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Alterations in Type I Hemidesmosome Components Suggestive of Epigenetic Control in the Salivary Glands of Patients With Sjögren's Syndrome

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***Objective*. Acinar cells in the salivary glands of patients with Sjögren's syndrome (SS) display severe alterations in anchorage to the basal lamina. Bioinformatics analysis of the BP230 gene sequence has revealed the presence of CpG islands that might be involved in epigenetic control of gene expression, and methylation of the BP230 promotor region may be implicated as an epigenetic control mechanism in salivary gland damage. Thus, the present study was undertaken to evaluate the protein BP230, as well as proteins BP180, $\alpha6\beta4$ integrin, and cytokeratin-18, for their expression levels, localization, and ability to form hemidesmosome adhesion complexes.**

***Methods*. Eighteen patients with primary SS and 14 healthy control subjects were studied. Levels of messenger RNA (mRNA) and protein were measured by reverse transcription–polymerase chain reaction and Western blotting, respectively. BP230 methylation was determined by methylation-sensitive polymerase chain reaction. Protein complexes were analyzed by immunoprecipitation and assessed for localization by immunofluorescence.**

***Results*. In patients with SS as compared with controls, BP230 mRNA levels were decreased while protein levels were increased, and the gene promotor region was hypermethylated. Augmented proteolysis of BP180 was detected, since levels of linear IgA disease fragment 1 were increased. The complex-forming ability of BP230, BP180, $\alpha6\beta4$ integrin, and cytokeratin-18 was maintained in patients with SS, in contrast to that in controls. BP230 and BP180 colocalized at the basal membrane of acinar cells, and cleavage of BP180 coincided with a loss of colocalization.**

***Conclusion*. The decrease in BP230 mRNA levels may be explained by gene hypermethylation. We postulate that local epigenetic modifications of BP230 are produced in response to factors present in the damaged salivary glands of patients with SS. Additionally, the paradoxical increase in BP230 protein levels and the formation of both normal and altered adhesion complexes may help avoid cell death induced by the loss of anchorage.**

Severe alterations in acinar cells and the extracellular matrix (ECM) are recurrent observations in the labial salivary glands (LSGs) of patients with primary Sjögren's syndrome (SS) (1–4). LSG epithelial cells are linked to the basal lamina (BL) through several transmembrane protein complexes that have important functions in adhesion and cell signaling (5). These adhesion complexes include $\alpha6\beta1$ and $\alpha6\beta4$ integrins as a central

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core, which connect the BL with the actin cytoskeleton and cytokeratin cytoskeleton, respectively (6). Significant changes both in gene expression and in subcellular distribution of these complexes in patients with SS have been reported recently by our group (4).

In patients with SS, increased proteolysis of nidogens and type I and type III collagens is the consequence of an imbalance of matrix metalloproteinase activity and tissue inhibitors of matrix metalloproteinases (TIMPs) (1,2,7). In addition, LSG samples from patients with SS display reduced immunodetection of laminins 111 and 211, which is paralleled by an increase in laminin 411 at locations distant from inflammatory foci (3). However, protein levels of laminins 111 and 332 were augmented, indicating that remodeling of the BL occurred (2). In acini with highly disorganized BL, the β4 integrin (laminin 332 receptor) protein levels were decreased and subcellular distribution was altered (4). These observations suggest that changes in the interactions between extracellular proteins with their cognate adhesion receptors can modify the role of these complexes, resulting in an impairment of the secretory function of LSGs in patients with SS (4).

The $\alpha 6\beta 4$ integrin localizes to the basal plasma membrane of acinar cells (4) and interacts with the protein BP180 (ColXVIIα1). The cytoplasmic domains of both proteins bind BP230, as well as plectin, and interact with cytokeratin filaments, while their extracellular domains bind laminin 332. This entire complex constitutes a group of proteins known as the type I hemidesmosomes (HDs) (8). Since HDs play key roles in differentiation, growth, survival, and cell migration, altered expression of HD constituents has been implicated in various diseases, such as bullous pemphigoid, in which autoantibodies to BP180 and BP230 have been detected (8).

BP180 is a type II glycoprotein that belongs to the transmembrane collagen family. The extracellular domain is particularly important, since this region contains the proteolytic site NC16, which, when cleaved by ADAM-17, generates linear IgA disease fragment 1 (LAD-1), a 120-kd proteolytic fragment that is a target for autoantibodies detected in linear IgA disease (9). BP230, a member of the plakin protein family, corresponds to the epithelial splice variant of the dystonin gene and contains interferon-γ (IFNγ) (10) as well as transforming growth factor β (11) response sites.

Changes in the expression of BP230 and BP180 may occur as a consequence of mutations, altered immune responses, and epigenetic modifications. Interestingly, autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and systemic sclerosis, have been linked to alterations in epigenetic control (12,13). Altered hypomethylation patterns in the DNA of T and B lymphocytes are associated with the pathogenesis of SLE and RA (12,14). The promoter of the death receptor 3 (DR-3) gene in synovial fibroblasts from RA patients contains one CpG island that is hypermethylated and causes downregulation of DR-3 (15). These observations suggest that promotor hypo- or hypermethylation is associated with epigenetic changes in autoimmune diseases. Recent findings also implicate microRNA (miRNA) in the posttranscriptional mechanisms of control in the LSGs of patients with SS. Moreover, miRNA are currently suggested to be relevant as biomarkers of SS (16). However, evidence linking epigenetic changes in salivary epithelial cells to the pathogenesis of SS is limited. Interestingly, bioinformatics analysis of the BP230 gene sequence has revealed the presence of CpG islands upstream of the transcription initiation site, which might be important for epigenetic control of gene expression.

