Agrin Binds to the N-terminal Region of Lrp4 Protein and Stimulates Association between Lrp4 and the First Immunoglobulin-like Domain in Muscle-specific Kinase (MuSK)∗§

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Background: How Agrin binds Lrp4 and stimulates MuSK kinase activity is poorly understood.

Results: We identify domains in Lrp4 important for Agrin to bind Lrp4 and MuSK and stimulate MuSK phosphorylation.

Conclusion: Different domains in Lrp4 bind Agrin and MuSK and stimulate MuSK phosphorylation.

Significance: Lrp4 functions as a ligand for MuSK, whereas Agrin promotes association between Lrp4 and MuSK.

Neuromuscular synapse formation depends upon coordinated interactions between motor neurons and muscle fibers, leading to the formation of a highly specialized postsynaptic membrane and a highly differentiated nerve terminal. Synapse formation begins as motor axons approach muscles that are prepatterned in the prospective synaptic region in a manner that depends upon Lrp4, a member of the LDL receptor family, and muscle-specific kinase (MuSK), a receptor tyrosine kinase. Motor axons supply Agrin, which binds Lrp4 and stimulates further MuSK phosphorylation, stabilizing nascent synapses. How Agrin binds Lrp4 and stimulates MuSK kinase activity is poorly understood. Here, we demonstrate that Agrin binds to the N-terminal region of Lrp4, including a subset of the LDLα-propeller domains, which promotes association between Lrp4 and MuSK on the surface of myotubes in the absence of the transmembrane and intracellular domains of Lrp4. Further, we demonstrate that the first Ig-like domain in MuSK, which shares homology with the NGF-binding region in Tropomyosin Receptor Kinase (TrKA), is required for MuSK to bind Lrp4. These findings suggest that Lrp4 is a cis-acting ligand for MuSK, whereas Agrin functions as an allosteric and paracrine regulator to promote association between Lrp4 and MuSK.

Neuromuscular synapse formation requires a complex exchange of signals between motor neurons and skeletal myofibers (1, 2). During development, motor axons approach muscles that are prepatterned, as acetylcholine receptor (AChR) transcription and AChR clustering are enhanced in the central, prospective synaptic region of muscle prior to and independent of innervation. Muscle prepatternning requires the low-density lipoprotein receptor-related protein 4 (Lrp4) and MuSK, a receptor tyrosine kinase expressed in skeletal muscle, and specifies the region where motor axons form synapses. Agrin, a ligand that is supplied by motor neurons and concentrated in the synaptic basal lamina, binds to Lrp4, leading to further MuSK activation and stabilizing nascent and labile synapses (3, 4). How Lrp4 and MuSK associate to promote muscle prepatternning and how Agrin binds Lrp4 and stimulates MuSK kinase activity are poorly understood. Once MuSK is tyrosine phosphorylated, MuSK recruits Dok-7, a muscle-specific adapter protein (5). The steps that follow Dok-7 recruitment and phosphorylation lead to synapse-specific transcription and the stable accumulation of postsynaptic proteins, including AChRs, in the postsynaptic membrane, which is necessary to ensure robust and reliable synaptic transmission (2, 6). In the absence of Agrin, Lrp4, MuSK, or Dok-7, neuromuscular synapses fail to form, leading to neonatal lethality (5, 7–9). Mutations that impair this signaling pathway lead to a reduced number of AChRs at synapses as well as presynaptic defects and are responsible for congenital myasthenia (10, 11). Moreover, autoantibodies to Lrp4, MuSK, or AChRs are responsible for myasthenia gravis (12, 13).

MuSK is an atypical “receptor” tyrosine kinase. Conventional receptor tyrosine kinases are activated by ligands presented by adjacent cells in a paracrine manner that bind directly to the receptor, stimulating dimerization and/or rearrangement of the receptor and leading to trans-phosphorylation and kinase activation (14). Atypical receptor tyrosine kinases, such as MuSK, Ret, and ErbB2, however, are activated by ligands that do not bind directly to the kinase. Instead, ligands bind to a separate component (Lrp4, GFRα, and ErbB3, respectively) that lacks kinase activity, and ligand-binding is relayed to stim-

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2 The abbreviations used are: AChR, acetylcholine receptor; AP, alkaline phosphatase; LDLR, low-density lipoprotein receptor.
ulate the kinase (3, 4, 15–17). The paradigms responsible for activation of these unconventional receptor tyrosine kinases, including MuSK, remain unclear (14).

