Pendred's syndrome is an autosomal recessive disorder characterized by sensorineural deafness, goiter, and impaired iodide organification. It is caused by mutations in the PDS/SLC26A4 gene that encodes pendrin. Functionally, pendrin is a transporter of chloride and iodide in Xenopus oocytes and heterologous mammalian cells and a chloride/base exchanger in β-intercalated cells of the renal corticomedullary collecting duct. The partially impaired thyroidal iodide organification in Pendred's syndrome suggests a possible role of pendrin in iodide transport at the apical membrane of thyroid follicular cells, but experimental evidence for this concept is lacking. The iodide transport properties of pendrin were determined in polarized Madin-Darby canine kidney cells expressing the sodium iodide symporter (NIS), pendrin, or NIS and pendrin using a bicameral system-permitting measurement of iodide content in the basolateral, intracellular, and apical compartments. Moreover, we determined the functional consequences of two naturally occurring mutations (L676Q and FS306D) in polarized Madin-Darby canine kidney cells. Wild type pendrin also mediates iodide efflux in transiently transfected cells. In contrast, both pendrin mutants lose the ability to promote iodide efflux. These results provide evidence that pendrin mediates apical iodide efflux from polarized mammalian cells loaded with iodide. Consistent with the partial organification defect observed in patients with Pendred's syndrome, naturally occurring mutations of pendrin lead to impaired transport of iodide.

Pendred's syndrome, an autosomal recessive disorder characterized by congenital sensorineural deafness, goiter, and impaired iodide organification (1), is caused by mutations in the PDS/SLC26A4 gene (2). It encodes pendrin, a member of the solute carrier family 26A, which contains several anion transporters and the motor protein prestin (3, 4). Mutations in the PDS gene displayed impressive allelic heterogeneity, and >75 mutations have been identified previously (2, 5–9). The majority of PDS mutations are missense mutations, and some of these mutants appear to be retained in the endoplasmic reticulum (10, 11). A smaller number of mutations resulted in premature truncations or in alterations of splice donor or acceptor sites. Individuals with Pendred's syndrome from consanguineous families are homozygous for PDS mutations, whereas sporadic cases typically harbor compound heterozygous mutations (12). Mutations in the PDS gene are not only found in patients with classic Pendred's syndrome but also in individuals afflicted with familial endolymphatic hydrops associated (13, 14).

The SLC26A family contains several transporters of sulfate or other anions. The chloride/bicarbonate exchanger SLC26A2, down-regulated in adenoma is expressed in the gastrointestinal tract, and mutations in this gene cause congenital chloride diarrhea (15, 16). SLC26A3/diastrophic dysplasia sulfate transporter is a sulfate transporter predominantly expressed in cartilage, and recessive alterations in this gene are the molecular basis of several forms of chondrodysplasias (17–19). An additional functionally distinct SLC26A5 family member is prestin (SLC26A5), a recently cloned motor protein found in outer hair cells, which does not seem to transport anions, but requires chloride for its electromotility (4, 20). Remarkably, the genes encoding pendrin, prestin, and down-regulated in adenoma are located in close vicinity on chromosome 7q21-31 and have a very similar genomic structure suggesting a common ancestral gene.

Initial functional studies of pendrin in Xenopus oocytes and Sf9 insect cells, as well as functional studies using thyrocytes from patients with documented Pendred's syndrome, revealed that pendrin is unable to transport sulfate (21, 22). In Xenopus oocytes, pendrin was shown to mediate uptake of chloride and iodide (21) and to act as a chloride/formate exchanger (23). Studies in transfected cells and renal tubuli from Pds-null mice indicate that pendrin is a chloride/base exchanger in β-intercalated cells of the cortical collecting duct in the kidney (24, 25). Based on the typical enlargement of the endolymphatic system in patients with Pendred’s syndrome and the Pds-null mouse (26, 27), pendrin is probably also involved in anion and fluid transport in the inner ear but its exact role in this organ remains to be defined.
In thyroid follicular cells, pendrin is inserted into the apical membrane (28, 29). Functional studies in transfected cells subsequently demonstrated that the protein can mediate iodide transport in mammalian cells (10, 30). These findings, together with the impaired iodide organization in patients with Pendred’s syndrome, suggest a possible role of pendrin in mediating apical iodide efflux from thyrocytes into the follicle. In this study, we determined the iodide transport properties of wild type pendrin in polarized and non-polarized mammalian cells, and the functional consequences of two naturally occurring mutations found in a sporadic patient with Pendred’s syndrome.

