Regulation of the CIC-2 Lung Epithelial Chloride Channel by Glycosylation of SP1

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Chloride channel-2 (CIC-2) is a pH- and voltage-activated chloride channel that is highly expressed in mammalian fetal airway epithelia during the period of maximal fluid secretion. A high level of luminal CIC-2 protein expression is maintained by the SP1 transcription factor until SP1 and CIC-2 decline rapidly at birth. Using fetal (preII-19) and adult (L2) rat lung Type 2 cell lines, we demonstrate that the active higher-molecular-weight 105-kD isoform of SP1 is phosphorylated and glycosylated. Exposure of either cell line to high-dose glutamine is sufficient to induce glycosylation of SP1 and to induce and maintain CIC-2. Exposure to tunicamycin to inhibit SP1 glycosylation reduces CIC-2 expression. We also demonstrate that *in vivo* CIC-2 expression is similarly regulated. SP1 from 6-wk-old murine lung (high CIC-2 expression) is hyperphosphorylated and hyperglycosylated compared with SP1 from 16-wk-old lung (low CIC-2 expression). Our results support the hypothesis that glycosylation of SP1 produces the 105-kD isoform of SP1 and is involved in regulating CIC-2 gene expression.

Keywords: chloride channel; transcription factor; cystic fibrosis; mouse; lung development

Chloride-driven fluid secretion by developing fetal airways is required for normal lung differentiation (1–3). Mammalian fetal airways express three chloride conductances: cAMP activated, calcium activated, and pH activated (4, 5). Loss of any single ion channel species is well tolerated because normal lung development occurs when cystic fibrosis transmembrane conductance regulator (CFTR) or chloride channel-2 (CIC-2) is eliminated (6–8). CFTR and CIC-2 are most highly expressed in the apical region and luminal membrane of airways during fetal gestation and are rapidly downregulated at birth (5, 9). We are interested in defining the regulatory elements that modulate CIC-2 expression as a potential therapeutic target in cystic fibrosis (10, 11).

The CIC-2 promoter has SP1 and SP3 domains that are important for gene regulation. We confirmed binding of SP1 and SP3 to GC box-containing sequences in the CIC-2 promoter region by gel super shift assay (9, 12). We have previously reported that modulation of SP1 and SP3 transcription factors in lung epithelial cells regulates CIC-2 chloride channel expression. We observed two isoforms of SP1, 95 kD and 105 kD, in fetal and adult rat type 2 cell lines (12). SP1, along with several other RNA polymerase II transcription factors, is known to be O-GlcNAc modified at multiple sites (13). The O-GlcNAc modification of SP1, in addition to regulating the expression of housekeeping genes, hormones, and metabolic enzymes such as acetyl-CoA carboxylase (14), leptin (15, 16), fatty acid synthase,

and ATP citrate-lyase (16, 17), enhances transcriptional activity of SP1. Functional effects of O-GlcNAc on SP1 include protection from protein degradation (18) and disruption of interaction with TATA binding-associated factor 110 and holoSP1 (19). Thus, although there is apparent consensus that O-GlcNAcylation modulates SP1 transcriptional activity, the mechanisms of regulation are controversial. We hypothesize the O-GlcNAcylation of SP1 is involved in regulating the CIC-2 gene expression.

Using a fetal rat lung Type 2 cell line (preII-19), which expresses high levels of CIC-2, and an adult rat lung Type 2 cell line (L2) that expresses low levels of CIC-2, we demonstrate that it is the amount and glycosylation state of SP1 that controls CIC-2 expression. We use tunicamycin to reduce the glycosylation level of SP-1, glutamine to induce glycosylation of SP1, and 2-deoxyglucose to hyperglycosylate and inactivate SP1. The glycosylation of SP1 transcription factor induced CIC-2 expression in preII-19 and L2 cell lines. SP1 and CIC-2 derived from C57BL6 murine lungs from 6-wk and 16-wk animals demonstrate a similar relationship. In mice, glycosylation of SP1 is associated with higher levels of CIC-2. These results support the hypothesis that induction and glycosylation of SP1 is required to stimulate CIC-2 gene expression.

