with the basement membrane zone [6]. Furthermore, the cytoplasmic plaque of the hemidesmosome acts as a cell-surface anchorage site for bundles of keratin-containing intermediate filaments [6]. The finding that hemidesmosome structure is perturbed in certain skin diseases such as BP and junctional epidermolysis bullosa provides evidence, albeit indirect, that hemidesmosomes play an important role in the maintenance of epidermal-connective tissue integrity [7,8].

It has become apparent that there are two major BP antigens of 180 and 230 kD in epidermal cells [9,10]. Klatte et al [5] affinity purified autoantibodies against BP180 and BP230 using a basal epithelial cell extract as an antigenic source. These workers were then able to show that BP180 and BP230 are immunologically distinct and that both are components of the electron-dense cytoplasmic plaque of the hemidesmosome. More recently, using fusion protein or peptide antibodies against BP230 and BP180, Tanaka et al [11] and Diaz et al [12] have confirmed that these polypeptides reside in the hemidesmosome.

Using BP autoantibodies, we have isolated clones encoding BP180 from a lambda gt11 keratinocyte expression library. We present a partial sequence of BP180 that overlaps with the sequence detailed by Giudice et al [13] and that is 5′ to the sequence encoding a certain collagen-like domain of this antigen. We have generated a BP180 fusion protein antibody that recognizes the cytoplasmic plaque of the hemidesmosome as determined by immunoelectron microscopy. Taken together, the results we present here and those of Giudice et al [13] suggest that BP180 is a transmembrane protein, with its N-terminus in the cytoplasm. We provide biochemical evidence that the cytoplasmic domain of BP180, which is recognized by our fusion protein antibody, is approximately 55 kD. We discuss the role of BP180 in the function of the hemidesmosome and contrast this with the proposed role of BP230.

MATERIALS AND METHODS

Cell Culture Normal human keratinocytes (NHEK) were purchased from Clonetics Corp. (San Diego, CA). SCC12 F2 cells were

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Abbreviations:
bp: base pair
BP: bullous pemphigoid
BP180: the 180-kD BP antigen
BP230: the 230-kD BP antigen
cDNA: complimentary DNA
KGM: keratinocyte growth medium
NHEK: normal human epidermal keratinocytes
PBS: phosphate-buffered saline
SCC: squamous cell carcinoma
SDS-PAGE: sodium dodecylsulfate–polyacrylamide gel electrophoresis

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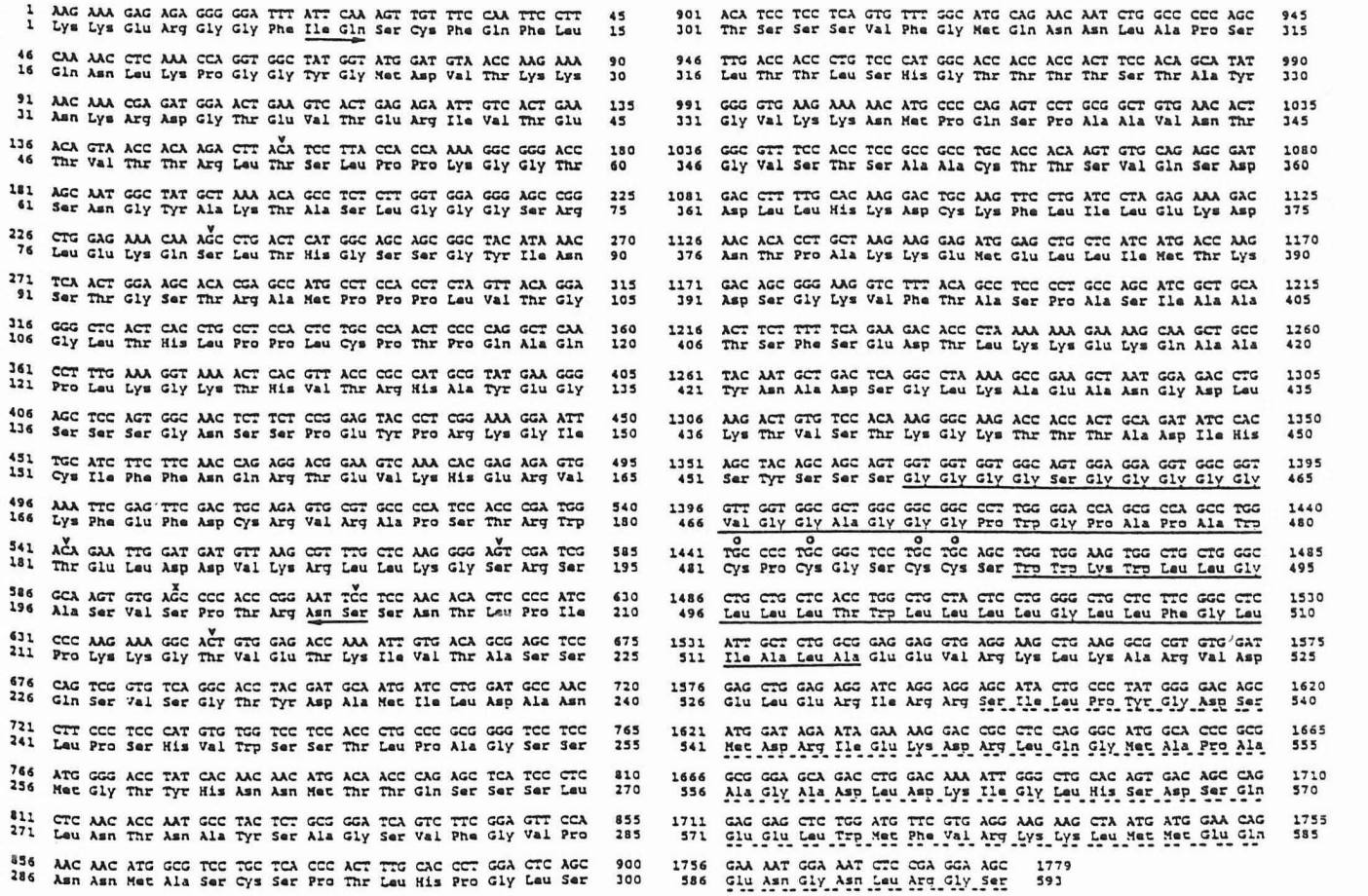

