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SHORT COMMUNICATION

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Identification of pathogenic mutations in the human rapsyn gene

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Abstract Rapsyn, a complex postsynaptic protein of the striated muscle, assembles acetylcholine receptors (AChR) at high density at the motor endplate (EP). Neuromuscular junctions of mice lacking rapsyn show no clusters of AChRs or other structural postsynaptic proteins such as β-dystroglycan and utrophin. Humans with mutations in the rapsyn gene (*RAPSN*) are affected with a postsynaptic form of congenital myasthenic syndrome (CMS) characterized by impairment of the morphologic development of the postsynaptic region. We have identified four patients from four different families with *RAPSN* mutations and CMS, confirmed in two cases by microelectrode and electron microscopy studies. The N88K mutation was present in all patients. One patient who was homozygous for N88K was only mildly affected, while the other three patients who were heterozygous for N88K and a second mutation (either L14P, 46insC, or Y269X) were severely affected. Mutations 46insC and Y269X predicts truncation of the protein. L14P predicts a conformational change at the N-terminus that may disrupt membrane association. N88K occurs within the putative leucine zipper motif potentially important for AChR clustering. These findings may explain the severe clinical involvement of compound heterozygous patients.

Keywords Rapsyn · Endplate · Acetylcholine receptor clustering · Congenital myasthenic syndrome · Postsynaptic

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Introduction

Neurally released agrin induces aggregation of acetylcholine receptors (AChRs) by interacting with both the receptor tyrosine kinase MuSK (muscle-specific kinase) and the 43-kDa postsynaptic muscle protein rapsyn (Froehner et al. 1990). Mutant mice lacking rapsyn show absence of aggregation of AChRs and lack of accumulation of cytoskeletal proteins such as β-dystroglycan, and utrophin (Guatam et al. 1995).

The three-dimensional structure of rapsyn is not known; however, its primary structure predicts the presence of several distinctive domains that participate in separate functions of the protein (Fig. 1). The N-terminus of rapsyn is myristolated and is involved in membrane association (Frail et al. 1988); amino acids 6–319 predict the formation of eight tetratricopeptides (TPRs) which are involved in rapsyn self association (Ponting and Phillips 1996); amino acids 297–330 form the putative coiled-coil domain that fuses to the long cytoplasmatic loop of each acetylcholine receptor (AChR) subunits clustering AChRs (Ramarao et al. 2001); the cysteine-rich C-terminus constitutes the RING-H2 domain that binds to the membrane protein β-dystroglycan (Bartoli et al. 2001); and amino acids 403–406 encompass a consensus sequence for protein kinase A and protein kinase C phosphorylation (Ramarao and Cohen 1998).

Congenital myasthenic syndromes (CMS) represent a heterogeneous group of disorders characterized by failure of neuromuscular transmission arising from presynaptic or postsynaptic defects (Engel et al. 1998). In humans, *RAPSN* mutations result in a postsynaptic form of CMS defined by deficiency of endplate AChRs and striking underdevelopment of junctional postsynaptic folds (Ohno et al. 2002). The severity of symptoms in this form of CMS is variable. All patients identified to date are either homozygous or heterozygous for the N88K mutation. In addition, the L14P mutation encountered in one of our patients was previously

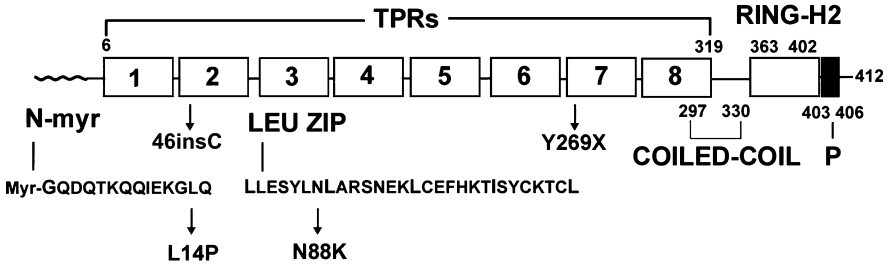

Fig. 1 Schematic view of the structural domains of rapsyn. Displayed are the myristolated N-terminus (N-myr), eight tetratricopeptide domains (TPRs), coiled-coil region, cysteine-rich domain (RING-H2), and phosphorylation site sequence (P). *Arrows* indicate the position of the four mutations. The L14P mutation is adjacent to the 10 amino acids that are conserved across species and the N88K mutation lies within the putative leucine zipper (LEU ZIP) motif. (Modified from Ramarao and Cohen 1998)

