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A change in gating mode leading to increased intrinsic Cl^- channel activity compensates for defective processing in a cystic fibrosis mutant corresponding to a mild form of the disease

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The effects of the mild cystic fibrosis (CF) mutation P574H were analysed and compared with those of three severe ones (ΔI507, ΔF508 and R560T). Immunochemical and functional analyses indicate that the rank order of CFTR expression at the cell surface is: wild type CFTR > P574H >> ΔF508 >> R560T ~ 0. Patch-clamp analysis indicates that the open probability of P574H Cl^- channels is almost twice as high as that of the wild type CFTR-Cl^- channel. This increased intrinsic activity of individual P574H CFTR-Cl^- channels compensates for the lower number of P574H CFTR-Cl^- channels reaching the cell surface, and probably explains the milder form of CF associated with the P574H mutation. NS004, a recently described activator, restores near normal CFTR activity in cells expressing the P574H-CFTR channel. The P574H mutation modifies the gating mode of the channel with a large increase (~×7) in the mean channel open time. Proline 574 might play an important role in the process connecting ATP hydrolysis at the nucleotide binding domain and opening and closing events of the CFTR-Cl^- channel.

Key words: CF/cystic fibrosis transmembrane conductance regulator/ion channel/mutation/NS004

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) gene encodes a cyclic AMP-regulated chloride channel that belongs to the super-family of the ATP binding cassette proteins (Riordan, 1993; Welsh and Smith, 1993). This 1480-residue protein is composed of five distinct domains. Two correspond to nucleotide binding domains, two correspond to a series of six successive transmembrane segments, and one to a regulatory domain (R) that contains numerous consensus sites for phosphorylation by protein kinases A and C (Riordan *et al.*, 1989). Opening of the channel requires phosphorylation (presumably at the R domain) by protein kinase A (Berger *et al.*, 1991; Tabcharani *et al.*, 1991), and binding of nucleotides to the nucleotide binding domain(s) (Anderson *et al.*, 1991; Baukrowitz *et al.*, 1994). The importance of the nucleotide binding domains in CFTR function is indicated by the deleterious effects of numerous missense mutations in these domains, which are associated with the development of cystic fibrosis (CF) (Tsui, 1992).

Manifestations of the disease (reviewed by Quinton, 1990) are observed in the gastro-intestinal tract [resulting in the formation of a meconium ileus for 17% of the newborns (Kristidis *et al.*, 1992)], the pancreas, the lungs, the sweat glands and the reproductive tract. The severity usually depends on which mutations are affecting the CFTR gene (Kristidis *et al.*, 1992). Severe CF mutations cause a drastic decrease or a total loss of the apical Cl^- permeability in these different tissues by several distinct mechanisms. Most of these mutations block the progress of the CFTR molecules through the biosynthetic pathway (Cheng *et al.*, 1990) in the endoplasmic reticulum, leading to their rapid degradation in a pre-Golgi non-lysosomal compartment (Yang *et al.*, 1993; Pind *et al.*, 1994). Deletion of phenylalanine at position 508 (ΔF508), in the first nucleotide binding domain, is the most common CF-associated mutation, which accounts for 68% of CF chromosomes in caucasian populations (Kerem *et al.*, 1989). ΔF508 has initially been associated with defective protein processing *in vitro* (Cheng *et al.*, 1990) as well as *in vivo* (Denning *et al.*, 1992; Kartner *et al.*, 1992; Puchelle *et al.*, 1993). However, when ΔF508 CFTR was expressed in cultured Vero cells (Dalemans *et al.*, 1991), residual Cl^- channel activity equal to ~3% of the activity observed after expression of the wild type CFTR was recorded, suggesting that under these conditions a small amount of ΔF508-CFTR is correctly processed to the cell membrane.

This work analyses the molecular properties of a CFTR protein expressed with a recombinant vaccinia virus, which carries the mutation P574H, previously shown to be associated with a mild form of the disease (Kristidis *et al.*, 1992). Mutations occurring in the first nucleotide binding domain and leading to a mild form of the disease have not been studied before. The properties of this mutation are compared with those of three severe mutations ΔI507, ΔF508 and R560T, also affecting the first nucleotide binding domain.

