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A novel CFTR disease-associated mutation causes addition of an extra N-linked oligosaccharide

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We have examined the influence of a novel missense mutation in the fourth extracytoplasmic loop (EL4) of CFTR detected in a patient with cystic fibrosis. This substitution (T908N) creates a consensus sequence (N X S/T) for addition of an N-linked oligosaccharide chain near the C-terminal end of EL4. Oligosaccharyl transferase generally does not have access to this consensus sequence if it is closer than about twelve amino acids from the membrane. However, the T908N site is used, even though it is within four residues of the predicted membrane interface and the oligosaccharide chain added binds calnexin, a resident chaperone of the ER membrane. The chloride channel activity of this variant CFTR is abnormal as evidenced by a reduced rate of $^{36}Cl^-$ efflux and a noisy single channel open state. This may reflect some displacement of the membrane spanning sequence C-terminal of EL4 since it contains residues influencing the ion pore.

Keywords: **CFTR, mutation, glycosylation, ion channel**

Abbreviations: **RIPA buffer: 0.1% sodium lauryl sulfate, 1% deoxycholate, 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4; IBMX: isobutylmethylxanthine; DBcA: dibutyryl cyclic AMP; DMSO: dimethyl sulfoxide; 894N, 900N, 908N, 910N indicate the positions of asparagine residues to which oligosaccharide chains are attached**

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a polytropic membrane glycoprotein which forms a chloride ion channel in the apical membrane of salt secreting or reabsorbing epithelial cells of many tissues [1–3]. Although a predominant single codon deletion mutant (ΔF508) resulting in biosynthetic misprocessing and intracellular mislocalization is responsible for most disease [4–6], more than 900 different mutations have been detected in patients (Cystic Fibrosis Genetic Analysis Consortium. http://www.genet.sickkids.on.ca/CFTR). These include all known types of mutations. However, while analyzing the functional impact of missense mutations in the putative extracytoplasmic loops (ELs) of the protein [7], we realized that one (T908N) created a consensus sequence (N X S/T) for N-glycosylation. This site is in the same loop (EL4) as the two sites known to be N-glycosylated at residues 894 and 900 of wild-type CFTR but is located within four residues of the predicted luminal interface of the membrane bilayer. Because it is generally considered that oligosaccharyl transferase cannot access such sites if they are closer than 12–14 amino acids [8–12] it was thought unlikely that the variant N908 would be used. However, reconstruction and heterologous expression in mammalian cells indicated that the site was used regardless of the occupancy of the other two native sites, forming a mature protein that reached the cell surface and functioned as a regulated chloride channel. Channel activity was modified, however. This provides the first example of a disease-associated mutation resulting in introduction of a novel N-glycosylated site into a glycoprotein and modulation of its function.

Materials and methods

Cell culture and stable transfection of CFTR

The BHK cells expressing wild type CFTR have been described previously [13,14]. BHK cells stably expressing T908N-CFTR were similarly established. Tunicamycin was added to the culture medium to a final concentration of 5 μg/ml where indicated.

In vitro mutagenesis of CFTR cDNA

A *Dra*III fragment in wild-type pNUT/CFTR [14] was replaced with the counterpart generated by PCR for N984D,

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N900D, T908N or T910N to produce a CFTR with different numbers or no consensus site for glycosylation on extracytoplasmic loop 4. The sequences of fragments generated by PCR were verified by DNA sequencing after insertion into the pNUT expression vector.

Metabolic labeling with [^{35}S]methionine

Cells were metabolically labeled with [^{35}S]methionine as described [15]. For the following chase, the [^{35}S]methionine containing medium was replaced by complete medium containing 5% fetal bovine serum and 1 mM methionine.

Cell lysis and immunoprecipitation

Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 20 mM $NaMoO_4$ and 0.09% N-P40) plus a mixture of protease inhibitors as previously described [15]. Following centrifugation at 4°C at 15 000 g for 15 min, the supernatant was incubated overnight with the monoclonal antibody M3A7 (Kartner *et al*., 1992) followed by a 4 h incubation with Protein G-agarose (Gibco-BRL). After 4 washings with RIPA buffer, attached complexes were dissolved in electrophoresis sample buffer.