Since cell–BL interactions and tight junctions are damaged in the LSGs of patients with SS (17), we evaluated messenger RNA (mRNA) and protein levels as well as localization of BP230 and BP180. We also quantified the methylation status of BP230. In addition, LAD-1 protein levels and the complex-forming ability of HD proteins were determined.

PATIENTS AND METHODS

Patients with primary SS and control subjects. A total of 32 individuals were informed about the aims and procedures of the study and signed a written informed consent form, which was previously approved by the Ethics Committee of the Faculty of Medicine, University of Chile. Eighteen subjects were diagnosed as having primary SS based on the American-European Consensus Group criteria (18). Fourteen control subjects were selected from among individuals who did not fulfill the classification criteria for SS. In lip biopsy specimens from controls, only mild, nonspecific chronic sialadenitis was detected (Chisholm and Mason grade 1 [19]). Table 1 lists the clinical and demographic characteristics of the patients with SS and controls.

Collection of biopsy samples. LSG samples were obtained from patients and controls using the method described by Daniels (20). Following surgery, samples were split into 2 portions. One portion was immediately frozen in liquid nitrogen and stored at −80°C until the time of extraction of DNA, RNA, and proteins. The second portion was processed for morphologic and immunohistochemical experiments.

RNA extraction and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR). Total RNA from the LSGs was extracted with the RNeasy kit (Qiagen). The concentration and purity of RNA were assessed

Table 1. Demographic, serologic, and histologic characteristics of the patients with Sjögren's syndrome and healthy control subjects*

	Healthy control subjects	Patients with Sjögren's syndrome
No. of subjects	14	18
Sex, no. male/no. female	0/14	0/18
Age, mean (range) years	46 (28–57)	52 (32–76)
USF, mean ± SD ml/15 minutes*	4.4 ± 2.1	0.68 ± 0.75†
Focus score, no. of subjects‡		
1	0	5
2	0	6
3	0	0
4	0	3
>5	0	4
Serologic feature, no. of subjects		
Ro antibodies	0	14
Ro/La antibodies	0	6
Antinuclear antibodies	0	14
Rheumatoid factor	0	5

* USF = unstimulated salivary flow.
† $P < 0.0001$ versus controls.
‡ Focus scores indicate the number of inflammatory foci/4 mm^2 of tissue.

by ultraviolet spectrophotometry. RNA quality was assessed by measuring the ratio of absorbance at 260 nm to that at 280 nm ($A_{260\ nm}/A_{280\ nm}$), and RNA with absorbance ratios between 1.8 and 2.0 were used in this study. RNA integrity was verified by electrophoresis in 1% agarose–formaldehyde gels. All RNA from controls and patients with SS passed quality and integrity control. One microgram of total RNA was reverse transcribed with oligo(dT), random primers, and the Superscript II enzyme (Invitrogen). In PCR amplification experiments, varying amounts of complementary DNA (cDNA) were used (equivalent to 100, 20, and 10 ng of total RNA for BP230, and 200, 50, and 10 ng of total RNA for BP180). In PCR assays for BP230 and BP180, 1-μl aliquots of cDNA template were used. Samples were denaturated at 94°C for 30 seconds, annealed at the optimal temperature of primers for 30 seconds, and polymerized at 72°C for 1 minute. The final extension step was at 72°C for 5 minutes. RT-PCR conditions for GAPDH have been previously described (4).

Primer sequences were as follows: for BP230 (accession no. NM_015548.3), forward 5′-GTC-CAA-CAC-TGA-TCA-ACT-C-3′ and reverse 5′-ACA-GGC-CAG-AAG-TCA-TAC-3′; for BP180 (accession no. NM_000494.3), forward 5′-GGA-AGC-CCT-GGC-CCT-AAA-GGT-GAC-3′ and reverse 5′-AAC-CTC-TCA-TGC-CAG-GCT-CGC-CTG-T-3′; and for GAPDH, forward 5′-ACT-ACA-CGA-ACA-ACA-GCC-3′ and reverse 5′-TCT-TGT-TGT-CTC-CTC-GTC-3′. Amplification products obtained after 30 cycles were analyzed by electrophoresis in a 1.5% agarose gel. PCR products of BP230 and BP180 were quantified by densitometry. Expression values were normalized to those of GAPDH. Results shown were averaged from 3 independent experiments.

DNA extraction and methylation-sensitive PCR. Genomic DNA was obtained from the LSG samples from patients with primary SS and control subjects using the QIAamp DNA kit Mini and Blood (Qiagen). The concentration and purity of the DNA were assessed by ultraviolet spectrophotometry at 260 nm and 280 nm. Samples with an $A_{260\ nm}/A_{280\ nm}$ absorbance ratio ranging from 1.8 to 2.0 were used in this study. DNA integrity was verified by electrophoresis in a 1% agarose gel. The MethylCode Bisulfite Conversion kit (Invitrogen) was used to assess genomic DNA methylation, in accordance with the manufacturer's protocol.

