Cell confluency-induced Stat3 activation regulates NHE3 expression by recruiting Sp1 and Sp3 to the proximal NHE3 promoter region during epithelial dome formation

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Su HW, Wang SW, Ghishan FK, Kiela PR, Tang MJ. Cell confluency-induced Stat3 activation regulates NHE3 expression by recruiting Sp1 and Sp3 to the proximal NHE3 promoter region during epithelial dome formation. *Am J Physiol Cell Physiol* 296: C13–C24, 2009. First published October 22, 2008; doi:10.1152/ajpcell.00263.2008.—Activation of signal transducer and activator of transcription-3 (Stat3) during cell confluence is related to its regulatory roles in cell growth arrest- or survival-related physiological or developmental processes. We previously demonstrated that this signaling event triggers epithelial dome formation by transcriptional augmentation of sodium hydrogen exchanger-3 (NHE3) expression. However, the detailed molecular mechanism remained unclear. By using serial deletions, site-directed mutagenesis, and EMSA analysis, we now demonstrate Stat3 binding to an atypical Stat3-response element in the rat proximal NHE3 promoter, located adjacent to a cluster of Sp cis-elements (SpA/B/C), within −77/−36 nt of the gene. SpB (−58/−55 nt) site was more effective than SpA (−72/−69 nt) site for cooperative binding of Sp1/Sp3. Increasing cell density had no effect on Sp1/Sp3 expression but resulted in their increased binding to the SpA/B/C probe along with Stat3 and concurrently with enhanced nuclear pTyr705-Stat3 level. Immunoprecipitation performed with the nuclear extracts demonstrated physical interaction of Stat3 and Sp1/Sp3 triggered by cell confluence. Stat3 inhibition by overexpression of dominant-negative Stat3-D mutant in MDCK cells or by small interfering RNA-mediated knockdown in Caco-2 cells resulted in inhibition of the cell density-induced NHE3 expression, Sp1/Sp3 binding, and NHE3 promoter activity and in decreased dome formation. Thus, during confluence, ligand-independent Stat3 activation leads to its interaction with Sp1/Sp3, their recruitment to the SpA/B/C cluster in a Stat3 DNA-binding domain-dependent fashion, increased transcription, and expression of NHE3, to coordinate cell density-mediated epithelial dome formation.

Cell confluence-induced Stat3 activation regulates NHE3 expression by recruiting Sp1 and Sp3 to the proximal NHE3 promoter region during epithelial dome formation.

CONFLUENT EPITHELIA IN CULTURE demonstrate sporadic hemicyst dome structures by actuating intracellular sodium influx and alkalization in patches of morphologically polarized cells (11, 13, 16, 34, 37, 50). This process is functionally relevant to in vivo epithelium differentiation (14, 29, 53) and is conceived by coordinated development of intracellular transepithelial transport systems, primarily involving differential distribution and activation of apical sodium transporters [sodium channels and Na+/H+/K+ ATPase, maintaining Na+ and fluid homeostasis (11, 16, 34, 37, 50). The molecular mechanisms underlying dome formation are not clear but are believed to be triggered by cell density-induced Stat3 signaling pathway involved in cell growth arrest- or survival-related physiological functions (42, 43, 46). Although Stat3 signaling in most instances promotes cell proliferation or transformation through cytokine or growth factor receptors (6, 15), its atypical, ligand-independent involvement triggered by cell density has been demonstrated in both cancer and normal epithelial cells (17, 42, 43, 46). The onset of domes with blunted expression of cell proliferating markers and limited influence of cytokines or growth factors provide ample evidence for such a unique biological signaling event (42, 43, 46).

Our recent study has unveiled a novel Stat3/NHE3 (Na+/H+ exchanger-3) signaling pathway induced by cell confluence that is accountable for modulating the initial stage of dome formation in Madin-Darby canine kidney (MDCK) or NMuMG cells (43). Cell confluence-induced nuclear translocation of pTyr705-Stat3 correlates with augmented NHE3 expression and NHE3 promoter activity in transiently transfected cells. Dome formation appears to result as a convergence of cell confluence and Stat3-mediated induction of NHE3. NHE3 expression reaches the highest level during the appearance of dome structures and declines with decreased Stat3 activity soon after dome formation. Most importantly, dome formation can be modulated in stable cell lines expressing constitutively active or dominant-negative Stat3 mutants and is adversely affected by NHE3 small interfering RNA (siRNA) or inhibitors during cell confluence (43).

The involvement of NHE3 in epithelial dome formation is consistent with the known physiological roles of NHEs, which are to maintain intracellular and systemic pH, transcellular absorption of NaCl and NaHCO3, and intracellular volume and body fluid balance (33, 40, 52). Among the nine NHE isoforms (31), NHE3 has been localized to the apical membrane of small intestine, colon, and renal tubular cells (31) and is the key transporter acting to reabsorb sodium and water across the epithelial cells of renal proximal tubule in the mammalian kidney (2, 40). The expression pattern, its physiological functions, and our recent observations (43) strongly suggest that NHE3 is transcriptionally regulated by pTyr705-Stat3 for sub-

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sequent expression and localization to the apical site of morphologically polarized epithelial cells where it can act to coordinate vectorial sodium transport and to promote dome formation.

