A Recombinant Form of the Human BP180 Ectodomain Forms a Collagen-like Homotrimeric Complex†

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ABSTRACT: BP180 is a glycoprotein constituent of the epidermal anchoring complex and a major antigenic target of autoantibodies associated with bullous pemphigoid, a blistering skin disease. The C-terminal extracellular domain of BP180 contains 15 domains composed of Gly-X-Y tandem repeats, which are predicted to form collagen-like triple helices. To facilitate the structural analysis of this protein, the extracellular region of human BP180 was expressed as a secreted protein (sec180e) in transiently transfected COS-1 cells. Gel filtration and sedimentation analyses demonstrated that sec180e exists in two forms: a globular monomeric form and a high-molecular mass multimeric form with an elongated conformation. Pulse−chase and cross-linking experiments established that the sec180e complex is a stable homotrimeric structure which assembles prior to secretion from the cell. On the basis of its calculated molecular mass, the oligomeric state of the sec180e complex is 3.25. With a Stokes radius of 13.6 nm, a sedimentation coefficent of 6.5 S, and a frictional ratio of 3.01, the sec180e protein appears to be highly extended (length to width ratio is between 52 and 60), yet is more flexible than a rigid rod. BP180 isolated from human epidermis was also shown to exist in a high-molecular mass complex which, like sec180e and other collagenous proteins, is SDS-stable but heat-labile. These findings strongly suggest that the BP180 ectodomain exists as an elongate, flexible homotrimer. This trimerization is likely to result from the formation of stable collagen triple-helical and coiled-coil type structures and does not depend upon the presence of the cytoplasmic or transmembrane domains of this protein.

Bullous pemphigoid (BP)[1] is an acquired blistering skin disease that is characterized by a detachment of basal epidermal keratinocytes from the basement membrane and by the presence of circulating and tissue-bound autoantibodies directed against basement membrane zone antigens (Korman, 1993; Zillikens et al., 1996). BP180, a 180 kDa glycoprotein also known as the α1 chain of type XVII collagen (Giudice et al., 1992; Li et al., 1993), was identified as one of the primary antigenic targets of BP autoantibodies and was shown to be a major component of the hemidesmosome, a plasma membrane-associated organelle located at the basal surface of stratified epithelia (Labib et al., 1986; Diaz et al., 1990). The identifying characteristic of the hemidesmosome is an electron dense intracellular plaque that serves as an insertion site for intermediate filaments (Selby, 1955; Kelly, 1966). Associated with the hemidesmosome are transmembranous constituents, an extracellular, sub-basal dense plate, and fibrillar components of the papillary dermis (Kelly, 1996; Shienvold & Kelly, 1976). These hemidesmosome-associated elements play a key role in the anchorage of basal epithelial cells to the extracellular matrix and may also function in transmembrane signaling (Staehelin, 1974; Schwartz et al., 1990).

Epitope mapping studies revealed the presence of an immunodominant autoantibody-reactive site, designated MCW-1, that mapped to the noncollagenous extracellular region of BP180 adjacent to the transmembrane domain (Giudice et al., 1993). The hypothesis that autoantibodies reacting with the MCW-1 region are involved in the initiation of subepidermal blistering has been supported by animal model studies [reviewed by Giudice et al. (1995)]. For use in these studies, rabbit antibodies were generated against the region of murine BP180 corresponding to the human BP180 MCW-1 epitope (Liu et al., 1993). Passive transfer of anti-murine BP180 antibodies into neonatal BALB/c mice resulted in the induction of a blistering skin condition that reproduced all of the key immunopathological features of the human disease, BP (Liu et al., 1993 and 1995). In addition to BP, there are two other blistering disorders, herpes gestationis and cicatricial pemphigoid, in which the patients exhibit an autoimmune response against the BP180 antigen. Autoantibodies from all three groups of patients react with a site at or near the MCW-1 epitope (Giudice et al., 1993, 1994; Balding et al., 1996), while cicatricial pemphigoid sera concomitantly show a pronounced reactivity with a distinct

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[1] Abbreviations: BP, bullous pemphigoid; DSP, dithiobis(succinimidyl propionate); PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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FIGURE 1: The BP180 protein. This figure shows a schematic diagram of the human BP180 protein with the sites recognized by rabbit antisera R594 and R136 indicated. The shaded oval represents the membrane-spanning domain. The extracellular C-terminal tail contains 15 collagenous domains, designated by the black rectangles.

epitope located near the C terminus of BP180 (Balding et al., 1996).

On the basis of cloning and sequence analysis of the BP180 cDNA (Giudice et al., 1991, 1992), the encoded protein was predicted to be a type II transmembrane glycoprotein with a single membrane-spanning domain and with a series of collagen-like, Gly-X-Y repeating sequences that encompass a majority of its extracellular region (see Figure 1). This domain organization has been confirmed by ultrastructural and biochemical studies (Giudice et al., 1993; Hopkinson et al., 1992; Ishiko et al., 1993). A recent immunoelectron microscopic study using domain-specific anti-BP180 antibodies has demonstrated that the extracellular region of BP180 spans the lamina lucida, with the C-terminal region of the protein colocalizing with laminin 5, an anchoring filament protein (Bedane et al., 1997). These data suggest that the BP180 protein exists in an elongated conformation, with its C terminus inserting into the lamina densa.

On the basis of its primary structure, we have previously hypothesized that the extracellular region of BP180 forms a collagen-like triple helix, a conformation consistent with the recent ultrastructural data. Structural analysis of the endogenous BP180 protein has been hampered due to its low abundance and its highly insoluble nature, which is apparently the result of its tight association with the cytoskeleton (Grekin et al., 1981). Very recently, Hirako *et al.* (1996) have reported that a putative BP180 homologue isolated from bovine cultured cells forms an asymmetrically elongated trimer with a central rod domain that is approximately 60−70 nm in length. In the present study, we have employed a molecular genetic approach to investigate the structural properties of the human BP180 ectodomain when expressed in the absence of the transmembrane and intracellular domains. We designed an expression system whereby the entire extracellular region of human epidermal BP180 is expressed as a secreted peptide (sec180e) in COS-1 cells. A similar strategy has previously been used by Krieger and co-workers to elucidate the structure and function of the extracellular domain of the macrophage scavenger receptor (Resnick et al., 1993).

