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ORIGINAL INVESTIGATION

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A neutral variant involved in a complex *CFTR* allele contributes to a severe cystic fibrosis phenotype

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Abstract In order to further elucidate the contribution of complex alleles to the wide phenotypic variability of cystic fibrosis (CF), we investigated the structure-function relationships of a severe CF-associated complex allele [p.S912L;p.G1244V]. To evaluate the contribution of each mutation to the phenotype, cystic fibrosis transmembrane conductance regulator (CFTR) mutants were expressed in HeLa cells and analysed for protein processing and Cl^- channel activity. Both p.G1244V and [p.S912L;p.G1244V] mutants had normal protein processing but markedly decreased Cl^- channel activity compared with wild-type. Notably, the double mutant displayed a dramatic decrease in Cl^- channel activity compared with p.G1244V ($P < 0.001$). p.S912L had normal protein processing and no detectable impact on CFTR function. In other respects, the p.S912L variation was identified in compound heterozygosity with p.R709X in a healthy fertile man. Together, these data strongly support the view that p.S912L in isolation should be considered as a neutral variant but one that might significantly impair CFTR function when inherited in *cis* with another *CFTR* mutation. Our data also further document the contribution of complex alleles to the wide phenotypic variability of CF. The results of functional studies of such complex alleles in other genetic diseases are discussed.

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Introduction

Cystic fibrosis (CF, MIM 219700) is an autosomal, recessively inherited disorder that affects the physiology of the lung, gastrointestinal tract, reproductive organs, and sweat ducts. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR* or *ABCC7*; MIM 602421) gene, which encodes a cAMP-regulated Cl^- channel located in the apical membrane of epithelial cells (Riordan et al. 1989). To date, over 1,300 mutations have been identified in the *CFTR* gene and, notably, several complex alleles further complicate the establishment of genotype–phenotype correlations (http://www.genet.sickkids.on.ca/cftr/). The combination of two missense mutations on the same chromo some has been described clinically to lessen ([p.R553Q;p.F508del], [p.R334W;p.R1158X]; Dork et al. 1991; Duarte et al. 1996) or worsen ([p.R74W;p.D1270N], [p.R347H;p.D979A]; Casals et al. 1995; Hojo et al. 1998) the phenotype of CF patients with regard to the commonest mutation alone (p.F508del, p.R1158X, p.D1270N, p.R347H). In vitro expression of some of these complex alleles has demonstrated that they alter Cl^- channel activity in a different way from their single mutant counterparts (Clain et al. 2001; Fanen et al. 1999; Teem et al. 1993; Wei et al. 2000).

The [p.S912L;p.G1244V] double-mutant allele is associated with severe CF (Savov et al. 1995). We report herein the functional analysis of this complex allele and evaluate the contribution of each mutation to the phenotype. In vitro experiments and identification of a healthy fertile man bearing the [p.R709X]+[p.S912L] genotype both indicate that the p.S912L mutation should be considered to be a polymorphism. Surprisingly, the combination of p.S912L and p.G1244V decreases

cAMP-dependent Cl^- channel activity compared with p.G1244V, highlighting the deleterious effect exerted by polymorphisms on the phenotype when combined in *cis* with a mutation. Functional studies of such a combination of complex alleles in other genetic diseases are discussed.

Materials and methods

Site-directed mutagenesis, cells and CFTR expression

CFTR mutants were constructed in the pTCFwt expression plasmid, a vector designed for the visual detection of transfected mammalian cells by means of the green fluorescent protein (GFP) with the GeneEditor kit (Promega, Charbonnières, France). The resulting mutant cDNAs were fully sequenced. Expression of the CFTR gene in pTCFwt is controlled by the CMV promoter, whereas synthesis of the GFP-Zeocin fusion protein is controlled by the SV40 promoter.

