Mutations Causing DOK7 Congenital Myasthenia Ablate Functional Motifs in Dok-7*

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Dok-7 is a cytoplasmic activator of muscle-specific receptor-tyrosine kinase (MuSK). Both Dok-7 and MuSK are required for neuromuscular synaptogenesis. Mutations in DOK7 underlie a congenital myasthenic syndrome (CMS) associated with small and simplified neuromuscular synapses likely due to impaired Dok-7/MuSK signaling. The overwhelming majority of patients with DOK7 CMS have at least one allele with a frameshift mutation that causes a truncation in the COOH-terminal region of Dok-7 and affects MuSK activation. Dok-7 has pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains in the NH2-terminal moiety, both of which are indispensable for MuSK activation in myotubes, but little is known about additional functional elements. Here, we identify a chromosome region maintenance 1-dependent nuclear export signal (NES) in the COOH-terminal moiety and demonstrate that the NES-mediated cytoplasmic location of Dok-7 is essential for regulating the interaction with MuSK in myotubes. The NH2-terminal PH domain is responsible for the nuclear import of Dok-7. We also show that the Src homology 2 target motifs in the COOH-terminal moiety of Dok-7 are active and crucial for MuSK activation in myotubes. In addition, CMS-associated missense mutations found in the PH or PTB domain inactivate Dok-7. Together, these findings demonstrate that, in addition to the NH2-terminal PH and PTB domains, the COOH-terminal NES and Src homology 2 target motifs play key roles in Dok-7/MuSK signaling for neuromuscular synaptogenesis. Ablation or disruption of these functional elements in Dok-7 probably underlies the neuromuscular junction synaptopathy observed in DOK7 CMS.

Dok-7 is the latest member of the Dok-family proteins, which share structural similarities characterized by the NH2-terminal PH3 and PTB domains followed by the SH2 target motifs in the COOH-terminal moiety, suggesting an adaptor function (1–8). Indeed, Dok proteins generally recruit other signaling molecules bearing the SH2 domains upon tyrosine phosphorylation by upstream kinases (2–5, 8). However, we previously identified Dok-7 as a muscle-intrinsic activator of the receptor protein-tyrosine kinase MuSK and further demonstrated that the Dok-7/MuSK signaling plays an essential role in the formation of neuromuscular junction (NMJ), a synapse between motor neuron and skeletal muscle (1). The contraction of skeletal muscle is controlled by the neurotransmitter acetylcholine released from the motor nerve terminal. To achieve efficient neuromuscular transmission, acetylcholine receptors (AChRs) must be densely clustered on the postsynaptic membrane of the NMJ. Indeed, impaired clustering of AChRs can underlie NMJ disorders that are both autoimmune (anti-MuSK antibody-positive myasthenia gravis) and genetic (CMS) in origin (9, 10). Therefore, the observation that mice lacking Dok-7 fail to cluster AChRs and do not form NMJs led us to screen DOK7 as a candidate gene for mutations associated with CMS (1).

CMS comprise a heterogeneous group of NMJ disorders associated with genetic defects in presynaptic, synaptic, and in most cases, postsynaptic proteins of the NMJ (10–12). Impaired synaptic transmission at the NMJ results in fatigable muscle weakness and typically may variably affect limb, ocular, bulbar, truncal, and respiratory muscles. CMS-associated genetic mutations had previously been identified in 10 genes: the acetylcholine receptor subunits (CHRNA1, CHRNA7, CHRNA9, CHRND, CHRNF, and CHRNQ), the choline acetyltransferase (CHAT), the collagen tail subunit of the asymmetric form of acetylcholinesterase (COLQ), rapsyn (RAPSN), MuSK (MUSK), and the skeletal muscle sodium channel Na1.4 (SCN4A) (13–23). However, in many CMS patients, including a major subgroup of those with a limb girdle pattern of muscle weakness, mutations had not been identified (11, 24, 25). Recently, we found biallelic mutations in DOK7 underlie a major subgroup of CMS with predominantly proximal muscle weakness that did

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not show tubular aggregates on muscle biopsy but were found to have normal AChR function but abnormally small and simplified NMJs (26). Patients with DOK7 mutations do not usually show long-term benefit from anticholinesterase medication (26–28), but ephedrine can be helpful (24, 27). The relatively late onset of presenting symptoms in some of these patients may indicate that Dok-7 plays a role not only in the formation but also in the maintenance of NMJ structure. Also of note, all cases of DOK7 CMS reported to date have been found to harbor at least one allele with a mutation in the COOH-terminal coding exon of DOK7, suggesting a pathophysiological importance for this region which has yet to be analyzed in detail for functional elements (26–28).

