Complete Abolition of the Retinal-Specific Guanylyl Cyclase (retGC-1) Catalytic Ability Consistently Leads to Leber Congenital Amaurosis (LCA)

Jean-Michel Rozet, Isabelle Perrault, Sylvie Gerber, Sylvain Hanein, Fabienne Barbet, Dominique Ducroq, Eric Souied, Arnold Munnich, and Josseline Kaplan

Purpose. Leber congenital amaurosis (LCA) is the earliest and the most severe form of all inherited retinal dystrophies. In 1996, the current investigators ascribed the disease in families linked to the *LCA1* locus on chromosome 17p13.1 to mutations in the photoreceptor-specific guanylyl cyclase (*retGC-1*) gene. So far, 22 different mutations, of which 11 are missense mutations, have been identified in 25 unrelated families. This is a report of the functional analyses of nine of the missense mutations.

Methods. cDNA constructs were generated that contained the *retGC-1* missense mutations identified in patients related to the *LCA1* locus. Mutants were expressed in COS7 cells and assayed for their ability to hydrolyze guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP).

Results. All mutations lying in the catalytic domain showed a complete abolition of cyclase activity. In contrast, only one mutation lying in the extracellular domain also resulted in a severely reduced catalytic activity, whereas the others showed completely normal activity.

Conclusions. More than half the mutations identified in patients related to the *LCA1* locus are truncating mutations expected to result in a total abolition of retGC-1 activity. Concerning missense mutations, half of them lying in the catalytic domain of the protein also result in the complete inability of the mutant cyclases to hydrolyze GTP into cGMP in vitro. In contrast, missense mutations lying in the extracellular domain, except one affecting the initiation codon, showed normal catalytic activity of retGC-1. Nevertheless, considering that all patients related to the *LCA1* locus displayed the same phenotype, it can be assumed that all missense mutations would have the same dramatic consequences on protein activity in vivo as truncation mutations. (*Invest Ophthalmol Vis Sci.* 2001;42: 1190–1192)

Leber congenital amaurosis (LCA; Mendelian Inheritance in Man [MIM] 204000) is the earliest and most severe form of all inherited retinal dystrophies. Originally described by Leber in 1869, LCA is an autosomal recessive condition distinct from other retinal dystrophies and is responsible for congenital blindness.[1] The diagnosis is usually made at birth or during the first months of life in an infant with total blindness or greatly impaired vision, normal fundus, and extinguished electroretinogram (ERG).[2] It is generally accepted that LCA accounts for 5% of all retinal dystrophies, but considering the high rate of consanguinity in LCA families, we assume that this condition is not uncommon in countries with a high rate of consanguineous unions.[3,4] A certain degree of clinical heterogeneity has long been recognized in LCA, but these clinical differences have largely been ignored.

Conversely, genetic heterogeneity has been accepted for a long time.[5] In 1995, we localized the first disease-causing gene, *LCA1*, to chromosome 17p13 and confirmed the genetic heterogeneity.[6,7] In 1996, we ascribed *LCA1* to mutations in the photoreceptor-specific guanylyl cyclase gene (*retGC-1*), which catalyzes the conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP) in the photoreceptor cells.[8] Twenty-two different mutations were identified in patients related to the *LCA1* locus. Half of them were truncating mutations expected to result in the total abolition of the cyclase activity of retGC-1,[9] whereas the other half were missense mutations. We report here the study of 9 of 11 of the missense mutations (M1I, W21R, L41F, N129K, R313C, R976, R995W, M1009L, and H1019P), as well as a mutation truncating the COOH end of the protein (Q1036Z), on the catalytic ability of the mutant proteins.

Methods

Patients

A total of 130 unrelated families fulfilling the minimum criteria for diagnosis of LCA[10] were ascertained from various genetic and ophthalmologic sources. Among these 130 families originating from various countries across the world, 44 were consanguineous, 41 were multiplex, and 18 were both multiplex and consanguineous.[9] All procedures were conducted in compliance with the tenets of the Declaration of Helsinki, and informed consent was obtained from all participants.