MATERIALS AND METHODS

Construction of Expression Constructs. The eukaryotic expression construct, pSVsec180e, encoding the sec180e protein was prepared using the pSVL vector (Pharmacia Biotech, Piscataway, NJ), which contains the SV40 late promoter region and the SV40 VP1 intron and polyadenylation signal as well as the SV40 origin of replication. To facilitate the construction, a *Not*I site was introduced into the multiple cloning region of pSVL using a *BamH*I/*Not*I adaptor. The cDNA encoding the N-terminal signal sequence and propeptide of human desmoglein-1, which includes the Kozak consensus translation initiation site, was obtained by PCR amplification (Mullis et al., 1986; Giudice et al., 1993) and subcloned into the *Sma*I/*Not*I sites on the modified pSVL vector. This construct, designated pSVdsg1-leader, was used as a negative control for the sec180e expression studies. The pSVsec180e construct was generated by inserting the cDNA encoding the entire BP180 extracellular domain into the *Not*I site of pSVdsg1-leader. The BP180 insert was assembled from two overlapping cDNA clones with a common *Kpn*I site at nucleotide position 2480. BP180 nucleotides 1570−2480 were obtained from clone BP180-EC1, which contained an engineered *Not*I site at the 5′ terminus, and nucleotides 2481−4665 were derived from clone BP180-FX, which contained an engineered 3′-terminal *Not*I-site. Clones BP180-EC1 and BP180-FX were digested with *Not*I and *Kpn*I, and the appropriate segments were ligated with *Not*I-digested pSVdsg1-leader. The final construct, pSVsec180e, was sequenced by standard methods (Sanger et al., 1977; Liu et al., 1992) to ensure that (a) the BP180 segments were in the correct orientation, (b) no sequence errors were introduced by PCR amplification, and (c) the BP180 reading frame was in register with that of the desmoglein-1 leader sequence.

Rabbit Antisera. Two rabbit antisera used in these studies, R594 and R136, react with distinct sites on the extracellular region of BP180. R594 was raised against a BP180 fusion protein containing a 42-amino acid stretch of extracellular BP180 beginning 18 amino acids from the membrane-spanning domain. R136 was raised against a BP180 fusion protein containing 49 amino acids of BP180 located near the C terminus of the protein. In addition, rabbit antiserum R58, which contains antibodies against recombinant glutathione *S*-transferase, was used as a negative control. All three rabbit antisera were generated as previously described (Giudice et al., 1993).

Transient Transfections and Metabolic Labeling. COS-1 cells were maintained with DMEM media containing penicillin/streptomycin (1 unit/mL) and 10% fetal bovine serum. Transient transfections were performed with either the control construct or the sec180e construct using the DEAE-dextran method of transfection as previously described (McCutchan & Pegano, 1968; Luthman & Magnusson, 1983; Zhang & Dahms, 1993; Emery et al., 1995). Forty-eight hours after DNA addition, the cultures were washed twice with PBS and fresh medium was added. For the experiment involving iodination of the transfected cell products, the fetal bovine serum was omitted at this final medium change. For metabolic labeling, the cells were cultured in methionine-free medium with 10% fetal bovine serum supplemented with 25 mCi/mL of [^{35}S]methionine. After 24 h of labeling, the conditioned medium was collected and stored at −80 °C. In the pulse−chase experiments, 24 h after DNA addition, a 100 mm plate of transfected cells was trypsinized and split into seven 35 mm dishes to ensure an equal transfection efficiency for each chase time point. Also, instead of 25 mCi/mL, 300 mCi of [^{35}S]methionine per milliliter of medium was used.

Immunoblotting and Immunoprecipitation. Immunoblotting was carried out using a standard protocol (Laemmli, 1970; Towbin et al., 1979; Giudice et al., 1993). Briefly, samples were electrophoresed on SDS−polyacrylamide gels

and electrotransferred to nitrocellulose. A standard transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol was used for all experiments except for those involving gels containing epidermal extracts, where 0.01% SDS was added to the transfer buffer to facilitate transfer of high-molecular mass proteins. Blots were blocked for 45 min with PTX buffer [0.01 M phosphate (pH 7.5), 0.2% Triton X-100, 0.15 M NaCl, and 1 mM EGTA] containing 4% BSA. The primary antibody was diluted 500-fold in the PTX/BSA solution and incubated with the blot for 2 h. The blots were washed five times, 5 min each, with GB buffer [50 mM triethanolamine (pH 7.4), 0.5% Triton X-100, 0.1 M NaCl, 1 mM EDTA, and 0.1% SDS]. After reblocking for 45 min, blots were incubated with [^{125}I]protein A, washed, and exposed to X-ray film.

Immunoprecipitation analysis was performed either directly on transfected cell-conditioned medium, on conditioned medium that was cross-linked, or on column fractions collected during sedimentation or gel filtration analyses. Prior to immunoprecipitation, the samples were centrifuged at 100000*g* for 1 h at 11 °C to remove insoluble aggregates. Five microliters of the primary antibody was added to a 0.5 mL aliquot of precleared, conditioned medium, and the sample was incubated on a rocker platform at room temperature. After 1 h, 15 μL of protein A-conjugated agarose beads (Sigma, St. Louis, MO; catalog number # P-0932) were added and incubated for an additional 1 h at room temperature. The agarose beads were pelleted in a microfuge and were washed five times with culture medium containing 1% sarcosyl but lacking fetal bovine serum. After the final wash, 25 μL of 5× SDS−PAGE sample buffer was added to the beads and the samples were vortexed and then boiled for 5 min (except where indicated in the text). The beads were pelleted, and 35 μL of the supernatant fraction was analyzed by SDS−PAGE and fluorography using the Entensify reagents (Dupont, Boston, MA).

Glycerol Gradient Sedimentation. To a 500 μL sample of metabolically labeled, sec180e-conditioned medium were added sarcosyl, deoxycholate, and Triton X-100 to final concentrations of 0.25, 0.25, and 1%, respectively. The samples were cleared of aggregates by centrifugation as described above. The sample was loaded onto an 11.5 mL, 10% to 30% (v/v) linear glycerol gradient prepared with the same detergent composition as the samples. The gradients were centrifuged for 16.5 h at 135000*g* at 16 °C using an SW41-Ti rotor. Fractions of 20 drops each were collected by puncturing the bottoms of the tubes, and sec180e was detected by immunoprecipitation followed by SDS−PAGE and fluorography. The gradients were calibrated using standards with known sedimentation coefficients. The standards were detected by SDS−PAGE followed by staining with Coomassie Blue.

Superose-6 Gel Filtration Chromatography. A Superose-6 preparative column (125 mL bed volume) was equilibrated with 0.25% sarcosyl, 0.25% deoxycholate, and 1% Triton X-100 in PBS. The void volume of the column was determined using blue dextran, and the column was calibrated with standards of known Stokes radii using a flow rate of 0.3 mL/min. The chromatographic profile of each standard was determined by SDS−PAGE analysis of the column fractions followed by staining with Coomassie Blue. A 500 μL sample of metabolically labeled, sec180e-conditioned medium containing 0.25% sarcosyl, 0.25% deoxycholate, and 1% Triton X-100 was cleared of aggregates by centrifugation and was loaded onto the Superose-6 column which was run at a flow rate of 0.3 mL/min. Fractions were collected beginning at the void volume, and each fraction (1.4 mL volume) was subjected to immunoprecipitation analysis, as described above. sec180e was detected by fluorography of the SDS−polyacrylamide gel.