Here, we use the AChR clustering pathway in C2 myotubes, where the Dok-7/MuSK signaling plays an essential role as in vivo, in association with DOK7 CMS mutations (1, 26–28), to reveal important functional modules in Dok-7. We show that a NES and two SH2 target motifs in the COOH-terminal domain of Dok-7 likely plays a role in regulating interaction with MuSK at the juxtamembrane part of cells. In addition, we show that CMS-associated missense mutations found in the PH or PTB domain inactivate Dok-7. Together, these findings suggest multiple pathogenic mechanisms underlying DOK7 CMS.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Antibodies, and Reagents—Constructions of human Dok-7, Dok-7-dupTGCC, Dok-7-RA (R158A, R159A, and R174A), Dok-7-dN (61–504), and mouse MuSK-myc expression plasmids were described elsewhere (1, 26). cDNAs encoding Dok-7 mutants (Dok-7-R158Q, -A33V, -R201X, -N110, -N143insC, -N420, -Y395F, -Y405F, -2YF, -2PA, -L241A, -L245A, -L248A) were generated by PCR to be inserted into pcDNA3.1-myc/His (Invitrogen) or pEGFP-N3 (Clontech), which is an expression plasmid for an Myc/His-tagged protein or enhanced green fluorescent protein (EGFP) fusion, respectively. cDNAs encoding NES and mutant NES (mNES) of Dok-7 were generated by PCR and inserted into pEGFP-C1 (Clontech). Mouse CrkII expression plasmids, pcDNA3-mCrkII and pcDNA3-mCrkII-R83K, were kindly provided from Dr. Hidesaburo Hanafusa (Osaka Bioscience Institute). The following antibodies were from commercial sources: anti-α-tubulin (DM1A), anti-Dok-7 (H-77), anti-MuSK (N-19 and C-19), anti-phosphorylated AChRβ1 (Tyr-390), anti-green fluorescent protein (B-2), anti-myosin heavy chain (F59), and horseradish peroxidase (HRP)-conjugated anti-goat IgG (Santa Cruz Biotechnology); anti-MuSK (AF562) (R&D Systems); anti-phosphotyrosine (4G10) (Upstate Biotechnology); HRP-conjugated anti-rabbit, rat, or mouse IgG (GE Healthcare); anti-Myc (9B11) (Cell Signaling Technology); anti-CrkII monoclonal antibody (BD Transduction Laboratories). Anti-Dok-7 rat antisera raised against peptide for the COOH-terminal portion of human Dok-7 (214–498) were prepared as described elsewhere (1). Leptomycin B was from Biomol. Alexa 594-conjugated α-bungarotoxin (Btx) and 4,6-diamidino-2-phenylindole were from Molecular Probes.

**Immunoprecipitation, Immunoblotting, Btx Pulldown, Fluorescent Microscopy, MuSK Phosphorylation, and AChR Clustering Assays—C2 myoblasts (C2C12) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum. After transfection with Dok-7 expression plasmids using FuGENE 6 (Roche Applied Science), C2 myoblasts were cultured in differentiation medium (Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum) for 5 days. We previously demonstrated that exogenous Dok-7 proteins were expressed in both undifferentiated myoblasts and differentiated myotubes (1). Differentiated myotubes were subjected to immunoprecipitation, immunoblotting, and Btx pulldown assays as described (1). Note that Btx-Sepharose was used to pull down the AChR pentamer complex, which includes the AChRβ1 subunit. For AChR clustering assays, 10 microscopic fields with the 40× objective were chosen at random, and the numbers of AChR clusters (>5 μm in their longest diameter) visualized with Alexa 594-conjugated Btx were counted using a DM6000B microscope (Leica). For fluorescent microscopy assays in Figs. 2 and 3, digital images were analyzed with Leica Deblur® software (AutoQuant) in a FW4000Z imaging system (Leica). For the MuSK phosphorylation assay in Fig. 4D, phosphorylated MuSK in C2 myotubes was visualized by immunoprecipitation and immunoblotting as in Fig. 4B, and levels of each phosphorylation were quantified with densitometry using NIH Image software (Version 1.63).