Spectrum of *retGC-1* Mutations

Forty-seven *retGC-1* mutations (22 different, 18 homozygous) were identified in 25 of 130 unrelated patients with LCA (18.5%).[9] Among the 22 different mutations identified, 11 were missense mutations, and 11 were truncating mutations. Interestingly, 15 of the 25 patients were found to carry mutations expected to result in the truncation of the retGC-1 protein[9] (11 homozygotes, 3 compound heterozygotes, and 1 single heterozygote).

Expression Studies

The full length *retGC-1* cDNA (3621 bp, GenBank accession number, M92432) was cloned into the eukaryotic expression vector PRK5 (Clonetech, Palo Alto, CA). PRK5 contains the early promoter and the polyadenylation signal of the simian 40 virus (SV40). Single-base substitutions (M1I, W21R, L41F, N129K, R313C, R976, R995W, M1009L, H1019P, and Q1036Z) were created by site-directed mutagenesis (Quickchange Site Directed Mutagenesis kit; Stratagene, La Jolla, CA), by using oligonucleotides specific for the various mutations (not shown, available on request). Mutant clones were transformed into DH5α bacteria cells and sequenced before expression studies.

From the Unité de Recherche sur les Handicaps Génétiques de l'Enfant, Insitut National de la Santé et de la Recherche Médicale U393, Hôpital des Enfants Malades, Paris, France.

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Corresponding author: Josseline Kaplan, Unité de Recherche sur les Handicaps Génétiques de l'Enfant, INSERM U393, Hôpital des Enfants Malades, 149 rue de Sèvres, 75743 Paris Cedex 15, France.
kaplan@necker.fr

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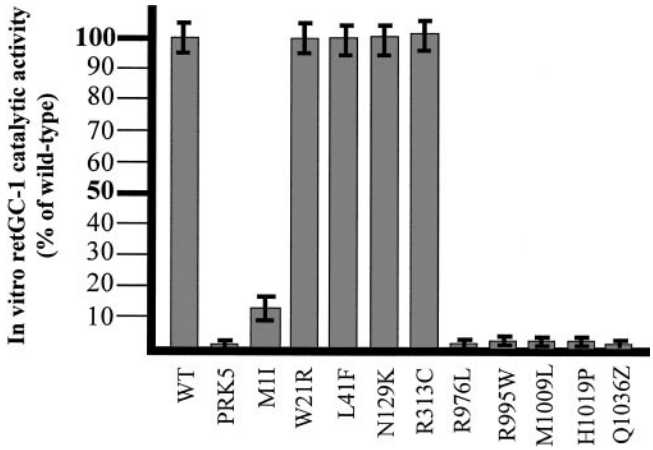

FIGURE 1. In vitro expression study of 11 mutant *retGC-1* cDNAs. Results are means ± SD of three distinct determinations.

