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Niemann-Pick C1 Disease: Correlations between *NPC1* Mutations, Levels of NPC1 Protein, and Phenotypes Emphasize the Functional Significance of the Putative Sterol-Sensing Domain and of the Cysteine-Rich Luminal Loop

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To obtain more information of the functional domains of the NPC1 protein, the mutational spectrum and the level of immunoreactive protein were investigated in skin fibroblasts from 30 unrelated patients with Niemann-Pick C1 disease. Nine of them were characterized by mild alterations of cellular cholesterol transport (the "variant" biochemical phenotype). The mutations showed a wide distribution to nearly all NPC1 domains, with a cluster (11/32) in a conserved NPC1 cysteine-rich luminal loop. Homozygous mutations in 14 patients and a phenotypically defined allele, combined with a new mutation, in a further 10 patients allowed genotype/phenotype correlations. Premature-termination–codon mutations, the three missense mutations in the sterol-sensing domain (SSD), and A1054T in the cysteine-rich luminal loop all occurred in patients with infantile neurological onset and "classic" (severe) cholesterol-trafficking alterations. By western blot, NPC1 protein was undetectable in the SSD missense mutations studied (L724P and Q775P) and essentially was absent in the A1054T missense allele. Our results thus enhance the functional significance of the SSD and demonstrate a correlation between the absence of NPC1 protein and the most severe neurological form. In the remaining missense mutations studied, corresponding to other disease presentations (including two adults with nonneurological disease), NPC1 protein was present in significant amounts of normal size, without clear-cut correlation with either the clinical phenotype or the "classic"/"variant" biochemical phenotype. Missense mutations in the cysteine-rich luminal loop resulted in a wide array of clinical and biochemical phenotypes. Remarkably, all five mutant alleles (I943M, V950M, G986S, G992R, and the recurrent P1007A) definitively correlated with the "variant" phenotype clustered within this loop, providing new insight on the functional complexity of the latter domain.

Introduction

Niemann-Pick type C (NPC [MIM 257220]) disease is a neurodegenerative lysosomal lipid storage disease, with autosomal recessive transmission, characterized by lysosomal/late-endosomal accumulation of endocytosed unesterified cholesterol (Pentchev et al. 1995; Patterson et al. 2001). The clinical manifestations of NPC are heterogeneous. Most patients have a progressive neurologic disease, but both age at onset, which ranges from early infancy to adulthood, and subsequent course vary (Vanier and Suzuki 1996; Patterson et al. 2001). We have also documented a wide variation in severity of the cellular cholesterol lesion, with typical severe alterations described as the "classic" biochemical phenotype and mild alterations as the "variant" phenotype (Vanier et al. 1991*b*).

Although the exact location and nature of the trafficking defect(s) are still under investigation (Liscum and Munn 1999; Blanchette-Mackie 2000; Cruz and Chang 2000; Davies et al. 2000; Ory 2000), substantial advances have occurred in our knowledge of NPC. Cell-hybridization studies and linkage analysis (Steinberg et al. 1994; Vanier et al. 1996) demonstrated the existence of two complementation groups. *NPC1,* the disease-causing gene in >95% of NPC patients, has been located to 18q11-q12 and is now fully characterized (Carstea

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et al. 1997; Morris et al. 1999). Very recently, a previously identified gene, *HE1,* mapped to chromosome 14q24.3, was recognized as the gene mutated in the minor NPC2 complementation group (Naureckiene et al. 2000). The finding of identical clinical, cellular, and biochemical phenotypes in patients belonging to the two groups (Vanier et al. 1996; Christomanou et al. 2000) led to the conclusion that both gene products may function in tandem or sequentially (Vanier et al. 1996; Carstea et al. 1997).

The *NPC1* cDNA sequence (see Genbank) predicts a protein of 1,278 amino acids. Topological analysis of NPC1 has revealed that the protein contains 13 transmembrane domains, three large and four small luminal loops, six small cytoplasmic loops, and a cytoplasmic tail (Davies and Ioannou 2000). The region located between amino acid residues 615 and 797 shows strong homology to the sterol-sensing domain (SSD) identified in several other integral membrane proteins that respond to endoplasmic reticulum cholesterol (Carstea et al. 1997). This putative SSD has the same orientation as those in HMG-CoA reductase and SCAP (sterol regulatory element binding protein [SREBP] cleavage activation protein) (Davies and Ioannou 2000) and appears to have important functional significance (Watari et al. 1999). The mature protein appears glycosylated, with a size of 170–190 kilodaltons (kD) (Higgins et al. 1999; Watari et al. 1999), and there is convincing evidence that NPC1 resides in late endosomes and interacts transiently with lysosomes and the trans-Golgi network (Higgins et al. 1999; Neufeld et al. 1999). A number of studies point toward a key role for the NPC1 protein in modulating vesicular trafficking of cholesterol and of glycolipids (Neufeld et al. 1999; Blanchette-Mackie 2000; Ory 2000; Zhang et al. 2001), but recent data suggest that NPC1 is a permease acting as a transmembrane efflux pump (Davies et al. 2000).

There are still only limited published studies on NPC1 mutations (Carstea et al. 1997; Greer et al. 1998, 1999; Millat et al. 1999; Yamamoto et al. 1999, 2000). The mutation prevalent in patients with Acadian Nova Scotian origins (Niemann-Pick type D) was identified as G992W (Greer et al. 1998), and that prevalent in Hispanic patients from the Upper Rio Grande area of the United States was identified as I1061T (Millat et al. 1999). The latter allele was further shown to be the most common NPC1 mutation among individuals of western European descent (Millat et al. 1999). Greer et al. (1999) pointed out that a majority of mutations were clustered in a luminal cysteine-rich loop, highlighting the potential functional importance of this domain. On the other hand, the study by Yamamoto et al. (2000), including mutational and immunoblotting studies, as well as information on the clinical presentation, disclosed a much wider distribution of the mutations. Preliminary results from the present study were presented in abstract form (Vanier et al. 1999; Vanier and Millat 2000).

