Original Paper

The Human Pendrin Promoter Contains two N₄ GAS Motifs with Different Functional Relevance

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Key Words

Interleukin 4 • Cytokine • ChIP • SLC26A4 • STAT6

Abstract

Background: Pendrin, an anion exchanger associated with the inner ear, thyroid and kidney, plays a significant role in respiratory tissues and diseases, where its expression is increased following IL-4 and IL-13 exposure. The mechanism leading to increased pendrin expression is in part due to binding of STAT6 to a consensus sequence (N₄ GAS motif) located in the pendrin promoter. As retrospective analyses of the 5' upstream sequence of the human pendrin promoter revealed an additional N₄ GAS motif (1660 base pairs upstream of the one previously identified), we set out to define its contribution to IL-4 stimulated changes in pendrin promoter activity. **Methods and Results:** Electrophoretic mobility shift assays showed that STAT6 bound to oligonucleotides corresponding to both N₄ GAS motifs *in vitro*, while dual luciferase promoter assays revealed that only one of the N₄ GAS motifs was necessary for IL-4 –stimulated increases in pendrin promoter activity in living cells. We then examined the ability of STAT6 to bind each of the N₄ GAS motifs *in vivo* with a site-specific ChIP assay, the results of which showed that STAT6 interacted with only the N₄ GAS motif that was functionally implicated in increasing the activity of the pendrin promoter following IL-4 treatment. **Conclusions:** Of the two N₄ GAS motifs located in the human pendrin promoter region analyzed in this study (nucleotides -3906 to +7), only the one located nearest to the first coding ATG participates in IL-4 stimulated increases in promoter activity.

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Introduction

Pendrin is an anion exchanger encoded by the solute carrier family 26A4 gene (*SLC26A4*) family and loss of function mutations in this protein can result in nonsyndromic or syndromic sensorineural hearing loss, termed Pendred Syndrome [1-7]. Pendrin is expressed in a multitude of tissues, including the airway epithelium, although its basal mRNA expression is relatively low in the latter [8-20]. However, in the presence of the T-helper type-2 (T_H2) cytokines, interleukin (IL)-4 and IL-13, pendrin expression is rapidly and robustly increased [11, 12, 18, 21-24].

IL-4 and IL-13 are two of many small glycoproteins acting as signaling molecules in the humoral immune system. Once bound to their membrane-located receptor, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction cascade is activated. This cascade involves activation of the receptor by phosphorylation, followed by the recruitment, binding, phosphorylation and activation of STAT monomers. Currently, seven STAT isoforms have been identified, and STAT6 is the isoform preferentially activated in the presence of IL-4. Once phosphorylated on tyrosine residue 641, STAT6 monomers dimerize, at which point they are able to translocate from the cytosol into the nucleus. The STAT6 homodimers then scan and preferentially bind to conserved DNA sequences referred to as interferon- γ activated sequences (GAS) motifs, which are defined as 5' TTC (N₄) GAA 3', where N is any nucleotide [25, 26].

It has been shown that pendrin regulation by IL-13 within the airway epithelium impacts the volume of the airway surface liquid (ASL) [21]. We initiated experiments aimed to define the mechanism of IL-4 and IL-13 stimulated increases in pendrin expression, and found that both cytokines increase pendrin promoter activity [23]. In addition, we discovered that an N₄ GAS motif that bound STAT6 was necessary for the increases in pendrin promoter activity. Further analysis of the 5' upstream sequence of the human pendrin promoter revealed another N₄ GAS motif. Therefore, in the present manuscript, we set out to investigate the contribution of this additional N₄ GAS motif to IL-4 stimulated changes in pendrin promoter activity.

Materials and Methods

Materials

Penicillin, streptomycin, glutamine, and base medias were from Sigma Aldrich (St. Louis, MO; USA). Fetal bovine serum (FBS) was from Lonza (Cologne, Germany). IL-4 was from Invivogen (San Diego, CA; USA) and prepared in distilled water. Blasticidin, zeocin and the HEK-Blue™ IL-4/IL-13 (HEK-Blue™) cells were from Invivogen. The rabbit anti-STAT6 (catalogue# sc-621X) antibody used for electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) assays was from SantaCruz Biotechnology (Europe). The normal rabbit IgG (catalogue# 2729) used for ChIP assays was from Cell Signaling (Boston, MA; USA). All antibodies conjugated to an infrared-dye were from LICOR (Lincoln, NE; USA). The pGL3B and pRL-TK vectors used for dual luciferase promoter assays were from Promega (Madison, WI; USA). Oligonucleotides for primers and EMSAs were purchased from Microsynth (Balgach, Switzerland) and the identities of all nucleotides corresponding to the human pendrin promoter were verified with sequencing (Microsynth) prior to use in experiments. Brightness and contrast adjustments were made to agarose gel and EMSA images with Adobe Photoshop.

Cell Culture

All cell lines were cultured at 37°C in a humidified incubator gassed with 5% CO₂ and maintained in plastic petri dishes. HEK-Blue™ cell media consisted of DMEM high glucose (4.5g/L) base media supplemented with 10% FBS, 10 μ g/mL blasticidin, 100 μ g/mL zeocin, 50U/mL penicillin, 50 μ g/mL streptomycin and 1.5% NaHCO₃. NCI-H292 cell media consisted of RPMI-1640 base media supplemented with 10% FBS, 4.5g/L D-glucose, 1.5g/L NaHCO₃, 2.38 g/L HEPES, 1mM sodium pyruvate, 50U/mL penicillin and 50 μ g/mL streptomycin. Media was replaced three times weekly for both cell lines.