**RESULTS**

A33V or R158Q Missense Mutations in the PH or PTB Domains Inhibit Dok-7-mediated Activation of MuSK—Previously we demonstrated that the PH and PTB domains of Dok-7 are essential for MuSK activation in cultured myotubes (1). However, it has remained unclear whether these domains play crucial roles in MuSK-dependent postsynaptic differentiation in vivo. Recently, two missense mutations, 98C→T and 473G→A, which produce mutant Dok-7 harboring A33V and R158Q substitutions in the PH and PTB domains, respectively, have been found in patients with DOK7 CMS (Fig. 1A and Refs. 27 and 28). Both Ala-33 and Arg-158 are conserved among vertebrates, and Dok-7-A33V and Dok-7-R158Q failed to induce MuSK activation, as judged by tyrosine phosphorylation of MuSK and its downstream target AChRβ1 and subsequent AChR clustering in myotubes (Fig. 1, B–E). We confirmed that the forced expression of Dok-7 does not affect the differentiation of myotubes with regard to morphology and expression of myosin heavy chain, a known differentiation marker (supplemental Fig. S1 and Ref. 29). Thus, these CMS-associated mutations inactivate Dok-7, which apparently is mediated through an effect on the PH or PTB domain. Indeed, Dok-7-R158Q showed the same defect, a failure to bind with MuSK in HEK 293T cells, as Dok-7-RA, which carries an inactivated PTB domain (Fig. 1F and Ref. 1). It should be noted that the PTB domain is necessary for Dok-7 to bind with MuSK (but not to activate MuSK) in heterologous cells, whereas it is required to activate MuSK in myotubes (Fig. 1, C, E, and F, and Ref. 1). Therefore, we conclude that the 473G→A mutation found in a
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FIGURE 1. CMS-associated mutations in the PH or PTB domain inhibit Dok-7 from activating MuSK in myotubes. A, CMS-associated missense mutations result in A33V and R158Q substitutions in the PH and PTB domains of Dok-7, respectively. Asterisks indicate each position of the substitution. YY indicates two SH2 target motifs in the COOH-terminal moiety. B–F, Dok-7-A33V (A), Dok-7-R158Q (RQ), Dok-7-RA (RA) that has an inactivated PTB domain, Dok-7 (WT), and/or MuSK were exogenously expressed in C2 myotubes (B–E) or in HEK 293T cells (F), and cells were subjected to IP and/or IB (B, C, E, and F) or AChR clustering assay, where number of AChR clusters (mean ± S.D.; n = 3) per 10 fields is shown (D and E). *, tyrosine-phosphorylated MuSK. PD, pull-down.

patient with DOK7 CMS disrupts the PTB domain. Together, these data suggest crucial roles of the PH and PTB domains of Dok-7 in humans.

Dok-7 Shuttles between the Cytoplasm and the Nucleus in Myotubes—601C→T is a nonsense mutation identified in a patient with DOK7 CMS that produces a mutant peptide (Dok-7-R201X), and/or MuSK were exogenously expressed in C2 myotubes (B–E) or in HEK 293T cells (F), and cells were subjected to IP and/or IB (B, C, E, and F) or AChR clustering assay, where number of AChR clusters (mean ± S.D.; n = 3) per 10 fields is shown (D and E). * , tyrosine-phosphorylated MuSK. PD, pull-down.

FIGURE 2. The NES-like sequence is required for the cytoplasmic retention of Dok-7 in myotubes. A, CMS-associated mutant Dok-7-R201X (R201X), but not Dok-7-dupTGCC, lacks the NES-like sequence (red box), which is shown in D, in the COOH-terminal moiety. YY and a green box indicate the SH2 target motifs and the polypeptide generated by the CMS-associated frameshift mutation, respectively. B and C, Dok-7-EGFP (WT), but not EGFP alone or fused with Dok-7-R201X (R201X), induces MuSK activation and AChR clustering in cultured myotubes. Dok-7 proteins indicated were exogenously expressed in C2 myotubes, and cells were subjected to IP and/or IB (B, C, E, and F) or AChR clustering assay (as in Fig. 1.)