described by Ohno et al. Because of the propensity of some of the *RAPSN* mutations to recur in different patients (e.g., L14P and N88K) we have developed restriction enzyme analysis for rapid identification of these frequent mutants. We are describing here the results of our studies.

Materials and methods

Patients

We studied four patients with CMS, two of which were confirmed by microelectrode and electron microscopy studies performed in anconeus muscle biopsy. The patients were 18- and 19-year-old females and 3- and 4-year-old boys with symptoms of hypotonia, ptosis, swallowing difficulties and proximal limb weakness since birth. The two females and the 4-year-old boy were severely affected, while the 3-year-old boy was mildly affected. In addition, the two female patients each had another sibling who died from respiratory complications of CMS. All patients were the product of full-term pregnancies from non-consanguineous parents. A detailed clinical description of these patients is provided elsewhere (Maselli et al., in preparation). The Institutional Review Board of Human Subjects of the University of California, Davis, approved all the protocols of this study, and a signed consent form was obtained from all patients and control subjects participating in the study.

PCR procedures

Genomic DNA was extracted from blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). PCR primers were designed to amplify all *RAPSN* coding regions and splice junctions using genomic and mRNA sequences deposited in GenBank (Accession number 008978 and Z33905). In addition, we amplified and sequenced all exons and splice junctions of the genes encoding AChR α-, β-, δ-, and ϵ-subunits and the ϵ-subunit promoter. A typical PCR reaction mixture included a 1× buffer that contained Tris-HCl, KCl and Triton X-100, 25 mM $MgCl_2$, 10 mM dNTPs, 100 pmol/μl sense primer, 100 pmol/μl antisense primer, 600 ng genomic DNA, and 2.5 U Taq DNA polymerase (Promega, Madison, WI) in 50 μl total volume. The cycling protocol for all reactions consisted of 3 min initial denaturing followed by 30 cycles of 94°C for 30 s, 60°C for 60 s and 72°C for 90 s, and a final extension at 72°C for 5 min. PCR products were run on a 1% agarose gel at 100 mV for 30 min, purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced using the ABI PRISM® 3100 Genetic Analyzer (Applied Bioscience, Foster City, CA).

Restriction analysis

To screen families of affected patients, restriction analysis was performed. For the L14P mutation in exon 1, a 320 bp fragment of genomic DNA was amplified with sense primer 5′CTGCTTTTGTTCCCACGTTTCG3′ and antisense primer 5′CGTTCGAGGGTCAGGCT3′ at a 60°C annealing temperature. The PCR product was run on 1% agarose gel and purified as described above. Restriction digest mixture included 10 U *Hae*III (New England Biolabs, Beverly, MA), 60 ng DNA from PCR product, and 1× NEBuffer 2 in 20 μl total volume. The DNA was digested at 37°C for 4 h then run on a 4% agarose gel with ethidium bromide and viewed with an ultraviolet light.

For the 46insC mutation also in exon 1, the same PCR product from above was used. Restriction digest mixture included 10 U *Alu*I (New England Biolabs, Beverly, MA), 60 ng DNA from PCR product and 1× NEBuffer 2 in 20 μl total volume. The DNA was digested under conditions described above.

For the N88K mutation in exon 2, a 458bp fragment of genomic DNA was amplified using a 56°C annealing temperature with a sense primer 5′ACCTGCCCACCGTCG3′ and antisense primer 5′GCTCTGGGTCTGAAAAGAAGC3′. Restriction digest mixture included 10 U *Bsr*I (New England Biolabs, Beverly, MA), 80 ng DNA from PCR product and 1× NEBuffer 3 in 20 μl total volume. The DNA was digested as described above.

