Published in final edited form as:

J Cell Physiol. 2007 September ; 212(3): 675–681. doi:10.1002/jcp.21064.

14-3-3 Sigma Isoform Interacts with the Cytoplasmic Domain of the Transmembrane BP180 in Keratinocytes

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Abstract

The protein bullous pemphigoid antigen-2 (BPAG2/BP180/collagen type XVII) plays a key role in attachment of basal keratinocytes to epidermal basement membrane. The binding of BP180 with either integrin $\alpha 6$, integrin $\beta 4$, or bullous pemphigoid antigen-1 (BPAG1/ BP230) is critical for this attachment in skin. The protein 14-3-3 σ , also known as stratifin and a marker for epithelial cells, is a member of a highly conserved small acidic 14-3-3 protein family naturally found in all eukaryotic cells. Here, we have used a 14-3-3 σ GST pull-down screening assay and showed that sigma (σ) isoform of the 14-3-3 protein family interacts with the cytoplasmic N-terminal domain of BP180. Analysis of a series of truncated or deleted 14-3-3 σ revealed that only intact 14-3-3 σ molecule, but not any of its fragments can interact with BP180. This finding suggests that conformation and possible dimerization of 14-3-3 σ is essential for this interaction. Further, a BP180 co-immunoprecipitation (IP) and its reverse IP assays were conducted and the results confirmed that 14-3-3 σ interacts with cytoplasmic domain, but not ecto-domain of the BP180. In conclusion, the finding of this study provides evidence that 14-3-3 σ isoform interacts with BP180 which is a major component of hemidesmosome involved in the attachment of epidermis to the basement membrane in skin. However, the significance of this interaction in hemidesmosome formation and/or attachment needs to be explored.

14-3-3 proteins are small acidic proteins naturally found in a dimeric form with a subunit MW of 28–33 kDa. They are expressed in all eukaryotic cells and are highly conserved in protein sequence from yeast to mammals (Fu et al., 2000). Seven isoforms, encoded by seven distinct genes, have so far been identified. Five of these isoforms named α – η are abundantly found in brain. Isoform σ is only expressed in the epithelial cells (Fu et al., 2000). These proteins originally were found as the cytoplasmic proteins. Nearly 200 proteins have been reported to interact with 14-3-3 proteins in vivo (Muslin et al., 1996; Yaffe et al., 1997; Cotellet et al., 2000; Fu et al., 2000). Among these, there are proteins involved in cell cycle control, such as Cdc25, Weel, P53, CDC2, and CDK2, as well as those proteins of cellular signaling and stress responses, such as Raf, IGF-1 receptor substrate, IRS-1, phosphatidylinositol-3 kinase (PI-3 kinase). 14-3-3 proteins also interact with protein kinase C, Cbl, Bcr, polyoma middle T antigen, MEKK-1 and 4, MLK2, BAD, ASK-1 and transcriptional regulatory proteins, such as FKHL1, DAF-16, TAZ, and TLX-2. In

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addition, cytoskeletal proteins such as keratin K18 and vimentin have also reported to bind to 14-3-3. Each of the 14-3-3 isoform regulates its partners through a variety of mechanisms, such as altering their catalytic activity, cellular localization, incorporation into protein complexes, or their susceptibility to proteases and phosphatases (Muslin et al., 1996; Yaffe et al., 1997; Cotellet et al., 2000; Fu et al., 2000).

Hemidesmosomes are cell–substrate adhesion junctions connecting the cytoskeleton network of epithelial cells to anchoring filaments and fibrils which are located in the basement membrane and the upper dermis, respectively (Hirako and Owaribe, 1998). In the past decade, at least five proteins have been identified as the major components of hemidesmosome, that is plectin and BP230 as cytoplasmic plaque proteins; integrin $\alpha 6$, integrin $\beta 4$, and BP180 (also known as collagen type XVII) as transmembrane proteins; and laminin 5 as anchoring filament-lamina densa component (Zillikens, 1999).

Collagen type XVII, also known as the 180 kDa bullous pemphigoid antigen 2 (BPAG2/BP180), is a hemidesmosomal transmembrane protein with a collagenous carboxyl-terminal extracellular domain and a globular, disulfide-linked, amino-terminal cytoplasmic domain (Giudice et al., 1992; Li et al., 1993). BP180 is expressed by the stratified squamous epithelia, the epithelial basement membranes of cornea, ocular conjunctiva, buccal mucosa, upper esophagus, placenta, umbilical cord, and transitional epithelium of the bladder (Fairley et al., 1995). BP180 is known to be one of the components of hemidesmosomes. There is accumulating evidence to suggest that BP180 is involved in the stable adhesion of epidermal cells to connective tissue element (Hirako and Owaribe, 1998). For example, mutations in BP180 are associated with pathogenesis of atrophic benign epidermolysis bullosa (GABEB) (McGrath et al., 1996), a blister skin disease. BP180 auto antibodies are known to be the causative agent in bullous pemphigoid (Liu et al., 1993; Balding et al., 1996).

