NF-κB Subtypes Regulate CCCTC Binding Factor Affecting Corneal Epithelial Cell Fate*

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CCCTC binding factor (CTCF) controls DNA imprinting, insulates important gene expression, and mediates growth factor- and stress-induced cell fate. However, regulatory mechanisms involved in intracellular CTCF activity are largely unknown. In this study, we show that epidermal growth factor (EGF)-induced increase and UV stress-induced decrease in CTCF activities mediate human corneal epithelial cell proliferation and apoptosis, respectively. CTCF is regulated by activation of different NF-κB subtypes via stimulation by EGF and UV stress. EGF-induced formation of a p65/p50 heterodimer activated CTCF transcription to promote cellular proliferation. This was accomplished by the heterodimer binding to a κ B site in the promoter region of CTCF gene. In contrast, UV stress induced formation of a p50/p50 homodimer, which suppressed CTCF expression leading to apoptosis. Thus, CTCF by itself plays a central role in mediating the dichotomous effects of growth factor- and stress-stimulated NF-κB activation on cell survival and death. These results suggest that CTCF is a downstream component of the NF-κB pathway involved in the core transcriptional network of cell fate.

Corneal epithelial wound healing plays a vital role in protecting eye structures from damages of environmental hazards. It’s implicated in corneal diseases, including corneal neovascularization, ulcers, and loss of transparency (1–6). In vivo and in vitro model systems have shown that epidermal growth factor (EGF)2 facilitates corneal epithelial wound repair by promoting migration and proliferation of the cells (7–10). In contrast, environmental stressors, such as UV irradiation, hypoxia, and infections, are biohazards that can directly affect the viability of actively dividing corneal epithelial progenitor cells. It has been shown that stress-induced cellular responses share common signaling mechanisms, including cytokine-induced apoptotic pathways. Stress-induced cell death can delay or retard corneal epithelial wound repair, resulting in the corneal epithelia being more susceptible to bacterial and viral infections (3, 8, 11).

Recent studies from our laboratory and others demonstrate that exposure of mammalian cells to different stresses causes activation of transcription factors CTCF, NF-κB, and other immediate early genes (6, 12–17). CTCF is a zinc finger protein and transcription factor. It plays important roles in epigenetic regulations of gene expression, DNA imprinting, and X chromosome inactivation. It also functions as methylation-sensitive insulator, transcription activator, and repressor (18–20). More recent studies have revealed that CTCF is involved in cancer cell proliferation, tumor suppression, and apoptosis (21–23). However, results obtained are contradictory, and the role of CTCF in cancer pathogenesis remains unclear. In corneal epithelial cells, CTCF is a downstream target protein of growth factor-induced pathways and is regulated by EGF and insulin through activation of ERK and AKT signaling cascades (6, 24). EGF-induced corneal epithelial cell proliferation through up-regulation of CTCF subsequently down-regulates Pax6, an important transcription factor in eye development, by binding to a repressor element between the EE enhancer and P0 promoter of the Pax6 gene (7, 25). Pax6 determines corneal epithelial terminal differentiation and activates corneal epithelial specific keratin 12 expression (7, 26, 27). In addition, overexpression of Pax6 triggers corneal epithelial cell apoptosis (26).

NF-κB is an important gene regulator in the Rel transcription factor family involving inflammatory responses, developmental processes, cellular growth, and apoptosis (28, 29). Inflammation induces immune responses and stimulates production of pro-inflammatory cytokine/chemokines (tumor necrosis factor-α and interleukins 1, 6, and 8), which leads to activation of the mitogen-activated protein kinase cascades, such as ERK, c-Jun N-terminal kinase (JNK), and p38 signal pathways (6, 11, 30–33). The downstream effect includes NF-κB phosphorylation and subsequent translocation to the nucleus (30, 32, 34). There are five NF-κB subtypes in mammalian cells, including RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52). NF-κB1 and NF-κB2 are synthesized as large precursors of 105 kDa (p105) and 100 kDa (p100), respectively. These are partially processed by proteolysis of their C-terminal peptides to produce the active NF-κB1 p50 and NF-κB2 p52 subunits. All NF-κB proteins share ~300-amino acid residues. These residues reside in a homologous domain near the N termini known as the Rel homology domain. The Rel homology domain is responsible for DNA binding, dimerization, inhibitor binding, and nuclear localization (35, 36).

