DOES THE SEVERITY OF THE LGMD2A PHENOTYPE IN COMPOUND HETEROZYGOTES DEPEND ON THE COMBINATION OF MUTATIONS?

AMETS SÁENZ, PhD,[1,2] YASUKO ONO, PhD,[3] HIROYUKI SORIMACHI, PhD,[3] MARIA GOICOECHEA, PhD,[1,2] FRANCE LETURCQ, PhD,[4] LOREA BLÁZQUEZ, PhD,[5] FEDERICO GARCÍA-BRAGADO, MD, PhD,[6] ALBERTO MARINA, PhD,[7] JUAN JOSÉ POZA, MD, PhD,[2,8] MARGARITA AZPITARTE, MD,[1] NAOKO DOI, MSc,[3] MIGUEL URTASUN, MD, PhD,[2,8] JEAN-CLAUDE KAPLAN, MD, PhD,[4] and ADOLFO LÓPEZ DE MUNAIN, MD, PhD[2,8]

[1] Biodonostia Institute, Hospital Donostia, Po. Dr. Begiristain s/n, 20014 San Sebastián, Basque Country, Spain
[2] CIBERNED, Instituto Carlos III, Madrid, Spain
[3] Calpain Project, Department of Advanced Science for Biomolecules, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan
[4] 5 Institut Cochin, CNRS UMR 8104, Inserm U 1016, Université Paris Descartes, Paris, France
[5] Department of Gene Therapy and Hepatology, Center for Investigation in Applied Medicine, Pamplona, Spain
[6] Department of Neuropathology, Hospital Virgen del Camino, Pamplona, Spain
[7] Instituto de Biomedicina de Valencia–CSIC, Valencia and CIBERER, Spain
[8] Department of Neurology, Hospital Donostia, San Sebastián, Spain

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ABSTRACT: *Introduction*: Limb-girdle muscular dystrophy type 2A (LGMD2A) is caused by a deficiency of calpain-3/p94. Although the symptoms in most LGMD2A patients are generally homogeneous, some variation in the severity and progression of the disease has been reported. *Methods*: We describe 2 patients who carry the same combination of compound heterozygous mutations (pG222R/pR748Q) and whose symptoms are exceptionally benign compared to homozygotes with each missense mutation. *Results*: The benign phenotype observed in association with the combined pG222R and pR748Q mutations suggested that it may result from a compensatory effect of compound heterozygosity rather than the individual mutations themselves. Our analyses revealed that these two mutations exert different effects on the protease activity of calpain-3, suggesting 'molecular complementation' in these patients. *Conclusion*: We propose several hypotheses to explain how this specific combination of mutations may rescue the normal proteolytic activity of calpain-3, resulting in an exceptionally benign phenotype.

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Limb-girdle muscular dystrophy type 2A (LGMD2A) is the most frequent autosomal recessive muscular dystrophy.[1–4] It is caused by a deficit of calpain-3/p94, a calcium-dependent protease whose exact physiological functions and substrates are poorly understood. Disease onset in most LGMD2A patients occurs in the second decade of life, beginning with proximal muscle weakness, and most patients become wheelchair-dependent after several years of disease progression. Despite the general uniformity of the symptoms in most LGMD2A patients, some differences in severity and progression have been reported. Indeed, these differences involve variation in the sites affected or the evolution of the disease (metabolic myopathy symptoms, clinical adult-type spinal muscular atrophy, and predominant distal involvement).[5–11]

Among patients in whom the disease has progressed for over 25 years, the number who are wheelchair-dependent is higher among those who carry two null mutations than those with at least one missense mutation.[12] The precise phenotype–genotype relationship is difficult to elucidate, as there appears to be some variability among patients with the same mutation(s), in some cases even within the same family.[9–12]

We found compound heterozygous patients who have exceptionally benign symptoms compared to homozygotes with each missense mutation. The different phenotypic consequences of this combination of missense mutations (in homozygous or compound heterozygous state) was analyzed both clinically and biochemically. Our results suggest that "molecular complementation" between specific calpain-3 missense mutants may result in a more benign form of the disease.

METHODS

Patients. Patients were recruited from the Muscular Dystrophy database of the LGMD2A Molecular Database of the Biodonostia Institute at the Donostia Hospital. For inclusion in the study, patients had to fulfill the following criteria: (1) confirmation of both missense mutations at *CAPN3*; (2) aged $\geq$30 years with a well-documented clinical status according to the Gardner–Medwin–Walton (GMW) functional scale[13]; and (3) atypically benign phenotype at 30 years of age, with a GMW score of <III.

