De-SUMOylation of CCCTC Binding Factor (CTCF) in Hypoxic Stress-induced Human Corneal Epithelial Cells*

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Background: CCCTC binding factor (CTCF) plays important roles in the epigenetic control of cell fate.

Results: Hypoxic stress suppressed a higher Mr form of CTCF by de-SUMOylation associated with lysine 74 and 689 residues, resulting in significantly inhibited PAX6 expression.

Conclusion: Hypoxic stress induces de-SUMOylation of CTCF to functionally regulate CTCF activity.

Significance: CTCF plays important roles in growth factor/stress-regulated cell fates through post-translational modulation to control gene expression.

Epigenetic factor CCCTC binding factor (CTCF) plays important roles in the genetic control of cell fate. Previous studies found that CTCF is positively and negatively regulated at the transcriptional level by epidermal growth factor (EGF) and ultraviolet (UV) stimulation, respectively. However, it is unknown whether other stresses modify the CTCF protein. Here, we report that regulation of CTCF by de-SUMOylation is dependent upon hypoxic and oxidative stresses. We found that stimulation of human corneal epithelial cells with hypoxic stress suppressed a high molecular mass form of CTCF (150 kDa), but not a lower molecular weight form of CTCF (130 kDa). Further investigation revealed that the hypoxic stress-suppressed 150-kDa CTCF was a small ubiquitin-related modifier (SUMO)-ylated form of the protein. Hypoxic stress-induced de-SUMOylation of human CTCF was associated with lysine 74 and 689 residues, but not to the phosphorylation of CTCF. Overexpression of SENP1 induced de-SUMOylation of CTCF. However, knockdown of SENP1 could not rescue hypoxic stress-induced CTCF de-SUMOylation. Overexpression of SUMO1 and SUMO2 increased SUMOylation of CTCF and partially blocked hypoxic stress-induced CTCF de-SUMOylation, suggesting that free cellular SUMO proteins play roles in regulating hypoxia-induced CTCF de-SUMOylation. In addition, hypoxic stress significantly inhibited PAX6 mRNA and protein expressions by suppression of PAX6-P0 promoter activity. The result was further supported by data showing that knockdown of CTCF significantly enhanced expression of PAX6 and abolished hypoxia-induced suppression of PAX6. Thus, we conclude that hypoxic stress induces de-SUMOylation of CTCF to functionally regulate CTCF activity.

The corneal epithelial cell layer, which forms a barrier at the front of the eye to protect structures behind from biological, chemical, and physical damage, undergoes a self-renewal process in 2–3 weeks. Growth factors and environmental stresses can alter corneal epithelial cell fates by affecting the corneal epithelial renewal process (1). It has been shown that hypoxic stress on the ocular surface leads to pathological conditions that result in corneal epithelial apoptosis, attenuation of re-epithelialization, and corneal neovascularization. For example, the extended wearing of contact lenses can induce hypoxic conditions, which increases susceptibility to corneal surface infections resulting in keratitis (2–4). Cellular responses to hypoxia in the corneal epithelium are complex and depend on degrees of altered oxygen tension (5). Hypoxic stress induces apoptosis in corneal epithelial cells through activations of JNK and Pololike-kinase 3 that further activate the AP-1 transcription complex (4).

CCCTC binding factor (CTCF)2 is a zinc finger protein and epigenetic factor highly conserved across species. CTCF is characterized as both a negative and positive regulator that binds to DNA motifs in promoter regions of different genes, including c-myc, chicken lysozyme, retinoblastoma (Rb), telomerase reverse transcriptase, and eye-specific PAX6 genes (6, 7). More studies indicate that CTCF is a multifunctional nuclear protein that involves transcriptional activation/repression, gene insulation, DNA imprinting, and X chromosome inactivation. CTCF has been defined as an insulator protein because it can specifically bind to unique DNA sequences in both globin locus and h19/lgf2 genes (8). The interaction of CTCF with DNA targets blocks communication between adjacent regulatory DNA elements in a position-dependent manner, and serves as a barrier to buffer transgenes from position effects caused by spreading repressive heterochromatin from adjacent sequences (9, 10). Recently a combination of computational methods and genome-wide screens revealed genomewide CTCF-binding sites, indicating that CTCF is a universal “master weaver” of diverged genomes. CTCF is important for coordinating the organization and regulation of a whole range of distinct genomic functions in three dimensions (11–13).

