

JB Review

Activation of receptor protein-tyrosine kinases from the cytoplasmic compartment

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It is widely accepted that receptor protein-tyrosine kinases (RTKs) are activated upon dimerization by binding to their extracellular ligands. However, EGF receptor (EGFR) dimerization per se does not require ligand binding. Instead, its cytoplasmic kinase domains have to form characteristic head-to-tail asymmetric dimers to become active, where one ‘activator’ domain activates the other ‘receiver’ domain. The non-catalytic, cytoplasmic regions of RTKs, namely the juxtamembrane and carboxy terminal portions, also regulate kinase activity. For instance, the juxtamembrane region of the RTK MuSK inhibits the kinase domain probably together with a cellular factor(s). These findings suggest that RTKs could be activated by cytoplasmic proteins. Indeed, Dok-7 and cytohesin have recently been identified as such activators of MuSK and EGFR, respectively. Given that failure of Dok-7 signaling causes myasthenia, and inhibition of cytohesin signaling reduces the proliferation of EGFR-dependent cancer cells, cytoplasmic activators of RTKs may provide new therapeutic targets.

Keywords: cancer/cytohesin/Dok-7/myasthenia/neuromuscular junction.

Abbreviations: ACh, acetylcholine; AChR, ACh receptor; CMS, congenital myasthenic syndrome; Dok, downstream of kinases; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; EGFR, EGF receptor; HER, human EGFR; HSA, human skeletal alpha-actin; MuSK, muscle-specific kinase; NMJ, neuromuscular junction; PH, pleckstrin homology; PTB, phosphotyrosine binding; PTK, protein-tyrosine kinase; RTK, receptor PTK; SH2, src homology 2; Tg, transgenic; WT, wild-type.,

Identification and Characterization of Dok-7 as a Cytoplasmic Activator of the Receptor Protein-tyrosine Kinase, MuSK

Dok-7, MuSK and the Neuromuscular Junction

Being a major substrate of many protein-tyrosine kinases (PTKs), the tyrosine-phosphorylated and

rasGAP-associated 62-kDa protein p62 was purified for study from cells transformed by v-Abl or Bcr-Abl by our group and another, respectively (1, 2). The cDNA sequence revealed that p62, now termed Dok-1, is an adaptor-like protein carrying N-terminal pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains followed by src homology 2 (SH2) target motifs present in its C-terminal moiety (Fig. 1A). This overall structure is similar to that of the insulin receptor substrate (IRS) family of adaptors, suggesting a role downstream of PTKs. Indeed, Dok-1 and its closest homologues Dok-2 and Dok-3 have been identified as negative regulators of many PTK-mediated signaling pathways (3). In addition, Dok-4, Dok-5 and Dok-6 have also been suggested to have adaptor functions (3).

On searching databases, we identified partial genomic and cDNA sequences of a new candidate member of the Dok family, which would be the seventh. On the basis of these partial sequences, we cloned human, mouse and fish Dok-7 cDNAs and confirmed it to be a member of the Dok family (Fig. 1) (4). Northern blot analysis showed it to be expressed in the skeletal and cardiac muscles, and immunoblot analysis identified a 55-kDa Dok-7 protein in these tissues. Furthermore, immunostaining of skeletal muscle highlighted accumulation of Dok-7 proteins at the post-synaptic region of the neuromuscular junction (NMJ), a synapse between a motor nerve and skeletal muscle.

The NMJ in mammals uses the neurotransmitter acetylcholine (ACh), which is released from the pre-synaptic motor nerve terminal to control skeletal muscle contraction. In order to achieve sufficient sensitivity to the neurotransmitter, ACh receptors (AChRs) on the muscle must be densely clustered in the post-synaptic region of NMJs (5, 6). Failure of AChR clustering is associated with disorders in neuromuscular transmission, including congenital myasthenic syndromes (CMSs) and myasthenia gravis (7, 8). The formation and maintenance of the NMJ, including the post-synaptic AChR clustering at the central, endplate region of each muscle, is orchestrated by the muscle-specific receptor PTK (RTK), MuSK (9–11). The pre-synaptic motor nerve terminal secretes the glycoprotein agrin to activate post-synaptic MuSK. This agrin-dependent activation of MuSK is essential to form NMJs during embryogenesis and for their post-natal maintenance (12–15). However, before innervation during embryogenesis, MuSK-dependent but nerve/agrin-independent AChR clusters can form along the endplate area of muscles (16, 17). Moreover, NMJs can form even in the absence of agrin in mice that lack ACh, which apparently antagonizes post-synaptic specialization (11). Thus, in