Co-immunoprecipitation of CFTR and Calnexin

CFTR expressing cells were lysed in 1 ml NP-40 lysis buffer. Cell lysates were incubated over night at 4°C with the monoclonal antibody M3A7 and the immune complexes were precipitated with 25 µl packed Protein G beads (Gibco-BRL). The protein G beads were washed four times with NP4O lysis buffer. Precipitated proteins were eluted by addition of 10 µl 10X electrophoresis sample buffer and incubated at room temperature for 1 h. Samples were analyzed by SDS-PAGE followed by immunoblot with a calnexin specific rabbit antibody (StressGen) or polyclonal CFTR specific antibody 155 [16] and enhanced chemiluminescence (Pierce).

Glycosidase digestion

Cells were lysed with NP-40 lysis buffer. These samples (20 µg total protein) were then incubated for 2 h at RT with either 0.25 U N-Glycosidase F (Boehringer Mannheim), 15 mU Endoglycosidase H (Boehringer Mannheim) or 15 mU Sialidase (CalBiochem). Reactions were stopped by adding electrophoresis sample buffer.

SDS-PAGE and immunoblotting

Immunoprecipitates or total cell lysates were electrophoresed on 6% acrylamide gel, transferred to nitrocellulose membranes (Bio-Rad) and probed with M3A7. Detection by enhanced chemiluminescence (Pierce) was according to the protocols described by Seibert *et al.* [13].

$^{36}Cl^-$-Efflux assay

Cells were grown to confluency in a 6 well microtitre dish and washed twice with efflux buffer (20 mM HEPES pH 7.4, 11 mM Glucose, 2 mM Ca_2NO_3, 2 mM Mg_2NO_3, 2 mM KNO_3, 135 mM $NaNO_3$). Cells were loaded for 1 h at RT with 0.5 ml efflux buffer containing 1 µlCi $^{36}Cl^-$. Loaded cells were washed three times with 1 ml efflux buffer at 1 min intervals. 0.5 ml samples were collected into 24 well Topcount plates and replaced with equal volume of efflux buffer. At time 0, cells were stimulated with efflux buffer containing 10 µM Forskolin, 1 mM IBMx, 100 µM DBcA or DMSO as control. 1 ml of Microscint scintillation fluid (Packard) was added to collected samples and analyzed in a Topcount scintillation counter (Packard).

Single channel records

Microsomal vesicles were isolated from BHK cells expressing wildtype and T908N CFTR and phosphorylated with protein kinase A [17]. CFTR single channels were incorporated into the lipid bilayer by fusion of the vesicles with a preformed planar bilayer. Single channel records were collected using symmetrical solutions containing 300 mM Tris-HCl pH 7.2, 1 mM EGTA, 3 mM $MgCl_2$. Na_2ATP (2 mM) was added to the cis compartment. Single channel currents were recorded using an Axopatch 200A amplifier (Axon Instruments) digitized at 50 Hz and recorded.

Results

Figure 1A schematically illustrates the location of residue T908 that is replaced by an asparagine due to this mutation, thereby forming an NST consensus sequence for N-glycosylation. Reconstruction and expression of the T908N variant in BHK cells resulted in the synthesis of a protein which ran with reduced mobility in SDS-PAGE (Figure 1B) consistent with the possibility that this additional N-glycosylation site had been used. To confirm that this mobility shift is due to increased N-glycosylation, we removed neuraminic acid from oligosaccharide chains by sialidase digestion (Figure 1B, lanes 2 and 6) and the entire chains by cleavage of asparagine-N-acetylglucosamine linkages with N-glycosidase F (Figure 1B, lanes 3 and 7). The sialidase treatment increased the mobility of both the wild-type and the T908N variant substantially, indicating that the oligosaccharide chains are highly sialylated. After N-glycosidase F treatment the mobility is increased further and the deglycosylated form of both the wild-type and T908N migrate to the same position. Similar results were obtained when rather than cleaving off oligosaccharide chains, N-glycosylation was inhibited with tunicamycin (Figure 1B, lanes 4 and 8). The difference in mobility between wild-type and T908N was entirely eliminated after cells were grown in presence of the inhibitor. Together these observations strongly indicate that the asparagine at residue 908 provides a third glycosylation site in EL4 which is recognized by

