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Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations

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Cystic fibrosis (CF) is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR). A single recessive mutation, the deletion of phenylalanine 508 (ΔF508), causes severe CF and resides on 70% of mutant chromosomes. Severe CF is also caused by premature stop mutations, which are found on 5% of CF chromosomes. Here we report that two common, disease-associated stop mutations can be suppressed by treating cells with low doses of the aminoglycoside antibiotic G-418. Aminoglycoside treatment resulted in the expression of full-length CFTR and restored its cyclic AMP-activated chloride channel activity. Another aminoglycoside, gentamicin, also promoted the expression of full-length CFTR. These results suggest that treatment with aminoglycosides may provide a means of restoring CFTR function in CF patients with this class of mutation.

The efficiency of translation termination varies as a function of the sequence context surrounding stop codons in a variety of organisms[1–4]. To determine whether context effects can cause the functional suppression of disease-causing premature stop mutations in human cells, we assayed for the production of full-length cystic fibrosis transmembrane conductance regulator (CFTR) from cDNAs containing two naturally occurring premature stop mutations that cause cystic fibrosis (CF). The mutations examined introduce an in-frame ochre (UGA) stop codon in place of glycine residue 542 (G542X) or arginine residue 553 (R553X) of CFTR. Each of these mutations occur near the end of the first nucleotide binding domain of CFTR[5,6]. HeLa cells infected with vaccinia-T7 were cotransfected with the plasmid vector pTM1 carrying the indicated *CFTR* allele under T7 promoter control[7,8]. Following transfection of a wild-type *CFTR* cDNA into vaccinia-T7-infected HeLa cells, CFTR expression was readily observed by immunoprecipitation, and its function was detected with an anion permeability assay utilizing the halide-sensitive fluorophore, 6-methoxy-*N*-(3-sulfopropyl) quinolinium (SPQ)[9]. However, we were unable to detect either full-length CFTR or an increase in anion conductance from cells transfected with *CFTR* cDNAs containing either the G542X or the R553X mutations. This indicates that readthrough of these premature stop mutations (to the extent detectable by these assays) does not occur under normal conditions.

We next examined whether the suppression of these premature stop mutations could be induced by pharmacological treatment. Treatment with a low concentration of aminoglycosides can stimulate the suppression of stop codons in various organisms[10–13]. To test initially whether aminoglycosides can stimulate the suppression of premature stop mutations within the *CFTR* messenger RNA, we incubated cells transfected with the *CFTR* R553X construct with different concentrations of the aminoglycoside G-418 for 8–12 hours. We observed a dose-dependent increase in the expression of full-length CFTR from the R553X mRNA as a function of G-418 concentration, indicating that G-418 stimulates readthrough of the R553X mutation (Fig. 1*a*). Quantification showed that the amount of full-length CFTR produced was as much as 25% of the level of protein expression obtained from the wild-type *CFTR* cDNA. Even more

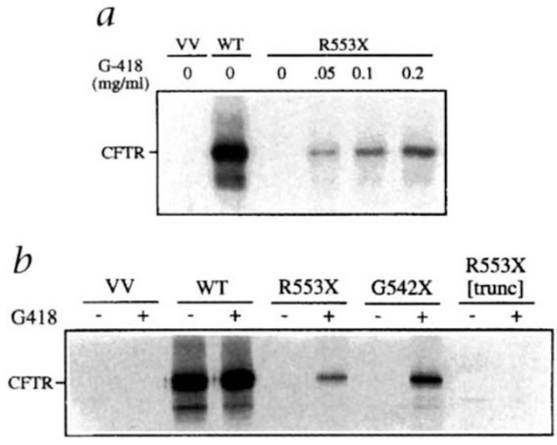

Fig. 1 Expression of full-length CFTR from the *CFTR* G542X and R553X cDNAs in the presence of G-418. *a*, Dose-dependent translational readthrough of the R553X mutation with increasing G-418 concentration. *b*, Suppression of the G542X and R553X mutations by 0.1 mg/ml G-418.

full-length CFTR (35% of wild type) was observed in cells transfected with the *CFTR* G542X cDNA (Fig. 1*b*), indicating that G-418 also promotes readthrough of this second *CFTR* mutation. G542X is the most common premature stop mutation found in CF patients[14,15]. In contrast, full-length CFTR was not detected in cells expressing a 5′ portion of the *CFTR* R553X cDNA truncated after the codon for amino acid 699 of CFTR. This confirmed that the intact *CFTR* cDNA was required for expression of the translation product observed upon G-418 treatment.