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2 The abbreviations used are: CTCF, CCCTC binding factor; HCE, human corneal epithelial; NEM, N-ethylmaleimide; SUMO, small ubiquitin-related modifier; IP, immunoprecipitation; SENP, sentrin/SUMO-specific protease.
We found that CTCF plays important roles in regulating proliferation, differentiation, and apoptosis of these cells in response to growth factor and stress stimulation (1, 14). Previous studies revealed that regulation of CTCF is dependent upon the altered ability of CTCF to interact with its partner proteins and to bind to target DNA sequences. Recent studies demonstrated that epidermal growth factor (EGF) and ultraviolet (UV) stress induce an increase and decrease in CTCF expression to mediate human corneal epithelial (HCE) cell proliferation and apoptosis, respectively (15, 16). More interestingly, CTCF gene transcription is regulated by different subtypes of NF-κB in response to stimulation by EGF and UV stress (14). The latter results provide novel information indicating that CTCF is regulated at the transcriptional level. These findings suggest that CTCF is capable of dynamically responding to stress stimulation. The CTCF protein can also be modified by post-translational modifications, such as phosphorylation, poly(ADP-ribose)ylation (PARylation), and SUMOylation (9). However, the molecular mechanisms underlying how the CTCF protein is SUMOylated and de-SUMOylated in response to stimuli and whether CTCF de-SUMOylation affects its function are still largely unknown. In the present study, we focus on investigating hypoxic stress-induced regulation of CTCF de-SUMOylation. We explore the molecular mechanisms behind stress-induced effects on CTCF function by examining the effects of different stresses, including UV irradiation, hypersmotic stress, hypoxia, and hydrogen peroxide, on CTCF modification and function in corneal epithelial cells. Our results demonstrate that CTCF modification is affected by hypoxic conditions through a hypoxia-sensitive de-SUMOylation process to affect downstream CTCF-controlled gene activities.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HCE cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (1:1) containing 10% fetal bovine serum and 5 μg/ml of insulin. Mouse pancreatic α tumor cell 1, clone 6 (α-TCT1–6) and HEK-293 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were maintained in an incubator supplied with 95% air and 5% CO₂ at 37 °C. The medium was replaced every 2 days, and cells were subcultured by treatment with 0.05% trypsin-EDTA. For hypoxia experiments, cells were placed in an incubator supplemented with 1% O₂, 5% CO₂ and 94% N₂ at 37 °C for 0.5–4 h. For UV irradiation experiments, HCE cells were exposed under UV-C light at an intensity of 45 mW/cm². For hyperosmotic stimulation, cells were exposed to culture medium containing 600 mm sorbitol. For heat shock and oxidative stress stimulation, HCE cells were incubated at 42 °C for 4 h and treated with 500 μM hydrogen peroxide, respectively.

**Antibodies and Reagents**—Antibody against CTCF (catalog number 07-729) was purchased from Millipore, Billerica, MA. Monoclonal anti-FLAG M2 antibody (catalog number F3165) was purchased from Sigma. Monoclonal antibody against HA (catalog number MMS-101R) was obtained from Covance, Princeton, NJ. Rabbit polyclonal antibody against PAX6 (catalog number 42-6600) was purchased from Invitrogen. Antibodies against SUMO1 (catalog number ab11672) and SUMO2/3 (catalog number ab3742) were from Abcam (Cambridge, MA). N-Ethylmaleimide (NEM, catalog number 128-53-0) was from Acros Organics (Fisher Scientific). Proteinase inhibitors, including phenylmethylsulfonyl fluoride (PMSF), aprotinin, and leupeptin, were purchased from Fisher Scientific. SDS was obtained from Sigma. Synthesized siRNA specific to human SENP1 (ON-TARGETplus SMARTpool SENP1, catalog number L-006357-00-0005) was purchased from Dharmacon RNAi Technologies (Lafayette, CO). Nonsilencing siRNA for the control was obtained from Qiagen (Valencia, CA).