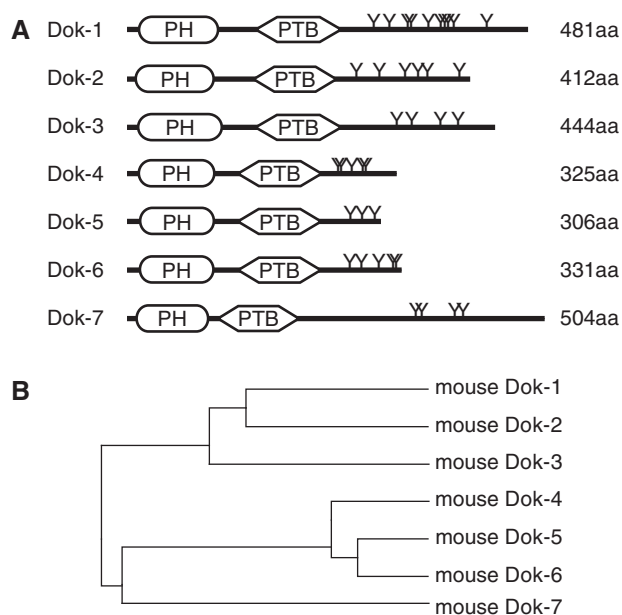


Fig. 1 Dok-7 is a member of the Dok family. (A) Schematic representation of mouse Dok proteins. Y denotes a tyrosine residue in the C-terminal region. Many of these tyrosines act as core residues in their respective SH2-binding motifs (3). (B) A phylogenetic tree of mouse Dok proteins. The phylogenetic tree was calculated using full-length sequences.

addition to agrin, another element seems to be present that achieves MuSK activation and subsequent post-synaptic specialization (Fig. 2A).

Dok-7 Induces MuSK Activation

As mentioned above, the cytoplasmic adaptor-like protein Dok-7 with its PH and PTB domains is localized to the post-synaptic region of the NMJ. Moreover, MuSK was known to be localized to the same region, with its PTB domain-binding motif encompassing the major autophosphorylation site (Tyr-553) in the juxtamembrane portion. Thus, it was presumed that activated and autophosphorylated MuSK recruits Dok-7 via its PTB domain to phosphorylate and activate Dok-7 as a downstream effector protein. However, when overexpressed in cultured C2C12 myotubes, Dok-7 induced the following molecular events: (i) tyrosine phosphorylation of MuSK, (ii) tyrosine phosphorylation of the AChR $\beta 1$ subunit and (iii) AChR clustering. Because these events are known to occur upon MuSK activation in myotubes, these unexpected findings suggested that Dok-7 activates MuSK at least in cultured myotubes, raising the possibility that Dok-7 could be the long-sought muscle-intrinsic activator of MuSK (4).

In order to examine if Dok-7 activates MuSK in living animals, we generated Dok-7 transgenic (Tg) mice, which express human Dok-7-enhanced green fluorescent protein (EGFP) fusion protein uniformly throughout skeletal muscle under the control of the human skeletal α -actin (HSA) promoter (18). The over-expression of Dok-7 in skeletal muscle increased the total AChR-clustering area in the central region of the muscle compared to wild-type (WT) controls