Recent studies suggest that the suppression of premature stop mutations occurs through a mechanism of near-cognate mispairing of an aminoacyl-tRNA with the premature stop codon[16]. Because the amino acid inserted by this mechanism may differ from the amino acid encoded in the wild-type protein, we next used the SPQ assay to determine whether CFTR's function as a cAMP-activated chloride channel was also recovered upon G-418 treatment. cAMP treatment of cells transfected with the wild-type *CFTR* cDNA caused a rapid increase in SPQ fluorescence (Fig. 2), consistent with stimulation of CFTR-mediated halide efflux. This response required CFTR expression, as no increase in fluorescence was observed when cells infected with vaccinia-T7 alone were treated with cAMP. As discussed above, cells expressing either the G542X or R553X cDNAs in the absence of aminoglycosides showed no cAMP-dependent increase in anion permeability. However, after incubation with G-418, cAMP induced a significant anion efflux in cells transfected with either the G542X or R553X cDNA. This indicates that the full-length CFTR expressed from these mutant constructs following aminoglycoside treatment also functions as a cAMP-stimulated anion channel.

To determine whether the truncated forms of CFTR produced by translation termination at either residue 542 or 553 might be activated to a functional state by aminoglycoside treatment, we next asked whether cAMP-activated anion efflux could be induced in cells transfected with the *CFTR* R553X cDNA truncated distal to the stop codon. We were unable to detect cAMP-dependent anion permeability in cells expressing this truncated cDNA in the presence of G-418 (Fig. 2*b*). Thus, the portion of the *CFTR* cDNA distal to the stop mutation is required for restoration of cAMP-activated chloride channel activity, indicating that this activity is attributable to the expression of full-length CFTR.

Currently, some aminoglycosides are aerosolized into the lungs of CF patients to treat bacterial infections. To determine whether these clinical aminoglycosides are also capable of inducing readthrough in human cells, we next used our vaccinia-based readthrough assay system to ask whether two commonly used compounds, tobramicin and gentamicin, could stimulate readthrough of the G542X or R553X mutations in HeLa cells. We were unable to detect full-length CFTR following treatment with tobramicin, but a small amount of full-length CFTR was observed by immunoprecipitation following treatment with gentamicin (Fig. 3). However, we were unable to reproducibly detect an increase in the cAMP-stimulated anion permeability by SPQ fluorescence, possibly because this assay is not sufficiently sensitive to quantify the small amount of CFTR produced.

It is possible that a general increase in the suppression of stop codons might lead to the accumulation of toxic, nonfunctional readthrough products. However, in these studies we did not find evidence that low level G-418 treatment significantly impaired normal cellular functions. Exposure of HeLa cells expressing wild-type CFTR to G-418 did not affect the total amount of CFTR synthesized (Fig. 1*b*), their functional response to cAMP stimulation (Fig. 2*b*), or total protein synthesis rates (data not shown).