We and others have previously demonstrated that 14-3-3 σ , also known as stratifin, is releasable from in stratified keratinocytes (Katz and Taichman, 1999; Ghahary et al., 2004). We also showed that secreted 14-3-3 σ stimulates the expression of MMP-1 in fibroblasts by enhancing the expression of c-fos, c-jun and by activation of P38 MAPK (Lam et al., 2005). These findings have led us to propose that the MMP-1 stimulatory effect may be mediated by some unknown cell surface receptors of 14-3-3 σ . In an attempt to identify these receptors, we carried out a series of experiments to screen cell surface proteins with a capacity to bind to 14-3-3 σ . In the present study, we surprisingly found that 14-3-3 σ is associated with BP180 in human skin keratinocytes. Further study found that only intact 14-3-3 σ molecule, but not its fragments, binds to the cytoplasmic domain of BP180.

Materials and Methods

Cell culture

The procedure of culture of primary epidermal keratinocytes has been previous established in our laboratory (Ghahary et al., 2004). Briefly, following informed consent, skin punch biopsies were obtained from patients undergoing elective reconstructive surgery, under the local anaesthesia using a protocol approved by the University of Alberta Hospitals Human Ethics Committee. Biopsies were then collected individually and washed three times in sterile Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotic–antimycotic preparation (100 ug/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B). Epidermal and dermal layer were then separated by treating samples with dispase (Roche Applied Science, Laval, QC, Canada). Human keratinocytes were then grown in serum-free keratinocyte medium (KSFM) (Invitrogen Life Technologies, Carlsbad, CA) supplemented with bovine pituitary extract (50 μ g/ml) and EGF (0.2 μ g/ml).

Production of recombinant proteins

The human full-length 14-3-3 σ gene was subcloned into the prokaryotic expression vector pGEX-6P-1 (Amersham/Pharmacia Bioscience, Piscataway, NJ) using the procedure described before (Ghahary et al., 2004). This plasmid encodes a fusion protein, glutathione S-transferase (GST)-tagged-14-3-3 protein, which consists of a 26 kDa GST-tag and 28 kDa of 14-3-3 σ . Fusion proteins were then expressed and purified as previously described (Ghahary et al., 2004).

Different regions of 14-3-3 σ fused to GST were also prepared in this study (Fig. 4A). Fusion proteins containing peptide fragments of 14-3-3 σ were referred as GST-ND50 (amino acids 51–246); GST-ND100 (amino acids 101–246); GST-CD98 (amino acids 1–150); GST-CD148 (amino acids 1–100). A cDNA clone of human 14-3-3 σ , as described previously (Ghahary et al., 2004) was used as the template to make all the above GST fusion fragments.

To prepare a purified 14-3-3 σ conjugated with Sepharose 4B matrix, GST fusion 14-3-3 σ was digested by PreScission protease (Amersham Bioscience), and purified 14-3-3 was isolated from the matrix by centrifugation. Purified 14-3-3 σ was then dialyzed against PBS and concentrated by Centricon (Millipore, Bedford, MA). Concentrated 14-3-3 σ was then coupled with CNBr-activated Sepharose 4B according to the manufacturer's instruction (Amersham/Pharmacia Bioscience). The coupled 14-3-3 σ Sepharose was used in GST pull-down assay.

Biotinylation of cell surface proteins and GST pull-down assay

EDTA detached keratinocytes were harvested. Cell surface proteins were labeled with Sulfo-NHS-Biotin in a concentration of 625 μ g/ml (Pierce, Rockfield, IL). Following an extensive washing with PBS, the cells were then disrupted by 50 mM Tris-HCl buffer (pH 7.50) containing 10 mM EDTA, 1% Triton X-100, 0.5% NP-40, and the protease inhibitor cocktail (Sigma Chemicals, Oakville, ON, Canada). The cell lysate was centrifuged at 13,000 rpm for 15 min. The supernatant was then pre-cleared with GSH-Sepharose 4B. Either GST, GST-fusion 14-3-3 proteins attached to glutathione-Sepharose, or purified 14-3-3 σ coupled Sepharose 4B matrix was incubated with the pre-cleared supernatant for 2 h at 4°C by rotating. The matrix mixtures were washed seven times with PBS containing 0.1% Triton X-100 to remove non-specifically bound proteins. Subsequently, the pull-down proteins were denatured by boiling in Laemmli buffer for 5 min, separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The biotin-labelled cell surface proteins bound to 14-3-3 was detected by using a 1: 3000 dilution of HRP-conjugated anti-biotin antibody (Cell Signalling Technology, Pickering, Ont, Canada).

Protein identification by MALDI mass spectrometry

Following silver staining, the recombinant protein bands, with molecular weight matched with purified 14-3-3 protein previously identified as biotin-labeled membrane protein were excised and digested by using trypsin. Digested products were desalted, eluted and analyzed on a Bruker REFLEX III time of flight mass spectrometer (MS) (Bremen/Leipzig, Serial # FM2413) using matrix-assisted laser desorption/ionization (MALDI) in positive ion mode according to a published procedure (Shevchenko et al., 1996). Obtained peptide maps were used for database searching to identify the protein. Furthermore, 1–2 selected peptides were used for further fragmenting and sequencing using MALDI MS/MS analysis on a PE Sciex API-QSTAR pulsar (MDS-Sciex).