For the methylation-specific PCR, two primer pairs were designed to amplify a 100-bp product from DNA sequences of interest. One of the two primer pairs amplified bisulfite-modified DNA. Methprimer Beta software version 1.1 (21) was utilized to design both the methylated primers (forward 5′-TTG-TAA-TTT-TAG-TAT-TTT-GGG-AGG-TC-3′ and reverse 5′-TTT-TAA-TAA-AAA-CGA-AAT-TTC-ACC-G-3′) and the unmethylated primers (forward 5′-GTA-ATT-TTA-GTA-TTT-TGG-GAG-GTT-GA-3′ and reverse 5′-ATT-TTT-AAT-AAA-AAC-AAA-ATT-TCA-CCA-3′). Standard conditions were used in the PCRs. The PCR products were resolved by electrophoresis in a 2.5% agarose gel. Bands were quantified with the UNI-SCAN-IT gel (Windows 4.1 version; Silk Scientific Corporation). The results were expressed as a methylation index (calculated as methylated DNA/[methylated DNA + unmethylated DNA]). To verify the sequence alteration in the unmethylated DNA and conservation of the original sequence in the methylated DNA after bisulfite treatment, all PCR products were sequenced after purification from the agarose gels using the Wizard SV gel and PCR Clean-Up Systems (Promega).

Western blotting. LSG samples were homogenized as previously described (17). Proteins were quantified using the Bradford method (22). Protein aliquots of 25–50 μg were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 6% acrylamide gels under nonreducing conditions for BP230, and in 8% gels under reducing conditions for BP180 and LAD-1, according to the Laemmli method (23). Proteins were transferred to nitrocellulose membranes that were blocked for 1 hour at room temperature in a 5% fat-free milk solution prepared in Tris buffered saline–Tween buffer (17), and then incubated with primary antibodies against either BP230 (a kind gift from Dr. K. Owaribe), BP180 (Abcam), β-actin (Zymed), β4 integrin (H-101), α6 integrin (BQ16), or cytokeratin-18 (DC10), using dilutions ranging from 1:50 to 1:10,000. After washing, blots were incubated with a horseradish peroxidase (HRP)–conjugated anti-mouse antibody, using a 1:5,000 dilution for BP230 and a 1:10,000 dilution for α6 integrin and cytokeratin-18 (Jackson ImmunoResearch). Alternatively, an HRP-conjugated anti-rabbit antibody was used (1:5,000 dilution for BP180 and β4 integrin and 1:10,000 dilution for β-actin; Jackson ImmunoResearch). Protein bands were visualized by enhanced chemiluminescence (Pierce), quantified by densitometry, and then normalized to the values for β-actin.

Immunoprecipitation of type I HD components. Anti–β4 integrin and anti-BP180 antibodies (kindly provided by Dr. J. C. Jones) were used to immunoprecipitate type I HD components, in accordance with previously described protocols (4).

Immunofluorescence detection of BP230, BP180, and laminin. Cryosections of the LSGs were fixed in cold acetone for 5 minutes, and then washed in phosphate buffered saline (PBS) buffer. Nonspecific antibody binding was blocked by

incubation for 1 hour at room temperature with a 0.25% casein solution prepared in PBS. Primary antibodies were separately added, using a 1:10 dilution for BP230, no dilution for BP180, and 1:25 dilution for laminin (Sigma). Samples were incubated for 20 hours at 4°C, and nonspecific binding was eliminated by washing with PBS. Secondary, Alexa Fluor–conjugated antibodies were diluted 1:250 in PBS, incubated for 45 minutes at room temperature, and washed with PBS. Preimmune serum obtained from the same animals as those used to prepare the antibodies was used as a negative control. Sections were mounted in Mowiol. Images were obtained with a Zeiss confocal microscope (model LSM-410 Axiovert-10.0) and stored in .tif format.

Statistical analysis. GraphPad Prism software version 5.00 was used for statistical analysis. Significant differences between the patients with primary SS and control subjects were assessed with the Mann-Whitney test for data not following normal distribution. *P* values less than 0.05 were used as the threshold for significance.

RESULTS

Levels of epithelial BP230 mRNA and protein. BP230 mRNA levels in LSG samples from 9 patients with primary SS and 9 control subjects were analyzed. As shown in Figures 1A and B, levels of BP230 mRNA were significantly decreased in patients with SS compared with controls ($P = 0.029$). However, as shown in Figures 1C and D, BP230 protein levels were significantly increased in patients with SS compared with controls ($P = 0.001$). These results suggest that BP230 mRNA and protein levels are regulated in an independent manner.

BP230 methylation index. Bioinformatics sequence analysis revealed the presence of putative methylation sites upstream of the BP230 transcription initiation site. Methylation assays with genomic DNA obtained from the LSGs of 18 patients with SS and 14 controls revealed that the methylation index was significantly higher in patients with SS compared with controls ($P = 0.018$) (Figures 1E and F). This result likely explains the reduced BP230 mRNA levels observed in patients with SS.

Levels of BP180 mRNA and protein and LAD-1 protein. As mentioned above, BP180 may undergo proteolysis, resulting in formation of the LAD-1 120-kd fragment (9). Relative BP180 mRNA levels were not significantly different between patients with SS and control subjects (n = 9 each; $P = 0.14$) (Figures 2A and B). Western blot experiments (Figure 2C) revealed the presence of both full-length BP180 (180 kd) and the LAD-1 (120 kd) proteolytic fragment in LSG protein extracts from 8 controls and 8 patients with SS. Individual densitometric analysis of BP180 and LAD-1 showed

