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Dok-7/MuSK signaling and a congenital myasthenic syndrome

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Skeletal muscle contraction is controlled by motor neurons, which contact the muscle at the neuromuscular junction (NMJ). The formation and maintenance of the NMJ, which includes the aggregation of densely packed clusters of acetylcholine receptor (AChR) opposite the motor nerve terminal, is orchestrated by muscle-specific receptor tyrosine kinase, MuSK. Recently, a MuSK-interacting cytoplasmic adaptor-like protein Dok-7 was identified and its localization at the postsynaptic region of the NMJ was revealed. Mice lacking Dok-7 have a phenotype indistinguishable from MuSK-deficient mice, and fail to form both AChR clusters and NMJs. In cultured myotubes, Dok-7 is required for MuSK activation and AChR clustering. Thus, Dok-7 is essential for neuromuscular synaptogenesis and it appears that the regulatory interaction of Dok-7 with MuSK is integrally involved in this process. In humans there are both autoimmune and genetic causes of defective neuromuscular transmission that gives rise to the fatigable muscle weakness known as myasthenia. *DOK7* has been found to be a major locus for mutations that underlie a genetic form of myasthenia with a characteristic 'limb girdle' pattern of muscle weakness (*DOK7* CMS). Patients with *DOK7* CMS have small, simplified NMJs but normal AChR function. The most common mutation causes a COOH-terminal truncation, which greatly impairs Dok-7's ability to activate MuSK. Recently, a series of differing *DOK7* mutations have been identified, which affect not only the COOH-terminal region but also the NH_2-terminal moiety. The study of these mutations may help understand the underlying pathogenic mechanism of *DOK7* CMS.

Key words: DOK7 congenital myasthenic syndrome, neuromuscular junction, protein tyrosine kinase, Dok-7, MuSK

Protein-tyrosine kinases (PTKs) play important roles in regulating cellular functions in multicellular organisms by transducing extracellular stimuli into intracellular signaling events, although some PTKs are also found in unicellular organisms (1, 2). In general, upon stimulation by extracellular cues, PTKs are activated and phosphorylate themselves or other substrates, including transmembrane receptors and intracellular docking/adaptor proteins, to recruit, or to inhibit in a few cases, various effectors. A receptor PTK MuSK (muscle-specific kinase) has proved essential for the postsynaptic specialization of the neuromuscular junction (NMJ), a synapse connecting the motor nerve terminals to muscle fibers (3, 4). A glycoprotein Agrin that is secreted from the motor nerve terminal induces activation of MuSK and is required for NMJ formation in mammals (3, 4). However, in the absence of neural Agrin or even in the absence of entire motor neurons, postsynaptic specialization, such as acetylcholine receptor (AChR) clustering in the plasma membrane, acetylcholinesterase (AChE) localization in the extracellular matrix, and localized expression of AChR mRNAs, was still observed in the central region of the skeletal muscle, where NMJs normally form (4, 5). Moreover, in the experimental absence of neuromuscular transmission through targeted disruption of the *CHAT* (choline acetyltransferase) gene, mice which also lack Agrin are able to form neuromuscular synapses (6, 7). Taken together, this data strongly suggested the existence of an as yet unidentified muscle-intrinsic activator of MuSK, which might play a role on the postsynaptic side of the NMJ in the central region of the developing skeletal muscle fibers.

Many receptor PTKs phosphorylate their cytoplasmic region to recruit downstream signaling molecules via the interaction of the SH2 (src homology 2) or phosphotyrosine binding (PTB) domain of such effectors with each target motifs that encompasses the autophosphorylation site (8). In general, the PTB domains preferentially bind with peptides of the form Asn-Pro-Xaa-Tyr (NPXY) upon tyrosine phosphorylation (8). MuSK has a PTB binding motif encompassing Tyr-553 in its cytoplasmic juxtamembrane region, which is indispensable for autophosphorylation of MuSK and subsequent clustering of AChRs in cultured myotubes treated with Agrin (9, 10). Furthermore, studies with MuSK-TrkA chimeric PTK strongly suggest that a region of only 13 cytoplasmic amino acids encompassing the PTB binding motif of MuSK are essential for postsy-

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naptic specialization and NMJ formation in vitro and in vivo (9-11). These observations suggest that there is an additional molecule which harbors a PTB domain, interacts with MuSK and is similarly crucial for postsynaptic specialization of the NMJ.