HeLa cells (7.5×10^5) were grown on 60-mm-diameter dishes at 37°C with 5% CO_2 in Dulbecco Modified Eagle's Medium (DMEM Glutamax, Invitrogen, Cergy-Pontoise, France) containing 10% fetal calf serum, 100 U/ml penicillin and 100 g/ml streptomycin (all from Invitrogen). Confluent cells (60%) were transfected by lipofection with 12 µl LipofectAMINE and 8 µl Plus reagent (Invitrogen) with 2 µg plasmid according to the manufacturer's instructions. Confluent monolayers were harvested and used 48 h post-transfection for functional assays or biochemical experiments.

Immunoprecipitation/cAMP-dependent protein kinase assay, pulse-chase experiments

The CFTR protein immunoprecipitation by a monoclonal antibody (MAB25031, R&D System, Oxon, UK) that recognizes the C-terminus of CFTR (aminoacid 1,377–1,480) has been previously described (Fanen et al. 1997). Briefly, transfected cell lysates from one 60-mm-diameter dish were mixed with 0.4 µg MAB25031 and Pansorbin (Calbiochem, San Diego, Calif., USA). The resulting proteins were phosphorylated in vitro with 5 U of the catalytic subunit of cAMP-dependent protein kinase (PKA; Promega) and 10 μCi [$\gamma-^{33}$ P]ATP (Amersham Pharmacia Biotech, Orsay, France), separated by 5% SDS-polyacrylamide gel electrophoresis, dried and autoradiographed. Radioactivity was quantified by radioanalytic scanning (Molecular Dynamics PhosphoImager, Sunnyvale, Calif., USA).

Pulse-chase experiments were performed by incubating cells for 30 min in DMEM lacking cysteine and methionine and then for 15 min in the same medium containing 100 μCi/ml [^{35}S]-labelled methionine and cysteine (Redivue Pro-mix [^{35}S], Amersham Pharmacia Biotech). CFTR was immunoprecipitated from cell lysates with MAB25031.

Fluorescence assay

Cells were grown on glass coverslips for 24 h and then loaded with the halide-indicator fluorescent dye 6-methoxy-*N*-ethylquinolinium (MEQ) as previously described (Fanen et al. 1997). MEQ fluorescence was excited at 345 nm (filter 345/40×, Chroma) and emitted fluorescence was passed through the dichromatic mirror, DM 400 nm, and the barrier filter, BA 420 nm (U-MWB box, Olympus, Rungis, France). The cells were incubated at room temperature for 4 min in hypotonic medium. The medium contained 20 mM MEQ prepared by diluting isotonic solution (138 mM NaCl, 2.4 mM K_2HPO_4, 0.8 mM KH_2PO_4, 10 mM HEPES, 1 mM $CaSO_4$, 10 mM glucose and 20 µM bumetanide, pH 7.4) 5:1 with water. The cells were then returned to isotonic buffer to recover. After 15 min of loading, the coverslip was placed in a perfusion chamber, continuously perfused at 37°C with isotonic Cl^- solution, on the stage of an inverted microscope (IX70 Olympus). The transfected cells were identified by visual detection of GFP fluorescence and, subsequently, intracellular MEQ fluorescence was measured. The Cl^- solution was replaced by nitrate (NO_3^-) solution after 2 min of perfusion. This solution was identical except that Cl^- was replaced by NO_3^-. As NO_3^- does not quench MEQ, fluorescence increases as intracellular Cl^- flows from the cell through the anion conductive pathways, providing that these pathways are functional in the cell membrane. Changes in fluorescence of stimulated (stimulatory cocktail: 500 µM 8-(4-chlorophenylthio)-cyclic AMP and 100 µM 3-isobutyl-1-methylxanthine) and non-stimulated cells were recorded at an excitation wavelength of 350 nm and an emission wavelength of >440 nm. The fluorescence of single cells was measured with a digital imaging system and a charge-coupled device camera (Gen IV, Prinston Instruments, Trenton, N.J., USA/Paris, France). Results were analysed by using Metafluor 3.0 software (Universal Imaging, Media, PA, USA/Paris, France).