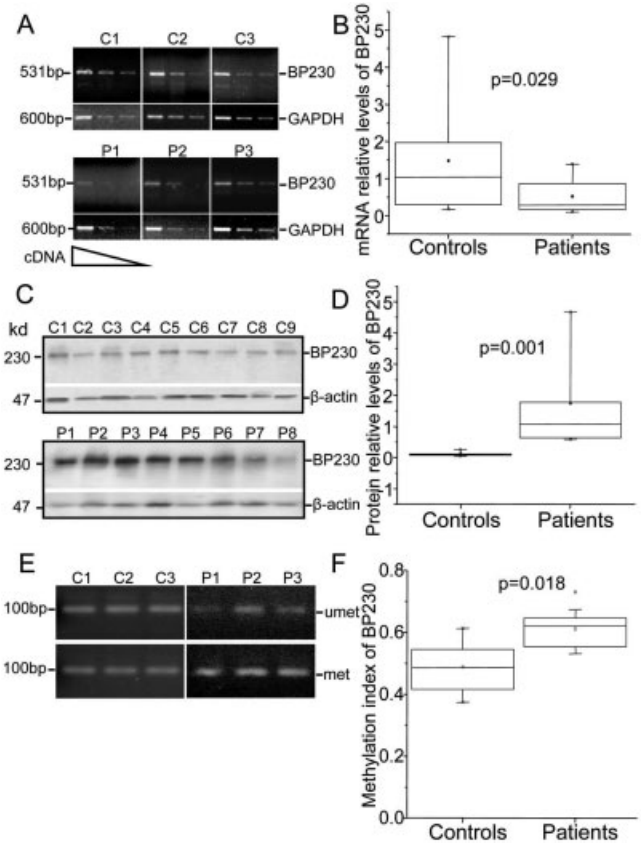

Figure 1. BP230 mRNA, protein, and methylation levels in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS) compared with healthy control subjects. **A** and **B,** LSGs from 9 patients with SS and 9 controls were assayed for BP230 mRNA, with results normalized to the values for GAPDH, by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR). For each subject, 3 different quantities of total RNA were used (100, 20, and 10 ng). BP230 was detected as a single PCR amplicon of 531 bp, and GAPDH as a band of 600 bp. Expression of BP230 mRNA was assessed by gel electrophoresis (data from 3 representative control samples [C1–C3] and 3 representative patient samples [P1–P3] shown) (**A**) and by ratiometric analysis of the densitometric data from all analyzed samples (**B**). **C** and **D,** LSGs from 9 patients with SS (P1–P9) and 9 controls (C1–C9) were assayed for BP230 protein, relative to the values for β-actin, by immunoblotting. Results were assessed by gel electrophoresis, with BP230 detected as a single band of 230 kd and β-actin as a band of 47 kd (**C**), and by ratiometric analysis of the densitometric data from all analyzed samples (**D**). **E** and **F,** LSGs from 18 patients with SS and 14 controls were assayed for methylation status of the BP230 gene. On gel electrophoresis, unmethylation-specific (umet) and methylation-specific (met) amplicons were visualized (data from 3 representative control samples [C1–C3] and 3 representative patient samples [P1–P3] shown) (**E**). Values for the BP230 methylation index were calculated using the densitometric data from ratiometric analysis of all analyzed samples (**F**). Results in **B, D,** and **F** are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles, using results from 3 independent experiments. *P* values less than 0.05 were considered significant.

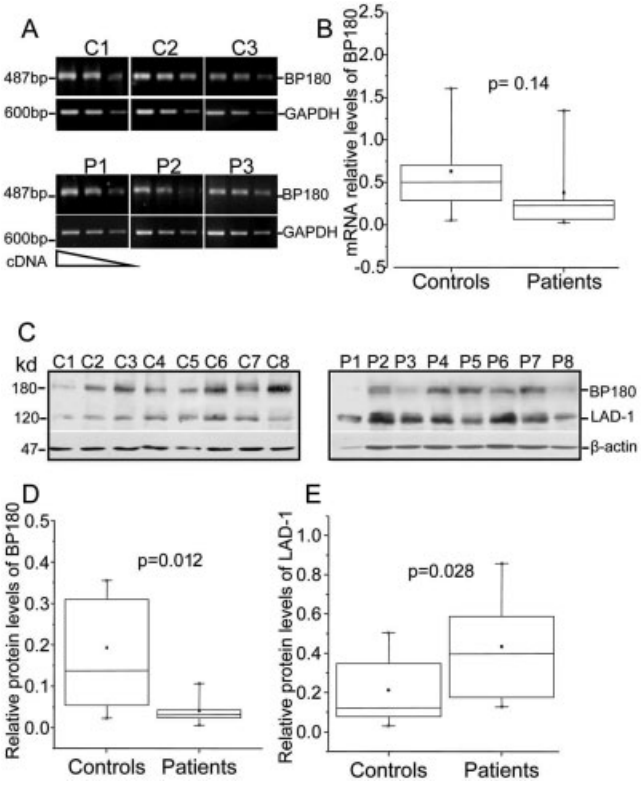

Figure 2. BP180 mRNA and protein levels in LSGs from patients with SS compared with healthy control subjects. **A** and **B,** LSGs from 9 patients with SS and 9 controls were assayed for BP180 mRNA, with results normalized to the values for GAPDH, by semiquantitative RT-PCR. For each subject, 3 different quantities of total RNA were used (200, 50, and 10 ng). BP180 was detected as a single PCR amplicon of 487 bp, and GAPDH as a band of 600 bp. Expression of BP180 mRNA was assessed by gel electrophoresis (data from 3 representative control samples [C1–C3] and 3 representative patient samples [P1–P3] shown) **(A)** and by ratiometric analysis of the densitometric data from all analyzed samples **(B)**. **C–E,** LSGs from 8 controls (C1–C8) and 8 patients with SS (P1–P8) were assayed for BP180 and linear IgA disease fragment 1 (LAD-1) protein, relative to the values for β-actin, by immunoblotting. Results were assessed by gel electrophoresis, with BP180 detected as a band of 180 kd (BP180 full-length), LAD-1 as a band of 120 kd, and β-actin as a band of 47 kd **(C)**, and by ratiometric analysis of the densitometric data from all analyzed samples **(D** and **E)**. Results in **B, D,** and **E** are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles, using results from 3 independent experiments. *P* values less than 0.05 were considered significant. See Figure 1 for other definitions.