Figure 1. cDNA and predicted amino acid sequence of a portion of BP180. *Tips of the arrows*, position of the original 588-bp cDNA that was isolated from the keratinocyte lambda gt11 library. The fusion protein used for antibody preparation is encoded by this sequence. The region of the sequence indicated by the *broken line* overlaps with the 3′ sequence detailed by Giudice et al [13]. *Solid lines*, regions of high hydrophobicity, v, possible phosphorylation sites; x, possible site of phosphorylation by $p34^{cdc2}$ kinase; o, four cysteine residues between the two regions of hydrophobicity.

originally derived by Dr. James Rheinwald and were a gift from Dr. Amy Paller, Children's Memorial Hospital, Chicago, IL. The NHEK and SCC12 cell lines were maintained in keratinocyte growth medium (KGM, Clonetics Corp.).

Tissue Material Normal human skin was provided by Dr. Frank Carone of Northwestern University Medical School. Rat tissues were provided by Dr. James Bartles of Northwestern University Medical School. Bovine material was purchased from Dutch Valley Veal of Dolton, IL. Frog skin was provided by Dr. Eugene Silinsky of Northwestern University Medical School.

Screening of a Human Keratinocyte Lambda gtll Library The human keratinocyte library was the generous gift of Dr. Peter Steinert of the NIH. It was screened with a BP serum sample containing autoantibodies against both BP180 and BP230 (see below), according to Huynh et al [14]. To further characterize positive clones, plaque lifts of nitrocellulose-bound fusion protein were used to epitope select antibodies[15]. The library was rescreened with ^{32}P-random-primer–labeled cDNA probes Multiprime DNA Labeling System (Amersham, Arlington Heights, IL).

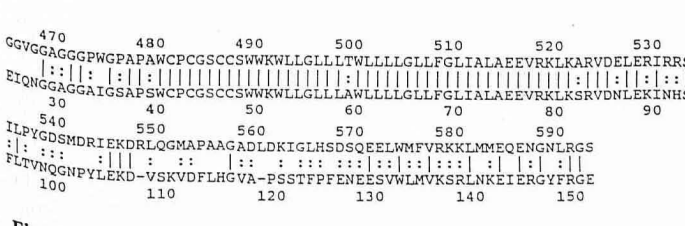

Figure 2. Comparison of a portion of BP180 sequence (indicated in a single letter code) (*upper*) with a region of the predicted amino acid sequence of a chicken collagen (*lower*) [25]. *Single lines* between residues indicate identity whereas *dots* between residues indicate similar residues.

Sequencing cDNA inserts were subcloned into M13 vectors and sequenced by the Sanger dideoxy chain termination method [16], using the USB Sequenase kit (USB, Cleveland, OH). Sequence analyses and secondary structural predictions were made using the GCG sequence analysis software package (University of Wisconsin Biotechnology Center, Madison, WI).

