Identification of a Switch in Neurotrophin Signaling by Selective Tyrosine Phosphorylation*

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Neurotrophins, such as nerve growth factor and brain-derived neurotrophic factor, activate Trk receptor tyrosine kinases through receptor dimerization at the cell surface followed by autophosphorylation and recruitment of intracellular signaling molecules. The intracellular pathways used by neurotrophins share many common protein substrates that are used by other receptor tyrosine kinases (RTK), such as Shc, Grb2, FRS2, and phospholipase C-γ. Here we describe a novel RTK mechanism that involves a 220-kilodalton membrane tetraspanning protein, ARMS/Kidins220, which is rapidly tyrosine phosphorylated in primary neurons after neurotrophin treatment. ARMS/Kidins220 undergoes multiple tyrosine phosphorylation events and also serine phosphorylation by protein kinase D. We have identified a single tyrosine (Tyr1096) phosphorylation event in ARMS/Kidins220 that plays a critical role in neurotrophin signaling. A reassembled complex of ARMS/Kidins220 and CrkL, an upstream component of the C3G-Rap1-MAP kinase cascade, is SH3-dependent. However, Tyr1096 phosphorylation enables ARMS/Kidins220 to recruit CrkL through its SH2 domain, thereby freeing the CrkL SH3 domain to engage C3G for MAP kinase activation in a neurotrophin dependent manner. Accordingly, mutation of Tyr1096 abolished CrkL interaction and sustained MAPK kinase activity, a response that is not normally observed in other RTKs. Therefore, Trk receptor signaling involves an inducible switch mechanism through an unconventional substrate that distinguishes neurotrophin action from other growth factor receptors.

Neurotrophins are a family of growth factors that exert their action through the Trk receptor tyrosine kinases and the p75 neurotrophin receptor (p75NTR). All neurotrophins bind to p75NTR, but nerve growth factor (NGF) binds specifically to TrkA, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) bind to TrkB, and neurotrophin-3 (NT-3) binds preferentially to TrkC (1, 2). Signaling mediated by neurotrophins produces several effects in the nervous system including survival of peripheral neurons during embryonic development and promotion of axonal growth and branching as well as modulation of synaptic activity (1–3).

Like other receptor tyrosine kinases, Trk receptors are activated after ligand-induced dimerization. Upon neurotrophin binding, activated Trk receptors provide several docking sites for different adaptor proteins and enzymes. Two tyrosine residues, Tyr490 and Tyr785, are principally responsible for triggering the majority of the different intracellular signaling pathways for neurotrophins. Phosphorylation of Tyr490 site is responsible for initiating MAPK and phosphatidylinositol 3-kinase/Akt activities, whereas the Tyr785 site engages phospholipase C-γ and protein kinase C activities (1, 2). These activities are promoted by a set of adaptor proteins, such as Shc, Grb2, Gab1, and FRS2. Two of these proteins, Shc and FRS2, interact directly with phosphorylated tyrosine residue Tyr490 (4, 5).

However, several reports suggest that the Shc-binding Tyr490 site does not fully account for the survival and differentiation activities associated with neurotrophin action. Mice harboring Y490F mutations in TrkB and TrkC receptors display only minimal neurotrophin-related deficits (6, 7), in contrast to mice lacking Trk tyrosine kinase activity entirely (8–10), which display significant losses of peripheral neurons during development. Hence, there are other Tyr490-independent mechanisms that also account for the effects of neurotrophins.

One recently reported Trk-associated molecule, the ankyrin-rich membrane-spanning (ARMS/Kidins220) protein (referred hereafter as ARMS), represents a prime candidate for a specific target of Trk receptor tyrosine phosphorylation (11). ARMS is a transmembrane protein highly expressed in many Trk receptor neuronal populations. The ARMS protein is closely associated with Trk receptors through its transmembrane domains but does not interact with the epidermal growth factor receptor (12). ARMS is a large protein that contains a SAM domain, multiple potential phosphorylation sites (>40), and a PDZ-binding motif at the C terminus. It does not contain classical domains of adaptor proteins, such as SH2, SH3, or pleckstrin homology (PH) domains. Recently, ARMS has been shown to be phosphorylated after neurotrophin treatment in PC12 cells and cortical neurons, leading to a prolonged MAP kinase response (12). It also serves as a substrate for protein kinase D (13).