Results

Immunolocalization of P574H-CFTR and other CFTR mutant proteins

Recombinant vaccinia viruses expressing wild type or mutant CFTR were constructed to study the effects of

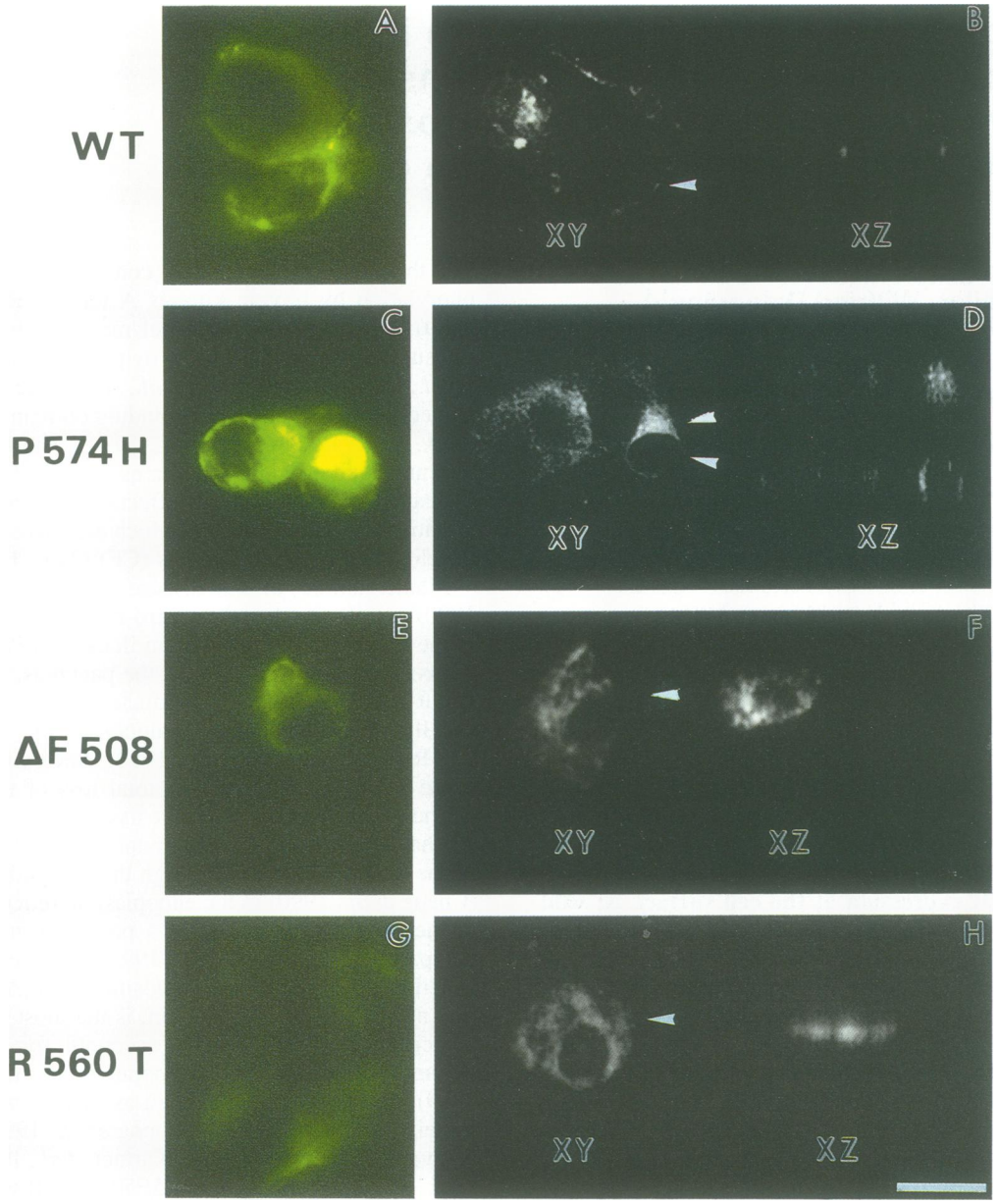

Fig. 1. Immunodetection of wild type, ΔF508-, R560T- and P574H-CFTRs in Vero cells. Standard fluorescence microscopic photomicrographs (**A**, **C**, **E** and **G**) and XY and XZ confocal optical section (**B**, **D**, **F** and **H**) of Vero cells expressing wild type (A and B), P574H- (C and D), ΔF508- (E and F) and R560T-CFTRs (G and H). All the microphotographs have been taken under identical conditions. Bar = 10 μm.