Environmental stress-induced signaling cascades in corneal epithelial cells are similar to those induced by inflammatory cytokines (6, 11, 30–33). We have shown that UV stress-induced NF-κB activation affects corneal epithelial wound healing. Interestingly, CTCF activity is significantly suppressed by
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UV stress-induced NFκB activation in apoptotic corneal epithelial cells (6). NF-κB is activated in corneal epithelial cells through infection- and trauma-induced inflammation (4, 30, 37–39). However, in our study, we also found that EGF-activated NF-κB is directly involved in the regulation of CTCF. This ultimately leads to corneal epithelial cell proliferation. It seems that the effect of NF-κB on cell fate via the regulation of CTCF is contrary and dependent upon the stimulant (growth factors or stressors), resulting in determination of corneal epithelial cell fate. In this study, we set out to investigate the molecular mechanism that is involved in the up-regulation of CTCF by NF-κB p65 and p50 heterodimer activation, and down-regulation of CTCF by NF-κB p50 homodimer formation in response to growth factor and stress stimulation affecting cell survival and death, respectively.

EXPERIMENTAL PROCEDURES

Cell Culture—Human corneal epithelial (HCE) cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen) with 10% fetal bovine serum and 5 ng/ml insulin in a humidified incubator supplied with 5% CO₂ at 37 °C. HCE cells were passaged by 0.05% trypsin-EDTA treatment and seeded at a cell density of 10⁵/ml. HCE cells were synchronized by serum-deprived culture for 48 h prior to experimental treatments. EGF was applied at concentrations from 5 to 20 ng/ml following various time courses. For stress-induction experiments, HCE cells were exposed to UV-C irradiation at 42 μJ/cm². Pyrrolidine dithiocarbamate (PDTC, 100 μM) was added to the cell culture to inhibit NF-κB activation 30 min prior to EGF treatment or UV exposure. Tetrazolium (MTT) assay was performed to measure EGF-induced cell proliferation. MTT solution (50 μl, 5 mg/ml in PBS) was added to each culture dish for 1 h, and acidic isopropanol solution (0.04 N HCl in absolute isopropanol) was added to each dish to dissolve colored crystals. Samples (0.4 ml per dish) were read using an enzyme-linked immunosorbent assay plate reader (Labsystems Multiskan MCC/340, Fisher Scientific) at a wavelength of 570 nm. UV stress-induced apoptosis was determined by measuring caspase 3 activity and poly(ADP-ribose) polymerase degradation by Western analysis. In addition, cell survival index was measured by MTT assay.

Western Analysis—Western blots were performed as described previously (6). In brief, HCE cells in 60-mm culture dishes were rinsed twice with ice-cold PBS and harvested in 0.3 ml of lysis buffer (137 mM NaCl, 1.5 mM MgCl₂, 2 mM EDTA, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aproatin, 10 mg/ml leupeptin, and 20 mM Tris, pH 7.5). After centrifugation (13,000 × g) for 15 min at 4 °C, cell lysates were denatured by adding equal volumes of 2 × Laemml buffer and by boiling for 5 min. Each sample containing 20 μg of protein was fractionated in a SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a Trans Blot SD Transfer Cell (Bio-Rad). The membrane was blocked with 5% fat free milk in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 1 h at room temperature, and hybridized with respective primary antibodies at 4 °C overnight. Secondary antibodies against rabbit, mouse, or goat immunoglobulin G conjugated with horseradish peroxidase (1:1000 in TBST with 5% milk, Santa Cruz Biotechnology, Santa Cruz, CA) were used to probe positive signals, respectively. Positive protein bands were visualized using a Luminol Reagent kit (Santa Cruz Biotechnology). Concentrations of primary antibodies in experiments were listed as follows: anti-CTCF (1:5,000, Upstate), anti-cleaved caspase 3 (1:1,000, Cell Signaling), anti-cleaved poly(ADP-ribose) polymerase (1:1,000, Cell Signaling), and anti-β-actin (1:10,000, Sigma).