**Plasmid Constructs**—The expression plasmid for FLAG-tagged CTCF was generated by subcloning human CTCF cDNA (NCBI reference number NM_006565.3) from pCDNA4-CTCF into p-Flag-CMV-3 vector (Sigma) (16). The PAX6-P0 promoter luciferase reporter construct (pGL2-PAX6-P0-Luc) was generated by subcloning a 4.2-kb DNA fragment located upstream of the PAX6 gene P0 promoter inserted in β-Gal-Pax6-P0 to a pGL2-Basic vector (Promega, Madison, WI) (17). SRA-HA-SUMO1 (plasmid 17359) and SRA-HA-SUMO2 (18) were purchased from Addgene (Cambridge, MA) (18). Expression vector of GFP-SENP1 was a generous gift from Dr. Thomas Pap (19). Plasmid GFP-SENP3 was a gift from Dr. Jing Yi (20). Plasmid GFP-SENP5 was kindly provided by Dr. Mary Dasso (21). The construct of SENP1-shRNA was received as a generous gift from Dr. Jinke Cheng (22). Mutants of human CTCF-K74R/K689R (it is in the site of Lys-698 in its mouse counterpart) and CTCF-ALA were generated by using a QuikChange® Lightning Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Primers used for generating mutants of CTCF were: CTCF-K74R, forward, 5’-CACCCCTTCTCCAGATGGCGACTTGAATAATGGAGG-3’/reverse, 5’-CTCTCTTATCCATGCTCAGCTCAGCATTGAGTGCTGGA-3’; CTCF-K689R, forward, 5’-GAACTTATAGTTGAACTCAGAGGAGCGACTGCG-3’/reverse, 5’-CTACAGCATTGCTGTCTTCTCTGAATCCTAATATTTTCTC-3’; CTCF-ALA, forward, 5’-GACCACAGTACTAAGAGATAGTTGACG-3’/reverse, 5’-ACATTTTCAGCTACGGACGGCAGTAAATGCT-3’.

**Lentiviral Infection of shRNAs**—Lentiviral particles, containing shRNAs of SENP1 or CTCF tagged with a turbo-GFP, were packaged in HEK-293T cells. The viral concentrations in the culture medium were titrated by PCR after co-transfecting cells with pCMV-VP64-pBabe-puro and pGIPZ-shRNA-CTCF (or pGIPZ-shRNA-SENP1) fused to TurboGFP for 72 h (Open Biosystems Products, Huntsville, AL). The culture medium containing lentivirus secreted from the HEK-293T cell was added to HCE cells, and infected clones stably expressing the shRNAs were selected by selective culture in the presence of G418 (800 μg/ml). HCE cells infected with a pGIPZ-shRNA-control vector packed in lentivirus served as the controls. In addition, expression of GFP from the pGIPZ-TurboGFP vector allowed to measure the efficiency of the viral infection, and to make a distinguished green from no green cells.

**DNA Transfection, siRNA, and Luciferase Reporter Assays**—HCE and HEK-293 cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen). Transfection of SENP1-specific siRNA was done by adding 25 nM SENP1-specific siRNA and 12 μl of HiPerFect (Qiagen) in 100 μl of serum-free culture.

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medium. The transfection mixture was incubated for 10 min at room temperature (RT). The mixture was evenly dropped onto cells. Transfected cells were cultured under normal growth conditions for 72 h prior to experiments. Control cells were transfected with nonsilencing-siRNA using the same method as described above.