The results were expressed as relative fluorescence F/F_0, where F is the change in fluorescence with time and F_0 the minimum fluorescence. This ratio was used rather than the absolute change in fluorescence, which could have been influenced by dye loading. We then calculated the slope of the dequenching curve under basal (ΔF_{basal}) and stimulatory (ΔF_{stim}) conditions and calculated the difference (ΔF_{stim}-ΔF_{basal}), which was used to quantify cAMP-dependent Cl^- transport across the cell membrane.

Statistical analysis

Statistical analysis was performed by using the Chi-square test. Differences were considered statistically significant for $P<0.05$.

Patient

A couple was referred to our laboratory for fetal meconium ascites diagnosed during a routine ultrasound scan at 28 weeks of gestation. No anomaly had been observed at 20 weeks of gestation. The two parents were healthy and had no family history of CF. Diagnostic investigations were requested after genetic counselling, with the couple's informed consent. *CFTR* molecular studies were performed on peripheral blood samples from the couple and on amniotic fluid by using the Applied-Biosystems Cystic Fibrosis Assay kit (Applied-Biosystems, Foster City, Calif., USA), which detects 31 of the most frequent CF mutations encountered in Caucasians, and a denaturing gradient gel electrophoresis/sequencing strategy focused on the coding regions as described elsewhere (Costes et al. 1993; Fanen et al. 1992). The diagnosis of CF was confirmed in the fetus by the presence of two allelic mutations, 711 + 1G → T, a splice mutation of intron 5 inherited from the mother, and p.R709X, a nonsense mutation of exon 13 inherited from the father; the father carried the p.S912L variant (not transmitted to the fetus) on the other chromosome. No other sequence variation was detected after scanning of the whole coding sequence in the father.

Results

Processing of CFTR mutants

To analyse the processing and Cl^- channel activity of p.S912L, p.G1244V and [p.S912L;p.G1244V] mutant proteins, wild-type and mutant CFTR alleles were expressed in HeLa cells. The processing of CFTR can be assessed by examining its glycosylation. Under our experimental conditions, electrophoresis of immunoprecipitated wild-type CFTR gave two bands (Fig.1a): a diffuse band with an approximate molecular mass of 170 kDa that represented mature, fully glycosylated protein that had migrated through the Golgi complex to the cell membrane (band C), and a thin band of about 140 kDa that represented the core-glycosylated protein located in the endoplasmic reticulum (band B). Immunoprecipitation experiments at steady-state showed that cells expressing the wild-type and the mutant CFTRs produced the mature protein (Fig.1a). Although the absolute amount of band C of the wild-type CFTR was the weakest (depending on transfection efficiency), the relative amount of band C (ratio C/B + C) was similar for the wild-type and mutant CFTRs (Fig.1b). The biosynthesis of wild-type and mutated CFTR proteins was further investigated by pulse-chase experiments (Fig.2). Band B (core-glycosylated form) appeared promptly, whereas band C (fully glycosylated form) was only detected after about 1 h of chase. The kinetics of core-glycosylated and mature forms of mutant CFTRs were nearly identical to those of the wild-type, indicating that p.S912L, p.G1244V and [p.S912L;p.G1244V] mutations did not affect maturation of the CFTR protein.

Functional assay of CFTR mutants

To characterise these mutant proteins further, the cAMP-stimulated Cl^- conductance of CFTR expressing cells was measured by using a halide-sensitive dye fluorescent assay. Synthesis of GFP allowed visual detection of transfected cells, so that all cells analysed were CFTR-transfected cells. Table 1 summarises the results of the MEQ fluorescence assay.

The mock-transfected cells displayed almost no fluorescence change under either basal or cAMP-stimulated conditions, whereas most cells expressing wild-type