a significant increase in LAD-1 ($P = 0.028$) and a concomitant decrease in BP180 ($P = 0.012$) in patients with SS (Figures 2D and E). For patients with SS, the BP180:LAD-1 proteolysis ratio was 1.7-fold higher than that for controls ($P = 0.0003$). To our knowledge, this proteolytic event has not been documented previously in LSGs.

Assessment of complex formation by detection of type I HD–forming proteins. The presence of BP230 and BP180, as detected by Western blotting, did not provide

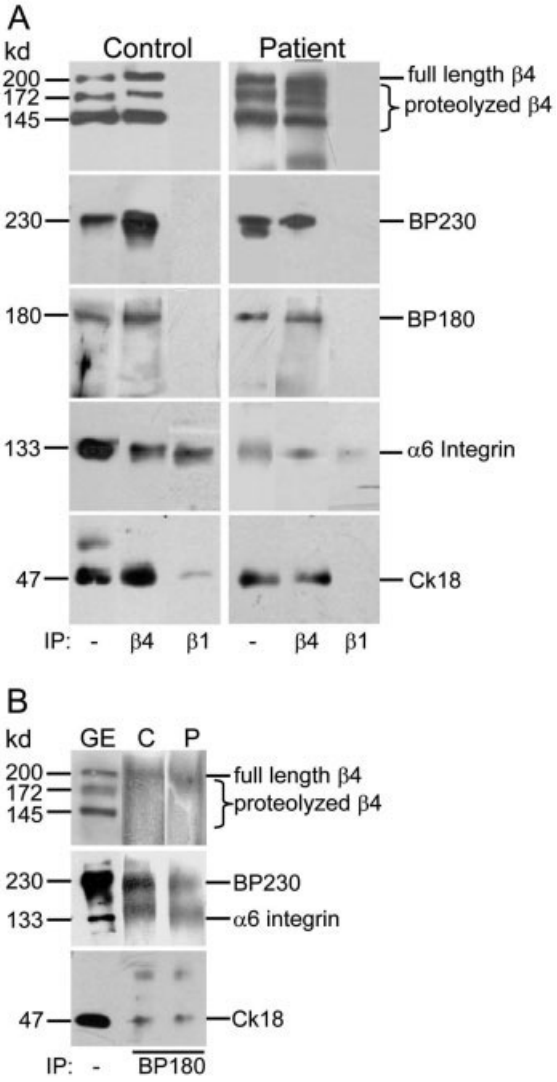

Figure 3. Immunoprecipitation (IP) of type I hemidesmosome–forming proteins in the LSGs of patients with SS compared with healthy control subjects. Pooled protein extracts of LSGs from 7 patients with SS and 6 controls were assayed by IP, with results analyzed by gel electrophoresis in assays with anti–β4 integrin antibodies **(A)** and anti-BP180 antibodies **(B)**. To assess the specificity of the IP, an anti–β1 integrin antibody was used in both assays. BP230, BP180, β4 integrin, α6 integrin, and cytokeratin-18 (Ck18) were separately detected by immunoblotting using the specific antibodies. Lanes labeled as "−" correspond to experiments performed with the total protein extract not subjected to IP. Representative results from 1 of 2 independent experiments are shown. GE = glandular extract (see Figure 1 for other definitions.)