For luciferase reporter assays, HCE cells were plated to a 12-well plate at a density of $7 \times 10^4$ per well 24 h prior to transfection experiments. A typical transfection experiment was carried out by adding 500 ng of reporter plasmid of pGL2-PAX6-P0 or pGL2-CMV. A plasmid of pRL-TK containing a transfection experiments. A typical transfection experiment were performed by using a Dual Luciferase® Reporter Assay System (Promega). PAX6-P0 promoter activity was analyzed by normalization of firefly luciferase activity with Renilla luciferase activity.

Immunoprecipitation and Immunoblotting—Immunoprecipitation (IP) experiments were performed as described in a previous publication (23). Briefly, cells were lysed in 200 μl of SDS buffer containing 62.5 mM Tris-Cl, 2% SDS, 10% glycerol, protease inhibitor mixture (containing 1 mM PMSF, 1 μg/ml of aprotinin, and 1 μg/ml of leupeptin), and 20 mM N-ethylmaleimide. Lysates were then sonicated 3× for 5 s to reduce viscosity followed by centrifugation at 15,000 × g for 15 min to remove cellular debris. Lysates were diluted 5-fold with a dilution buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40) and incubated with 1 μg of primary antibody at 4 °C overnight. Immunocomplexes were recovered by incubation of the lysates with 40 μl of A/G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplex beads were washed with a wash buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40), boiled in 2× SDS-PAGE loading buffer, and then subject to immunoblotting assays.

For immunoblotting assays, protein samples prepared in SDS-PAGE loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromphenol blue) were fractionated in SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a TransBlot 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 RT, and hybridized with the respective primary antibodies at 4 °C overnight. Secondary antibodies conjugated with horseradish peroxidase (1:3000 in TBST with 5% milk, Santa Cruz Biotechnology) were used to probe positive signals, respectively. Positive protein bands were visualized using a Luminol reagent kit (Santa Cruz Biotechnology).

RNA Extraction, Reverse Transcription, and Real-time PCR—Total RNAs extracted from cells were used in reverse transcription reactions. The cDNA samples were diluted 50-fold and subjected to real-time PCR assays using the Power SYBR® Green PCR Master Mix (catalog number 4368708, Applied Biosystems, Carlsbad, CA). Primers used in real-time PCR experiments were: 1) human SENP1, forward, 5′-GCATTCTTCTCTTCTTTTTGCTTTGTCA-3′; reverse, 5′-CGGACCTACAGTAC-3′; 2) human PAX6, forward, 5′-GAGCTGGCCGTCACCTTTTGC-3′; reverse, 5′-GATCCTTTGACCCCACTT-ACCA-3′; 3) human β-glucuronidase, forward, 5′-GAAGATCCCCTTTTA-3′; and 4) human GAPDH, forward, 5′-TGGGAAAGGTTAAGGTCGG-3′; reverse, 5′-CTGAAGATGGTGATGGGA-3′.

RESULTS

Suppression of Higher Molecular Weight CTCF by Various Stresses—Previous studies found in corneal epithelial cells that CTCF expression is up-regulated and down-regulated by EGF and UV stress, respectively. To further study the role of CTCF in various environmental stress-induced cellular responses, we challenged corneal epithelial cells with different stresses, including heat shock, UV irradiation, hypersomotic pressure, hydrogen peroxide (H$_2$O$_2$), and hypoxia (Fig. 1). Expression of the CTCF protein was determined by Western analysis 4 h after stimulation. There were two forms of CTCF proteins visualized in a 6% SDS-PAGE gel: a primary form with a molecular mass of 130 kDa and a secondary form with a higher mass of 150 kDa. Interestingly, upon exposure of corneal epithelial cells to hypoxia and H$_2$O$_2$, expression of the 150-kDa CTCF was diminished, whereas expression of the 130-kDa CTCF was not affected (Fig. 1A). UV irradiation and hypersomotic stresses down-regulated expression of both forms of CTCF. However, there was no change in CTCF expression found in cells treated with heat shock. Thus, our study focuses on oxidative stress-induced effects on CTCF modification in corneal epithelial cells. Following a time course of treatments, hypoxic stress and H$_2$O$_2$ significantly diminished the 150-kDa CTCF within 0.5 h without affecting the 130-kDa CTCF (Fig. 1, B and C). The effect of hypoxic stress on CTCF expression was also examined in other cell types. We found that hypoxic stress also markedly suppressed the 150-kDa CTCF in human telomerase-deficient immortalized corneal epithelial, HEK-393 (human embryonic kidney 293), and α-TC-1–6 (mouse pancreatic α tumor) (Fig. 1D). The results indicate that cellular CTCF activities are regulated by stimulation of various stresses. UV and hypersomotic stress inhibited CTCF expression, whereas hypoxic stress and H$_2$O$_2$ suppressed the higher molecular mass 150-kDa CTCF, suggesting a novel mechanism in hypoxia-sensitive CTCF modification.