Transient Transfection and Luciferase Assays

NCI-H292 cells were seeded in white 96-well Nunclon™ Δ Surface plates (Nunc; Langensfeld, Germany) and transiently transfected on the following day with polyethylenimine (PEI). A transfection mix containing 120 μg/mL PEI (pH 7.0), pGL3B vector (containing fragments of the human pendrin promoter subcloned upstream of the firefly luciferase gene), and pRL-TK vector (containing the thymidine kinase promoter upstream of the *Renilla* luciferase gene) was prepared in PEI buffer (150 mM NaCl, 10 mM HEPES, pH 7.0). Transfection mix was added to culture media at a 100 μL:2500 μL ratio. The final concentrations of pGL3B and pRL-TK vectors were 0.183 fM and 0.076 fM, respectively. 24 hours later, media containing transfection mix was replaced with media containing vehicle (distilled water), IL-4 (40 ng/mL) or TNFα (40 ng/mL). 48 hours later, the cells were prepared for the dual luciferase assay using the Dual Luciferase® Assay System (Promega). Media was removed and cells were washed once with phosphate buffered saline (PBS, 136.89 mM NaCl, 2.69 mM KCl, 3.21 mM Na₂HPO₄, pH 7.4). 20 μL of passive lysis buffer (Promega) were added to each well and incubated at room temperature (RT) for 15 minutes with agitation. 50 μL of luciferase assay reagent (LAR) II was added and luminescence from the Firefly luciferase was detected for 10 seconds. 50 μL of STOP & Glo reagent was then added to simultaneously quench the Firefly luciferase signal and activate the *Renilla* luciferase signal. The *Renilla* luciferase luminescence was also detected for 10 seconds. The experimental design was as follows: for each 96-well plate, at least 4 replicate wells were transfected and treated in the same manner. This was then repeated at least 3 times (each time, a newly passaged batch of cells was used). Addition of the LAR II and STOP & Glo buffers, as well as detection of the luminescence was performed using the VICTOR™ X3 2030 multi-label reader from Perkin Elmer (Vienna, Austria).

3'-end biotin labeling

The following oligonucleotides were commercially synthesized (Microsynth) and biotinylated using the Biotin 3' End DNA Labeling (Pierce; Rockford, IL; USA) kit according to the manufacturers instructions. The oligonucleotide sequences used for the wildtype N₄ GAS motif 1 were: (forward) 5' GATGCATCTATTCTTTTGGAAAGAAGTCCTGA 3' and (reverse) 5' TCAGGACTTCTTTCCAAAAGAAATAGATGCATC 3', and for the mutant N₄ GAS motif 1 were: (forward) 5' GATGCATCTATT**ATTTTGGAAAGAAGTCCTGA** 3' and (reverse) 5' TCAGGACTTCTTTCCAAA**ATA**ATAGATGCATC 3'. Nucleotides belonging to the N₄ GAS motif 1 are underlined, and the mutated nucleotides are in bold-face font. Mutation of N₄ GAS motifs in this way has been previously demonstrated to abrogate STAT6 binding [27, 28]. Following biotinylation, equal amounts of complementary oligonucleotides were annealed by heating to 95°C for 5 minutes and cooling down to RT over time.

Nuclear Extract Preparation & Electrophoretic Mobility Shift Assays (EMSAs)

HEK-Blue™ cells were treated for 15 minutes with either vehicle (water) or IL-4 (40 ng/mL) at 37°C and fractionated into nuclear and cytoplasmic extracts using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce) according to the manufacturer's instructions, except the following ratio of reagents were used: 500 μL:27.5 μL:40 μL CER I:CER II:NER. Halt protease inhibitor cocktail (HPIC) was added to CER I and NER to a final concentration of 1X. Protein concentrations were determined by the RCDC protein assay (BioRad; Vienna, Austria).

The binding reaction contained 20 mM HEPES-KOH pH 7.9, 0.5 mM Tris pH 8.0, 50 mM KCl, 10% glycerol, 50 ng/μL poly dI•dC, 5 mM dithiothreitol, and 0.15 mM EDTA pH 8.0. Nuclear extract (5 μg total) from vehicle or IL-treated cells was added and incubated on ice for 20 minutes. 25 nM non-biotinylated (cold) oligonucleotides or 0.6 μg anti-STAT6 antibody were then added and incubated on ice for 15 minutes. 1.2 nM biotinylated oligonucleotides were then added and incubated for another 15 minutes on ice. Orange loading dye (LICOR) was added to the samples to a final concentration of 1X (to stop the reaction), and the entire reaction was then separated on 5% polyacrylamide native gels at 120 V for 45 minutes. Samples were transferred to Biotinylated nylon membranes (Pierce) for 45 minutes at 380 mA with stirring. All electrophoreses were carried out with ice-cold buffers. The membranes were cross-linked at 120 mJ/cm² for 1 minute with UV light and subsequently blocked for 15 minutes with 1X Nucleic Acid Detection Blocking Buffer (Pierce). Membranes were then incubated for 15 minutes with a 1:1000 dilution of anti-streptavidin-IRDye-800, followed by six, five minute washing steps with 1X Nucleic Acid Detection Wash Buffer. All incubations and washing steps were carried out with gentle agitation at RT and in the absence of light. The membranes were visualized with the ODYSSEY® infrared imaging system (LICOR).