For the Y269X mutation in exon 5, a 250bp fragment of genomic DNA was amplified using sense primer 5′TGGAGGTCACCGGGATGC3′ and antisense primer 5′GCCAGTAGGTTAGCCGGC3′ at a 58°C annealing temperature. Restriction digest mixture included 8 U *Afa*I (Amersham Pharmacia Biotech Inc., Piscataway, NJ), 40 ng DNA from PCR product, 1× Amersham Buffer T and 1× BSA in 20 μl total volume. The DNA was digested as described above.

Statistical analysis

Statistical analysis using the chi-squared test was performed to compare observed versus expected frequencies of two linked polymorphisms (456T/C and 1143T/C) in the N88K homozygous individuals reported in this paper and three additional homozygous patients reported elsewhere (Maselli et al., in preparation).

Results

Mutational analysis

No mutations were found in the AChR α-, β-, δ-, or ϵ-subunit genes; however, direct sequencing of the eight exons of *RAPSN* revealed four mutations in four patients as well as three polymorphisms (172C/T, 456T/C,

and 1143T/C) previously described by Ohno et al. (2002). All patients displayed the N88K mutation, with only Patient 3 homozygous for the mutation. Patient 1 was heterozygous for L14P, Patient 2 was heterozygous for 46insC, and Patient 4 was heterozygous for Y269X (Table 1). None of the encountered polymorphisms were found to be significantly associated with the N88K mutation using the chi-squared test.

Table 1 Mutational analysis of *RAPSN* in four patients with CMS

Patient and nucleotide		Exon	Amino acid	Domain
1.	41 T/C[a]	1	L14P	TPR 1
	264 C/A[a]	2	N88K	TPR 3
2.	46insC[b]	1	frameshift	TPR 2
	264 C/A[a]	2	N88K	TPR 3
3.	264 C/A (homozygous)[a]	2	N88K	TPR 3
4.	264 C/A[a]	2	N88K	TPR 3
	807 C/A[b]	5	Y269X	TPR 7

[a]Previously described by Ohno et al. (2002)
[b]Novel mutations

Restriction enzyme analysis

Restriction analysis with *Bsr*I of family 1 showed a heterozygous father and brother for the N88K mutation. Digestion with *Hae*III revealed that the unaffected mother was heterozygous for the L14P mutation. Restriction analysis with *Bsr*I in family 2 showed a heterozygous mother for the N88K mutation, and digestion with *Alu*I displayed heterozygosity in the father and brother for the 46insC mutation, which was confirmed by direct sequencing. In family 3, restriction digestion with *Bsr*I showed that the father and sister were heterozygous for N88K, while the unaffected mother was homozygous for this mutation. Restriction analysis in family 4 with *Bsr*I showed a heterozygous father for the N88K mutation, and digestion with *Afa*I revealed a heterozygous mother for the Y269X mutation (Fig. 2).