Hypoxic Stress-induced De-SUMOylation of CTCF—As mentioned previously, CTCF is modified by phosphorylation, PARylation, and SUMOylation. However, which CTCF modifications are affected by hypoxic stress is unknown. We performed IP experiments with standard lysis buffer and found that anti-CTCF antibodies were able to pulldown 130-kDa CTCF proteins, but not 150-kDa CTCF, suggesting that modification of the 150-kDa CTCF might be altered by specific cellular enzymes that are active in the normal IP lysis buffer. We tested the effects of three components added to the standard IP lysis buffer, including NEM (inhibitor of cysteine peptidases), proteasine inhibitor mixture, and SDS (anionic surfactant), on preservation of the 150-kDa CTCF protein. The 150-kDa CTCF was preserved in the modified IP lysis buffer by adding NEM or SDS. However, the 150-kDa CTCF was diminished in the presence of a protease inhibitor mixture in the lysis buffer (Fig. 2A). These results indicate that hypoxic stress-induced suppression of the 150-kDa CTCF could be the result from loss of protein modification by SUMOylation or monoubiquitination. Fur-
thermore, we performed a denaturing IP by using a lysis buffer containing both NEM and SDS. We found that the 150-kDa CTCF was present in the IP products pulled down by anti-CTCF antibody and recognized in Western analysis by anti-SUMO1 and anti-SUMO2/3 antibodies (Fig. 2B), but not by the anti-ubiquitin antibody (data not shown). The possibility of the ubiquitination of CTCF was further investigated by the addition of MG132, a proteasome inhibitor. De-SUMOylation of CTCF still occurred in the presence of MG132 following a time course (no ubiquitinated-CTCF was observed) (Fig. 2C). Both SUMO1- and SUMO2/3-conjugated CTCF proteins were suppressed by hypoxic stress in 4 h. The results also demonstrated that the 150-kDa CTCF was suppressed by hypoxic stress when CTCF proteins were pulled down by anti-SUMO2/3 antibodies and recognized by anti-CTCF antibody (Fig. 2D). In addition, the time course showed a consistent pattern in hypoxic stress-induced suppression of 150-kDa CTCF proteins within 0.5 h in whole cell lysates, detected by immunoblotting and immunoprecipitation with an anti-SUMO2/3 antibody (Fig. 2E).

Accordingly, HEK-293 cells were co-transfected with FLAG-tagged CTCF (Flag-CTCF) and HA-tagged SUMOs (HA-SUMO1 and HA-SUMO2) for 48 h, and then exposed to hypoxic stress. Expressed exogenous CTCF proteins were precipitated by an anti-FLAG antibody and detected for HA-SUMO-conjugated CTCF by an antibody against HA tags (Fig. 2F). Consistent with data on endogenous CTCF modification, the exogenous CTCF proteins were modified by SUMO1 and SUMO2. Both of the SUMOylation modifications were markedly decreased following treatment of the hypoxic condition for 4 h. These results demonstrate hypoxic stress-induced de-SUMOylation of CTCF proteins in the stimulated cells.