Here we demonstrate a new mechanism for receptor tyrosine kinases that involves the phosphorylation of ARMS at a single tyrosine residue to produce a switch between short term and long term signaling. Mutation of this amino acid impairs prolonged MAPK activation and differentiation of PC12 cells in response to NGF treatment. Our data support a model in which a constitutive association of Trk receptors, ARMS and CrkL, is altered by a switch in the motif recognition of adaptor molecules after neurotrophin treatment. The phosphorylation of a specific tyrosine residue in ARMS allows the adaptor protein CrkL to engage C3G and activate Rap1. These results provide a new mechanism that goes beyond established models of neurotrophin signaling (1, 2, 14, 15).
and indicate that Trk receptor tyrosine phosphorylation is capable of altering its signaling potential through a non-conventional downstream substrate.

**EXPERIMENTAL PROCEDURES**

Subcellular Fractionation—Subcellular fractionation was performed as described previously (16), using iodoxanol density gradient centrifugation. Cortical neurons (DIV11) were homogenized using a Dounce homogenizer in buffer H (250 mM sucrose, 20 mM Tricine-NaOH, pH 7.8, 1 mM EDTA, 2 mM MgCl₂, with protease and phosphatase inhibitors). Membrane fractions (P2 and P3) were prepared by sequential centrifugation (800, 16,000, and 200,000 × g). P2 was then adjusted to 25% iodoxanol (OptiPrep; Accurate, Westbury, NY) and overlaid with 20, 15, 10, and 5% iodoxanol in buffer H. Gradients were centrifuged either in a SW40Ti rotor (Beckman, Fullerton, CA) at 27,000 rpm for 18 h or in a TLS55 rotor (Beckman) at 38,000 rpm for 5 h at 4 °C. After gradient centrifugation, membrane fractions were collected, and equal volumes were analyzed by SDS-PAGE and immunoblotting with different antibodies.

Preparation of GST Fusion Proteins and in Vitro Binding Assays—For the production of tyrosine-phosphorylated recombinant proteins, TKX1 bacteria (Stratagene) were used. We followed the instructions recommended by the manufacturer. Briefly, expression of recombinant proteins in TKX1 bacteria was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37 °C. After collecting the cells the expression of the tyrosine kinase gene was induced in tryptophan starvation media containing indoleacrylic acid, and 2 h later bacteria were harvested, and recombinant tyrosine-phosphorylated GST fusion proteins were purified. In vitro binding experiments were performed as described previously (12).

In Vitro Protein Translation—The wild type, R39N and W160L CrkL constructs, were in vitro translated and [³⁵S]-labeled with the TNT-coupled reticulocyte lysate system using pDual GC vector (Promega, Madison, WI). Following translation, [³⁵S]-labeled CrkL proteins were incubated with HEK293 cell extracts in 1% Nonidet P-40 lysis buffer at 4 °C and then extensively with Tris-buffered saline containing 0.1% Tween 20, and antibodies were then eluted with 0.1 M glycine, pH 2.5. Fractions were collected from the eluate, neutralized with Tris buffer, pH 9.5, and then tested for specificity by blotting ARMS immunoprecipitates from neurotrophin treated cells.

**RESULTS**

**ARMS Is Tyrosine-phosphorylated at Tyr¹⁰⁹⁶ in Response to Neurotrophins**—ARMS is phosphorylated on many tyrosine residues in response to neurotrophins (11, 12). However, no specific tyrosine residues have been mapped in the ARMS protein. We therefore scanned the amino acid sequence of ARMS to search for consensus tyrosine motifs. One sequence, Y₁₀⁹₆SQP, was found in the polyproline-rich region of ARMS that conforms to the consensus sequence previously described for binding to the SH2 domain of CrkL (for a review see (19)). CrkL was previously found to interact through its SH3 domain with a polyproline-containing peptide (20). We developed phosphoantibodies against a peptide carrying this phosphorylated residue. PC12 cells and cortical neurons were stimulated with NGF and BDNF, respectively. ARMS protein was immunoprecipitated with an anti-ARMS serum and subjected to Western blots with the purified phospho-ARMS antibody. An increase in the level of phosphorylated ARMS protein was observed upon NGF treatment in PC12 cells (2.6-fold) and BDNF treatment of cortical neurons (3.1-fold) (Fig. 1A). This result indicated that the Tyr¹⁰⁹₆ residue in ARMS was phosphorylated upon neurotrophin treatment.