Calculations. The molecular mass (M_r) and frictional ratio (f/f_0) of sec180e were calculated using the following equations (Siegel & Monty, 1966):

$$M_r = 6\pi N\eta r_s s_{20,\omega}/(1 - \bar{\nu}\rho) \quad (1)$$

$$f/f_0 = r_s(4\pi N/3M_r\bar{\nu})^{1/3} \quad (2)$$

where N is Avagadro's number (6.022×10^{23} mol^{-1}), η is the viscosity of water at 20 °C (1.0×10^{-3} kg m^{-1} s^{-1}), $\bar{\nu}$ is the partial specific volume, ρ is the density of H_2O at 20 °C (0.998 g/mL), r_s is the Stokes radius, and $s_{20,\omega}$ is the sedimentation coefficient. The axial ratio, P, of the length to width of sec180e was calculated using successive approximations from the frictional ratio by fitting the protein as a cylinder using the equation $f/f_0 = (2/3)^{1/3}[P^{2/3}/-0.3 + \ln(2P)]$ and by fitting the protein as a prolate ellipsoid using the equation $f/f_0 = P^{-1/3}(P^2 - 1)^{1/2}/\ln[P + (P^2-1)^{1/2}]$ (van Holde, 1985).

Cross-Linking with DSP. Cross-linking of sec180e using DSP [dithiobis(succinimidyl propionate); Pierce, Rockford, IL], a thiol-cleavable cross-linking reagent, was performed on 1 mL samples of metabolically labeled, sec180e-conditioned medium containing 1% sarcosyl that was precleared of aggregates by centrifugation. The DSP reagent was dissolved in DMSO at a concentration of 100 mM and added to the sec180e samples which were incubated at room temperature for 30 min. The cross-linking reaction was quenched by adding 150 μL of 1.5 M Tris at pH 7.5 and incubating for an additional 15 min. The samples were immunoprecipitated and subjected to SDS−PAGE. One-half of each sample was electrophoresed under reducing conditions, which cleaves the cross-linker, while the other half was electrophoresed under nonreducing conditions, leaving the cross-links intact. Radiolabeled sec180e was detected by fluorography.

Iodination of sec180e. Ten milliliter samples of sec180e and control conditioned media were concentrated to 200 μL using a Centricon 30 (Amicon Inc., Beverly, MA). The samples were iodinated using a chloramine T protocol as previously described (Bailey, 1984; Olague-Alcala & Diaz, 1993). Briefly, 1 mCi of ^{125}I was added in the presence of chloramine T for 2 min at room temperature, and the reaction was stopped by the addition of sodium metabisulfite followed by potassium iodide. The samples were washed five times with PBS and brought to a volume of 1 mL. Sarcosyl was added to 1%, and the samples were spun at 100000*g* as before.

RESULTS

Expression and Immunological Detection of the Recombinant sec180e Protein. COS-1 cells were transiently transfected with the pSVsec180e and control constructs. Indirect immunofluorescence analysis using R136, a rabbit antiserum directed against the C-terminal region of the BP180 ectodomain, revealed a punctate staining in the

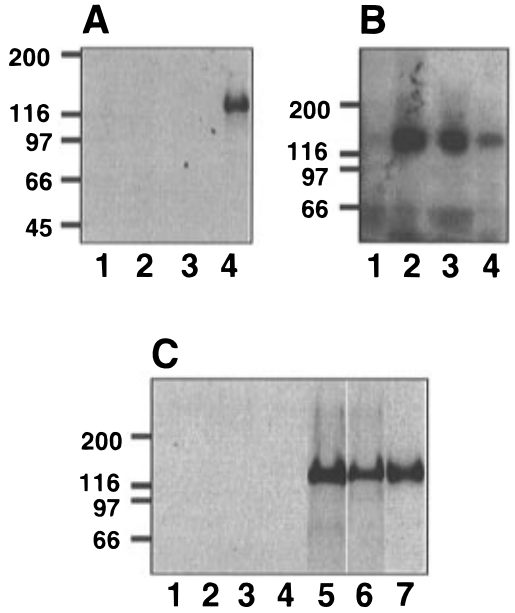

FIGURE 2: Characterization of sec180e expression and solubility. (A) Immunoblots containing culture supernatants from COS-1 cells transfected with either the pSVdsg1-leader construct (lanes 1 and 3) or the pSVsec180e construct (lanes 2 and 4). The blots were labeled with either antiserum R136 (lanes 3 and 4) or the corresponding preimmune serum (lanes 1 and 2). (B) sec180e-conditioned medium was subjected to a 100000*g* centrifugation step in the presence of either 1% *n*-octyl glucopyranoside (lanes 1 and 2) or 1% sarcosyl (lanes 3 and 4). The supernatant (lanes 1 and 3) and pellet fractions (lanes 2 and 4) from each sample were run on a 7% SDS−polyacrylamide gel, transferred to nitrocellulose, and labeled with serum R136. (C) SDS−PAGE and fluorographic analysis of immunoprecipitated sec180e. Metabolically labeled culture supernatants from COS-1 cells transfected with pSVdsg1-leader (lanes 1−3) and pSVsec180e (lanes 4−6) were clarified by high-speed centrifugation in the presence of 1% sarcosyl and subjected to immunoprecipitation with one of the following antisera: negative control rabbit antiserum R58 (lanes 1 and 4), R594 (lanes 2 and 5), or R136 (lanes 3 and 6).

sec180e-transfected cells, while the control transfected cells were negative. The conditioned media and total cell extracts were tested for the presence of sec180e by immunoblotting with R136. An immunoreactive band with an apparent molecular mass of approximately 120 kDa was detected in both the conditioned medium (Figure 2A, lane 4) and the cell extract (not shown) from the sec180e transfectants, while no immunoreactive product was detected in the preparations from control transfected cells. After centrifugation of the sec180e-conditioned medium for 1 h at 100000*g*, immunoblot analysis revealed that nearly all of the sec180e protein was in the pellet (data not shown). In an attempt to dissociate the sec180e aggregates, a variety of detergents, both nonionic and anionic, were added to the sec180e-conditioned medium. The percentage of sec180e found in the aggregate state was unaffected by treatment with nonionic detergents, such as Triton X-100 (not shown) and *n*-octyl glucopyranoside (Figure 2B, lanes 1 and 2). However, after addition of a mild anionic detergent, *n*-lauryl sarcosine (sarcosyl), to the pSVsec180e-transfected cell medium, most of the sec180e protein was found in the supernatant fraction after a 100000*g* centrifugation step (Figure 2B, lanes 3 and 4). A combination of nonionic and anionic detergents similar to that used by Resnick et al. (1993), which contains 1% Triton X-100, 0.25% sarcosyl, and 0.25% deoxycholate, was found to be as effective as 1% sarcosyl for dissociating the sec180e aggregates and for maintaining efficient immunoprecipitation of sec180e (data not shown).