NHE3 is a highly regulated transporter, both at the protein level (through phosphorylation and trafficking) and at the level of gene transcription (24). Transcriptional modulation of NHE3 expression has been documented in response to a variety of stimuli, including glucocorticoid hormones (20), thyroid hormone (9), or sodium butyrate (23). Both human and rat NHE3 gene promoter, which share a significant homology in their proximal region, are under control of a family of Sp (specificity protein) transcription factors (3, 4, 22, 23). However, it is unknown whether Sp proteins and Stat3 coordinately regulate NHE3 promoter activity in response to cell confluence. While the family of mammalian SP/KLF transcription factors continues to grow, four major Sp transcription factors from the Cys2-His2 zinc finger class have been best characterized. Among them, Sp2 has a consensus-binding site (GT-box) distinct from the other Sp transcription factors, whereas Sp4 expression is restricted primarily to neuronal cells (26, 44). Sp1 and Sp3 are ubiquitously expressed in mammalian cells and are involved in the regulation of a relatively large subset of genes (26), including housekeeping genes and growth factors, and their importance is highlighted by embryonic or perinatal lethality in respective knockout mouse models (5, 32). In intestinal epithelial cells, contribution of Sp1 and Sp3 to regulating NHE3 gene transcription is subject to modulation by sodium butyrate, a differentiating stimulus that results in post-translational modifications (phosphorylation or acetylation) of these two transcription factors (22). In addition, GATA-5 regulates NHE3 transcription via synergistic interaction with Sp1 and Sp3 (23). To date, there has been no evidence addressing how Stat3 and Sp proteins may regulate NHE3 promoter activity.

In the present study, we report that cell density induces NHE3 gene transcription in MDCK and Caco-2 cells, a phenomenon mediated by Stat3 activation (pTy705-Stat3) via specific binding to an atypical Stat3 cis-element in the NHE3 promoter. This Stat3 response element was mapped to a minimal region around position −77/−36 nt, which contains a cluster of three Sp binding sites (SpA/B/C). SpA and SpB sites were further demonstrated to be critical for binding of Sp1/Sp3 recruited by Stat3 and for the induction of NHE3 promoter activity in MDCK cells. Finally, the novel functional interaction of Stat3 with Sp1/Sp3 in response to cell density was demonstrated and verified by functional assays. These results provide new insight into the molecular mechanism of cytokine-independent, Stat3-mediated increase of NHE3 expression leading to epithelial dome formation.

MATERIALS AND METHODS

Cell culture and stable transfection. Madin-Darby canine kidney (MDCK) cells (subclone II 3B5) were maintained in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). Human colon adenocarcinoma (Caco-2) cells were maintained in high-glucose DMEM supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 20% FCS. Both cell lines were maintained at 37°C in a 5% CO2-95% air incubator. The hemagglutinin (HA)-tagged Stat3-D plasmid was kindly provided by Dr. T. Hirano (36). Stat3-D is a dominant-negative form of Stat3 with mutated DNA binding domain. The MDCK cell line stably expressing Stat3-D, designated as D3, was selected as described previously (43). The expression level of transfected Stat3-D was screened by immunoblotting with anti-HA monoclonal antibody.

Preparation of cytoplasmic and nuclear extracts. Cytoplasmic and nuclear extracts were prepared according to the method of Wang et al. (49) with minor modifications. Cells grown to various degrees of density on 6-cm culture dishes were washed three times with phosphate-buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM dibasic sodium phosphate, and 2 mM monobasic potassium phosphate) and scraped off the plate after hypotonic lysis with 400 μl buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, and 10 mM KCl) on ice for 10 min. Cells were pelleted by centrifugation at 7,500 g for 30 s, and the resulting supernatant was collected and designated as cytoplasmic fraction. The pellet was resuspended in 100 μl buffer C (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 420 mM NaCl) and centrifuged at 7,500 g for 2 min. The obtained supernatants were collected as nuclear fraction for further studies. Buffers A and C contained 0.5 mM dithiothreitol, 2 μg/ml leupeptin, 1 mM orthovanadate, 2 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride.

DNA affinity precipitation assay. DNA affinity precipitation assay (DAPA) was performed according to the method of Wang et al. (49) with minor modifications. The binding assay was conducted by mixing 200 μg nuclear extract proteins, 2 μg biotinylated oligonucleotide (wild-type WTABC sequence: 5′-AGCGGGTGCGCGGAGGGGAAAGCCCCCCTGGGCAGGAGGGGCAG-3′ and mutant MutABC sequence: 5′-AGC GGTTGGGAAGGGGCACTG-GCGCGTTAAAGGGGACAG-3′) designed from the −77/−36 nt region of NHE3 promoter (23), and 20 μg streptavidin-agarose beads in Tris-EDTA buffer (pH 7.9). The mixture was then incubated at room temperature for 1 h with constant rotation; the DNA-protein complexes were then precipitated at low speed in a microcentrifuge, and washed three times with cold PBS. The bound proteins were eluted, and separated by SDS-polyacrylamide gel electrophoresis (PAGE), followed by Western blot analysis using Sp1 and Sp3 antibodies (Upstate Biotechnology, Lake Placid, NY) and Stat3 antibody (Cell Signaling Technology, Danvers, MA).