Of 295 LGMD2A patients, 2 fulfilled all three criteria, both of whom had compound heterozygous pG222R and pR748Q missense mutations. The clinical characteristics of these patients are summarized in Table 1, including ages at onset and results of muscle biopsies. Mutation screening was carried out according to Richard et al.[14]

To evaluate the benign phenotype, the patients who fulfilled the following criteria were also

Abbreviations: *Capn3*$^{CS/CS}$, calpain-3 knock-in mice; EDTA, ethylenediamine tetraacetic acid; EF-3, third EF-hand motif; GMW, Gardner–Medwin–Walton; LGMD2A, limb-girdle muscular dystrophy type 2A; mCL, m-calpain catalytic subunit; SDS, sodium dodecylsulfate; WT, wild-type

Key words: calpain-3, calpainopathy, LGMD2A, phenotype, protein activity

Correspondence to: A. Sáenz; e-mail: amets.saenzpena@osakidetza.net

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Table 1. Patients' clinical information.

Patient	Age at onset (current age)	Current clinical status and Western blot analysis of biopsy sample	CK (U/L)	Predominant MRI findings	Current GMW scale
P1*: Female	23 (43 years)	Ambulant with a myopathic gait, hyperlordosis, unable to climb stairs or get up from a chair. Normal cardiac echography. Western blot data not available.	3370	Selective involvement of the posterior compartment of the thighs and posteromedial compartment of the legs.	II
P2: Male	20 (34 years)	Ambulant with moderate weakness of the pelvic girdle. Hypertrophy of quadriceps and mild pseudo-hypertrophic features of scapular winging. Pseudometabolic clinical pattern. Western blot analysis revealed normal/borderline protein bands.	5000–8000	Severe impairment of the hamstring and hip adductors. Quadriceps and gracilis spared.	II

**Originally included in a previous study (Urtasun et al., 1998).*

screened: (1) at least one allele containing a pG222R or pR748Q missense mutation on the *CAPN3* gene; and (2) ≥30 years of *LGMD2A* history with a well-defined clinical status according to the GMW scale. The phenotypes of patients identified by this screening were compared with the benign phenotype patients.

Western Blotting of Tissue. Western blotting was performed as previously described,[15] with minor modifications. Frozen tissue samples were weighed and homogenized with 19% (w/v) of treatment buffer [125 mM Tris/Cl (pH 6.8), 4% sodium dodecylsulfate (SDS), 10% (v/v) glycerol, 0.1 mol/L ethylene-diamine tetraacetic acid (EDTA), and 5% (v/v) β-mercaptoethanol] in a mixer-mill disruptor (TissueLyser; Qiagen) and loaded onto a SDS–polyacrylamide gel. The membranes were probed with the following antibodies in duplex immunoblot analysis: NCL-2C4 (for calpain-3; Novocastra) and Ad1/20A6 (for α-sarcoglycan; Novocastra).

cDNA Constructs. Expression vectors for human calpain-3 and its mutants were constructed as described previously[16] and, after verification by full-length DNA sequencing, they were transfected into COS7 cells by electroporation using a Gene Pulser (Bio-Rad). Cells were incubated for 60–72 hours at 37°C and harvested for further analysis.

Western Blotting of Transfected COS7 Cells. Cells were harvested in homogenizing buffer [20 mM Tris/Cl (pH 8.0), 1 mM EDTA (pH 8.0), and 1 mM dithiothreitol]. Equal amounts of protein from each sample were resolved by SDS–polyacrylamide gel electrophoresis, and transferred onto an Immobilon-P transfer membrane (Millipore). Gels were also stained by Coomassie brilliant blue to ensure the equality of loaded protein amount. Membranes were incubated with goat anti–calpain-3–pIS2 antibody (for calpain-3),[17] or a rabbit anti–150K-fodrin–Nterm antibody (for the 150-kDa α-fodrin fragment specifically proteolyzed by calpain).[18]

RESULTS

Two of 295 patients showed significantly benign clinical features (Table 1), both of whom carried the pG222R and pR748Q mutations, and exhibited compound heterozygosity. To examine the potential link between this genotype and the benign phenotype, patients from previous studies with at least one of these mutations (pG222R or pR748Q) were screened according to the same criteria described earlier but with GMW <III. There were 11 such patients, including 2 who were homozygous for the pG222R mutation and 2 who were homozygous for the pR748Q mutation. These patients exhibited little phenotypic variation. All but 2 of them were wheelchair-bound, most by the third decade of life, reflecting the severity of the phenotype associated with the pG222R and pR748Q missense mutations.

