ABSTRACT

Myosin X (MyoX) is an unconventional myosin that is known to induce the formation and elongation of filopodia in many cell types. MyoX-induced filopodial induction requires the three PH domains in its tail region, although with unknown underlying molecular mechanisms. MyoX’s first PH domain is split into halves by its second PH domain. We show here that the PH1N-PH2-PH1C tandem allows MyoX to bind to phosphatidylinositol (3,4,5)-triphosphate [PI(3,4,5)P3] with high specificity and cooperativity. We further show that PH2 is responsible for the specificity of the PI(3,4,5)P3 interaction, whereas PH1 functions to enhance the lipid membrane–binding avidity of the tandem. The structure of the MyoX PH1N-PH2-PH1C tandem reveals that the split PH1, PH2, and the highly conserved interdomain linker sequences together form a rigid supramodule with two lipid-binding pockets positioned side by side for binding to phosphoinositide membrane bilayers with cooperativity. Finally, we demonstrate that disruption of PH2-mediated binding to PI(3,4,5)P3 abolishes MyoX’s function in inducing filopodial formation and elongation.

INTRODUCTION

Myosin X (MyoX), encoded by Myo10, is an actin-based unconventional myosin localized at the tips of filopodia (Berg et al., 2000; Berg and Cheney, 2002; Tokuo and Ikebe, 2004; Zhang et al., 2004; reviewed in Sousa and Cheney, 2005). Overexpression of MyoX in heterologous cell lines, such as HeLa and COS7, induces the formation of large numbers of elongated filopodia (Bohil et al., 2006; Tokuo et al., 2007; Kerber et al., 2009; Watanabe et al., 2010). In endothelial cells, MyoX, in response to bone morphogenetic protein (BMP)–mediated up-regulation of expression, promotes filopodial extension and is required for BMP-dependent directed cell migration (Pi et al., 2007). The functional role of MyoX in directed cell migration has also been demonstrated in vivo in the cranial neural crest cells of Xenopus embryos (Hwang et al., 2009; Nie et al., 2009). The role that MyoX plays in filopodial formation is likely to be directly linked to the motor’s function in directed cell migrations, as filopodia act as cell antenna that integrate various cellular signals to regulate actin cytoskeletal structures (Gupton and Gertler, 2007; Mattila and Lappalainen, 2008).

From its N- to C-termini, MyoX contains a motor domain, a neck region consisting of three IQ motifs, a single α-helix domain followed by a coiled-coil domain, three PH domains, a motor tail homology (MyTH4) domain, and a band 4.1/ezrin/radixin/moesin (FERM) domain (Figure 1A). The motor domain binds to bundled actin filaments and powers the processive movement of MyoX (Nagy et al., 2008; Kerber et al., 2009). A GCN4-forced dimer of MyoX containing its head, neck, and predicted coiled-coil domains (known as MyoX-HMM) is sufficient for the filopodia tip localization and intrafilopodial movement of the motor (Berg and Cheney, 2002; Kerber et al., 2009). However, substrate-attached filopodial “buds” induced by MyoX-HMM often fail to elongate, and thus the PH repeats, MyTH4, and FERM domains of the motor are also required for the formation of elongated filopodia (Bohil et al., 2006; Tokuo et al., 2007; Plantard et al., 2010). Recently the MyTH4 and FERM domains were shown to act as a structural and functional supramodule in binding to deleted in colorectal cancer (DCC), a transmembrane MyoX cargo mediating directed axonal migrations (Zhu et al., 2009; Hirano et al., 2011; Wei et al., 2011).
Enrichment of phosphatidylinositol (3,4,5)-triphosphate [P(3,4,5)P$_3$] in the leading edges is important for the dynamics of filopodium (Luikart et al., 2008; Ketschek and Gallo, 2010). MyoX contains three consecutive PH domains immediately N-terminal to its MyTH4-FERM supramodule (Figure 1A). The PH domains of the motor couple with PI3 kinase activities by directly binding to the kinase product P(3,4,5)P$_3$ during MyoX-mediated cellular processes, including phagocytosis (Cox et al., 2002) and filopodial extension (Plantard et al., 2010). The organization of the MyoX PH domains is highly unusual: the second PH domain is inserted in the predicted β3/β4 loop of PH1, splitting PH1 into halves and forming a PH1N-PH2-PH1C tandem (Berg et al., 2000; Figure 1A). The amino acid sequences of PH1 and PH2, as well as all linker sequences connecting them, are highly conserved, and the splitting site of PH1 is identical in MyoX orthologues from different species, indicating that the PH1N-PH2-PH1C tandem is likely to have specific functions in addition to the individual functions of PH1 and PH2. In vitro biochemical assays, as well as in silico amino acid sequence analysis, demonstrated that the isolated PH2 is capable of specifically binding to P(3,4,5)P$_3$ (Isakoff et al., 1998; Plantard et al., 2010; Umeki et al., 2011). Using a loss-of-function approach, it was shown that the PH2 domain is required for filopodia induction as well as phagocytosis (Cox et al., 2002; Plantard et al., 2010). It was shown recently that the PH domains are involved in the direct binding to the motor head, thus maintaining MyoX in an autoinhibited conformation. Binding of P(3,4,5)P$_3$ to the PH domains can release the autoinhibited conformation of MyoX (Umeki et al., 2011). However, neither the biochemical properties nor the functional role of the PH1N-PH2-PH1C tandem of MyoX were directly tested.