EMSA. EMSA was performed according to the method of Chang et al. (12) with minor modifications. The Sp binding oligonucleotide WTABC: 5′-AGCGGGTGCGCGGAGGGGAAAGCCCCCCTGG-GCGCGTTAAAGGGGACAG-3′ and the Stat3-specific binding oligonucleotide M67: 5′-TAGGGATTTACCGAAATGAGCT-3′ were labeled with [α-32P]dCTP by the filled-in reaction with Klenow DNA polymerase (Promega, Madison, WI). Unlabeled double-stranded oligonucleotides used as competitors and their sequences including Sp, SpM, SpA, SpB, SpC, WTABC, MutABC, MutB, and MutAC were described elsewhere (23). Human inhibitor of apoptosis protein-1 (iAP-1) sequence (5′-GCCGAGTGGCCGCGCGCTGTTAG-3′) was used as negative control (12). Assays were conducted by incubating 1 μg nuclear proteins with 20,000 counts/min of the labeled probe for 20 min. Competitors were added in excess 20 min before the addition of the labeled probe. Additionally, in Sp gel shift assays, Sp1 and Sp3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with the reaction mixture at 4°C for 30 min before the addition of the labeled WTABC probe. DNA-protein complexes were resolved on 6% polyacrylamide gel (acylamide/bisacrylamide 29:1) in 0.5× Tris borate-EDTA buffer) at 10 V/cm for 2.5 h. The gels were then dried and analyzed by autoradiography.

Reverse transcription, semiquantitative PCR, and real-time quantitative PCR. Total cellular RNA was extracted with RNeasy Mini kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 0.2–1 μg total RNA with an oligo(dT) primer and the Moloney murine leukemia virus reverse transcriptase (Promega). Both semi- and real-time quantitative PCR methodologies were used to demonstrate the NHE3 mRNA expression levels between cells cultured at

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low cell density (LCD) and high cell density (HCD). The primer sequences designed for these assay are listed in Table 1. For semiquantitative PCR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The PCR protocol performed to amplify the canine and human NHE3 cDNA was as follows: 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s (35 cycles followed by 72°C for 7 min). The PCR protocol performed to amplify the canine and human GAPDH cDNA was as follows: 94°C for 30 s, 60°C for 45 s, and 72°C for 1 min (28 cycles followed by 72°C for 10 min). Note that the amplification efficiency of each primer pair has been tested and was not saturated at those PCR amplification cycles. For real-time PCR, quantification of the target NHE3 cDNA was conducted in an ABI Prism 7900 apparatus (ABI Prism 7900HT; Applied Biosystems) using SYBR Green I PCR Master Mix (Applied Biosystems) as detection dye. The final reaction volume was 20 μl, which contains 2 μl total (20 μl) cDNA and 18 μl master mix with SYBR Green I and a primer set specific to either canine or homo sapiens NHE3. The real-time PCR protocol was as follows: heat activated at 95°C for 10 min followed by 40 cycles of amplification step at 95°C for 15 s and 60°C for 1 min. GAPDH amplified by the same real-time PCR protocol was used as an internal control. The RNA sample derived from each LCD and HCD preparation before RT-reaction was used as a negative control. All samples were analyzed in triplicate, and the level of expression was characterized by cycle threshold (Ct) values. The canine (MDCK) and mouse (NMuMG) epithelial cells (43).

**RESULTS**

Cell density triggers Stat3 activation and upregulates NHE3 gene transcription. We previously demonstrated that cell density-activated nuclear pTyr705-Stat3 enhances NHE3 expression and gene promoter activity and that induced NHE3 expression is required for epithelial dome formation in confluent canine (MDCK) and mouse (NMuMG) epithelial cells (43). However, it was unclear whether this scenario is applicable to human cells or epithelia other than renal, and whether Stat3

### Table 1. NCBI accession numbers and primer sequences used in the semiquantitative RT-PCR detection of dog and human NHE3

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<th>Target</th>
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<tr>
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GAPDH expression served as an internal control. NHE3, Na+/H+ exchanger isomorph 3; NCBI, National Center for Biotechnology Information.
The involvement of Stat3/NHE3 signaling in cellular response to confluency was compared between MDCK and Caco-2 cells. Western blot analysis of the cell lysates indicated that the protein levels of pTyr705-Stat3 and NHE3, but not of total Stat3, were markedly higher in these two cell lines cultured at HCD as compared with LCD conditions (Fig. 1A). The NHE3 mRNA levels were also elevated in both cell types cultured at HCD as determined by quantitative real-time RT-PCR, which showed a fourfold and a twofold increase in MDCK and Caco-2 cells, respectively (Fig. 1B). These results indicate that phosphorylation of Stat3 and transcriptional regulation of NHE3 gene are promoted by cell density in MDCK as well as in Caco-2 cells. As opposed to MDCK cells, NHE3 mRNA was easily detectable in both LCD and HCD cultures of Caco-2. As canine and human NHE3 promoter sequences were not immediately accessible, cells transfected with a well-characterized rat NHE3 promoter constructs were used to confirm the effects of confluency (LCD vs. HCD) on NHE3 gene transcription. The activity of the rat NHE3 promoter in Caco-2 was somewhat lower than that in MDCK cells, but in both cases it was significantly increased in HCD compared with LCD culture (Fig. 1C). These results signify the positive correlation between cell density and NHE3 gene transcription in both renal and intestinal epithelial cells and support the involvement of Stat3 in the transcriptional control of the NHE3 gene promoter during cell confluency.