Western blot analysis of muscle biopsies from patient 2 (P2) with the pG222R/pR748Q mutations revealed an almost normal level of the 94-kDa band for calpain-3 (data not shown; Table 1). The same trend was observed for one of the patients who is homozygous for the pG222R mutation from our previous series (data not shown).

To address the apparent discrepancy in the phenotype–genotype correlation observed between the compound heterozygotes and the homozygotes for the pG222R and pR748Q missense mutations, calpain-3 proteins with these mutations were expressed in COS7 cells. When wild-type (WT) calpain-3 was

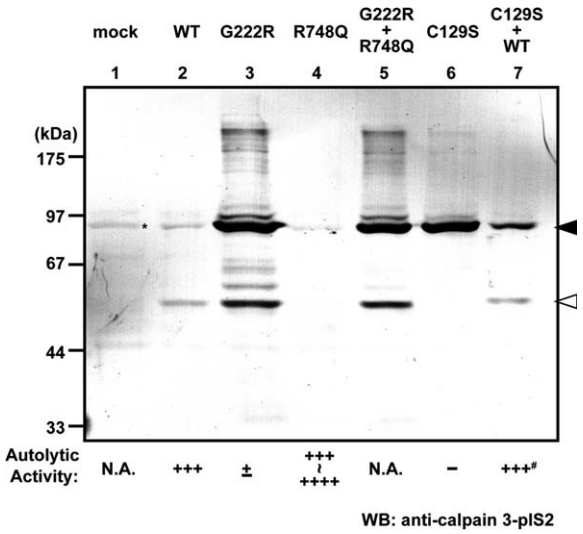

FIGURE 1. Effects of LGMD2A pathogenic mutations on calpain-3 autolysis. Closed and open triangles indicate the full-length form and autolyzed/proteolyzed fragment of calpain-3. Asterisks indicate non-specific signals. The autolytic activity of the proteins expressed is qualitatively shown by '+' and '–' below the blot (WT = +++; #including proteolysis of pC129S by WT calpain-3). The pG222R but not the pR748Q mutation inactivates calpain-3 autolytic activity. The inactive pC129S mutant produced a stable 94-kDa band. 'Mock' indicates the negative control transfected with the empty vector.

expressed, it underwent rapid and exhaustive autolysis, and only a weak 94-kDa band and a faint 55-kDa autolytic fragment were detected in Western blots, as previously reported.[19] The full-length pG222R mutant calpain-3 protein was stably expressed, and its autolytic activity was almost totally abolished (Fig. 1). Conversely, only a very faint 94-kDa pR748Q mutant calpain-3 band was evident. Moreover, this pR748Q mutant calpain-3 may have enhanced autolytic activity compared with the WT form. Both the full-length 94-kDa protein and the 55-kDa autolytic fragment were detected more weakly than the WT calpain. However, autolysis of these proteins occurred too rapidly to compare them precisely.

WT calpain-3 but not the mutant forms, such as pC129S and pR769Q, are reported to proteolyze endogenous fodrin when they are expressed in COS7 cells.[16] By contrast, pG222R showed no proteolytic activity against fodrin, and only very weak activity was observed for the pR748Q mutant when compared with the WT control. Surprisingly, when pG222R and pR748Q were coexpressed, they exhibited greater fodrinolytic activity than that observed when each mutant was expressed alone. These findings suggest that the combination of the pG222R and pR748Q mutations provokes acquisition of unexpected activity in a gain-of-function manner (Fig. 2).

DISCUSSION

The two compound heterozygous cases described here are clearly more benign than other cases analyzed to date, both in terms of disease progression and the GMW functional score in relation to patient age. Despite the exceptionally benign clinical findings, magnetic resonance images (data not shown) were highly suggestive of an LGMD2A type in both cases, and pathogenic mutations at *CAPN3* were confirmed. For patients in whom clinical examination reveals very benign symptoms and Western blot analysis of calpain-3 shows an almost normal 94-kDa band, patients are likely to be misdiagnosed as not having LGMD2A. This raises the possibility of the existence of more undiagnosed patients with this benign form of LGMD2A.