Dok-7: an essential cytoplasmic activator of MuSK

Since a 62 kDa cytoplasmic protein Dok-1 was identified as a common substrate of many PTKs, the Dok-family has been expanded to seven members, Dok-1 to Dok-7, which share structural similarities characterized by the NH_2-terminal pleckstrin homology (PH) and PTB domains, followed by the SH2 target motifs in the COOH-terminal moiety, suggesting an adaptor function (12-14). Indeed, Dok-1 and Dok-2 recruit p120 rasGAP, which has the two SH2 domains, upon tyrosine phosphorylation to suppress Ras/Erk signaling (15, 16). Unlike the other members of the Dok-family proteins, Dok-7 is preferentially expressed in muscle tissues, and immunohistochemical studies further demonstrate that Dok-7 is colocalized with AChRs at the postsynaptic area of NMJ in skeletal muscle (14). Because MuSK is also known to be colocalized with AChRs at the postsynaptic area, these results suggested that Dok-7 may interact with MuSK via the PTB domain of Dok-7 and its target motif in the juxtamembrane region of MuSK. Indeed, forced expression of these proteins revealed that they bind via the interaction of the PTB domain and its target motif (14). Moreover, the forced expression of Dok-7 also activated MuSK both in heterologous cells and in cultured myotubes (14). Given that receptor PTKs are thought to be activated by extracellular cues and transduce such stimuli into cytoplasmic signaling, this observation is surprising. However, downregulation of Dok-7 using RNA interference technique demonstrated that Dok-7 is required for activation of MuSK at least in cultured myotubes (14). Furthermore, the RNA interference experiments revealed that even neural Agrin requires Dok-7 to activate MuSK in myotubes (14). Indeed, E18.5 embryos of mice lacking Dok-7 do not form AChR clusters nor NMJs in the diaphragm muscles as was observed in MuSK-deficient mice (14, 17). It is of note that both mutant mice showed abnormal extension of motor nerve axons at the medial area of the skeletal muscle possibly due to the lack of retrograde signaling from the postsynaptic apparatus (14, 17). Together, these data indicate that Dok-7 is a cytoplasmic activator of MuSK essential for MuSK-dependent postsynaptic specialization of NMJ (Fig. 1).

As previously stated, a skeletal muscle-intrinsic activator of MuSK was predicted due to existence of i) aneural, but MuSK-dependent, clustering of AChRs in mouse embryos; and ii) neuromuscular synapse formation in mice lacking both Agrin and CHAT (5-7). Although there is no definitive proof that Dok-7 is this muscle-intrinsic activator of MuSK, there is supporting evidence: i) mice lacking Dok-7 or MuSK form no AChR clusters while those lacking Agrin can form aneural, MuSK-dependent AChR clusters (14, 17); ii) Dok-7 transcripts were preferentially expressed in the central region of the diaphragm muscle of mouse E14.5 embryos, where the aneural, MuSK-dependent AChR clusters normally form (14). However, as we will discuss later, if Dok-7 plays a role