Antibodies The BP serum, which contains autoantibodies against both BP180 and BP230, was provided by Dr. Ruth Frienkel of Northwestern University Medical School (see below). BP230 autoantibodies from a BP serum have been detailed previously [5,17].

A cDNA insert encoding 588 bp of 180BP antigen was subcloned into the Eco R1 site of the pATH1 vector and transfected into HB101 cells [18]. A trpE fusion protein was induced as previously described [18] and the cells containing the fusion protein were processed for SDS-PAGE (see below). The fusion protein was identified on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel following staining in Coomassie Brilliant Blue (Sigma Chemical Co., St. Louis, MO) and was excised. The gel pieces were homogenized and injected into a rabbit for the generation of a polyclonal rabbit serum. In addition, some of the gel slices

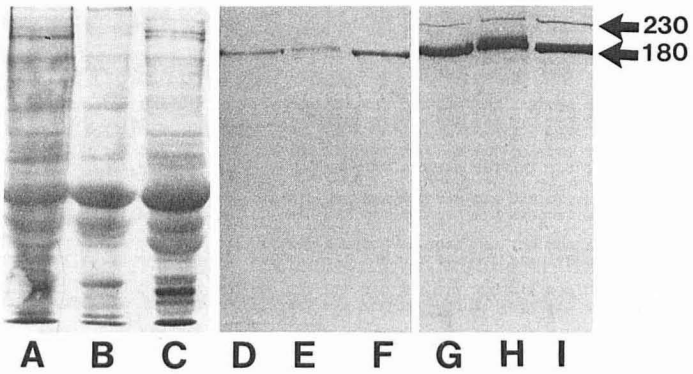

Figure 3. Western immunoblotting analysis of the BP180 fusion protein antibody. Extracts of NHEK (*lanes A, D,* and *G*) and SCC12 cells (*lanes B, E,* and *H*) and a bovine tongue basal epithelial cell extract (*lanes C, F,* and *I*) (at approximately 20 μl per *lane*) were processed for SDS-PAGE and the separated polypeptides were transferred to nitrocellulose. *Lanes A–C* are the amido black stains of the transferred proteins. *Lanes D–F* and *lanes G–I* are immunoblots using the BP180 fusion protein antibody and a BP serum sample, respectively. Note that the fusion antibodies recognize a single polypeptide of 180 kD, whereas the BP serum sample contains autoantibodies that recognize both 230- and 180-kD polypeptides.

were used to immunize mice and an IgM monoclonal antibody was prepared against the fusion protein as detailed elsewhere.*

Protein Preparations The basal cell extract enriched in hemidesmosomal components was prepared from bovine tongue epithelium as detailed in Klatte et al [5]. The extracts of cultured cells were prepared as detailed in Riddelle et al [19].

Immunofluorescence Cells grown on glass coverslips were fixed for 2 min in −2-°C acetone and air dried. Tissue material was snap frozen in liquid nitrogen, 8-μm-thick sections were prepared using a Tissue Tek cryo microtome, and the sections placed on microscope slides. The sections were fixed for 5 min in −20°C acetone and air dried. A 1:50 dilution of the rabbit fusion protein antiserum was overlaid on the cells or tissue sections, which were incubated at 37°C for 1 h. After thorough washing, the cells on coverslips and sections were incubated for a further 1 h at 37°C in fluorescein-conjugated goat anti-rabbit IgG (Kirkegard and Perry). For double labels, the cells on coverslips were first incubated in a mixture of primary antibodies and then, after washing, were incubated in a mixture of fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-human IgG (Kirkegard and Perry). The sections and cells on coverslips were viewed using either a Zeiss Photomicroscope III fitted with epifluorescence and phase optics or a Zeiss laser scan confocal microscope (LSM10). Photomicrographs were made using Kodak Plus X film, which was developed in a Diafine two-stage developer.

Immunoelectron Microcopy Cryostat sections of normal human skin were prepared, placed on glass slides, fixed in −20°C acetone for 5 min, and then air dried. The fusion antibody diluted 1:20 in phosphate-buffered saline (PBS) was overlaid on the sections, which were then incubated for 1 h at 37°C. After thorough washing, the sections were incubated in 5-nm gold conjugated goat anti-rabbit antibody for 6 h at room temperature. The sections were then processed for electron microscopy as previously detailed [5]. Thin sections of material were prepared using an Ultracut E ultramicrotome and viewed in a JEOL 100CX electron microscope at an accelerating voltage of 60 kV.