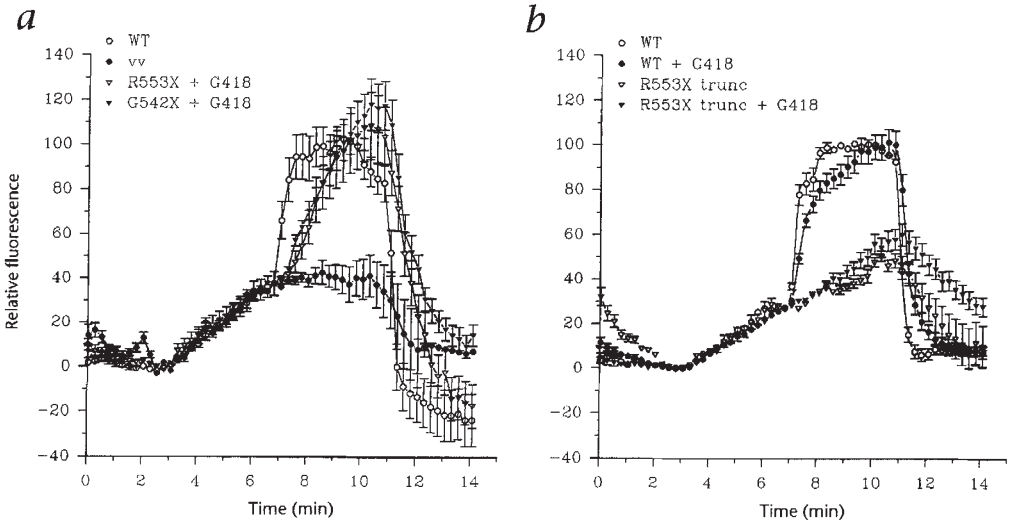

Fig. 2 Functional CFTR expression monitored as cAMP-induced anion efflux using the halide-sensitive fluorophore, SPQ. A cAMP stimulation cocktail was added to the bath at 6 min, and iodide was returned to the bath at 10 min (see Methods section for further details). *a*, G-418 increases cAMP-stimulated anion channel activity in cells expressing the G542X or R553X cDNAs. *b*, G-418 stimulation of cAMP-stimulated anion channel activity requires an intact *CFTR* cDNA.

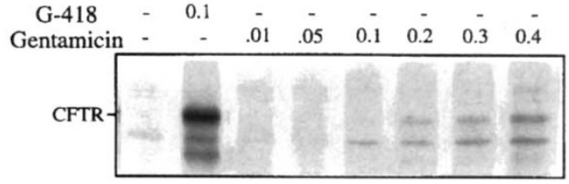

Fig. 3 Dose-dependent stimulation of full-length CFTR synthesis from the *CFTR* R553X cDNA with gentamicin. Gentamicin concentrations used are indicated in milligrams per milliliter.

Furthermore, it is well documented that relatively efficient suppressor tRNAs that promote readthrough of stop mutations can be maintained in several organisms (including human cell lines) without adverse affects[17–19].

Premature stop mutations account for approximately 5% of the total mutant alleles in CF patients[14,15]. However, in certain subpopulations the incidence of this class of mutation is much higher. For example, the W1282X mutation is the most common CF-causing mutation in the Ashkenazi Jewish population, where it is present on 60% of all CF chromosomes[20]. Our findings raise the possibility that the aerosolized delivery of aminoglycosides to the airway may promote the production of full-length CFTR through the suppression of premature stop mutations in lung epithelia. If successful, this approach would represent the first clinical treatment capable of correcting CF by restoring the expression of functional, endogenous CFTR in a specific genotypic subgroup of CF patients.

Methods

Infections and transfections. HeLa cells were infected with vaccinia-T7 (cTF7-3) at a multiplicity of infection (MOI) of 10. Transfection of vaccinia-T7 infected cells with pTM1 carrying the indicated *CFTR* allele were carried out using lipofectin (BRL) as described[7,8].

Immunoprecipitation of CFTR. Cells were treated with aminoglycosides for 8 h at the concentrations indicated, then labeled with [^{35}S]methionine for 1 h before cell lysis. CFTR was immunoprecipitated using a monoclonal antibody to the extreme carboxy terminus (Genzyme). The proteins were then resolved by SDS–PAGE and visualized by fluorography.

Halide efflux assays. HeLa cells grown on glass coverslips were loaded for 10 min in a hypotonic iodide buffer containing 10 mM SPQ; iodide quenches SPQ fluorescence[9]. This hypotonic buffer was made by diluting an isotonic iodide buffer (130 mM NaI, 4 mM KNO_3, 1 mM $Ca(NO_3)_2$, 1 mM $Mg(NO_3)_2$, 1 mM Na_2HPO_4, 10 mM glucose, 20 mM Hepes, pH 7.4) 1:1 with water. Cells were then returned to the isotonic iodide buffer to recover for 5 min. The experiment measuring SPQ fluorescence was initiated in this same buffer. NaI in the bath was replaced by $NaNO_3$ at 2 min; because nitrate does not interact with SPQ, fluorescence increases as cell iodide is lost to the bath[9]. A cAMP stimulation cocktail (10 µM forskolin, 100 µM cpt-cAMP and 100 µM IBMX) was added at 6 min. Fluorescence was then quenched again by returning NaI to the bath at 10 min. Functional CFTR expression was monitored as the dequenching of SPQ fluorescence caused by cAMP-induced iodide efflux.

Truncation of the *CFTR* cDNA. The plasmid carrying the *CFTR* R553X cDNA was truncated distal to the premature stop mutation by digestion with *Eco*RI and *Sac*I. The cohesive ends were removed by treatment with the Klenow fragment of DNA polymerase I and the plasmid was then religated. This treatment removed the *CFTR* structural gene from the *Eco*RI site at position 2230 through the *Sac*I site at position 4651 (76 nucleotides beyond the natural termination codon). This resulted in the loss of the distal 2346 nucleotides of the coding sequence in the *CFTR* cDNA.

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