Determining NF-κB Translocation to Nuclei—To analyze translocation of NF-κB to nuclei, nuclear proteins were isolated and analyzed by Western blots. Cells were washed twice with ice-cold PBS and suspended in 5 × volumes of ice-cold homogenization buffer containing (mM): 10 HEPES-KOH, 1.5 MgCl₂, 10 KCl, 0.5 dithiothreitol, and 0.5 phenylmethylsulfonyl fluoride, pH 7.9, and homogenized with 20 strokes in a Dounce homogenizer. Intact nuclei released from cells were collected by centrifugation at 250 × g for 10 min at 4 °C and lysed directly in 200 μl of 1 × Laemml buffer with three cycles of sonication. Primary antibody concentrations were listed as follows: anti-p50 (1:1000, Invitrogen), anti-p65 (1:2000, Santa Cruz Biotechnology), anti-RelB (1:2000, Santa Cruz Biotechnology), anti-c-Rel (1:2000, Santa Cruz Biotechnology), and anti-p100/p52 (1:1000, Cell Signaling).

Immunocoprecipitation of NF-κB Subtypes—To analyze NF-κB subtypes, immunocoprecipitation experiments were performed using protein-A-agarose beads cross-linked with rabbit anti-p50 (Invitrogen) or rabbit anti-p65 (Santa Cruz Biotechnology). Anti-p50 or anti-p65 antibody was mixed with protein-A beads on a rotator for 1 h at room temperature. Disuccinimidyl suberate solution (2 mg of disuccinimidyl suberate in 80 μl of DMSO) was added to the antibody and protein-A bead mixture, and equilibrated for 60 min at room temperature. The cross-linked mixtures were centrifuged, washed, and resuspended in 100 μl of binding/wash buffer. After adding 10 μl of anti-p50 beads or anti-p65 beads to 0.5 ml of cell lysates, the mixture was gently rotated at 4 °C overnight. The mixture was boiled for 5 min and centrifuged to separate precipitated proteins from antibody beads.

Knockdown of p50 and p65—Synthesized siRNAs specific to human p50/105 and p65 mRNAs were purchased from Santa Cruz Biotechnology. In addition, a control double strand siRNA (sense: aacauucgguagauuccucgc and antisense: aagcgag-gaauccggagau) was synthesized as described in a previous study (7). The sequence homologies of siRNA primers were examined by using the National Institutes of Health Blast program. Cells were transfected with siRNAs using a siPORT Lipid system (Ambion, Austin, TX). Briefly, sub-confluent cells in 6-well culture plates were washed twice with PBS and treated with a transfection mixture containing: siRNAs (25 nm) and siPORT lipid (10 μl) in 100 μl of serum-free medium at 37 °C for 6 h. Transfected cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal bovine serum for 2 days.

Electrophoretic Mobility Shift Assay—Isolated nuclei collected as described above were suspended in 1 ml of nuclear suspension buffer (400 mM KCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% aproatin, 40 mM HEPES-KOH, pH 7.9). Nuclear extracts were obtained by centrifuga-
binding site in hCTCF-pGL2 was also made by PCR with a pair of primers: forward, 5′-ACCCAGC-GGGAGCCAGGACC-3′ (−297 to −270); and reverse, 5′-CTGG- GCGGAGCCGCGCC-3′ (−306 to −325). To detect CTCF promoter activity, HCE cells were transfected by using Lipofectamine Plus Reagent (Invitrogen) mixed with 0.5 µg of hCTCF-pGL2, NF-κB-hCTCF-pGL2, pGL2 basic vector, or pGL2 control vector containing an SV40 promoter, respectively. Cells were synchronized by serum deprivation for 48 h before treatment of EGF or UV irradiation. A Dual-luciferase® Reporter Assay kit (Promega) was used to detect reporter activity. For each samples, reporter activity (Firefly luciferase in pGL2) and internal control activity (Renilla luciferase in pRL-TK) were measured sequentially following a time course. Promoter activity was normalized with a ratio of Firefly luciferase activity/Renilla luciferase activity.