**EXPERIMENTAL PROCEDURES**

**Clinical Studies**—The female patient was born in 1968 and had severe congenital sensorineural deafness (Fig. 1). She had a normal somatic development and was euthyroid. Her parents are of distinct ethnic origin. Her mother is Vietnamese, and her father is Swiss. Her mother also has a goiter, but she has a normal auditory function. The husband of the patient is also severely hearing impaired, but the two offspring of the couple have no hearing impairment. Already in childhood, the patient was found to have a large goiter. On ultrasonographic examination at age 29 years, the thyroid was diffusely enlarged and had an estimated volume of 50–60 g. There were no major lesions but several small cysts and nodules. At the age of 29, her thyroid function tests confirmed a euthyroid metabolic state. Her thyrotropin was 1.6 units/liter (0.2–4.8), and her free thyroxine was 17.2 pmol/liter (10.0–22.3). Serum thyroglobulin was markedly elevated to 627 ng/ml (26–46). Anti-thyroglobulin and anti-thyroidperoxidase antibodies were negative. Six months after initiating a treatment with 100-μg levothyroxine, her free thyroxine was 19.6 pmol/liter, her thyrotropin was 0.16 milliunits/liter, and her thyroglobulin decreased into the normal range with a value of 41.7 ng/ml.

A thyroid scan with 131I documented diffuse hyperplasia and a normal uptake. The perchlorate test was positive with a discharge of 22% of the incorporated iodide 15 min after administration of 0.6 g of KClO4. Magnetic resonance of the inner ear revealed the presence of an enlarged endolymphatic sac (Fig. 1).

**DNA Sequencing**—After obtaining informed consent, blood was collected and DNA was extracted from peripheral leukocytes using standard techniques. Exons 2–21 of the PDS gene were amplified using primers and conditions reported previously (2). The PCR products were purified with Centricon 100 columns (Amicon, Beverly, MA), and both strands were sequenced directly using FS AmpliTaq DNA polymerase with an ABI Prism rhodamine dye primer cycle-sequencing kit followed by direct DNA sequencing using FS AmpliTaq DNA polymerase with an ABI Prism rhodamine dye primer cycle-sequencing kit following the protocol of the supplier. Sequencing products were analyzed on a 373A Sequencer (Applied Biosystems).

**Construction of Plasmids and Recombinant Adenoviral Vectors**—The human wild type PDS cDNA was generated by reverse transcriptase– PCR using total RNA from normal human thyroid tissue and Pfu polymerase (Stratagene, La Jolla, CA). After PCR amplification with primers containing appropriate linkers, the PDS cDNA was subcloned into the XhoI and BamHI sites of pCMX (31). Construction of the two PDS mutants identified in this patient was performed using the overlap extension method with Pfu polymerase (32). The cDNAs encoding the wild type and the two mutations were also subcloned in-frame and without stop codon into the vector pEGFPN1 (Clontech, Palo Alto, CA) to create fusion proteins with a carboxyl-terminal green fluorescent protein (GFP) tag. The PDS cDNA was also cloned into an amino-terminal His6 epitope vector to create fusion proteins with a carboxyl-terminal His6 tag, opossum kidney cells were transfected with the Ad5 vector, a cassette containing the PDS cDNA. One of the selected MDCK clones stably expressing PDS was infected with 10 plaque-forming units/cell of recombinant adenoviral vector for functional assays in a bicameral system using Costar 3490 Transwell cell culture chambers (Corning Costar, Cambridge, MA). After formation of a monolayer, electric resistance was measured with a Millicell-ERS volthometer (Millipore, Bedford, MA) to assure the formation of an intact polarized monolayer. Cells were assayed after reaching a resistance >200 ohms/cm2. The solution in the lower chamber consisted of Hank’s buffered saline solution containing 410 μM cold NaI and was labeled with Na125I (20 mCi/mmol). Unlabeled uptake solution with 10–6 M cold iodide was added to the upper chamber. Radiolabeled iodide was measured in the lower chamber, the intracellular compartment after lysis of the cells in buffered Hank’s buffered solution containing 1% Triton X-100, and the upper chamber after 5 min.

