Molecular Mechanism of Proinflammatory Cytokine-Mediated Squamous Metaplasia in Human Corneal Epithelial Cells

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PURPOSE. The cornified envelope protein small proline-rich protein 1B (SPRR1B) is a biomarker for squamous metaplasia. Proinflammatory cytokines IL-1 β and IFN- γ are potent inducers of ocular surface keratinization and SPRR1B expression. Here the molecular mechanisms controlling *SPRR1B* gene expression in response to IL-1 β and IFN- γ are elucidated.

METHODS. A 3-kb fragment of the *SPRR1B* gene 5'-flanking region was amplified from human chromosome 1, sequentially deleted, and cloned into a luciferase vector. Constructs were transiently transfected into human corneal epithelial cells, and activity was assessed in response to IL-1 β , IFN- γ , or basal medium. Functional *cis*-elements responding to IL-1 β and IFN- γ were characterized by site-directed mutagenesis and gel mobility shift assay. Effects of mitogen-activated protein kinases p38, ERK, and JNK were assessed using inhibitors and dominant-negative mutants. Results were validated by real-time RT-PCR.

RESULTS. The first 620 bp of the *SPRR1B* 5'-flanking region regulated constitutive expression and increased promoter activity in response to IL-1 β and IFN- γ . Corresponding *cis*-elements for IL-1 β and IFN- γ were bound by cAMP response element binding protein (CREB) and zinc-finger E-box binding homeobox 1 (ZEB1), respectively. Inhibition of p38 abolished the stimulatory effects of IL-1 β and IFN- γ on *SPRR1B*, whereas inhibition of JNK and ERK had no effect. Dominant-negative mutants targeting p38 α and p38 β 2 blocked cytokine-induced *SPRR1B* promoter activity and mRNA expression.

CONCLUSIONS. *SPRR1B* is upregulated by the proinflammatory cytokines IL-1 β and IFN- γ via p38 MAPK-mediated signaling pathways that lead to the activation of transcription factors CREB and ZEB1, respectively. These results identify key intracellular signaling intermediates involved in the pathogenesis of immune-mediated ocular surface squamous metaplasia. (*Invest Ophthalmol Vis Sci.* 2010;51:2466–2475) DOI:10.1167/iops.09-4677

Pathologic keratinization of the ocular surface is a hallmark of autoimmune-mediated ocular surface diseases. It represents a pathologic process whereby the nonkeratinized, strat-

ified, ocular mucosal epithelium is replaced by a keratinized, epidermal-like epithelium.^{1–4} This process, known as squamous metaplasia, is a serious and challenging clinical problem that can cause severe discomfort and vision loss. Although the molecular mechanisms triggering squamous metaplasia in the setting of autoimmunity are not clear, considerable evidence links pathologic keratinization to T lymphocyte-mediated chronic inflammation of the ocular surface.^{5–8}

To explore the pathogenesis of squamous metaplasia, we and others^{8–11} have established cornified envelope-specific proteins, small proline-rich proteins (SPRRs), as surrogate biomarkers for squamous metaplasia in both human patients with Sjögren's syndrome and mouse models of dry eye. *SPRR* genes rank among those most highly upregulated during inflammation-mediated remodeling of mucosal epithelia in a broad range of tissues, including lung, skin, and intestine (for review, see Ref. 12). In the ocular epithelia, *SPRR1B* and *SPRR2A* expression are upregulated in conjunctival cytology specimens obtained from dry eye patients with Sjögren syndrome.^{11,13} Moreover, we identified a direct link between autoimmune-mediated chronic inflammation and *SPRR1B* expression in the ocular epithelia using a mouse model of ocular surface keratinization.^{11,14} Although a vital role for T cell-mediated inflammation in the pathogenesis of squamous metaplasia has been established, the specific effector cytokine(s) and inflammatory mechanism(s) that direct the response are not fully understood.

Although many cytokines have been shown to induce the expression of cornified envelope proteins, IL-1 β and IFN- γ directly induce SPRR protein and gene expression in vitro¹¹ and have been consistently implicated in the pathologic changes associated with keratinizing ocular surface disease.^{4,8,13} IL-1 β , a master regulator of the immune response, is a potent inducer of inflammation and stimulates the production of many other proinflammatory cytokines, including IL-6, IL-8, TNF- α , and interferons. On the ocular surface, IL-1 is increased at the protein level in both the ocular surface epithelium and the tear fluid of humans and animals with dry eye.^{15–17} It is released by corneal epithelial cells in response to multiple environmental insults^{18–20} and provides an important role in directing the inflammatory response.²¹ The pathogenic role of IL-1 β in immune-mediated squamous metaplasia was substantiated in the lung, where its secretion from metaplastic epithelial cells was found to be the most pronounced proinflammatory-inducer of squamous metaplasia.²² Moreover, IL-1 regulates a characteristic set of genes that define its specific contribution to inflammation and aberrant differentiation in skin diseases.²³ The significance of IFN- γ in the pathogenesis of squamous metaplasia is also well established. IFN- γ is found in subepithelial infiltrating cells in the corneal epithelium after conjunctivalization in patients with Stevens-Johnson syndrome.²⁴ It upregulates the expression of cornified envelope precursors in keratinocytes,²⁵ corneal epithelial cells,¹¹ and