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additional functional element in the COOH-terminal region between Arg-201 and Ala-377. By searching databases, we noticed a potential NES sequence composed of 10 amino acids (240–249 amino acids) including Leu-241, Leu-245, and Leu-248 in human Dok-7, which are conserved among vertebrates (Fig. 2, A and D). We, therefore, investigated subcellular localization of Dok-7-R201X in myotubes to determine whether Dok-7 shuttles between the cytoplasm and the nucleus. Dok-7-R201X showed prominent accumulation in the nucleus of cultured myotubes, whereas the wild-type control was mainly in the cytoplasm (Fig. 2E), suggesting that the NES-like sequence is functional. Indeed, leptomycin B (LMB), a specific inhibitor of the CRM1-dependent nuclear exporter, induced nuclear accumulation of Dok-7 (Fig. 2F). Although we failed to find a canonical nuclear localization signal sequence in Dok-7, a mutant harboring a large NH2-terminal deletion in the PH domain (dN-EGFP) was cytoplasmic even in the presence of LMB (Fig. 2F). Conversely, the PH domain (1–110 amino acids) of Dok-7 fused with EGFP (N110-EGFP) strongly accumulated in the nucleus, indicating that the PH domain of Dok-7 has a nuclear localization activity. Taken together, these findings suggest that Dok-7 shuttles between the cytoplasm and nucleus by way of the nuclear localization activity in the PH domain and the NES in the COOH-terminal moiety.

The Nuclear Export Signal of Dok-7 Is Critical for MuSK Activation—To confirm that the potential NES sequence in the COOH-terminal moiety of Dok-7 is active, we generated Dok-7 mutants fused with EGFP carrying a Leu/Ala substitution at each core residue of the NES-like sequence, namely Leu-241, Leu-245, or Leu-248 (Fig. 2D). Forced expression of each protein in cultured myotubes demonstrated that intact Dok-7 and the Dok-7-L245A mutant were localized mainly in the cytoplasm; however, Dok-7-L241A was localized mainly in the nucleus, and Dok-7-L245A was in both of the compartments (Fig. 3A). EGFP fused with a Dok-7 peptide (225–251), which includes the potential NES sequence, termed NES-EGFP, was localized mainly in the cytoplasm in the absence but not in the presence of LMB (Fig. 3B). By contrast, its mutant form (mNES-EGFP) harboring a substitution corresponding to L241A mutation of Dok-7 showed virtually the same localization pattern as intact EGFP (Fig. 3B). Together, these findings indicate that the potential NES sequence in the COOH-terminal moiety of Dok-7 is functional.

Nuclear localization of Dok-7-R201X and L241A suggests that inability of Dok-7-R201X to activate AChR clustering may be attributed at least in part to the loss of the NES (Fig. 2E and 3A). We examined if Dok-7-L241A can activate MuSK and induce AChR clustering in myotubes and found that its ability to activate MuSK and cluster AChRs was greatly reduced (Fig. 3, C and D). Thus, the NES in the COOH-terminal moiety of Dok-7 has a role to play in the Dok-7/MuSK signaling pathway. Most likely, Dok-7 requires the NES to move from the nucleus to the cytoplasm, where it can activate MuSK at the juxtaembrane part of cells.

The SH2 Target Motifs in Dok-7 COOH-terminal Motiy Are Crucial for MuSK Activation—As is the case in other members of the Dok family, Dok-7 has SH2 target motifs in the COOH-terminal moiety; that is, two Tyr-Xaa-Xaa-Pro motifs encompassing Tyr-395 or Tyr-405 of human Dok-7. These motifs are conserved in Dok-7 among vertebrates (Fig. 4A). Because the 1124_1127dupTGCC mutation found in the majority of patients with DOK7 CMS eliminates these motifs (26–28), we examined the effects of Tyr/Phe substitution(s) in the SH2 target motifs on Dok-7-induced MuSK activation in cultured myotubes. As expected, Dok-7 mutants harboring Y395F/Y405F substitutions (Dok-7-2YF) showed a significantly
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Reduced ability to activate MuSK and to induce AChR clustering (Fig. 4, B and C). Dok-7 mutants that have only the Y405F, but not the Y395F substitution showed reduced activities but to a lesser extent than the double mutant Dok-7-2YF. Pro/Ala substitutions in the SH2 target motifs, Dok-7-2PA, showed virtually the same effects on the Dok-7 function as Y395F/Y405F substitutions (Fig. 4, D and E). Thus, the SH2 target motifs play important roles in the Dok-7/MuSK signaling.

As previously stated, the common frameshift mutation, 1124_1127dupTGCC, produces mutant Dok-7 (Dok-7-dupTGCC) lacking the COOH-terminal peptide from Ala-377 to 1124_1127dupTGCC, produces mutant Dok-7 (Dok-7-1143insC), which again lacks the SH2 target motifs (Fig. 4A and Ref. 26), also showed reduced ability to induce MuSK activation and AChR clustering (Fig. 4, F and G). By contrast, a truncation of Dok-7 (from Pro–421 to the COOH-terminus) at only 11 amino acids COOH-terminal to the SH2 target motifs, which produces Dok-7-2PA, showed virtually the same effects on the Dok-7 function as Y395F/Y405F substitutions (Fig. 4, A and D). These findings together suggest that the loss of the two SH2 target motifs underlies, at least in part, the abnormal NMJ structure associated with the common mutation 1124_1127dupTGCC.