Western blot

Proteins which purified by GST pull-down assay were transferred to PVDF membrane with Mini Trans-Blot Cell (Bio-Rad, Hercules, CA, USA) after separation by SDS-PAGE.

Immunoblotting against BP180 was carried out. Blots were initially incubated with rabbit anti-human cytoplasmic region of BP180 polyclonal antibody (Hopkinson et al., 1992) at a concentration of 1:3000, subsequently with HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA). Protein bands were visualized by an ECL detection system (Amersham/Pharmacia Biosciences Inc., QC, Canada).

Immunoprecipitation

The EDTA detached cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5% NP 40, and protease inhibitor cocktail (Sigma). Lysates were cleared by centrifugation, and immunoprecipitated by overnight incubation with 5 µg/ml of either non-immune goat IgG or goat anti-14-3-3 σ polyclonal antibody (N-14) (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C, followed by an incubation with protein A/G (Santa Cruz Biotechnology) for another 2 h. After extensive washing with PBS containing 0.1% Triton X-100 and 0.025% NaN₃, the immunocomplex was denatured by boiling in Laemmli sample buffer, separated by SDS-PAGE and analysed by Western blot using anti-BP180 antibody as described above. For a reverse IP, 1 ml of cell lysate was immuno-precipitated by overnight incubation with either 5 µl of non-immune rabbit serum or 5 µl of rabbit anti-BP180 serum. Immunocomplex was blotted with mouse anti-14-3-3 σ antibody (Medicorp).

Enzyme digestion of cell surface proteins

For assessment of binding domain of BP180 with 14-3-3 proteins, several approaches were carried out according to a report by Schacke et al. (1998). To evaluate the interaction of 14-3-3 σ protein with endodomain of BP180, biotin-labeled keratinocytes were incubated with 40 µg/ml of *Clostridium histolyticum* collagenase (Sigma) for 40 min at 37°C in KSFM. Reaction was terminated by addition of 10 µl/ml of 0.5 M EDTA. Cells were then collected by centrifugation, washed twice with PBS, and disrupted by 50 mM Tris-HCl (pH7.50) buffer containing 10 mM EDTA, 1% Triton X-100, 0.5% NP-40, and protease inhibitor cocktail. Following centrifugation, pre-cleared cell lysates (containing endodomain of BP180) and collagenase digestion medium were used for GST pull-down assay. To achieve the ectodomain of BP180 for evaluating the interaction of 14-3-3 σ with ectodomain of BP180, the conditioned medium from biotin labeled keratinocyte were collected and concentrated by Centricon (Millpore). The biotin labeled cell lysates were then digested with 10 µg/ml sequence grade trypsin (Promega) for 2 minute at room temperature followed by an addition of 10 µg/ml of trypsin inhibitor (Sigma). Conditioned medium and cell lysates were then subjected to either GST-beats or GST-14-3-3 σ beats pull down assay. After separation of proteins by SDS-PAGE, they were immunoblotted onto PVDF membrane. Signals were detected by using either HRP-conjugated anti-biotin or rabbit anti-BP180 cytoplasmic domain antibody using a procedure previously used (Lam et al., 2005).

Results

Identification of BP180 as a 14-3-3σ binding protein

To identify any keratinocyte membrane proteins with ability to interact with 14-3-3 σ, a bacterial expression vector was designed to express full-length 14-3-3σ as glutathione S-transferase (GST) fusion protein. The fusion protein was purified by affinity chromatography on glutathione-agarose beads (Ghahary et al., 2004). In another set of experiments, keratinocyte cell surface proteins were labeled by impermeable biotin. Cell lysates were then incubated with beads containing either GST or 14-3-3σ-GST fusion proteins. As shown in Figure 1A, a protein with apparent molecular mass about 180 kDa was identified by the beads containing 14-3-3σ GST fusion protein. This protein was also visualized by a recombinant 14-3-3σ coupled Sepharose 4B matrix. The result in Figure 1B

showed that this protein is the major detectable 14-3-3 associated protein in our pulled down assay. A GST-14-3-3 fusion protein was also seen in the protein pulled down assay. To identify this 180 kDa protein, protein band was cut after silver staining. Candidate peptides were extracted after trypsin-digestion of the gel plug and subjected to MALDI MS/MS analysis. The results of the peptide mapping identified 180 kDa protein as being the BP180 protein, a transmembrane protein mainly expressed in epithelial cells. To further verify this result, Western blot with the anti-BP180 antibody was performed. As shown in Figure 2, the 180 kDa protein was recognized by BP180 antibody.

To test whether endogenous 14-3-3 σ is associated with BP180 protein, proteins from cultured keratinocytes were immunoprecipitated by anti-14-3-3 σ antibody. As shown in Figure 3A, indeed, BP180 was co-precipitated with anti-14-3-3 σ antibody. To further confirm this finding, a reverse co-precipitation assay was conducted. The result shown in Figure 3B revealed that 14-3-3 σ can also be co-precipitated by anti-BP180 antibody (lane 3). The apparent size of this protein was the same as that identified in keratinocyte cell lysate used as a positive control (lane 1). However, this protein was not detectable in IP: rabbit IgG samples used as negative control (lane 2).