**Iodide Transport in Transiently Transfected Non-polarized Cells**—JEG-3 choriocarcinoma, COS, and TSA-201 cells, a clone of human embryonic kidney 293 cells (34) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were split into 12-well plates the day before transfection and grown to 80% confluency. pcDNA plasmids containing the wild type or mutant PDS cDNAs or the NIS cDNA, were transfected (500 ng/well) using the calcium-phosphate method. The empty pcDNA vector was included as negative control. In cotransfection experiments of PDS & NIS constructs, the total amount of added plasmid was kept constant by adding empty vector. Iodide uptake was performed 48 h after transfection. Iodide uptake was performed using Hank’s balanced salt solution, 10 μM Hepes, pH 7.4, and 10–5 M cold NaI and was labeled with Na125I (20 mCi/mmol). Intracellular iodide uptake was determined by measuring radiolabeled iodide in the cell lysates, and iodide efflux was determined by measuring iodide release as described previously (35). Perchlorate (100 mM) was used as inhibitor as indicated.

**Immunohistochemistry and Expression of Green Fluorescent Proteins**—For immunohistochemical experiments, MDCK cells were grown as monolayers on Transwell cell culture chambers. After fixation in 4% paraformaldehyde, the polycarbonate membrane was removed and paraffin-embedded in vertical orientation. For the detection of pendrin, slides were rehydrated and mounted with a primary chicken IgY antibody against the carboxyl-terminal epitope CZETELTEEELDVQDEAMRT (Aves, Tigard, OR) or a rabbit anti-NIS antibody kindly provided by Dr. Nancy Carrasco (New York, NY). The secondary antibody was a biotin-streptavidin-conjugated donkey anti-chicken antibody (Jackson ImmunoResearch laboratories) or a mouse anti-rabbit (DAKO) labeled with horseradish peroxidase. Cells were counterstained with Mayers hematoxylin.

For the detection of pendrin fused to a amino-terminal or carboxyl-terminal His tag, opossum kidney cells were transfected with the respective cDNAs. The cells were fixed in 1% formaldehyde with or without the addition of 1% saponin incubated with a primary anti-His antibody and subsequently with a fluorescein-labeled secondary goat anti-rabbit antibody. Expression of fusion proteins was confirmed using an anti-His antibody and a fluorescein-labeled secondary goat anti-rabbit antibody. Expression of fusion proteins was confirmed using an anti-His antibody and a fluorescein-labeled secondary goat anti-rabbit antibody.
Results

Identification of Compound Heterozygous Mutations in the PDS Gene—Sequence analysis revealed that the patient was compound heterozygous for a novel and a recently described PDS gene mutation. One allele was found to harbor insertion 916insG in exon 7 (Fig. 1). This alteration results in a frameshift beginning at codon 306 and leads most likely to a premature stop in codon 309 (FS306→309X). Alternatively, it could result in altered splicing. The second allele had a transversion 2027T→A in exon 17, resulting in the substitution of leucine at position 676 by glutamine (L676Q).

Pendrin-mediated Apical Iodide Transport in Polarized Cells—A bicameral system using polarized MDCK monolayers was used to study pendrin-mediated iodide transport at the apical membrane. MDCK cells expressing NIS, pendrin, or NIS...
and pendrin were exposed to a solution containing 10^{-6} \text{M} \text{NaI} labeled with 20 mCi/mmol 125I in the lower chamber (Fig. 2D). Untransfected MDCK cells served as a negative control. Cells stably transfected with NIS showed a significant uptake in intracellular iodide transport (Fig. 2D). The release into the apical chamber was higher than in untransfected MDCK cells, most probably because of unspecific transport across the apical membrane following the electrochemical gradient. In contrast, cells expressing NIS and infected with the PDS adenovirus showed significant iodide transport into the apical chamber and a significant drop in intracellular iodide content. Cells expressing only pendrin did show lower intracellular iodide levels than wild type MDCK cells but higher levels in the upper chamber. Values are the means of triplicates ± S.E.

Pendrin-mediated Iodide Transport in Non-polarized Cells—Transiently transfected JEG-3 cells expressing NIS alone showed a significant perchlorate-sensitive increase in the uptake of iodide compared with cells transfected with empty vector (Fig. 3). In cells transfected only with PDS, iodide uptake was not increased in comparison to control cells at concentrations in the physiologic range of 10^{-6} \text{M} \text{NaI}. Consistent with our findings in the bicameral system (Fig. 2D), pendrin reduced NIS-mediated intracellular iodide accumulation to control levels in coexpression experiments. In contrast, coexpression of the two mutants (L676Q + NIS; FS306->309X + NIS) did not result in a decrease in intracellular iodide accumulation in comparison to cells transfected with NIS only (Fig. 3).
4). Similar results were observed in TSA-293 cells (results not shown).