Here we demonstrate that the PH1N-PH2-PH1C tandem of MyoX specifically binds to P(3,4,5)P$_3$-containing lipid membranes. The high-resolution structure of the PH1N-PH2-PH1C tandem reveals that the two split halves of PH1 adopt an intact PH domain fold. The two PH domains form a structural supramodule with their phosphoinositide-binding pockets juxtaposed with each other for synergistic binding to lipid membranes. The structure of the PH1N-PH2-PH1C tandem, together with detailed biochemical analysis, demonstrates that PH2 provides the P(3,4,5)P$_3$-binding specificity of MyoX, whereas the split PH1 functions to enhance its lipid membrane–binding avidity via nonspecific binding to phosphoinositide. We further demonstrate that the lipid-binding properties of both PH1 and PH2 are required for MyoX to function in filopodial induction, indicating that the PH1N-PH2-PH1C tandem acts as a functional supramodule in sensing acute increases in P(3,4,5)P$_3$ concentration induced by PI3 kinase at the tip of filopodia.

**RESULTS**

The PH1N-PH2-PH1C tandem of MyoX specifically binds to P(3,4,5)P$_3$-containing liposomes with high affinity

We attempted to determine the lipid membrane–binding properties of PH1N-PH1C and PH2 individually by removing the PH2 sequence from the predicted β3/β4 loop of the split PH1, as our earlier studies of split PH domains from α-syntrophin, phospholipase C$_{7}$, Rho kinase, and PI3 kinase enhancer showed that covalently fusing two halves of a split PH domain gives rise to a well-structured canonical PH domain fold (Yan et al., 2005, 2008; Wen et al., 2006, 2008). To our disappointment, neither PH1N-PH1C nor PH2 could be purified as soluble proteins despite extensive trials using different domain boundaries, implying the two domains in the tandem may not act as separately functional PH domains. In contrast, the PH1N-PH2-PH1C tandem can be expressed as a highly soluble protein that adopts a stable...
monomeric conformation at a wide range of concentrations (up to 0.5 mM tested; Supplemental Figure S1).

The purified PH1N2-PH2-PH1C tandem robustly binds to liposomes prepared from total bovine brain lipid extracts in a cosedimentation-based lipid membrane–binding assay (Figure 1B). We tested the binding specificity of the PH1N2-PH2-PH1C tandem for different phosphatidylinositol phosphates (PIPs) using reconstituted defined liposomes, each composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and one isofrom of PIP at a ratio of 77:20:3 using the same assay. We kept the PIP concentration low (3%) in the defined liposomes to ensure assay stringency. The results demonstrate that the PH1N2-PH2-PH1C tandem has a high selectivity for PIP(3,4,5)P3 and a modest selectivity for PIP(3)P (Figure 1C). Because PIP(3)P is known to be present only in endosomes and multivesicular bodies at relatively constant levels (Lemmon, 2008), we decided not to further address its interaction with the PH1N2-PH2-PH1C tandem. Instead, we focused on the interaction between PIP(3,4,5)P3-containing liposomes and the PH1N2-PH2-PH1C tandem, as PIP kinase–mediated signaling is coupled with the functions of MyoX (Cox et al., 2002; Plantard et al., 2010; Umeki et al., 2011).