The Stat3-responding element of NHE3 promoter is located at −89 nt to −34 nt. To locate the putative Stat3-response element in the NHE3 promoter, a series of 5′-deletion constructs of the rat NHE3 promoter were tested in transiently transfected MDCK and D3 cells, the latter being a selected MDCK clone stably expressing dominant-negative form of Stat3, with mutated DNA-binding domain (Stat3-D; Fig. 2). Reporter gene activity in cells transfected with the constructs containing −89 nt and above of the NHE3 gene promoter was significantly blunted in D3 cells compared with MDCK. Shorter constructs (−34 and −18 nt) that exhibited minimal yet detectable activity were not affected by the expression of Stat3-D. These results indicated that the major regulatory region of NHE3 promoter responding to Stat3 was located between −89 nt and −34 nt, relative to the transcription start site.

In MDCK cells, SpA and SpB sites are critical for Sp1/Sp3 binding within −89/−34 nt of the NHE3 promoter. A cluster of three Sp sites (A/B/C) located within the Stat3-responsive −89/−34 nt region of the NHE3 promoter is critical for its basal activity driven by Sp1 and Sp3 in Caco-2 cells (23). Therefore, we examined whether the same sites were critical for the binding of Sp1 and Sp3 in the renal MDCK cells (Fig. 3). As determined by EMSA, a [32P]-labeled WTABC probe spanning

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**Fig. 1.** Cell density triggers signal transducer and activator of transcription-3 (Stat3) activation and upregulates Na+/H+ exchanger (NHE) isoform 3 (NHE3) gene transcription in MDCK and Caco-2 cells. MDCK and Caco-2 cells were cultured under low cell density (LCD; 1 × 10⁶ cells/10-cm dish) or high cell density (HCD; 6 × 10⁶ cells/10-cm dish) for 1 and 2 days, respectively. A: whole cell lysates were harvested and subjected to Western blot analysis with indicated antibodies to correlate the expression levels of pTyr705-Stat3 and NHE3 proteins with cell density. β-Actin was used as a loading control. Molecular mass (in kDa) markers are indicated. B: RT-PCR and real-time RT-PCR. NHE3 and GAPDH mRNA levels were assessed by RT-PCR using sequence-specific primers for canine (MDCK) or human (Caco-2) transcripts. The level of NHE3 mRNA was further determined by real-time RT-PCR and presented as fold changes of NHE3 mRNA expression between cells cultured at LCD and HCD. Each bar represents the mean ± SE from three independent experiments in triplicate. *P < 0.05; **P < 0.01; Student’s t-test (n = 3). C: NHE3 promoter activity was assessed by dual luciferase assay. NHE3 promoter construct (−1360/+58 nt; pNHE3) and pCMV-FL plasmid were cotransfected into MDCK or Caco-2 cells. The activity of Renilla luciferase, normalized to that of the internal firefly luciferase control, was presented as fold-induction over the reporter activity in cells transfected with promoterless phRG-B vector. Each bar represents the mean ± SE from six independent experiments, each performed in triplicate. *P < 0.05; **P < 0.01; Student’s t-test (n = 6).
the conserved SpA/B/C sites within the −77/−36 nt sequence of the rat NHE3 promoter formed specific DNA-protein complexes with MDCK nuclear extracts. These complexes contained Sp1 and Sp3 as determined by supershift analysis with polyclonal antibodies specific to Sp1 and/or Sp3 (Fig. 3A). There were no supershifted complexes found in the mock or IgG controls, indicating that both Sp1 and Sp3 bound specifically to the NHE3 promoter. On the basis of this supershift analysis, Sp1 appeared to be the predominant protein binding to WTABC probe.

To evaluate which of the three Sp sites (SpA, SpB, or SpC) was involved in the Sp1/Sp3 binding, an EMSA competition assay was conducted by including an excess of cold competitor probes (Fig. 3B, left). Formation of Sp complexes with the WTABC probe was entirely eliminated by the unlabelled generic Sp probe with consensus GC-rich motif (Sp) and by unlabelled WTABC but not by SpM or nonspecific hIAP-1 probe. Furthermore, the cold SpB probe and MutAC probe containing preserved site B only almost completely eliminated Sp1/3 binding to WTABC probe, thus suggesting a predominant or prerequisite interaction of Sp1/3 to the SpB site in the NHE3 promoter. Unlabeled SpA probe was only partially effective as a competitor, whereas very little or no competition effect was observed for the cold SpC probe. Site B mutant probe (MutB) was much less effective as a competitor in comparison to the MutAC probe with double mutation at sites A and C (Fig. 3B). Therefore, SpA site likely plays a supportive role for the interaction with Sp1/Sp3, and together with the predominant SpB site, it accounts for all of the Sp1/3 DNA interaction within −77/−36 nt of NHE3 promoter in MDCK cells.