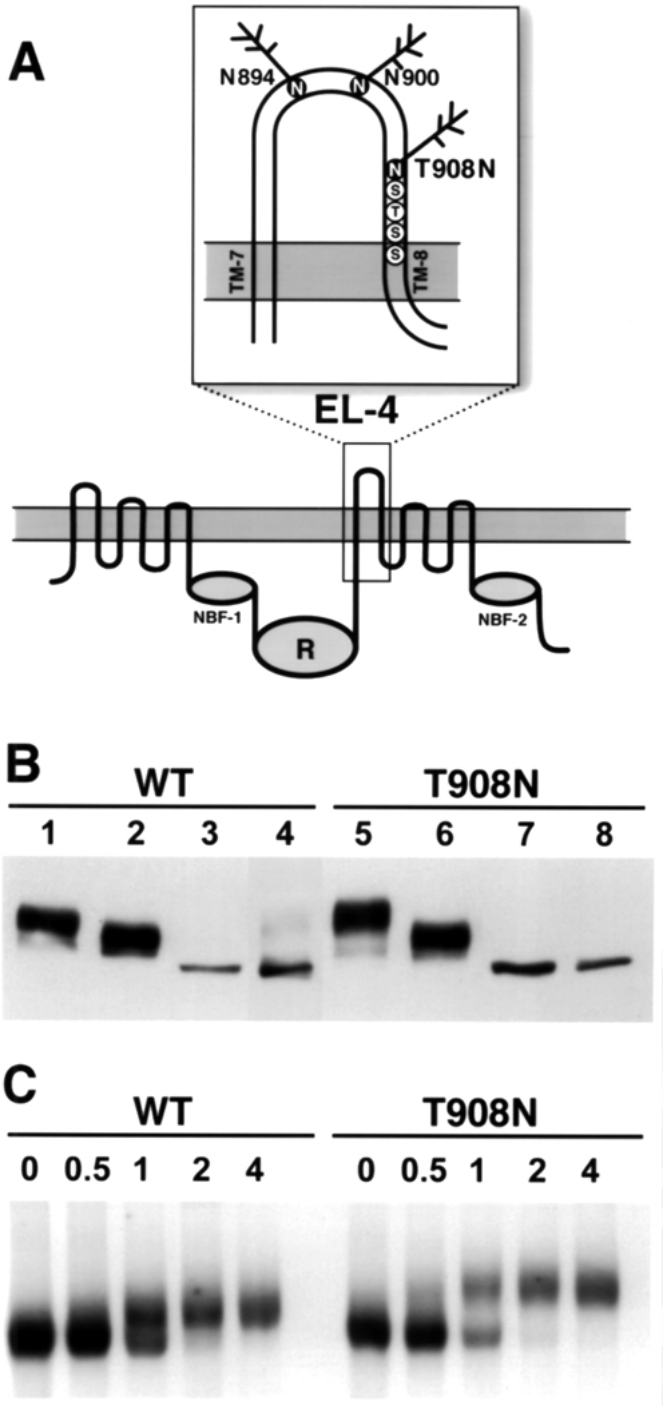

Figure 1. Novel mutant N-glycosylation site at residue N908 is used despite proximity to the membrane. (A) Sketch of CFTR topology indicating position of T908N relative to the membrane and native glycosylation sites at N894 and N900. (B) Cells stably expressing wild type and T908N-CFTR were lysed in NP-40 lysis buffer (see Materials and methods). Total lysates were incubated for 2 h at RT without (lanes 1 and 5) or with the glycosidases (Sialidase: lane 2 and 6: N-Glycosidase F: lane 3 and 7) or cells expressing different CFTR variants were pretreated with tunicamycin (5 μg/ml) for 20 h (lanes 4 and 8). Subsequently 20 μg of total protein samples were analyzed by Western blotting, using M3A7 as the primary antibody [24]. (C) The tri-oligosaccharide chain CFTR matures with the same kinetics as wild type. Cells expressing wild-type and T908N-CFTR were pulse labeled and chased as described in Methods.