Here, we show that Agrin binds to a region in Lrp4 that includes three of the eight LDLa domains and the first four β-propeller domains. Moreover, we demonstrate that Agrin stimulates a dramatic increase in association between Lrp4 and MuSK, which requires two additional LDLa repeats and two additional β-propeller domains in Lrp4. In addition, the extracellular region of Lrp4, which binds both Agrin and MuSK, is sufficient to restore Agrin-stimulated MuSK phosphorylation in lrp4 mutants. This, the demonstration of a functional signaling complex in the absence of the transmembrane and intracellular domains of Lrp4. Further, we demonstrate that a solvent-exposed region in the first immunoglobulin-like domain of MuSK, which is similar to the region in TrkA that binds NGF, is required for MuSK to bind Lrp4, consistent with the idea that Lrp4 functions as a ligand for MuSK. These findings indicate that Lrp4 is a cis-acting ligand for MuSK, whereas Agrin functions as an allosteric regulator to promote association between Lrp4 and MuSK.

EXPERIMENTAL PROCEDURES

Human placental alkaline phosphatase (AP)-ecto-Lrp4 fusion proteins were generated by subcloning sequences from the extracellular region of Lrp4 into pcDNA3 (supplemental Table S1) and expressed from transiently transfected COS-7 cells that were grown in Opti-MEM (Invitrogen), containing 1 mM CaCl2, at 31–33 °C. The Lrp4 mutant constructs are listed in supplemental Table S1 and expressed at their anticipated molecular sizes (supplemental Fig. S1). The media were concentrated 20- to 40-fold using an Amicon ultracentrifugation filter (Millipore, Billerica, MA), and a constant level of AP activity, corresponding to 5 nM fusion protein, was added to 96-well MaxiSorp plates (Thermo Scientific, Rochester, NY) that were coated with BSA, non-neural Agrin, or neural Agrin (20 μg/ml). Following several washes, the amount of AP-ecto-Lrp4 bound to the plate was measured using p-nitrophenyl phosphate as a substrate, as described previously (18, 19). Neural and non-neural Agrin were isolated from SF9 cells as described previously (3). MuSK-Fc fusion proteins were generated by subcloning sequences encoding the extracellular region of MuSK in pFUSE-hIgG1-Fc1 (InvivoGen, San Diego, CA). Fc, MuSK-Fc or MuSK I96A (0.3 μM) were added to 96-well protein A-coated plates (Thermo Scientific). The amount of Fc fusion protein was determined by Western blotting or ELISA using purified human Fc as a standard. The structures of DNA constructs were confirmed by DNA sequencing.

BaF3 cells expressing MuSK-GFP or Lrp4-mCherry BaF3 cells were grown and analyzed as described previously (3). Wild-type and lrp4 mutant muscle cells were grown and allowed to differentiate as described previously (3). MuSK was immunoprecipitated from lrp4 mutant myotubes, treated with 10 nM AP-ecto-Lrp4 and 10 nM Agrin for 8 h, and then MuSK phosphorylation was measured as described previously (3).

RESULTS

Agrin Binds to the N-terminal Region of Lrp4—To identify sequences in the extracellular region of Lrp4 that mediate association with Agrin and MuSK, we expressed the extracellular region of Lrp4 (ecto-Lrp4) in mammalian cells and used a solid-phase binding assay to measure association between Agrin and Lrp4. We found that most ecto-Lrp4, expressed by cells grown at 37 °C, was poorly secreted and instead was retained within cells. We reasoned that improper folding might be the cause for poor secretion, so we shifted the cells to 31–33 °C and found that lowering the temperature increased the amount of ecto-Lrp4 found in the media by 10- to 50-fold. As such, we routinely harvested media from cells that were grown at 31–33 °C.

We used a solid-phase binding assay to measure binding between human AP-ecto-Lrp4 and Agrin B0, a neural isoform of Agrin (Fig. 1A). As negative controls, we adsorbed either Agrin B0, a non-neural isoform of Agrin that fails to bind Lrp4, or BSA to the plate (3, 4). We found that AP-ecto-Lrp4 bound selectively to neural Agrin (Fig. 1A).