SDS-PAGE and Western Immunoblotting The bovine basal epithelial cell preparation and cultured cell extracts were processed for SDS-PAGE on 7.5% polyacrylamide gels according to Laemmli [20]. Proteins were transferred from the gels to nitrocellulose according to Towbin et al [21]. Immunoblotting was carried out as detailed in Zackroff et al [22].

RESULTS

Isolation of BP180 cDNA and Sequence Determination Twenty-five positive clones were isolated from a keratinocyte lambda gt11 library using a BP serum (see below for the immunoblotting reactivity of this particular serum) and plaque purified. Epitope selection revealed that, of these clones, two contained inserts encoding BP230 whereas one contained a 588-bp cDNA insert encoding a portion of the BP180. This 588-bp fragment was used to rescreen the library. Several overlapping clones were identified that further extended the sequence to a total of 1779 bp. The entire sequence that we have obtained to date is shown in Fig 1.

Translation of this sequence reveals that it contains an open reading frame extending the entire 1779 bp. A possible ATG start codon is located at bases 73–75 (Fig 1). Secondary structural analyses were performed using GCG software. There are two regions of hydrophobicity in the predicted amino acid sequence stretching from amino acid 456 through 480 and 489 through 514. Both are 5′ to the portion of our sequence that overlaps with the partial sequence of BP180 detailed in Giudice et al [13] (Fig 1). We indicate six possible substrate recognition sites for protein kinases [23] (Fig 1). In addition, there is a possible site for phosphorylation by $p34^{cdc2}$ protein kinase at serine residue 199 [24] (Fig 1). A 125-amino-acid stretch of BP180 beginning at residue 469 shows 55% identity and 80% homology with a portion of the predicted amino acid sequence of a chicken corneal protein [25] (Fig 2).

Immunochemical Characterization of the Fusion Protein Antibody To confirm that the clones we identified encode BP180, the 588-bp cDNA insert of our first clone was expressed as a fusion protein (see *Materials and Methods*) against which we prepared both monoclonal and polyclonal antibodies. The monoclonal antibody will be described elsewhere.* Polyclonal rabbit serum that was produced recognizes a single 180-kD polypeptide on immunoblots of human epidermal cell extracts as well as a preparation of bovine tongue basal epithelial cells enriched in hemidesmosome components (Fig 3). Furthermore, this 180-kD polypeptide is recognized on comparable blots by autoantibodies in a BP serum sample (Fig 3). It should be noted that the same BP serum also contains autoantibodies against the 230-BP antigen (Fig 3). The fusion protein antibody, on the other hand, fails to recognize BP230.

We have undertaken a limited tissue and species immunofluorescence survey using this fusion antibody. The antibody recognizes the interface between the epithelium and connective tissue of human skin and bovine tongue mucosa (Fig 4). The antibody generates a similar staining pattern in frog skin (Fig 4). In rat, the antibody recognizes the epithelium-connective tissue interface in skin, trachea, bladder and cornea but not intestine (results not shown). The antibody also recognizes basement-membrane components in human mammary tissue (result not shown).

In the cultured human epidermal cell line SCC12 the fusion protein antibody generates staining along the substrate-attached surface of cells (Fig 5). Figure 5 shows a confocal microscope image of SCC12 cells processed for double labeling using BP230 autoantibodies and the fusion protein antibody. D″ in Fig 5 is a computer-generated z-section of the cells revealing that BP180 antigen is distributed along the region where the cell is in contact with the glass coverslip. It is remarkable that the surface-staining patterns generated by both the fusion protein and BP230 antibodies show continuity from cell to cell (Fig 5).

At the electron-microscopic level, the fusion protein antibody binds to the cytoplasmic portion of the plaque of hemidesmosomes of human skin (Fig 6). Gold particles are concentrated over the

* Riddelle, Hopkinson, Jones (submitted for publication).