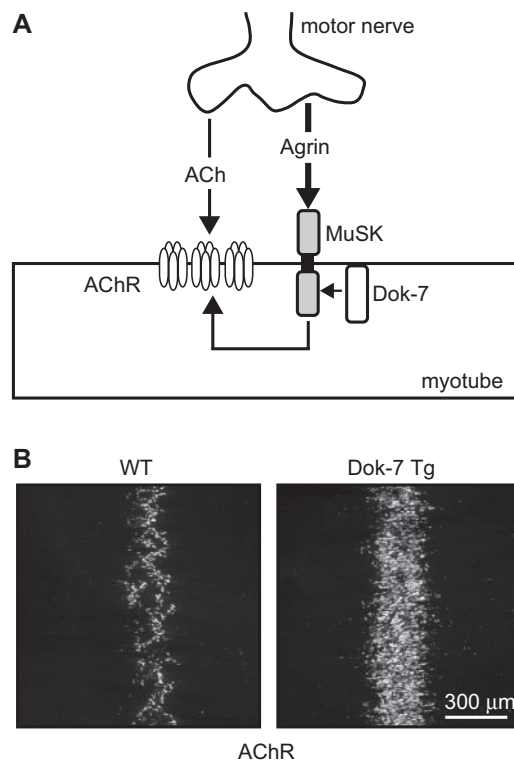


Fig. 2 A simplified model of neuromuscular synaptogenesis and augmented AChR clustering in Dok-7 Tg mice. (A) Dok-7 and agrin activate MuSK to promote clustering of AChRs at the post-synaptic site. While agrin is dispensable for MuSK activation, Dok-7 is required (18). However, in the physiological setting, agrin is required to protect NMJs from ACh-mediated dispersal of the post-synaptic AChR clusters (13, 14). (B) Diaphragm muscles from wild-type (WT) or Dok-7 Tg mouse embryos at E18.5 were stained with fluorescently labelled α -bungarotoxin to visualize post-synaptic AChR clusters. Note that a single AChR cluster is formed in the central region of each myotube in general. Numerous AChR clusters of diaphragm muscles collectively represent an area, termed endplate zone, in the central region, which includes more and larger AChR clusters in Dok-7 Tg than in WT mice.

(Fig. 2B). In most types of mammalian skeletal muscle, each myotube has a single NMJ, where the motor nerve terminal is apposed to an endplate bearing a single AChR cluster. However, four AChR clusters were formed on average per myotube in Dok-7 Tg mice at embryonic day 18.5 (E18.5), suggesting increased NMJ formation. Indeed, myotubes in the Dok-7 Tg embryos contained 1.7-fold more NMJs than those from WT controls, although the majority of these post-synaptic AChR clusters were not innervated in the Tg mice. Together, these data suggested that over-expression of Dok-7 in living animals induces activation of MuSK, which leads to enhanced post-synaptic specialization and subsequent NMJ formation (18).

In order to see MuSK activation in skeletal muscle of Dok-7 Tg mice, we examined tyrosine phosphorylation of MuSK and the AChR $\beta 1$ subunit (18), which is indicative of MuSK activity as mentioned above (19). Although tyrosine phosphorylation of MuSK in limb muscle from WT embryos (E18.5) was undetectable, robust phosphorylation of MuSK was observed in Dok-7 Tg muscle. Similarly, tyrosine phosphorylation of AChR was increased in Dok-7 Tg embryos

compared to WT controls. Collectively, these data demonstrated that Dok-7 is a muscle-intrinsic activator of MuSK.

Dok-7 is an Essential Activator of MuSK

To address Dok-7's contribution for MuSK activation, we examined effects of small interfering RNA (siRNA)-mediated inhibition of Dok-7 in cultured myotubes and found that it suppressed tyrosine phosphorylation of MuSK in the absence or presence of recombinant agrin in the culture medium (4). This suggests that Dok-7 is required for MuSK activation and that even agrin cannot activate MuSK in the absence of Dok-7. Thus, we generated mice lacking Dok-7 to see if they lack AChR clusters before and after innervation, which supplies agrin to skeletal muscle. Like mice lacking MuSK, Dok-7-deficient mice do not form any AChR clusters on myotubes irrespective of innervation and thus have no NMJs (4). In addition, AChR was not tyrosine phosphorylated even after innervation (18). Moreover, consistent with the trend seen in the Dok-7 knockdown experiments, agrin cannot activate MuSK in myotubes derived from Dok-7-deficient mice unlike those from WT controls (18). Collectively, these data demonstrate that Dok-7 is an essential muscle-intrinsic activator of MuSK and that Dok-7-mediated activation of MuSK is a prerequisite for full activation of MuSK by agrin.