Identification of mutations and studies of the mutated protein in cultured skin fibroblasts of selected patients with mutations in NPC1 was initiated primarily to obtain more information on functional domains of the NPC1 protein. Although we attempted to cover the entire spectrum of clinical and biochemical phenotypes, we were particularly interested in studying two subsets of patients. Children with infantile neurological onset invariably showed pronounced cellular cholesterol abnormalities (Vanier et al. 1988, 1991*a*); they clearly corresponded to the most severe form of the disease and therefore were best suited to pinpoint the most critical regions of the protein. Among patients with other clinical phenotypes, a majority share the same "classic" biochemical pattern, but about one-fifth show moderate alterations of cellular cholesterol trafficking, described as the "variant phenotype" (Vanier et al. 1991*b*). The biochemical subtype has been shown to be constant within a sibship (Vanier et al. 1991*b*) and thus is most likely defined by the genotype. Whether the variant phenotype is correlated with alterations of a specific domain of the protein is not yet known. To address this question, we have studied patients with late onset showing either severe or mild cholesterol-trafficking abnormalities. Since no biochemical marker has been found, so far, to correlate with a given clinical phenotype, the second aim of this study has been to evaluate correlations between genotype and clinical phenotype. Overall, our findings allow assignment of existing or new allelic combinations to specific phenotypes and provide new information on the functional significance of two particular domains of the NPC1 protein.

Subjects and Methods

Subjects

Cultured skin fibroblasts were obtained from 30 unrelated NPC patients from various ethnic backgrounds, including French (13), German (4), British (4), Dutch (1), Gypsy (1), Tunisian (3), Turkish (2), Pakistani (1), and African American (1). Close consanguinity was certain or likely in 10 families. The methods used for diagnosis by evaluation of cellular cholesterol by filipin staining and of LDL-induced cholesteryl ester formation were those described by Vanier et al. (1991*b*). Genetic-complementation analysis was performed prior to molecular studies to ensure that all patients belonged to the main (NPC1) complementation group (Vanier et al. 1996). Classification of patients with respect to their clinical and biochemical characteristics (table 1) was as proposed by us elsewhere (Vanier et al. 1988, 1991*a*,

Table 1

Clinical Summary of the 30 Families Included in the Study

Family	Cell Line Code	Biochemical Phenotype[a]	Clinical Phenotype[b]	Age at Death (years)	Age at Last Follow-up (years)	Ethnicity	Consanguinity	Parents Available	Reference
1	98119	Classic	Infantile		2.83	African American	No	No	Dawson et al. 1971
2	80001	Classic	A: Infantile	3.83		Gypsy	Yes	No	Vanier et al. 1988, case 2
			B: Infantile	3.33					
			C: Cholestatic	.30					
3	87024	Classic	A: Infantile	2.58		Tunisian	Yes	Yes	Kanoun et al. 1989
			B: Cholestatic	.25					
4	91029	Classic	A: Infantile	5.16		French	Yes	Yes	
			B: Infantile	4.92					
5	81057	Classic	A: Cholestatic	.04		French	Yes	No	Vanier et al. 1988, case 10
			B: Infantile	3.16					
6	83024	Classic	Infantile	5.08		Turkish	Likely	No	
7	83049	Classic	Infantile	5.33		French/West Indies	No	No	Vanier et al. 1988, case 13
8	82052	Classic	Infantile	3.5		French	No	No	Vanier et al. 1988, case 8 Vanier et al. 1999
9	79011	Classic	Infantile	5.0		Tunisian	No	No	Vanier et al. 1988, case 14
10	96115	Classic	Severe late infantile		4.50	French/West Indies	No	Yes	
11	82042	Classic	Late infantile	5.75		French	No	No	Vanier et al. 1988, case 16
12	95085	Classic	A: No neurological signs (cardiac problem)	1.00		Pakistani	Yes	No	
			B: Late infantile		6.00				
			C: Late infantile		5.75				
13	90104	Variant	Late Infantile	10		German	No	Yes	
14	93094	Classic	A: Late infantile	11.66		French	No	Yes	Millat et al. 1999, family 8
			B: Late infantile	8.00					
15	91048	Classic	A: Juvenile		24	French	No	Yes	Millat et al. 1999, family 1
			B: Juvenile		20				
			C: Juvenile		20				
			D: Juvenile	16					
16	98009	Classic	Juvenile		32	French	No	No	Millat et al. 1999, family 15
17	90096	Variant	A: Juvenile	18		British	No	Yes	
			B: Juvenile		24				
18	94139	Variant	Juvenile		25	British	No	No	
19	92114	Variant	A: Juvenile		28	German	No	No	
			B: Juvenile		22				
20	95070	Variant	Juvenile		13	British	No	No	
21	97089	Variant	Juvenile		20	Dutch	Yes	Yes	
22	96017	Variant	Adult		43.5	French	No	Yes	
23	96157	Classic	A: Adult	37		German	No	No	
			B: Adult		41				
24	90002	Variant	Adult without neurological symptoms (simple psychic structure)		66	German	Yes	No	Fröhlich et al. 1990
25	97014	Classic	Adult without neurological symptoms		50	British	No	No	Fensom et al. 1999
26	96084	Classic	No neurological symptoms		7.5	French	No	Yes	
27	91144	Variant	No neurological symptoms		9.0	French	No	Yes	
28	86065	Classic	Cholestatic	1.08		Tunisian	Yes	No	Vanier et al. 1988, case 39
29	97114	Classic	Cholestatic	.25		Turkish	Yes		Baumkötter et al. 1998
30	97119	Classic	Cholestatic	.5		French	No	No	

NOTE.—Families 1–25 are listed in order of decreasing severity of neurological phenotypes. Patients 26–30 could not be classified neurologically (too limited follow-up or early death resulting from liver failure).