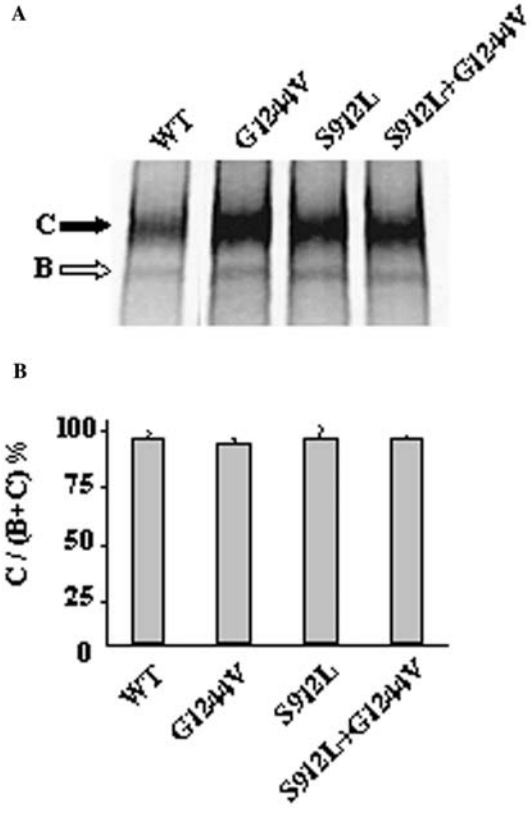

Fig. 1 Processing of wild-type and mutant CFTR assessed by protein glycosylation states. **a** Immunoprecipitation of PKA-phosphorylated (with [$\gamma-^{33}$ P]ATP) CFTR proteins by using anti-CFTR monoclonal antibody MAB25031. Band B represents the core-glycosylated immature form of the CFTR protein located in the endoplasmic reticulum, and band C the mature fully glycosylated protein that has migrated through the Golgi complex to the cell membrane. Bands B and C are indicated by *arrows*. **b** CFTR maturation efficiency calculated as the amount of mature CFTR (band C) relative to the total amount of CFTR produced (bands B + C). Radioactivity was quantified by radioanalytic scanning. Data are the means ± SEM of at least three independent experiments

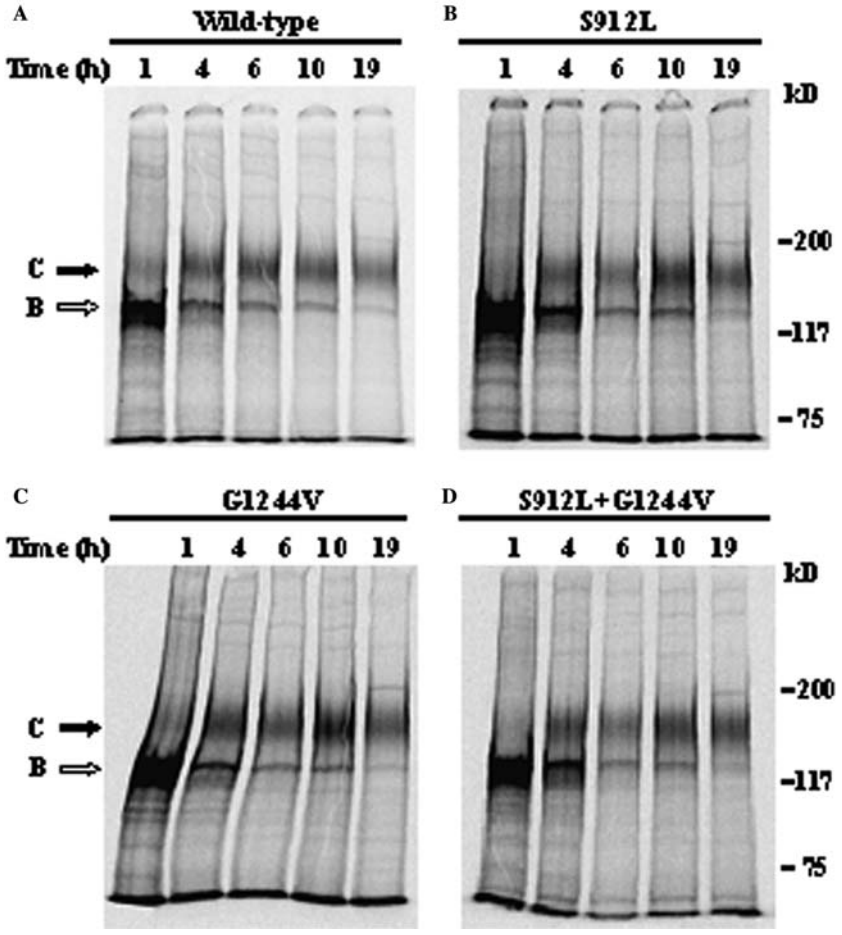

Fig. 2 a–d Pulse-chase experiments showing the turnover of the immature and mature forms of wild-type and the mutant CFTR proteins (representative of two independent experiments)