Tyry-395 and Tyr-405 of Dok-7 Are Phosphorylation Targets of MuSK—Although several SH2 domains can bind to their target peptides generated by the frameshift mutations, respectively. WT: wild type, h.m. f, human, mouse, and fish, respectively. B–E, the core residues of the SH2 target motifs are crucial for MuSK activation and AChR clustering in myotubes. Dok-7 proteins indicated were exogenously expressed in C2 myotubes, and cells were subjected to IP/IB (B) or a AChR clustering assay (C and E) as in Fig. 1 or to a MuSK phosphorylation assay (D). Relative intensity of MuSK phosphorylation (mean ± S.D.; n = 3) is shown (D), where the intensity of MuSK phosphorylation in C2 myotubes transfected with empty plasmid was defined as 1.0 in the arbitrary units. Tub, tubulin. F and G, the polypeptide (421–504) COOH-terminal to the SH2 target motifs is dispensable for Dok-7 to activate MuSK in myotubes. Dok-7-1143insC was subjected to IP/IB (B) or AChR clustering assay (C) and Ref. 26), also showed reduced ability to induce MuSK activation and AChR clustering (Fig. 4, F and G). By contrast, a truncation of Dok-7 (from Pro–421 to the COOH-terminus) at only 11 amino acids COOH-terminal to the SH2 target motifs, which produces Dok-7-2PA, showed virtually the same effects on the Dok-7 function as Y395F/Y405F substitutions (Fig. 4, A and D). These findings together suggest that the loss of the two SH2 target motifs underlies, at least in part, the abnormal NMJ structure associated with the common mutation 1124_1127dupTGCC.
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**DISCUSSION**

Twelve of 18 mutations identified in patients with DOK7 CMS were found to result in truncations in the COOH-terminal region (26–28). For example, the most prevalent mutation 1124_1127dupTGCC produces a mutant (Dok-7-dupTGCC) that retains the PH and PTB domains but lacks a part of the COOH-terminal region including the two SH2 target motifs (Fig. 4A). Dok-7-dupTGCC reduces but does not eliminate activation of MuSK in cultured myotubes, suggesting that the COOH-terminal region is involved in regulation of the Dok-7/MuSK signaling. Here we demonstrated that the two SH2 target motifs of Dok-7 are functional and required for the full-activation of MuSK in myotubes. Also, we demonstrated that Tyr-395 and Tyr-405 in the motifs are the phosphorylation target sites of MuSK. Given that SH2 domains generally require tyrosine phosphorylation of their targets to bind, these data strongly suggest that the Dok-7 SH2 target motifs do indeed play a role in recruitment of an SH2-containing molecule(s) that is important for the Dok-7-mediated activation of MuSK and AChR clustering in myotubes. It is of note that Dok-7-dupTGCC induced the same, limited levels of MuSK activation and AChR clustering as Dok-7-2YF or -2PA (Fig. 4, D and E). In addition, Dok-7-N420, which lacks almost all amino acids COOH-terminal to the SH2 target motifs (Fig. 4A), induced the normal levels of MuSK activation and AChR clustering (Fig. 4, F and G), suggesting that the loss of the SH2 target motifs significantly contributes to the pathogenicity of the common mutation 1124_1127dupTGCC.

We previously demonstrated that the COOH-terminal moiety is dispensable for Dok-7 to activate MuSK in heterologous cells or myoblasts, but not in myotubes, suggesting a myotube-specific, as yet unknown negative regulation against Dok-7-mediated activation of MuSK (1). It is tempting to speculate that SH2-containing protein(s) expressed in myotubes is recruited to the phosphorylated SH2 target motifs of Dok-7 to facilitate activation of MuSK in full. Identification of the hypothetic modulator may advance our understanding of the molecular mechanisms underlying the Dok-7/MuSK signaling, an essential driving force of neuromuscular synaptogenesis. Conversely, it would also help understand the underlying pathogenic mechanism of CMS-associated DOK7 mutations that eliminate the SH2 target motifs. So far the role of CrkII in Dok-7/MuSK signaling is unclear.