Chromatin Immunoprecipitation—Cells in 100-mm culture dishes were fixed in 1% formaldehyde for 10 min and incubated with 125 mM glycine for an additional 5 min at room temperature. After rinsing with PBS, cells were harvested in 1 ml of cell lysis buffer with a policeman. Cell nuclei were isolated and further lysed in 1 ml of nuclear lysis buffer. Genomic DNA was fragmentized to 1- to 2-kb fragments by sonication. DNA samples (25 µg each) were used for ChIP. Anti-p50 or anti-p65 antibody was added to lysates in the dilution buffer, and the samples were incubated overnight at 4 °C. Controls were done in two parallel reaction tubes by additions of unrelated antibody and no antibody, respectively. The antibody-protein-DNA complexes were pulled down by adding protein A/G beads that were pre-absorbed with single strand DNA to block nonspecific binding. The complex of antibody-protein-DNA was washed twice with PBS and eluted with eluting buffer. Samples were treated with proteinase K at 55 °C for 6 h. The resulting DNA fragments were precipitated with ethanol and resuspended in 20 µl of TE buffer for PCR reactions. A TaqDNA polymerase kit (Qiagen) was used to perform the PCR reaction. Purified ChIP-DNA (1 µl per sample) or 0.1 µg of control DNA was used in PCR reaction with a pair of primers (sense, 5′-TAAGGTCAACGCGACTGGA-3′; and antisense, 5′-GGGAAAGAGTTGAG-3′). The PCR reaction was carried out by a protocol with a starting temperature of 95 °C for 30 s, and followed by 30 cycles at 56 °C (1 min) and 72 °C (30 s).
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RESULTS

In previous studies we found that EGF stimulates HCE cell proliferation through activation of CTCF. EGF-induced CTCF activation subsequently suppresses Pax6 expression, which is required for corneal epithelial proliferation (7). However, the signaling mechanism involved in EGF-induced CTCF activation still remains a mystery. In the present study we found that application of EGF induced a strong nuclear binding activity of NF-κB by PDTC on UV stress-induced CTCF suppression. The effect of inhibiting NF-κB by PDTC on UV stress-induced CTCF induction and enhanced following increases in EGF concentration, inhibition of NF-κB with PDTC (Fig. 1D). The results suggest that NF-κB may be involved in mediating EGF-induced regulation of CTCF and cell proliferation.

Effect of Suppressing UV Irradiation-induced NF-κB Activation on CTCF Expression and Apoptosis—Previous studies have demonstrated that environmental stresses such as UV irradiation and hyperosmotic pressure have strong effects on the suppression of CTCF activity resulting in apoptotic responses in various cell types (6). In our study, we set out to investigate the signaling mechanisms behind stress-induced suppression of CTCF. We found that UV irradiation induced a strong activation in NF-κB nuclear binding activity detected by EMSA (Fig. 2A). The UV-induced increase in NF-κB nuclear binding was abolished in competitive EMSA experiments by using a non-radioactive NF-κB-specific DNA probe. However, it was not affected by competition of nonspecific probes. The results demonstrated the specificity of UV-induced NF-κB nuclear binding activity in these cells (Fig. 2B). To investigate the effect of UV-induced NF-κB activation on CTCF activity, PDTC was applied to HCE cells with/without exposure to UV irradiation. UV irradiation induced a strong suppression of CTCF activity resulting in CTCF expression and cell proliferation. UV irradiation induced a strong suppression of CTCF activity as detected by MTT assays in these cells (Fig. 1D). The results suggest that NF-κB may be involved in mediating EGF-induced regulation of CTCF and cell proliferation.