To confirm the specificity of the phosphoantibody, we transfected HEK293 cells with wild type ARMS and ARMSY¹⁰⁹₆F together with TrkA. TrkA activation resulted in a stronger signal detected by the phospho-ARMS antibody of wild type ARMS compared with ARMSY¹⁰⁹₆F (Fig. 1B). However, reprobing with a general p-Tyr anti-

FIGURE 1. ARMS is tyrosine-phosphorylated at Tyr¹⁰⁹₆ in response to neurotrophins. A. PC12 cells and primary cortical neurons were stimulated with NGF (100 ng/ml) or BDNF (50 ng/ml), respectively, for 5 min. After ARMS immunoprecipitation Western blotting was performed with specific phospho-ARMS antibodies, and the membrane was re-probed with 4G10, an anti-phosphotyrosine antibody, and with 892 ARMS antibody. Quantification was performed with ImageJ software (National Institutes of Health) and the data normalized to signal in the non-stimulated condition. A representative experiment is shown. B. HEK293 cells were transfected with TrkA and FLAG-ARMS or FLAG-ARMSY¹⁰⁹₆F and stimulated with NGF (50 ng/ml) for 5 min, and extracts were immunoprecipitated with FLAG antibodies. Western blotting was performed with p-ARMS antibodies as described for A.
body revealed a smaller decrease in the total tyrosine phosphorylation in ARMSY1096F. These results indicated that the phospho-ARMS antibody displays enhanced specificity for recognizing phosphorylated Tyr1096.

Tyrosine-phosphorylated ARMS Can Bind to SH2 Domain of CrkL—To assess the functional consequences of the sequence Y1096SQP in ARMS, we produced recombinant tyrosine-phosphorylated GST fusion proteins carrying ARMS sequences (Fig. 2A). The recombinant proteins were phosphorylated on tyrosine using the bacterial strain TKX1, which contains an inducible tyrosine kinase activity (see “Experimental Procedures”). The proteins were purified and used to carry out protein interaction assays. The GST fusion proteins were first analyzed for their ability to bind to CrkL, a known binding partner of ARMS (12). The tyrosine-phosphorylated GST-ARMS7.2 protein bound more CrkL protein compared with the non-phosphorylated protein (Fig. 2B, compare lanes 3 and 4). To distinguish between the binding of GST-ARMS7.2 to the SH2 and the SH3 domains of CrkL, a deletion of the polyproline region was analyzed (GST-ARMS7.2ΔP). Interestingly, an association between CrkL and tyrosine-phosphorylated GST-ARMS7.2ΔP deletion protein was observed, whereas the interaction did not occur when the GST-ARMS7.2ΔP was not tyrosine-phosphorylated (Fig. 2B, lanes 5 and 6). This implies that ARMS can bind to the SH2 domain of CrkL through a phosphotyrosine residue of ARMS.

To investigate this possibility further, the Tyr1096 was mutated to phenylalanine. The interaction between the GST-ARMS proteins and CrkL was abolished (Fig. 2B, lanes 7–10). Binding to CrkL was not detected with the tyrosine-phosphorylated proteins GST-ARMS7.3 and GST-ARMS7.6 that do not contain the Tyr1096 residue, verifying the specificity for this tyrosine (Fig. 2B, lanes 12 and 14). Surprisingly, the interaction between non-tyrosine phosphorylated ARMS7.2 and CrkL was also disrupted by mutation of Tyr1096 (Fig. 2B, lane 7). These results suggest that constitutive binding of the SH3 domain of CrkL to ARMS may also require this tyrosine residue for proper association. The involvement of flanking amino acids of the polyproline consensus motif