To functionally examine the contribution of SpA and SpB sites on the induction of NHE3 promoter activity in MDCK cells, reporter assay was conducted with cells transfected with NHE3 promoter constructs harboring individual or composite Sp site-specific mutations within the SpA/B/C cluster (Fig. 3C). Consistent with the EMSA results, NHE3 promoter activity was reduced more significantly in cells transfected with SpB site-specific mutant than with SpA site-specific mutant, while combined mutation of sites A and B decreased NHE3 promoter activity to the residual levels. Collectively, these data support the predominant role of SpB site and the subsidiary role of SpA site in the regulation of NHE3 promoter activity by Sp1 and Sp3 transcription factors.

Sp1/Sp3 and Stat3 coordinately bind to the −77/−36 nt sequence of the rat NHE3 promoter. Since we determined that Sp1 and Sp3 are critical in NHE3 gene transcription in both intestinal and renal epithelium, we aimed to verify whether the expression or nuclear localization of the two transcription factors is affected by cell density and whether they regulate NHE3 promoter activity coordinately with Stat3 during confluence. For this purpose, the cytoplasmic and nuclear distribution of Sp1 and Sp3 in MDCK cells cultured at LCD or HCD was first investigated by immunoblotting. Two species of Sp1 proteins, namely p95 and p105, were detected. The appearance of p105 Sp1 might be a result of posttranslational modifications. Three isoforms of Sp3 were detected at Mr 110, 60 and 58 kDa. The two short isoforms of Sp3 are products of differential translational initiation lacking one transactivation domain (19a, 19b). All species of Sp1 and Sp3 were detectable only in the nucleus, and their expression levels were not changed in cells cultured at LCD or HCD conditions (Fig. 4A), thus suggesting that neither Sp1 or Sp3 protein expression nor their cellular localization was altered in HCD cultures.

We then examined whether DNA binding affinity of Sp1 or Sp3 to NHE3 promoter was affected by cell density. DAPA assay with wild-type or mutant SpA/B/C probe was applied as an approach more quantitative than EMSA. WTABC probe pulled down detectable quantities of Sp1 and Sp3 only from the nuclear extracts obtained from HCD culture of MDCK cells. No detectable Sp1 or Sp3 binding to biotinylated MutABC probe was observed (Fig. 4B). Intriguingly, Stat3 was also pulled down by the WTABC probe, and its DNA binding affinity was not affected by mutation of all three Sp cis-elements in the MutABC probe. Stat3 binding to either probe was significantly enhanced with nuclear extract from HCD cultures, consistent with previously described activation and nuclear translocation of Stat3 with increasing confluency and dome formation process.

Fig. 2. 5′-Deletion analysis of rat NHE3 promoter activity in MDCK cells: effects of dominant-negative Stat3 mutant of the DNA binding domain (Stat3-D). Reporter plasmids containing different lengths of the rat NHE3 promoter were transfected into MDCK or D3 clone to locate Stat3-response element(s). MDCK cells and an MDCK clone (D3) stably expressing Stat3-D were cultured for 1 day. A series of 5′-NHE3 promoter deletion constructs (from −18/+58 to −1360/+58 nt, relative to the transcription start site) were cotransfected with a pcMV-FL, and 24 h later, dual luciferase (Luc) assay was performed. The activity of Renilla luciferase, normalized to that of the internal firefly luciferase control, was presented as fold-induction over the reporter activity in cells transfected with promoterless phRG-B vector. Each bar represents the mean ± SE from six independent experiments, each performed in triplicate. ***P < 0.001; Student’s t-test (n = 6).
This observation strongly indicated that Stat3 not only binds to the proximal NHE3 promoter, but also that it does so independently of the three Sp binding sites and that it does not interfere with Sp1/3 binding. It also suggested a potential for a cooperative control of the NHE3 gene transcription by Sp1/Sp3 and Stat3 during confluency and in the process of dome formation. However, because both probes did not contain a typical Stat3 response element (GAS or ISRE), it was unclear whether Stat3 alone can physically bind to this region of the NHE3 promoter. We addressed this question by EMSA analysis with recombinant Stat3 (Fig. 4, C and D). Indeed, recombinant Stat3 protein was found to form a dose-dependent complex with either radio-labeled M67 [Stat3 high-affinity binding probe (6)] or WTABC (Fig. 4C). Although Stat3 bound less effectively to WTABC than to the high-affinity M67 probe, the binding was not only easily detectable, but also specific since it could be effectively competed by an excess of unlabeled M67 probe (Fig. 4, C and D). This further signifies the existence and specificity of coordinate binding of Sp1/Sp3 and Stat3 within the −77/−36 nt sequence of the proximal rat NHE3 promoter.