Intact 14-3-3 σ is required for BP180 binding

To examine whether the whole or a specific sequence of 14-3-3 σ protein is needed for this interaction, we made a series of GST fusion 14-3-3 σ fragments containing either the N-terminal domain amino acids from 2 to 100 (Cd148), N-terminal domain amino acids from 2 to 150 (Cd98), C-terminal domain amino acids from 51 to 248 (Nd50), or C-terminal domain amino acids from 101 to 248 (Nd100) (Fig. 4A). Using the method of protein expression described above, these GST fusion fragments were successfully expressed in bacteria and purified by affinity column (Fig. 4B). These chimera GST fusion fragments were then used in GST pull down assay. As shown in Figure 4C, none of these GST fusion fragments exhibited any binding capacity with BP180 as indicated by using both anti-biotin and anti-BP180 in Western blot analysis. This finding indicates that the entire sequence of the 14-3-3 σ is needed to mediate its interaction with BP180 protein.

14-3-3 σ binds to the cytoplasmic domain of BP180

BP180 protein is a transmembrane protein with a large extracellular collagen domain (designated as COL1-15), a short non-helix domain (NC16A) linked with a transmembrane domain (NC16B), and the intracellular domain (NC16C) (Van den Bergh and Giudice, 2003). It has previously been demonstrated that the extracellular portion of BP180 can be shed from the cell surface as a result of proteolytic cleavage (Hirako et al., 1998). In conditioned medium of cultured keratinocytes, a 120 kDa soluble ectodomain of BP180 has been reported (Schacke et al., 1998). To test whether 14-3-3 σ binds to the ectodomain of BP180, three different strategies were used. First, cell surface proteins in keratinocytes were labeled with biotin, and cells were cultured for another 48 h. Cell culture medium was then collected and concentrated by Centricon. The concentrated medium was incubated with GST fusion 14-3-3 σ beads as described above. When total labeled proteins were separated by SDS-electrophoresis, Western blot analysis was performed using anti-biotin antibody. By using this approach, a protein band with apparent molecular mass of 180 was found only in GST-14-3-3 σ (lane 1), but not GST alone (lane 2), pull-down assay (Fig. 5A). As shown in this panel (lane 3), when concentrated conditioned medium was subjected to GST-14-3-3 σ pull-down assay, no protein band was identified. This finding indicates that 14-3-3 σ interacts only with cytoplasmic domain, but not ectodomain of BP 180. In the second approach, cell lysates from biotin labeled keratinocytes were digested with trypsin according to a previous report revealing that a 90 kDa extracellular domain of BP180 is resistant to limited trypsin treatment (Schacke et al., 1998). As shown in Figure 5B, non-treated cell

lysates were pulled-down by either GST beads (lane 1) or GST 14-3-3 σ fusion protein beads (lane 2). In consistent with the results of Figure 5A, a protein band with apparent molecular mass of 180 kDa was detected only in untreated cell lysate subjected to GST 14-3-3 σ fusion protein, but not GST alone, pull down assay (lane 2 versus lane 1). However, when trypsin-digested cell lysates were subjected to the same procedure of pull down assay by either GST beads (lane 3) or GST 14-3-3 σ fusion protein beads (lane 4), no detectable BP180 was detected. This finding further confirmed that 14-3-3 σ did not interact with trypsin resistant ectodomain of the BP180. In a third approach, the cell surface proteins in keratinocytes were labeled, and cells were incubated with either purified GST fusion 14-3-3 σ or GST alone for 1 h. Following removal of free ligand from conditioned medium, cells were washed extensively and lysed. Both GST and GST fusion 14-3-3 σ binding proteins were then immunoprecipitated by anti-GST antibody. Immune complex was picked up by protein A plus G, and BP180 was detected by Western blot analysis using anti-biotin and anti-BP180 antibodies. By using this approach, we also did not detect any protein that interacts with 14-3-3 σ (data not shown). The findings of these experiments suggest that it would be unlikely that 14-3-3 σ interacts with the ectodomain of the BP 180 in keratinocytes.

To determine whether the intracellular domain of BP180 binds to 14-3-3 σ , biotin-labeled keratinocytes were digested with collagenase to remove the collagen domain of BP180 as described previously (Schacke et al., 1998). Medium was collected and concentrated by Centricon and cells were lysed. The collagenase digested fragments of BP180 from medium or cell lysates were used to bind with GST fusion 14-3-3 σ beads. BP180 was detected by Western blot analysis as described above. As expected, after collagenase treatment, BP180 was degraded and a protein peptide with an apparent molecular mass of 60 kDa with weak biotin labeling was visualized (Fig. 6A). The 60 kDa protein peptide was strongly recognized by anti-BP180 antibody which directed against the N-terminus of BP180 (Fig. 6B). Previous data have also demonstrated that the BP180 fragment of 60 kDa consists of a cytoplasmic and transmembrane fragment (Schacke et al., 1998). Therefore, this result strongly indicates that 14-3-3 σ binds to the cytoplasmic domain of BP180.