We performed a PIP(3,4,5)P3 density-dependent binding assay between the PH1N2-PH2-PH1C tandem and defined liposomes. In this experiment, the ratio of PIP(3,4,5)P3 in the PC/PS/PIP(3,4,5)P3 was increased incrementally from 0 to 7.5%, and the PC ratio was proportionally changed from 80 to 72.5%, with the PS concentration fixed at 20%. We observed a highly sigmoidal PIP(3,4,5)P3 density–dependent binding curve, with a mid PIP(3,4,5)P3 density point of ~3% (Figure 1D). Only a basal level of membrane binding could be detected when the PIP(3,4,5)P3 density was <2.5, a condition corresponding to the low-activity state of PI3 kinase. In contrast, the PH1N2-PH2-PH1C tandem is essentially saturated when PIP(3,4,5)P3 concentration in response to PI3 kinase activation (Papayannopoulos et al., 2005). Taken together, these biochemical data indicate that the PH1N2-PH2-PH1C tandem of MyoX is ideal as a sensor for cellular PI3 kinase activity.

We then performed a liposome concentration–dependent PH1N2-PH2-PH1C tandem binding assay. In this assay, we fixed the PIP concentration in the defined liposome at 3% (i.e., a PC:PS:PIP(3,4,5)P3 ratio of 77:20:3) and varied total liposome concentration. We found PIP(3,4,5)P3 concentration in the defined liposome at 3% (i.e., a PC:PS:PIP(3,4,5)P3 ratio of 77:20:3) and varied total liposome concentration. We found PIP(3,4,5)P3 concentration in response to PI3 kinase activation (Papayannopoulos et al., 2005). Taken together, these biochemical data indicate that the PH1N2-PH2-PH1C tandem of MyoX is ideal as a sensor for cellular PI3 kinase activity.

The PH1N2-PH2-PH1C tandem forms a structural supramodule

To elucidate the mechanistic basis governing the specific and cooperative binding of the PH1N2-PH2-PH1C tandem to PIP(3,4,5)P3-containing membranes, we tried to determine the atomic structure of the PH1N2-PH2-PH1C tandem using NMR spectroscopy and x-ray crystallography. The poor quality of NMR spectra of the wild-type PH1N2-PH2-PH1C indicated that the protein is not amenable to NMR-based structure determination. Despite numerous trials, we failed to obtain crystals of the wild-type PH1N2-PH2-PH1C with or without the PIP(3,4,5)P3 head group bound to the protein. However, we were able to obtain high-quality crystals of a PH1N2-PH2-PH1C mutant in which three positively charged residues (K1187 in the PH2 insert site (i.e., the segment linking PH1 and PH2, and the stretch connecting 3 of PH1 and 4 by the insertion of the PH2 domain. Like all other known split PH domains (Tao et al., 2006; Yan et al., 2005, 2008; Wen et al., 2006, 2008), the two halves of MyoX PH1 (PH1N and PH1C) interact with each other to form an intact PH domain fold.

The unique and most important feature of the PH1N2-PH2-PH1C tandem with respect to the other split PH domains is that the split PH1 domain and the inserting PH2 domain are physically integrated into a structural supramodule by two highly conserved linking sequences at the PH2 insertion site (i.e., the segment linking 3 of PH1 and 4 of PH2, and the stretch connecting 3 of PH2

| Table 1: Data collection and refinement statistics for MyoX PH12_3KNN. |

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aThe values in parentheses refer to the highest-resolution shell.
bRmerge = ΣIi − l̄/Σl̄, where Ii is the intensity of the measured reflection and l̄ is the mean intensity of all symmetry-related reflections.