Cell confluency promotes physical interaction between Stat3 and Sp1/Sp3. To further verify whether Stat3 physically interacts with Sp1 and or Sp3 during cell confluency, but before their DNA binding, coimmunoprecipitation (co-IP) was performed with Sp1, Sp3, and Stat3-specific antibodies and chromatin-depleted nuclear extract from MDCK cells. As depicted in Fig. 5, two Sp1 and three Sp3 isoforms were coimmunoprecipitated with Stat3, and their binding appeared to be significantly enhanced when Stat3 was precipitated from the HCD sample (Fig. 5A). In support of this observation, antibodies against Sp1 or Sp3 also coimmunoprecipitated detectable amounts of Stat3, increasingly from nuclear extracts obtained from HCD cultures (Fig. 5, B and C). There was no detectable interaction between Sp1 and Sp3 in the nuclear extracts. Collectively, these results indicate that the increasing cell
density most likely promotes physical interaction of Stat3 with Sp1 or Sp3, a phenomenon that may be independent of the chromatin context. It remains to be determined whether Stat3 has preference for interaction with either Sp1 or Sp3. Although co-IP data suggest similar affinities, more precise measurement by competition may be required to fully address this question.

**Stat3 DNA binding is required for Sp1/Sp3 recruitment to the NHE3 promoter.** Functional promoter analysis in cells transfected with dominant-negative form of Stat3 with mutated DNA binding site (Fig. 2) as well as the demonstrated physical interaction of Stat3 with Sp1 and Sp3 in HCD cultures were highly suggestive of a prerequisite role of Stat3 as a transcription factor independently binding to the NHE3 promoter and recruiting Sp1 and Sp3 to augment NHE3 gene transcription during cell confluency. To test this hypothesis, we again used the DAPA assay using WTABC probe from NHE3 gene promoter and nuclear extract obtained from wild-type MDCK and D3 cells, the latter stably transfected with HA-tagged Stat3-D mutant with defective DNA binding domain. Expression of Stat3-D mutant and total Stat3 in wild-type and D3 clone of MDCK cells was first verified by Western blotting with anti-HA or anti-Stat3 antibodies (Fig. 6A). Nuclear proteins derived from MDCK and D3 cells were then used in DAPA assay to monitor recruitment of Sp1 and Sp3 to the NHE3 promoter in relation to Stat3 binding (Fig. 6B). Comparable amounts of Sp1 and Sp3 were detected in the nucleus of confluent MDCK and D3 cells, indicating that Stat3 or Stat3-D levels did not influence the expression or nuclear localization of Sp1 or Sp3. Both Sp1 and Sp3 could be specifically pulled down from control MDCK cells by biotin-labeled WTABC probe but not MutABC probe or beads alone. More importantly, the amounts of Sp1 and Sp3 pulled down by biotin-labeled WTABC alone or together with 50× molar excess of unlabeled M67 competitor. SC, specific Stat3/ DNA complex.

**Fig. 4.** HCD triggers association of Sp1 and Sp3 with the SpA/B/C cluster without concomitant changes in their nuclear expression. Cytoplasmic and nuclear proteins from MDCK cells cultured under LCD or HCD for 1 day were subjected to Western blot analysis (A) and DNA affinity precipitation assay (DAPA) (B) to investigate the mode of regulation of Sp1/Sp3 with Stat3 on NHE3 promoter in response to cell density. The corresponding molecular mass (in kDa) of the markers is indicated. A: immunoblotting analysis was performed with indicated antibodies. Tubulin and lamin A were used as loading controls for cytoplasmic and nuclear protein fractions, respectively. B: interacting nuclear proteins were pulled down by biotin-labeled probes (WTABC and MutABC) and were immunoblotted with antibodies against Sp1, Sp3, Stat3, and lamin A. Internal control is shown as 10% input from total nuclear extracts. Since WTABC probe pulled down Stat3 despite the lack of a typical Stat3 binding element, an EMSA with recombinant Stat3 was performed. C: purified Stat3 was incubated with [32P]-labeled probes (M67, consensus Stat3-binding site; WTABC, −77/−36 nt region of NHE3 promoter). D: recombinant Stat3 (1 μg) was incubated with [32P]-labeled WTABC alone or together with 50x molar excess of unlabeled M67 competitor. SC, specific Stat3/DNA complex.
to reduce Sp1/Sp3 binding to NHE3 promoter strongly support the hypothesis that DNA binding domain of Stat3 is required for tethering and recruitment of Sp1 and Sp3 to the proximal NHE3 promoter.

Stat3 knockdown suppresses NHE3 expression, dome formation, Sp1/Sp3 recruitment, and NHE3 promoter activity in Caco-2 cells. To provide further evidence for the functional relevance of the presented interactions, we knocked down expression of Stat3 with specific siRNA in the dome-forming human colonic adenocarcinoma Caco-2 cells (Fig. 7). Compared with cells transfected with the scrambled siRNA control, the amounts of Stat3 protein were depressed by Stat3 siRNA in a dose-dependent fashion (Fig. 7A). Although the suppression of Stat3 expression was incomplete, it correlated well with the expression of NHE3 protein (Fig. 7A) and with the ensuing inhibition of dome formation, which were reduced by approximately 30% and 50% by 50 and 100 nmol transfected siRNA, respectively (Fig. 7B). These observations in Caco-2 cells are in accordance with our previously published results with MDCK cells (43).