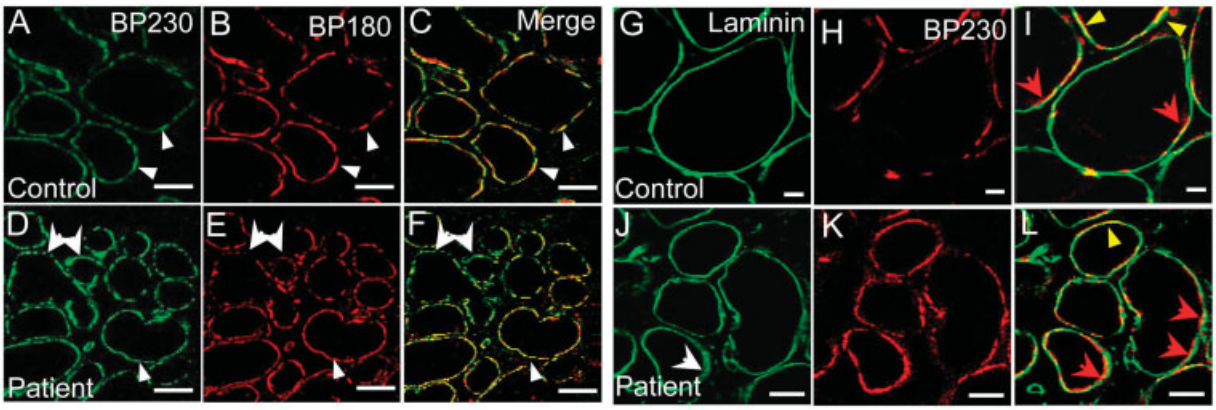

Figure 4. Localization of BP230/BP180 **(A–F)** and laminin 111/BP230 **(G–L)** in LSG sections from control subjects and patients with SS. Immunofluorescence experiments were performed in LSG samples from 6 controls (top panels) and 8 patients with SS (bottom panels). **A–F,** BP230 and BP180 localized to the basal surface in a patchy pattern in control LSGs **(A–C)**, while distribution was preserved in patients with SS **(D–F). Arrowheads** indicate colocalization of both proteins, while **arrows** indicate the loss of localization, mostly attributable to decreased BP180 staining. **G–L,** Homogeneous distribution of laminin 111 **(G)** and patchy distribution of BP230 **(H)** were observed in control LSGs, and BP230 colocalized with laminin 111 **(I)** (**yellow arrowheads**). **Red arrows** in **I** indicate small areas without colocalization. In patient LSGs, laminin 111 was distributed in a diffuse pattern **(J)** (**arrow**) that was accompanied by an increase in BP230 signal **(K)**. Colocalization **(yellow arrowhead)** and loss of colocalization **(red arrows)** were observed in diffuse areas **(L)**. Bars in **A–C** and **K–L** = 25 μm; bars in **D–F** = 50 μm; bars in **H–J** = 10 μm. See Figure 1 for definitions.

information related to complex formation between proteins composing the type I HDs. To address this issue, immunoprecipitation assays were done in pooled protein extracts of LSGs from 6 controls and 7 patients with SS. Antibodies against the extracellular domain of β4 integrin, α6 integrin, BP230, BP180, and cytokeratin-18 were used in Western blot experiments. The results of these experiments, as shown in Figure 3A, indicate that the aforementioned proteins formed a complex with β4 integrin in LSG samples both from controls and from patients with SS. Higher protein band intensity was observed in the pooled samples from controls compared to the pooled samples from patients with SS. The specificity of immunoprecipitation procedures was assessed using an antibody against β1 integrin, and, as expected, only complexes with α6 integrin were detected.

We have previously observed increased proteolysis of β4 integrin in LSGs of patients with SS (4), and other studies have revealed that proteolytic fragments of β4 integrin no longer contain binding sites for BP230 and BP180. Therefore, to analyze the complex-forming ability of intact β4 integrin with the above-mentioned proteins, an anti-BP180 antibody was used in immunoprecipitation experiments. As shown in Figure 3B, only full-length β4 integrin coimmunoprecipitated with BP180 both in samples from controls and in samples from patients with SS. These experiments suggest that BP180 and BP230 do not bind to the 172-kd and 145-kd proteolytic fragments.

Localization and subcellular distribution of BP230 and BP180 proteins. Immunolocalization experiments were done with LSG samples from 6 controls and 8 patients with SS. In the LSGs of controls, a patchy distribution of BP230 in the basal region of acini was observed. In the LSGs of patients with SS, the distribution of BP230 in some acini was similar to that in controls, while in other acini, higher levels of staining, as compared with that in controls, combined with a more homogeneous distribution over the cell surface were observed (Figures 4A and D). The localization and distribution of BP180 were similar to those of BP230 in controls and patients (Figures 4B and E). Extensive colocalization of both proteins was observed at the basal surface of LSG acini in controls and patients with SS (arrowheads in Figures 4C and F). However, colocalization was lost in some acini of patients with SS, concomitant with a decrease in BP180-specific immunofluorescence (Figure 4F, arrows).

Colocalization of BP230 and laminin 111. Previous work in our laboratory has shown that BL changes are correlated with alterations in the localization and distribution of β4 integrin (4). To assess whether changes observed in the BL coincide with alterations in the BP230 distribution pattern, colocalization with laminin 111 was evaluated. As shown in Figures 4G and

J, localization of laminin 111 in the acini differed between controls and patients with SS. Homogeneous distribution of laminin 111 was observed surrounding the acinus in control individuals. In patients with SS, distribution of laminin 111 was discontinuous and diffuse in some regions of the BL (Figure 4J, arrow).

In addition, the localization of BP230 in the acini was compared between controls and patients with SS, as shown in Figures 4H and K. A distribution pattern similar to that observed in Figures 4A and D was detected. BP230 and laminin 111 colocalized in the acini of controls and patients with SS (Figures 4I and L, yellow arrowheads). However, in the acini of patients with SS, the distribution of BP230 and laminin 111 was diffuse in the BL and no colocalization was observed (Figure 4L, red arrows).

DISCUSSION

In the present study, the expression, interaction, and localization of BP230 and BP180 in LSG acini of patients with SS were assessed for the first time. Their relationship with type I HD–forming proteins was also explored. In addition, BP230 gene methylation was determined in the acini. In patients with SS, the BP230 gene was hypermethylated and the mRNA levels were decreased, while protein levels were increased. In contrast, a decrease in the levels of full-length BP180 protein was observed and this was paralleled by an increase in the levels of the LAD-1 proteolytic fragment, without changes in BP180 mRNA levels.

BP230 and BP180 retained their complex-forming ability with HD components. Both proteins colocalized in the basal region of acini but displayed alterations in the fluorescence intensity, whereby BP230 increased and BP180 decreased. Reduction of BP230 mRNA levels may be influenced by gene hypermethylation and IFNγ, a cytokine produced both by epithelial cells and by lymphocytic infiltrates in the LSGs of patients with SS (24). Indeed, the BP230 gene promoter contains IFNγ response elements (10,25). Furthermore, activation of the IFNγ signaling pathway in keratinocytes inhibits BP230 transcription via the IFN regulatory factors IRF-1 and IRF-2, which bind to the IFNγ inhibitory element (10,25).