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TABLE 1. Primer Nucleotide Sequences for *SPRR1B* Promoter/Reporter Constructs

Promoter	Primer
Upstream*	
–3.0 kb/+12	5'-CGACGCGTGTCTATTGTTCCCA-3'
–2.0 kb/+12	5'-CGACGCGTGAATGAAAGTCC-3'
–1.0 kb/+12	5'-CGACGCGTTCATGAGAGGTGAC-3'
–620 b/+12	5'-CGACGCGTCCAGAGTTTCACTG-3'
–450 b/+12	5'-CGACGCGTGAACCGAAATAATC-3'
–300 b/+12	5'-CGACGCGTTTCTGGTTCAGCA-3'
–200 b/+12	5'-CGACGCGTATAAAGCCAGGTG-3'
–150 b/+12	5'-CGACGCGTATCACAAAAGTTGA-3'
–96 b/+12	5'-CGACGCGTACAGGTAAGGAATG-3'
Downstream†	
All constructs	5'-CACTCGAGTCCCTTAGAACTGG-3'

* All upstream primers contained an *MluI* site at the 5'-end (underlined).

† All downstream primers contained an *XbaI* site at the 5'-end (underlined).

conjunctival specimens from patients with Sjögren syndrome²⁶ and has been shown to mediate ocular surface keratinization in a mouse model of desiccating stress dry eye.⁸ Interestingly, the expression of these genes often precedes the squamous phenotype, further validating their use as surrogate biomarkers.^{8,25,27}

Although the roles of IL-1 β and IFN- γ are well established in the pathogenesis of squamous metaplasia, intracellular intermediates involved in the signaling cascade from cytokine activation at the cell surface to aberrant production of cornified envelope proteins in the ocular mucosal epithelia are largely unknown. In the present study, we sought to define the events that connect these mediators to keratinization. Our results demonstrate p38 mitogen-activated protein kinase (MAPK) as a common intermediate shared by the IL-1 β and IFN- γ signaling cascades. The p38 MAPK cascade initiates *SPRR1B* promoter activation through the recruitment of transcription factors cAMP response element binding protein (CREB) and Zinc finger E-box-binding homeobox 1 (ZEB1), respectively. Molecular details of these pathways provide information relevant to the identification of novel targets for drug development in the treatment of immune-mediated ocular surface keratinization.

MATERIALS AND METHODS

Cell Culture and Cytokine Treatment of SV40-Transformed Human Corneal Epithelial Cells

SV40-transformed human corneal epithelial (HCE) cells were grown in SHEM-X medium supplemented with 10% fetal bovine serum (FBS), 0.5% DMSO, insulin (5 μ g/mL), epithelial growth factor (0.01 μ g/mL), cholera toxin (0.1 μ g/mL), and penicillin/streptomycin and were maintained at 37°C in a 5% CO₂ incubator. For stimulation studies, IL-1 β and IFN- γ were added to the cultures at 20 ng/mL when the cells reached approximately 80% confluence and were maintained for 12 hours. The cytokines were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation and Culture of Human Corneal Epithelial Cells

Human tissue was handled in accordance with the Declaration of Helsinki. Human limbal epithelial sheets were isolated directly from six corneoscleral rims obtained immediately after penetrating keratoplasty for three independent experiments of cytokine-induced *SPRR1B* expression. As previously reported, limbal epithelial sheets were surgically dissected after enzymatic digestion of the basement membrane using 10 mg/mL neutral protease grade II (Dispase II; Roche, Indianapolis,

Ind., IN) in keratinocyte serum-free defined medium (KSFM; Invitrogen, Carlsbad, CA) at 4°C for 16 hours.²⁸ Isolated limbal epithelial sheets were further rendered into single cells by digestion with 0.05% trypsin/0.53 mM EDTA at 37°C for 10 minutes. Cells were seeded on 12-well plates at the density of 1.0×10^4 cells per cm² and were cultured in KSFM. When they were 70% confluent, primary cells were treated with cytokine in the presence and absence of the MAPK inhibitor, as described in experiments using SV40 HCE cells. Calcium concentration in KSFM (0.07 mM) was adjusted to (0.9 mM) during the treatment period to mimic the calcium level in SHEM-X medium.

Construction of *SPRR1B* Promoter/Reporter

A segment of *SPRR1B* genomic DNA corresponding to –3000 to +12 bp in relation to the transcription start site was amplified from human chromosome 1 by PCR with the upstream primer containing the *MluI* site and the downstream primer containing the *XbaI* site (Table 1). DNA polymerase (PfuTurbo DNA polymerase; Stratagene, La Jolla, CA) was used in the PCR reaction. The PCR product was restriction digested and cloned into luciferase reporter vector pGL3. This construct is referred to as –3 kb/+12. A variety of putative transcription factor binding sites was mapped to this fragment by DNA sequence alignment using programs including <http://www.cbil.upenn.edu/cgi-bin/tess/tess>, <http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl>, <http://www.cbrc.jp/research/db/TFSEARCH.html>, and <http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>. A series of 5'-end deletion constructs was generated by PCR using the primers listed in Table 1. Nucleotide sequences and orientations of the inserts were confirmed by DNA sequencing and restriction enzyme digestion. In site-directed mutagenesis, mutations were introduced into the construct –620/+12 using a PCR-based method of primer overlap extension. Primers containing desired bases were listed in Table 2.