Western blot analysis indicated no influence of Stat3 siRNA on the nuclear expression of Sp1 or Sp3 proteins (Fig. 7C). However, when DAPA assay was performed with biotinylated WTABC probe and nuclear extract isolated from Caco-2 cells transfected with scrambled or Stat3 siRNA, significantly lower amounts of Sp1 and Sp3 were pulled down in Stat3 knockdown samples (Fig. 7D). Furthermore, when NHE3 promoter constructs (wild-type −89/+58 nt or MutA/B/C) were transfected into Stat3 or scrambled siRNA-transfected Caco-2 cells, partial knockdown of Stat3 reduced NHE3 promoter activity by half (Fig. 7E). The activity of the rat NHE3 promoter −89/+58 MutA/B/C construct was predictably significantly lower than that of wild-type promoter construct and was not further decreased by Stat3 knockdown (Fig. 7E). These results provide strong and conclusive evidence for the involvement of Stat3 in the recruitment of Sp1 and Sp3 to the proximal NHE3 gene promoter and for the functional cooperation of these three transcription factors in cell density-induced NHE3 gene expression and epithelial dome formation.

DISCUSSION

We have previously demonstrated that Stat3 activated by cell confluency triggers epithelial dome formation via augmentation of NHE3 expression and activity in confluent canine (MDCK) and mouse (NMuMG) cells (43). This activation process is highly dependent on cell density, and the molecular control modulating dome formation is dominated by the activity of NHE3-mediated transepithelial Na⁺ transport. In the present study, the same mechanism was confirmed in human intestinal cell line Caco-2, in which we describe a similar cell...
density-activated process triggering nuclear translocation of Stat3, induction of NHE3 expression, and dome formation. Furthermore, we provide a mechanistic explanation of this phenomenon, whereby a nonclassical Stat3 response element within the proximal NHE3 promoter binds Stat3, which, in turn, by means of physical interactions, recruits Sp1 and Sp3 to the SpA/B/C cluster to induce NHE3 gene transcription. The functional relevance of these interactions was confirmed through interference with Stat3 activity in MDCK cells expressing dominant-negative mutant of Stat3 (Stat3-D) and in siRNA-mediated knockdown of Stat3 in Caco-2 cells. Our findings strongly suggest that this represents a universal mechanism underlying the dome formation process in NHE3-expressing epithelia, since the molecular interplay between Stat3 and NHE3 promoter as well as its morphological consequences were comparable in all the tested dome forming-derived human, canine, and mouse cells.

The rat NHE3 promoter was initially cloned in 1996 (8, 19), but it was not thoroughly characterized until 2003 (23), when a functional model of basal transcriptional regulation through GATA-5 and Sp1/Sp3 was proposed to be involved in a gradient of intestinal NHE3 gene expression along the crypt-villus axis (23). As eukaryotic gene regulation requires coordinated signaling and binding of multiple transcription factors to promoter-enhancer regions, an array of factors in responding to extra- and intracellular ligands have also been implicated in transcriptional regulation of NHE3 gene expression (1, 8, 9, 20, 21). However, there has been no information regarding the involvement of Stat3 in the regulation of NHE3 gene transcription. Our current results point to a region within the proximal NHE3 promoter (−1/170 to +68 nt) containing an atypical Stat3-binding site. Although this Stat3-responsive element was not found by prediction analysis by TFSEARCH software (data not shown), it bound recombinant Stat3 specifically and in a dose-dependent manner, suggesting an uncommon but functional cis-element for the regulatory control of NHE3 promoter by Stat3. On the other hand, the consensus Sp sites residing within the proximity of the Stat3-binding site have been previously mapped in both human and rat NHE3 promoter (4, 23) and shown to interact with both Sp1 and Sp3 (3, 4, 22, 23). Functional interactions of Stat3 with Sp1 or Sp3 were previously demonstrated in transcriptional regulation of genes con-

Fig. 7. Evaluation of the functional role of Stat3 in the dome formation, Sp1/Sp3-NHE3 promoter interaction, and NHE3 gene promoter activity by Stat3 small interfering RNA (siRNA) knockdown in Caco-2 cells. Stat3 siRNA- and scrambled siRNA-transfected Caco-2 cells were cultured either for 4 days (A and B) or for 1 day (C–E). A: expression levels of Stat3 and NHE3 proteins were characterized by immunoblotting analysis with indicated antibodies. β-Actin was used as a loading control. B: dome formation was quantified by counting domes from at least 20 nonoverlapping microscopic fields (×100) and averaged from three independent experiments. C: effects of siRNA transfection on nuclear expression of Stat3, Sp1, and Sp3 proteins were characterized by immunoblotting analysis with indicated antibodies. Lamin A was used as a loading control. D: nuclear proteins from siRNA-transfected cells were precipitated by biotinylated WTABC probe (DAPA assay) and characterized by immunoblotting analysis with indicated antibodies. E: wild-type (WTABC) and mutant (MutABC) NHE3 (−89/+58 nt) promoter constructs were cotransfected with pCMV-FL plasmid. The activity of Renilla luciferase, normalized to that of the internal firefly luciferase control, was presented as fold-induction over the reporter activity in cells transfected with promoterless pHG-B vector. Each bar represents the mean ± SE from six independent experiments in triplicate. *P < 0.05; ***P < 0.001; Student’s t-test (n = 6). Molecular mass (in kDa) markers are indicated.