Rcryst = Σ(Fobs − Fcalc)/ΣFcalc, where Fobs and Fcalc are observed and calculated structure factors, respectively.

Rfree = Σ(Fobs − Fcalc)/ΣFcalc, where T is a test data set of ~10% of the total reflections randomly chosen and set aside prior to refinement.

*Root-mean-square deviation from ideal values.
and β4 of PH1; see Figure 2, A, B, and E). The relative orientations of the two PH domains of MyoX are fixed, although the two PH domains make minimal contact with each other, with the exception of the Arg-1210–Asp-1254 salt bridge (Figure 2D; see later discussion for more details). In sharp contrast, the orientations of the split PH domains are highly flexible with respect to the inserting domains in other split PH domains (Yan et al., 2005, 2008; Wen et al., 2006, 2008).
The interface between PH1 and the linker region is primarily hydrophobic, with a total buried surface area of ~420 Å² (Figure 2C). A number of highly conserved hydrophobic residues from the first β-sheet of PH1 (β1–β4) with their side chains facing outside of the PH domain β-barrel (e.g., Phe-1180, Trp-1198, Val-1200, Leu-1207, and Phe-1209) pack with conserved Ala-1326, Pro-1328, Ala-1331, and Lys-1212 in the linker region (Figure 2, C and E). The interface between PH2 and the linker region is more extensive and involves residues from the first β-sheet of PH2 (β1’–β4’) and the α1’ helix, burying ~680 Å² of surface area through hydrophobic interactions, hydrogen bonding, and two salt bridges between Asp-1315 and Arg-1260 and between Asp-1315 and Arg-1266 (Figure 2, D and E).

Mutation of any single hydrophobic residue in the PH1/linker interface (Figure 2C) invariably leads to heavy aggregation of the PH1N-PH2-PH1C tandem mutant protein (unpublished data). We also tested the role of the salt bridge between Asp-1254 and Arg-1210 in the PH2/linker interface by substituting Asp-1254 with an Arg. Although the Asp-1254–Arg-1210 salt bridge is at the edge of the PH2/linker interface, the D1254R-PH1N-PH2-PH1C mutant was eluted with a highly broadened low-molecular weight peak and a heavy aggregated high-molecular weight peak on an analytical gel filtration column (Supplemental Figure S1), indicating that the mutant protein is conformationally heterogeneous, presumably due to the destabilized PH1/PH2 interface caused by the D1254R substitution. The foregoing biochemical data are consistent with the structure-based prediction that the highly conserved residues in the PH1/PH2 interface maintain the conformation of the PH1N-PH2-PH1C supramodule.

**PH2 is responsible for P(3,4,5)P3-binding specificity, whereas the split PH1 increases general membrane-binding avidity**

A number of PH domains—for example, PH domains from general receptors for phosphoinositides (Grp1), Bruton’s tyrosine kinase (Btk), a dual specificity for P(3,4,5)P3 and P(3,4,5)P2 to PH domains of the dual adaptor for phosphotyrosine and 3-phosphoinositides (DAPP1), and protein kinase B (PKB/Akt)—are known to bind selectively to P(3,4,5)P3 (Lemmon, 2008). The structures of these PH domains in complex with Ins(1,3,4,5)P4, the soluble head group of PI(3,4,5)P3, essentially eliminate the lipid membrane–binding capacity of the PH1N-PH2-PH1C tandem (Figure 3D), consistent with the predicted nonspecific avidity-enhancing role of the split PH1 in lipid membrane binding. In contrast, replacements of Lys-1237 and Arg-1239 of PH2 with Ala (denoted PH12_2M) essentially eliminated the lipid membrane–binding capacity of the PH1N-PH2-PH1C tandem (Figure 3D), supporting our structure-based prediction that PH2 is chiefly responsible for specific binding to P(3,4,5)P3-containing membranes. The direct and specific binding of P(3,4,5)P3 to the PH2 domain in the PH1N-PH2-PH1C tandem was further probed using an isothermal titration calorimetry (ITC)–based binding assay. We found that a water-soluble head group of phosphoinositide, Ins1(3,4,5)P4, binds to PH1N-PH2-PH1C with a KD of ~32.9 ± 4.2 μM (Figure 3G). No binding could be detected between Ins1(3,4,5)P3 and PH1N-PH2-PH1C (Figure 3G). In addition, we could not detect any binding between the Lys1237Ala/Arg1239Ala mutant of PH1N-PH2-PH1C and Ins1(3,4,5)P4 (unpublished data).