[a] Defined by the degree of severity of alterations of intracellular cholesterol processing (Vanier et al. 1991*b*).

[b] Classification of clinical phenotypes by age at onset of neurological symptoms (except for the cholestatic rapidly fatal form) (Vanier and Suzuki 1996).

Table 2

Sequence of Primers Used for RT-PCR

Primer	Sequence (5′→3′)	cDNA Coordinates[a]
NPC-As	GAG CCC AAC CAG CCG AAC	−51/−34
NPC-Aas	CCG AAC ATC ACA ACA GAG ACT GAC	234/211
NPC-Bs	CCA TTG CCA AAG GAT GGA TAT G	148/169
NPC-Bas	GCC TTG TCA TTA CTT GAG GGG G	515/494
NPC-Cs	CTA CGT CGG ACA GAG TTT TGC C	438/459
NPC-Cas	GCT ACA TGG TGC TGT GAC CTC ATC	717/694
NPC-Ds	CAA TGG ACA GGC ACC TTT TAC C	591/612
NPC-Das	CCG TTT TCT GTA GCA CCA CAC TG	888/866
NPC-Es	GCT TGG ACG CCA TGT ATG TCA TC	791/813
NPC-Eas	CCT GAC GAA CAC GCA GTA ATG AAG	1,100/1,077
NPC-Fs	CAG CAT TTG AGG GCT GCT TG	986/1,005
NPC-Fas	AAG CGG AGG TCC AAA GGG TAC ATC	1,305/1,282
NPC-Gs	CAA CCA ATC CAG TTG ACC TCT GG	1,121/1,143
NPC-Gas	AGC ACG GAA TGG CTG TTC TG	1,484/1,465
NPC-Hs	CAC GAA CTG CAC CAT TTT GAG TG	1,428/1,450
NPC-Has	TTG ACA GGG AAG GTA ATC ACA AGG	1,703/1,680
NPC-Is	TGT GTT GGG AGG CTA TGA TGA TC	1,635/1,657
NPC-Ias	CCT GCG ACA GCT TTT GAT GTG	1,941/1,921
NPC-Js	GTG ACA GTG ATG TCT TCA CCG TTG	1,850/1,873
NPC-Jas	AGG ATG ACA GGA ACA TAC TGG GAG	2,218/2,195
NPC-Ks	CCA GAG AGA TGA ACG TCT TCA AGG	2,127/2,150
NPC-Kas	CCC CAA GAG ACT CAC GAA ACA G	2,352/2,331
NPC-Ls	CGG GAT TGG CAG TCT TCA TTG	2,291/2,311
NPC-Las	TCC ACC ATG TAG GAG TCA TCT GG	2,621/2,599
NPC-Ms	AGC ATC GCA GTC CTG AAC AAA G	2,545/2,566
NPC-Mas	GAT ATT GTC CAC TCG ACA GCA AGA C	2,886/2,862
NPC-Ns	TTT CGA CTG GGT GAA GCC ACA G	2,838/2,859
NPC-Nas	GCG TCA ATA AAG TCA GCA GAG GTC	3,161/3,138
NPC-Os	AAG TGT GGC AAA GGG GGA CAT G	3,028/3,049
NPC-Oas	ACC GAG GTT GAA GAT AGT GTC GTC	3,309/3,286
NPC-Ps	ATA GCC AGT AAT GTC ACC GAA ACC	3,181/3,204
NPC-Pas	TTC ATG CTC ACC GTG AAC GCT C	3,539/3,518
NPC-Qs	AAC CTG GTG ATG AGC TGT GGC ATC	3,466/3,489
NPC-Qas	GTA TCG CTC TTC AGT GGC ACA AC	3,801/3,779
NPC-Rs	CTC CGT GTT CAG TGG AAT CAC AC	3,588/3,610
NPC-Ras	ACA CAG TTC AGT CAG GAT GCC C	3,869/3,848

[a] Counted from the adenosine residue of the initiation codon.

1991*b*; Vanier and Suzuki 1996; Millat et al. 1999). In summary, patients with neurological symptoms were categorized, by type and age at onset of first neurological symptoms, as having either a severe infantile form (onset at age <2 years), a late infantile form (onset at age 3–5 years), a juvenile form (onset at age >5–16 years), or an adult form (onset at age >16 years). The denomination "rapidly fatal cholestatic form" was applied to the disease of patients who died from liver failure in the first months of life. On the basis of the severity of impairment in intracellular cholesterol processing in cultured fibroblasts (Vanier et al. 1991*b*), patients were also classified into either a classic or a variant biochemical phenotype. The classic phenotype refers to patients with a striking accumulation of free cholesterol in lysosomes assessed by filipin staining, together with a severe block in LDL-induced cholesteryl ester formation. In cells from biochemically variant patients, cholesterol storage may be evidenced only after challenge with pure LDL, and the rate of cholesteryl ester formation may not be affected. Cultured skin fibroblasts or peripheral blood samples were also available from the parents of 11 patients. Genomic DNA was obtained from a control population of 95 unrelated unaffected subjects and from 30 additional patients with mutations in NPC1, of whom 24 showed a variant biochemical phenotype.

DNA and RNA Isolation

Genomic DNA was isolated from skin fibroblasts or from whole blood, according to the protocol of Jeanpierre et al. (1987). Total RNA was extracted from fibroblasts monolayers using the Trizol reagent (Gibco BRL).

Reverse-Transcription and SSCP Analysis

Reverse transcription was performed using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. *NPC1* cDNA was amplified in 18 overlapping fragments, using primers described in table 2. Each PCR was performed in a 20-μl reaction volume containing 0.4 μl of cDNA, 200 μM of each dNTP, 1.5 mM $MgCl_2$, PCR buffer, 1 U of Eurobio*Taq*, 4 pmol of each primer, 0.1 μl α[^{33}P]dATP, and 3,000 Ci/mmol (Amersham Pharmacia Biotech). Two microliters of the radiolabeled PCR product were mixed with 15 μl stop solution (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol) and denatured for 5 min at 95°C. The sample was loaded on 0.5 × MDE gels (Biowhittaker Molecular Applications), with or without 10% glycerol. Electrophoresis was performed overnight in 0.6 × TBE running buffer, either at 20 W at 4°C or at 6W at room temperature.