**Effect of Altering SUMOylation on 150-kDa CTCF**—Hypoxic stress-induced changes in de-SUMOylation of the 150-kDa CTCF were further verified. First, we tested whether the higher Mr CTCF is sensitive to sentrin/SUMO-specific proteases (SENPs). HEK-293 cells were transfected with GFP-vector only (control) and cDNAs encoding full-length SENP1, -3, and -5. Expression of the 150-kDa form of CTCF was significantly decreased in cells overexpressing SENP1 compared with the control \((p < 0.05, n = 3)\), but was not altered by overexpression of either SENP3 or SENP5 (Fig. 3A). Second, two SUMOylation sites in the human CTCF protein were mutated by replacing individual lysine 74 or 689 residues with arginine (K74R or K689R), and by replacing both lysine 74 and 689 residues with arginine residues (K74R/K689R) (Fig. 3B). In HEK-293 cells, overexpression of the wild type and K74R/K689R mutant of CTCF
were compared with cells transfected with the FLAG vector. In addition, the cells were co-transfected with HA vector, HA-SUMO1, and HA-SUMO2. SUMOylation of CTCF was examined by Western analysis. Increases in SUMO levels resulted in an increase in the 150-kDa wild type CTCF, but had no effect on the CTCF K74R/K689R mutant (Fig. 3C). Next, both wild type and mutants of CTCF proteins expressed in transfected HCE cells were detected by immunostaining with an anti-FLAG antibody. As shown in Fig. 3D, both exogenous wild type CTCF and CTCF mutants demonstrated a similar pattern of subcellular distribution and remained in the nucleus. Taken together, these results provide direct evidence to support a mechanism in which regulation of 150-kDa CTCF by hypoxic stress is due to SUMOylation of CTCF.

Mechanism Involving Hypoxic Stress-induced De-SUMOylation of CTCF—To verify the role of SENP1 in de-SUMOylation of CTCF, SENP1 mRNA was knocked down by two approaches in HCE cells: 1) a SENP1-specific siRNA was transiently transfected by lipofection; and 2) a SENP1-specific shRNA was stably expressed by infecting cells with SENP1-shRNA using a lentiviral delivery system, followed by culturing cells in a selection medium containing antibiotic G418. Results of the real-time PCR demonstrated that SENP1 siRNA transfection and shRNA infection significantly reduced the mRNA level of endogenous SENP1 compared with the nonsilencing-siRNA/shRNA controls (Fig. 4, A and D). Knockdown of SENP1 mRNA also markedly suppressed SENP1 protein expression (Fig. 4, B and E). However, knockdown of SENP1 did not block hypoxic stress-induced de-SUMOylation of CTCF, indicating that SENP1 is not involved in hypoxic stress-induced de-SUMOylation of CTCF (Fig. 4, C and F). Furthermore, a human CTCF mutant (CTCF-ALA) was established by replacing four serine residues (604Ser-Lys-Lys-Glu-609Ser-610Ser-Asp-612Ser-Glu) that consist of major phosphorylation sites with alanine residues to study the effect of CTCF phosphorylation on hypoxic stress-induced CTCF deSUMOylation. In HEK-293 cells that were co-transfected with wild type CTCF and CTCF-ALA mutant plus HA-SUMO1 and HA-SUMO2, wild type and mutant CTCF were equally modified by SUMO1 or SUMO2, suggesting that phosphorylation of CTCF occurring in these serine sites did not affect the SUMOylation of CTCF (Fig. 5A). Next studies were aimed toward answering the question of whether deficiency of free SUMO proteins contributes to hypoxic stress-induced de-SUMOylation of CTCF. Levels of free SUMO1 and SUMO2 were markedly increased in control and hypoxic stress-induced cells by overexpression of HA-SUMO1 and HA-SUMO2. Overexpression of HA-SUMO1 and HA-SUMO2 partially reversed hypoxia-induced de-SUMOylation of CTCF (Fig. 5B). The role of hypoxic stress in altering SUMOylation of cellular proteins was further examined by monitoring the global SUMO conjugation pattern in HCE cells and by Western analysis with anti-SUMO1- and anti-SUMO2/3-specific antibodies. Hypoxic stress decreased free SUMO1 and SUMO2/3 and altered the global patterns of SUMO conjugations. In particular, rapid accumulation of the endogenous SUMO2/3 conjugates was...
observed (Fig. 5C). These results suggest that cellular free SUMO proteins (especially SUMO2) indeed play key roles in regulating hypoxia-induced de-SUMOylation of CTCF.