FIGURE 2. Tyrosine-phosphorylated ARMS interacts with CrkL SH2 domain. A, the polyproline-rich region of ARMS contains a consensus sequence, YXXP (bold), for binding to the SH2 domain of CrkL (19). B, GST-ARMS fusion proteins non- or tyrosine-phosphorylated were produced in bacteria, incubated with HEK293 cell extracts, and subjected to Western blotting (WB) analysis with anti-CrkL (top panel) and anti-phosphotyrosine (middle panel). A Coomassie-stained gel of the input GST fusion proteins is shown (bottom panel). C, CrkL SH2 domain is responsible for binding to tyrosine-phosphorylated ARMS. Wild type CrkL, SH2 mutant (R39N), and SH3 mutant (W160L) were in vitro translated and [35S]Met-labeled and incubated with the tyrosine-phosphorylated and non-phosphorylated GST-ARMS fusion proteins.
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in protein interactions has been previously reported (for a review, see Ref. 19). Interactions between ARMS and CrkII were not detected using recombinant proteins (data not shown). These findings indicate that the binding of ARMS to CrkL can be accomplished by the phosphorylation of ARMS specifically at Tyr1096.

To confirm the participation of SH2 domain of CrkL in ARMS-CrkL interaction [35S]methionine CrkL proteins were synthesized and tested for association with ARMS. In vitro translated wild type CrkL interacted with GST-ARMS fusion proteins (Fig. 2C, top panel). A SH2 mutant CrkL protein, CrkLR39N, that abolished the interaction with previous known interactors as Cbl or paxillin, (20), was not capable of binding to tyrosine-phosphorylated GST-ARMS fusions (Fig. 2C, middle panel). On the other hand, an SH3 CrkL mutant, CrkLW160L, that disrupted the binding of Abl, Sos, or C3G (20), did not display any constitutive association but maintained the interaction with phosphorylated GST-ARMS proteins (Fig. 2C, bottom panel). As a control, binding of GST-ARMS7.2DPY1096 did not display any binding to the CrkL mutant proteins. These data support an interaction of the ARMS and CrkL proteins mediated by the phosphorylation of ARMS at residue Tyr1096 and the SH2 domain of CrkL.

Trk Receptors, ARMS, CrkL, C3G, and Rap1, Are Present in Endosomal Membranes—To study the association of ARMS, CrkL, and Trk receptors further, we utilized Optiprep gradient centrifugation of membranes isolated from primary cortical neurons. This will better resemble the in vivo situation as compared with using cell lines. The Optiprep gradient effectively separates intracellular membrane compartments, including organella, vesicles, and endosomal membranes (16). Membrane fractions were prepared by sequential centrifugation and then adjusted to 25% iodixanol. The P2 pellet was subjected to discontinuous Optiprep gradient centrifugation (Fig. 3A). Fractions from each interface were then subjected to SDS-PAGE and immunoblotted for Trk and Trk-related proteins.

After centrifugation, ARMS, CrkL, C3G, and Rap1 were present in the same fractions as EEA1, an endosomal marker (Fig. 3B). Co-localization of ARMS and EEA1 using indirect immunofluorescence (Fig. 3A) also confirms these results. ARMS, CrkL, C3G, and Rap1 were not found in the endoplasmic reticulum or Golgi membranes, as assessed by ribophorin and GM130 co-migration, respectively (Fig. 3B). Trk receptors were found predominantly in endosomal membranes but displayed a broader distribution (Fig. 3B). Treatment with BDNF of these primary neurons resulted in a slight shift of Trk receptors to heavier membranes.
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without affecting the mobility of the other proteins in these fractions (Fig. 3B). Similar results have been observed in the localization of TrkA and ARMS in PC12–615 cells (21). These data suggest that ARMS, CrkL, C3G, and Rap1 reside in similar endosomal membrane compartments. Endosomes serve as a meeting point for the formation of signaling complexes for a wide variety of ligands and their receptors, including transforming growth factor-β (22) and epidermal growth factor (23). This would be consistent with experiments indicating that Trk and ARMS exist in a complex previous to its activation (12, 24) in “signaling endosomes” (25).

Another specialized membrane compartments implicated in cell signaling are lipid rafts (for a review, see Ref. 26). Previously, it has been reported that Trk receptors are recruited to lipid rafts in response to neurotrophins in cortical neurons (27). To address whether ARMS and CrkL are present in these specialized membranes we isolated lipid rafts from primary cortical neurons in presence or absence of BDNF treatment using Triton X-100 extraction and centrifugation in an Optiprep gradient. ARMS and TrkB are slightly enriched in the lipid raft fractions. CrkL levels in that fraction are very low, and we do not find an enrichment of this protein in lipid rafts as compared with fractions 3–6, as it is clear for TrkB and ARMS. BDNF consistently induced the translocation of a small fraction of TrkB receptors to lipid rafts without changing the location of ARMS and CrkL. (Fig. 4B). Fyn and transferrin receptor were used as positive and negative controls, respectively, for lipid raft association (Fig. 4B).