Subtype-specific Activation of NF-κB by EGF and UV Irradiation—We questioned how NF-κB activated by EGF and UV stress could lead to opposite effects on CTCF expression and cell destinations. To begin answering this question, EGF and UV irradiation-induced activation of NF-κB subtypes were detected by Western analysis using specific antibodies against p52, RelA, c-Rel, p65, and p50 (Fig. 3A). HCE cells were treated with EGF, UV irradiation, and EGF plus UV irradiation. Nuclear translocation of NF-κB p50 was markedly increased in cells treated with EGF, UV irradiation, and EGF plus UV irradiation. Nuclear translocation of NF-κB p65 was also increased in EGF- and EGF plus UV irradiation-treated cells, but not in UV irradiation-induced cells. This indicates that there is a distinctive pattern for EGF and UV irradiation-activated NF-κB
subtypes. Further experiments were done to verify the effect of EGF and UV irradiation on both p50 and p65 nuclear translocations. The effect of UV irradiation on p50 nuclear translocation was analyzed by Western analysis following a time course (Fig. 3, B and C). DNA-binding activities of NF-κB subtypes induced by EGF and UV irradiation in HCE cells were measured by EMSA and supershift assays (Fig. 3D). Supershift bands in EMSA verified EGF-induced increases in DNA-binding activities of p50 and p65. There was a UV irradiation-induced supershift band in the p50 antibody-treated sample. However, samples treated with nonspecific antibodies did not result in any supershift bands. The composition of NF-κB subtypes in EGF- and UV irradiation-induced formation of NF-κB dimers was examined by immunocoprecipitation experiments. NF-κB p65 was pulled down by adding p50 antibody, and p50 was pulled down by adding p65 antibody in EGStimulated HCE cell lysates, respectively (Fig. 3E). The interaction between p65 and p50 was not found in UV irradiation-induced cells, indicating that p65 and p50 dimers were formed only in EGF-induced cells.

Effects of Knockdown p65 and p50 on CTCF Expression and Cell Survival—We then asked whether the effects of EGF and UV irradiation on NF-κB subtypes could cause different responses and interactions in downstream events in HCE cells. To answer this question, we knocked down p65 and p50 mRNAs using specific siRNAs (Fig. 4, A and B). Our results showed that NF-κB subtypes were effectively inhibited by p65- and p50-specific siRNAs in transfected HCE cells within 48 h. In EGF-induced cells, knockdown of p50 mRNA expression blocked the effect of EGF on increases in CTCF expression and cell proliferation (Fig. 4, C and E). A similar response was also observed in p65 knockdown cells in which EGF failed to stimulate CTCF expression and cell proliferation (Fig. 4, D and F). These results suggest that both NF-κB p65 and p50 subtypes formed a functional heterodimer mediating EGF-induced CTCF activation and cell proliferation. Knockdown of p50 mRNA markedly inhibited UV irradiation-induced suppression of CTCF expression and apoptosis as detected by measuring caspase 3 activity and MTT levels (Fig. 5, A–C). In contrast to the EGF effect, knockdown of p65 mRNA had no effect on either UV irradiation-induced suppression of CTCF or apoptosis. The results indicate that NF-κB p65 is not involved in UV stress-stimulated down-regulation of CTCF (Fig. 5, D–F).

Effects of NF-κB Activation on Regulating CTCF Transcription—NF-κB is a transcription factor, and it regulates gene expression through binding to a specific DNA sequence termed
kB site in the promoter region of these genes. Through analyzing DNA sequences of the CTCF gene, we found that there is a kB site in CTCF gene promoter region (−293 to −576). A CTCF reporter construct was established by cloning a 1000-bp DNA fragment upstream from axon 1 of the CTCF gene, including the kB site. The reporter construct was termed hCTCFp containing the CTCF promoter and luciferase (Luc) reporter gene (Fig. 6A). In addition, a mutant CTCF reporter construct was also established by deleting the kB site from the CTCF reporter construct, termed hCTCFp-del. Both wild-type and mutant constructs were subcloned into a pGL2 vector for transfection experiments. Application of EGF to HCE cells transfected with CTCF reporter stimulated a significant higher activity following a time course (Fig. 6, B and C). In addition, cells that were transfected with pGL2-basic (without inserts) and pGL2-control (with Luc gene) served as negative and positive controls. Inhibition of NF-κB with PDTC diminished EGF-induced increases in CTCF reporter activity (Fig. 6D). We also wanted to compare EGF-induced CTCF reporter activities between cells transfected with wild-type and deletion mutant constructs. Our results showed EGF induced a significant higher Luc activity in wild-type CTCF reporter-transfected cells, whereas EGF had no effect on CTCF reporter activity in cells that were transfected with the deletion mutant of CTCF reporter (Fig. 6E). As shown in the figures above, UV stress-induced activation of NF-κB in HCE cells resulted in suppression of CTCF expression. Furthermore, UV irradiation in CTCF reporter-transfected cells induced an inhibitory effect on CTCF reporter activity following a time course (Fig. 7, A and B). Inhibition of UV irradiation-induced NF-κB activation by PDTC abolished UV stress-induced suppression of CTCF reporter activity (Fig. 7C). In addition, CTCF reporter activity was not found in UV irradiation-induced cells transfected with the mutant reporter construct (Fig. 7D). These results provide evidence that EGF- and UV stress-activated NF-κB suppression of CTCF expression is through control of CTCF promoter activity at the transcriptional level.