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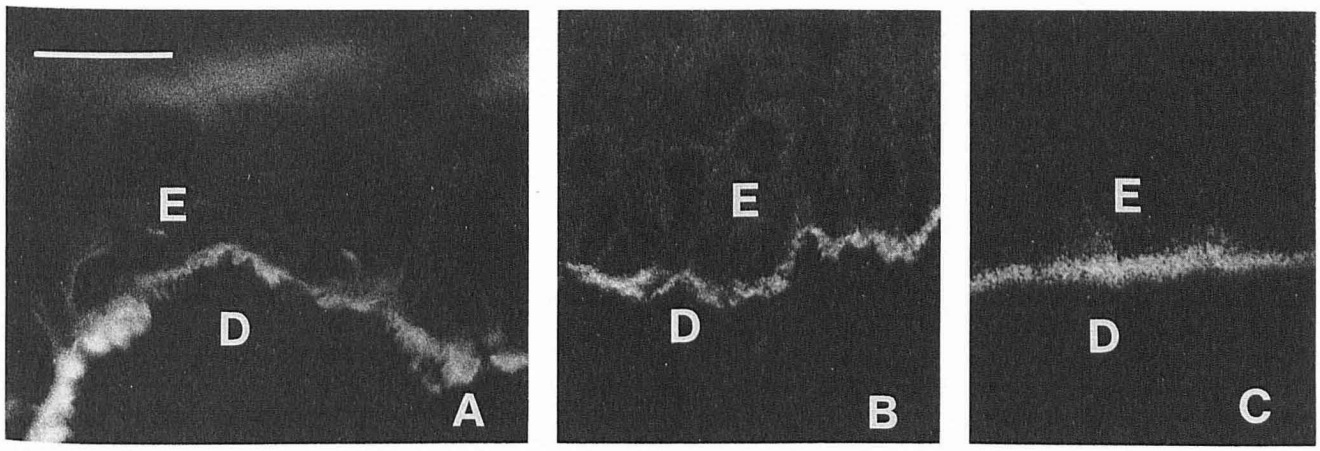

Figure 4. Cryosections of human skin (*A*), bovine tongue (*B*), and frog skin (*C*) were processed for immunofluorescence microscopy using the fusion protein antibody. Note that in each of these tissues the antibodies generate staining along the epithelial (E), dermal (D) border. *Bar*, 30 μm.

region of the plaque to which keratin bundles attach (Fig 6). No gold particles are observed at the extracellular face of the hemidesmosome (Fig 6).

Trypsin Digestion of BP180 in Epithelial Cells We have followed the fate of both BP180 and BP230 following trypsinization of human epidermal cells. Confluent epidermal cells maintained on tissue-culture plastic were rinsed briefly in PBS and then treated for 5 min in 0.05% trypsin in PBS. The cells were removed from the dish by aspiration and collected by centrifugation. The trypsin solution was inactivated by briefly washing the cells in tissue-culture medium containing 10% fetal calf serum. Over 95% of the cells are viable following such treatment as assessed by vital dye staining.

Extracts of freshly trypsinized epidermal cells and, as a control, extracts of intact confluent monolayers of cells, were processed for immunoblotting using the fusion-protein antibody and a BP serum (Fig 7). The BP serum recognizes BP230 in both the trypsinized and monolayer extract. However, this same serum fails to recognize BP180 in the trypsinized preparation (Fig 7). The latter is also the case for the fusion protein. Indeed, the fusion protein antibodies and autoantibodies in the BP serum recognize a 55-kD species in the trypsinized cell extract (Fig 7). This low–molecular-weight species is absent in the extract of the cell monolayer (Fig 7).

DISCUSSION

In this study, we detail the sequence for a portion of the epidermal autoantigen BP180. We have confirmed that the clones we have isolated encode for this polypeptide by the production of a fusion protein antibody. Giudice et al [13] have already described a partial sequence for BP180 and the 3′ region of the BP180 sequence that we present here overlaps with their 5′ sequence. Giudice (personal communication) has recently derived another 2000 bp of sequence 3′ to the published data. All told, this makes about 4800 bp of open reading frame. This would encode a polypeptide of approximately 160 kD, 20 kD short of the apparent molecular weight of BP180 on polyacrylamide gels. It is possible that some of this missing molecular weight may be accounted for by post-translational modifications such as glycosylation, particularly as our data supports the idea that BP180 possesses an extracellular domain (see below). Certainly, there is a potential start codon (ATG) towards the 5′ of our sequence. Furthermore, G residues in the −9, −3, and +4 positions flanking this codon are consistent with the consensus sequence for the initiation of translation according to Kozak [26]. However, we cannot rule out the possibility that we may still lack 5′ sequence. We are currently attempting to purify BP180 so that we can chemically derive the sequence of its N-terminus.