ARMSY1096F Impairs Prolonged MAPK Activation and Differentiation in PC12 Cells—The biological effects of trophic factors require that signals are conveyed over long distances from the nerve terminal to the cell body. It has been proposed that signaling endosomes containing the receptor and associated proteins are delivered to the cell body, where signals are transduced to ensure trophic effects and differentiation (25).

To address whether phosphorylation of ARMS at Tyr1096 is directly involved in neurotrophin signaling, we assessed the ability of NGF to signal through the Trk receptor. A well established response is the induction of MAP kinase activity. Therefore, PC12 cells were transiently transfected with GFP, GFP-ARMS, and GFP-ARMSY1096F, and the activation of MAP kinase was followed in response to NGF. Our results indicated that the late phase activation of MAP kinase (40 min treatment) was impaired with the GFP-ARMSY1096F mutant compared with the wild type ARMS or GFP alone (Fig. 5B). This can be readily observed when comparing the p-MAPK staining at 5 versus 40 min of NGF treatment (Fig. 5A). No effects were observed in transient MAPK activation at 5 min after NGF treatment (Fig. 5B).

We also noticed a detectable decrease in the number of PC12 cells transfected with GFP-ARMSY1096F that underwent NGF-dependent differentiation, in comparison with GFP and GFP-ARMS cells (Fig. 6A). A 40% decrease in the number of differentiated cells was observed (Fig. 6B). These data support the involvement of ARMS tyrosine phosphorylation at Tyr1096 residue in sustained MAPK activation and in the differentiation of PC12 cells elicited by neurotrophins.

It was previously reported that C3G binding to CrkL increased with neurotrophin treatment (12). To test whether the Tyr1096 residue in ARMS was required for this association, we performed in vitro binding studies with GST-ARMS fusion proteins. In contrast to GST-wild type ARMS protein (7.2), a decrease in binding of C3G to CrkL was observed with GST-ARMSY1096F fusion protein (Fig. 7A). Quantitation of the levels of C3G indicated that there was 112-fold more binding in the presence of wild type ARMS versus ARMSY1096F. As well, the ΔP deletion of ARMS bound 20-fold better than the ΔPY1096F mutation. These
results verify that the Tyr\textsuperscript{1096} residue of ARMS is important for the association of C3G to CrkL.

**DISCUSSION**

Neurotrophin signal transduction through Trk receptor tyrosine kinases is well understood (1, 2, 15), in part from the similarity between other receptor tyrosine kinases to Trk receptors. A common set of cytoplasmic proteins is generally recruited to receptor tyrosine kinases. At the heart of Trk receptor function is the activation of phospholipase C-γ, MAP kinase and phosphatidylinositol 3-kinase activities, which are utilized by nearly all members of the RTK family.

Here we describe an unusual protein substrate for neurotrophin receptors, the ARMS protein, which is a very large protein with four closely spaced transmembrane domains. To our knowledge, this type of protein structure has not been observed in other tyrosine kinase substrates. Another analogous substrate, LAT (linker for activation of T cells) protein, is a single transmembrane protein that is utilized by the T cell receptor in mediating immune responses (28). Among over forty potential phosphorylation sites, we have identified a key tyrosine phosphorylation event in ARMS protein that serves to recruit CrkL through its SH2 domain. In vitro binding assays established that this interaction is of a higher affinity than the polyproline sequence for the SH3 domain of CrkL (Fig. 2). Disruption of this phosphorylation event impairs the prolonged MAPK activation and the differentiation of PC12 cells in response to neurotrophins (Figs. 5 and 6).