Identifying NF-κB Interactive Motifs in CTCF Promoter Region—DNA binding motifs for NF-κB (kB sites) are composed of 9- to 10-bp consensus sequences such as 5′-GGGRNYWYYCC-3′ (40, 41). As shown in Fig. 6A, a putative kB site was found in the CTCF promoter region (−293 to −576) by analyzing DNA sequences of CTCF gene and by expressing the CTCF Luc reporter construct. To further identify the binding motif for NF-κB, two 32P-labeled probes were made from a DNA fragment of the human CTCF promoter (hCTCFp-κB-site) containing NF-κB binding DNA sequence and from synthesized oligonucleotides of NF-κB binding sequence (consensus-NF-κB). Stimulation of HCE cells with either EGF or UV irradiation induced a strong NF-κB-DNA binding as detected by competitive EMSA, applications of cold hCTCFp-κB-site and consensus-NF-κB probes competitively inhibited EGF- and UV irradiation-in-
duced activations of NF-κB binding (Fig. 8, C and D). However, EGF- and UV irradiation-induced NF-κB-binding activities were not affected by the nonspecific probe (Oct-1). These results indicate that the CTCF promoter indeed contained NF-κB binding motifs, because the hCTCFp-κB-site and consensus-NF-κB probes were able to compete with each other for NF-κB binding. In vivo studies were done to further verify NF-κB binding motifs in the human CTCF promoter. Chromatin in EGF- and UV irradiation-induced HCE cells were isolated for use in ChIP and probed using specific antibodies against NF-κB p50 and p65 subtypes. Two primers were designed in the ChIP-based PCR experiment that generated a 250-bp DNA fragment according to consensus DNA sequences upstream from the CTCT promoter (Fig. 8E). Results from ChIP-based PCR demonstrated markedly enhanced bands in both EGF- and UV irradiation-induced HCE cells when the p50-specific antibody was used to pull down chromatin. This suggests the p50 antibody pulled down the CTCF promoter in both EGF and UV irradiation-induced cells (Fig. 8F). However, there was only a single enhanced band presented in EGF-induced cells while p65-specific antibody was used in ChIP and ChIP-based PCR. This result suggests that the anti-p65 antibody did not pull down chromatin containing the CTCF promoter in UV irradiation-induced cells (Fig. 8G). Results from ChIP and ChIP-based PCR experiments are consistent with the data obtained from the EMSA and immunocoprecipitation experiments, indicating that the NF-κB p50 subtype was activated in both EGF- and UV irradiation-induced cells. However, the p65 subtype was only activated to form NF-κB heterodimers with the p50 subtype in EGF-induced cells.

DISCUSSION

CTCF functions as an epigenetic regulator and transcription factor that controls important gene expression and cell fate. In the previous study, we found that CTCF plays an important role in corneal epithelial cell proliferation and differentiation by regulating homeobox Pax6 gene expression (7, 42, 43). Further downstream, Pax6 possesses unique functions controlling corneal epithelial lineage-specific differentiation marker keratin 12 expression (44, 45). Suppression of Pax6 expression by overexpression of CTCF results in small eye defects in mice (42). In contrast, enhanced Pax6 activities by overexpression of Pax6 or by knocking down CTCF caused corneal epithelial cell apoptosis (7, 26). However, there is still no information available regarding how CTCF is regulated at transcription levels. In the present study we have demonstrated for the first time that CTCF is positively and negatively regulated by different NF-κB dimers stimulated by EGF and UV irradiation, respectively. We have also shown through binding assays and ChIP-based PCR that activation of the NF-κB p65 subtype is necessary for positive regulation of CTCF transcription through binding to a κB site located in the CTCF promoter region. Furthermore, deletion of the κB site in the CTCF promoter region abolished the effects of NF-κB subtypes on CTCF transcription reporter
activity. This provides direct evidence of NF-κB/H9260 activation-mediated regulation of CTCF expression. These results support the conclusion that CTCF plays a key role in mediating EGF- and UV stress-induced corneal epithelial cell fate downstream of the NF-κB pathway.