Table 1. Sequence and expected size in base pairs (bps) of PCR products from Figure 4

	Name	Primer Sequence (5'-3')	Expected Size (bps)
Digestion Control Primers	HindIII	Fwd: CATCAATTATAACATGAACCTGATAAAATGTG	106
		Rev: GTGCTGGCTGTGGGTACAATGATG	
	BglII	Fwd: GTCGCATGCAAAACCTTGGAGTG	416
		Rev: CTCAGAACTCCTCCCAGAGAACCC	
	XbaI	Fwd: GTAAGAAAGGGCCACCTGGACGG	218
		Rev: GTGCTCTCATACCACATGGGGAG	
Target Primers	N ₄ GAS motif 1	Fwd: CATCATTTGTACCCACAGCCAGCAC	294
		Rev: CTGTTTCACACAGAGGTGGTGGTTC	
	N ₄ GAS motif 2	Fwd: CAAGGCAGGTGGATTACGGAAGG	218
		Rev: GAGGGAGGAGGAGCTGGAGCTATC	
CYP2D6		Fwd: GCCTTCGCCAACCCTCCG	303
		Rev: GCCCTGCAGAGACTCCTCGG	

Chromatin Immunoprecipitation (ChIP)

ChIP experiments were executed with the ChIP-IT® Express kit (Active Motif; La Hulpe, Belgium). 1x10⁷ HEK-Blue™ cells on plastic 10cm Ø petri dishes were treated with IL-4 (40 ng/mL) or vehicle (water) for 15 minutes. Nucleoprotein complexes were cross-linked by treating the cells with 1% formalin (Sigma Aldrich) in serum free HEK-Blue™ media for 10 minutes at RT. To stop cross-linking, the formaldehyde containing media was replaced with 1X glycine-PBS (Active Motif), and cells were incubated for 5 minutes at RT. After removing the glycine-PBS solution, the fixed cells were collected by scraping in 1mL of PBS containing 5 µL of phenylmethanesulfonyl fluoride (PMSF). Cells were centrifuged at 600 *x g* at 4°C for 10 minutes, and the supernatant discarded. Nuclei were isolated from cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce), according to the manufacturer's instructions with the exceptions described above. Nuclei were centrifuged at 1200 *x g* for 10 minutes at 4°C, and the supernatant discarded. All recovered chromatin was fragmented by simultaneous digestion with 20 Fast Digest Units each of Fast Digest restriction enzymes HindIII, BglII and XbaI (ThermoFisher Scientific) overnight at 37°C. Complete fragmentation was verified via PCR. Samples were centrifuged at 1200 *x g* for 5 minutes at 4°C and the supernatant discarded. Nuclei were then lysed with 350µL sample buffer (1% SDS, 10 mM EDTA, pH 8.0 in 50mM Tris HCl, pH 8.1) to recover cleaved chromatin. 10µL of this solution (input DNA) was set aside for later use. 10 to 150µL cleaved chromatin were then subjected to immunoprecipitation using 1 µg ChIP-validated anti-STAT6 antibody or rabbit IgG (negative control), together with 25µL protein G magnetic beads (Active Motif) overnight on a rotating wheel at 4°C. Samples were then washed once with 800µL ChIP Buffer 1 (Active Motif) and twice with 800µL ChIP Buffer 2 (Active Motif). Elution was performed using 50µL of Elution Buffer AM2 (Active Motif) for 15 minutes on a rotating wheel at RT. Next, reverse cross-linking was performed with 50µL Reverse Cross-link Buffer (Active Motif), and protein digestion was realized with the addition of 0.01 mg/mL Proteinase K (Active Motif). Protein digestion was then halted with 2µL Proteinase K stop solution (Active Motif). DNA from all samples, as well as that from the input DNA, was then subjected to PCR.

PCR reactions were performed with the KOD Hot Start DNA polymerase kit (Novagen). Each 50 µL reaction was composed of 1X PCR KOD Hot Start DNA polymerase buffer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 1.5 mM (or 1.25 mM for the N₄ GAS motif 1 reaction) MgSO₄, 1 µM forward primer, 1 µM reverse primer, 0.02 U/µL KOD Hot Start DNA Polymerase and 1 µL template chromatin (or 0.1 µL for the input chromatin). The PCR parameters were as follows: 95°C for 2 minutes, followed by 35 cycles of 95°C for 20 seconds, 59.7°C for 10 seconds and 68°C for 3 seconds (HindIII and XbaI) or 5 seconds (BglII). PCR primer sequences and expected product lengths are shown in Table 1.

Bioinformatic Analyses

The ~5000 base pair sequence upstream of the first coding ATG in the human pendrin gene (GenBank accession no. AC078937.1) was analyzed for transcription factor binding consensus motifs using MatInspector software (a module of the Genomatix software suite).

Fig. 1. Schematic of the human pendrin promoter constructs. The first two exons (E1 and E2) of the human pendrin promoter separated by an intron are shown. A fragment containing 3912 base pairs upstream and including the sequence ATGGCAG (ATG is the pendrin open reading frame and the A is designated +1) was subcloned upstream of the firefly luciferase gene (in the pGL3B vector) for promoter activity experiments. This construct includes two N₄ GAS motifs. Their positions relative to the open reading frame are indicated. The nucleotides corresponding to the GAS motifs are underlined.