MATERIALS AND METHODS

Materials

The L2 cell line was originally derived from type II-like alveolar pneumocytes from normal adult rat lung (ATCC, CCL-149). The pre-type II cell line was derived from 19-d-gestation fetal rats (kindly provided by Dr. G.W. Hunninghake). The cell lines were maintained as previously described (9) using tissue culture reagents from GIBCO (Invitrogen, Carlsbad, CA). The pCMV-SP1 plasmid (kind gift from Dr. Robert Tjian) and pCMV (Promega, Madison, WI) control vector were transfected in L2 and preII-19 cells using Lipofectamine 2000 (Invitrogen). The rabbit anti-rat CIC-2 polyclonal antibody (S787) has been described previously (12). The rabbit anti-CIC-2 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) detects human and mouse CIC-2. The monoclonal anti-phosphoserine (Biosource, Camarillo, CA), anti-phosphothreonine (Sigma, St. Louis, MO), and anti-O-GlcNAc (Affinity Bioreagents, Golden, CO) were used to detect post-translational modification of SP1. The rabbit anti-SP1 polyclonal antibody was from Santa Cruz Biotechnology Inc. The anti-mouse, -rabbit, or -goat HRP antibodies were from Amersham (Piscataway, NJ). Glutamine, 2-deoxyglucose, and tunicamycin were from Sigma.

Cell Culture and Transfection

The L2 and preII-19 cell lines were maintained in Ham's F12K and Dulbecco's modified Eagle's medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 10% FBS (Invitrogen), respectively. The cells were transfected for 48 h with pCMV control vector or pCMV-SP1 construct using Lipofectamine 2000 (Invitrogen) as described by the manufacturer.

Animals

The C57BL6 mice were housed in a pathogen-free facility and treated according to policies approved by the Animal Care and Use Committee, Johns Hopkins University.

(Received in original form December 2, 2005 and in final form January 20, 2006)

This work was supported by grant RO1 HL 59410.

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Am J Respir Cell Mol Biol Vol 34, pp 754–759, 2006

Originally Published in Press as DOI: 10.1165/rcmb.2005-04420C on February 2, 2006

Internet address: www.atsjournals.org

Immunoprecipitation and Immunoblotting

Cells were lysed directly on plates using M-PER (Pierce Biotechnology Inc., Rockford, IL) protein lysis buffer containing protease inhibitor cocktail (Roche Diagnostic Corporation, Indianapolis, IN) after three washes with ice-cold PBS. The murine lungs were collected in T-PER tissue extraction reagent (Pierce Biotechnology Inc.; 20 ml/g of tissue) containing 1× Halt protease inhibitor mixture (Pierce Biotechnology Inc.), and samples were homogenized on ice. Tissue debris was removed by centrifugation for 5 min at 10,000 rpm. The protein concentration was determined by the BCA protein assay (Pierce Biotechnology Inc.). For immunoprecipitation, 500 µg/ml total protein extracts were incubated with 50 µl of protein A/G plus agarose beads (Santa Cruz Biotechnology Inc.) for 3 h at 4°C. After preclearing, 5 µg of respective primary antibody or preimmune sera (negative control) was added to each tube. After 1 h, protein A/G agarose beads (50 µl) were added to each tube, and tubes were incubated overnight at 4°C. Beads were washed once with lysis buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.5% Triton X-100, and 10 µM PMSF) followed by two washes with PBS. The beads were suspended in Laemmli's sample buffer (30 µl) containing β-mercaptoethanol, vortexed for 1 min, resolved by 4–10% SDS-PAGE, and transferred to a 0.4-µm pore size nitrocellulose membrane. Proteins were detected using respective primary and secondary antibodies.