CFTR produced an intense cAMP-dependent fluorescence change, indicating cAMP-dependent Cl^- conductance. CFTR-expressing cells for which the ΔF_{stim}-ΔF_{basal} difference was higher than the maximum ΔF_{stim}-ΔF_{basal} difference observed for mock-transfected cells (0.0052; Table 1) were considered responsive. p.S912L-transfected cells displayed a similar proportion of responsive cells to wild-type cells (92% of responsive cells vs 83%; $P > 0.2$; Fig.3), suggesting that Cl^- channel function was not affected by this mutation.

Unlike p.S912L, the p.G1244V and [p.S912L; p.G1244V] CFTR transfected cells showed a marked decrease in cAMP-stimulated Cl^- conductance compared with wild-type (43% and 2.4% of responsive cells, respectively vs. 83%; $P < 0.001$; Fig.3). As both of these mutants displayed normal processing, these data indicated defective channel properties.

Surprisingly, whereas the p.S912L mutation had no detectable impact on CFTR function, the [p.S912L;p.G1244V] complex allele showed an almost 20-fold reduction in cAMP-dependent conductive pathway compared with the p.G1244V deleterious mutant (2.4% of responsive cells vs. 43%; $P < 0.001$; Fig.3).

Discussion

Clinical data suggest that the combination of p.S912L and p.G1244V mutations in *cis* ([p.S912L;p.G1244V] complex allele) produces a severe CF phenotype similar to that of p.F508del homozygotes. Our functional data support this observation. Thus, the poor Cl^- channel activity measured in vitro (2.4% of the wild-type) and the severity of the associated disease observed in vivo (pancreatic insufficiency, severe lung disease and elevated sweat electrolytes) both indicate that the [p.S912L;p.G1244V] complex allele should be considered to be a severe allele.

We have described the contribution of each mutant to this double-mutant phenotype. In this study, we have

Table 1 Summary of MEQ fluorescence assay results from mock-transfected, wild-type and mutant cells

Statistic ΔF_{stim}- ΔF_{basal}					
	Mock	Wild-type	p.S912L	p.G1244V	[p.S912L;p.G1244V]
n	28	64	36	53	42
Mean	0.002	0.023	0.036	0.005	0.001
SEM	0.001	0.017	0.028	0.005	0.001
Range	0–0.005	0–0.340	0–0.140	0–0.034	0–0.006

provided functional and clinical evidence that the p.S912L mutation is not a disease-causing mutation on the basis of the following data. (1) In vitro experiments have shown that the p.S912L mutation has no detectable impact on CFTR processing and Cl^- channel function. (2) Non-conservation of this residue in various CFTR species (Chen et al. 2001) suggests that there is no specific requirement at this position (junction of extracellular loop IV and transmembrane segment 8) for CFTR processing and function. (3) This mutation was found several times in isolation: twice in our laboratory (Ghanem et al. 1994 and this study) and in Northeastern Italy during carrier screening (C. Castellani, personal communication). Notably, the healthy father of a CF fetus carrying the p.S912L mutation has been identified in our laboratory in the framework of prenatal diagnosis, whereas the CF-associated p.R709X mutation (Bonizzato et al. 1995) has been detected on his other allele. However, no symptoms related to cystic fibrosis have been identified in the father, not even hypofertility, which could be expected given the finding that the most sensitive tissues, in terms of embryological development, are the epididymal duct and vas deferens. Together, these data strongly support the view that the p.S912L mutation should be considered to be a neutral variant.