Discussion

In this study, we provided evidence that BP180 is the major membrane protein identified in human keratinocytes in a 14-3-3 σ GST pull-down screening assay. This finding indicates the presence of a specific interaction between BP180 and 14-3-3 σ . This interaction was further verified by co-immunoprecipitation experiments. Analysis of a series of truncated or deleted 14-3-3 σ revealed that BP180 can only bind to the intact 14-3-3 σ molecule. This finding suggests that conformation and possible dimerization of 14-3-3 σ is essential for this interaction.

The finding that 14-3-3 proteins associated with BP180 was surprising and unexpected. BP180 does not contain either RSXpSXP or RXYFXpSXP motifs that are known to be important for interaction of 14-3-3 σ with its binding proteins (Yaffe et al., 1997). In these consensus, the sequences pS denote both phosphoserine and phosphothreonine. However, it is well established that there exists an alternative mechanism by which 14-3-3 isoforms interact with their ligands through phosphorylation-independent bindings. For example, Bax protein which is one of the main components of the apoptosis pathway interacts with 14-3-3 σ and 14-3-3 θ by a phosphorylation-independent mechanism. It is, therefore, very likely that 14-3-3 σ would interact with BP180 in this fashion. However, more studies are needed to clarify this issue. Several proteins including BP230, integrin β 4, and integrin α 6 have binding sites on BP180 and the interaction of these proteins play a critical role in assembly of hemidesmosomes (Borradori et al., 1997; Hopkinson and Jones, 2000; Koster et al., 2003). Although, our results support the notion that 14-3-3 σ primarily binds to

cytoplasmic domain of the BP180, further studies are also needed to explore not only the mechanism through which these two proteins interact but also examine the biological significance of this interaction.

The result showing that only intact 14-3-3 σ protein, but not its fragments is needed for this interaction is not surprising. This is because the crystal structures of the τ and ξ isoforms of 14-3-3 (Liu et al., 1995; Xiao et al., 1995) show that they are highly helical and dimeric proteins. The dimeric structure of 14-3-3 proteins is important for their interaction of members of this protein family with other binding partners. In fact, it is now well known that 14-3-3 proteins function as adapters, where two different target proteins bind simultaneously to each monomer of the same 14-3-3 dimer. This notion is supported by a number of observations. For example, 14-3-3 proteins mediate the pairings between Raf-1 and B-cell receptor (Brasemann and McCormick, 1995), Raf-1 and A20 (Vincenz and Dixit, 1996), as well as Raf-1 and PKC (Van der Hoeven et al., 2000). It is, therefore, possible that 14-3-3 proteins may act as a bridge to link other components of hemidesmosome with BP180. Similarly, it has been shown that 14-3-3 proteins bind to keratin 18 (Liao and Omary, 1996) expressed by keratinocytes and that has been associated with BP180 (Aho and Uitto, 1999). The dimeric 14-3-3 proteins mediate the macrophage stimulating protein-dependent formation of a Ron/ $\alpha 6\beta 4$ complex. This in turn induces disassembly of hemidesmosomes and $\alpha 6\beta 4$ relocation in lamellipodia (Santoro et al., 2003). Furthermore, previous studies found that when epithelial sheets were placed on the intact basal laminae and incubated in the presence of the tyrosine kinase inhibitor genistein, hemidesmosome formation was impaired (Payne et al., 2000).

Here, several different approaches were taken to determine whether 14-3-3 σ interacts with either extracellular domain, cytoplasmic tail of BP180 or both. The findings clearly indicate that 14-3-3 σ interacts with BP180 in keratinocytes. This is because the result of BP180 and 14-3-3 σ co-precipitation and its reverse IP experiments confirmed this finding. Using different experimental approaches such as trypsin and collagenase digestion assays of the keratinocytes, it becomes clear that 14-3-3 σ interacts only with intracellular domain, but not ectodomain of the BP180 protein. Consider the fact that BP180 is a transmembrane protein that functions as an attachment receptor as well as stimulation of the synthesis and release of some cytokines, one could speculate that interaction of 14-3-3 σ to its cytoplasmic domain plays a functional regulatory role for BP180 in keratinocytes.

Previous studies demonstrated the involvement of BP180 in cell attachment and suggested the possible role of this protein as a novel type of adhesion receptor (Hirako et al., 1998). It is also shown that treatment of cultured cells derived from a human squamous carcinoma with TPA (12-O-tetradecanoylphorbol-13-acetate) causes serine phosphorylation of BP180 and subsequent rearrangement of hemidesmosomes (Kitajima et al., 1992, 1995). Cleavage of the extracellular domain of BP180 by autoantibodies induces the release of IL-6 and IL-8, as well as tissue-type plasminogen activator from cultured human keratinocytes (Schmidt et al., 2000, 2004). Considering the fact that BP180 contains a large and unique collagen domain in its extracellular part with several putative phosphorylation sites in its cytoplasmic domain (Hopkinson et al., 1992), it is possible that 14-3-3 σ interaction with cytoplasmic domain of BP180 plays a potential yet unidentified biology role of this protein in keratinocytes.