ΔI507, ΔF508, R560T and to compare them with the P574H mutation. Figure 1 shows typical immunofluorescence labelling of CFTR in Vero cells infected with these five recombinant vaccinia viruses. Cells were labelled with a monoclonal antibody raised against the carboxyl terminus of CFTR (mAb C24-I, Genzyme, Framingham, USA), which did not give any background in Vero cells infected with control vaccinia virus (not shown). Cells expressing wild type CFTR exhibited strong cell surface labelling (Figure 1A and B), as previously reported (Dalemans *et al.*, 1992). In cells expressing ΔF508-CFTR, the labelling was weak and mainly restricted to the cytosol (Figure 1E and F), although in a small number of cells, discontinuous and patchy plasma membrane labelling could be identified (not shown). The R560T mutation resulted in very low immunolabelling of R560T-CFTR expressing cells, which was strictly located within the cytosol. For this mutant, the number of negative cells (i.e. not labelled) was very high, and no plasma membrane labelling was detectable (Figure 1G and H). In cells expressing P574H-CFTR, the immunolabelling was mainly intracellular, and preferentially localized in the Golgi region, although some cells also exhibited focal CFTR labelling on the plasma membrane (Figure 1C and D). Because qualitative differences were observed between cells expressing the different mutant proteins, a quantification of the immunofluorescence experiments was carried out (Figure 2). The number of cells that exhibited labelling of the plasma membrane was chosen as an index for the maturation of the different CFTR proteins. Cells were considered as positive when cell-surface labelling could be detected, even if it was focal or incomplete. Figure 2 shows the percentage of cells that were positive for membrane immunolabelling after incubation with the

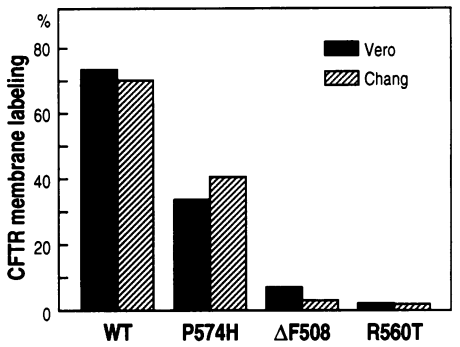

Fig. 2. Analysis of the cell surface expression of wild type, ΔF508-, R560T- and P574H-CFTRs. The number of Vero and Chang cells used for the analysis was equal to 125 and 223 for wild type CFTR, 59 and 288 for ΔF508, 169 and 317 for R560T, 88 and 479 for P574H respectively. Identical results were obtained in two independent experiments and were pooled for the quantification.

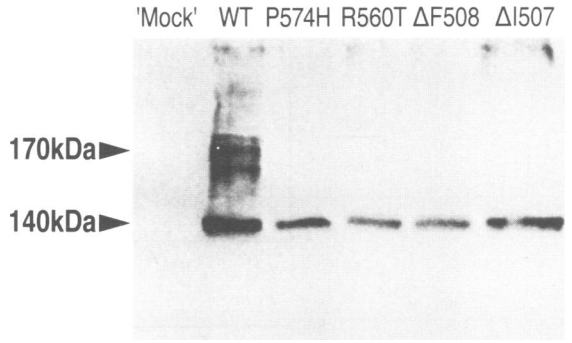

Fig. 3. Western blotting analysis of the 'mock'-infected, wild type, ΔF508-, R560T- and P574H-CFTRs. Chang cells were infected with 5 p.f.u./cell VVTG 1193 [encoding T7 polymerase alone ('mock')] or in combination with 5 p.f.u./cell of vaccinia virus expressing different CFTR proteins. Protein extracts were prepared and analysed 16 h later. The size of core-glycosylated (140 kDa) and fully glycosylated (170 kDa) forms of CFTR are indicated.

monoclonal antibody C24-1. The experiment was performed on Vero and Chang cells, and gave very similar results in both cell types. In wild type CFTR-expressing cells, 70–80% of the WT-CFTR infected cells exhibited cell surface labelling, but also displayed significant labelling of the cytosol. The remaining 20–30% probably represents cells not infected by the viruses. The percentage of cells with positive plasma-membrane labelling was lower for P574H and ΔF508, but remained significantly higher than zero (20–30% for P574H and 2–6% for ΔF508). No R560T-expressing cells exhibited plasma-membrane immunolabelling. These results were not due to any differences in the level of expression of the different constructions, as shown in Figure 3.

Expression and glycosylation of CFTR mutant proteins

Cell extracts prepared from Chang cells infected with recombinant vaccinia viruses expressing wild type or mutant CFTR proteins were analysed by Western blot, using the same monoclonal antibody that was used in immunohistochemistry experiments. Figure 3 shows that a band of 140 kDa, that is known to correspond to an immature form of CFTR, was obtained after infection with all vaccinia viruses. A diffuse band of 160–170 kDa corresponding to the fully glycosylated CFTR was only detected in cells infected with the recombinant virus expressing wild type CFTR, but in some experiments small amounts of 160–170 kDa glycosylated protein could be observed in cells expressing ΔF508 or P574H (not shown). The 140 kDa form of the protein was expressed in similar levels in lanes 2–6, indicating that differences in cell-surface labelling (Figure 2) cannot be explained by differences in protein synthesis. In addition, the absence of fully glycosylated forms of CFTR in cells expressing ΔI507, ΔF508, R560T and P574H mutant proteins confirms that these mutations are associated with defective processing.