Fig. 2. A nucleoprotein complex composed of STAT6 and oligonucleotides containing the N₄ GAS motif 1 sequence is formed in HEK-Blue™ cells treated with IL-4. Nuclear extracts from HEK-Blue™ cells treated for 15 minutes with vehicle (distilled water) or IL-4 (40ng/mL) were incubated with biotinylated (labeled) or a 20.8 molar excess of unlabeled oligonucleotides (oligo) corresponding to the human pendrin promoter and containing either the wild-type or mutant N₄ GAS motif 1 in the presence or absence of an anti-STAT6 antibody. The image is representative of three individual experiments. The wild-type and mutated N₄ GAS motif 1 sequence is shown above the image. The mutated nucleotides are underlined.

Statistics

Significant differences between 2 experimental groups were determined using the two-tailed Student's t-test, whereas significant differences between 3 or more experimental groups were determined using a one-way ANOVA followed by Bonferroni's Multiple Comparison Test. Differences were considered statistically significant where the p-value ≤ 0.05. Statistical analyses and the generation of graphs were performed with GraphPad Prism 5.0 software.

Results

We recently identified an N₄ GAS motif located 1803 nucleotides upstream of the pendrin open reading frame [23]. Further bioinformatical analysis of the 5' upstream region of the pendrin promoter revealed an additional N₄ GAS motif located 3463 nucleotides upstream of the coding ATG (Fig. 1). The N₄ GAS motif located furthest from the ATG will heretofore be referred to as N₄ GAS motif 1 and the one closest to the ATG will be referred to as N₄ GAS motif 2.

We previously demonstrated that STAT6 binding to N₄ GAS motif 2 is necessary for IL-4 stimulated increases in human pendrin promoter activity and mRNA expression [23]. Therefore, we first set out to investigate if STAT6 also interacts with N₄ GAS motif 1. Using EMSAs, a single nucleoprotein complex (shift) was detected in HEK-Blue™ cell nuclear

Fig. 3. N₄ GAS motif 1 is not necessary for IL-4 stimulated increases in pendrin promoter activity. NCI-H292 cells were transiently transfected with A) the pGL3B control vector (no promoter), pendrin promoter construct 3912 containing either i) the two wild-type (wt) N₄ GAS motifs, ii) the mutant (mt) N₄ GAS motif 1, iii) the mutant N₄ GAS motif 2, or iv) the two mutant N₄ GAS motifs or B) the pGL3B control vector (no promoter), pendrin promoter construct 3912 containing either i) the two wild-type (wt) N₄ GAS motifs or ii) the mutant (mt) N₄ GAS motif 1, or the pendrin promoter construct 3462 (containing only the wt N₄ GAS motif 2). Both N₄ GAS motifs were mutated as indicated in Figure 2. Cells were then treated with vehicle (distilled water) or IL-4 (40ng/mL) for 48 hours and assayed for firefly and *Renilla* luciferase expression. The firefly luciferase luminescence was normalized to that of *Renilla* luciferase, and represents the promoter activity. For each vector construct, the promoter activity in cells treated with IL-4 was then normalized to that of the vehicle treated cells. These values represent the fold changes in promoter activity and are shown as bars representing mean values $\pm$ S.E.M of 108 or 50 experiments for panel A and B, respectively. * $p < 0.001$ compared to the respective vehicle-treated cells as indicated by a one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

extracts stimulated with IL-4 and incubated with a biotinylated oligonucleotide probe containing the wild-type N₄ GAS motif 1 sequence (Fig. 2). This interaction was specific since the shift was not visible in the presence of a molar excess of unlabeled, wild-type oligonucleotide. As expected, no prominent nucleoprotein complexes were detected when using a biotinylated oligonucleotide probe in which N₄ GAS motif 1 was mutated. In addition, a single nucleoprotein complex was detected in HEK-Blue™ nuclear extracts treated with IL-4 in the presence of an anti-STAT6 antibody (supershift). From these experiments, we can conclude that STAT6 binds to an oligonucleotide bearing the sequence of N₄ GAS motif 1 *in vitro*.

Next, the functional relevance of N₄ GAS motif 1 in human pendrin promoter activity was assessed using dual luciferase reporter assays. Taking the aforementioned EMSA results into account, we hypothesized that N₄ GAS motif 1 contributes to IL-4 stimulated increases in pendrin promoter activity similarly to that observed with N₄ GAS motif 2. To this

Fig. 4. STAT6 interacts with N₄ GAS motif 2 *in vivo*. (A) Site-specific ChIP design. An ~4000 base pair region immediately upstream of the human pendrin open reading frame is shown. Complete digestion with HindIII, BglII and XbaI, will physically separate the two N₄ GAS motifs into individual fragments. Small regions (target amplicons) of each fragment (containing either of the two N₄ GAS motifs) amplified by the target primers are indicated in black. Small regions (digestion control amplicons) overlapping the restriction sites amplified by the digestion control primers are indicated in gray. (B) HEK-Blue™ cells were treated with vehicle (water) or IL-4 (40ng/mL) for 15 minutes and processed for site-specific ChIP analysis. Collected nuclei were lysed and chromatin was cleaved by restriction enzyme digestion. PCR-based analysis of undigested (upper panel) or digested (lower panel) chromatin amplified with either the digestion control or target primer pairs is shown. The expected product size for each fragment is indicated in Table 1. NTC = no template control. (C) Specifically digested chromatin was immunoprecipitated with either normal rabbit IgG or a rabbit anti-STAT6 antibody. Either non-immunoprecipitated (input) or immunoprecipitated chromatin was subjected to PCR using the target primers designed to amplify a 294 base pair or 218 base pair region of fragments containing either N₄ GAS motif 1 or N₄ GAS motif 2, respectively. A 303 base pair amplicon of cytochrome P450 family 2 subfamily D polypeptide 6 (CYP2D6) was used as a control. The image is representative of 2 independent experiments.