Dok-7 Directly Activates the Cytoplasmic Region of MuSK

As mentioned earlier, it is widely accepted that extracellular ligands bind to RTKs to activate them as PTKs, which triggers intracellular signaling cascades. Therefore, it is puzzling how the cytoplasmic Dok-7 could activate the RTK MuSK. Although Dok-7 might induce an as yet unidentified extracellular activator of MuSK in myotubes, we found that the extracellular and transmembrane portion of MuSK is not required for Dok-7 to activate it when overexpressed in HEK 293T cells (18). In other words, Dok-7 activates the cytoplasmic region of MuSK at least in these cells. To test if Dok-7 directly activates MuSK, we further performed *in vitro* kinase assays with the purified, bacterially expressed cytoplasmic region of MuSK (MuSK-cyt) and Dok-7. When MuSK-cyt was subjected to the assay, its autophosphorylation was faintly detectable. However, it was markedly elevated when Dok-7 was added to the *in vitro* reaction (18). In addition, tyrosine phosphorylation of Dok-7 was also clearly detected, and increased in parallel with that of MuSK-cyt. By contrast, the Dok-family protein Dok-1, which cannot activate MuSK in cells, failed to activate MuSK in this assay. Together, these data demonstrate that Dok-7 directly interacts with the cytoplasmic region of MuSK and activates the RTK (Fig. 3A).

Stable Binding of Phosphorylated MuSK and Dok-7 is Not Required for MuSK Activation in Myoblasts, but is Required in Myotubes

As already mentioned, Dok-7 has the PH and PTB domains in its N-terminal moiety and multiple

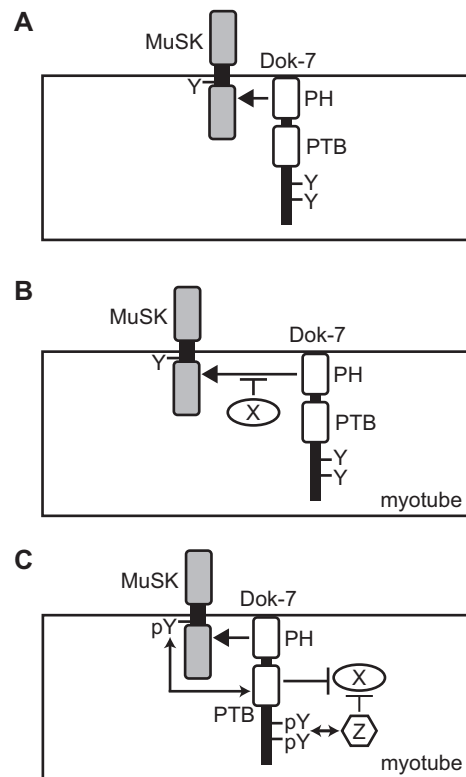


Fig. 3 Simplified models of MuSK activation by Dok-7. (A) Dok-7 directly interacts with the cytoplasmic region of MuSK and activates the RTK. The PH domain of Dok-7 is required for activation. (B) In myotubes, as yet unidentified negative regulator(s) (denoted by X) may prevent MuSK activation. (C) Phosphorylation of MuSK and Dok-7 in myotubes might allow stable binding with the Dok-7 PTB domain and as yet unidentified positive regulator(s) (denoted by Z) having SH2 domain(s), respectively. These events may counteract the negative regulator(s) X. Y and pY denote the core tyrosine residue and its phosphorylated form in each binding motif of the SH2 or PTB domain.

SH2-binding motifs in its C-terminal portion. We found that the PTB domain binds to the PTB target motif in the juxtamembrane region of MuSK when its Tyr-553 was phosphorylated. However, even when Tyr-553 in the PTB target motif was substituted with phenylalanine, Dok-7 activated the mutant (MuSK-Y553F) to an extent comparable to intact MuSK in HEK 293T cells (4). Consistent with this, when core arginines of the PTB domain were substituted with alanines, the mutant Dok-7 (Dok-7-RA) continued to activate MuSK to the same extent as intact Dok-7 in HEK 293T cells and cultured myoblasts. MuSK-Y553F and Dok-7-RA failed to bind to intact Dok-7 and phosphorylated MuSK, respectively, and so stable binding of the two molecules is not required for MuSK activation at least in these heterologous (non-myotube) cells.