A significant increase in BP230 protein levels ($P = 0.001$) (Figures 1C and D) may appear paradoxical. However, findings in a recent study suggest that there is a significant negative correlation between the relative translation efficiency and changes in transcription levels in *Saccharomyces* (26). Those authors suggested that the cell preserves a global equilibrium between transcription and translation, with a tendency to increase translation when transcription decreases, and vice versa (26). In addition, a recent study by Vogel et al (27) in a medulloblastoma cell line indicates that translation efficiency can be increased by a number of factors, including shortening of the 3′-untranslated region ends, loss of secondary structures to enhance ribosome movement, increased binding of translation factors and other RNA-binding proteins, which may promote translation, and covering of upstream open-reading frames or alternative translation initiation sites so that the main open-reading frame can be better translated. Whether any of the above-mentioned mechanisms are specifically involved in increasing BP230 protein levels, despite reduced mRNA levels, represents an interesting avenue of future research. Mechanistic studies using specific DNA-methylating agents in animals or for treatment of glandular explants may be helpful in shedding light on this paradoxical observation.

In acinar cells, severe loss of interactions with the BL of LSG acini in patients with SS has been reported (28). An effective rescue mechanism may prevent or at least delay cell death by increasing the half-life of mRNA and/or protein. In our system, high levels of BP230 protein may improve anchorage to the BL, thus allowing BP180 binding. As demonstrated in the retina, this adhesion complex is sufficient to preserve laminin binding (29). The SPARC protein is expressed by endometrial cancer cells and displays traits similar to those of BP230 (30). Low levels of SPARC mRNA are correlated with aberrant gene hypermethylation in CpG islands. An increase in SPARC protein may be the consequence of changes in the kinetics of synthesis and degradation via the mechanisms described above (30).

The development of autoimmune diseases has recently been linked to alterations in epigenetic silencing patterns (13,14). Indeed, we detected BP230 gene hypermethylation in the LSGs of patients with SS ($P = 0.018$), which suggests that BP230 mRNA levels are controlled by epigenetic mechanisms. Global and gene-specific (CD70 and interleukin-2 [IL-2]) hypomethylation have been determined in circulating T lymphocytes from patients with SLE and patients with RA (12,13,31–33). In synovial fibroblasts from the joints of RA patients, hypermethylation in promoters of proapoptotic genes was observed. These observations may be related to increased cell survival (14,34,35). Alterations in the expression of apoptosis-related genes, observed using cDNA microarrays for epithelial cells of LSGs from patients with SS (36), might also reflect differential methylation or additional epigenetic mechanisms (37).

Interestingly, as reported for the DR-3 gene in

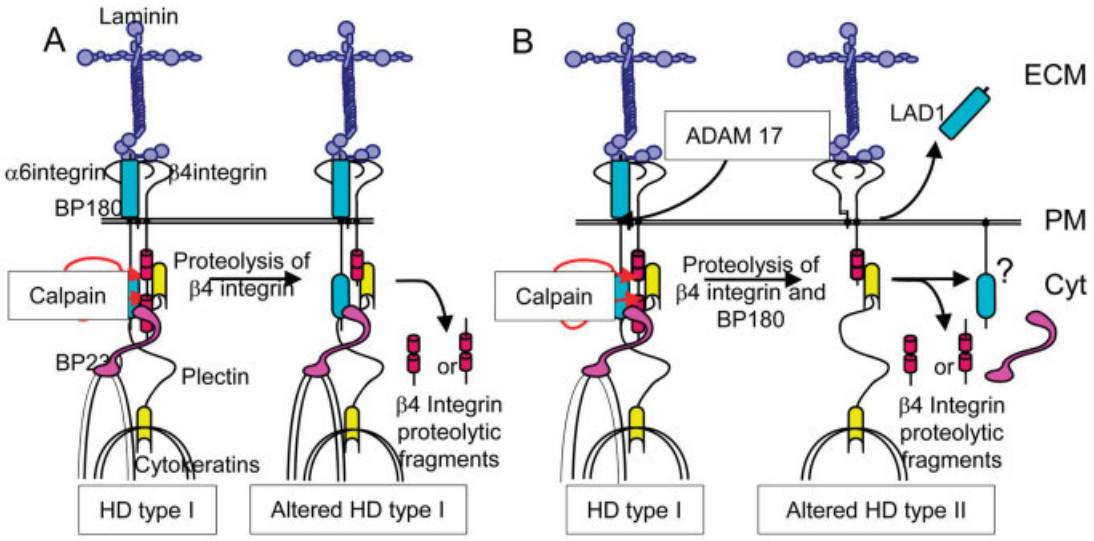

Figure 5. Model of the changes in assembly of type I hemidesmosomes (HDs) in labial salivary glands from patients with Sjögren's syndrome. Type I HDs are formed when BP230 is incorporated into the complex by binding to both β4 integrin and BP180. Both BP230 and plectin can bind to cytokeratins via interaction sites present in their respective COOH-termini (43), and α6β4 integrin and BP180 have binding sites to the extracellular matrix (ECM) protein laminin 332. **A,** Calpain degrades the cytosolic domain of β4 integrin, forming 2 proteolytic fragments, which lack the binding sites for BP180 and BP230, thereby producing altered type I HDs. **B,** Concomitantly, ADAM-17 degrades BP180, generating linear IgA disease fragment 1 (LAD-1) (120 kd), which no longer binds to α6 integrin. The residual components may constitute altered type II HDs, which remain associated with plectin, laminin 332, and cytokeratins. High levels of BP230 protein may improve anchorage to the basal lamina via BP180 binding, as has been demonstrated in the retina (27). Alternatively, exacerbated proteolysis of BP180 by ADAM-17 is expected to completely disrupt the adhesion complex. PM = plasma membrane; Cyt = cytoplasm.