Given that patients who are homozygous for the pG222R or pR748Q mutations show a very severe phenotype, the benign phenotype observed for the compound heterozygotes is unlikely to be due to the mutations themselves. This is the first report to show that the combination of these mutations, both of which are associated with a severe phenotype in homozygotes, results in a benign phenotype. Although several benign phenotypes have been associated with specific mutations,[7,9–11] in most cases missense mutations were found in at least one of the alleles, suggesting that calpain-3 function was partially retained in skeletal muscle.

When their expression was assessed using COS7 cells, the pG222R mutation almost completely abolished the autolytic activity of calpain-3, whereas the pR748Q mutant showed autolytic activity comparable with (or possibly greater than) that

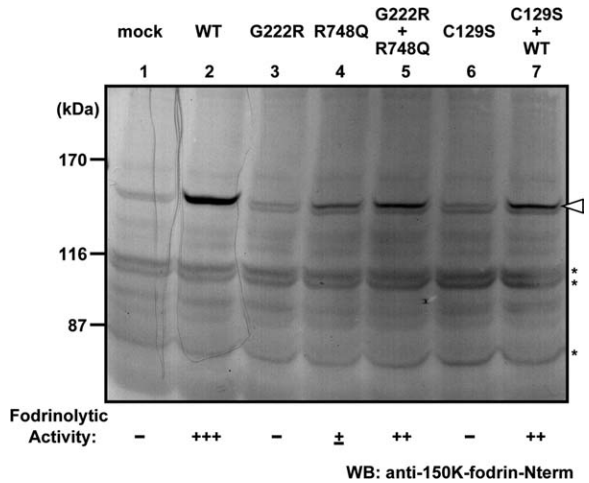

FIGURE 2. Protease activity against fodrin. The same sample as that described in Figure 1 was analyzed with an anti–150K-fodrin–Nterm antibody that specifically detects the N-terminus of fodrin proteolyzed by calpain. Open triangles indicate the 150-kDa fodrin fragment proteolyzed by calpain-3. Fodrinolytic activity is indicated qualitatively by '+' and '–,' according to the intensity of the proteolyzed fodrin bands (WT = +++). Note that the C129S, G222R, and R748Q mutants showed little or no activity. 'Mock' and asterisks indicate the negative control transfected with the empty vector and non-specific signals, respectively.

of the WT control. Gly222 of calpain-3 corresponds to Gly198 in the rat m-calpain catalytic subunit (mCL), which plays an important role in breaking/turning the α-helix close to the junction of the protease subdomains IIa and IIb (also known as domains I and II, respectively) in the three-dimensional (3D) structure of active m-calpain.[20,21] Thus, the conformation of subdomains IIa and IIb is probably misaligned by the pG222R mutation, resulting in inactivation of calpain-3. Arg748 is located in the "F-helix" (the helix after the Ca^{2+}-binding loop) of the third EF-hand motif (EF-3) in domain IV, and it corresponds to Arg628 in the rat mCL. In the 3D protein structure, Arg628 is very close to D362 in domain III (the distance between the closest N and O atoms of Arg628 and Asp362, respectively, is 3.03 Å), indicating that these residues generate a salt bridge. Thus, the pR748Q mutation of calpain-3 most likely disrupts the interaction between domains III and IV, resulting in an incorrect conformation of these domains.

When expressed in COS7 cells, the pR748Q mutant demonstrated significantly less fodrinolytic activity than the WT calpain-3. As previously described for other domain IV mutations in LGMD2A patients, such as pR744G and pR769Q, pR748Q may have greater autolytic activity than the WT protein, which in turn may produce functional defects.[16]

The structure–function relationships of calpain-3 mutants demonstrate that both mutations are deleterious, consistent with the severe phenotypes of the pG222R and pR748Q homozygotes. Thus, it could be hypothesized that the benign effect of the pG222R/pR748Q mutation combination is due to functional complementation between these specific mutations. Indeed, fodrinolytic activity in COS7 cells that coexpress pG222R and pR748Q mutations is greater than that of cells that express either pG222R or pR748Q alone, and it is comparable to that of cells that coexpress the WT form and the pC129S mutant. Hypothetical scenarios of molecular complementation between pG222R and pR748Q are described in what follows.