Monkey COS7 cells (American Type Culture Center, Rockville, MD [ATCC]) were grown in minimum essential medium (MEM) supplemented with 10% fetal calf serum. Before transfection, the cells (10^6/80-cm^2 tissue culture flask) were grown for 24 hours in MEM with 10% fetal calf serum. For transfection, cells were incubated in MEM chloroquine with either normal or mutant PRK5 retGC-1 (9 μg). After 3 hours, the cells were treated with 10% dimethyl sulfoxide (DMSO) in Hanks' balanced salt solution (2 ml) for 2 minutes. The DMSO medium was discarded, and the cells were washed twice with MEM and incubated in fresh medium for 48 hours to allow expression of the transfected constructs. The luciferase cDNA was cloned into a PSG5 vector and systematically cotransfected (1 μg) as a test of transfection efficiency. After 48 hours of culture, the cells were washed twice in 5 ml ice-cold 0.02 M HEPES (pH 7.4) and 0.15 M NaCl; harvested in a centrifuge tube in a total of 1.5 ml of 20 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol; and broken by passage 10 times through a 22-gauge needle. The homogenate was spun for 15 minutes at 5000*g*. Supernatant protein concentration was adjusted to 4 mg/ml, and luciferase activity was measured according to the manufacturer's protocol (Luciferase Assay System; Promega, Madison, WI). The pellet was washed in 1.5 ml of the same buffer. Membranes were solubilized for 30 minutes on ice in 250 μl of 20 mM HEPES (pH 7.4), 100 mM NaCl, 1% Triton X-100, 10% glycerol, and 1 mM dithiothreitol. After centrifugation for 5 minutes at 5000*g*, supernatant fluid protein was adjusted to 4 mg/ml. Two hundred micrograms of membrane protein was assayed to determine guanylyl cyclase activities in a total reaction volume of 150 μl containing 20 mM HEPES (pH 7.4), 0.1 mM GTP, 4 mM $MnCl_2$, 0.2 mM 3-isobutyl-1-methylxantine (IBMX), and 1 μCi [α-32P]GTP. Incubations were for 20 minutes at 37°C and were terminated by the addition of 750 μl of 120 mM zinc acetate and 600 μl 144 mM sodium carbonate. The produced [α-32P]cGMP was purified by neutral alumina chromatography and quantitated by liquid scintillation counting, as described.[11]

RESULTS

Expression Analysis of Mutant Alleles

Figure 1 shows the retGC-1 activity in COS7 cell homogenates after transient expression of wild-type and mutant cDNA constructs. Each experimental value is the mean (±SD) of four independent triplicate experiments and is expressed as percentages of wild-type retGC-1 activity. Experimental values were corrected for differences in transfection efficiencies by normalizing for luciferase activity and were subtracted with background from nonrecombined PRK5-transfected cells.

All mutations lying in the catalytic domain (R976L, R995W, M1009L, H1019P, and Q1036Z) result in the total abolition of the ability of the mutant cyclases to hydrolyze GTP into cGMP. Conversely, except the M1I mutation, which affects the initiation codon and results in severely reduced catalytic activity (13% compared with the wild-type), all mutations lying in the extracellular domain (W21R, L41F, N129K, and R313C) showed a normal cyclase activity compared with the wild-type.

TABLE 1. *RetGC-1* Mutations Identified in Patients Related to the *LCA1* Locus and Deduced or Measured Residual Catalytic Activities of the Mutant Cyclases Encoded by Each Allele

Family#	Allele 1	Activity (%)	Allele 2	Activity (%)
34	**620delC**	**0**	**620delC**	**0**
23	**620delC**	**0**	**620delC**	**0**
3	**387delC**	**0**	**387delC**	**0**
90	**387delC**	**0**	**387delC**	**0**
91	**387delC**	**0**	**387delC**	**0**
121	**387delC**	**0**	**387delC**	**0**
110	**226-239del14bp**	**0**	**226-239del14bp**	**0**
85	**52-99dup48bp**	**0**	**52-99dup48bp**	**0**
20	**1805-1829del25pb**	**0**	**1805-1829del25pb**	**0**
33	**3078-3079delGA**	**0**	**3078-3079delGA**	**0**
70	**IVS9+2T/A**	**0**	**IVS9+2T/A**	**0**
11	**M1I^{EC}**	**13 ± 5**	**M1I^{EC}**	**13 ± 5**
51	**M1I^{EC}**	**13 ± 5**	**IVS9-1G/T**	**0**
31	**R976L^{CD}**	**0**	**R976L^{CD}**	**0**
56	**H109P^{CD}**	**0**	**H1019P^{CD}**	**0**
88	**M1009L^{CD}**	**0**	**IVS9-1G/A**	**0**
82	**R995W^{CD}**	**0**	N129K^{EC}	100
52	**S448Z**	**0**	R313C^{EC}	100
72	**Q1036Z^{CD}**	**0**	?	—
60	W21R^{EC}	100	W21R^{EC}	100
89	L41F^{EC}	100	?	—
117	R540C	—	?	—
7	F565S^{EC}	100	F565S^{EC}	100
17	F565S^{EC}	100	F565S^{EC}	100
36	F565S^{EC}	100	F565S^{EC}	100

Family numbers refer to Perrault et al.[9] Mutations resulting in abolition of the catalytic activity of the mutant cyclases are in bold type. F565S and R540C were not analyzed in this study. EC, extracellular domain; CD, catalytic domain; ?, mutation not identified.