Functional characterization of P574H-CFTR as compared with other CFTR mutant proteins

The functional activity of CFTR mutant proteins was measured in two different experimental conditions that permit activation of the CFTR-Cl⁻ channels. In the first protocol, cells were treated with forskolin, a molecule that activates the catalytic subunit of adenylate cyclase, and thus raises the level of cyclic AMP, leading to phosphorylation of the CFTR protein by protein kinase A (Dalemans *et al.*, 1991). In the second protocol, cells were treated with substituted benzimidazolone NS004, a molecule which has recently been described as an activator of the CFTR-Cl⁻ channel (Gribkoff *et al.*, 1994). Because NS004 is active on excised membrane patches, it has been proposed that a direct interaction might exist between the molecule and the CFTR protein. The effects of the two drugs being additive, maximal activation measured by $^{125}I^-$ efflux and by electrophysiology of wild type CFTR-Cl⁻ channels and of ΔF508-CFTR-Cl⁻ channels was obtained by treating the cells with a mixture of forskolin and NS004 (Gribkoff *et al.*, 1994). Figure 4 analyses the cAMP activated anionic permeabilities detected on Vero cells expressing the different CFTR mutant proteins. The activities of wild type and ΔF508 constructions have been characterized elsewhere with identical experimental conditions (Dalemans *et al.*, 1991; Gribkoff *et al.*, 1994). The techniques used for these characterizations consist of radioisotopic effluxes with anions permeant through the Cl⁻ channel and electrophysiology. When $^{125}I^-$ efflux were performed on Vero cells expressing ΔI507 and R560T CFTRs, no significant stimulation of the efflux was observed, even in conditions permitting a maximal activation of wild type CFTR or of ΔF508 mutant protein, i.e. 10 μM forskolin + 20 μM NS004 (Gribkoff *et al.*, 1994). As expected, a very low cAMP-activated Cl⁻ current was observed on the same cells by electrophysiological measurements in the whole cell configuration (Figure 4D). Very different results were observed for P574H (Figure 4C–E). In this case, 10 μM forskolin alone, 20 μM NS004 alone and 10 μM forskolin + 20 μM NS004 increased the maximal stimulation factor (i.e. the maximal value of the ratio: stimulated efflux rate on basal efflux rate) up to 4.75 ± 1.5, 2.45 ± 0.61 and 6.71 ± 1.85 (mean ± SD) respectively. In the presence of forskolin alone, the time-course of efflux was intermediate between those previously reported for the wild type and the ΔF508 CFTR (Gribkoff *et al.*, 1994) (1 min 15 s, 2 min 45 s and 4 min 15 s for