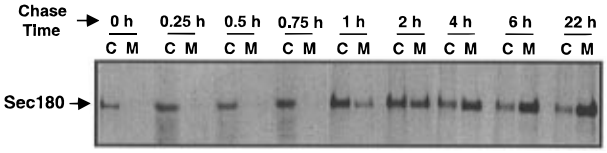

FIGURE 3: Pulse−chase labeling of sec180e. COS-1 cells transfected with pSVsec180e were metabolically labeled with [^{35}S]-methionine for 30 min and then chased with media lacking radiolabeled methionine for the time points indicated in hours. The conditioned medium (M) and the cell extracts (C) were prepared as described in Materials and Methods, cleared of aggregates by centrifugation in the presence of 1% sarcosyl, and immunoprecipitated with R594. sec180e is first detected in the culture supernatant at the 1 h chase time point (lane 10).

For use in an immunoprecipitation protocol, pSVsec180e and control transfected COS-1 cells were metabolically labeled with [^{35}S]methionine, and the conditioned medium was precleared of aggregates by the addition of sarcosyl followed by centrifugation at 100000*g*. The supernatant fractions were incubated with one of two anti-BP180 rabbit antisera or a negative control rabbit serum, and the immune complexes were precipitated with protein A-conjugated agarose beads. A 120 kDa radiolabeled band was specifically immunoprecipitated from the pSVsec180e-conditioned medium by both anti-BP180 antisera, but not by the control rabbit serum (Figure 2C). Immunoprecipitates of the control transfected cell medium exhibited no specific bands.

Pulse−Chase Labeling of sec180e. To determine the time course of sec180e secretion, we performed a pulse−chase labeling experiment using a 30 min pulse of [^{35}S]methionine and various chase times with media containing unlabeled methionine. Both the conditioned medium and total cell extract from each time point were immunoprecipitated as before, and the results are shown in Figure 3. sec180e is first detectable in the conditioned medium at the 1 h chase time point and accumulates in the medium with increasing chase times. The secreted sec180e polypeptide appears to be quite stable during an overnight incubation.

Cross-Linking of sec180e. To begin to investigate the oligomeric state of sec180e, a cross-linking analysis was performed using the thiol-cleavable cross-linker, DSP (Pierce). DSP at various concentrations was added to metabolically labeled, sec180e-conditioned medium which had been precleared of supermolecular aggregates by centrifugation at 100000*g*. After the cross-linking reaction was quenched with excess Tris, the cross-linked samples were subjected to immunoprecipitation, SDS−PAGE, and fluorography. As seen in the nonreducing gel in Figure 4, at low concentrations of DSP, sec180e runs at the monomeric position (136 kDa) and as a cross-linked product of approximately 292 kDa, which corresponds in size to a sec180e dimer. At higher concentrations of DSP (Figure 4A, lanes 4 and 5), sec180e is detected in a high-molecular mass cross-linked complex of approximately 442 kDa, consistent with a sec180e trimer. When the cross-linked products are analyzed by SDS−PAGE under reducing conditions, which cleave the disulfide linkage in DSP, all of the radiolabeled sec180e migrates at the monomeric position, as expected (Figure 4B).

Superose-6 Gel Filtration Chromatography of sec180e. Gel filtration chromatography was performed on metabolically labeled, sec180e-conditioned medium that had been

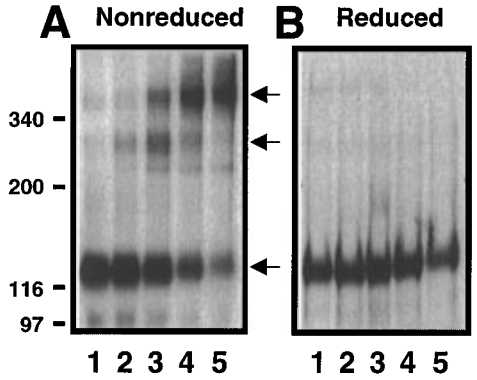

FIGURE 4: Cross-linking analysis shows that sec180e forms a trimeric complex. Clarified, conditioned medium from COS-1 cells transfected with pSVsec180e cross-linked with DSP, a thiol-cleavable cross-linker. Cross-linking was performed with 0, 0.1, 0.5, 1.5, and 4.0 mM concentrations of DSP shown in lanes 1−5, respectively, in both panels A and B. After the cross-linking reactions were stopped, the samples were immunoprecipitated with R594 and electrophoresed on an SDS−polyacrylamide gel under either nonreducing conditions (A), keeping the cross-links intact, or reducing conditions (B), which cleave the cross-linker. Bands corresponding to the monomer, dimer, and trimer forms of sec180e are indicated by the arrows to the right of panel A.

precleared of aggregates by centrifugation after the addition of 1% Triton X-100, 0.25% sarcosyl, and 0.25% deoxycholate. Fractions were collected, and sec180e was detected by immunoprecipitation. Two distinct peaks of sec180e immunoreactivity were identified at fractions 6 and 15 (Figure 5). On the basis of the standard curve shown in Figure 5A, the first sec180e peak was determined to have a Stokes radius of 13.6 nm and the second peak a Stokes radius of 8.4 nm. Chromatography of a denatured preparation of sec180e on the Superose-6 column resulted in the disappearance of the first peak, while the second peak, corresponding to the smaller Stokes radius, remained (Figure 5C).

To further investigate the two forms of sec180e, the pSVsec180e-transfected COS cell medium was subjected to cross-linking with 4 mM DSP prior to separation by gel filtration. After immunoprecipitation, the column fractions were subjected to SDS−PAGE followed by fluorography. As described above, the sec180e was detected in two peaks. When the SDS−polyacrylamide gels were run under nonreducing conditions (to retain the cross-links), sec180e present in the first chromatographic peak migrated as a cross-linked complex near the top of the SDS−polyacrylamide gel while sec180e from the second peak, which comigrated on the Superose-6 column with denatured sec180e, ran at the monomeric sec180e position on the SDS gel, with an apparent molecular mass of approximately 120 kDa (Figure 5D).