Our previous studies demonstrate that the effect of NF-κB activity on corneal epithelial function is dependent upon the nature of the stimuli. Tumor necrosis factor-α (TNF-α) does not induce corneal epithelial cell apoptosis, but instead increases expression of p21 and cell cycle attenuation in the G1 phase. This in turn promotes cell survival (32). However, the functional role of NF-κB in regulating corneal epithelial cell proliferation and apoptosis is largely undefined. The most important findings of the present study are that EGF- and UV irradiation-induced activation of NF-κB leads to forming different compositions of NF-κB dimers. These
dimers differentially regulate CTCF expression resulting in corneal epithelial cell proliferation or apoptosis. It has been shown that NF-κB is activated to form dimers and transferred into the nucleus to modulate gene expression (46, 47). Active NF-κB dimers are composed of homodimers or heterodimers with at least 15 combinations from 5 subtypes known in mammalian cells (48). The conflicting effects of NF-κB, promoting or inhibiting apoptosis, have been observed in various cell types, including corneal epithelial cells (6). The nature of the effects of NF-κB appears to be influenced by the cell type and the inducing stimulus. Recent studies suggest that certain types of stimuli may determine the composition of active NF-κB dimers affecting cell proliferation and apoptosis (29, 48). It has been shown that activation of p65 (RelA) is necessary for activation of anti-apoptotic genes (49). Activation of NF-κB can prevent apoptosis through activating transcription of pro-inflammatory genes and anti-apoptotic genes that include cIAP1, XIAP, c-FLIP, Bcl-2, and Bcl-XL. Consistent with the results from previous studies, we have shown that EGF-induced activation of p65 leads to increased activity of CTCF promoting proliferation in corneal epithelial cells.

It has been suggested that the pro-apoptotic effect of NF-κB in some cell types is perhaps due to the production of pro-inflammatory cytokines and reactive oxygen species (50, 51). These products may interact with NF-κB activation in stimulated cells (52–54). We found that UV stress-induced NF-κB p50 subtype activation promotes apoptosis through suppression of CTCF activity in corneal epithelial cells. This effect is independent of p65. Thus, our results reveal that dichotomous effects of NF-κB on CTCF expression in EGF- and UV irradiation-induced corneal epithelial cells are due to activations of different NF-κB subtypes, namely p65 and p50, without activation of other subtypes (Fig. 3A). We believe that EGF-induced activation of p65 and p50 forms heterodimers to up-regulate CTCF thus promoting cell survival. Formation of the p50/p50 homodimer that suppresses CTCF transcription determines UV stress-induced apoptosis. Blockade of NF-κB activity by using PDTC suppressed UV stress-induced down-regulation of CTCF, resulting in protection of cells from apoptosis. This was consistent with other findings: 1) knockdown of NF-κB p65 and p50 mRNA effectively abolishes EGF-induced proliferation and 2) knockdown of NF-κB p50 mRNA blocked UV stress-induced apoptosis. Taken together,

FIGURE 8. Identifying interactions of NF-κB and human CTCF promoter in vivo. A, EGF-stimulated NF-κB binding activities. B, competitive inhibition of EGF-induced NF-κB binding activities. C, UV stress-induced NF-κB binding activities. D, competitive inhibition of UV stress-induced NF-κB binding activities. The probes used in competitive inhibition experiments were consensus-NF-κB, hCTCFp-NF-κB, and Oct1, respectively. E, scheme of design for chromatin immunoprecipitation (ChIP) and ChIP-based PCR. F, ChIP-based PCR to detect CTCF promoter binding region by using anti-NF-κB p50 antibody to immunoprecipitate the target chromatin. G, ChIP-based PCR to detect CTCF promoter binding region by using anti-NF-κB p65 antibody to immunoprecipitate the target chromatin. The chromatin was isolated 1 h after treating cells with EGF (20 ng/ml) and UV irradiation.
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we found that CTCF plays a fundamental role in the EGF- and UV stress-activated downstream effects of the NF-κB pathway, which modulates expression of target genes responsible for cell death and survival.

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