end, we subcloned the 3912 base pair fragment of the human pendrin promoter shown in Figure 1 upstream of the firefly luciferase gene within the pGL3B vector. The N₄ GAS motifs were systematically mutated, and the ability of the constructs to drive firefly luciferase expression in the presence and absence of IL-4 was evaluated. Firstly, Figure 3A shows that IL-4 significantly increased firefly luciferase expression in cells transiently transfected with the 3912 base pair fragment containing the two wild-type N₄ GAS motifs. As expected, this increase was blocked after mutating N₄ GAS motif 2, as well as after mutating both N₄ GAS motifs within the same fragment. Interestingly, however, mutation of N₄ GAS motif 1 alone did not alter the response of the promoter fragment to IL-4. This was unexpected since STAT6 binds to this N₄ GAS motif *in vitro* (refer to Fig. 2). For verification, we deleted N₄ GAS motif 1 entirely and assessed the sensitivity of this new fragment (referred to as fragment 3462) to IL-4. Figure 3B shows that this fragment behaved similarly to the 3912 base pair promoter fragment containing both wild-type N₄ GAS motifs, as well as to the 3912 base pair fragment containing mutated N₄ GAS motif 1.

Since STAT6 bound to both N₄ GAS motifs *in vitro* (as determined by the EMSAs), but only N₄ GAS motif 2 was required for IL-4 stimulated increases in pendrin promoter activity,

we wondered whether STAT6 bound either of the two N₄ GAS motifs *in vivo*. To do this, we turned to site-specific chromatin immunoprecipitation (ChIP), which combines the benefits of EMSAs and *in vivo* footprinting, thereby allowing detection of the simultaneous association of transcription factors with individual sequences on chromatin. Following the design reported by Schuch et al. [29], we engineered a site-specific ChIP assay to simultaneously assess the *in vivo* interaction of STAT6 with both N₄ GAS motifs present in the human pendrin promoter (Fig. 4A). Complete and specific digestion of the chromatin (with the use of specific restriction enzymes) is an essential prerequisite for site-specific ChIP, since this step should physically separate the regions of interest (the nucleotides expected to interact with a transcription factor; in this case, the two N₄ GAS motifs with STAT6). This aspect was verified using primer pairs designed to amplify regions overlapping the critical restriction enzyme sites (digestion control amplicons), as well as regions within the fragments containing the two N₄ GAS motifs (target amplicons). Figure 4B shows that only the target amplicons were generated following digestion of the chromatin, confirming that the isolated chromatin was completely digested and that the N₄ GAS motifs were located on separate, individual chromatin fragments. Figure 4C shows that STAT6 was immunoprecipitated only from the chromatin fragment encompassing N₄ GAS motif 2 and only from cells treated with IL-4. These results are consistent with those from the dual luciferase reporter assay and confirm that N₄ GAS motif 1 does not contribute to IL-4-stimulated increases in pendrin promoter activity.

Discussion

At least 10 transcripts of varying sizes from the *SLC26A4* gene are listed in the Ensemble database (www.ensembl.org, gene ID# ENSG00000091137), only five of which code for proteins. Of these 5 protein coding transcripts, only the one (Ensemble transcript ID# ENST00000265715) encoding a 780 amino acid long protein (from 21 exons) was originally linked to Pendred Syndrome [4]. Since then, the promoter controlling this particular open reading frame has been the subject of many investigations [18, 23, 30-38], as well as the present study.

It is becoming increasingly accepted that pendrin, an anion exchanger associated with the inner ear, thyroid and kidney, also plays a significant role in respiratory tissues/diseases [11, 12, 18, 21-24, 39-42]. We previously demonstrated that increases in pendrin promoter activity following IL-4 stimulation required the N₄ GAS motif 2 and involved binding of STAT6 [23]. Subsequent bioinformatical analyses of the human pendrin promoter revealed another N₄ GAS motif (referred to as N₄ GAS motif 1 in the present study), and we therefore set out to define the role of this motif in T_H2-type cytokine-induced increases in pendrin promoter activity.

While EMSAs demonstrated STAT6 binding to oligonucleotides bearing the wild-type sequence of either N₄ GAS motif *in vitro*, promoter activity assays clearly showed that only N₄ GAS motif 2 is necessary for IL-4 stimulated increases in promoter activity. Data from ChIP experiments showed that STAT6 associates only with N₄ GAS motif 2 *in vivo*, and only in cells stimulated with IL-4. These data are consistent with previous data from our group showing that active STAT6 (phosphorylated at Tyr residue 641) localizes to the nucleus only after IL-4 treatment [23]. While STAT6 is able to bind N₄ GAS motif 1 *in vitro*, the ChIP data suggest a different scenario *in vivo*.