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Discussion

LCA is the earliest and most severe form of all inherited retinal dystrophies.[1] The genetic heterogeneity of LCA has been accepted for a long time. Conversely, the subtle clinical heterogeneity of the disease was largely ignored until it was shown that *retGC-1* mutations account for congenital and stationary cone–rod dystrophy that results in neonatal blindness, which represents the most severe form of all LCA clinical subtypes.[12]

Among the 130 unrelated LCA patients of our series, 25 were found to harbor mutations in the *retGC-1* gene. For 16 (67%) of them, the predicted residual cyclase activity resulting from the combination of the activities of the mutant proteins encoded by each *retGC-1* allele was dramatically reduced (Table 1). Indeed, 11 of 16 were found to be homozygous for a truncating mutation (patients 34, 23, 3, 90, 91, 121, 110, 85, 20, 33, and 70; Table 1), 3 of 16 harbored a homozygous missense mutation resulting in a dramatic reduction of the cyclase activity (patients 11, 31, and 56; Table 1), and 2 of 16 were compound heterozygotes for a truncating mutation and a missense mutation leading to the same dramatic reduction in catalytic activity (patients 51, and 88; Table 1). In addition, among the nine remaining patients, three were found to carry one mutation resulting in the complete abolition of the enzyme activity (patients 72, 82, and 52; Table 1). One of these three patients was a single heterozygote for a mutation truncating the carboxyl terminus of the protein. The other two were found to carry one mutation residing in the extracellular (EC) domain of the protein. Finally, six of nine patients were found to carry mutations in this domain only (two single heterozygotes, patients 89 and 117; four homozygotes, patients, 60, 7, 17, and 36; Table 1).

It is worth noting that four of the six different EC mutations that we studied resulted in normal cyclase activity compared with the wild-type. However, all patients related to *LCA1* displayed the same phenotype, whatever the nature of the mutations they carried. Consequently, we can speculate that these last mutations should have deleterious consequences on protein activity in vivo. One explanation could have been that these mutations might result in the inability of the mutant protein to be activated by the guanylate cyclase–activating proteins (GCAPs).[13–15] However, this hypothesis is unlikely, because it has been demonstrated that the EC domain of *retGC-1* is not a critical region for the activation by GCAPs.[16,17] Therefore, the most likely explanation is that extracellular mutations might result in misfolding of the mutant *retGC-1* protein during biosynthesis and subsequent degradation in the endoplasmic reticulum (ER). Indeed, it has already been shown that potentially functional mutant proteins can be retained in the ER because of minor structural defects.[18] For instance, some patients with α_1-antitrypsin deficiency produce mutant molecules that, although functionally intact, are retained in the ER and degraded.[19] In fact, in a large number of diseases, expressions of mutant proteins are targeted to the ER and fail to reach their intended cellular location, often displaying an ER storage phenotype with aggregated material accumulating in the ER.[20] This situation has been well documented in cystic fibrosis, in which the most common mutation in the *CFTR* gene, ΔF508, leads to the disease, whereas the same mutation introduced into a recombinant *CFTR* channel does not abolish the biologic activity.[21]

Nevertheless, this hypothesis of a misfolding of the mutant cyclase encoded by a *retGC-1* gene carrying mutations in the EC domain could not be studied in our experimental conditions. Immunochemistry experiments indicate that most of the overexpressed wild-type retGC-1 protein resides in the ER (data not shown, available on request). This observation has already been described for other membrane proteins, such as the retinal specific adenosine triphosphate (ATP)-binding cassette (*ABCR*).[22]

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