An analysis of the predicted amino acid sequence of BP180 indicates that it contains two regions of high hydrophobicity beginning at residues 456 and 489. Both these are potential transmembrane domains or regions of close BP180 apposition to the plasma membrane. Giudice et al [13] have shown that the carboxy terminus of BP180 possesses collagen-like domains. Furthermore, an antibody that these workers have generated against a region of BP180 close to these domains appears to localize to the lamina lucida side of the hemidesmosome.* Our antibody recognizes an epitope of BP180 that is located towards the N-terminus of the molecule, separated from the collagen-like domains by the regions of high hydrophobicity. Moreover, our fusion protein antibody binds to the cytoplasmic face of the hemidesmosome. Together these results argue against the possibility that there are two membrane-spanning domains with both the N- and C-terminus being cytoplasmic. Rather, they strongly suggest that the N-terminus of BP180 is cytoplasmic, whereas the C-terminus is extracellular. This is the orientation of only about 5% of transmembrane proteins. Other examples include the type I and type II macrophage scavenger receptors. Remarkably, the latter proteins, like BP180, possess extracellular collagen-like domains [27,28].

The results from our trypsin-digestion experiments support that BP180 is an integral membrane with both cytoplasmic and extracellular domains. There are many possible trypsin digestion sites in the putative extracellular domain of BP180. Some of these are located close to the predicted transmembrane region of the molecule. We presume that the only portion of BP180 that would be sensitive to trypsin in the experiments we have undertaken would lie outside the cell. Thus the residue of trypsin digestion of BP180 would be the cytoplasmic domain and the membrane-spanning region, which together have a predicted molecular weight of either 50 or 54 kD, depending upon which of the two hydrophobic domains actually spans the membrane. Because the molecular weight of the trypsin product is closer to the higher of these two figures, we propose that the transmembrane domain begins at residue 489, rather than 456.

It should be noted that, in contrast to BP180, BP230 is apparently unaffected by trypsin treatment. This strongly suggests that BP230 is entirely cytoplasmic. If this is the case, then the small 17 hydrophobic amino acid stretch that certain authors have noted towards the C-terminus of BP230 is more likely to function as a membrane anchor for the molecule rather than a membrane-spanning region, as Sawamura et al [29] have suggested.

Within the putative cytoplasmic domain of BP180 there are sev-

* Giudice (personal communication).