The extracellular region of Lrp4, like the LDLR and other Lrp family members, contains LDLa, EGF-like, and β-propeller domains (20). In the LDLR, the LDLa domains, disulfide-linked calcium-binding repeats, bind ApoB and ApoE together with bound cholesterol (21). Once the LDLR is internalized and acidified within endosomes, the single β-propeller domain associates with the LDLa repeats, displacing ApoB/E, releasing cholesterol, and allowing the LDLR to recycle to the cell surface (22, 23). In Lrp5 and Lrp6, receptors for Wnts, the β-propeller domains, rather than the LDLa domains, are responsible for ligand binding (24, 25).

To determine which domains in Lrp4 are responsible for binding neural Agrin, we generated truncated forms of Lrp4 and used the solid-phase binding assay to identify domains in Lrp4 that are necessary and sufficient to bind neural Agrin. Fig. 1 shows that an approximately 50-kDa fragment, composed of LDLa domains 6–8, the first two EGF-like domains, and the first of four β-propeller domains, is necessary and sufficient to bind Agrin (Fig. 1A and supplemental Fig. S1). In contrast, the four β-propeller domains alone or the LDLa domains with or without the EGF-like domains were insufficient to bind Agrin (Fig. 1A and supplemental Fig. S1).

Although the second through fourth β-propeller domains in Lrp4 are dispensable for Agrin binding, the fourth β-propeller domain in Lrp4 restrains Agrin binding, as deletion of this β-propeller domain caused a 4-fold increase in Agrin binding (Fig. 1).

The First β-Propeller in Lrp4 Is Required for Agrin Binding and Is Unique among the β-Propeller Domains in Lrp4—The first β-propeller domain is necessary for Agrin binding (Fig. 1A). We next asked whether the other β-propeller domains, which share 50–60% sequence identity with propeller domain 1, could replace the first β-propeller domain or whether the first β-propeller domain had a unique role in binding Agrin. We substituted β-propeller domain 2, 3, or 4 for β-propeller domain 1 and found that only β-propeller domain 1, together with the LDLa and EGF-like domains, bound neural Agrin (Fig. 1B and supplemental Fig. S1). These data indicate that β-pro-
peller domain 1 contains sequences that uniquely facilitate Agrin-binding rather than only fulfilling a space-filling function to position the LDLa domains.

Agrin Promotes Binding between Lrp4 and MuSK—Binding of Agrin to Lrp4 stimulates MuSK kinase activity (3, 4). In principle, Agrin might stimulate MuSK activity by promoting association between Lrp4 and MuSK, reorienting MuSK without altering binding between Lrp4 and MuSK, or a combination of these mechanisms. To determine whether Agrin alters the association between Lrp4 and MuSK, we measured binding between ecto-Lrp4 and MuSK in the presence or absence of Agrin.

We used a solid-phase binding assay in which the extracellular region of MuSK, fused to Fc, was attached to a multi-well plate. In the absence of Agrin, full-length AP-ecto-Lrp4 bound specifically but weakly to MuSK (Fig. 2A). Importantly, simultaneous incubation with neural Agrin led to a selective and substantial increase in binding between full-length AP-ecto-Lrp4 and MuSK (Fig. 2A). In addition, we used a cell aggregation assay to determine whether Agrin stimulated association between Lrp4 and MuSK (3). Previously, we showed that Lrp4-expressing cells self-associate and coaggregate with MuSK-expressing cells, demonstrating that Lrp4 and MuSK interact (3). Fig. 2B shows that the extent of aggregation between Lrp4- and MuSK-expressing cells was substantially increased by treatment with neural Agrin (Fig. 2B). Thus, Agrin increases association between Lrp4 and MuSK, suggesting that MuSK activation is achieved, at least in part, by an increase in association with Lrp4 (see below and “Discussion”).
MuSK Activation by Lrp4

To identify the sequences in Lrp4 that are required for association with MuSK, we deleted LDLα or β-propeller domains from Lrp4 and measured the ability of the truncated forms of Lrp4 to bind MuSK following Agrin stimulation. Fig. 3 shows that the first three LDLα domains and the last LDLα domain in Lrp4 are dispensable for binding between Lrp4 and MuSK. Indeed, deletion of the fourth LDLα repeat as well as the third β-propeller domain, which are not required to bind Agrin (Fig. 2), are critical for Lrp4 to bind MuSK.