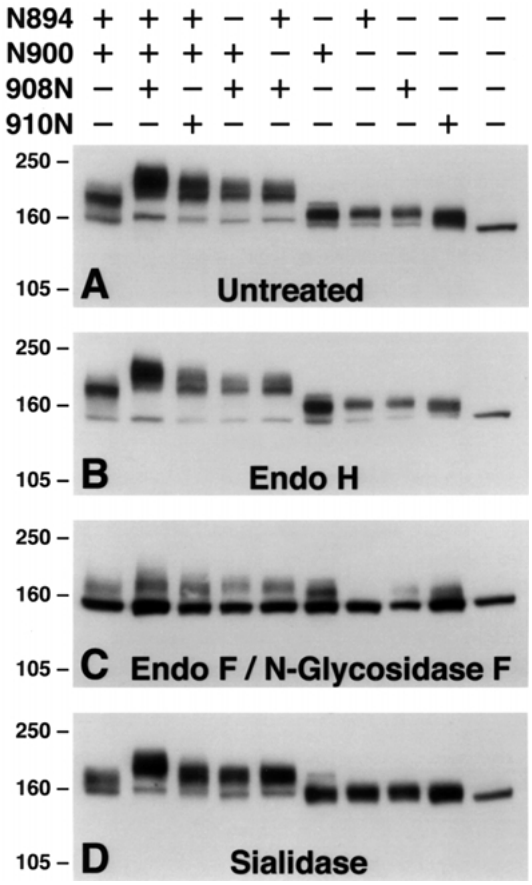

Figure 2. N-glycosylation at multiple or single sites in EL4. (A) Western blots of untreated lysates; (B) after digestion with endoglycosidase H; (C) with a mixture of endoglycosidase F and N-glycosidase F; (D) with sialidase. Cells expressing the different glycosylation variants of CFTR were lysed in NP-40 lysis buffer. 20 μg total lysate protein were incubated for 2 h at RT with or without the indicated enzymes. Subsequently the samples were analyzed by Western blotting, using M3A7 as the primary antibody.

oligosaccharyl transferase and is an acceptor of an N-linked oligosaccharide chain.

To determine the influence of the addition of this third chain on the maturation and stability of CFTR, pulse-chase experiments were performed. Figure 1C indicates that the smaller core-glycosylated precursor band disappears at approximately the same rate as the wild-type so that it is undetectable by 2 h of chase. The larger mature product with complex oligosaccharide chains also appears at a similar rate in both cases. Conversion of precursor to product occurs with low efficiency (~ 30%) but this also is similar with wild-type and the T908N variant. Hence the additional oligosaccharide in T908N does not have a profound effect on the biosynthetic processing of CFTR.

To further characterize the utilization of these three glycosylation sites in EL4 as well as the possible use of a fourth artificially introduced even closer to the predicted membrane interface at residue 910, each was evaluated individually. The mobilities in SDS-PAGE of CFTR variants with zero, one, two or three of these four sites occupied are