**The PH domains of MyoX are localized to P(3,4,5)P3-enriched basolateral membranes in polarized MDCK cells**

We used polarized MDCK cells as a model to assess the lipid membrane–binding properties of the MyoX PH domains under cellular conditions. It is known that P(3,4,5)P3 and P(4,5)P2 are restricted to the basolateral and apical membranes, respectively, of polarized MDCK cells (Gassama-Diagne et al., 2006). Confocal images of the MyoX PH repeats show that the wild-type PH12 is selectively targeted to basolateral membranes with no detectable apical membrane localization (Figure 4, A1 and B1). A PH12 mutant with Lys-1237 and Arg-1239 in PH2 substituted with Ala (PH12_2M) is almost completely diffused in cytosol.
(Figure 4, A3, B3, and C), indicating that the MyoX PH repeats selectively bind to P(3,4,5)P₃-containing phosphatidylinositol membranes, and PH2 in the PH123 repeats plays an essential role for the basolateral membrane localization in MDCK cells. Substitutions of Lys-1195 and Arg-1197 in the split PH1 domain with Ala weaken the membrane localization of PH123, and a significant proportion of the mutants are diffused in cytoplasm (Figure 4, A2, B2, and C). It is noted that, compared with PH123_2M, the PH123_1M mutant is still partially enriched at the basolateral membranes, consistent with the idea that the split PH1 plays an accessory role in the lipid membrane binding of the MyoX PH1N-PH2-PH1C tandem. We also tested the role of potential lipid-binding properties in PH3 in mediating basolateral localization of MyoX PH123 by substituting the two positively charged residues in the predicted second and fourth residues of the domain's B2 strand (K1417A and R1419A-, denoted PH123_3M). The PH123_3M mutant is also partially enriched at the basolateral membranes (Figure 4, A4, B4, and C), suggesting that the PH3 domain plays at most a minor role in the lipid membrane binding of MyoX. Because the MyoX PH1N-PH2-PH1C tandem is more diffused in polarized MDCK cells than the PH123_3M mutant (Figure 4, A5), we reasoned that the PH3 domain might facilitate basolateral membrane binding via a lipid-unrelated mechanism (e.g., possibly by binding to a protein target). Consistent with this hypothesis, a recent study showed that its two mutants, PH12_1M and PH12_2M, to defined liposomes containing 3% P(3,4,5)P₃.

(E, F) Surface electrostatic potential representation of the PH12 domain (blue to red, +5 to −5 times kBTɛₓ⁻¹, where k is the Boltzmann constant, T is temperature, and ɛₓ is the elementary charge constant), showing two prominent positively charged lipid head group–binding pockets in the tandem. Ins(1,3,4,5)P₄ (shown in the stick model) is modeled into the PH2 lipid-binding pocket, and Ins(1,4,5)P₃ is modeled into the lipid-binding pocket of PH1. It is also shown in F that the two PIP lipid-binding pockets of the PH12 tandem are juxtaposed to lipid membrane bilayers. (G) ITC curves showing that MyoX PH12 can specifically interact with Ins(1,3,4,5)P₄ with a KD of \(32.9 \pm 4.2 \mu M\) [the error represents the fitting of the experimental data with each PH12 containing one Ins(1,3,4,5)P₄ binding-site model]. No detectable binding can be found between PH12 and Ins(1,4,5)P₃.
MyoX PH3 does not bind to PIP lipids (Cox et al., 2002; Plantard et al., 2010).