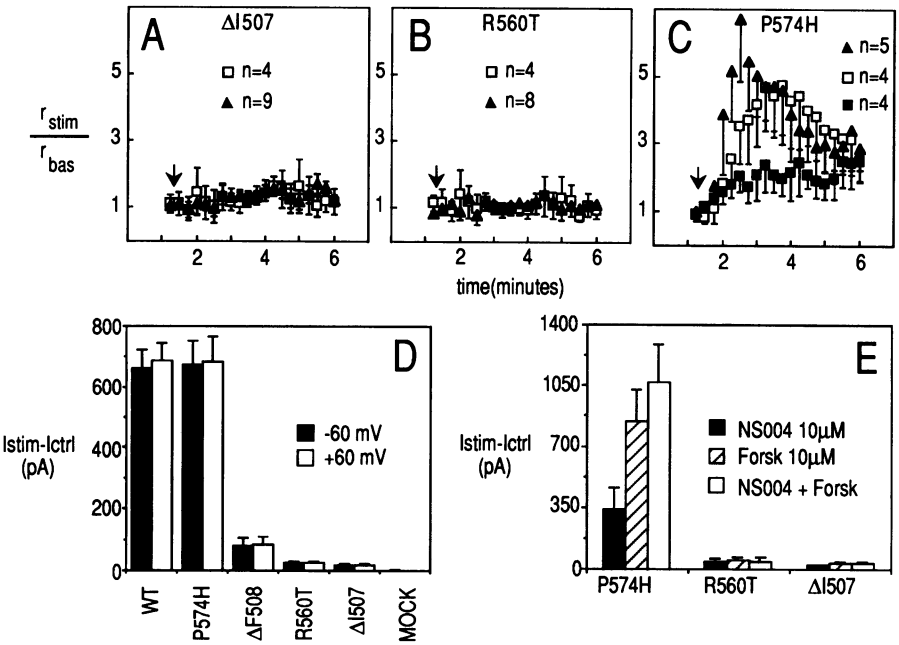

Fig. 4. Functional analysis of the ΔI507-, R560T- and P574H-CFTRs. (**A**) Time-course of the activation of the ΔI507-CFTR mediated $^{125}I^-$ efflux by 10 μM forskolin (□) and 10 μM forskolin + 20 μM NS004 (▲). (**B**) Time-course of the activation of the R560T-CFTR mediated $^{125}I^-$ efflux by 10 μM forskolin (□) and 10 μM forskolin + 20 μM NS004 (▲). (**C**) Time-course of the activation of the P574H-CFTR mediated $^{125}I^-$ efflux by 10 μM forskolin (□), 20 μM NS004 (■) and 10 μM forskolin + 20 μM NS004 (▲). Vertical arrows indicate addition of the effectors. Results are shown as mean ± SD (**D**) Whole cell patch-clamp studies of wild type, ΔI507, ΔF508, R560T and P574H after stimulation by 10 μM forskolin. (**E**) Activation of the ΔI507, R560T and P574H -Cl^- currents (I_{stim}) by 20 μM NS004 and/or forskolin 10 μM. I_{stim} is the difference between the current measured in the presence of the drug and in control condition. n = number of experiments.

wild type, P574H and ΔF508 respectively). Co-treatment with forskolin and NS004 stimulated the $^{125}I^-$ efflux up to values previously reported for the wild type CFTR (Gribkoff *et al.*, 1994).

Electrophysiological recordings in the whole-cell configuration were performed before and after treatment with forskolin (Figure 4D). Values of the Cl^- current did not differ very significantly between P574H and the wild type proteins, suggesting that in that configuration there might be no significant difference in activity between the two different channel proteins. Figure 4E shows that NS004 can stimulate the CFTR-Cl^- current in P574H-CFTR expressing cells, either when used alone or in combination with forskolin. A Cl^- current in R560T and ΔI507 expressing cells was stimulated by forskolin (Figure 4D), but its amplitude remained always very small in comparison with the Cl^- current generated by the expression of wild type CFTR (2.6 and 3.7% of the wild type, for ΔI507 and R560T respectively), and the stimulating effect of NS004 remained modest (Figure 4E).

The mutant protein P574H has a higher intrinsic Cl^- channel activity than wild type

In order to understand why the P574H mutant protein displayed a high residual Cl^- channel activity, despite its defective cell-surface expression, the properties of the P574H-Cl^- channel were compared with those of the wild type CFTR using the cell-attached patch-clamp technique. When cells expressing wild type or P574H Cl^- channels were exposed to NS004, an increase in the open probability was noticed in both cases (not shown), but the comparison shown here was performed after stimulation by a 'cocktail' of forskolin (5 μM), 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (cpt-cAMP, 100 μM) and isobutylmethylxanthine (IBMX, 100 μM) and in the absence of NS004. Long lasting recordings of P574H mutant CFTR and wild type CFTR show the typical difference observed between the activity of the two Cl^- channels (Figure 5A). Although the unitary current amplitude was not modified by the P574H mutation (Figure 5B), the mean number of active Cl^- channels was reduced by a factor of ~2 (i.e. 46% of the wild type CFTR) (Figure 5C) and the open probability of the channel was increased by 59% (Figure 5D). The conductance of the P574H Cl^- channel is 5.1 pS, very similar if not identical to that found for the normal CFTR channel (5.3 pS) under the same conditions. Figure 5E presents a comparative analysis of the mean open and mean closed times of P574H CFTR and normal CFTR after stimulation by the cocktail of forskolin, cpt-cAMP and IBMX. The P574H mutation led to a large increase (a factor of 6.8) in the mean open time of the Cl^- channel to a value of 10.4 s. The mean closed time was also increased but by a lesser factor (2.6).