Acknowledgments

This research was supported by the Canadian Institute for Health Research (CIHR-MOP-13387).

Abbreviations

BP	bullous pemphigoid
DMEM	Dulbecco's modified Eagle's medium
KSEFM	keratinocyte serum-free medium
GST	glutathione S-transferase
PVDF	polyvinylidene difluoride
MS	mass spectrometer
MALDI	matrix-assisted laser desorption/ ionization

Literature Cited

- Aho S, Uitto J. 180-kD bullous pemphigoid antigen/type XVII collagen: tissue-specific expression and molecular interactions with keratin 18. *J Cell Biochem* 1999;72:356–367. [PubMed: 10022517]
- Balding SD, Prost C, Diaz LA, Bernard P, Bedane C, Aberdam D, Giudice GJ. Cicatricial pemphigoid autoantibodies react with multiple sites on the BP180 extracellular domain. *J Invest Dermatol* 1996;106:141–146. [PubMed: 8592065]
- Borradori L, Koch PJ, Niessen CM, Erkeland S, van Leusden MR, Sonnenberg A. The localization of bullous pemphigoid antigen 180 (BP180) in hemidesmosomes is mediated by its cytoplasmic domain and seems to be regulated by the beta4 integrin subunit. *J Cell Biol* 1997;136:1333–1347. [PubMed: 9087447]
- Brasemann C, McCormick F. Bcr and Raf form a complex in vivo via 14-3- proteins. *EMBO J* 1995;14:4839–4848. [PubMed: 7588613]
- Cotelle V, Meek SE, Provan F, Milne FC, Morrice N, MacKintosh C. 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved *Arabidopsis* cells. *EMBO J* 2000;19:2869–2876. [PubMed: 10856232]
- Fairley JA, Heintz PW, Neuburg M, Diaz LA, Giudice GJ. Expression pattern of the bullous pemphigoid-180 antigen in normal and neoplastic epithelia. *Br J Dermatol* 1995;133:385–391. [PubMed: 8546992]
- Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol* 2000;40:617–647. [PubMed: 10836149]
- Ghahary A, Karimi-Busheri F, Marcoux Y, Li Y, Tredget EE, Kilani RT, Li L, Zheng J, Karami A, Keller BO, Weinfeld M. Keratinocyte-releasable stratifin functions as a potent collagenase-stimulating factor in fibroblasts. *J Invest Dermatol* 2004;122:1188–1197. [PubMed: 15140222]
- Giudice GJ, Emery DJ, Diaz L. Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180. *J Invest Dermatol* 1992;99:243–250. [PubMed: 1324962]
- Hirako Y, Owaribe K. Hemidesmosomes and their unique transmembrane protein BP180. *Microscopy Res Tech* 1998;43:207–217.
- Hirako Y, Usukura J, Uematsu J, Hashimoto T, Kitajima Y, Owaribe K. Cleavage of BP180, a 180-kDa bullous pemphigoid antigen, yields a 120-kDa collagenous extracellular polypeptide. *J Biol Chem* 1998;273:9711–9717. [PubMed: 9545306]
- Hopkinson SB, Jones JCR. The N terminus of the transmembrane protein BP180 interacts with the N-terminal domain of BP230, thereby mediating keratin cytoskeleton anchorage to the cell surface at the site of the hemidesmosome. *Mol Biol Cell* 2000;11:277–286. [PubMed: 10637308]
- Hopkinson SB, Riddelle KS, Jones JCR. Cytoplasmic domain of the 180-kD bullous pemphigoid antigen, a hemidesmosomal component: molecular and cell biologic characterization. *J Invest Dermatol* 1992;99:264–270. [PubMed: 1512461]
- Katz AB, Taichman LB. A partial catalog of proteins secreted by epidermal keratinocytes in culture. *J Invest Dermatol* 1999;112:818–821. [PubMed: 10233778]
- Kitajima Y, Owaribe K, Nishizawa Y, Jokura Y, Yaoita H. Phorbol ester- and calcium-induced reorganization of 180-kDa bullous pemphigoid antigen on the ventral surface of cultured human