Glycerol Gradient Sedimentation of sec180e. Glycerol gradient sedimentation analysis was performed on precleared, metabolically labeled sec180e-conditioned medium containing 1% Triton X-100, 0.25% sarcosyl, and 0.25% deoxycholate. For calibration purposes, a gradient containing standards with known sedimentation coefficients was run in parallel. The standards were detected by Coomassie Blue staining on an SDS−polyacrylamide gel, while fractions

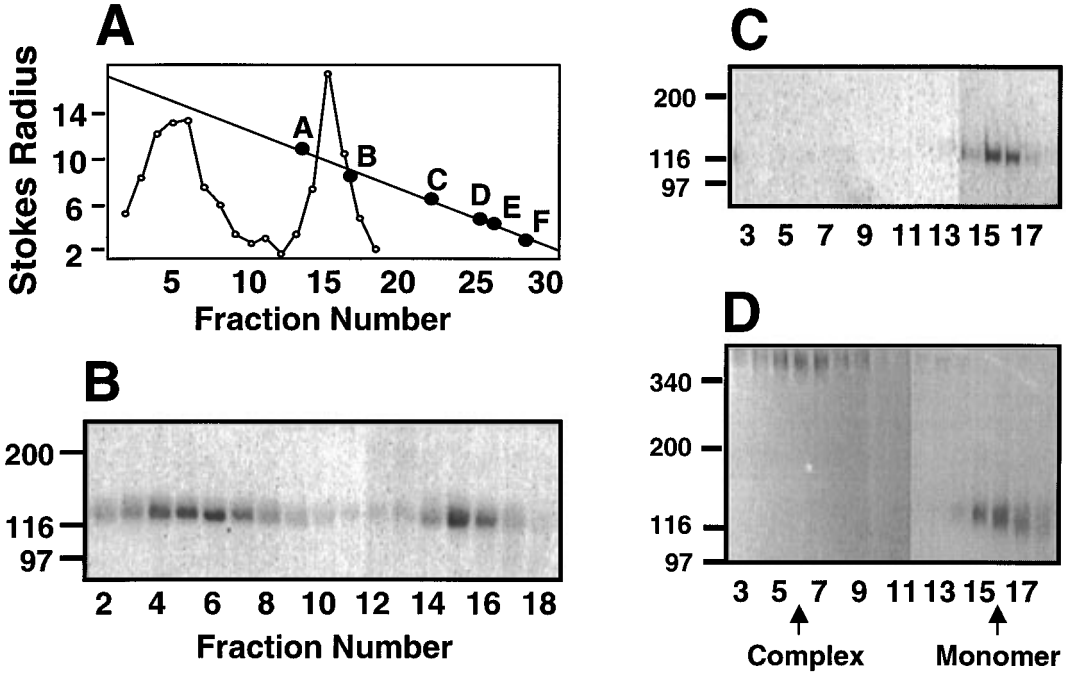

FIGURE 5: Superose-6 gel filtration chromatography reveals two forms of sec180e. (A) Clarified, conditioned medium of [^{35}S]methionine-labeled COS-1 cells transfected with pSVsec180e was fractionated on a Superose-6 gel filtration column. Sec180e was detected in the column fractions by immunoprecipitation with an anti-BP180 antiserum, and the chromatographic profile, as determined by densitometry scanning, shows two distinct peaks (open circles). The standard curve (closed circles) was generated using proteins of known Stokes radii. Standards used were fibrinogen (A), thyroglobulin (B), ferritin (C), catalase (D), aldolase (E), and bovine serum albumin (F), which have Stokes radii of 10.7, 8.6, 6.7, 5.1, 4.8, and 3.5 nm, respectively. (B) Immunoprecipitation analysis of sec180e gel filtration column fractions shown in panel A. Sec180e runs with an apparent molecular mass of 120 kDa. (C) Superose-6 gel filtration analysis of a sec180e preparation which was boiled for 5 min prior to chromatography. The column fractions were processed as in panel B. Under these denaturing conditions, sec180e ran as a single peak corresponding to the second peak (lower Stokes radius) seen in panels A and B. (D) Superose-6 gel filtration analysis of a DSP-cross-linked preparation of sec180e. Column fractions were immunoprecipitated with an anti-BP180 antibody and then analyzed by SDS−PAGE under nonreducing conditions, which keeps the cross-links intact. As anticipated, the first and second chromatographic peaks corresponded to the multimeric and monomeric forms of sec180e, respectively.

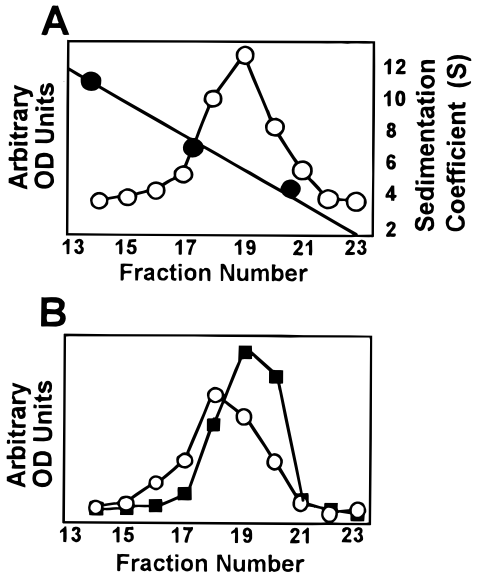

FIGURE 6: Multimeric and monomeric forms of sec180e have very similar glycerol gradient sedimentation properties. (A) A preparation of sec180e expressed by transfected COS-1 cells was fractionated by glycerol gradient sedimentation. The gradient fractions were analyzed by immunoprecipitation using R594, an anti-BP180 antibody, and the radiolabeled 120 kDa sec180e was detected by SDS−PAGE and fluorography. Open circles show the densitometric profile of the sec180e band in sedimentation fractions 14−23. Also shown is a plot of the positions of protein standards within the glycerol gradient (filled circles) versus their previously documented sedimentation coefficients. Standards (from left to right) include catalase, aldolase, and bovine serum albumin, which have sedimentation coefficients of 11.4, 7.4, and 4.3 S, respectively. (B) Glycerol gradient sedimentation analysis of a DSP-cross-linked preparation of sec180e. The gradient fractions were immunoprecipitated with R594 and fractionated by SDS−PAGE under nonreducing conditions, and the precipitated bands were detected by fluorography. Shown on this plot are the profiles of the densitometric readings of bands corresponding to the high-molecular mass sec180e complex (open circles) and the monomeric form of sec180e (solid squares). On the basis of the standard curve (shown in panel A), the sec180e multimeric complex has a sedimentation coefficient of 6.5 S and monomeric sec180e has a value of 5.3 S.

containing sec180e were identified by immunoprecipitation and fluorography. A single peak of sec180e was detected, as shown in Figure 6A.