What is the significance of two separate binding interactions between ARMS and CrkL? This is an intriguing question, since the Tyr\textsuperscript{1096} is within two amino acids of the polyproline sequence P\textsuperscript{1089}PRPP in ARMS used to bind the CrkL SH3 domain. A model can be proposed in which a constitutive association of Trk-ARMS-CrkL occurs through the polyproline sequence in ARMS and SH3 domain of CrkL. Activation of Trk receptors by neurotrophins induces the phosphorylation of the

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**FIGURE 7.** CrkL/C3G association with GST-ARMS fusion proteins and proposed model. A, C3G is pulled down together with CrkL when ARMS is tyrosine-phosphorylated at Y1096F. Tyrosine-phosphorylated GST-ARMS fusion proteins were incubated with HEK293 cell extracts as described in the legend to Fig. 2B. C3G (top panel) and CrkL (middle panel) Western blots were performed to assess the association with ARMS proteins. A Coomassie-stained gel of the input GST fusion proteins is shown (bottom panel). B, model of Trk-ARMS-CrkL signaling upon neurotrophin treatment.
Tyr1096 residue in ARMS that binds to the SH2 domain of CrkL. This switch in the binding requirement would allow for the release of the SH3 domain of CrkL to recruit C3G and trigger the activation of MAPK (Fig. 7). Thus, the spatial fidelity of signal transduction may be maintained by a constitutive interaction of ARMS and CrkL (SH3-dependent) while allowing for an NGF-inducible cascade of MAP kinase activation, once ARMS becomes tyrosine-phosphorylated (SH2-dependent).

Support for this model comes from evidence that C3G recruitment is enhanced to the Trk-ARMS-CrkL complex upon NGF-induced phosphorylation (12). A preformed Trk-ARMS complex has also been consistently observed in co-immunoprecipitation experiments (11, 12, 24). Previous studies indicated that conformational changes occur intramolecularly in Crk proteins that modify the binding specificities of Crk to other signaling proteins (29–31). An implicit prediction is that tyrosine-phosphorylated ARMS would facilitate CrkL to use its SH3 domain for recruitment of C3G or other adaptor proteins. Other adaptor proteins include c-Abl, DOCK2, IRS-4, and STAT5 (19). Therefore, regulation of neurotrophin signaling could be dependent upon the specific phosphorylation status of ARMS and the expression of other signaling proteins.

In this study, the Tyr1096 residue was found to be critical for the activation of a key signaling pathway, MAP kinase. Indeed, disruption of Tyr1096 phosphorylation in ARMS abolished CrkL interaction (Fig. 2) and impaired prolonged MAPK activation and differentiation of PC12 cells (Fig. 3 and 6). The switch in domain requirements for ARMS-CrkL is directly influenced by the binding affinity and phosphorylation status of ARMS and CrkL (Fig. 2, B and C).

Another key parameter in neurotrophin signaling is the intracellular localization of Trk receptors and their effectors. Activation of TrkA in PC12 cells leads to its internalization and presence in early endosome compartments (32). It has been demonstrated that activated Trk receptors can signal during the retrograde transport after internalization (33–35) and in intracellular organelles such as the Golgi (17). We have detected the ARMS, CrkL, C3G, and Rap1 proteins in the same membrane compartments as Trk receptors with EEA1, a marker for early endosomes (Fig. 3B). The co-localization of ARMS with EEA1 (Fig. 4A), consistent with the localization of TrkA in endosomal compartments (32). Consistent with a specialized location, ARMS has been also been found associated in lipid raft compartments in neurons (36), where TrkB receptors have been found to be recruited in response to neurotrophins (27). Lipid rafts are specialized microdomains in membranes rich in low density lipids, such as cholesterol and sphingolipids, implicated in many signaling processes at the cell surface (for a review, see Ref. 26). It is tempting to speculate that the presence of ARMS in lipid rafts has a role in the neurotrophin signaling after recruitment of Trk receptors to these microdomains.

The ARMS protein is a member of an atypical family of P-loop NTPases, KAP (Kidins220/ARMS and PifA), with a predicted function as NTP-dependent regulator of the assembly of membrane-associated signaling complexes (37). It should be noted that ARMS can serve as a substrate for ephrin-induced tyrosine phosphorylation (11). In fact, recent experiments indicate that ARMS/Kidins220 interact with alpha-syntrophin and modulate ephrin-mediated signaling (38). The function of ARMS in signal transduction has yet to be fully understood, but it is likely to play a fundamental role in the response to extracellular cues.