By contrast, both the intact PTB domain of Dok-7 and its target motif in MuSK were required in myotubes, which were differentiated from myoblasts (4). These observations indicate that stable binding of phosphorylated MuSK and Dok-7 is required for MuSK activation in myotubes but not in myoblasts, further implying that as yet unidentified mechanisms operating in myotubes may interfere with MuSK

activation by Dok-7 (Fig. 3B and C). Of note, Hubbard and colleagues recently solved the crystal structure of the Dok-7 PH and PTB domains (Dok-7 PH-PTB) in complex with a tyrosine-phosphorylated peptide including the PTB target motif of MuSK, and reported that binding of the PTB domain to the PTB target motif induces dimerization of Dok-7 PH-PTB (20). Based on this and other biochemical data, they concluded that dimeric arrangement of Dok-7 PH-PTB facilitates transautophosphorylation of the kinase activation loop of MuSK. Taken together, stable binding of Dok-7 and MuSK may overcome the aforementioned as yet hypothetical negative regulation in myotubes by inducing dimerization of MuSK, although it is not required for MuSK activation in myoblasts or HEK 293T cells.

The SH2-binding Motifs in the C-terminal Portion of Dok-7 are Important But Not Required for MuSK Activation in Myotubes

With regard to the SH2-binding motifs in the C-terminal portion of Dok-7, we found that the core tyrosine residues in these motifs are major phosphorylation sites and that the SH2 domain of Crk binds them in a manner dependent on tyrosine phosphorylation, indicating these SH2-binding motifs are active (21). In addition, substitution of core tyrosines (Tyr-395 and Tyr-405) or prolines (Pro-398 and Pro-408) with phenylalanines or alanines, respectively, significantly inhibits Dok-7's ability to activate MuSK in cultured myotubes. Given that loss of the entire C-terminal portion of Dok-7 does not affect MuSK activation in myoblasts, the SH2 domain-containing protein(s) may help Dok-7 to overcome the above-inferred negative regulation in myotubes (Fig. 3C). Recently, Burden and colleagues also found that Dok-7 binds to Crk proteins when tyrosine phosphorylated (22). The authors further generated mice lacking Crk and Crk-L selectively in skeletal muscle and demonstrated that these mutant mice showed severe defects in neuromuscular synapses, providing evidence for the physiological importance of these two adaptor proteins. However, the biological significance of their binding to phosphorylated Dok-7 remains to be studied.

The PH Domain is Indispensable for Dok-7 to Activate MuSK

Although the need for the PTB domain and the SH2 target motifs of Dok-7 in MuSK activation apparently differs between myotubes and heterologous cells, N-terminal deletion of the PH domain disables Dok-7 from activating MuSK irrespective of cell type (4). In addition, amino acid substitutions such as Ala-33 to Val in this domain abolish Dok-7's ability to activate MuSK in myotubes (21). However, how the PH domain contributes to activation of MuSK is unclear. The molecular mechanisms underlying Dok-7-mediated MuSK activation await further genetic, biochemical and biophysical studies.

Identification and Characterization of Cytohesins as Cytoplasmic Activators of ErbB Family RTKs

Cytohesins Activate ErbB Family RTKs

Many RTKs are activated upon transautophosphorylation of the kinase activation loop in the catalytic domain, which is believed to be triggered by dimerization. However, RTKs of the ErbB family including EGFR/c-erbB/HER1, lack the requirement for activation loop phosphorylation (23). For example, HER3 is known to be a catalytically inactive member of this family, but can activate other members upon heterodimerization. Crystal structure analyses of the EGFR kinase domain in the active conformation revealed that active kinase domains form an asymmetric, head-to-tail dimer, where one kinase domain binds and activates the other kinase domain (23–25). This offers an explanation of how the kinase inactive HER3 can activate the other ErbB RTKs upon heterodimerization. In addition, the recent single-molecule tracking study by Mellman and colleagues revealed that EGFRs spontaneously form finite-lifetime dimers in the catalytically inactive conformation (26), implying that cytoplasmic factor(s) could modulate and activate these EGFR dimers (27).