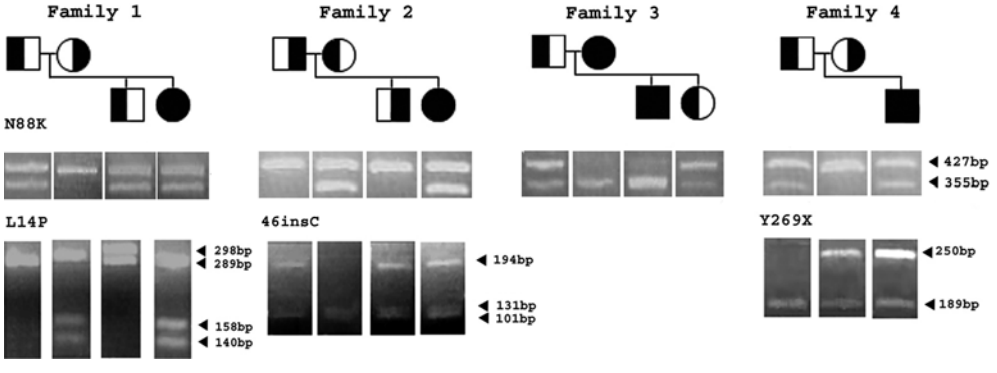

Fig. 2 Restriction enzyme analysis. *Upper panel*: BsrI restriction analysis of genomic DNA from families of patients. The N88K mutant allele results in a restriction site yielding two fragments of 72 bp and 355 bp, while the wild-type produces an undigested 427 bp fragment. Homozygous wild-type individuals display only one 427 bp fragment, homozygous mutant individuals show two bands at 72 and 355 bp in size (though only the 355 bp fragment is visible), and the heterozygous individuals display all three bands (though only the 355 and 427 bp fragments are visible). *Lower panel (left)*: HaeIII restriction digest of exon 1. The L14P mutant allele results in a restriction site yielding two fragments of 158 and 140 bp each, while the wild-type allele results in a 289 bp undigested fragment. An extra band at 298 bp is produced in lane 3 by polymorphism 172T/C. Homozygous wild-type individuals display one band of 289 bp in size. Heterozygous patients and relatives show three bands of 140, 158, and 289 bp in size. *Lower panel (center left)*: AluI restriction digest of exon 1. The 46insC mutant allele eliminates a restriction site producing a 194 bp undigested band, while the wild-type allele contains a restriction site that yields a 131 bp band. Both wild-type and mutants also produce a 101 bp fragment unrelated to the mutation site. Homozygous wild-type individuals display two bands of 101 and 131 bp in size, and heterozygous patients and relatives show three bands of 101, 131, and 194 bp in size. *Lower panel (right)*: AfaI restriction digest of exon 5. The Y269X mutant allele eliminates a restriction site, and yields a 250 bp undigested fragment. The wild-type allele contains a restriction site that results in a 189 bp fragment. Homozygous wild-type individuals display one band of 189 bp in size, and heterozygous patients and relatives show two bands of 189 and 250 bp in size

Discussion

We have identified four *RAPSN* mutations in four patients with CMS in whom no mutations were encountered in AChR subunit genes. Two of these mutations (L14P and N88K) have been previously reported by Ohno et al. (2002), who demonstrated that both mutants fail to cluster the AChR in HEK cells co-transfected with *RAPSN* and AChR subunit transcripts. The two other mutations were novel (46insC and Y269X) and predicted truncation of the protein upstream from the coiled-coil domain, which is fundamental to AChR clustering. An important observation is that the N88K mutation has been present in all patients with *RAPSN* mutations reported to date, suggesting a common founder effect. In our study, however, no significant association of any observed SNPs with the N88K mutation was found to support the common founder effect hypothesis. A second observation concerns the phenotype–genotype correlation. The homozygous N88K genotype resulted in mild symptoms in patient 3 and no symptoms in his 33-year-old mother, who carried the

same genotype. In contrast, the compound heterozygous L14P and N88K patient was severely affected.

A possible explanation for the severity of the L14P/N88K genotype is that the L14P mutant may alter membrane attachment, while the N88K mutant disrupts AChR clustering. It has been suggested that myristolated proteins require a stretch of positively charged residues either contiguously (Murray et al. 1997) or spatially (Ramarao et al. 2001) aligned with the N-terminus for membrane association. The introduction of a proline within this region, which is known to change protein conformation and destabilize protein folding (Barlow and Thornton 1988), may alter the alignment of these residues and therefore disrupt rapsyn membrane attachment. The N88K mutation resides within the leucine zipper motif, which is comprised of seven leucine/isoleucine repeats organized as an α-helix and functions as a leucine zipper coiled-coil motif important for AChR clustering (Phillips et al. 1991).

In the present study, we have provided evidence that mutations in the rapsyn gene result in potentially severe forms of CMS. The N88K mutation is prevalent and in combination with L14P or a nonsense mutation results in a severe phenotype probably as a consequence of a combined disruption of membrane association and AChR clustering.

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