A Solvent-exposed Region of the Ig1 Domain in MuSK Is Required for Interaction with Lrp4—MuSK contains three Ig-like domains and a single frizzled-like, cysteine-rich domain in the extracellular region (26–29). Previous studies established that the first Ig-like domain in MuSK is required for Agrin to stimulate MuSK phosphorylation (30). The crystal structure of Ig-like domains 1 and 2 of the MuSK extracellular region reveals that the first Ig-like domain forms a homodimer mediated by a hydrophobic face, including Leu-83 (28). Mutation of Leu-83 prevents Agrin-stimulated MuSK activation, consistent with the idea that formation of a MuSK dimer is necessary for MuSK activation (28).

Another region in the first Ig-like domain in MuSK, which is opposite to the hydrophobic face, resembles the NGF-binding region in TrkA, a related receptor tyrosine kinase (28, 31, 32). Specifically, the first Ig-like domain in MuSK and the second Ig-like domain in TrkA, which binds NGF, contain an additional disulfide bond that is absent from nearly all other Ig-like domains (28). Thus, we sought to determine whether this solvent-exposed region in the first Ig-like domain of MuSK was required to associate with Lrp4. To this end, we mutated Ile-96, a solvent-exposed hydrophobic residue adjacent to the unique disulfide bond, and introduced MuSK I96A-GFP, MuSK L83R-GFP, or MuSK-GFP into BaF3 cells. We mixed the MuSK-GFP-expressing cells, which grow as solitary cells in the absence of Lrp4-expressing cells (3), with BaF3 cells expressing Lrp4-mCherry and found that cells expressing wild-type MuSK or MuSK L83R coaggregate with Lrp4-expressing cells, whereas cells expressing MuSK I96A aggregate poorly with Lrp4-expressing cells. The mean ± S.E. (n = 4) are indicated. C, we used a solid-phase binding assay to measure binding between AP-ecto-Lrp4 and Fc, Fc-MuSK, or Fc-MuSK I96A in the presence of neural Agrin B8. Mutation of Ile-96 in MuSK severely impairs binding to AP-ecto-Lrp4. The mean ± S.E. (n = 3) are indicated.

FIGURE 3. Binding of MuSK to Lrp4 requires multiple LDLα repeats and β-propeller domains in Lrp4. We used a solid-phase binding assay to measure binding of AP-ecto-Lrp4 and Fc or MuSK-Fc (M) in the presence of neural Agrin B8. Deletion of β-propeller domain 4 in Lrp4 increased binding between Lrp4 and MuSK, suggesting a role for β-propeller domain 4 in restraining association between Lrp4 and MuSK. β-propeller domain 3 is essential for binding between Lrp4 and MuSK. LDLα repeats 4 and 5 contribute to binding between Lrp4 and MuSK. Binding between ecto-Lrp4 and MuSK was assigned a value of 100%, and all other values are expressed relative to ecto-Lrp4. The mean ± S.E. (n = 3) are indicated.
MuSK Activation by Lrp4

FIGURE 5. The extracellular region of Lrp4 is sufficient to stimulate MuSK phosphorylation in myotubes. Addition of the soluble, extracellular region of Lrp4 to Ipr4* mutant myotubes restores Agrin-stimulated MuSK phosphorylation. Deletion of the fourth β-propeller domain in Lrp4, which increased binding between Agrin and Lrp4 and enhanced Agrin-dependent association between Lrp4 and MuSK, reduced Agrin-stimulated MuSK phosphorylation by 2-fold (p = 0.055). Additional deletion of the third β-propeller domain in Lrp4, which abolishes binding between Lrp4 and MuSK, prevents Agrin-stimulated MuSK phosphorylation. IP, immunoprecipitation.