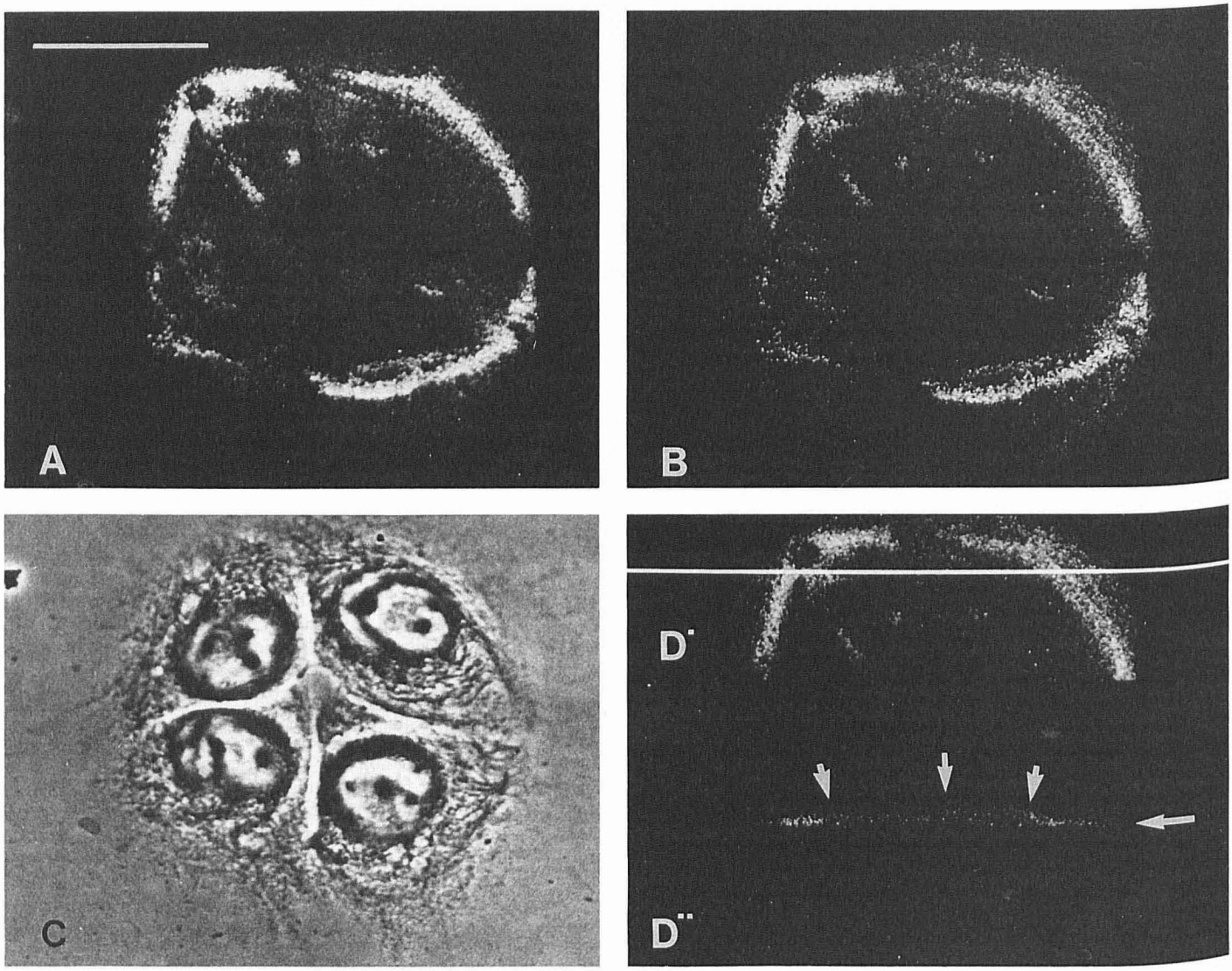

Figure 5. SCC12 cells were processed for double-label immunofluorescence using BP230 autoantibodies (*A*) and the BP180 fusion protein antibody (*B,D*). The preparation was viewed in a confocal microscope. *C*, phase-contrast image of the cells for orientation. The staining patterns in *A* and *B* are essentially identical. *D'* shows a portion of the image shown in *C*. *White line*, region where a computer-generated z-section was made and this is shown in *D''*. Note that the staining generated in the cell is close to the substrate (*large arrow*). The apical surface of the cell (i.e., the surface of the cell opposite the glass coverslip) is indicated by *small arrows*. *Bar*, 10 μm.

eral potential phosphorylation sites as well as a consensus sequence for recognition by $p34^{cdc2}$ kinase. The latter enzyme is involved in the initiation of mitotic and meiotic M phase [24]. In this regard, it is likely that as a cell enters the M phase of the cell cycle, there may be modifications in cytoskeletal-hemidesmosome linkage and/or hemidesmosome-matrix interactions. Moreover, hemidesmosomes may even disassemble. It will now be of interest to determine whether phosphorylation of BP180 by $p34^{cdc2}$ kinase plays a role in these phenomena in much the same way that it is involved in nuclear lamina disassembly and reorganization of the cytoskeleton [24].

The fusion protein antibody recognizes those tissues, such as epidermis, bladder, trachea, and mammary glands, that contain hemidesmosomes. The antibody fails to recognize tissues such as the intestine, which do not possess bona fide hemidesmosomes. The antibody shows considerable species cross-reactivity, as it recognizes amphibian as well as mammalian epidermis. This suggests to us that the N-terminus of BP180 is conserved and may play a crucial role in the function of BP180. It presumably contributes to the structure of the plaque and may be involved in BP180 interactions with other elements in the plaque such as BP230, the cytoplasmic domains of $\alpha_6\beta_4$ integrins, or a hemidesmosomal polypeptide of 200 kD characterized by Kurpakus and Jones [17]. Of course, the cytoplasmic domain of BP180 may be involved in the linkage of intermediate filaments to the hemidesmosomal plaque, a role that has already been suggested for BP230 and the cytoplasmic tail of β_4 integrin [30,31]. It should be noted that four cysteine residues are located close to the putative membrane-spanning domain of the molecule and these may be involved in cross linking BP180 to other hemidesmosomal plaque elements. Alternatively, these cysteine residues may play a role in the homophilic association of BP180 molecules.

A portion of the putative cytoplasmic region and the extracellular domain of BP180 shows a high amino acid sequence homology with the partial sequence presented for a collagen characterized at the molecular level in chick cornea [25]. In fact this chick protein may well be the avian form of BP180.