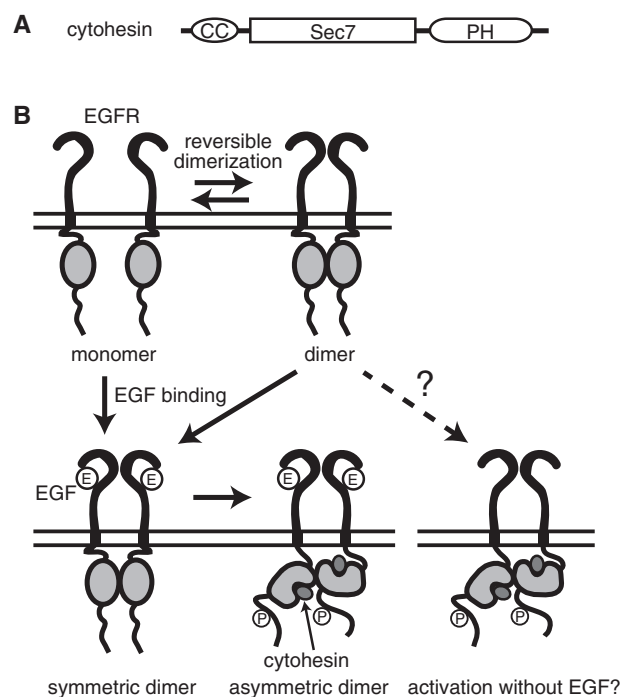


Fig. 4 A simplified model of EGFR activation by cytohesin.

(A) Schematic representation of mouse cytohesin protein. CC, Sec7 and PH denote the coiled-coil, the Sec7 homology, and the PH domains, respectively. (B) A simplified model of EGFR activation by EGF and cytohesin. Even in the absence of the extracellular ligand EGF, EGFRs can spontaneously form finite-lifetime dimers in the catalytically inactive conformation, which requires EGF to be activated (26). Addition of EGF induces dimerization of EGFRs and also primes them for cytohesin-mediated activation by conformational rearrangement of the intracellular domains. Whether EGF can activate EGFRs in the absence of cytohesins or whether cytohesins can activate EGFRs in the absence of EGF remains to be studied.

Cytohesins are a family of guanine nucleotide exchange factors (GEFs) for ADP ribosylation factors (ARFs) that belong to the family of Ras-like small GTPases (Fig. 4A). The cytohesin family includes cytohesin-1, cytohesin-2/ARNO, cytohesin-3/Grp1 and cytohesin-4, and they play important roles in regulation of cytoskeletal dynamics by controlling ARFs' activities (28). Upon exploring the possible role of cytohesins in ErbB receptor signaling, which can also regulate cytoskeletal dynamics, Famulok and colleagues found that inhibition and overexpression of cytohesin reduces and up-regulates ErbB receptor activation, respectively, in the presence of the receptors' corresponding ligands (29). Interestingly, GEF activity of cytohesin-2 was dispensable for its roles in EGFR activation. Although the cytohesin inhibitor SecinH3 could not fully inhibit ErbB receptor activation under the experimental conditions (29), it remains unclear if the RTKs can be activated by their ligands in the absence of cytohesins.

Cytohesin-2 Facilitates a Conformational Rearrangement of the Cytoplasmic Domains of Dimerized EGFRs

Because GEF activity is not essential for cytohesin-2-mediated EGFR activation and it does not induce dimerization of the receptors, Famulok and colleagues further investigated if cytohesin-2 induces a conformational change of the EGFR dimers (29). First, they examined if cytohesin-2 acts on dimerized EGFRs and found that EGFRs experimentally dimerized using a leucine zipper and a disulfide bond were activated by cytohesin-2 and inactivated by its inhibitor, respectively. Since EGFRs can form dimers in the absence of EGF as mentioned above, these data raised the possibility that cytohesins might activate EGFRs in the absence of EGF. However, EGFR activation requires EGF even in a dimer form (26), implying that EGF not only dimerizes but also primes EGFRs for cytohesin-mediated activation (Fig. 4B). Since overexpression of EGFRs can lead to their activation in the absence of EGF, the overexpression-mediated activation might be triggered by cytohesins. In addition, it also remains possible that overexpression of cytohesins can activate EGFRs in, for instance, tumor cells.

Next, they examined if cytohesin-2 induces a conformational change of the cytoplasmic domains of the experimentally dimerized EGFRs using the fluorescent protein mCitrine as a reporter in homo-FRET analysis, which probes both the distance and the orientation of the fluorophores. As expected, their observation demonstrated that cytohesin-2 induces a conformational change of the cytoplasmic domains. In addition, when the purified cytohesin-2 was added to an autophosphorylation reaction of the purified cytoplasmic domain of EGFR (EGFR-ICD), increased autophosphorylation was observed. Taken together, these data strongly suggest that cytohesin-2 acts on intracellular domains of dimerized EGFRs as a cytoplasmic activator.