Quantitative RT-PCR

The L2 cells were treated with 200 or 500 mM of glutamine and 1 µg/ml tunicamycin. After overnight incubation, total RNA was isolated using TRIzol reagent (Invitrogen). Real-time RT-PCR was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA (100 ng) was mixed with a Superscript Platinum One-Step qRT-PCR kit (Invitrogen) and specific primers according to the manufacturer's instructions. The FAM-labeled primers were selected based on the reference sequences from Genbank for CIC-2 (X64139), SP1 (D12768), and GAPDH (NM_008084, certified LUX primer set from Invitrogen). The primers sequences were as follows: CIC-2 (forward primer, 5'-CGGTTGTCTTGGCAGTGTGGAACCG; reverse primer, 5'-GTGCGAGTCGGAACCGAGTTT), SP1 (forward primer, 5'-CGGTTTCATCATTCGGACACCAACCG; reverse primer, 5'-CAAGGTGATTGTCTGGGCTTGT). The real-time RT-PCR reaction was performed as follows: 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 1 min with initial 48°C for 30 min for reverse transcription. The data were collected and analyzed with Sequence Detection Software 2.0 (Applied Biosystems). The threshold cycle difference $C_t = (C_t \text{ of CIC-2/SP1} - C_t \text{ of GAPDH}) - (C_t \text{ of CIC-2/SP1 no template control} - C_t \text{ of GAPDH no template control})$. The threshold cycle was used to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. The relative expression was calculated according to manufacturer's direction using the following formula: $2^{-\text{difference } C_t}$. The ΔC_t validation experiments showed similar amplification efficiency for all templates used (difference between linear slopes for all templates < 0.1). Three independent experiments were performed, and the average ($\pm$ SD) results are presented in graph (Figure 6).

Statistical Analyses

Samples were compared by Student's *t* test using two-tailed distribution between two samples with equal variance. A *P* value ≤ 0.05 was considered to have statistical significance.

RESULTS

Post-translational Modification of SP1 in preII-19 Cells

We have previously reported that there are two isoforms of endogenous SP1, 95 kD and 105 kD, in preII-19 epithelial cell line (12). To elucidate the post-translational modifications of SP1 resulting in two different isoforms, we immunoprecipitated SP1 from preII-19 cells and immunoblotted for anti-O-GlcNAc

and anti-p-threonine (Figure 1, upper panel). The 105 kD isoform of SP1 was preferentially phosphorylated and glycosylated (Figure 1). We hypothesized that the 105 kD SP1 isoform is the one that regulates CIC-2 gene expression.

Regulation of CIC-2 Protein Expression by Glutamine and 2-deoxy-α-Glucose

To determine the importance of glycosylation of SP1 in activation of CIC-2 gene expression, preII-19 and L2 cell lines were exposed to 200 mM glutamine or 1 M 2-Deoxy-α-glucose. The glutamine is known to produce glycosylation of SP1, and 2-Deoxy-α-glucose is predicted to inhibit SP1 activity by hyperglycosylation of the transcription factor (20). CIC-2 protein expression was induced by glutamine and inhibited by 2-Deoxy-α-glucose (Figure 2), indicating SP1 glycosylation induces CIC-2 gene expression. The CIC-2 induction was best appreciated in L2 cells which express low levels of CIC-2, whereas CIC-2 inhibition was easily visualized in preII-19, which express abundant CIC-2 (12).

Tunicamycin Inhibits SP1 Glycosylation and CIC-2 Protein Expression

To confirm that glycosylation of SP1 is required for CIC-2 induction, we used tunicamycin to block glycosylation. SP1 was immunoprecipitated from tunicamycin treated preII-19 cells and immunoblotted with anti-O-GlcNAc antibody. We found a time-dependent decrease in SP1 glycosylation by tunicamycin treatment (Figure 3, middle panel), whereas β-actin remained the same or increased slightly (Figure 3, lower panel). Moreover, CIC-2 expression was decreased with inhibition of glycosylation by tunicamycin (Figure 3, upper panel). Our results demonstrate that SP1 glycosylation is required for CIC-2 gene expression.