Cell Transfection and Luciferase Assay

HCE cells were plated in 24-well plates at 5×10^4 cells/well in 0.5 mL culture medium. When the cells reached approximately 60% confluence, transient transfection was performed using a reagent (FuGENE HD; Roche) according to the manufacturer's recommendations. Briefly, 20 μ L transfection mixture was prepared with serum-free medium containing 0.4 μ g *SPRR1B* promoter/reporter construct, 2 ng *Renilla* luciferase plasmid (which served as internal control), and 1.2 μ L reagent (FuGENE HD; Roche). For experiments with p38 mutants (gifts from Jian-Dong Li, University of Rochester, Rochester, NY), either dominant-negative mutant (DNM) p38 α or DNM p38 β was cotransfected with the *SPRR1B* promoter construct (–620/+12 bp) into HCE cells. After incubation at room temperature for 15 minutes, the mixture was added drop-wise to cells. The transfection was maintained for 48 hours. Cells in control wells were transfected with the basic pGL3 plasmid, a promoterless vector. For luciferase assays, the transfected cells were washed twice with PBS (pH 7.4) and lysed in 60 μ L buffer (Passive Lysis; Promega, Madison, WI). After two cycles of freeze-thaw

TABLE 2. PCR Primers Used for Generating Mutations in the *SPRR1B* Promoter and Probes Used for EMSA

Primers	
ZEB1	
Mutation	Upper: 5'-CACTATATTCGGGAGCAAAGGGTG-3' Lower: 5'-CTCCCGAATATAGTGTTCACAGG-3'
EMSA	WT: 5'-TGTAACACTACACCTGGGAGCAAAG-3' Mut: 5'-TGTAACACTATATTCGGGAGCAAAG-3'
CREB	
Mutation	Upper: 5'-CTGATGCTATCCTGCCCTATG-3' Lower: 5'-CATAGGGCAGGATAGCATCAG-3'
EMSA	WT: 5'-ACAGACTGAGGTGAGCCTGCCCTAT-3' Mut: 5'-ACAGACTGATGCTATCCTGCCCTAT-3'

Mut, mutation.

at -80°C , 10 μL cell lysate was analyzed for reporter luciferase activity and internal control *Renilla* luciferase activity by using a dual luciferase assay kit (Promega) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Reporter luciferase activity of individual samples was normalized against *Renilla* luciferase activity. *SPRR1B* promoter activity was expressed as fold-induction of the empty vector alone. Results were averaged from three separate transfections. In stimulation studies, cytokines IL-1 β and IFN- γ (20 ng/mL) were added to the HCE cells 24 hours after transfection and were maintained for an additional 12 hours before cell lysis.

Nuclear Extract and Gel Mobility Shift Assay

Nuclear extracts (NE) were isolated from control and stimulated HCE cells using reagents (NE-PER; Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. In stimulation studies, IL-1 β and IFN- γ were added to the cells ($\sim 80\%$ confluence) at 20 ng/mL and were maintained for 30 minutes. DNA probes were synthesized containing ZEB1- and CREB-binding elements (Sigma). Mutant probes contained the same mutated base pairs as in the mutant promoters. Nucleotide sequences for both wild-type and mutant DNA probes are listed in Table 2. These probes were end-labeled with digoxigenin (Dig; DIG Gel Shift Kit; Roche Applied Science, Germany) according to the manufacturer's instruction. Protein-DNA binding reactions were carried out under optimized conditions. Briefly, nuclear extracts (5 μg) were mixed with Dig-labeled probe (60 fM) in 20 μL binding buffer containing 20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 1 mM dithiothreitol, 1% Tween-20 (wt/vol), and 30 mM KCl. After incubation at 15°C to 25°C for 15 minutes, the reaction mixtures were resolved in 4% to 20% gradient acrylamide gels in $0.5\times$ Tris-borate-EDTA buffer. After electrophoresis at 150 V for 2.5 hours, the gel was transferred to a positively charged nylon membrane by electroblotting. The blot was incubated with anti-digoxigenin-alkaline phosphatase. Chemiluminescence was developed with the addition of CSPD (a substrate of alkaline phosphatase). For binding competition, 125 $\times$ unlabeled (cold) probe was added to the reaction mixture before adding the Dig-probe.

Characterization of MAPK Pathways

Three MAPK inhibitors were used, including JNK inhibitor (SP60012), ERK inhibitor (PD98059) and p38 inhibitor (SB202190). They were purchased from Calbiochem and dissolved in DMSO. HCE cells were transfected with the *SPRR1B* gene promoter ($-620/+12$) for 24 hours, starved in 1% FBS medium for 12 hours, and exposed to serum-free medium containing MAPK inhibitors for 45 minutes at the final concentrations SP60012 10 μM , PD98059 25 μM , and SB202190 10 μM . To examine the effect of the inhibitors on cytokine-induced *SPRR1B* promoter activity, IL-1 β , IFN- γ , or basal medium was added to the cells and maintained for 12 hours. *SPRR1B* promoter activity was assessed by luciferase assay. Toxic effects of the inhibitors on cell viability were examined (TACS MTT assays; R&D Systems, Inc., Minneapolis, MN).