Mutation Analysis

Sequences of PCR products with aberrant SSCP patterns were determined on cDNA and on genomic DNA using the Thermosequenase cycle sequencing kit, with primers labeled with γ[^{33}P]dATP, 3,000 Ci/mmol (Amersham Pharmacia Biotech). Because of the numerous known polymorphisms of the *NPC1* gene (Morris et al. 1999; Yamamoto et al. 1999, 2000), for each new point mutation identified, genomic DNA from the patient and from 95 control samples was amplified using specific primers. PCR products were laid as a dot-blot on a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). Membranes were hybridized with either wild-type or mutated γ[^{33}P]dATP labeled oligoprobes. Whenever possible (table 1), the mutation was also studied in the parents.

Detection of the P1007A Mutation by Introduction of an NheI *Restriction Site*

Genomic DNA was amplified using the sense primer 5′-TTGACCCTGCCTGCGTTC-3′ and the antisense primer 5′-CTTTGCCACACTTGGGGCT-3′. The 40 PCR cycles each included steps of 45 s at 92°C for denaturation, at 60°C for annealing, and at 72°C for extension. The 126-bp PCR product was digested for 2 h at 37°C by *Nhe*I (Roche Diagnostics) and was analyzed on a 12% polyacrylamide gel. The mutation gave rise to a 105-bp product.

Western Blot

Western blotting was performed as described by Yamamoto et al. (2000). Pellets from confluent fibroblast cultures (in MEM or DMEM, supplemented with 10% fetal calf serum) were homogenized and briefly sonicated on ice in buffer A (50 mM Tris (pH8.0), 150 mM NaCl, 100 μg/ml PMSF, and 1 μg/ml aprotinin). The lysates were centrifuged for 20 min at 100,000 rpm (+4°C). After centrifugation, the supernatants were discarded. The pellets were sonicated again with buffer A containing 0.4% SDS and were centrifuged for 20 min at 13,000 rpm. The protein content of the supernatants was determined by the Coomassie Plus Protein Assay Reagent Kit (Pierce). The lysates (10 μg protein) were subjected to 7.5% SDS/PAGE. After transfer overnight to nitrocellulose membranes (BioRad), NPC1 protein was detected with a polyclonal NPC1 rabbit antibody raised against a peptide corresponding to a 19–amino acid residues (1256–1274) peptide in the C-terminus of human NPC1, used at 1:500 dilution. Final detection was done by chemiluminescence using the ECL detection kit (Amersham Pharmacia Biotech).

Results

Spectrum of NPC1 Mutations

Analysis of cDNA and genomic DNA from 30 patients belonging to the NPC1 complementation group led to the identification of 32 different mutations, 26 of which were novel. A compendium of the mutant alleles is presented in table 3, and their location on a model of the NPC1 protein is represented schematically in figure 1. The largest number (25) of mutant alleles contained point mutations that led to single–amino acid substitutions. None of the novel substitutions was found in the control population of 95 individuals tested. Other abnormalities included two nonsense mutations, two 1-bp deletions, one 381-bp insertion, one 14-bp duplication, and one splice mutation. Mutations were widely scattered and affected all domains of the protein except for the NPC1 domain in the N-terminal end. Three of the five frameshift mutations were in the N-terminal luminal loop, leading to complete or near-complete truncation of the protein, and three missense mutations were located in the putative SSD. Among them, the Q775P allele was found to be homozygous in one family (5) and heterozygous in another (20). It was noteworthy that 11 (i.e., one-third) of the 32 identified mutations were located on the luminal cysteine-rich loop between transmembrane domains 8 and 9. Except for a nonsense mutation, these mutations all resulted in a single–amino acid substitution.Two different changes of the same nucleotide, G2974C (24) and G2974A (27) were observed, both resulting in the G992R substitution. Because of our particular selection of families, the I1061T allele, documented elsewhere as the most common *NPC1* mutation (Millat et al. 1999), constituted only 7 of the 60 alleles, but P1007A (Greer et al. 1999) appeared as the second-most-recurrent allele (4/60) in this study.

Table 3

Mutations in 30 Unrelated Patients with NPC1

Patient Identification (Phenotype)	Location	Nucleotide Change[a]	Effect on Protein	
			Amino Acid Change	Affected Domain[b]
1 98119 (Cl)	Exon 2	72delC	Frameshift from codon 24[c] PTC+33 aa	Stop at the end of signal peptide
		72delC or deletion		
2 80001 (Cl)	Exon 6	763dupl 14 bp[c] (GCCCCAGCCCCCAC)	Frameshift from codon 255[c] PTC+59 aa	Stop before TM1
3 87024 (Cl)	Exon 6	845delT[c]	Frameshift from codon 282[c]PTC+27 aa	Stop within TM 1
4 91029 (Cl)	Exon 19	C2819A[c]	S940X[c]	Stop within the cysteine-rich luminal loop between TM 8 and 9
5 81057 (Cl)	Exon 15	A2324C[c]	Q775P[c]	Sterol-sensing domain– TM7; residue conserved in SCAP
6 83024 (Cl)	Exon 21	G3160A[c]	A1054T[c]	Cysteine-rich luminal loop between TM 8 and 9
7 83049 (Cl)	Exon 6	C709T[d]	P237S	N-terminal loop
	Exon 8	G1211A	R404Q	Luminal, between TM 2 and 3
8 82052 (Cl)	Exon 12	T1892G	M631R	TM3
	?	?	?	?
9 79011 (Cl)	Exon 9	G1553A[d]	R518Q+splicing mutation	Between TM 2 and 3
	?	?	?	?
10 96115 (Cl)	Exon 14	T2171C	L724P	Sterol-sensing domain
	Intron 24	IVS24+1G→C	Frameshift after codon 1197 PTC+3 aa	Exon 24 skipping+frameshift
11 82042 (Cl)	Exon 23	G3557A[e]	R1186H	Between TM 11 and 12
	?	?	?	?
12 95085 (Cl)	Exon 23	G3503A[c]	C1168Y[c]	Between TM 11 and 12 (cytosolic loop)
13 90104 (Va)	Exon 20	C3019G	P1007A	Cysteine-rich luminal loop between TM 8 and 9
	Exon 22	G3428A	W1143X	Between TM 11 and 12
14 93094 (Cl)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 6	G724C	D242H	N-terminal loop