Discussion

CF mutations which have been studied up until now fall into four different classes (Welsh and Smith, 1993): class I mutations produce premature termination signals (splice site abnormalities, frameshifts caused by deletions or insertions, nonsense mutations), class II mutations affect trafficking of the protein to the correct cellular location of CFTR at the cell surface, class III mutants have defective regulation and class IV mutants have defective conduction. All mutations affecting the first nucleotide binding domain that have been described so far lead to a

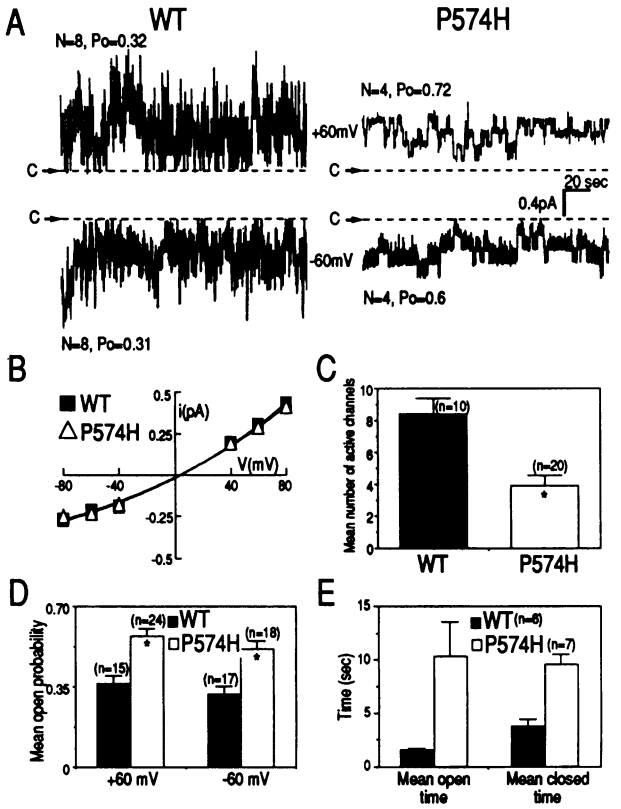

Fig. 5. Single channel comparison between wild type and P574H mutant Cl^- channels. (**A**) Typical cell-attached recordings on forskolin-stimulated WT-CFTR-infected cell (left) and P574H-CFTR infected cell (right); c = closed state of the channels. (**B**) Mean *I–V* relationships for wild type and P574H mutant CFTR. (**C**) Histograms showing the mean number of active CFTR-Cl^- channels in wild type and P574H CFTR expressing cells. (**D**) Histograms showing the mean open probability of active CFTR-Cl^- channels in wild type and P574H CFTR expressing cells at ±60 mV. *, $P < 0.05$ using Student's unpaired *t*-test. (**E**) Histograms showing the mean open and closed time for wild type and P574H Cl^- channels at –60 mV.

severe form of the disease either because they prevent or suppress the incorporation of CFTR at the cell surface (class II) or because they block the normal activation of CFTR-Cl^- channels (class III).

Severe- and mild-disease-associated mutations studied in this paper affect the first nucleotide binding domain. No significant Cl^- channel activity was observed in cells expressing the two mutant proteins ΔI507 and R560T-CFTR corresponding to severe mutations. This was explained by the absence of a significant cell-surface expression in immunolabelling experiments. A different situation was observed for ΔF508, the main CF mutation. Immunodetection of some CFTR in the plasma membrane of the cells expressing the ΔF508 mutant protein (Figure 2) correlates closely with our previous electrophysiological measurements. Using the same expression system, Dalemans *et al.* (1991) reported that the number of Cl^- channels measured by cell-attached patch clamp recording in ΔF508 expressing cells was between 1.9 and 3.9% of the number of Cl^- channels in wild type CFTR expressing cells, a value similar to the 2–6% shown in Figure 2.

Since no ΔI507 or R560T proteins could be detected at the plasma membrane, and since their expression generated <4% of the whole-cell Cl^- current generated by the wild type (Figure 4D), these mutant channels can be considered as members of class II. It may be that in addition to their severe sorting defect, ΔI507-CFTR and R560T-CFTR also have altered Cl^- channel properties, as has been reported for the ΔF508 mutant protein (Dalemans *et al.*, 1991). Their very low expression level did not permit an accurate analysis of that point.

A different situation was observed with the P574H mutation. Although it is associated with a cystic fibrosis phenotype (Kristidis *et al.*, 1992), relatively high residual Cl^- channel activity was measured in cells expressing this mutant form of CFTR (Figures 4 and 5). In this case the anti-CFTR monoclonal antibody labelled the cell surface of 20–30% of P574H-CFTR expressing cells (Figures 1 and 2). This percentage is lower than for wild type CFTR, for which a value between 70 and 79% was observed. This alteration in the traffic toward the cell surface of the P574H mutant protein was confirmed by functional studies. The number of active channels per patch (estimated by assuming independence between the channels present in a patch) was about half for P574H as compared with wild type (Figure 5C and D). Immature P574H-CFTR accumulated mainly in the Golgi apparatus and in the cytoplasm, whereas diffuse labelling restricted to the cytoplasm was observed for ΔF508-CFTR. These differences may be due to different retrieval sites that would depend on the mutations or to different half-lives of the different mutant proteins, as previously suggested for the ΔF508 mutant protein (Lukács *et al.*, 1993).