Dimer Hypothesis. The p94 protein may form a homodimer in specific situations, for example, very soon after being activated. This is witnessed by the formation of a p94 domain IV homodimer.[22] Domains III–IV are important for substrate recognition and/or titin binding, and this region is affected/altered in the pR748Q mutant, but not the pG222R mutant. We previously demonstrated that, in some cases, titin can act as a scaffold for calpain-3 proteolysis of substrates.[23] Although the pG222R mutant form of calpain-3 cannot proteolyze substrates, it can recognize substrates and/or bind to titin, whereas the protease domain of dimerized pR748Q mutant calpain-3 can proteolyze substrates. Thus, the "canonical" functions of calpain-3 may be partially restored by the formation of a pG222R/pR748Q heterodimer.

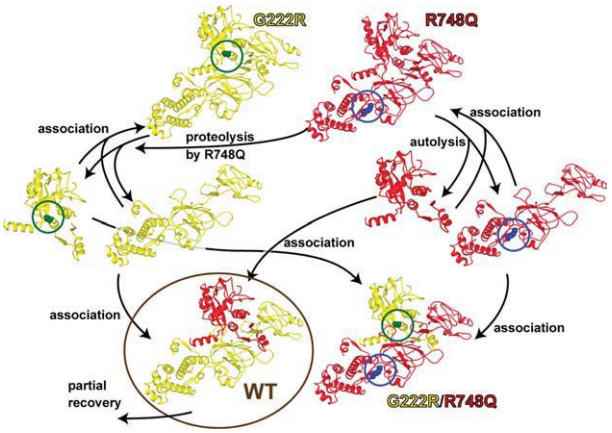

FIGURE 3. A schematic representation of our hybrid hypothesis. The loci of G222R and R748Q mutations in calpain-3 molecules are represented in green and blue, respectively. The calpain-3 molecule is illustrated by the ribbon model, based on the reported 3D structure of human m-calpain (1KFX[25]). All associations can be reversed (dissociation), which is not indicated in the figure. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Inhibitor Hypothesis. The pC129S and pG222R calpain mutants may also act as competitive inhibitors of autolysis. If pR748Q causes unregulated/upregulated autolytic activity by acting as a substrate for pR748Q autolytic activity, the pG222R mutant could suppress the rate of autolytic turnover of pR748Q to a level similar to that of WT p94, resulting in normal calpain-3 function.

Hybrid Hypothesis. Autolysis of calpain-3 occurs at the NS, IS1, and IS2 regions. Nicking in IS1 does not cause immediate dissociation of the molecule, but it causes it to retain its active molecular state.[17] Similarly, association of the N-terminal (34–274aa, from NS to IS1) and C-terminal (323–821aa, from IS1 to C-term) autolyzed fragments has been described.[24] Given that Gly222 and Arg748 reside in the N-terminal and C-terminal autolyzed fragments, respectively, and supposing that pG222R is proteolyzed intermolecularly by pR748Q, it is possible that the other halves of each mutant molecule co-associate to reconstitute an intact (WT) calpain-3 molecule (Fig. 3). Titin has plural adjacent binding sites for calpain-3, which could facilitate such an exchange by acting as a scaffold for calpain-3. Furthermore, the propensity of calpain-3 to form a dimer may also be significant.[23]

These hypotheses were constructed to explain how the specific combination of mutations rescues, at least in part, the proteolytic activity of calpain-3. It is also possible that the compensatory effect of this combination of mutations affects other functions of calpain-3. Recent studies highlighting the

novel functions of calpain-3 have suggested that the structural integrity of calpain-3 plays certain roles in skeletal muscle.[26,27] In addition, it is not an excluded possibility that deficits at the levels other than protein, such as mRNA metabolism, are caused by mutations in *CAPN3*. In other words, the severity of LGMD2A could vary due to the combination of secondary effects of the mutations that are primarily abrogating protease activity of calpain-3 protein.

Obviously, additional genetic and biochemical data are required to ascertain the relevance of these hypotheses. Rescue of the phenotypes of calpain-3 knock-in ($Capn3^{CS/CS}$) mice, which express a structurally intact but protease-dead calpain-3:C129S mutant, by other mutants theoretically competent for intermolecular compensation would be one of the approaches to validate our hypotheses *in vivo*. However, our results suggest that certain combinations of missense mutations undergo molecular compensation, thereby ameliorating disease symptoms. This compensatory effect is ascribed to the domain structure of calpain-3 with sequence insertions, where we propose that two mutations located before and after the IS1 region complement each other. As the exact functions and substrates of calpain-3 remain unknown, it will be necessary to elucidate further roles for calpain-3 to determine the validity and significance of the hypotheses proposed.

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