15 91048 (Cl)	Exon 21	T3182C[c,f]	I1061T[c]	Cysteine-rich luminal loop between TM 8 and 9
16 98009 (Cl)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 12	C1814T	A605V	Loop between TM 2 and 3
17 90096 (Va)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 20	C3019G[g]	P1007A	Cysteine-rich luminal loop between TM 8 and 9
18 94139 (Va)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 20	C3019G[g]	P1007A	Cysteine-rich luminal loop between TM 8 and 9
19 92114 (Va)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 20	C3019G[g]	P1007A	Cysteine-rich luminal loop between TM 8 and 9
20 95070 (Va)	Exon 20	G2956A	G986S	Cysteine-rich luminal loop between TM 8 and 9
	Exon 15	A2324C	Q775P	Sterol-sensing domain – TM7
21 97089 (Va)	Exon 19	C2829G[c]	I943M[c]	Cysteine-rich luminal loop between TM 8 and 9
22 90002 (Va)	Exon 19	G2848A[c]	V950M[c]	Cysteine-rich luminal loop between TM 8 and 9
23 96157 (Cl)	Exon 16	A2474G	Y825C	Cytosolic loop between TM 7 and 8
	?	?	?	?
24 96157 (Va)	Exon 20	G2974C[c]	G992R[c]	Cysteine-rich luminal loop between TM 8 and 9
25 97014 (Cl)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 8	T1133C	V378A	Loop between TM 2 and TM3
26 96084 (Cl)	Exon 18	A2621T[c]	D874V[c]	Cysteine-rich luminal loop between TM 8 and 9
27 91144 (Va)	Exon 20	G2974A	G992R	Cysteine-rich luminal loop between TM 8 and 9
	Junction exon 1/exon 2	57ins381bp	Frameshift from codon 19 PTC+19 aa	Stop within signal peptide
28 86065 (Cl)	Exon 19	G2801A[c,g]	R934Q[c]	Cysteine-rich luminal loop between TM 8 and 9
29 97114 (Cl)	Exon 6	T815G[c]	M272R[c]	TM1
30 97119 (Cl)	Exon 19	G2830A	D944N	Cysteine-rich luminal loop between TM 8 and 9
	Exon 22	T3425C	M1142T	TM 10

NOTE.—PTC = premature stop codon. TM = transmembrane domain. Cl = classic biochemical phenotype. Va = variant biochemical phenotype.

[a] Counted from the adenosine residue of the initiation codon.

[b] The putative spanning membrane domains of the NPC1 protein were determined according to Davies and Ioannou (2000).

[c] Mutation was homozygous in the patient.

[d] Yamamoto et al. 1999.

[e] Carstea et al. 1997.

[f] Millat et al. 1999.

[g] Greer et al. 1999.

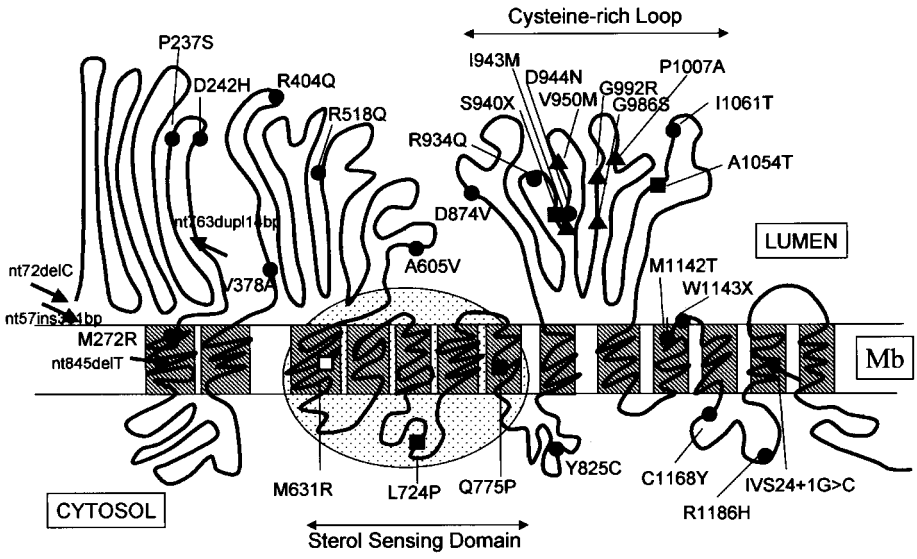

Figure 1 Topology of the mutations identified in 30 patients on an NPC1 protein model and genotype/phenotype correlations. The schematic NPC1 protein model is drawn as proposed by Davis and Ioannou (2000). The hatched areas indicate the putative transmembrane domains. The dotted oval frame delimits the SSD. →, Frameshift mutations, all of them observed in patients with a severe infantile neurological form. ■, Missense or nonsense mutation definitively associated to a severe infantile neurological form. □, missense mutation likely correlated with a severe infantile neurological form. ▲, Missense mutation definitively correlated with a variant biochemical phenotype. ●, Other missense mutation.