An interesting property of the P574H mutant protein is that it is associated with defective sorting and increased intrinsic activity of the Cl^- channel (Figures 4 and 5). This increased Cl^- channel activity is not due to a change of the channel conductance (5.1 pS and 5.3 pS, for P574H- and WT-CFTR, respectively) but is associated with an increase in the time during which the channels remain open. This is the first example of an association between a CF mutant protein and increased channel activity. The open probability of the P574H-CFTR Cl^- channel is nearly 60% higher than normal.

An initial conclusion from these observations is that mutations associated with mild CF: (i) can lead to partial defective processing, less severe than previously observed for severe mutations, and (ii) are not necessarily associated with a decrease in intrinsic channel activity as previously reported for R117H, R334W and R347P. These three mutations, identified in mildly affected patients, are located at the external end of the second (R117H) and the sixth (R334W, R347P) putative membrane-spanning sequences. These mutants are correctly processed but have reduced single channel conductances (Sheppard *et al.*, 1993).

A second conclusion is that the 'quality control' which prevents the trafficking of mutated CFTR-Cl^- channels to the plasma membrane does not only eliminate mutants with reduced or abolished Cl^- channel activity, since the intrinsic activity of the P574H mutant protein is higher than normal. Therefore it seems clear that structural elements of the CFTR protein that are involved in the traffic control (Yang *et al.*, 1993; Pind *et al.*, 1994) are not identical to those which are essential for Cl^- channel function, even if the two nucleotide binding domains seem to be crucially involved in the sorting and control of the Cl^- channel activity.

The P574H mutation has been identified in compound heterozygotes where it is associated with other mutations such as ΔF508 (Kristidis *et al.*, 1992). The clinical status of these patients clearly depicted it as a mild CF mutation characterized by pancreatic sufficiency. Results from $^{125}I^-$ efflux measurements (Figure 4) indicate that the P574H-Cl^- channel activity is ~70% of the activity measured with wild type CFTR-expressing Vero cells (as measured by the ratios of the maximal stimulation factors). This would mean, assuming that the activity observed in Vero cells can be extrapolated to polarized epithelial cells, that residual *in vivo* Cl^- transport activity for patients bearing P574H mutation would be at most 35% of the normal value. An expression of the Cl^- current representing ~35% of normal would then lead to development of a mild form of the disease. However, atypical or asymptomatic forms of the disease associated with <35% Cl^- current activity have been described (Rigot *et al.*, 1991; Sheppard *et al.*, 1993). As a comparison, since the percentage of ΔF508-CFTR at the surface membrane is only 2–5% and since, in addition, the ΔF508-Cl^- channel is also characterized by altered kinetics resulting in one third of the normal open probability (Dalemans *et al.*, 1991), it could be expected that the value of Cl^- transport in ΔF508-CFTR expressing cells would represent ~1% of the current observed for wild type. It would not be surprising that such a low value would lead to the development of a severe form of the disease.

The association of the P574H mutation with a mild clinical status of the disease is certainly explained by the relatively high intrinsic activity of the corresponding CFTR-Cl^- channel. This of course suggests that increasing the Cl^- channel activity of a mutant protein might be able to compensate for the reduced cell-surface expression attenuating the manifestation of the disease. The fact that some compounds, such as substituted benzimidazolone NS004, possibly interact directly with the CFTR-Cl^- channel, and lead to its activation, may therefore provide an alternative, and/or complementary approach to gene therapy (Gribkoff *et al.*, 1994). Compounds like NS004 might then be worth investigation in the case of such mild CF mutations.

Finally, as always, diseases provide useful indications on structure–function relationships of target protein. The ΔF508 mutation has been previously shown to alter the gating system of the CFTR-Cl^- channel. It does not change the open time kinetics but it increases (at –60 mV) the mean closed time by a factor of 5 (Dalemans *et al.*, 1991). The work presented in this paper clearly shows that proline 574 is also involved in the control of the opening properties of the CFTR-Cl^- channel. The P574H mutation also increases the mean closed times at –60 mV but only by a factor of 2.6. Its most spectacular effect was to increase drastically mean open times (a factor of nearly 7) up to values of 10.4 s.