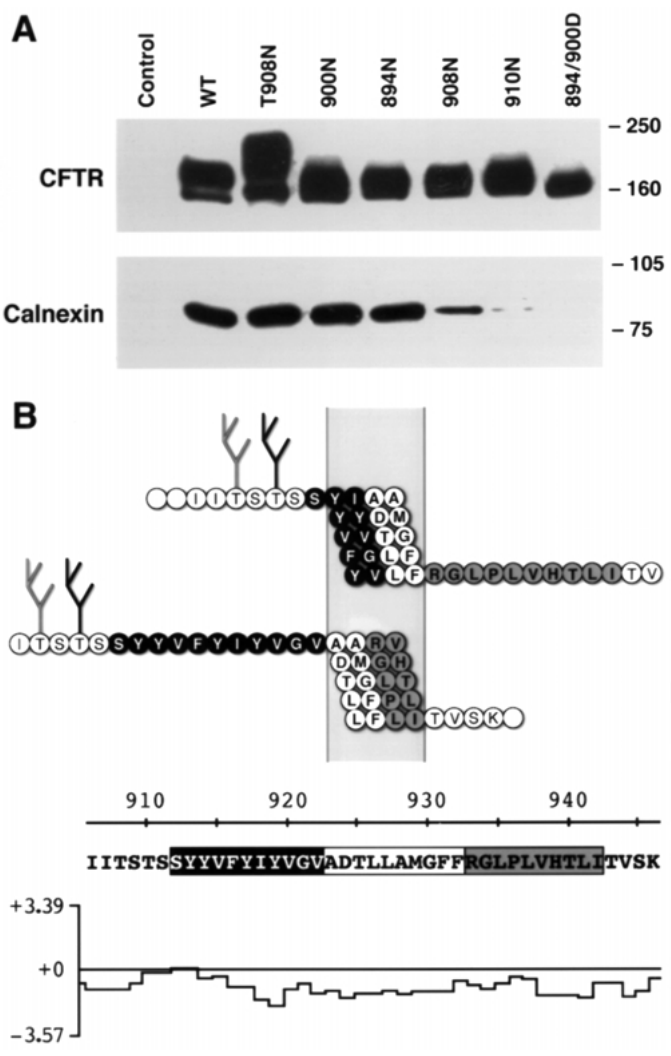

Figure 3. Calnexin association with CFTR variants glycosylated at different sites. (A) Lysates of cells expressing the different CFTR variants were immunoprecipitated with M3A7. The precipitated proteins were separated in a 6% acrylamide gel, transferred to nitrocellulose (BioRad) and probed with either a rabbit polyclonal antibody to CFTR or to calnexin and detected by enhanced chemiluminiscence (Pierce). Calnexin binds only to the most rapidly moving band which is due to the smaller core-glycosylated precursors present in the sample. The Western blot in Figure 3A is exposed for a longer period than the blots in Figures 1 and 2. (B) Sketch indicating position of EL4 and TM8 with oligosaccharide chains added to 908N (light shading) and 910N (black). The upper figure represents the usual predicted topology of CFTR [19, 25] in which the residues in black are the N-terminal half of TM8, the open balls are the C-terminal half of TM8 and the grey balls are residues in the cytoplasmic loop. The lower figure is a suggested alternative topology in which the N-terminal half of TM8 is completely in the extracytoplasmic loop. At the bottom of the figure is a Kyte/Doolittle hydrophilicity plot with relative values shown on the ordinate (window = 7). All residues attributed to TM8

compared in Figure 2A. As already noted in Figure 1 the T908N variant in the wild-type background has substantially decreased mobility. However, the mobility of the T910N variant also is somewhat slower compared to wild-type suggesting that it also can serve as oligosaccharyl transferase acceptor. Interestingly when 908N is available for use in combination with either one of the two native sites, 894N or 900N, the mobility of the mature band is still somewhat less than wild-type where 894N and 900N are used. This might be interpreted to mean that the chain attached to residue 908 could be larger than those at 894 or 900. However when only 894, 900 or 908 are occupied individually each of these three variants migrates with similar mobility, substantially greater than when two sites are occupied but slightly less than that when only N910 is used. Hence there is a suggestion that a smaller chain may be added at the 910 position both in the presence and absence of chains at the two native sites. After endoglycosidase H digestion which removes core but not complex oligosaccharide chains the smaller deglycosylated bands of all of the variants run with the same mobility whereas the larger complex glycosylated bands are unaffected (Figure 2B). This effect is most readily appreciated by noting the greater separation between the larger and smaller bands in Figure 2B than in Figure 2A.

To confirm that the bands with diminished mobility were due solely to the presence of carbohydrate, the wild-type and the glycosylation site variants were digested with N-Glycosidase F, which removes complex as well as core oligosaccharide chains. Figure 2C shows that, indeed, in all cases there was conversion to a band with the mobility of the unglycosylated