Correlations between Genotypes and the Classic or Variant Biochemical Phenotypes

One aim of our study was to pinpoint possible differences between mutations associated with the classic or the variant biochemical phenotypes (severe or mild impairment of cellular cholesterol trafficking, respectively) on the basis of the observation that the biochemical phenotype was constant within a sibship (Vanier et al. 1991*a*). In the homozygous state, all frameshift, splice or nonsense mutations and a number of missense mutations (M272R, Q775P, D874V, R934Q, A1054T, I1061T, and C1168Y) led to the classic biochemical phenotype (fig. 1 and table 3). This was also the case for several combinations of two missense mutations (patients 7, 14, 16, 25, and 30) or of L724P associated with a frameshift mutation (patient 10) (table 3).

Among the nine patients with the less common variant phenotype, four were found to carry one P1007A allele, combined either with I1061T (patients 17, 18, and 19) or to a nonsense mutation, W1143X (patient 13). I1061T has definitively been correlated with a classic phenotype when in the homozygous state (Millat et al. 1999), and one P1007A allele thus appeared sufficient to maintain some degree of cholesterol trafficking. These data suggested that the P1007A mutation was a common mutant allele associated with the variant biochemical phenotype.

This hypothesis was confirmed by investigation of genomic DNA in a total population of 55 patients with NPC1, using a simple PCR test based on introduction of an *Nhe*I restriction site. The P1007A allele was never observed in the 22 patients with a classic biochemical phenotype but constituted 15/66 alleles (22.7%) in the 33 patients studied who had the variant phenotype (9 from the present mutational study and 24 additional ones).

Four additional mutant alleles could clearly be ascribed to a variant phenotype: I943M (patient 21), V950M (patient 22) and G992R (patient 24) were all found in the homozygous state, and G986S (patient 20) was found in combination with Q775P which is clearly associated with a classic phenotype (patient 5). An intriguing observation is that these four mutations, as well as P1007A, are clustered within a small region of the cysteine-rich luminal loop between transmembrane domains 8 and 9 (fig. 1).

Correlations between Genotypes and Mutant NPC1 Protein Studied by Western Blot

Selected cell lines with missense mutations, most of them homozygous or associated with a frameshift mutation, were studied by immunoblotting (fig. 2). The homozygous 845delT cell line, which gives rise to very early premature termination of the protein, was tested to establish the specificity of the affinity-purified polyclonal antibody, and, as expected, no detectable band was seen in the region of interest. Normal fibroblasts gave a double band of ~170 and ~190 kD. Essentially no detectable

protein was repeatedly found in three cell lines with missense mutations: homozygous Q775P and L724P, both located in the SSD, but also A1054T in the cysteine-rich region. Furthermore, a clearly diminished amount of the protein was found in cells with the C1168Y mutation. In all other mutations studied, including the classic I1061T, a substantial amount of NPC1 protein could be detected. Definite conclusions about an apparent small reduction of protein in some mutations are difficult to draw, since repeated experiments on different cell pellets could show a variation of at least twofold in levels of expression of the protein, in both normal cells and mutant cells. We observed that the protein was unstable in dilute solution, but variations under strict experimental conditions suggest the influence of yet-undefined factors that regulate expression of NPC1. Interestingly, NPC2 cells carrying the mutation E20X on the *HE1* gene (fig. 2) or another mutation (Yamamoto et al. 2000) invariably showed NPC1 protein levels well above those observed in the control cells.

Correlations with the Clinical Phenotype

The three patients with a frameshift mutation and premature stop codon (patients 1, 2, and 3) or a nonsense mutation (patient 4) on both alleles all had an early neurological onset and a short life span. This was also the case for all patients with missense mutations for which immunoblotting revealed a lack of NPC1 protein. Patients 5 and 6 carried a homozygous missense mutation in the SSD or the cysteine-rich luminal loop, respectively. Patient 10, who had a SSD missense mutation associated with a splice mutation leading to exon-24 skipping, had a slightly later neurological onset, but the disease is progressing very rapidly. For patient 8, one allele carried a mutation in the SSD of an amino acid conserved in the *patched* gene, but the second allele remained unidentified. The significantly reduced amount of NPC1 protein observed in fibroblasts of patient 12, who had the C1168Y mutation, also correlated with the relatively severe late-infantile neurological phenotype.

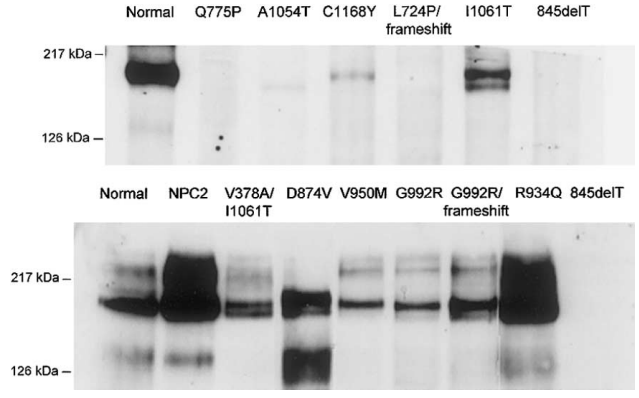

Figure 2 Western blot of NPC1 protein in cultured fibroblasts from control subjects, 12 patients with NPC1, and 1 patient with NPC2. A C-terminal affinity-purified polyclonal antibody was used. Cells from a patient with a 845delT frameshift mutation served as a negative control. Eight patients with NPC1 had a homozygous missense mutation (lanes with a single label), and three were compound heterozygotes (lanes with a dual label). The NPC2 cell line carried a homozygous E20X mutation of the *HE1* gene.