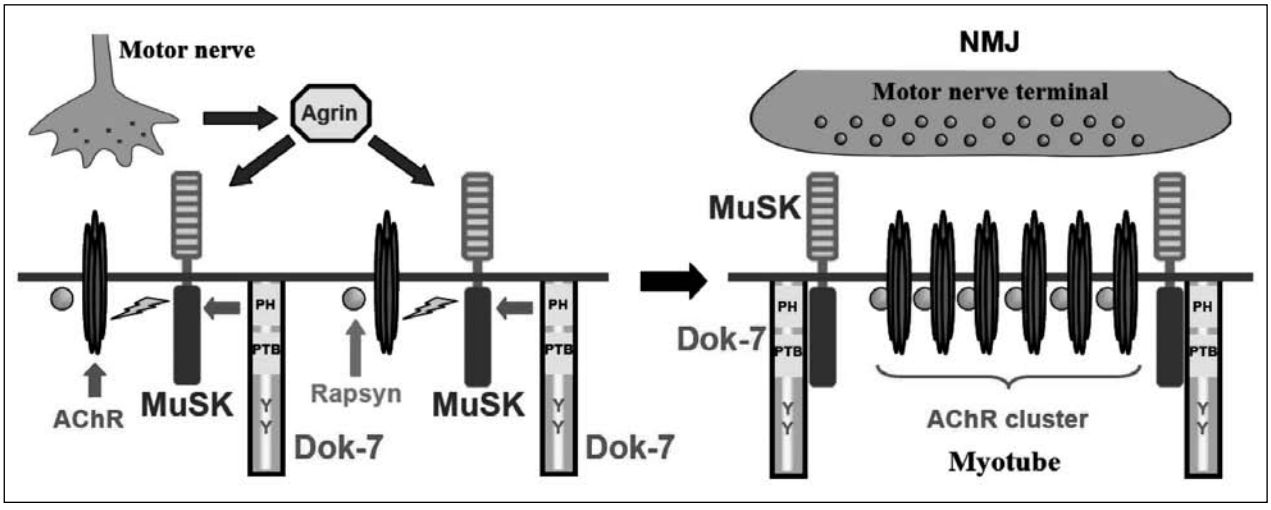

Figure 1. A greatly simplified model of the Dok-7/MuSK pathway for postsynaptic specialization of the mammalian NMJ. Dok-7 can activate MuSK and induce Rapsyn-dependent AChR clustering even in the absence of neural Agrin; however MuSK requires both Dok-7 and Agrin to form functional NMJs in vivo. Many of the molecular mechanisms underlying these signaling events have yet to be determined. AChR, acetylcholine receptor; PH, pleckstrin homology; PTB, phosphotyrosine binding; Y, tyrosine residue in each SH2 target motif of Dok-7.

as an essential signaling molecule downstream of MuSK, the same defects could be observed in Dok-7-deficient mice. Therefore, careful examination of kinase activity of MuSK in the skeletal muscle during embryogenesis of Dok-7-deficient mice would be important.

The Dok-7/MuSK pathway

How does a cytoplasmic adaptor-like protein Dok-7 activate a receptor PTK MuSK? Although the definitive answer awaits further studies, several interesting observations have been found (14). In heterologous cells, which do not express Dok-7 nor MuSK, the forced expression of these two proteins resulted in robust activation of MuSK. In addition, when ectopically expressed in heterologous cells, these proteins form a stable complex that requires the intact PTB domain of Dok-7 and its target motif encompassing Tyr-553 in the juxtamembrane region of MuSK. These data suggest that Dok-7 directly interacts with MuSK and activates it. However, this PTB-dependent stable complex formation of Dok-7 with MuSK is not prerequisite for Dok-7-mediated activation of MuSK in the heterologous cells or even in cultured C2C12 myoblasts. Interestingly, in addition to the PTB domain, the entire COOH-terminal region, but not PH domain, is also dispensable for MuSK activation in these cells. However, when myotubes were fully differentiated from C2C12 myoblasts, both the PTB domain and the COOH-terminal region were indispensable for MuSK activation and subsequent AChR clustering. The data suggests that a negative regulatory mechanism preventing MuSK activation is established upon differentiation from myoblasts into myotubes. Note that C2C12 myotube differentiation is accompanied by increasing expression of MuSK and Dok-7 (14). To counteract the hypothetical negative regulation, Dok-7 may need to be stably complexed with MuSK via the PTB domain and may also need an as yet unidentified function of the COOH-terminal moiety. For example, trace phosphorylation of MuSK in myotubes might allow physical interaction with Dok-7, in turn facilitating dimerization and/or conformational changes in MuSK that are necessary for its sustained activation in myotubes.