The promoters of other genes, including the thymus- and activation-regulated chemokine (TARC/CCL17) and adhesion receptor P-selectin promoters both harbor two N₄ GAS motifs, and the suppressors of cytokine signaling-1 (SOCS-1) promoter contains at least three N₄ GAS motifs [27, 28, 43]. For all of these promoters, the multiple N₄ GAS motifs cooperate to induce maximal transcriptional activation following IL-4 treatment. The two N₄ GAS motifs in the P-selectin promoter are separated by 87 nucleotides and are located only ~140 nucleotides upstream of the coding ATG; in the TARC/CCL17 promoter, the two

N₄ GAS motifs are only 46 base pairs apart and ~190 base pairs upstream of the translational start site; and for SOCS-1, the three N₄ GAS motifs are separated from each other by 35 and 20 nucleotides, and are located ~600 base pairs upstream of the first open reading frame. In the human pendrin promoter, the two N₄ GAS motifs are separated by more than 1600 nucleotides, and located more than 1800 (N₄ GAS motif 2) and 3400 (N₄ GAS motif 1) base pairs upstream of the open reading frame (refer to Fig. 1). Therefore, the proximity of the N₄ GAS motifs to the coding ATG, as well as to each other, may impact their relevance for functional changes in promoter activity.

It is also possible that other nuclear proteins (transcriptional factors) may regulate the *in vivo* binding of STAT6 to either of the N₄ GAS motifs in the human pendrin promoter. It is well known that efficient induction and regulation of gene expression involving N₄ GAS motifs/STAT6 is highly dependent on the total transcriptional environment (both enhancer and repressor transcription factors), the activity of which can be altered by exogenous stimuli and cell type specific expression [44]. As such, potential transcription factor binding sites neighboring either of the N₄ GAS motifs in the pendrin promoter may explain the functional difference (in terms of promoter activity) we observed. The sequences that flank both N₄ GAS motifs in the human pendrin promoter do differ from one another, and the presence of potential transcription factor binding sites flanking these motifs and their contribution to IL-4 stimulated increases in pendrin promoter activity and gene expression could be of significant clinical value.

Although STAT6 has been described as having the highest affinity for the N₄ GAS motif, other STAT isoforms (STAT1, for example) have been shown to interact with these motifs [25, 26]. Therefore, N₄ GAS motif 1 could represent a site for binding of STAT isoforms other than STAT6. We have not detected activation of any other STAT isoform following IL-4 stimulation in HEK-Blue™ cells (data not shown), but stimuli that activate other STAT family members may interact with N₄ GAS motif 1 to regulate pendrin promoter activity. Further experimentation is required to answer these conjectures.

Conflicts of Interests

There are no conflicts of interest or disclosures.

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References

- 1 Dossena S, Rodighiero S, Vezzoli V, Nofziger C, Salvioni E, Boccazzi M, Grabmayer E, Botta G, Meyer G, Fugazzola L, Beck-Peccoz P, Paulmichl M: Functional characterization of wild-type and mutated pendrin (SLC26A4), the anion transporter involved in Pendred syndrome. *J Mol Endocrinol* 2009;43:93-103.
- 2 Dossena S, Vezzoli V, Cerutti N, Bazzini C, Tosco M, Sironi C, Rodighiero S, Meyer G, Fascio U, J FUR, Ritter M, Fugazzola L, Persani L, Zorowka P, Storelli C, Peccoz PB, Botta G, Paulmichl M: Functional characterization of wild-type and a mutated form of SLC26A4 identified in a patient with Pendred syndrome. *Cell Physiol Biochem* 2006;17:245-256.
- 3 Everett LA, Belyantseva IA, Noben-Trauth K, Cantos R, Chen A, Thakkar SI, Hoogstraten-Miller SL, Kachar B, Wu DK, Green ED: Targeted disruption of mouse Pds provides insight about the inner-ear defects encountered in Pendred syndrome. *Hum Mol Genet* 2001;10:153-161.