FIGURE 6. Domains in Lrp4 required for binding to Agrin and MuSK. LDLa repeats 6–8, the first β-propeller domain, and the two intervening EGF-like domains in Lrp4 are sufficient to bind neural Agrin. LDLa repeats 4–8, β-propeller domains 1–3, and the two intervening EGF-like domains in Lrp4 are sufficient to bind MuSK.

independently of Agrin (3, 4, 31), these data suggest that Lrp4 acts as a ligand for MuSK.

The Extracellular Region of Lrp4 Is Sufficient for Lrp4 to Activate MuSK—The extracellular region of Lrp4 binds both Agrin and MuSK. To determine whether the extracellular region of Lrp4, devoid of the transmembrane and intracellular regions, is sufficient for Agrin to stimulate MuSK phosphorylation in muscle, we added soluble AP-ecto-Lrp4, together with Agrin, to Ipr4* mutant myotubes and measured MuSK phosphorylation (Fig. 5). Lrp4 mutant myotubes express MuSK, but Agrin fails to stimulate MuSK phosphorylation (3). Soluble, AP-ecto-Lrp4 restored Agrin responsiveness to Ipr4* mutant myotubes, demonstrating Agrin responsiveness to Ipr4* mutant myotubes, demonstrating the formation of an Agrin-dependent signaling complex, mediated by the extracellular region of Lrp4, on the myotube surface (Fig. 5). Deletion of the fourth β-propeller domain, which increased association between Lrp4 and MuSK (Fig. 3), reduced Agrin-stimulated MuSK phosphorylation by 2-fold (Fig. 5), suggesting that the fourth β-propeller domain contributes to MuSK activation. Further removal of the third β-propeller domain, which prevented binding between Lrp4 and MuSK, blocked Agrin-stimulated MuSK phosphorylation. Together, these findings demonstrate that a soluble form of Lrp4, once activated by neural Agrin, can form a complex with MuSK on the myotube cell surface and stimulate MuSK kinase activity.

DISCUSSION

Our studies reveal that Agrin stimulates association between Lrp4 and MuSK and suggest a model for MuSK activation following Agrin binding to Lrp4. In this model, Lrp4 is in dynamic equilibrium between a “closed” configuration, which is not capable of binding and activating MuSK, and an “open” configuration, which binds MuSK. In the absence of Agrin, Lrp4 has a low probability of adopting the open conformation. This open conformation is not strictly dependent upon Agrin, however, as Lrp4 can activate MuSK in the absence of Agrin, and muscle prepatterning depends upon Lrp4 and MuSK but not Agrin. Agrin, however, shifts the equilibrium to favor the open configuration, promoting binding between Lrp4 and the first Ig-like domain in MuSK, which may reconfigure MuSK and orient the hydrophobic faces of the Ig1-like domain in a favorable arrangement to form MuSK dimers and facilitate trans-phosphorylation of the kinase domains. Structural studies will be required to learn how Agrin-binding reconfigures Lrp4, exposes the MuSK-binding region in Lrp4, and alters the arrangement of MuSK (Fig. 6).

Deletion of the fourth β-propeller domain in Lrp4 increased association between Agrin and Lrp4, which likely underlies the increase in Agrin-dependent association between Lrp4 and MuSK. These findings suggest that the fourth β-propeller domain normally restrains binding between Agrin and Lrp4. Deletion of the fourth β-propeller domain in Lrp4, however, reduced Agrin-stimulated MuSK phosphorylation. These findings raise the possibility that the fourth β-propeller domain becomes rearranged following Agrin-binding and facilitates MuSK activation.

We show that the extracellular region of Lrp4 is sufficient to restore Agrin-dependent MuSK phosphorylation in Ipr4* mutant myotubes. These experiments not only show that an Agrin-Lrp4-MuSK signaling complex can form on the extracellular cell surface of myotubes but demonstrate that the intracellular region of Lrp4 is dispensable for activating MuSK. In this respect and in this context, Lrp4 acts distinctly from LDLR and other Lrps, such as ApoER2, very low density lipoprotein receptor (VLDLR), or Lrp5/6, which require their intracellular domains to function in signal transduction (33–35).