At least seven mutations, including 1263insC, have been identified in patients with DOK7 CMS that either truncate or affect amino acids COOH-terminal to the SH2 target motifs, suggesting a functional element in this particular region (26–28). However, Dok-7-N420 is functional with respect to MuSK activation and AChR clustering when overexpressed in cultured myotubes, indicating that peptide sequences COOH-terminal to the SH2 target motifs are functionally dispensable (Fig. 4, A, F, and G). Therefore, these particular mutations may affect levels of DOK7 transcripts or translation or the stability of Dok-7 protein. Indeed, expression of Dok-7-1263insC, resulting from mutation 1263insC, was not detected in immunoblot.

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**FIGURE 5.** The SH2 target motifs of Dok-7 have phosphorylation targets of MuSK and are required for binding to CrkII via the SH2 domain. MuSK, Dok-7, and/or CrkII proteins indicated were exogenously expressed in HEK 293T cells (A and C) or C2 myotubes (B), and cells were subjected to IP and/or IB. Asterisks and arrowheads indicate tyrosine-phosphorylated MuSK and Dok-7, respectively. Tub, tubulin.

| A | MuSK-myc | WT | 2YF | 2PA |
|---|---------|----|-----|-----|
| Dok-7 | WT | + | + | + |
|      | Y395F | + | + | + |
|      | Y405F | + | + | + |
|      | 2PA   | + | + | + |
| IP: α Dok-7 | pY | + | + | + |
| IB: α pY  | + | + | + | + |

| B | Dok-7 | WT | 2YF | 2PA |
|---|------|----|-----|-----|
| IP: α Dok-7 | pY | + | + | + |
| IB: α pY  | + | + | + | + |

| C | MuSK-myc | WT | 2YF | 2PA |
|---|---------|----|-----|-----|
| Dok-7 | WT | + | + | + |
|      | 2YF | + | + | + |
| Crk II | WT | + | + | + |
|      | R38K | + | + | + |
| IP: α Dok-7 | α Crk | + | + | + |
| IB: α Crk | + | + | + | + |

The SH2 target motifs of Dok-7 have phosphorylation targets of MuSK and are required for binding to CrkII via the SH2 domain. MuSK, Dok-7, and/or CrkII proteins indicated were exogenously expressed in HEK 293T cells (A and C) or C2 myotubes (B), and cells were subjected to IP and/or IB. Asterisks and arrowheads indicate tyrosine-phosphorylated MuSK and Dok-7, respectively. Tub, tubulin.

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Although biological significance of the Dok-7-CrkII complex is as yet unclear.
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analysis of cultured myotubes after transfections with expression plasmid for this construct (data not shown).

Mice lacking Dok-7 failed to form the NMJs and showed neonatal lethality apparently due to respiratory failure, suggesting that complete loss of Dok-7 activity is also likely to be lethal in humans (1). CMS due to mutations in DOK7 shows recessive inheritance with several patients homozygous for the most commonly identified mutation, 1124_1127dupTGCC. The resultant mutant protein Dok-7-dupTGCC has a reduced but significant ability to activate MuSK and cluster AChRs in myotubes (Fig. 4, D and E). Likewise, mutation 1143insC identified in at least three unrelated patients truncates Dok-7 and partially reduces the ability of Dok-7 to activate MuSK (Fig. 4, F and G, and Ref. 26). Thus, DOK7 CMS patients may harbor a series of different COOH-terminal truncations. By contrast, the 601C → T mutation ablates not only the SH2 target motifs but also the NES to generate Dok-7-R201X and results in the complete loss of Dok-7’s ability to induce MuSK-dependent AChR clustering in myotubes (Fig. 2, A–C). We would predict that harboring this mutation in both alleles is lethal, and this would also be the case for reported truncating mutations 548_551delTCCT and 555delC, which also ablate the NES and SH2 target motifs (26–28). Indeed, no patients have been reported with DOK7 CMS who are homozygous or compound heterozygotes for these mutations. Likewise, we would predict that homozygous inheritance of Dok-7 missense mutations A33V or R158Q would be lethal since these point mutations completely inhibited Dok-7 from activating MuSK in myotubes (Fig. 1, A–E).

Here we identified the NES and the SH2 target motifs in the COOH-terminal moiety of Dok-7 as functional elements important for MuSK activation and AChR clustering in myotubes. The loss of these motifs is likely to contribute to the NMJ synaptopathy observed in DOK7 CMS. Additional studies of Dok-7 in relation to disease should further our understanding of how its interaction with MuSK plays such a crucial role in the formation and maintenance of NMJ structure in humans.

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