- 4 Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis AD, Sheffield VC, Green ED: Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). *Nat Genet* 1997;17:411-422.
- 5 Kopp P, Pesce L, Solis SJ: Pendred syndrome and iodide transport in the thyroid. *Trends Endocrinol Metab* 2008;19:260-268.
- 6 Pendred V: Deaf mutism and goiter. *Lancet* 1896;2:532.
- 7 Pera A, Dossena S, Rodighiero S, Gandia M, Botta G, Meyer G, Moreno F, Nofziger C, Hernandez-Chico C, Paulmichl M: Functional assessment of allelic variants in the SLC26A4 gene involved in Pendred syndrome and nonsyndromic EVA. *Proc Natl Acad Sci U S A* 2008;105:18608-18613.
- 8 Alesutan I, Daryadel A, Mohebbi N, Pelzl L, Leibrock C, Voelkl J, Bourgeois S, Dossena S, Nofziger C, Paulmichl M, Wagner CA, Lang F: Impact of bicarbonate, ammonium chloride, and acetazolamide on hepatic and renal SLC26A4 expression. *Cell Physiol Biochem* 2011;28:553-558.
- 9 Bidart JM, Lacroix L, Evain-Brion D, Caillou B, Lazar V, Frydman R, Bellet D, Filetti S, Schlumberger M: Expression of Na⁺/I⁻ symporter and Pendred syndrome genes in trophoblast cells. *J Clin Endocrinol Metab* 2000;85:4367-4372.
- 10 Bronckers AL, Guo J, Zandieh-Doulabi B, Bervoets TJ, Lyaruu DM, Li X, Wangemann P, DenBesten P: Developmental expression of solute carrier family 26a member 4 (Slc26a4/pendrin) during amelogenesis in developing rodent teeth. *Eur J Oral Sci* 2011;119:185-192.
- 11 Di Valentin E, Crahay C, Garbacki N, Hennuy B, Gueders M, Noel A, Foidart JM, Grooten J, Colige A, Piette J, Cataldo D: New asthma biomarkers: Lessons from murine models of acute and chronic asthma. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L185-197.
- 12 Kuperman DA, Lewis CC, Woodruff PG, Rodriguez MW, Yang YH, Dolganov GM, Fahy JV, Erle DJ: Dissecting asthma using focused transgenic modeling and functional genomics. *J Allergy Clin Immunol* 2005;116:305-311.
- 13 Lacroix L, Mian C, Caillou B, Talbot M, Filetti S, Schlumberger M, Bidart JM: Na⁺/I⁻ symporter and Pendred syndrome gene and protein expressions in human extra-thyroidal tissues. *Eur J Endocrinol* 2001;144:297-302.
- 14 Rillema JA, Hill MA: Pendrin transporter carries out iodide uptake into MCF-7 human mammary cancer cells. *Exp Biol Med (Maywood)* 2003;228:1078-1082.
- 15 Royaux IE, Suzuki K, Mori A, Katoh R, Everett LA, Kohn LD, Green ED: Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells. *Endocrinology* 2000;141:839-845.
- 16 Scott DA, Wang R, Kreman TM, Sheffield VC, Karniski LP: The Pendred syndrome gene encodes a chloride-iodide transport protein. *Nat Genet* 1999;21:440-443.
- 17 Suzuki K, Royaux IE, Everett LA, Mori-Aoki A, Suzuki S, Nakamura K, Sakai T, Katoh R, Toda S, Green ED, Kohn LD: Expression of PDS/Pds, the Pendred syndrome gene, in endometrium. *J Clin Endocrinol Metab* 2002;87:938-941.
- 18 Lee A, Nofziger C, Dossena S, Vanoni S, Diasio R, Paulmichl M: Methylation of the human pendrin promoter. *Cell Physiol Biochem* 2011;28:397-406.
- 19 Adams KM, Abraham V, Cohen N, Kolls JK, Kreindler JL: Pendrin is a part of antimicrobial responses in the lung. *Am J Respir Crit Care Med* 2013;187:A4741.
- 20 Garnett JP, Hickman E, Burrows R, Hegyi P, Tiszlavicz L, Cuthbert AW, Fong P, Gray MA: Novel role for pendrin in orchestrating bicarbonate secretion in cystic fibrosis transmembrane conductance regulator (CFTR)-expressing airway serous cells. *J Biol Chem* 2011;286:41069-41082.
- 21 Nakagami Y, Favoreto S Jr, Zhen G, Park SW, Nguyenvu LT, Kuperman DA, Dolganov GM, Huang X, Boushey HA, Avila PC, Erle DJ: The epithelial anion transporter pendrin is induced by allergy and rhinovirus infection, regulates airway surface liquid, and increases airway reactivity and inflammation in an asthma model. *J Immunol* 2008;181:2203-2210.
- 22 Nakao I, Kanaji S, Ohta S, Matsushita H, Arima K, Yuyama N, Yamaya M, Nakayama K, Kubo H, Watanabe M, Sagara H, Sugiyama K, Tanaka H, Toda S, Hayashi H, Inoue H, Hoshino T, Shiraki A, Inoue M, Suzuki K, Aizawa H, Okinami S, Nagai H, Hasegawa M, Fukuda T, Green ED, Izuhara K: Identification of pendrin as a common mediator for mucus production in bronchial asthma and chronic obstructive pulmonary disease. *J Immunol* 2008;180:6262-6269.
- 23 Nofziger C, Vezzoli V, Dossena S, Schonherr T, Studnicka J, Nofziger J, Vanoni S, Stephan S, Silva ME, Meyer G, Paulmichl M: STAT6 links IL-4/IL-13 stimulation with pendrin expression in asthma and chronic obstructive pulmonary disease. *Clin Pharmacol Ther* 2011;90:399-405.