The sedimentation properties of sec180e were further investigated by subjecting the sec180e preparation to cross-linking with DSP prior to the glycerol gradient analysis. The gradient fractions were then analyzed by immunoprecipitation with anti-BP180 antibodies followed by nonreducing SDS−PAGE and fluorography. As shown in Figure 6B, the single sec180e peak that was identified in the previous experiment was now resolved into two overlapping peaks consisting of different forms of sec180e. SDS−PAGE analysis of the immunoprecipitated fractions revealed that the form of sec180e exhibiting the higher sedimentation coefficient (6.5 S) ran as a high-molecular mass cross-linked complex on nonreducing gels, while the peak having a lower sedimentation coefficient (5.3 S) consisted of a form of sec180e that was unable to be cross-linked even with high concentrations of DSP.

Compositional Analysis of the sec180e Multimeric Complex. The results described above clearly demonstrated that a substantial fraction of the sec180e produced by transfected COS cells is secreted in the form of a high-molecular mass complex which has a Stokes radius of 13.6 nm and a

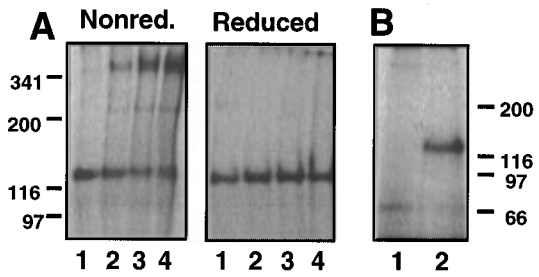

FIGURE 7: Sec180e forms a homomultimer which assembles prior to secretion. (A) Cross-link analysis of the sec180e protein within 1 h of synthesis, i.e., prior to secretion from the cells. Sec180e expressed by COS-1 cells was detected by immunoprecipitation, followed by SDS−PAGE and fluorography. Prior to immunoprecipitation, the transfected cells were metabolically labeled for a brief period (30 min pulse plus 30 min chase), and the clarified culture supernatant was subjected to cross-linking with 0, 0.5, 1.5, and 3 mM DSP as shown in lanes 1−4, respectively. Samples were electrophoresed either in the absence (gel on the left) or in the presence of a reducing agent (gel on the right). The heavy band migrating slower than the 341 kDa marker (lanes 2−4 in the left-hand gel) corresponds to the cross-linked trimeric sec180e complex. (B) Immunoprecipitation analysis of radioiodinated conditioned medium from COS-1 cells transfected with either pSVdsg1-leader (lane 1) or pSVsec180e (lane 2). Both samples were immunoprecipitated with antiserum R594 and subjected to SDS−PAGE and autoradiography. No specific bands other than that for the 120 kDa sec180e are detected in lane 2.

sedimentation coefficient of 6.5 S. The cross-linking experiments showed that sec180e was the only metabolically labeled constituent of the multimeric complex, indicating that this complex consisted either of a sec180e homotrimer or of sec180e complexed with an unlabeled molecule(s). Possible sources of the unlabeled molecules include the exogenous factors added to the cultured cells (e.g., fetal bovine serum) and cellular products that do not contain detectable levels of [^{35}S]methionine (e.g., proteins with unusually long half-lives). The next series of experiments were aimed at resolving whether the sec180e complex was homo- or heterotypic.

The pulse−chase experiments described above showed that sec180e is first detected in the COS cell culture medium 1.5 h after the initiation of the pulse (30 min pulse plus 60 min chase). Therefore, to determine whether the sec180e complex is formed prior to secretion into the medium, cross-linking experiments were performed on fractions of cells labeled with [^{35}S]methionine for 30 min followed by a 30 min chase. The metabolically labeled pSVsec180e-transfected cells were extracted in PBS containing 1% sarcosyl, cleared of high-molecular mass aggregates by high-speed centrifugation, and cross-linked with DSP. As shown in Figure 7A, the sec180e multimeric complex was detected with concentrations of cross-linker comparable to those used for the metabolically labeled sec180e-conditioned medium.

As a second approach, a sample of sec180e-conditioned medium that had not been metabolically labeled was radioiodinated by the chloramine T method. In this way, cellular proteins that were not metabolically labeled and exogenously added proteins would be detectable by autoradiography. Immunoprecipitation of this iodinated sec180e complex with anti-BP180 antibodies yielded a single band corresponding to sec180e (Figure 7B). There was no evidence of coprecipitation of other proteins.

Determination of the Oligomeric State and Shape of sec180e. The trimeric nature of the sec180e complex was

demonstrated by the above cross-linking experiments. With the additional information that the complex is composed solely of sec180e, we can apply the biochemical data to calculate the molecular mass of the complex, thereby determining its actual oligomeric state. In addition to the sedimentation coefficient and the Stokes radius, which we have obtained experimentally, the partial specific volume was calculated from the known amino acid sequence. Taking into account the single N-linked polysaccharide chain that is predicted to be present on sec180e, the value of the partial specific volume is 0.70 mL/g. The calculated molecular mass of sec180e, including the 2.4 kDa for the average-sized N-linked polysaccharide chain, is 102.3 kDa. Using the sedimentation coefficient and Stokes radius values corresponding to the multimeric form of sec180e, the calculated molecular mass of the complex, using eq 1, is 332.9 kDa. Thus, the calculated oligomeric state of sec180e is 3.25. In arriving at this value, we did not account for the water of hydration because of the technical difficulties associated with accurately determining this parameter. Associated water would tend to decrease the calculated oligomeric state of the sec180e complex from the current value of 3.25, but the general conclusion would likely remain the same; i.e., sec180e appears to exist in a homotrimeric state.

The frictional ratio, f/f_0, was calculated using the experimentally determined molecular mass of 332.9 kDa and the calculated partial specific volume, 0.70 mL/g. These values were used in eq 2 from Materials and Methods, and the frictional ratio for sec180e was determined to be 3.01 (for spherical molecules, the frictional ratio would be 1.0). If sec180e is cylindrical, the axial ratio, P, of the length to width would be 60, but if it is modeled as a prolate ellipsoid, the P value is 52. Thus, sec180e is a highly elongated protein, with a length to width ratio estimated to be between 52 and 60.

Sec180e and Endogenous Epidermal BP180 Exist as SDS-Stable but Heat-Labile Complexes. In general, many collagens have a tendency to undergo melting into the monomeric subunits at relatively low temperatures (Boedtker & Doty, 1956). We suspected that the dissociation of the sec180e multimeric complex that was observed during SDS−PAGE analysis might be heat-induced and that this complex might be stable to SDS treatment. To investigate this possibility, we performed immunoprecipitation on precleared, metabolically labeled sec180e-conditioned medium as described above. After the addition of Laemmli sample buffer (1% SDS, final concentration), one aliquot was heated to 100 °C for 5 min, as usual, while a second aliquot was kept at room temperature. Both samples were then subjected to SDS−PAGE and fluorography. The trimeric form of sec180e was detected in the sample that had not been boiled, while the boiled sample migrated as a monomer as expected (Figure 8A, lanes 1 and 2). The unboiled trimeric form of sec180e comigrated on the SDS gel with the cross-linked trimeric complex shown in lane 4 of Figure 8A. Thus, the sec180e homotrimer is stable to SDS but heat-labile.