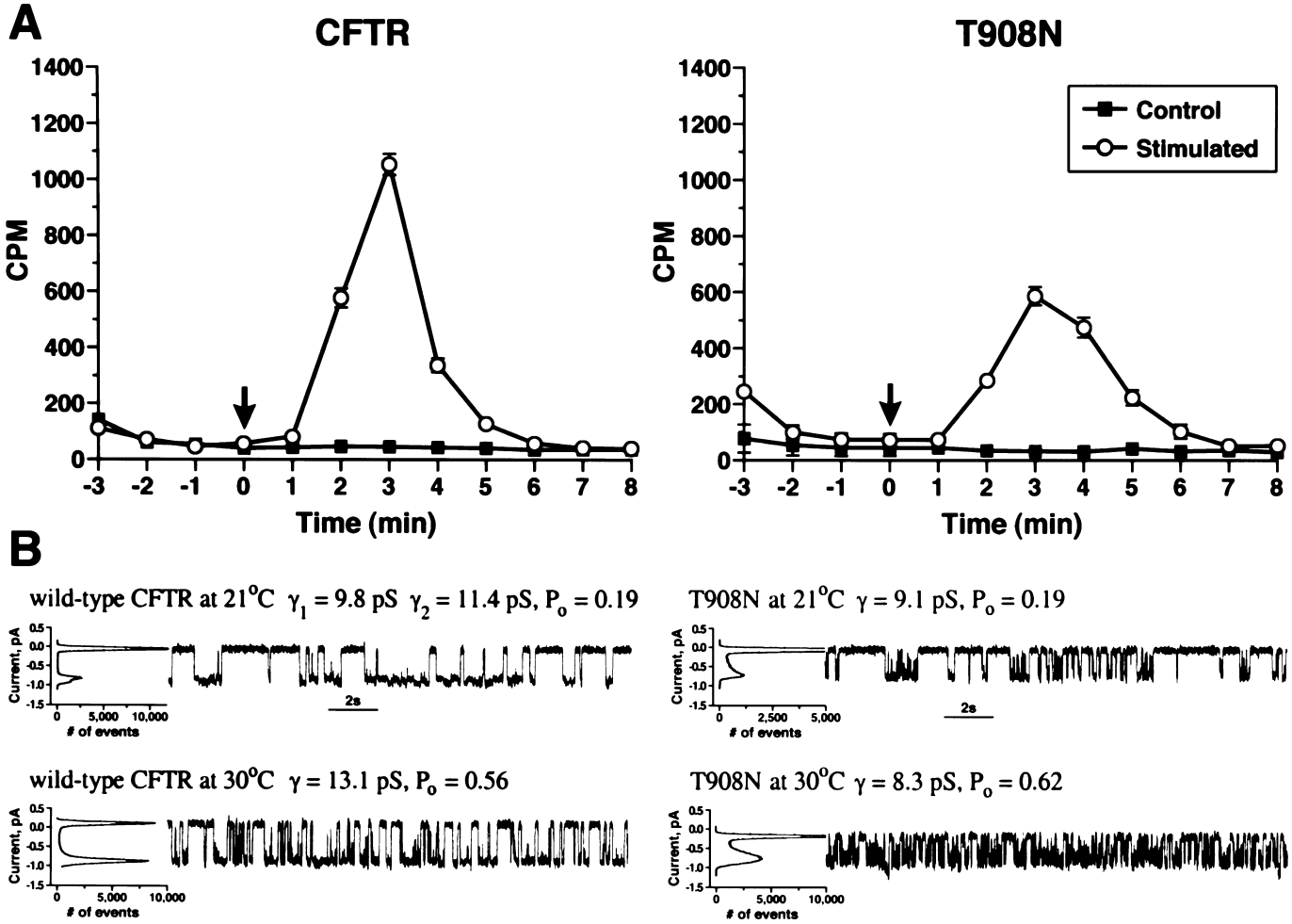

Figure 4. T908N reduces the rate of $^{36}Cl^-$ efflux and alters both the single channel gating kinetics and conductance of CFTR. (A) Cells were incubated with loading buffer containing 1 μCi $^{36}Cl^-$ for 1 h at room temperature. The cells were washed three times with efflux buffer without sodium chloride. From time 0 the efflux buffer contained 10 μM Forskolin, 1 mM IBMX and 100 μM DbcA. The efflux of $^{36}Cl^-$ was determined with a Topcount scintillation counter (Packard). Each point is the average of 3 independent samples. (B) Phosphorylated CFTR single channels were incorporated into the lipid bilayer by fusion of microsomal vesicles with a preformed planar bilayer. Representative

894D/900D. Interestingly, however, the extent of oligosaccharide removal from 894N, 900N, 908N and 910N was markedly different. Removal from position 894 was complete within 2 h (Figure 2C) while removal from positions 900, 908, or 910 remained incomplete even after 24 h or even with a three fold higher enzyme concentration (data not shown).