We have already discussed the evidence that our original BP180 cDNA clone encodes a cytoplasmic portion of BP180. This clone was identified using BP180 autoantibodies. Klatte et al [5] also showed that BP autoantibodies appear to recognize cytoplasmic

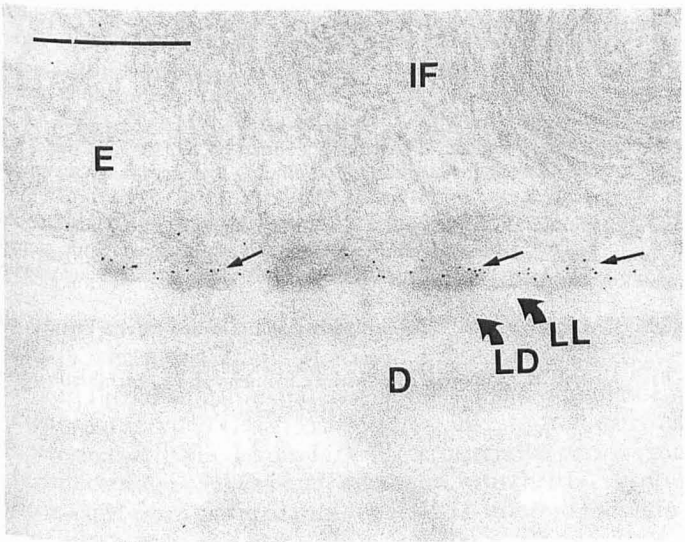

Figure 6. Immunogold localization of the fusion protein antibody on a cryosection of human skin. Gold particles are concentrated on the cytoplasmic aspect of hemidesmosomal plaques (*small arrows*). IF, keratin intermediate filaments; E, epidermal cell; D, Dermis; LL, Lamina lucida; LD, lamina densa. *Bar*, 250 nm.

epitopes of BP180 by immunoelectron microscopy. On the other hand, the BP180 cDNA clones that Diaz et al [12] and Giudice et al [13] identified using BP autoantibodies encode the putative extracellular domains of the molecule. This is of interest because it implies that either BP patients possess distinct immuno-reactivities with BP180 or that their immune response to BP180 is a polyclonal one.

BP autoantibodies may play a causative role in BP pathogenesis although this remains controversial. Certainly, it is difficult to believe that BP230 autoantibodies and those BP180 autoantibodies which recognize cytoplasmic epitopes, could play a role in blister formation [5]. Indeed, much more likely candidates for causative agents in the disease are those BP180 autoantibodies against the extracellular domains of BP180 that Diaz et al [12] and Giudice et al [13] have identified.

In summary, we have undertaken a cell and molecular study of BP180. This protein appears to be a type II transmembrane protein with a conserved cytoplasmic domain. It is now our goal to analyze the protein-protein interactions of both the cytoplasmic and extracellular domains of BP180. This should provide new insights into the structure of the hemidesmosome and its role as a connector of the epidermis to the basement membrane zone.

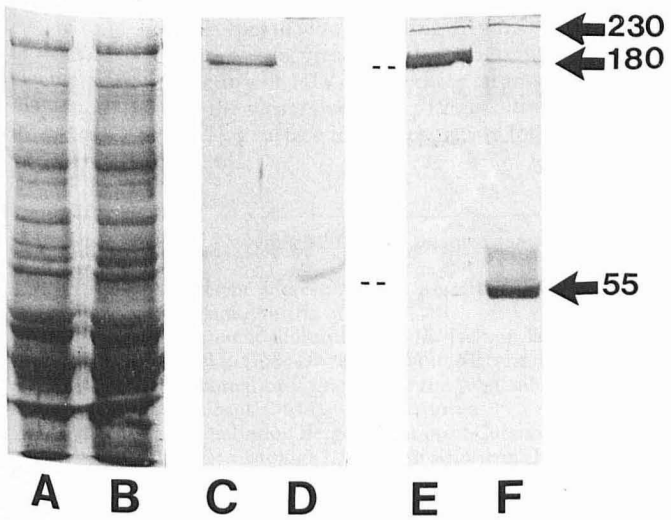

Figure 7. Extracts of SCC12 cells prepared from an intact cell monolayer (*A*, *C*, and *E*) or freshly trypsinized SCC12 cells (*B*, *D*, and *F*) (about 20 μl per lane) were processed for SDS-PAGE and subsequently transferred to nitrocellulose. *Lanes A* and *B* show amido black stains of transferred proteins. *Lanes C* and *D* and *lanes E* and *F* are immunoblots using the BP180 fusion protein antibodies and a BP serum, respectively. Note that the fusion protein antibodies and the BP autoantibodies strongly stain a 180-kD polypeptide in the SCC12 cells in *lanes C* and *E*. However, both antibody samples only weakly recognize a comparable polypeptide in *lanes D* and *F*. Rather, in the latter lanes, they recognize a polypeptide of around 55 kD. Note that the BP autoantibodies show comparable reactivity with BP230 in both the intact and trypsinized cell preparations (*lanes E* and *F*).

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