From this result, we hypothesized that BP180 endogenously expressed in epidermal tissue also forms a homotrimeric complex via the same mechanism used by sec180e. To test this hypothesis, an SDS extract of normal human epidermis was prepared. As before, one aliquot was boiled for 5 min, while the other was kept at room temperature. Immunoblot analysis of the two samples using a rabbit anti-

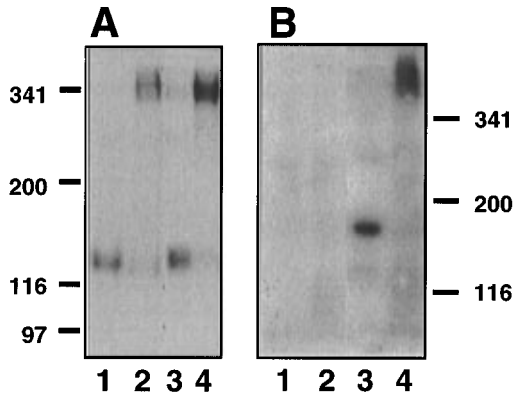

FIGURE 8: Both sec180e and endogenous epidermal BP180 form complexes that are SDS-stable but heat-labile. (A) Metabolically labeled, sec180e-conditioned medium was immunoprecipitated with R594 and then boiled (lane 1) or not boiled (lane 2) prior to SDS−PAGE and fluorography. As controls, the sec180e preparation was cross-linked with either 0 (lane 3) or 4 mM (lane 4) DSP and then subjected to immunoprecipitation with R594 and nonreducing SDS−PAGE followed by fluorography. In the absence of boiling (lane 2), most of the sec180e runs as a high-molecular mass complex, comigrating with the DSP-cross-linked sec180e (lane 4). (B) Immunoblot analysis of an SDS extract prepared from human epidermis labeled with either R594 (lanes 3 and 4) or the corresponding preimmune serum (lanes 1 and 2). If the SDS epidermal extract is boiled prior to SDS−PAGE (lanes 1 and 3), BP180 runs as a single band at 180 kDa (lane 3). In the absence of boiling (lanes 2 and 4), epidermal BP180 runs as a high-molecular mass complex on the SDS−polyacrylamide gel (lane 4).

BP180 antiserum (R594) showed that BP180 in epidermal tissue does exist as a complex with a very high apparent molecular mass, consistent with a trimeric complex (Figure 8B).

DISCUSSION

In this study, we have expressed the extracellular region of BP180 as a recombinant secreted polypeptide (sec180e) in COS-1 cells to facilitate the elucidation of the oligomeric structure of the extracellular region of BP180 when expressed in the absence of the transmembrane and intracellular domains. On the basis of the results of this investigation, sec180e was found to exist in two forms: a monomeric form and a high-molecular mass multimeric form. The two forms were easily separable by gel filtration but exhibited very similar glycerol gradient sedimentation properties. The multimeric form of sec180e was shown to have a Stokes radius of 13.6 nm and a sedimentation coefficient of 6.5 S. These data suggested that the sec180e monomer exists in a globular conformation while the multimeric form has an elongated structure. The pulse−chase and cross-linking experiments showed that sec180e achieved its stable multimeric form prior to secretion into the culture medium.

The secreted sec180e present in the culture medium of transfected COS cells exists largely in the form of insoluble aggregates; i.e., most of it is found in the pellet after a 100000*g* centrifugation. Using various combinations of detergents, the aggregates can be dissociated into soluble, multimeric and monomeric forms of sec180e. Cross-linking studies revealed that a pool of monomeric sec180e remained even after subjecting the preparation to very high cross-linker concentrations. These data suggest that a certain fraction of sec180e may be secreted by the transfected COS cells in the monomeric form. Alternatively, a subset of the trimeric

sec180e may be dissociated to the monomeric form as a result of the extraction procedure (which includes the use of anionic detergents), during storage (e.g., freeze−thaw cycles) or during room-temperature incubations that are part of the immunoprecipitation procedure.

Several independent lines of evidence indicate that the high-molecular mass multimeric form of sec180e corresponds to a homotrimer. In the cross-linking experiments, we have shown that the sec180e complex migrates in SDS−PAGE at a rate that is consistent with that of a trimeric complex. With the additional information gained from the iodination experiment, the fact that the complex is composed only of sec180e, we can apply the biochemical data to calculate the molecular mass of the complex, thereby determining the actual oligomeric state of sec180e. The molecular mass of the multimer was found to be 3.25-fold higher than that of the monomeric form of sec180e, providing strong support for the conclusion that the multimeric sec180e complex is a homotrimer.

Our data revealed that the trimeric sec180e complex is highly elongated. If the sec180e complex is modeled as a cylinder, it would be approximately 60 times longer than it is wide. If it is modeled as a prolate ellipsoid, it would be roughly 52 times longer than it is wide. These data are consistent with very recent ultrastructural data obtained by our laboratory (Bedane et al., 1997). Using a postembedding immunoelectron microscopic technique with multiple antibodies prepared against specific sites on the BP180 molecule, we showed that the NC16A domain of this protein is located immediately subjacent to the plasma membrane of the epidermal basal keratinocyte, while the carboxyl terminus of BP180 localizes to the junction of the lamina lucida and lamina densa of the basement membrane. Thus, the extracellular region of BP180 spans the entire lamina lucida, a distance of roughly 50 nm. The data we present in this paper are also consistent with the data of Hirako et al. (1996), from which the frictional ratio of a bovine BP180-like protein was estimated to be 2.8. This value was based on an experimentally derived sedimentation coefficient and a molecular mass and partial specific volume that were based on the assumption that the primary structure of the bovine mammary gland protein is highly similar to that of human epidermal BP180. The slightly higher value that we obtained for sec180e ($f/f_0 = 3.01$) could be due to the absence of the transmembrane and intracellular domains or may be due to structural differences exhibited by human epidermal BP180 and the bovine mammary gland protein. There is currently no sequence information available for the BP180-like proteins expressed by the various cell lines that were used by Hirako et al. (1996).

Our biochemical data suggest that, while sec180e is highly elongate, it has more flexibility than a rigid rod. The sedimentation coefficient of the trimeric sec180e was shown to be 6.5 S. This result is somewhat higher than that which would be expected for a molecule of this size with a highly rigid rod-like structure [a sedimentation coefficient of 3−4 S (Boedtker & Doty, 1956)] and is consistent with results obtained for the putative bovine BP180 homologue (Hirako et al., 1996). An extended, yet flexible, conformation for the BP180 ectodomain is also consistent with our recent immunoelectron microscopic data described above (Bedane et al., 1997). The observed span between the keratinocyte plasma membrane and the C-terminal region of BP180 (50−60 nm) is significantly less than what would be expected if the BP180 collagenous tail were fully extended.