It has been reported that an adaptor protein SH2-B, which has the PH and SH2 domains, binds via the SH2 domain to multiple receptor PTKs including insulin receptor (IR) and NGF receptor (TrkA). In addition, the forced expression of IR and SH2-B in CHO cells enhanced IR-mediated signaling upon stimulation with insulin; however, it did not affect IR activity in the absence of insulin (18). Similarly, the forced expression of SH2-B in PC12 cells enhanced TrkA-mediated signaling upon NGF treatment, but again it did not affect TrkA activity in the absence of NGF (19). By contrast, Dok-7 does not require Agrin to activate MuSK in myotubes, and furthermore, Dok-7 does not require the PTB domain, which is essential for stable binding with MuSK, to activate MuSK in 293T cells (14). Given that Agrin requires Dok-7 to activate MuSK at least in cultured myotubes, Dok-7 appears to be a cytoplasmic activator of MuSK rather than a signal enhancer of it. Increase of expression of both MuSK and Dok-7 upon differentiation of myoblasts into myotubes may trigger the Dok-7-mediated activation of MuSK in the central region of the developing skeletal muscle, where preferential expression of AChR, MuSK and Dok-7 together with aneural, Agrin-independent AChR clustering are observed (14). Then, Agrin and Dok-7 may cooperate to induce full activation of MuSK to orchestrate NMJ formation. Since patients with NMJ disorders due to genetic mutations of *DOK7* (see below) often only present with symptoms at least 18 months after birth, it suggests that Dok-7-mediated activation is essential not only for NMJ formation but also for its maintenance (20-22). This seems to be consistent with the postsynaptic localization of Dok-7 at fully formed NMJ in adult mice.

As mentioned, although Dok-7 appears to be an essential cytoplasmic activator (it could be referred to as a cytoplasmic ligand) of MuSK, it is possible that Dok-7 also plays a role downstream of MuSK. The forced expression of Dok-7 and MuSK, but not its kinase-inactive mutant, results in activation of MuSK and tyrosine phosphorylation of Dok-7 (14). In addition, treatment of cultured myotubes with Agrin induced autophosphorylation of MuSK and Dok-7 phosphorylation synchronously (14). Because Dok-7 retains all characteristic domains/motifs for adaptor proteins, namely the PH and PTB domains and the SH2 binding motifs, the data implies that Dok-7 can function as an adaptor protein in MuSK-mediated signaling.

DOK7 congenital myasthenic syndrome

Skeletal muscle contraction is controlled by the motor nerves via the NMJ. In patients, defects of neuromuscular transmission characteristically present as fatigable muscle weakness, known as myasthenia. This can be autoimmune (such as myasthenia gravis) or genetic (congenital myasthenic syndromes (CMS)) in origin, or on occasion can arise from botulism or snake bites (23, 24). CMS can stem from genetic defects in presynaptic, synaptic and, in most cases, postsynaptic proteins of the NMJ (24, 25). In these disorders, impaired neuromuscular transmission results in fatigable weakness at various levels in the limb, ocular, bulbar, truncal and respiratory muscles. CMS-associated genetic mutations had previously been identified in ten genes that encode essential component of the NMJ:

the acetylcholine receptor subunits (*CHRNA1, CHRNB1, CHRND*, *CHRNE*, and *CHRNG*), choline acetyltransferase (*CHAT*), the collagen tail subunit of acetylcholinesterase (*COLQ*), rapsyn (*RAPSN*), MuSK (*MUSK*), and the skeletal muscle sodium channel $Na_V1.4$ (*SCN4A*) (25-27). However, in many CMS patients, including a major subgroup with a limb girdle pattern of muscle weakness, mutations had not been identified (25, 28, 29). Given that Dok-7 was newly recognized as an important NMJ protein, the *DOK7* locus of these patients was investigated and found to be a major locus for mutations underlying 'limb girdle' type CMS (20).