It has been established recently that opening and closing of CFTR-Cl^- channels are coupled to ATP hydrolysis (Baukrowitz *et al.*, 1994; Hwang *et al.*, 1994). Therefore in light of the results presented in this paper it should now be expected that some mutations in the first nucleotide binding domain can alter this ATP hydrolysis step and/or conformational changes that link it to opening and closing events of the channel. Two gating modes of the CFTR-Cl^- channel have recently been observed (Fisher and Machen, 1994) corresponding to short or long open states. The P574H mutation described in this work favours the long open state situation, and as such might become useful for further mechanistic studies of the Cl^- channel activity as have been recently carried out with the normal CFTR protein (Fisher and Machen, 1994).

Materials and methods

Construction and expression of the different CFTR mutants

Recombinant vaccinia virus construction and infection were carried out as previously described (Dalemans *et al.*, 1991). Briefly, mutations were introduced in the first nucleotide binding domain by site-directed mutagenesis in M13 (Kunkel *et al.*, 1987). The mutated nucleotide binding domain sequences were used to replace the corresponding sequences in pTG5959, in which the CFTR cDNA is under the control of the T7 promoter (Dalemans *et al.*, 1991). Recombinant viruses were obtained by homologous recombination with the vaccinia virus genome (Moss, 1991).

In all expression experiments, cells were infected with both VVTG1193, encoding T7 polymerase, and one of the CFTR-expression vaccinia viruses, at 5 p.f.u./virus/cell. 'Mock' infected Vero cells were infected by VVTG1193 only. Expression of CFTR mutant proteins was examined by Western blot analysis as described (Dalemans *et al.*, 1992). Monoclonal antibodies MAb 13.1, MAb 24.1 (Genzyme) and MATG1104 were used at a 1:1000 or 1:10 000 (MATG1104) dilution.

Light fluorescence microscopy

Vero and Chang cells were grown on Lab Tek chamber slides and were infected using a recombinant vaccinia virus expression system carrying either the wild type or the mutant (ΔF508, R560T or P574H CFTR) cDNAs under the control of the T7 promoter. Cells were fixed for 30 min with methanol at –20°C and then rinsed in PBS 0.1 M pH 7.2. The slides were incubated with 1% bovine serum albumin (PBS–BSA) for 5 min and further incubated for 60 min with a 1:200 dilution of the monoclonal antibody (mAb 24-1 from Genzyme) generated against the COOH terminal end of the CFTR protein. Cells were then incubated with a goat anti-mouse IgG biotinylated complex (1:50 in PBS–BSA) and subsequently for 30 min with streptavidin-fluorescein isothiocyanate (FITC 1:50). The slides were mounted in citifluor glycerol-PBS (Agar Science Ltd) to prevent photobleaching. Cells were observed under a Zeiss Axiophot microscope equipped with epifluorescence illumination. Photomicrographs were taken using Ektachrome 160 ASA films. The absence of labelling was controlled in the two cell lines either non-infected or infected with control vaccinia viruses.

In order to quantify the percentage of infected cells exhibiting a CFTR membrane labelling, successive adjacent fields were video recorded using a low level SIT camera (Lhesa) and printed on a Sony video printer. This allowed analysis of the distribution of CFTR on a high number of cells that was used to calculate the percentage of cells displaying membrane labelling.

Scanning laser confocal microscopy

The different infected cells were examined with an MRC-600 Bio-Rad confocal system mounted on a Zeiss Axioplan microscope equipped with a 63, 1.4 NA planapochromat objective. A 25 mW argon ion laser was used as the light source producing an excitation line at 488 nm. From the serial sections collected at steps of 0.4 μm, XY and XZ sections were selected, and used to visualize the preferential distribution of CFTR, i.e. at the plasma membrane or in the cytosol.

Functional expression of the mutant CFTRs

Iodide efflux experiments were performed as described previously (Dalemans *et al.*, 1991; Gribkoff *et al.*, 1994), according to the protocol initially described by Venglarik *et al.* (1990).

Electrophysiological recordings from Vero cells

Currents were measured at room temperature using the whole-cell patch-clamp technique (Hamill *et al.*, 1981). The pipette solution contained in mM: 120 *N*-methyl-D-glucamine chloride (NMDGCl), 4 $MgCl_2$, 4 EGTA/NaOH (free Ca^{2+} < 1 nM) : 2 Na_2ATP, 10 HEPES (pH 7.2). The cell was perfused with (in mM): 140 NMDGCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH 7.2). To determine *I–V* relationships, the membrane

potential was held at −20 mV and stepped to levels between −100 mV and +100 mV in 20 mV increments. For single channel recordings from cell-attached patches, outward currents refer to the flow of cations from the pipette (cytosolic side) into the bath (external side). Single channel analysis (open probability, mean dwell times) was performed with biopatch software (Biologic).

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