Immunofluorescence

SPRR1B expression in primary corneal epithelial cells was visualized by immunofluorescence. Primary anti-*SPRR1B* antibody at a 1:800 dilution (gift from Reen Wu, University of California, Davis, CA) was placed on cells overnight, followed by secondary antibody (FluoroLink Cy3-conjugated; Amersham Bioscience, Piscataway, NJ). Nuclei were counterstained with DAPI. Negative controls included omission of the primary antibody or isotype control.

Real-Time RT-PCR

HCE cells grown in six-well plates (2×10^5 cells/well) were treated with IL-1 β or IFN- γ in the presence and absence of p38 inhibitor (SB202190). For experiments using p38 mutants, RNA samples were isolated from control HCE cells and DNM p38 α - or DNM p38 β -transfected cells at 2 μg /well. Briefly, at approximately 70% conflu-

ence, the cells were starved in 1% FBS medium for 12 hours and then were treated with either vehicle (DMSO) or SB202190 at a final concentration of 10 μM in serum-free medium for 45 minutes. Fetal bovine serum was added to the cultures to a final concentration of 1%, after which IL-1 β or IFN- γ was added at 20 ng/mL and was maintained for 12 hours. Total RNA was extracted using reagent (TRIzol; Invitrogen). Reverse transcription was carried out with a reverse transcription kit (TaqMan RT Kit; Applied Biosystems, Foster City, CA) at 5 ng RNA/ μL . Human *SPRR1B* mRNA was detected with gene expression assays (TaqMan; Applied Biosystems). The C_T value was normalized to that of the housekeeping gene GAPDH. Cytokine induction of *SPRR1B* transcription in the presence and absence of p38 inhibitor was expressed as fold-induction over control cells using the $\Delta\Delta C_T$ calculation.

Statistical Analysis

Student's *t*-test was used to analyze differences between control and cytokine-treated groups. $P \leq 0.01$ was considered statistically significant. All assay samples were performed in triplicate, and each experiment was repeated at least three times. Data are expressed as mean $\pm$ SE.

RESULTS

Analysis of the *SPRR1B* Promoter

A variety of putative transcription factor binding sites were aligned to 3 kb of the *SPRR1B* 5'-flanking region by DNA sequence comparisons. Several with high scores are schematically shown in Figure 1A, including the cAMP response element for CREB and zinc-finger E-box binding homeobox 1 for ZEB1, located at $-585/-576$ and $-227/-220$, respectively.

Nine *SPRR1B* promoter/reporter constructs and the empty pGL3-basic vector were transiently transfected into HCE cells and treated with IL-1 β or IFN- γ . Cell lysates from control and stimulated cultures were measured by dual luciferase assay. As shown in Figure 1B, the $-620/+12$ construct revealed the highest promoter activity at both the basal level and after stimulation with cytokines. HCE cells transfected with this construct in the absence of cytokine challenge showed a 32-fold increase in luciferase activity (vs. mock control), whereas the addition of IL-1 β and IFN- γ drastically increased *SPRR1B* promoter activity up to 84- and 64-fold, respectively. These results imply that the -620 bp region contains critically important *cis*-acting elements capable of inducing *SPRR1B* expression. Putative *cis*-responding elements—including CREB, ER- α , Ets-1, and ZEB1—bound by several transcription factors with relatively high scores were mapped to this region by DNA sequence alignment. Notably, extending the promoter region upstream to include -1kb to -3kb led to decreased activity, indicating the presence of putative inhibitory elements in these regions.

CREB- and ZEB1-Binding Elements Respond to IL-1 β and IFN- γ Stimulation

To analyze the $-620/+12$ region of the *SPRR1B* promoter, mutations were introduced into CREB-binding element (ctgag-gtcag, at $-585/-576$) and ZEB1-binding element (acacctgg, at $-227/-220$) (Table 2; Fig. 2A). Luciferase activity of HCE cells transfected with wild-type promoter or its mutant variants and exposed to IL-1 β - or IFN- γ -stimulated cells is shown in Figure 2A. Compared with the wild-type promoter, mutation of the CREB-binding site, decreased IL-1 β -induced luciferase activity from 85-fold to 35-fold, whereas mutating the ZEB1-binding site significantly decreased IFN- γ -induced luciferase expression from 68-fold to 36-fold. Deletion of both CREB and ZEB1 sites resulted in promoter activity similar to that of basal medium control. Interestingly, these mutations did not affect basal

FIGURE 1. Characterization of the *SPRR1B* gene promoter and the effects of IL-1 β and IFN- γ on promoter activity. (A) Schematic representation of 3 kb *SPRR1B* gene 5'-flanking region. Bent arrow: transcription start site. Several putative transcription factor-binding sites were mapped on this fragment. (B) Cloning, cell transfection, and luciferase assay results. The promoter activity of sequentially deleted *SPRR1B* promoter/reporter constructs is shown. For each assay, 0.4 μ g *SPRR1B* construct and 2 ng *Renilla* luciferase plasmid (an internal control) were transiently cotransfected into HCE cells. *SPRR1B* promoter activity was assessed 48 hours after transfection and expressed as fold induction of relative luciferase activity (RLU) compared with promoterless vector. *SPRR1B* promoter activity in response to IL-1 β or IFN- γ (20 ng/mL) was assessed 12 hours after the addition of cytokine (24 hours after transfection). Cells exposed to basal medium in the absence of cytokine were used as a control. Data are expressed as mean $\pm$ SE RLU. * P < 0.01, significant induction versus basal medium control.