Conversely, the finding of a near-normal amount of NPC1 protein was of limited predictive value. This pattern was shared by patients with a neurological juvenile onset (patient 15, I1061T), a neurological adult form (patient 22, V950M), or a nonneuronopathic adult form (patient 24, G992R, and patient 25, I1061T/V378A). On the other hand, correlations could clearly be drawn concerning the clinical expression of some missense mutations or combination of missense mutations. The combination of the two most common alleles, I1061T/P1007A, was encountered in three families (17, 18, and 19; five patients) and resulted in a juvenile onset of symptoms and a significantly slower progression of the disease than in homozygous I1061T patients. P1007A combined with a nonsense mutation (patient 13) resulted in a late-infantile neurological form. Homozygous V950M corresponded to an adult onset of neurological symptoms. Study of two adults with splenomegaly and no neurological symptoms (patients 24 and 25) led to the conclusion that G992R and V378A were responsible for this atypical mild phenotype. Interestingly, G992R had only a mild effect on cholesterol trafficking, whereas V378A was associated with severe biochemical alterations, demonstrating that the block in cholesterol transport does not correlate with clinical severity, as discussed elsewhere (Vanier et al. 1991*b;* Vanier and Suzuki 1998). Future studies should disclose whether correlations would be tighter with the newly discovered, possibly more relevant permease function of the NPC1 protein (Davies et al. 2000).

Patients 26 and 27 are still too young for conclusions to be drawn about their potential neurological subtyping. Mutations found in patients 28–30, initially studied to facilitate prenatal diagnosis, are reported because M272R and M1142T (which affects an amino acid conserved in *patched*) were the only two missense mutations found in our study in transmembrane domains outside of the SSD. R934Q was reported elsewhere in association with I1061T (Greer et al. 1999). But since the first affected child in those families died early, of liver failure, the complete phenotype associated with these mutations remains unknown.

Discussion

General Distribution of NPC1 *Mutations*

Although the number of known *NPC1* mutations appears to be ~100, taking into account data presented by

seven groups at international meetings (Patterson et al. 2001), few studies have been published except in abstract form. In a survey of 14 Japanese patients with NPC1, with a majority of late-infantile cases, the 14 missense mutations and one in-frame deletion were widely distributed on *NPC1* cDNA (Yamamoto et al. 1999, 2000). This was at variance with the initial data on nine patients reported by Carstea et al. (1997), where most mutations were found to affect the carboxy-terminal third of the protein. A later survey of 13 families originating from Canada and Maryland, restricted to the last six *NPC1* exons and boundaries, indeed allowed identification of 20/26 mutant alleles (Greer et al. 1999). Greer and colleagues further observed that six of the eight missense mutations found in these patients—including G992W, which is characteristic of Nova Scotian patients—were clustered in a conserved NPC1-specific cysteine-rich loop. More recent topological analysis of the NPC1 protein by Davies and Ioannou (2000) indicated that the most common missense mutation, I1061T (Greer et al. 1999; Millat et al. 1999), initially thought to affect a transmembrane domain, also located to this luminal loop. In the present study, which included mostly white patients but covered a wider clinical spectrum, nearly all domains of the NPC1 protein—except for the leucine-zipper region—were affected. Eleven of the twenty-five identified missense mutations and four frameshift mutations were located in the first two-thirds of the protein, suggesting that the discrepancy between previous results in Japanese versus North American populations might be explained, in part, by differences in phenotypes of the patients. Mutagenesis studies indicated that both the leucine-zipper motif and the putative SSD were particularly critical regions (Watari et al. 1999). Mutations in the SSD have been identified by Yamamoto et al. (2000) (F703S) and in this report (M631R, L724P, and Q775P), but no naturally occurring mutations have been reported so far that affect the NPC1 leucine-zipper domain. In the present study, 14 of the 30 patients showed a proven or almost certain homozygous mutation, which greatly facilitated establishment of genotype/phenotype correlations.

Perinatal Liver Disease Independent of the NPC1 Mutation

It is well known that the neonatal, severe cholestatic form of NPC and neurological forms of the disease may occur in the same sibship (Vanier et al. 1991*a*); this is further illustrated in this study by families 2, 3, and 5. Most of the published families have shown an association with an infantile neurological form (Patterson et al. 2001), but one of the sibs in the family described by Jaeken et al. (1980) had a neurological juvenile-onset form. We recently found one patient with fetal hydrops/ascites to be homozygous for the I1061T mutation (M. T. Vanier and G. Millat, unpublished data). His affected elder brother, now 24 years old, presented with the expected neurological juvenile form (Millat et al. 1999). Thus, fatal liver disease in a first affected child (patients 28–30) gives no clue as to the possible neurological course in other sibs, unless the mutations correspond to a known neurological subtype. Remarkably, the rapidly fatal neonatal hepatic forms we studied so far all showed severe alterations of cellular cholesterol trafficking (M. T. Vanier, unpublished data).

Missense Mutations in SSD as well as Frameshift and Nonsense Mutations Lead to Absence of Stable NPC1 Protein and Severe Neurological Phenotype

In patient 1, initially described as having "lactosylceramidosis" (Dawson et al. 1971) and later reclassified as having NPC (Wenger et al. 1975; Vanier et al. 1988), a frameshift occurs at the end of the putative signal peptide. Patients in families 2 and 3 (Vanier et al. 1988; Kanoun et al. 1989) also have a very early NPC1 termination caused by a frameshift mutation, whereas those in family 4 have a nonsense mutation. The very severe neurological and biochemical phenotype observed in these families thus is well in line with the nature of the molecular lesion. The Q775P missense molecular lesion found for family 5 is of particular interest, both as the first naturally occurring homozygous mutation described in the NPC1 SSD (amino acids 615–797) and because it affects a SCAP-conserved residue. NPC1 protein (fig. 2) was undetectable both in fibroblasts of this patient and in those of patient 10, in whom another SSD-located substitution, L724P, was associated with a truncation of the C-terminus that should result in abnormal targeting of the protein (Watari et al. 1999). In patient 8, who had a M631R alteration, the second allele remained unidentified, but this patient also had a very severe course of the disease. Absence of detectable levels of NPC1 protein was reported by Yamamoto et al. (2000) in a Japanese patient with a F703S/S813X combination. Our results are in agreement with those of the latter authors, who were the first to point out a correlation between absence or near absence of NPC1 protein and the most severe clinical presentations (Yamamoto et al. 2000). Conversely, SSD mutants created by site-directed mutagenesis (Y643S, Y643C, and P691S), studied after transfection in CT-60 CHO cells, showed the presence (although in reduced amount) of NPC1 protein (Watari et al. 1999). Since we obtained similar results after transfection of Q775P cDNA to mutant FR4 CHO cells (K. Chikh, G. Millat, C. Tomasetto, K. Ohno, and M. T. Vanier, unpublished data), a likely explanation would be that overexpression masks the instability of the native protein. Brain glycolipids, studied in patients