Effect of CTCF De-SUMOylation on Downstream PAX6 Gene Activity—Previous studies found that CTCF controls eye development through regulation of the eye-specific PAX6 gene by
binding to a repressor element between the EE enhancer and P0 promoter of the PAX6 gene (17). We explored the functional role of CTCF de-SUMOylation in HCE cells by comparing the effect of human wild type CTCF and the CTCF-K74R/K689R mutant on the downstream PAX6 gene. We found that overexpression of wild type CTCF inhibited PAX6-P0 promoter reporter activity, whereas overexpression of the CTCF-K74R/K689R mutant further enhanced the inhibitory effect on PAX6-P0 promoter reporter activity (Fig. 6A). Neither wild type CTCF nor the CTCF-K74R/K689R mutant affected pGL2CMV activity in control experiments (Fig. 6B), indicating that the impact of CTCF de-SUMOylation on the downstream PAX6 gene is specific. Real-time PCR experiments were performed on hypoxic stress-induced cells to investigate the effect of hypoxic stress that de-SUMOylates CTCF on the downstream CTCF promoter. The CTCF-ALA mutant was established by replacing four serine residues with glycine residues. HEK-293 cells were co-transfected with FLAG-wild type CTCF and FLAG-CTCF-ALA mutant with HA-SUMO1 or HA-SUMO2 for 48 h. The CTCF-ALA mutant was established by replacing four serine residues with glycine residues. HEK-293 cells were co-transfected with FLAG-wild type CTCF and FLAG-CTCF-ALA mutant with HA-SUMO1 or HA-SUMO2 for 48 h. The CTCF-ALA mutant was established by replacing four serine residues with glycine residues. HEK-293 cells were co-transfected with FLAG-wild type CTCF and FLAG-CTCF-ALA mutant with HA-SUMO1 or HA-SUMO2 for 48 h. We found hypoxic stress induced significant decreases in PAX6 mRNA expression starting within 1 h, and that the PAX6 mRNA level continued decreasing to reach greater than a 50% reduction at 4 h. The mRNA level of β-glucuronidase was detected by real-time PCR in parallel to PAX6 mRNA measurements as a control (Fig. 6C). It has been shown that the mRNA level of β-glucuronidase is unchanged in hypoxic stress-induced cells (24). The effect of hypoxic stress on PAX6 protein expression was determined by Western analysis. PAX6 expression was significantly suppressed by hypoxic stress in 4 h in corneal epithelial cells (Fig. 6D). To further determine whether hypoxia affects PAX6 expression exclusively through CTCF de-SUMOylation, HCE cells were infected by lentiviral CTCF-specific shRNA to knockdown the CTCF mRNA. The stable expression of CTCF-specific shRNA in the G418 selective medium resulted in a marked decrease in CTCF protein expression (Fig. 6E). Real-time PCR and Western analysis data revealed that knockdown of CTCF effectively abolished hypoxia-induced suppression of PAX6 mRNA and protein expressions, respectively (Fig. 6, F and G). These results indicate that hypoxic stress-induced suppression of PAX6 is mediated by CTCF and de-SUMOylation of CTCF enhanced CTCF capability to suppress PAX6 expression.