SPRR1B promoter activity. This finding suggests that CREB and ZEB1 function as downstream transcription factors that modulate *SPRR1B* expression in response to IL-1 β - and IFN- γ -activated signaling networks, respectively. In the screening of IL-1 β and IFN- γ response elements, mutagenesis of other putative elements, such as putative ER- α - and Ets-1-binding elements, did not lead to any notable effects on *SPRR1B* promoter activity (data not shown).

CREB and ZEB1 Are Inducible by IL-1 β and IFN- γ

To confirm CREB and ZEB1 as critical transcriptional mediators of *SPRR1B* expression in HCE cells, we conducted competitive electrophoretic gel mobility shift assays using digoxigenin-labeled double strand-DNA oligos (26 bp) as probes. Probes containing the nucleotide sequence of CREB- or ZEB1-binding elements are shown in Table 2, indicated as wild-type probes and their mutant variants. Unlabeled DNA oligos were used as competitors.

As shown in Figure 3A, we detected a faint protein-DNA complex when nuclear extracts from control cells were added to the binding reaction (lane 2). In contrast, we noted significantly more protein-DNA complexes in IL-1 β -stimulated cells (lane 3), indicating substantial induction of CREB. Complex formation was significantly reduced when the CREB site was mutated (lane 4), suggesting high specificity of the protein-DNA interaction. We were able to compete away both specific and nonspecific protein-DNA interactions using a high concentration of wild-type, cold oligo (lane 5), and a mutant oligo removed nonspecific interactions (lane 6). These results provided further evidence of an IL-1 β -mediated interaction between nuclear CREB and its binding element on the *SPRR1B* promoter.

As shown in Figure 3B, we used an identical approach to study the ZEB1-binding site of the *SPRR1B* promoter. A strong protein-DNA complex was formed in IFN- γ -stimulated cells (lane 3) compared with control cells (lane 2). Mutation of the

FIGURE 2. Characterization of transcription factor binding elements by mutagenesis and luciferase assay. (A) HCE cells were transfected with wild-type (WT) *SPRR1B* promoter (-620/+12), two mutant variants (mutated at ZEB1 site and CREB site, respectively), and the -200/+12 construct. Relative luciferase activity (RLU), expressed as fold induction relative to promoterless vector, were measured in the presence and absence of IL-1 β and IFN- γ . Data are expressed as mean $\pm$ SE RLU. * P < 0.01, significant induction versus basal medium control. (B) CREB and ZEB1 transcription factor-binding elements are underlined. Mutated base pairs are highlighted using boldface type.

FIGURE 3. Competitive EMSA analysis of transcription factors on the *SPRR1B* promoter that mediate the stimulatory effects of IL-1 β and IFN- γ . A digoxigenin-labeled, double stranded-DNA probe (60 fm) was added to 5 μ g nuclear extract (NE) in protein-DNA binding buffer. Protein-DNA complexes were separated using a gradient (4%–20%) TBE gel, transferred to a nylon membrane, and detected by chemiluminescence. Unlabeled double-stranded DNA was used as competitor at 125 $\times$. *Lane 1*, no NE; *lane 2*, NE alone; *lanes 3–6*, cytokine-stimulated NE; *lane 4*, mutant (mut) probe was added; *lane 5*, wild-type (wt) competitor was added; *lane 6*, mutant competitor was added. **(A)** Dig-labeled probe containing CREB-binding element in the presence and absence of IL-1 β . **(B)** Dig-labeled probe containing ZEB1-binding element in the presence and absence of IFN- γ . Images are representative of three independent experiments. NS, nonspecific binding.

ZEB1 element significantly reduced the binding reaction to control levels (lane 4), and the use of wild-type cold oligo competed away both specific and nonspecific protein-DNA interaction (lane 5). Using mutant oligo, we provided further evidence of the specific interaction between the ZEB1-binding element and the nuclear protein induced by IFN- γ (lane 6).

p38 MAPK Transduces Cytokine-Stimulated *SPRR1B* Transcription

To investigate the potential signaling intermediates that connect IL-1 β and IFN- γ to *SPRR1B* expression in squamous metaplasia, we assessed *SPRR1B* promoter activity in the presence or absence of inhibitors directed against MAPKs. Extracellular signals, such as those induced by proinflammatory cytokines, generate intracellular signals to coordinated cellular responses. The MAPK cascades include three distinct kinase families identified in mammalian cells: mitogen-responsive ERKs, stress-responsive JNK/p54, and p38 MAPKs.²⁹ We used inhibitors specific for ERK (PD98059), JNK (SP600125), and p38 (SB202190) to determine whether MAPKs were involved in the induction of *SPRR1B* by IL-1 β or IFN- γ . Interestingly, inhibitors targeting JNK and ERK had no effect on *SPRR1B* promoter activity in response to either cytokine (Figs. 4A, 4B), whereas the p38 inhibitor, SB202190, largely suppressed *SPRR1B* promoter activity in response to both IL-1 β and IFN- γ (Fig. 4C). There was no inhibitor toxicity at the working concentrations (data not shown). Consistent with this, we demonstrated a robust decrease in endogenous *SPRR1B* mRNA when SB202190 was added to IL-1 β - or IFN- γ -treated HCE cells (Fig. 5). Among the three isoforms of p38 (α , β , δ) expressed in keratinocytes,³⁰ SB202190 selectively inhibited the α and β isoforms. To confirm the involvement of p38 in cytokine-induced *SPRR1B* regulation, we measured *SPRR1B* promoter activity and endogenous mRNA levels in the presence and absence of dominant-negative mutants (DNMs) directed at the p38 α and β isoforms. Results showed that forced expression