Figure 1. Post-translational modification of SP1. SP1 was immunoprecipitated from preII-19 cells (500 µg/ml) and probed by immunoblotting with anti-O-GlcNAc and anti-p-threonine (upper panel) and SP1 (lower panel). The 105-kD isoform of SP1 is the predominant form and is phosphorylated and glycosylated.

Figure 2. Regulation of CIC-2 protein expression by glutamine and 2-deoxy- α -glucose. prell-19 and L2 cell lines were exposed to 200 mM glutamine or 1 M 2-Deoxy- α -glucose. Total protein extract (50 μ g) from each cell line were loaded in each lane and immunoblotted with anti-CIC-2 antisera as described in MATERIALS AND METHODS. CIC-2 protein expression was induced by glutamine and inhibited by 2-Deoxy- α -glucose. Densitometric quantification of immunoblots is shown in the lower panel. Data ($n = 3 \pm$ SD) are shown as the percentage of expression level in each sample relative to L2 control (* $P < 0.05$).

CIC-2 Overexpression in SP1 Transfected Cells Is O-GlcNAcylation Dependent

To further confirm that CIC-2 overexpression is O-GlcNAcylation dependent, we immunoprecipitated SP1 from preII-19 and L2 cells transfected with pCMV (mock vector) or pCMV-SP1 and immunoblotted with anti-O-GlcNAc antibody. We observed elevated CIC-2 protein levels in pCMV-SP1-transfected cells as compared with pCMV mock controls. Moreover, preII-19 cells have elevated basal CIC-2 levels as compared with L2 cells (Figure 4, upper panel). We also observed that SP1 was hyperglycosylated in preII-19 cells as compared with L2 cells (Figure 4, middle panel). Our results indicate that induction of CIC-2 expression was dependent on the glycosylation state of SP1.

The *In Vivo* Differences in the Glycosylation State of SP1

To identify the functionally relevant form of SP1 *in vivo*, lung extracts of 6- and 16-wk-old C57BL6 mice were immunoblotted with anti-CIC-2, anti-SP1, and anti- β -actin antibody. The 6-wk-

old murine lungs have higher SP1 protein levels as compared with 16-wk-old mice, and the CIC-2 protein expression in 6-wk-old murine lungs are higher (Figure 5, upper panel). Moreover, the 6-wk-old mice had higher levels of 105 kD glycosylated form of SP1 as compared with 16-wk-old mice (Figure 5, lower panel). The *in vivo* differences in the glycosylation state of SP1 reflected the CIC-2 expression levels and confirmed our earlier work in fetal and adult rat type 2 cell lines (12).

Transcription Regulation of CIC-2 Gene Expression

To further confirm the effect of SP1 glycosylation on CIC-2 gene expression, we treated L2 cells with glutamine to induce SP1 glycosylation or tunicamycin as an inhibitor of SP1 glycosylation. The quantitative PCR showed that glutamine mediated upregulation of CIC-2 transcript ($P < 0.05$) with no change in SP1 mRNA levels, whereas tunicamycin had minimal effect on CIC-2 relative transcript levels and caused no change in SP1 (Figure 6). Our results further confirmed that post-transcriptional changes in SP1 induce CIC-2 gene transcription.

Figure 3. Inhibition of CIC-2 protein expression by tunicamycin. SP1 was immunoprecipitated from prell-19 (500 μ g/ml) and probed by immunoblotting with anti-O-GlcNAc (middle panel). Fifty micrograms of these total protein samples were separated on polyacrylamide gels and immunoblotted for CIC-2 (upper panel) and β -actin (lower panel). There was a decrease in O-GlcNAc modified SP1 and CIC-2.