In this study, we have also shown for the first time that, like recombinant sec180e, BP180 extracted from epidermal tissue exists in the form of a high-molecular mass multimeric complex. Both the sec180e and the epidermal BP180 complexes were shown to be SDS-stable and heat-labile. On the basis of these observations and on the primary structural analysis of BP180, it is highly likely that the epidermal form of this protein exists in a trimeric, and possibly a homotrimeric, complex. The multimeric structure of BP180 is likely to be the result of the formation of multiple collagen-like triple helices.

We, and others, have previously pointed out that the several known membrane-associated collagens (BP180 being among this group) exhibit the rather unusual type II transmembrane orientation; i.e., the N terminus is intracellular, and the C terminus is extracellular. Pihlajaniemi and Rehn (1995) have suggested that, unlike the other members of the collagen family, the membrane-associated collagens may undergo triple helix formation in the N terminus (membrane proximal) to C terminus direction. It was further suggested that the presence of the cytoplasmic and/or transmembrane domains may be essential for initiating the trimerization process or in subunit alignment in this collagen subfamily. Within this context, it is significant that sec180e, which lacks both the cytoplasmic and transmembrane domains, was capable of forming a collagen-like homotrimer with structural and solubility properties similar to those of endogenous BP180. Another structural motif has been identified at the N terminus of the BP180 ectodomain which may play an important role in assembly of the trimer. Computer analysis of the BP180 primary structure revealed that there is a very high probability that the N-terminal region of the extracellular noncollagenous NC16A domain forms a three-stranded coiled coil (Lupas et al., 1991). As shown in Figure 9, the heptad repeat of hydrophobic residues extends from this stretch of NC16A through the adjacent membrane-spanning domain. This coiled-coil region could play a role in aligning the multiple BP180 polypeptides, thus facilitating the formation of the collagen-like triple helices. It is worth noting that two isoforms of another membrane-associated collagen, the macrophage scavenger receptor, form a coiled-coil structure in the region between the transmembrane domain and the collagen triple helix (Kodama et al., 1990). On the basis of our findings, we propose a model which shows the BP180 ectodomain as a rod-like, yet flexible, homotrimeric structure (see Figure 9). The rod-like sections correspond to the coiled-coil and collagen-like triple helices, and the flexibility in this multimeric structure is due to the presence of the multiple noncollagenous stretches interspersed throughout the long collagenous domain.

BP180 has long been hypothesized to function in cell-matrix adhesion. The structural features of BP180 documented in this report and our recent ultrastructural findings (Bedane et al., 1997) support the notion that the C-terminal region of BP180 interacts with a component of the basal lamina. Further support for the adhesive role of this molecule comes from molecular analyses of pathological conditions that affect the basement membrane zone. Experimental data indicate that a humoral autoimmune response

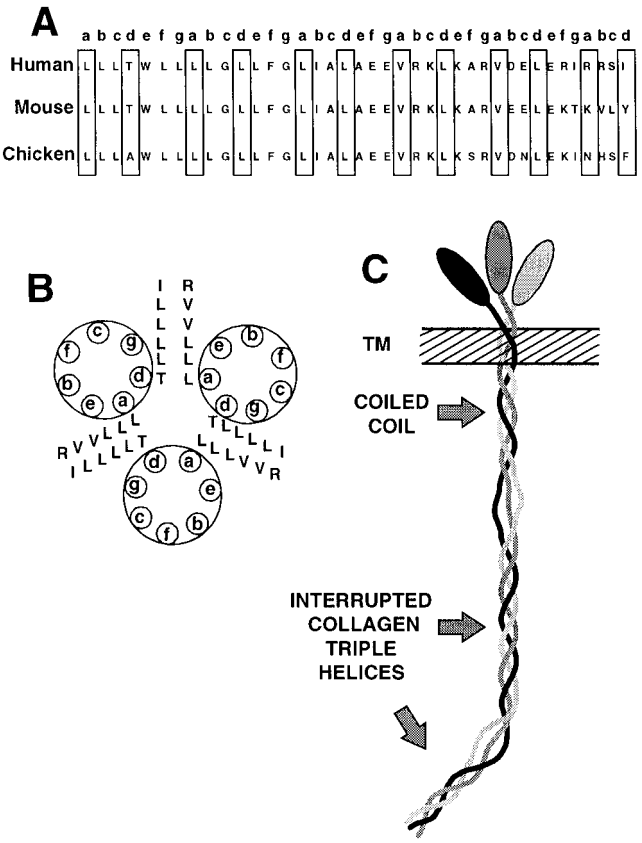

FIGURE 9: Three subunits of the sec180e complex are likely to be stabilized by both collagen triple-helical and coiled-coil type interactions. (A) Computer analysis of the BP180 protein sequence revealed a region within the extracellular NC16A domain which is predicted to form a coiled coil. The amino acid sequences corresponding to the coiled-coil domain from human, mouse, and chicken BP180 are shown. The a and d residues of the heptad repeat, important for the formation of the three-stranded coiled-coil structure, are enclosed in rectangles. The conservation of these amino acid positions across species is evident. (B) A helical wheel representation of the three-stranded, coiled-coil domain of human BP180. The membrane-spanning domain was shown to contain a heptad repeat containing mostly leucines at the a and d positions, which is in frame with the heptad repeat of the NC16A domain. (C) A hypothetical structural model of human BP180 based on our data. Various structural features of the protein, such as the transmembrane (TM), coiled-coil, and collagen triple-helical domains, are indicated. In order to show the general features of the coiled-coil and collagenous domains, this representation of BP180 was not drawn to scale.

directed against the BP180 ectodomain is responsible for the detachment of basal keratinocytes from the basal lamina in the acquired human diseases bullous pemphigoid and herpes gestationis (Giudice et al., 1993, 1995; Liu et al., 1993). In addition, it has very recently been shown that an inherited subepidermal blistering disease, generalized atrophic benign epidermolysis bullosa, is caused by a genetic defect within the BP180-coding region (Jonkman et al., 1995; McGrath et al., 1995, 1996; Pohla-Gubo et al., 1995).

In summary, we have presented evidence that the human BP180 ectodomain is capable of forming a highly elongate, flexible, homotrimeric complex, in a process that does not depend upon the presence of the cytoplasmic or transmembrane domains of this protein. The structural properties of this recombinant protein are similar to those of endogenous BP180 and are consistent with an interrupted collagen-like conformation. Our experimental strategy of expressing the extracellular domain of BP180 as a secreted protein should facilitate future studies that address the structure and function of this hemidesmosome-associated transmembrane molecule under both normal and pathological conditions.

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