Research groups including the authors have already identified *DOK7* mutations in 27 patients from 24 kinships (20, 21). The most common mutation, 1124_1127dupTGCC, was present in 20 of the 24 reported kinships and all patients were found to have at least one allele with a frameshift mutation in *DOK7* exon 7, which encodes a large part of the COOH-terminal moiety (20-22); however, mutations were identified in other exons such as those that correspond to the PH and PTB domains (21, 22). When DNA from family members was available, it was observed that the disease co-segregated with recessive inheritance of *DOK7* mutations. The 1124_1127dupTGCC mutation produces truncated Dok-7 (p.Pro376ProfsX30), which lacks a large part of the COOH-terminal moiety. This mutant form of Dok-7 showed impaired, but still significant ability to induce MuSK activation and consequent AChR clustering in myotubes, suggesting that impaired Dok-7/MuSK signaling in the skeletal muscle causes malformation of the neuromuscular synapse and consequent muscle weakness (20). Consistently, it was also reported that patients harboring the *DOK7* mutations have abnormally small, simplified NMJs that show normal AChR and AChE function (20, 28). Taken together, these findings indicate that biallelic *DOK7* mutations underlie an NMJ synaptopathy that causes a new disease entity, *DOK7* CMS.

DOK7 CMS patients have some distinct clinical features that provide clues for diagnosis (20-22). As mentioned above, proximal muscles are usually more affected than distal ones. As with other CMS, ptosis is often present from an early age; however, eye movements are rarely involved. In general, patients with *DOK7* CMS do not show long-term benefit from anticholinesterase medication but frequently responded to ephedrine. Interestingly, this phenotype can be distinguished from limb girdle myasthenia associated with tubular aggregates in the skeletal muscle, where *DOK7* mutations were not detected and patients do respond to anticholinesterase medication. Although these clinical features help diagnosis and appropriate managements, molecular mechanisms underlying these characteristic phenotypes of *DOK7* CMS are as yet unclear.

Are additional components of the Dok-7/MuSK pathway involved in CMS?

DOK7 CMS has been established as a newly recognized disorder. Although CMS-associated genetic mutations have been identified in 11 genes, in many patients causative mutations cannot be found. There is an aforementioned subgroup of CMS patients with a limb girdle pattern of muscle weakness, who have tubular aggregates when muscle biopsies are analyzed in whom genetic mutations have not yet been identified (28). It is likely that additional elements will be identified that play important roles in AChR assembly, and NMJ formation and maintenance. For example, Low-density lipoprotein receptor-related protein 4 (Lrp4) has recently been identified as an essential molecule for the postsynaptic specialization of NMJ (30). Defects found in the skeletal muscle of mice lacking Lrp4 are indistinguishable from those in MuSK- or Dok-7-deficient mice; namely, failure to cluster AChRs and exuberant growth of motor nerve axons in embryos. However, how this receptor-like transmembrane protein plays a role in Dok-7 and MuSK-dependent AChR clustering is unknown. A comprehensive understanding of the molecular mechanisms of NMJ formation and maintenance, in which the Dok-7/MuSK pathway is central, may contribute to identification of other causative mutations of CMS and to the discovery of potential therapeutic targets.

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We have recently identified a CRM1-dependent nuclear export signal (NES) in the COOH-terminal moiety of Dok-7 and demonstrated that the NES as well as the SH2 target motifs are critical for MuSK activation in myotubes (31).

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