Figure 4. SP1 overexpression in preII-19 and L-2 cells. PreII-19 and L2 cells were transfected with pCMV or pCMV-SP1 as described in MATERIALS AND METHODS. SP1 was immunoprecipitated (500 μ g/ml) and probed by immunoblotting with anti-O-GlcNAc (middle panel). Fifty micrograms of these total protein samples were separated on polyacrylamide gels and immunoblotted for CIC-2 (upper panel) and β -actin (lower panel). Induction of CIC-2 expression is dependent on the glycosylation state of SP1. Densitometric quantification of immunoblots is shown in the lower panel. Data ($n = 3 \pm$ SD) are shown as percentage of expression level in each sample relative to L2-pCMV CIC-2 control (* $P < 0.05$). Data have been normalized using β -actin as standard. Lower panel: open bars, anti-CIC-2; shaded bars, anti-O-GlcNAc.

DISCUSSION

Our results demonstrate that glutamine may regulate the expression of CIC-2 chloride channels through glycosylation of the SP1 transcription factor that stimulates the transcription of the CIC-2 gene. To our knowledge, this is the first report demonstrating the requirement for O-GlcNAc glycosylation of SP1 for expression of the CIC-2 gene. CIC-2 is a pH- and voltage-activated chloride channel that is coexpressed in epithelial cells with

CFTR. We are interested in the potential of CIC-2 to serve as an alternative chloride channel in cystic fibrosis. Our earlier work demonstrated that CIC-2 was highly expressed in the fetal lung epithelial cell line preII-19 in association with two isoforms of SP1, 95 kD and 105 kD. CIC-2 was present but in lower levels in the adult lung epithelial cell line L2 with a single 105-kD SP1 isoform. Based on these and other results, we hypothesized that the lower-molecular-weight form (95 kD) of SP1 is responsible for CIC-2 induction (12). We showed that the CIC-2 promoter contains a consensus binding site for SP1 transcription factor and that modulation of SP1 in lung epithelial cells regulates CIC-2 gene expression. We also confirmed the binding of SP1 to CIC-2 promoter by the gel super shift assay (9). Both cells responded to transient transfection with a pCMVSP1 cDNA by increasing CIC2 protein expression (12).

SP1 can be regulated by different stimuli, such as growth factors, lipopolysaccharides, cAMP, or hyperglycemia (18, 21–23). Most of these effects were shown to occur through post-translational changes (i.e., phosphorylation and glycosylation) (19, 24, 25). The O-glycosylation of SP1 was shown to stimulate or inhibit its transcriptional activity (13, 19, 26). There is apparent consensus that O-GlcNAcylation modulates SP1 transcriptional activity, but the mechanisms of regulation are controversial. SP1 glycosylation is recognized to play an important role in regulating signal transduction and gene transcription. Our results strengthen the notion that O-glycosylation may activate SP1 DNA binding and hence CIC-2 gene transcription. We demonstrate that induction of SP1 glycosylation stimulates CIC-2 expression, whereas inhibition of SP1 glycosylation suppresses CIC-2 expression. It has been reported that besides its role in stimulation of SP1 transcriptional activity, O-glycosylation of SP1 plays a key role in its translocation to the nucleus (27). Moreover, O-glycosylation of SP1 has been known to prevent its degradation (18).

Figure 5. Glycosylation state of the 105-kD SP1 isoform in murine lungs. To identify the functionally relevant form of SP1, protein extracts from native lung of 6- and 16-wk-old mice were probed by immunoblotting with anti-CIC-2, anti-SP1, and anti- β -actin (top panel). SP1 immunoprecipitates of lung extracts (500 μ g/ml) were immunoblotted with anti-O-GlcNAc (bottom panel). Duplicate experiments are shown. The 6-wk-old murine lungs have higher amounts of glycosylated SP1 (105 kD) as compared with 16-wk-old mice, and the CIC-2 protein expression in the younger mice is also higher. The *in vivo* differences in glycosylation state of SP1 reflected the CIC-2 expression levels.