of p38 α and β DNMs blocked IL-1 β - and IFN- γ -induced *SPRR1B* promoter activity (Fig. 6A). These results were confirmed by real-time RT-PCR in which the induction of endogenous *SPRR1B* mRNA was blocked in the presence of p38 α and β DNMs (Fig. 6B).

To confirm the involvement of p38 in cytokine-driven *SPRR1B* expression, we used primary corneal epithelial cells isolated from human corneal rims to validate results obtained with SV40 HCE cells. Among a panel of proinflammatory cytokines, IL-1 β is the strongest inducer of *SPRR1B* protein and mRNA expression in primary corneal epithelial cells.¹¹ Using quantitative PCR, we showed significant upregulation of *SPRR1B* in response to IL-1 β that was completely abolished in the presence of the p38 inhibitor SB202190 (Fig. 7A). Similarly, we used immunofluorescence microscopy to visualize *SPRR1B* protein in primary cells after exposure to IL-1 β . Cytoplasmic *SPRR1B* was localized in cell clusters after 24 hours of IL-1 β treatment (Fig. 7B; IL-1 β +/-SB202190-) but was scarcely observed in response to IL-1 β when SB202190 was added to the culture medium (Fig. 7B; IL-1 β +/-SB202190+). As expected, controls containing vehicle or inhibitor alone had no effect on *SPRR1B* mRNA (Fig. 7A) or on protein expression (not shown). These data confirm that primary and SV40-transformed human corneal epithelial cells share a cytokine-induced pathway that regulates *SPRR1B* expression through p38 MAPK signaling.

DISCUSSION

Pathologic keratinization in autoimmune-mediated ocular surface disease is characterized by lymphocytic infiltration of the conjunctiva^{7,24,31,32} and increased expression of proinflammatory cytokines.^{4,33,34} Using the *aire*-deficient mouse model of autoimmune, T cell-mediated ocular surface keratinization and human cytology specimens of patients with Sjögren syndrome, we identified the cornified envelope protein *SPRR1B* as a

FIGURE 4. The involvement of MAPK signaling in IL-1 β - and IFN- γ -induced *SPRR1B* promoter activity. HCE cells transfected with *SPRR1B* –620bp/luciferase reporter construct were exposed to IL-1 β or IFN- γ in the presence and absence of chemical inhibitors directed against (A) JNK (SP60012, 10 μ M), (B) ERK (PD98059, 25 μ M), and (C) p38 (SB202190, 10 μ M). Data represent the fold-induction of relative luciferase activity (mean $\pm$ SE). * P < 0.01, significant induction.

biomarker for quantifying pathologic keratinization.¹¹ Although inappropriate, T cell-mediated inflammation is an established driving force for pathologic keratinization in a variety of inflammatory diseases,^{24,35,36} the specific mediators and molecular mechanisms that drive the process are complex and likely composed of multiple mediators released from a variety of cell types. We chose to study the mechanism whereby two well-established mediators of inflammation, IL-1 β and IFN- γ , alter the normal course of corneal epithelial cell differentiation. Both IL-1 β and IFN- γ are effective inducers of *SPRR1B*.¹¹ IL-1 β can be released by mucosal epithelial cells in response to multiple environmental stressors,^{18–20} whereas IFN- γ is the principal Th1 cytokine released from infiltrating T cells known to induce squamous metaplasia in humans and animal models.^{24,25,37} Through detailed analysis of the *SPRR1B* promoter,

we identified cAMP response element binding protein (CREB) and zinc-finger E-box binding homeobox 1 (ZEB1) as the regulatory mediators for IL-1 β - and IFN- γ -induced *SPRR1B* gene transcription, respectively (Fig. 8).

To connect IL-1 β and IFN- γ to their downstream response elements on the *SPRR1B* promoter, we sought to identify key intermediates of the intracellular signaling pathway. p38 MAPK is a well-known integrator of multiple stimuli, including environmental stressors and proinflammatory cytokines, particularly the prototypical IL-1 cytokine family.³⁸ Although MAPKs are involved in a wide variety of cellular processes, such as proliferation, differentiation, development, and regulation of stress responses,^{39,40} stress-induced activation of p38 δ (but not p38 α or p38 β) played a central role in upregulating the cornified envelope protein involucrin in epidermal keratinocytes through the transcription factors AP1 and C/EBP.^{41–43} By comparison, our study provides evidence that ocular keratinocytes make use of both p38 α and p38 β as signaling intermediates to aberrantly express *SPRR1B* in the setting of inflammation. Intriguingly, Li et al.⁵ used an ex vivo culture model of limbal epithelium to induce squamous metaplasia with aberrant expression of the cornified envelope protein filaggrin by airlifting explant tissue in the culture medium. Similar to our observation, the aberrant expression of filaggrin in the airlifting model was abolished by adding the p38 α/β inhibitor SB202190 to the culture medium.