In contrast, our functional data indicate that residue Gly-1244 is important for CFTR Cl^- channel function, consistent with previous observations. It is the first residue of the Walker A motif in the second nucleotide-binding domain (NBD), which is strictly conserved throughout CFTR evolution (Chen et al. 2001), and also in 23 of 24 NBDs from various other proteins (Riordan et al. 1989). At the same location, the severe CF-associated p.G1244E mutation (Devoto et al. 1991) exhibits a defect in the open state probability of the CFTR protein (Anderson and Welsh 1992). Taken together, these data support the view that the p.G1244V mutation is a disease-causing mutation.

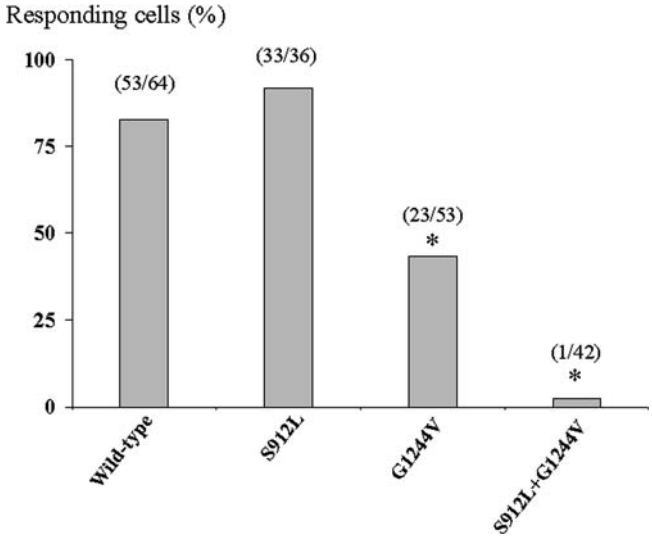

Fig. 3 cAMP-dependent Cl^- channel activity of transfected HeLa cells expressing wild-type and mutant CFTRs determined with the MEQ fluorescence assay. Responding cells are expressed as the percentage of all cells analysed. Cells were scored as responsive when the ΔF_{stim}-ΔF_{basal} difference was greater than the maximum value for mock-transfected cells (* significantly different from the other cell types; $P < 0.01$)

Surprisingly, the p.G1244V and [p.S912L;p.G1244V] alleles affect in vitro Cl^- channel function in different ways. This might indicate that p.S912L and p.G1244V mutations occurring on the same allele cause severe structural alterations worsening the effect of the p.G1244V mutation on CFTR function. However, we cannot exclude the possibility that the p.S912L mutation may have a slight, but undetectable, effect on CFTR function and that a threshold is reached when these two mutations are combined in *cis*. An important consequence of our observation is that when a patient or an asymptomatic relative carries the p.S912L mutation, the p.G1244V mutation should be sought in order to exclude a CF allele; this is important for genetic counselling. These results have important implications for CF because they suggest that "neutral" missense mutations, such as p.S912L, might significantly alter CFTR function when inherited in *cis* with another mutation.

With more than 1,300 alterations having been described in the *CFTR* gene, CF provides a good model to study complex alleles in which two different DNA alterations coexist in the same *CFTR* allele. Several mechanisms have been described in which the mutation at the second site can modulate the effect of the principal mutation. The length of a polythymidine tract in the 3′ splice site of intron 8 modulates the clinical presentation of the p.R117H missense mutation because of a dramatic decrease in the amount of p.R117H-*CFTR* transcript (Kiesewetter et al. 1993). Another mechanism involves an alteration located in the minimal promoter region in *cis* with a missense mutation in exon 11. The sequence change $-102T \rightarrow A$ attenuates the effects of the severe p.S549R ($T \rightarrow G$) missense mutation by causing a significant increase in CFTR expression in vitro. This explains the striking differences in genotype–phenotype correlations between patients carrying mutation p.S549R alone, who presented severe disease, and patients carrying the complex allele [$-102T \rightarrow A$; p.S549R], who exhibit milder forms of CF (Romey et al. 2000).