The LDLa repeats in the LDLR are necessary and sufficient to bind ApoB and ApoE, apolipoproteins that promote cholesterol uptake (36). The requirements for binding these two ligands are distinct, however, because the fourth and fifth LDLa
repeats in the LDLR are necessary and sufficient to bind ApoE, whereas all seven LDLa repeats in the LDLR participate in binding ApoB (36). In this respect, Agrin binding to Lrp4 closely resembles ApoE binding to the LDLR because only a subset of LDLa repeats in Lrp4, including the sixth LDLa repeat in Lrp4, the equivalent of the fifth LDLa repeat in the LDLR, is essential for Agrin binding. Nonetheless, unlike LDLR, which requires only the LDLa repeats for ligand-binding, or Lrp5/6, which also binds Wnt proteins, Lrp4 requires a subset of the LDLa repeats as well as the first β-propeller domain to bind Wnt proteins. Lrp4 appears to bind Agrin in a manner resembling Agrin or MuSK and induces conformational changes in Lrp4 that allow it to bind additional partners in these tissues. Moreover, because LDLRs, EGF-like, and β-propeller domains are present in all Lrp family members, the autoinhibitory and activation mechanisms described here may be shared with other family members.

Previous studies demonstrated that the first Ig-like domain in MuSK is necessary for Agrin to stimulate MuSK phosphorylation (28, 30). One face of this Ig-like domain is hydrophobic and mediates the formation of MusK dimers, providing an explanation for the importance of the first Ig-like domain in MuSK activation (28). The experiments described here demonstrate that another solvent-exposed face of this same Ig-like domain is required to bind Lrp4, illustrating a second function for this Ig-like domain. Because a similar region in the second Ig-like domain of TrkA binds NGF (31) and because Lrp4 binds the Ig-like domain of TrkA, it is likely that Lrp4 may reconfigure the architecture of Lrp4 and expose the MusK-binding region on Lrp4.

Conventional receptor tyrosine kinases bind directly to ligands that are presented by adjacent cells in a paracrine manner. MusK, however, binds a cis-acting ligand, Lrp4, and the competence of Lrp4 to bind MusK is regulated by Agrin, acting in a paracrine manner. This relay mechanism, which combines paracrine and autocrine signaling, may represent a novel means for controlling the formation of a signaling complex or might contribute to the formation of other receptor complexes. For example, although Wnts bind independently to Lrp5/6 and Frizzled, bridging the two receptors, Wnt-binding could also reconfigure Lrp5/6 or Frizzled, stimulate their direct association and further stabilize the signaling complex. In the absence of ligand, typical receptor tyrosine kinases, such as the EGF receptor, are autoinhibited. Ligand binding induces a conformational change that facilitates receptor dimerization and trans-phosphorylation in the cytoplasmic domains (37). In this sense, MusK activation resembles the EGF receptor, as Agrin-binding induces a conformational change in Lrp4 that stimulates association with MusK. A similar stepwise paradigm may control the activation of Ret and other kinases that are activated by ligands that bind to a separate subunit.

Mathematical modeling suggests that gial cell line-derived neurotrophic factor initiates a stepwise assembly of GFRα and Ret (38), but the domains in GFRα and Ret that mediate assembly of a signaling complex and how assembly is regulated by GDNF are poorly understood. Lrp4 is widely expressed in the mouse embryo and required not only for neuromuscular synapse formation but also for digit formation and the development of multiple organs, including lung, kidney, and bone. Hypomorphic mutations in human lrp4, which reduce Lrp4 expression, are responsible for Cenani Lenz syndrome, typified by defects in distal limb development and abnormal kidney differentiation (39). The ligands for Lrp4 in other tissues have not been well defined, but Lrp4 binds Dickkopf, Sclerostin, and Wnt modulator in surface ectoderm (WISE), modulators of Wnt and bone morphogenetic protein (BMP) signaling (40, 41), and genetic evidence supports the idea that Lrp4 can regulate Wnt and BMP signaling pathways (42). Although Lrp4 may simply sequester these regulators of Wnt and BMP signaling (41), these and other ligands may bind Lrp4 in a manner resembling Agrin or MuSK and induce conformational changes in Lrp4 that allow it to bind additional partners in these tissues. Moreover, because LDLRs, EGF-like, and β-propeller domains are present in all Lrp family members, the autoinhibitory and activation mechanisms described here may be shared with other family members.

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