Figure 6. Transcription regulation of CIC-2 gene expression by glutamine and tunicamycin. The L2 cells were treated overnight with 200 mM and 500 mM glutamine (glycosylation inducer) or 1 μ g/ml tunicamycin (glycosylation inhibitor). RNA was isolated, transcribed to cDNA, and subjected to quantitative PCR using CIC-2 and SP1 primers. The graph demonstrates a dose-dependent upregulation of CIC-2 mRNA by glutamine in the absence of a change in SP1 mRNA levels. Tunicamycin did not modulate either mRNA species. The post-transcriptional changes in SP1 regulate CIC-2 expression. The data ($n = 3 \pm$ SD) are shown as the percentage of expression level in each sample relative to CIC-2 control (* $P < 0.05$). Open bars, CIC-2; shaded bars, SP1.

O-GlcNAc is a modification of serine and threonine residues of nuclear and cytoplasmic proteins with O-linked- β -N-acetylglucosamine. It is one of a growing number of post-translational protein modifications that modulate the action of these intracellular proteins (25). It has been proposed that O-GlcNAc modification of proteins meets the requirements of a legitimate modifier of the pathways of signal transduction. This concept is supported by the observations that (1) O-GlcNAc modification is dynamic (for the proteins that have been examined, the O-GlcNAc half life is much shorter than that of modified polypeptide chain) (28, 29), (2) O-GlcNAc protects the peptides from degradation (18), and (3) addition or removal of the O-GlcNAc modification of the peptide is inducible by certain stimuli and prompt enough to participate in the rapid events of signal transduction (29). Most of the known O-GlcNAcylated proteins can also be phosphorylated (29, 30). Because O-GlcNAc and O-phosphate can be attached to a serine or threonine on the same or closely related species (29), it has been proposed that a reciprocal relationship between O-GlcNAcylation and O-phosphorylation may be involved in regulating biological functions in eukaryotes (31).

O-GlcNAc has been detected in a number of transcription factors. The ubiquitous transcription factor SP1 is modified extensively by O-GlcNAc (25), but whether this modification regulates SP1 function is controversial. The O-GlcNAc may positively regulate some genes while negatively regulating others. We observe that the glycosylation state of SP1 is related to CIC-2 expression levels. The contradictory conclusions from different laboratories can be explained by a recently proposed model in which O-glycosylated SP1 binds to the DNA, is deglycosylated and phosphorylated, and activates gene transcription (32).

The results from our study suggest that, unlike low glucose, 2-deoxyglucose treatment causes downregulation of SP1-mediated CIC-2 induction. It has been proposed that 2-deoxyglucose inhibits SP1 activity through hyperglycosylation (20). To further confirm the SP1 glycosylation mediated CIC-2 induction, we used glutamine as a glycosylation inducer and tunicamycin as an inhibitor of glycosylation. Glutamine induced SP1 glycosylation and CIC-2 protein levels, whereas tunicamycin inhibited SP1 glycosylation, and hence CIC-2 protein levels. Moreover, glutamine induced CIC-2 mRNA/protein levels with no change

in SP1 transcript/protein levels, further confirming our hypothesis that glycosylation of SP1 is required for CIC-2 gene induction.

We previously reported prenatal expression of SP1 and CIC-2 in rat lungs (9). We show here that 6-wk-old mice, as compared with 16-wk-old mice, not only have elevated levels of SP1, but also that it is the 105-kD glycosylated isoform of SP1 that is associated with higher CIC-2 protein levels. Our results support the hypothesis that the 105-kD isoform of SP1 induces CIC-2 expression instead of the previously reported 95-kD isoform (12). Other methods, such as a chromatin immunoprecipitation assay (33), may be used for further confirmation of the hypothesis. Because SP1 regulates several other genes, some of which are growth regulated, it is likely that the glycosylation state of SP1 may be critical in organogenesis and maturation of lung. Further investigation of the role of SP1 glycosylation during lung development is required.

Conflict of Interest Statement: Neither author has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Dr. G.W. Hunninghake for the prell-19 cell line and Dr. Robert Tjian for the pCMV-Sp1 construct.

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