In iodide efflux studies, cells transfected with NIS alone demonstrated a time-dependent iodide efflux (Fig. 5). In contrast, cells cotransfected with wild type PDS and NIS exhibited a very rapid efflux of iodide. Cells transfected with mutant PDS and NIS showed an impaired efflux compared with the cells expressing wild type pendrin, and the efflux was similar to cells expressing only NIS (Fig. 5).

Immunohistochemistry and Expression of Green Fluorescent Proteins—MDCK cells expressing NIS grown as monolayers on polycarbonate membranes showed immunopositivity at the basolateral membrane (Fig. 6B). In contrast, pendrin immunopositivity in cells expressing pendrin was localized at the apical membrane (Fig. 6C).

Staining of cells expressing fusion proteins of wild type pendrin with an amino-terminal or a carboxyl-terminal His6 epitope revealed that the amino terminus is located intracellularly (Fig. 7, A and B) and confirmed a previous observation that the carboxyl terminus, initially proposed to be extracellular (2), is also facing the cytosol (Fig. 7, C and D) (28).

A fusion protein of pendrin with a carboxyl-terminal green fluorescent protein showed a staining pattern consistent with membrane insertion (Fig. 8). In contrast, the two naturally occurring mutations displayed a markedly distinct staining pattern suggesting retention of the proteins in intracellular compartments (Fig. 8).

DISCUSSION

Pendrin, a member of the solute carrier family 26A, has been shown to transport chloride and iodide but not sulfate into Xenopus oocytes (21). Subsequent studies demonstrated an exchange of chloride and formate in oocytes (23) and an exchange of chloride with bicarbonate, hydroxide, and formate in transfected human embryonic kidney cells (24). The absence of bicarbonate secretion was also demonstrated in renal tubuli isolated from mice with targeted disruption of the Pds gene (25). More recently, studies performed in non-polarized mammalian cells proposed a role for pendrin in mediating iodide efflux (10, 30).

Iodide uptake at the basolateral membrane into the thyroid follicular cell was critically dependent on the sodium-iodide symporter NIS and the sodium gradient created by the Na,K-ATPase (36, 37). After entrance into the follicular compartment, iodide was oxidized and organified, steps that were catalyzed by thyroperoxidase and an H2O2-generating system that involved two recently cloned NADPH oxidases (38–40). Although there is formal evidence for the existence of iodide channels at the apical membrane (41), their exact identity has not been established. In thyroid follicular cells, pendrin was inserted into the apical membrane (28, 29). The apical localization, together with the impaired iodide organization observed in patients with Pendred’s syndrome and the previous demonstration of the ability of pendrin to transport iodide in oocyte or non-polarized cell systems, suggested a possible role in iodide transport into the thyroid follicle (3, 10, 28, 30). Using a polarized cell system, the experiments presented here provides formal evidence that pendrin can mediate iodide efflux at the apical membrane (Fig. 2D).

The kinetics of iodide transport in thyroid cells are complex because they include various compartments (blood, cell, and lumen) and concomitant influx, efflux, and organification events (36). Consistent with observations in thyroid follicles (36), MDCK cells, cultured in a dual chamber system and expressing both NIS and pendrin, concentrate iodide in the upper chamber (Fig. 2D). Because of the electrochemical gra-
Pendrin-mediated Iodide Transport

and D meabilization of the cells (amino-terminal His6 epitope only showed immunopositivity after per -

FIG. 6. Immunohistochemistry of MDCK cells grown on polycarbonate membranes. A, unstained untransfected MDCK cells. B, cells expressing NIS stained with a polyclonal anti-NIS antibody show immunopositivity at the basolateral membrane. C, cells expressing pendrin stained with a polyclonal anti-pendrin antibody show immunopositivity at the apical membrane (magnification ×630).

dient existing between the negatively charged cytosol and the positively charged follicular lumen (36), it seems more likely that pendrin is exerting its function as an anion channel rather than as an outward rectifying transporter.