- keratinocytes as studied by immunofluorescence and immunoelectron microscopy. *Exp Cell Res* 1992;203:17–24. [PubMed: 1426040]
- Kitajima Y, Owada MK, Fujisawa Y, Seishima M, Yaoita H, Hirako Y, Owaribe K. A hemidesmosomal transmembrane collagenous molecule, the 180-kDa bullous pemphigoid antigen (BPA II), is phosphorylated with 12-O-tetradecanoylphorbol-13-acetate in a human squamous cell carcinoma cell line (DJM-1). *Epithelial Cell Biol* 1995;4:70–75. [PubMed: 8688920]
- Koster J, Geerts D, Favre B, Borradori L, Sonnenberg A. Analysis of the interactions between BP180, BP230, plectin and the integrin $\alpha 6 \beta 4$ important for hemidesmosome assembly. *J Cell Sci* 2003;116:387–399. [PubMed: 12482924]
- Lam E, Kilani RT, Li Y, Tredget EE, Ghahary A. Stratifin-induced matrix metalloproteinase-1 in fibroblast is mediated by *c-fos* and p38 mitogen-activated protein kinase activation. *J Invest Dermatol* 2005;125:230–238. [PubMed: 16098031]
- Li K, Tamai K, Tan EM, Uitto J. Cloning of type XVII collagen. Complementary and genomic DNA sequences of mouse 180-kilodalton bullous pemphigoid antigen (BPAG2) predict an interrupted collagenous domain, a transmembrane segment, and unusual features in the 5'-end of the gene and the 3'-untranslated region of the mRNA. *J Biol Chem* 1993;268:8825–8834. [PubMed: 8473327]
- Liao J, Omary MB. 14-3-3 proteins associate with phosphorylated simple epithelial keratins during cell cycle progression and act as a solubility cofactor. *J Cell Biol* 1996;133:345–357. [PubMed: 8609167]
- Liu Z, Diaz LA, Troy JL, Taylor A, Emery DJ, Fairley JA, Giudice GJ. A passive transfer model of the organ-specific autoimmune disease, bullous pemphigoid, using antibodies generated against the hemidesmosomal antigen, BP180. *J Clin Invest* 1993;92:2480–2488. [PubMed: 7693763]
- Liu D, Bienkowska J, Petosa C, Collier RJ, Fu H, Liddington R. Crystal structure of the zeta isoform of the 14-3-protein. *Nature (London)* 1995;376:191–194. [PubMed: 7603574]
- McGrath JA, Darling T, Gatalica B, Pohla-Gubo G, Hintner H, Christiano AM, Yancey K, Uitto J. A homozygous deletion mutation in the gene encoding the 180-kDa bullous pemphigoid antigen (BPAG2) in a family with generalized atrophic benign epidermolysis bullosa. *J Invest Dermatol* 1996;106:771–774. [PubMed: 8618019]
- Muslin AJ, Tanner JW, Allen PM, Shaw AS. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* 1996;84:889–897. [PubMed: 8601312]
- Payne J, Gong H, Trinkaus-Randall V. Tyrosine phosphorylation: a critical component in the formation of hemidesmosomes. *Cell Tissue Res* 2000;300:401–411. [PubMed: 10928271]
- Santoro MM, Gaudino G, Marchisio PC. The MSP receptor regulates $\alpha 6 \beta 4$ and $\alpha 3 \beta 1$ integrins via 14-3-3 proteins in keratinocyte migration. *Develop Cell* 2003;5:257–271.
- Schacke H, Schumann H, Hammami-Hauasli N, Raghunath M, Bruckner-tuderman L. Two forms of collagen XVII in keratinocytes. A full-length transmembrane protein and a soluble ectodomain. *J Biol Chem* 1998;273:25937–25943. [PubMed: 9748270]
- Schmidt E, Reimer S, Kruse N, Jainta S, Brocker EB, Marinkovich M, Giudice GJ, Zillikens D. Autoantibodies to BP180 associated with bullous pemphigoid release interleukin-6 and interleukin-8 from cultured human keratinocytes. *J Invest Dermatol* 2000;115:842–848. [PubMed: 11069622]
- Schmidt E, Wehr B, Tabengwa EM, Seimer S, Brocker EB, Zillikens D. Elevated expression and release of tissue-type, but not urokinase-type, plasminogen activator after binding of autoantibodies to bullous pemphigoid antigen 180 in cultured human keratinocytes. *Clin Exp Immunol* 2004;135:497–504. [PubMed: 15008985]
- Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996;68:850–858. [PubMed: 8779443]
- Van den Bergh F, Giudice GJ. BP180 (type XVII collagen) and its role in cutaneous biology and disease. *Adv Dermatol* 2003;19:37–71. [PubMed: 14626817]
- Van der Hoeven PCJ, Van der Wal JCM, Ruurs P, Van Dijk MCM, Van Blitterswijk WJ. 14-3-3 isotypes facilitate coupling of protein kinase C-zeta to Raf-1: negative regulation by 14-3-phosphorylation. *Biochem J* 2000;345:297–306. [PubMed: 10620507]

- Vincenz C, Dixit VM. 14-3-3 proteins associate with A20 in an isoform-specific manner and function both as chaperone and adapter molecules. *J Biol Chem* 1996;271:20029–20034. [PubMed: 8702721]
- Xiao B, Smerdon SJ, Jones DH, Dodson GG, Soneji Y, Aitken A, Gamblin SJ. Structure of a 14-3-3 protein and implications for coordination of multiple signalling pathways. *Nature (London)* 1995;376:188–191. [PubMed: 7603573]
- Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, Gamblin SJ, Smerdon SJ, Cantley LC. The structural basis for 14-3-3: phosphopeptide binding specificity. *Cell* 1997;91:961–971. [PubMed: 9428519]
- Zillikens D. Acquired skin disease of hemidesmosomes. *Dermatol Sci* 1999;20:134–154.