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taining both Stat3-binding site (or acute phase response element) and Sp cis-elements, such as IL-6-mediated induction of cellular enhancer-binding protein-8 (10), as well as in regulation of genes lacking the canonical Stat3-binding sites. Examples of the latter are provided by leptin-induced transcription of metalloproteinase 1 (27), IL-6-mediated induction of VEGF gene (28), or the recently described IL-6-induced transcription of calcium-sensing receptor CaSR from the P2 promoter (7).

To our knowledge, this is the first report implicating ligand-independent Stat3 involvement in the transcriptional regulation of a gene without the canonical Stat3 cis-element.

Sp1 and Sp3 are structurally similar and are known to interact with other proteins that help with their recruitment or stabilization of their DNA binding to activate or repress the expression of target genes (e.g., 25, 39, 45, 48, and 54). Therefore, cooperation of Sp1/Sp3 with other nuclear proteins, such as Stat3 to enhance NHE3 promoter activity in responding to certain cellular signals was not inconceivable, and the presented data provide strong support for such molecular interplay. We provide several lines of evidence for such interaction: 1) the cooperative binding of Stat3 and Sp1/Sp3 to the −771−36 nt region of NHE3 promoter, 2) the requirement for the intact DNA-binding domain of Stat3 for Sp1/3 recruitment, and 3) the physical interaction of Stat3 with both Sp1 and Sp3 without the presence of DNA. Most importantly, however, we demonstrate that this Stat3/Sp1/Sp3 interaction was enhanced by cell density, signifying the coordinated effects of these three transcription factors in induction of NHE3 gene expression following cell confluence leading to epithelial dome formation.

Despite the experimental evidence for recruitment of Sp1 and Sp3 by Stat3, and their cooperative activation of NHE3 promoter, the lack of physical interaction between Sp1 and Sp3 observed in our immunoprecipitation studies was somewhat unexpected. Several reports suggest that Sp1 and Sp3 heterodimerize to regulate the expression of target genes, which is fine-tuned by the ratio of these two proteins (30, 38, 51). It remains unclear whether this is a universal phenomenon and our experimental approach was insufficient to confirm it, or whether it is dependent on the context of the gene promoter or physiological status of the cell.

The prerequisite role of Stat3 to recruit Sp1/Sp3 for cooperative control of NHE3 promoter during cell confluence was further evidenced by experiments in which Stat3 was inhibited by overexpression of dominant-negative Stat3-D in MDCK cells or by siRNA-mediated knockdown in Caco-2 cells. Although Stat3 function was not completely abolished in either system, the effects were specific, dose dependent, and effectively reduced the ensuing molecular and morphological consequences, i.e., Sp1/Sp3 recruitment to NHE3 promoter, NHE3 expression, and dome formation. It is of importance that, in siRNA-transfected Caco-2 cells, aside from smaller and fewer domes in Stat3 knockdown cells, transfection and decreased Stat3 expression did not cause an aberrant cell growth, growth arrest, or cell death. These result are also in agreement with our previous finding in MDCK cells expressing the constitutively active form of Stat3, which displayed enhanced dome size and their number (43).

Although dome formation is believed to mimic the terminal differentiation of renal epithelium during epithelial cell remodeling in vivo (14, 29, 53), this ligand-independent Stat3/NHE3 signaling may be actively involved during cell growth arrest or survival related to physiological or developmental processes in epithelial cells in general (42, 46). Despite the existing evidence for enhanced NHE3 expression during epithelial cell differentiation as well as during postnatal renal or intestinal development (18, 35, 41), the involvement of Stat3 and its relationship with Sp1/Sp3 or other transcription factors, such as GATA, AP-2, or NF-1 (19), in these processes has yet to be explored. Interestingly, sodium butyrate, a well-known prodifferentiating factor for intestinal epithelial cells in culture, also induces NHE3 expression via modulating Sp1 and Sp3 affinities to the SpA/B/C cluster in the proximal NHE3 gene promoter (21, 22). Epigenetic mechanisms, such as chromatin remodeling, a function of histone deacetylase inhibition by butyrate, may also be a factor in the cell density- and differentiation-induced NHE3 gene transcription to increase accessibility of the endogenous gene to Sp transcription factors. It is thus plausible that the Stat3-dependent induction of NHE3 expression during cell confluence is also a subject of modulation by chromatin structure in terminally differentiating epithelial cells. Moreover, posttranslational modifications of Sp proteins may affect their DNA binding affinity for differential regulation of NHE3 promoter activity (3, 4, 22, 23). It has been reported that phosphorylated Stat3 can bind only with phosphorylated Sp1 for the induction of tissue inhibitor of metalloproteinase-1 gene (27). Therefore, many questions remain about the role of Stat3 in modulating NHE3 gene transcription in different epithelia or at cellular differentiation stages induced by cell-cell contact.

In summary, we extend our previous observations on the contribution of NHE3 to Stat3-mediated dome formation in canine and mouse renal epithelial cells to human intestinal epithelium, and we provide a novel mechanism for a ligand-independent activation of Stat3 leading to its physical interaction with Sp1 and Sp3 and their recruitment to the proximal NHE3 promoter. This series of events is critical for induction of NHE3 expression and activity in differentiating epithelium, increased transepithelial Na\(^+\) and water transport, and dome formation.

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