synoviocytes from the damaged joints of RA patients (15), hypermethylation of the BP230 gene in patients with SS was found in the damaged tissue, i.e., the LSG. Links between genetic and epigenetic factors have recently been observed in circulating T lymphocytes (38). In particular, a polymorphism in the methyl-CpG–binding protein 2 (MECP-2) gene appeared to be associated with primary SS (38). MECP-2 is a transcriptional repressor that binds to methylated CpG dinucleotides, resulting in tighter winding of the chromatin coil and reduced transcription (37).

Decreases in BP180 protein levels and increases in LAD-1 protein levels may be the consequence of the enhanced proteolytic activity of ADAM-9, ADAM-10, and, particularly, ADAM-17 (9). These proteases cleave BP180 and generate LAD-1 (9). Accordingly, the activity of TIMP-1, an ADAM-9 and ADAM-10 inhibitor, is decreased in patients with SS (7). Also, TIMP-3 regulates ADAM-17 activity and is down-regulated in keratinocytes treated with IL-1β (39), a cytokine whose levels are increased in patients with SS (40). Thus, further BP180 cleavage would be expected. Notably, BP180 extracellular proteolysis does not block its binding to the cytoplasmic domain of β4 integrin (41). However, BP180 lacking the extracellular domain, which normally binds laminin 332, would generate unstable type I HDs (42). BP180 is suggested to be essential for correct organization of laminin 332 at the BL. This idea is based on the observation that the laminin 332–containing matrix in keratinocytes lacking BP180 is of poor quality (43).

BL disorganization has been observed in the skin of patients with junctional epidermolysis bullosa. This condition is correlated with BP180 gene mutations and the presence of reduced levels of a truncated protein in the basal stratus of the epidermis (44). Our findings suggest that low amounts of full-length BP180 in the basal region of acini, in addition to increased degradation of nidogens 1 and 2, as demonstrated in our previous study (2), might contribute to BL disorganization in the acini of patients with SS.

In immunoprecipitation assays, complex formation between β4 integrin and BP180 with type I HD–organizing proteins was observed. This is the first experimental evidence that α6β4 integrin, BP230, BP180, and cytokeratin-18 are components of adhesion complexes formed in the LSGs of patients with SS (Figures 3A and B). The α6β4 integrin constitutes the core of the complex, while the cytoplasmic domain of the β4 subunit contains 2 pairs of fibronectin type III repeats, separated by a connecting segment (45), that interact with BP230,

BP180, and plectin (46) (Figures 5A and B). Our results suggest that both in controls and in patients with SS, only $\alpha6\beta4$ dimers containing full-length $\beta4$ integrin are able to form complexes with all of the proteins involved in the formation of type I HDs (Figures 5A and B).

The cytoplasmic domain of $\beta4$ integrin is cleaved by calpain, to yield 172-kd and 145-kd fragments (4), which have also been identified in the LSGs of patients with SS. These fragments retain the ability to bind $\alpha6$ integrin, resulting in altered $\alpha6\beta4$ dimers that participate in the formation of both altered type I HDs and altered type II HDs (47) (Figures 5A and B). Concomitantly, ADAM-17 degrades BP180, thus generating LAD-1 (120 kd), which no longer binds to $\alpha6$ integrin. The residual components may constitute altered type II HDs, which remain associated with plectin, laminin 332, and cytokeratins. All proteins immunoprecipitated with anti–$\beta4$ integrin appeared to be less prevalent in patients with SS compared with controls (Figures 3A and B). This finding may be explained by previous studies from our laboratory showing that decreases in full-length $\beta4$ integrin levels observed in the LSGs of patients with SS are attributable to proteolysis (4). As a consequence, the number of binding sites for BP230 and BP180 decreased.

In the acini of patients with SS, augmented immunofluorescence staining for BP230 correlated with an increase in protein levels, as measured by Western blotting in whole gland extracts. In contrast, the weak signal observed for BP180 correlated with low BP180 protein levels, as measured with the same method. Colocalization of BP230 and BP180 was lost in some acini of patients with SS, possibly due to decreased BP180 availability. Therefore, both the colocalization and coimmunoprecipitation results suggest that binding of BP230 and BP180 is preserved, but is decreased in magnitude. In addition, the observed lack of colocalization of BP230 and laminin 111 may be explained by BL acini disorganization.

In summary, changes detected in BP230 and BP180, in conjunction with our results demonstrating BL disorganization and modified localization and distribution of $\alpha6\beta4$ integrin, suggest that significant type I HD alterations impede ECM–acinar cell communication. This mechanism is proposed to contribute to alterations in cell death and survival responses, as well as SS pathogenesis. Further studies analyzing genetic and epigenetic factors that may explain the altered gene expression observed in LSG acini of patients with SS are required to uncover the molecular basis of SS. The identification of epigenetic targets and subsequent development of strategies that permit specific modification of these epigenetic control mechanisms may open up novel therapeutic approaches for the treatment of SS.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. S. González had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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