Fig. 1.

Analysis of 14-3-3 binding proteins by GST pull-down assay in keratinocytes. A: Keratinocyte cell surface proteins were labeled with biotin, and lysates were subjected to GST pull-down assay with either GST beads (lane 1), GST fusion 14-3-3 σ beads (lane 2) or 14-3-3 σ coupled Sepharose 4B matrix (lane 3). Purified proteins were analyzed by immunoblotting with anti-biotin. B: Lysates from keratinocytes were subjected to GST pull-down assay with GST beads (lane 2) or GST fusion 14-3-3 σ beads (lane 4). Purified proteins were visualized by silver staining. Lanes 1 and 3 which present equal amount of GST beads and GST fusion 14-3-3 σ beads, respectively, used as controls. The arrows indicate the purified cell surface protein with molecular weight approximately 180 kDa. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2. Interaction of 14-3-3 σ with BP180. Cell surface proteins were purified by GST pull-down assay as described in Figure 1, purified proteins were then analyzed by immunoblotting with anti-BP180 antibody. The arrows indicate BP180.

Fig. 3.

Immunoprecipitation and reverse IP for BP180 and 14-3-3 σ proteins. For IP of BP180 by 14-3-3 σ antibody, keratinocytes were disrupted in lysis buffer and lysates were subjected to either non-immune IgG (Part A, lane 1) or anti-14-3-3 σ antibody (Part A, lane 2). Immunocomplex was picked up by protein A/G agarose. Purified proteins were analyzed by immunoblotting with anti-BP180 antibody (Part A). The arrow indicates BP180 band. For reverse IP, 14-3-3 σ was co-immunoprecipitated by anti-BP180 serum. Keratinocytes were disrupted in lysis buffer and lysates were subjected to either non-immune IgG (lane 2) or anti-BP180 serum (lane 3). Immunocomplex was picked up by protein A/G agarose.

Purified proteins were analyzed by immunoblotting with anti-14-3-3 σ antibody (Part B). The arrow indicates the 14-3-3 σ protein. A cell lysate was also included as a positive control for 14-3-3 σ (lane 1).

Fig. 4.

Deletion of either N-terminus or C-terminus of 14-3-3σ abrogates its binding capacity to BP180. **A**, Schematic representation of truncated 14-3-3σ. Nd50 represents the deletion of 1–50 amino acids from the N-terminus; Nd100 represents the deletion of 1–100 amino acids from the N-terminus; Cd98 represents the deletion of 151–248 amino acids from the C-terminus; Cd148 represents the deletion of 101–248 amino acids from the C-terminus. **B**: The truncated 14-3-3σ were expressed as GST fusion proteins in bacteria and purified by glutathione affinity chromatography, 5 μg of GST fusion proteins were run on SDS-PAGE, gel was stained by brilliant blue R-250. **C**: Keratinocyte cell surface proteins were labeled by biotin, lysates were subjected to GST fusion 14-3-3σ either wild type protein (WT), Nd50, Nd100, Cd98 or Cd148 beads, purified proteins were analyzed by immunoblotting with either anti-biotin (left part) or anti-BP180 (right part). The arrow indicates BP180. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 5.

14-3-3 σ does not bind with the ectodomain of BP180. In part A, cell surface proteins of keratinocytes were labeled by biotin and maintained in culture for another 48 h. Cells were then harvested, lysed and the corresponding conditioned media were collected and concentrated by Contricon. Cell lysate was subjected to either GST-14-3-3 σ (lane 1) or GST-alone (lane 3) pull down assay. In parallel, the concentrated conditioned medium was also subjected to GST-14-3-3 σ (lane 3) pull-down assay. In panel B, the cell surface proteins of keratinocytes were labeled by biotin. Cell lysates were treated with either nothing or 10 μ g/ml of trypsin for 2 min at room temperature. The activity of trypsin was terminated by adding 10 μ g/ml of trypsin inhibitor to cell lysates and the lysates were then subjected to GST-pull down assay. Non-treated cell lysates were pulled-down by either GST beads (lane 1) or GST 14-3-3 σ fusion protein beads (lane 2). Trypsin-digested cell lysates were also pulled-down by either GST beads (lane 3) or GST 14-3-3 σ fusion protein beads (lane 4). The arrow indicates BP180 band.

Fig. 6. 14-3-3 σ binds with cytoplasmic region of BP180. Biotin-labeled keratinocytes were incubated with 40 μ g/ml of collagenase in serum-free keratinocyte medium (KSFM) at 37°C for 45 min. KSFM fractions were collected and cells were lysed in lysis buffer. KSFM fractions (lanes 2 and 3) and lysates with (lanes 4 and 5) or without (lane 1) collagenase treatment were subjected to GST pull-down assay with either GST (lanes 2 and 4) or GST fusion 14-3-3 σ (lanes 1, 3, and 5). Purified proteins were analyzed by immunoblotting with either anti-biotin (Part A) or anti-BP180 (Part B). The filled arrow indicates BP180 with 180 kDa, and open arrow indicates the fragment of BP180 with 60 kDa.