Thyrotropin rapidly stimulates iodide efflux in FRTL-5 cells, which lack a distinct polarity, and in polarized porcine thyrocytes (42–44). In the latter system, bidirectional measurements indicate that thyrotropin stimulates iodide efflux in apical direction, leaving efflux in the basolateral direction unchanged, thus facilitating vectorial transport of iodide into the

FIG. 7. Expression of pendrin with an amino-terminal or carboxyl-terminal His6 epitope. A and B, wild type pendrin fused to an amino-terminal His6 epitope only showed immunopositivity after per-

FIG. 8. Expression of wild type and mutant pendrin. The expression of wild type (wt) and mutant pendrin was determined using green fluorescent fusion proteins. In comparison to wild type pendrin, which shows a staining pattern consistent with membrane insertion, the expression pattern of both mutants (L676Q; FS306→309) was distinct and suggestive of retention in intracellular compartments.

The expression of NIS and pendrin in the heterologous polarized system used in this study will permit further characterization of the regulation and kinetics of pendrin-mediated iodide efflux.

Despite the complete loss of iodide transport of the two studied mutations (Figs. 4 and 5), the organification defect is only partial in this sporadic patient with Pendred’s syndrome, a finding that is typical for the majority of patients with this disorder (7). Moreover, this patient is euthyroid, as are most individuals with Pendred’s syndrome with sufficient nutrient iodide intake and without other forms of thyroid disease. These observations indicate that iodide may reach the follicular lumen independent of pendrin, as also indirectly supported by the absence of a thyroid phenotype in mice with a targeted disruption of the Pds gene (25). In addition to pendrin-mediated transport, the efflux of iodide into the follicular lumen is facilitated by the electrochemical gradient and could occur through unspecific channels, e.g. chloride channels, or a second iodide channel. It is noteworthy that studies performed with plasma membrane vesicles suggested the existence of two apical iodide channels (41). One of these channels appears to display a high permeability and specificity for iodide ($K_m$ = 70 μM), whereas the second anion channel has a lower affinity for iodide ($K_m$ = 33 mM) (41). The pendrin-mediated apical iodide transport shown here, together with the loss of function of pendrin mutations, strongly suggest that pendrin corresponds to one of these channels. Whether the recently identified cloned channel referred to as human apical iodide transporter SCL5A8 was indeed involved in apical iodide efflux in thyroid follicular cells is currently unknown (45).

PDS gene mutations displayed significant allelic heterogeneity and included missense, nonsense, and splice site mutations (2, 5–9). Several mechanisms could account for the loss of function associated with these mutations. They include mRNA instability, retention of the mutated proteins in intracellular compartments, or inactivation of domains that are essential for anion transport despite insertion into the plasma membrane. One of the two novel mutations characterized here, 916insG, leads to a frameshift and premature truncation of pendrin (Fig. 1). Therefore, it is not surprising that it is retained within intracellular compartments (Fig. 8). The second mutation (2027T>A, L676Q) is located within a highly conserved region of the sulfate transporter and anti-$\sigma$ factor antagonist (STAS) domain found in the transporters of the SCL26 family, a region that may have a NTP binding function (46). This mutation is predicted to disrupt an $\alpha$-helical domain and also results in inactivation of the protein by intracellular retention (Fig. 8). Thus, as previously shown for other pendrin mutations (10, 11), both mutations result in altered protein trafficking and at least some of the pendrin mutations appear to result in an endoplasmic reticulum storage disorder (47). As in other forms of endoplasmic reticulum storage disorders, a subset of the mutants retained in intracellular compartments may retain biological
activity, although their processing is altered (Type A), whereas others have no functional activity (Type B) (47). Among the particularly prevalent missense mutations of pendrin, some may fall into the Type A category and become amenable to therapeutic strategies that modify the quality control in the endoplasmic reticulum, thus allowing potentially functional molecules to reach the plasma membrane.

In conclusion, this study presents evidence for pendrin-mediated apical iodide efflux from polarized mammalian cells. Moreover, we demonstrate complete loss of function of two novel pendrin mutations that are both retained in intracellular compartments, most likely the endoplasmic reticulum and intracellular location of the amino terminus. Aside from further studies addressing the kinetics of pendrin-mediated iodide transport, the characterization of pendrin mutants will be of great importance in establishing structure-function relationships of the transporter and determining genotype-phenotype correlations.

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