Alternatively, expression of *SPRR1B* in response to IL-1 β and IFN- γ might not have occurred solely through a p38-dependent pathway. Hypertonic stress, a known inducer of SPRR proteins and squamous metaplasia, induces ocular surface IL-1 β and TNF- α expression and activates all three MAPK pathways (JNK, ERK, and p38) in a mouse model of dry eye.^{44,45} Although some have shown cytokines released from limbal epithelial cells to be p38-dependent,⁴⁶ others have demonstrated an important role for JNK in mediating the downstream effects of hypertonic stress, with p38 having a relatively

FIGURE 5. Effect of the p38 MAPK inhibitor SB202190 on *SPRR1B* gene expression in response to IL-1 β and IFN- γ . Endogenous *SPRR1B* mRNA was examined by real-time RT-PCR using gene expression assay. Total RNA was isolated from HCE cells pretreated for 45 minutes with DMSO (vehicle) or p38 inhibitor (10 μ M), followed by 12-hour exposure to IL-1 β (20 ng/ μ L) or IFN- γ (20 ng/ μ L), either alone or in combination with the inhibitor. mRNA expression level data represented as fold-induction (mean $\pm$ SE) compared with 1-fold defined by DMSO-treated control. * P < 0.01, significant induction.

FIGURE 6. Effects of DNM p38 α and p38 β 2 on *SPRR1B* gene expression. **(A)** Effect of DNM p38 (α and β 2) on *SPRR1B* promoter activity was examined by dual luciferase assay. HCE cells were cotransfected with the *SPRR1B* promoter construct (620 bp) and with DNM p38 α , DNM p38 β 2, or empty vector control. Relative luciferase activity of the *SPRR1B* promoter was examined in the presence and absence of IL-1 β and IFN- γ (20 ng/mL). Data are expressed as mean $\pm$ SE RLU. * P < 0.01, significant induction. **(B)** Endogenous *SPRR1B* mRNA was examined by real-time RT-PCR using gene expression assays. HCE cells were transfected with p38 α or β 2 DNM or empty vector control. Basal medium with and without IL-1 β or IFN- γ (20 ng/mL) was added to HCE cells 36 hours after transfection and was maintained for 12 hours. Fold change in *SPRR1B* mRNA expression in the presence and absence of DNM was determined compared with control. * P < 0.01, significant induction.

minor role.^{47,48} In the setting of hypertonic stress, several proinflammatory mediators are released, and these mediators may act by different, yet interdependent, mechanisms to alter epithelial differentiation. Both JNK and p38 MAPK cascades are strongly activated by cellular stressors, including proinflammatory agents such as IL-1,⁴⁰ though the functional role of each pathway is highly dependent on the cell type and stimulus.⁴⁹ Interestingly, different members of the MAPK family have been shown to participate in unique interactions that mediate the activation of differentiation-associated genes. For example, the previously mentioned p38 δ -dependent pathway mediating involucrin expression in response to okadaic acid was found to

involve a physical interaction between p38 δ and ERK1/2 that suppressed Erk1/2 phosphorylation.⁴¹ Although one must consider the complexity of MAPK signaling in each system, p38

FIGURE 7. p38-Dependent regulation of *SPRR1B* mRNA and protein expression in primary human corneolimbic epithelial cells. Primary cells were pretreated with p38 inhibitor SB202190 (10 μ M) or vehicle (DMSO) alone in KSEF for 45 minutes, followed by 6- and 24-hour exposures to IL-1 β (20 ng/mL) alone or in combination with the inhibitor for mRNA and protein studies, respectively. **(A)** *SPRR1B* mRNA was examined by quantitative real-time PCR using gene expression assay (as in Fig. 5). With the $\Delta\Delta C_T$ method, *SPRR1B* mRNA expression was normalized using endogenous GAPDH and is shown as relative fold increase (using control as 1-fold). *SPRR1B* transcription was upregulated 1.77-fold compared with control (DMSO) in response to IL-1 β . * P < 0.05, significant induction. Data are representative of three independent experiments using six corneolimbic rims. **(B)** *SPRR1B* protein was visualized by immunofluorescence. Cytoplasmic *SPRR1B* (red) was well defined in clusters of cells stimulated by IL-1 β cytokine in the absence of SB202190 (IL-1 β +/SB202190-) but not in the presence of SB202190 (IL-1 β +/SB202190+). Scale bar, 10 μ m.

FIGURE 8. Model depicting cytokine regulation of squamous metaplasia biomarker *SPRR1B* expression in human corneal epithelial cells. Ligation of cytokines IL-1β and IFN-γ to their cell surface receptors led to the activation of p38α/β2 MAPKs. Downstream of p38, IL-1β and IFN-γ signals recruit their individual downstream transcription factors to activate CREB- and ZEB1-response elements on the *SPRR1B* promoter, respectively.

has a central role in both the induction of proinflammatory cytokines in response to stress and the initiation of aberrant differentiation in response to these cytokines. Multiple studies highlight the importance of p38 signaling in coupling proinflammatory stimuli to abnormal epidermal differentiation and suggest the potential to block the process of pathologic keratinization through the inhibition of p38 or other downstream regulators that mediate abnormal differentiation.⁵