We and other authors have previously reported that the combination of two missense mutations reverses (Teem et al. 1993; Wei et al. 2000) or aggravates in vitro CF phenotypes (Clain et al. 2001; Fanen et al. 1999). Such combinations of missense mutations have also been described in other genetic diseases. Remarkably, the few alleles that have been studied functionally reveal a "phenotype-modifying" effect (Table 2). A biochemical and clinical study of sickle-cell-disease patients has demonstrated that haemoglobin S Antilles (a variant that combines the p.E6V and p.V23I β–globin gene mutations) has a lower solubility than haemoglobin S (p.E6V). This complex allele produces sickle-cell disease in heterozygotes in contrast with p.E6V, which is disease-causing only

Table 2 Complex alleles in autosomal recessive disorders: impact on disease phenotype

Inherited disorders[a]	Complex allele[b]	Phenotype[c]		References[d]
		Clinical status	Functional status	
Cystic fibrosis	[**p.F508del**;p.R553Q]	–	–	Teem et al. (1993)
	[**p.S1235R**;p.M470V]	–	–	Wei et al. (2000)
	[**p.R347H**;p.D979A]	+	+	Clain et al. (2001)
	[p.R74W;**p.D1270N**]	+	+	Fanen et al. (1999)
	[**p.S912L**;p.G1244V]	+	+	This study
Gaucher disease	[**p.E326K**;p.L444P]	+	+	Grace et al. (1999)
	[p.D140H;**p.E326K**]	Id	Id	Grace et al. (1998))
Sickle-cell disease	[**p.E6V**;p.V23I]	+	+	Monplaisir et al. (1986)
Brugada syndrome	[p.R1232W;**p.T1620M**]	+	+	Baroudi et al. (2002)
GM1 gangliosidosis type II	[p.R201C;**p.L436F**]	+	+	Caciotti et al. (2003)

[a]Only monogenic autosomal recessive disorders are considered
[b]Combination of two missense mutations in *cis*; the more frequent mutation in each complex allele is given in *bold*
[c]Compared with the *bold* mutation alone, – indicates an attenuation and + an aggravation of the phenotype
[d]Only functional studies are considered

in homozygotes (Monplaisir et al. 1986). In Gaucher disease, two complex alleles involving the p.E326K mutation ([p.E326K;p.L444P] and [p.D140H;p.E326K]) have different impacts on clinical presentation; this has further been confirmed by an in vitro study of the mutant proteins. In combination with either p.D140H or p.L444P, the resulting mutant proteins have different residual enzymatic activities. The [p.D140H;pE326K] protein has sufficient activity to produce a type 1 phenotype; in contrast, the [p.E326K;p.L444P] protein has little residual acid β-glucosidase activity resulting in a severe type 2 phenotype (Grace et al. 1999). Another lysosomal storage disorder, GM1 gangliosidosis, is caused by the deficiency of β-galactosidase. The complex allele [p.R201C;p.L436F] has recently been reported (Caciotti et al. 2003); expression studies have revealed a deleterious effect of the p.L436F polymorphism on the known p.R201C mutation resulting in a late infantile subtype of type-II GM1, instead of the juvenile form classically associated with p.R201C.

The identification of these complex alleles therefore has several important consequences for recessive disorders: (1) the prognosis of a severe phenotype should lead to improved follow-up of patients; (2) adapted genetic counselling should be offered to the relatives of affected children; (3) knowledge of these complex alleles may contribute to a better understanding of genotype–phenotype relationships. Moreover, the impact of these alleles on the CF population must be determined, as their frequency remains unknown. Various studies have reported vastly different frequencies, ranging from 0% to 7.7% of CF chromosomes (Bombieri et al. 1998; Casals et al. 1995; Claustres et al. 2000; Mercier et al. 1995; Savov et al. 1995). These discrepancies indicate that no search for additional mutations is carried out when a patient is found to have two allelic mutations known to be disease-causing and/or a sampling effect. Our results have important implications for CF, as they show that "neutral" missense mutations might significantly alter CFTR function when inherited in *cis* with a CF-causing mutation. This may partly account for the difficulties in establishing genotype–phenotype correlations and certainly highlights the need to screen for additional mutations when a discrepancy between phenotype and genotype is observed.

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