As shown in Figure 1B, removal of sialic acid from complex glycosylated CFTR results in a higher mobility of wild-type and T908N. Therefore, we investigated the effect of sialidase digestion on the mobility of the different glycosylation variants in SDS-PAGE (Figure 2D). The mobility of the core glycosylated bands of all variants and the unglycosylated N894D/N900D were unaffected by the sialidase treatment. The complex glycosylated bands of all variants had a higher mobility after treatment with the enzyme. There was no significant difference in the mobility of the different single glycosylated species of CFTR after sialidase treatment.

To determine if the location of oligosaccharide chain attachment in EL4 influenced binding to calnexin, the ability of the chaperone to be coimmunoprecipitated with each of the variants was tested. Similar amounts of calnexin were detected in CFTR immunoprecipitates containing wild-type and T908N, and 894N and 900N alone whereas much less was detected when single oligosaccharide chains were present at 908N and especially at 910N (Figure 3). Hence while oligosaccharyl transferase is apparently able to access the latter two non-native sites as well as the native sites, calnexin associates weakly with the monoglucosylated oligosaccharide at 908N and almost not at all with that at 910N.

To assess how the presence of a third oligosaccharide chain influences CFTR function, the rate of $^{36}Cl^-$ efflux from cells expressing the T908N variant was compared with wild-type and was found to be reduced about 50% (Figure 4A). At the single channel level, neither the open probability nor the conductance was much altered at 21°C (Figure 4B). However when recorded at 30°C, conductance is reduced approximately 40% and the open state appears less stable. Thus both measures of the T908N CFTR chloride channel indicate that while still active, it is substantially altered from the wild-type.

Discussion

Although all types of mutations have been detected in the CFTR gene of patients with cystic fibrosis, T908N is the only example of a single residue substitution that introduces a consensus site for glycosylation. When expressed in mammalian cells, this site is used regardless of whether one or both of the wild-type sites in the same EL are occupied. Occupation of this additional or alternative site increases the apparent molecular weight of CFTR but has little or no effect on the synthesis and maturation of the molecule.

In addition to being a novel "gain of structure" mutation this change results in addition of a carbohydrate chain closer to the luminal side of the ER membrane than would have been predicted. As a consequence the chloride channel activity is compromised, possibly because addition of the oligosaccharide in this position may prevent complete incorporation of the next transmembrane segment (TM8; Figure 3B) into the bilayer. Since residues within TM8 have been reported to contribute to the properties of the pore [18] this might be expected to influence its ability to conduct ions.

Although we have no direct evidence that the degree of membrane integration of TM8 may vary as implied in Figure 3B, it is possible that this may occur transiently during translation or even later. Alternatively, it may just be that the predicted boundaries of TM8 are incorrect and extracytoplasmic loop 4 is larger than proposed and cytoplasmic loop 3 between TM8 and TM9 smaller [19]. While the membrane integration of some TMs of CFTR have been studied using *in vitro* translation with microsomal membranes [20], there is no published work specifically with TM8. Tector and Hartl [21] focused on TM6 that contains several charged residues which contribute to the ion pore formed by CFTR. Using a model construct consisting of the N-terminal membrane domain fused to invertase in yeast, they found that residues R334 and K335 in TM6 contributed to limited integration of TM6. TM8 has a single charged residue (D924) located in the middle of the putative helix in the original model but near the interface with the ER lumen when the 908N site is moved 12 residues into the lumen (Figure 3B). Since there is some evidence that D924 may form a salt bridge with R347 near the cytoplasmic end of TM6 [22], this more luminal location of D924 would not seem very likely. Perhaps a location intermediate between the two extremes depicted in Figure 3B may exist or there may be a degree of movement perpendicular to the plane of the membrane.

While oligosaccharyl transferase can utilize the membrane proximal sites, 908N and 910N as effectively as the more distal native sites (N894 and N900), once the core oligosaccharide chains are present the ones closer to the membrane are less reactive with calnexin. This apparent ability of calnexin to access chains closest to the membrane differs from the findings of Andersson *et al*. [23] in another system where the chaperone was found to bind to N-linked glycans quite close to the ER membrane.

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