1 (Dawson et al. 1972) and 8 (Vanier 1999), showed striking abnormalities. Taken together, these data clearly show that mutations affecting the putative SSD of the NPC1 protein are particularly deleterious, since they invariably correspond to the most severe neurological form of the disease.

Most missense alterations located to other regions of the NPC1 protein also severely affect cholesterol processing. A majority have a lesser impact on the stability of the protein as seen from immunoblotting, and a varying effect on the clinical picture, as illustrated by C1168Y (late-infantile neurological onset, patient 12), I1061T (juvenile neurological onset; Millat et al. 1999), and V378A, which apparently underlines the nonneurological adult phenotype observed in patient 25.

In spite of the fact that recent data show that NPC1 has an unexpected transmembrane molecular-pump activity and suggest that it is a permease that can transport certain lipids, but apparently not cholesterol, across membranes (Davies et al. 2000), studies in patients definitively highlight a key role of the SSD for the in vivo function of the protein.

Cysteine-Rich Luminal Loop Appears as a Functionally Complex Domain

Greer et al. (1999) were the first to highlight the functional significance of the cysteine-rich domain on the basis of mutational studies in patients. This domain has resemblance to the RING-finger motif regulatory domain of protein kinase C. Watari et al. (2000) further showed that this domain can bind zinc. From the studies of Greer et al. (1999), Yamamoto et al. (2000), and ourselves (Millat et al. 1999 and the present work), no fewer than 16 different missense mutations have already been ascribed to the cysteine-rich domain, which spans amino acids 855 through 1098 (Davies and Ioannou 2000). The first mutation identified, G992W, characterizes patients from Nova Scotia (former Niemann-Pick type D), with a juvenile onset and slowly progressing neurological disease (Greer et al. 1998). Despite the fact that Nova Scotian patients are of Acadian extraction, G992W has not been found so far in French patients. We identified two other base alterations on the same codon, both leading to the G992R substitution. Patient 24, who has isolated splenomegaly (Frölich et al. 1990) and is homozygous for this mutation, still has no neurological signs of NPC at age 66 years (K. Harzer, unpublished data). Cultured fibroblasts from this patient; from patient 27, who has G992R and a frameshift mutation on the other allele; and from patients from Nova Scotia (Byers et al. 1989) all behaved as biochemical "variants," leading to the conclusion that codon 992 is not essential for cholesterol transport. On the other hand, the most recurrent I1061T mutation, initially described by us (Millat et al. 1999) and by Greer et al. (1999), which constitutes 15%–18% of mutated alleles in countries like the United Kingdom, France, or the United States, is correlated with severe alterations of cholesterol trafficking (the classic phenotype) and a juvenile-onset neurological disease. Immunoblotting studies revealed a quite good level of NPC1 protein in cells homozygous for both the G992R and I1061T mutations. Conversely, the present study has identified the A1054T mutation, for which no NPC1 protein was detectable, and which correlated with a severe infantile neurological onset form (patient 6). Thus, at variance with what was seen for the SSD, consequences of naturally occurring missense mutations affecting the cysteine-rich luminal loop appeared highly variable.

P1007A is a Common Mutated Allele Associated with the Variant Biochemical Phenotype

One patient homozygous for the P1007A mutation, categorized as having the biochemically variant phenotype, was described by Greer et al. (1999). We found four additional patients with the variant phenotype in whom this allele was combined with I1061T or a nonsense mutation and further showed a high prevalence of this allele, 22.7%, in a total population of 33 patients with the variant phenotype. Systematic screening for this mutation thus appears justified in patients with the variant phenotype.

Seven Variant Mutant Alleles So Far Identified Locate to the Cysteine-Rich Luminal Loop

From the study of patients with the P1007A and G992R mutations, we concluded that a single variant allele apparently leads to a variant phenotype, irrespective of the nature of the second allele. Apart from G992W, G992R, and P1007A discussed above, I943M, V950M, G986S (identified in this report) and V889M (Yano et al. 1996; Yamamoto et al. 2000) could all be classified as variant alleles. Although a recent study in Portuguese patients indicated that some variant mutations may affect another domain (Ribeiro et al., in press), those reported so far all located to the cysteine-rich loop, and most of them were clustered within a small region (codons 943–1007) of this loop. The present observation suggests that part of the cysteine-rich domain could be associated with a NPC1 function not tightly associated with cholesterol trafficking. The NPC1 protein seems to be implicated in glycolipid transport (Blanchette-Mackie 2000; Zhang et al. 2001), and, since glycolipid abnormalities have been found in brain of patients with the variant phenotype (Martin et al. 1984; Vanier 1999), more-detailed studies are indicated. Future studies on the three-dimensional structure of the cysteine-luminal loop might also provide new insights on the functional

complexity of this domain. Above all, how variant mutations specifically relate to the molecular-pump activity of NPC1 recently described by the group of Ioannou (Davies et al. 2000) is a particularly intriguing question.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for NPC1 cDNA [accession number AF002020])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for Niemann-Pick C [MIM 257220]

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