- 24 Pedemonte N, Caci E, Sondo E, Caputo A, Rhoden K, Pfeffer U, Di Candia M, Bandettini R, Ravazzolo R, Zegarra-Moran O, Galletta LJ: Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: Role of pendrin and anion channels. *J Immunol* 2007;178:5144-5153.
- 25 Ehret GB, Reichenbach P, Schindler U, Horvath CM, Fritz S, Nabholz M, Bucher P: DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. *J Biol Chem* 2001;276:6675-6688.
- 26 Schindler U, Wu P, Rothe M, Brasseur M, McKnight SL: Components of a STAT recognition code: Evidence for two layers of molecular selectivity. *Immunity* 1995;2:689-697.
- 27 Hebenstreit D, Luft P, Schmiedlechner A, Regl G, Frischauf AM, Aberger F, Duschl A, Horejs-Hoeck J: IL-4 and IL-13 induce SOCS-1 gene expression in A549 cells by three functional STAT6-binding motifs located upstream of the transcription initiation site. *J Immunol* 2003;171:5901-5907.
- 28 Wirnsberger G, Hebenstreit D, Posselt G, Horejs-Hoeck J, Duschl A: IL-4 induces expression of TARC/CCL17 via two STAT6 binding sites. *Eur J Immunol* 2006;36:1882-1891.
- 29 Schuch R, Agelopoulos K, Neumann A, Brandt B, Burger H, Korsching E: Site-specific chromatin immunoprecipitation: A selective method to individually analyze neighboring transcription factor binding sites in vivo. *BMC Res Notes* 2012;5:109.
- 30 Adler L, Efrati E, Zelikovic I: Molecular mechanisms of epithelial cell-specific expression and regulation of the human anion exchanger (pendrin) gene. *Am J Physiol Cell Physiol* 2008;294:C1261-1276.
- 31 Blomqvist SR, Vidarsson H, Fitzgerald S, Johansson BR, Ollerstam A, Brown R, Persson AE, Bergstrom G, Enerback S: Distal renal tubular acidosis in mice that lack the forkhead transcription factor Foxi1. *J Clin Invest* 2004;113:1560-1570.
- 32 Blomqvist SR, Vidarsson H, Soder O, Enerback S: Epididymal expression of the forkhead transcription factor Foxi1 is required for male fertility. *EMBO J* 2006;25:4131-4141.
- 33 Dentic M, Luongo C, Elefante A, Ambrosio R, Salzano S, Zannini M, Nitsch R, Di Lauro R, Rossi G, Fenzi G, Salvatore D: Pendrin is a novel in vivo downstream target gene of the TTF-1/Nkx-2.1 homeodomain transcription factor in differentiated thyroid cells. *Mol Cell Biol* 2005;25:10171-10182.
- 34 Jonard L, Niasme-Grare M, Bonnet C, Feldmann D, Rouillon I, Loundon N, Calais C, Catros H, David A, Dollfus H, Drouin-Garraud V, Duriez F, Eliot MM, Fellmann F, Francannet C, Gilbert-Dussardier B, Gohler C, Goizet C, Journal H, Mom T, Thuillier-Obstoy MF, Couderc R, Garabedian EN, Denoyelle F, Marlin S: Screening of SLC26A4, FOXI1 and KCNJ10 genes in unilateral hearing impairment with ipsilateral enlarged vestibular aqueduct. *Int J Pediatr Otorhinolaryngol* 2010;74:1049-1053.
- 35 Xing M, Tokumaru Y, Wu G, Westra WB, Ladenson PW, Sidransky D: Hypermethylation of the Pendred syndrome gene SLC26A4 is an early event in thyroid tumorigenesis. *Cancer Res* 2003;63:2312-2315.
- 36 Yang T, Vidarsson H, Rodrigo-Blomqvist S, Rosengren SS, Enerback S, Smith RJ: Transcriptional control of SLC26A4 is involved in Pendred syndrome and nonsyndromic enlargement of vestibular aqueduct (DFNB4). *Am J Hum Genet* 2007;80:1055-1063.
- 37 Rozenfeld J, Efrati E, Adler L, Tal O, Carrithers SL, Alper SL, Zelikovic I: Transcriptional regulation of the pendrin gene. *Cell Physiol Biochem* 2011;28:385-396.
- 38 Rozenfeld J, Tal O, Kladnitsky O, Adler L, Efrati E, Carrithers SL, Alper SL, Zelikovic I: The pendrin anion exchanger gene is transcriptionally regulated by uroguanylin: A novel enterorenal link. *Am J Physiol Renal Physiol* 2012;302:F614-624.
- 39 Ishida A, Ohta N, Suzuki Y, Takehata S, Okubo K, Ikeda H, Shiraishi H, Izuhara K: Expression of pendrin and periostin in allergic rhinitis and chronic rhinosinusitis. *Allergol Int* 2012;61:589-595.
- 40 Yick CY, Zwinderman AH, Kunst PW, Grunberg K, Mauad T, Dijkhuis A, Bel EH, Baas F, Lutter R, Sterk PJ: Transcriptome sequencing (RNA-seq) of human endobronchial biopsies: Asthma versus controls. *Eur Respir J* 2013;42:662-670.
- 41 Nofziger C, Dossena S, Suzuki S, Izuhara K, Paulmichl M: Pendrin function in airway epithelia. *Cell Physiol Biochem* 2011;28:571-578.
- 42 Wu D, Ahrens R, Osterfeld H, Noah TK, Groschwitz K, Foster PS, Steinbrecher KA, Rothenberg ME, Shroyer NF, Matthaei KI, Finkelman FD, Hogan SP: Interleukin-13 (IL-13)/IL-13 receptor alpha1 (IL-13Ralpha1) signaling regulates intestinal epithelial cystic fibrosis transmembrane conductance regulator channel-dependent Cl⁻ secretion. *J Biol Chem* 2011;286:13357-13369.
- 43 Khew-Goodall Y, Wadham C, Stein BN, Gamble JR, Vadas MA: STAT6 activation is essential for interleukin-4 induction of P-selectin transcription in human umbilical vein endothelial cells. *Arterioscler Thromb Vasc Biol* 1999;19:1421-1429.
- 44 Goenka S, Kaplan MH: Transcriptional regulation by STAT6. *Immunol Res* 2011;50:87-96.