The Cytoplasmic RTK Activators Provide New Therapeutic Targets

Dok-7 Mutations Underlie the NMJ Disease DOK7 Myasthenia

The identification of Dok-7 and cytohesins as cell-intrinsic activators of RTKs makes it plausible that the expression level of cytoplasmic activators determines the cell-specific amplitude of specific RTK-signaling pathways, usually in cooperation with cognate extracellular ligands. Indeed, loss of Dok-7 resulted in unresponsiveness of MuSK to its extracellular activator agrin, as mentioned earlier. This prompted us to examine if reduction-of-function mutations of Dok-7 cause NMJ disorders known as CMS with fatigable muscle weakness. In collaboration with Beeson and colleagues, we found that biallelic mutations of *DOK7* cause a limb-girdle type of CMS, termed *DOK7* myasthenia (30). Slater and colleagues found that this type of CMS is associated with smaller and simplified NMJs compared to healthy controls, suggesting Dok-7's impaired ability to activate MuSK in the patients (31). Indeed, the mutations Ala-33 to Val substitution in the PH domain, Arg-158 to Gln substitution in the PTB domain, and the nonsense mutation of Arg-201, which causes the entire loss of the C-terminal region, inactivate Dok-7 with regard to activation of MuSK and consequent AChR cluster formation in cultured myotubes (21, 30). By contrast, the most prevalent mutation, 1124_1127dupTGCC, partially inactivates Dok-7. Of note is that no patient has been found to possess loss-of-function mutations homozygously or in combination, supporting the idea that loss of Dok-7 activity results in the lethal loss of NMJs. Indeed, a homozygous *DOK7* splice mutation, 331+1G>T, was identified in a family with three children affected pre-natally with a lethal type of foetal akinesia deformation (32). This splicing mutation causes skipping of at least exon 3, resulting in the loss of the PH domain and inactivation of Dok-7 in MuSK activation in cultured myotubes (33). Taken together, these findings identify the Dok-7-MuSK-signaling pathway as a new therapeutic target not only for *DOK7* myasthenia but also other NMJ synaptopathies.

Cytohesins are Essential for EGFR-dependent tumor Proliferation

Enhanced EGFR signaling in many cancers raises the possibility that cytohesins might contribute to tumorigenesis by activating EGFRs in tumor cells. Indeed, up-regulation of cytohesin expression was observed in a large fraction of lung adenocarcinomas, and the inhibition of cytohesins with the antagonistic chemical compound SecinH3 reduced the proliferation of the EGFR-dependent lung cancer cell line PC9 (29). Moreover, the chemical inhibition of cytohesins reduced *in vivo* proliferation of PC9 tumor xenografts. These findings indicate pathophysiological significance for intracellular EGFR activators and thus provide a new therapeutic target for ErbB family RTK-dependent cancers.

Implications

Given the physiological and pathophysiological importance of Dok-7 and cytohesins, it seems possible that the local levels of Dok-7, cytohesins, or other as yet unidentified cytoplasmic activators control RTK-mediated signaling in individual tissues. For example, at the early stage of NMJ formation Dok-7 expression is constrained to the central region of myotubes, where MuSK is activated to initiate post-synaptic differentiation of the NMJ. Experimentally, in the absence of Dok-7, MuSK is not activated and its expression together with other NMJ components like AChR is dispersed throughout the myotubes (18). Thus, the Dok-7-mediated activation of MuSK appears to set the stage for the centrally restricted formation of NMJs in skeletal muscle.

Ye and colleagues recently reported that overexpression of phosphoinositide 3-kinase enhancer A (PIKE-A) enhances tyrosine kinase activity of insulin receptor in the presence, but not significantly in the absence, of insulin (34). Furthermore, insulin receptor is not responsive to insulin in the absence of PIKE-A, resulting in the development of type2 diabetes *in vivo*. These data suggest that PIKE-A is another cytoplasmic RTK activator. To fully understand the physiological and pathophysiological roles of RTKs, it now seems important to investigate how their activities are intracellularly controlled.

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Conflict of interest

None declared.

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