DISCUSSION

CTCF is a multifunctional transcription factor that plays important roles in epigenetic regulation of gene expression. Recently, it has been shown that CTCF is a leading candidate for mediating a network of local and long-range intra-chromosomal loops and inter-chromosomal contacts (25). Therefore, CTCF must be capable of dynamically responding to stresses and mediating stress-induced alteration of chromatin structures. Several stress-related proteins such as PUMA (p53 up-regulated modulator of apoptosis) have been reported to be regulated by CTCF (26). However, it is still unclear how CTCF is regulated in response to environmental stresses. We previously demonstrated that UV and hyper-osmotic stresses down-regulated CTCF by reducing its expression at the transcription level. Here, we continue to investigate the response of CTCF to environmental stresses by challenging corneal epithelial cells with multiple stimuli including UV irradiation, hyper-osmotic pressure, heat shock, hydrogen peroxide (oxidative stress), and hypoxia. In addition to down-regulated CTCF expression induced by UV and hyper-osmotic stresses, the study was aimed at addressing the question of whether there is modification of the CTCF protein in response to hypoxic and oxidative stresses. We found that a higher molecular mass form of CTCF at 150 kDa disappeared after stimulation with hypoxic and oxidative stresses, indicating that there are certain types of
environmental stresses that can alter CTCF activity through post-translational modifications at the protein level.

Three post-translational modifications of CTCF have been previously characterized, including phosphorylation (27, 28), PARylation (29–31), and SUMOylation (23, 32). CTCF phosphorylation that occurs in the carboxyl terminus of CTCF is responsible for attenuation of CTCF activity toward c-myc promoter activity (27). PARylation in the N-terminal domain of CTCF can also regulate CTCF function. PARylation is essential for CTCF to function as an insulator for the Igf2/h19 imprinting gene and as a chromatin boundary upstream of the p16 gene (10, 30). Recently, it has been reported that CTCF can be modified by SUMO proteins and the SUMOylation sites identified are at lysine 74 and 698 of the mouse CTCF protein (23). In the present study, we identified that lysine 74 and 689 in human CTCF protein can be modified by SUMO, which is different from lysine 698 in mouse CTCF.

It is not known how the CTCF protein is de-SUMOylated and whether de-SUMOylation of CTCF affects its function. In the present studies, we observed hypoxia-sensitive de-SUMOylation of CTCF. Our results demonstrate that the CTCF protein has two forms. A higher molecular weight form of CTCF is regulated by hypoxic stress, whereas the major form of CTCF protein at 130 kDa is not sensitive to hypoxic stress. The difference between the two forms, as analyzed in a 6% SDS-PAGE gel, is in accordance with the size of a SUMOylated protein. More evidence revealed that hypoxic stress-induced de-SUMOylation of CTCF can be blocked in the presence of NEM, resulting in preservation of the 150-kDa CTCF. NEM is commonly used as an inhibitor in protein de-SUMOylation or de-ubiquitination studies. In other reports, PARylated CTCF is characterized as a 180-kDa protein, as detected by Western analysis. However, we did not detect the PARylated 180-kDa CTCF in either immunoprecipitation or Western blot experiments in HCE cells. Instead, we found that there is a 150-kDa SUMOylated form of CTCF in HCE, HEK-293, and pancreatic islet H9251 cells.

One possible explanation for this is that the methods of immunohistochemistry assays that we have used in the study were different from those reports (29, 30). In this study, all cell lysis buffers for immunohistochemistry assays contained 2% SDS followed by a 5-fold dilution with 1% Triton X-100 buffer to optimize preservation of SUMOylated CTCF.

Most SUMO targets like CTCF itself are nuclear proteins, including transcription factors, transcription co-regulators, and chromosome remodeling regulators. SUMOylations of these proteins can affect their localization in different cellular compartments as well as their biological activities. SUMO conjugation is dynamically catalyzed by SUMO-specific E1, E2, and E3 ligases and reversed by a family of the SENPs (33–35). Ubc9-deficient cells lacking SUMO-conjugation enzymes showed severe defects in nuclear organization including chromosome condensation and segregation, nuclear envelope dysmorph, and disruption of nucleoli and PML nuclear bodies. All these findings indicate a functional role of SUMOylation in regulat-
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