Although stress-induced cell surface receptor activation and its corresponding intracellular signal transduction is often a prerequisite of squamous metaplasia, the final squamous metaplastic “phenotype” depends on the activation of downstream transcription factors that directly regulate the expression of target genes mediating corneal-to-epidermal differentiation. Keratinization of the ocular epithelium is a multistep disease, including de novo synthesis of epidermal keratinocyte-specific cytoskeleton, cell granules, cornified envelope proteins, and activation of a key enzyme, transglutaminase 1 (TGase 1), that cross-links these proteins.⁵⁰ In the present study, we identified CREB as the effector transcription factor acting downstream of IL-1β-p38MAPK to regulate the expression of the cornified envelope protein *SPRR1B*. CREB, AP1, and C/EBP belong to the bZIP family of transcription factors that have a common basic region-leucine zipper domain. As the best studied of transcription factors, they are activated by a variety of extracellular stimuli and play a well-established role in regulating cornified envelope proteins in keratinocytes.⁵¹ The mechanism of CREB activation is effected by diverse signals, including those regulating the intracellular levels of cAMP, Ca²⁺, growth factors, and cellular stress. Accordingly, CREB-mediated transcription regulates diverse cellular responses, including intermediary metabolism, neuronal signaling, cell proliferation, and apoptosis.⁵² Our goal was first to demonstrate that CREB mediates corneal-to-epidermal differentiation in response to proinflammatory cytokines by directly binding the *SPRR1B* promoter and activating *SPRR1B* transcription (Figs. 2A, 3A). Interest-

ingly, CREB has also been reported to regulate *TGase 1* gene expression in maturing cells of the epidermis.⁵³ Similar to *SPRR1B*, TGase 1 is overexpressed in ocular squamous metaplasia induced by hyperosmolarity through a JNK-dependent pathway.⁴⁸ The coincidence that two functionally correlated cornification proteins share the same transcription factor not only reflects the potential importance of CREB in the pathogenesis of squamous metaplasia but implies an efficient pathologic mechanism by which a single transcription factor can simultaneously regulate several components of transdifferentiation.

Alternatively, the induction of *SPRR1B* through the IFN-γ-p38MAPK pathway was dependent on the ZEB1 response element on the *SPRR1B* promoter. ZEB1 is a recently discovered, and less studied, transcription factor that has at least three synonyms, EF-1, AREB6, and TCF8 (PubMed GeneID: 6935). It has a wide spectrum of biological functions with multiple conformational states that can lead to positive and negative regulatory functions.^{54,55} Although the particular function of ZEB1 in the corneal epithelium remains unclear, a zinc finger protein of the same family, basonuclin, is a key regulator of keratinocyte proliferative potential (stemness).⁵⁶ Whether ZEB1 plays a role in keratinocyte reprogramming is unclear, but several studies have identified a role for ZEB1 in guiding differentiation in normal epithelium and in facilitating epithelial-to-mesenchymal transition (EMT) in cancer with inflammation. EMT is a characteristic of embryogenesis and cancer in which epithelial cells convert from their differentiated state to undifferentiated mesenchymal cells. Knockdown of ZEB1 can completely reverse EMT.

Less is known regarding the role of ZEB1 in the eye, particularly in the setting of chronic inflammation. Interestingly, almost 50% of patients with posterior polymorphous corneal dystrophy type 3 (PPCD) have heterozygous frameshift and nonsense mutations in the *ZEB1* gene.^{57,58} PPCD is an autosomal-dominant disease characterized by the abnormal transition of the corneal endothelium to an epithelial phenotype. *ZEB1* has also been implicated in the regulation of multiple immune response genes through the repression of the T cell-specific IL-2 gene.⁵⁵ To our knowledge a connection between IFN-γ and ZEB1 has not been reported. Given the role of ZEB1 in the transcriptional regulation of both abnormal differentiation and inflammation, we suggest a p38-dependent mechanism whereby IFN-γ promotes squamous metaplasia through the ZEB1-mediated upregulation of the cornified envelope protein *SPRR1B*. Further insight into the functional significance of ZEB1 in diseases that involve altered differentiation and inflammation (e.g., squamous metaplasia and tumor formation) may open an interesting avenue of research that ultimately suggests targeted therapies to prevent aberrant differentiation during chronic inflammation.

In the midst of complex signaling networks mediating keratinizing ocular surface diseases, SPRRs provide a convenient end point by which to study its pathogenesis because they are minimally expressed in nonkeratinized mucosal tissues and quickly become overexpressed in response to stress or inflammation.^{9,11,59} Although thought to occur as a mechanism of host defense, the induction of SPRRs by inflammatory cytokines can have vision-threatening consequences. To understand how the initiating events lead to pathologic differentiation of the mucosal surface, it is necessary to decipher the molecular characteristics of the multiple signaling pathways likely to be involved. In the present study, we characterized the *SPRR1B* promoter structure and identified the *cis*-acting elements and transcription factors that mediate its upregulation in response to two known inducers of abnormal differentiation. The stimulatory effects of IL-1β and IFN-γ on *SPRR1B* by CREB and ZEB1, respectively, provide clues for the investi-

gation of novel therapeutics to manage keratinizing ocular surface disease. Moreover, the intersection of these pathways at the level of p38 MAPK suggests the possible coordination of complex networks in the induction of squamous metaplasia.

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