We also tested the membrane localization properties of the PH123-MyTH4-FERM fragment of MyoX (i.e., the entire cargo-binding tail of the motor) using MDCK cells. Like the PH123 repeats of the motor, the wild-type PH123-MyTH4-FERM is membrane enriched (Supplemental Figure S3A). Disruption of the lipid-binding capacity of PH2 or the split PH1 eliminates or weakens the membrane localization of PH123-MyTH4-FERM, respectively (Supplemental Figure S3, B and C), indicating that PH repeat–mediated lipid membrane binding is essential for proper membrane localization of the motor.

**PH repeat–mediated membrane binding is essential for MyoX to induce filopodial formation**

MyoX is known to induce filopodia formation, and both endogenous and overexpressed MyoX localize at the tips of filopodia (Berg and Cheney, 2002; Bohil et al., 2006; Tokuo et al., 2007; Plantard et al., 2010). We tested the role of PH repeat–mediated PI(3,4,5)P3-membrane binding on MyoX's function in filopodial formation. Consistent with reported findings (Berg and Cheney, 2002; Bohil et al., 2006; Tokuo et al., 2007), HEK293T cells expressing the wild-type, full-length MyoX display a large number of green fluorescent protein (GFP) puncta at filopodial tips and massive numbers of substrate-attached elongated filopodia (Figure 5, A1, B1, and C). In line with a recent report, removal of all three PH repeats eliminated the filopodia tip localization and filopodial-inducing activities of MyoX (Plantard et al., 2010). Of importance, disruption of the PH2-mediated PI(3,4,5)P3 binding of MyoX (i.e., the Lys1237Ala/Arg1239Ala mutant of the full-length MyoX) also eliminated the filopodia tip localization and filopodial-inducing activities of MyoX (Plantard et al., 2010). Consistent with our in vitro lipid membrane–binding
experiments, the lipid binding–deficient mutations in the split PH1 also have significant effects on the filopodial-inducing activities of MyoX (Figure 5, A2, B2, and C), although the impact of the mutations is not as large as that caused by the mutations in PH2. The M10FL_3M mutant has an even smaller effect on the filopodia induction activity of the motor (Figure 5, A4, B4, and C). We repeated the MyoX-induced filopodia formation assay of the wild-type and various mutants of the full-length MyoX shown in Figure 5 in COS7 cells. Strikingly similar filopodia induction patterns were found for these MyoX constructs in COS7 cells (Supplemental Figure S4) and in HEK293T cells (Figure 5). In an earlier study, it was shown that mutation of one amino acid residue in the predicted lipid binding pocket of PH1 (K1179A, equivalent of K1187 in our study, which is the last instead of the penultimate residue in the β1 strand of PH1) or PH3 (K1395A, equivalent of K1403 in our study, which is the penultimate residue in the β1 strand of PH3) had no detectable impact on cellular localization of MyoX (i.e., both MyoX mutants are localized at filopodia tips) (Plantard et al., 2010). In contrast, a large portion of cytoplasm localized M10FL_1M or M10FL_3M was detected in the current study (Figure 5, A2 and A4). A likely explanation for this discrepancy is that the PH1 and PH3 mutations used in our study (K1195A/R1197A in PH1 and K1417A/R1419A in PH3) are expected to completely disrupt their lipid-binding capacities. The single point mutation in PH1 used in the Plantard et al. (2010) study is the last amino acid residue of the β1 strand, and this residue is known not to be critical for lipid binding of PH domains (Lemmon, 2008). The single point mutation of the penultimate residue in the β1 strand of PH3 used by Plantard et al. (2010) is likely to have a smaller effect than the double mutation used in our study. Therefore the PH1 and PH3 mutants of MyoX described by Plantard et al. (2010) are not likely to be significantly different from the wild-type MyoX. It is also possible that different experimental conditions between the two studies (e.g., different protein expression levels) might account for the observed differences. To minimize such variations possibly introduced by experimental conditions, we chose to compare both total amount of filopodia and the amount of elongated filopodia induced by different forms of MyoX expressed at a comparable level in our study.

DISCUSSION
Split PH domains constitute a unique subfamily among the hundreds of PH domains in eukaryotic genomes (Lemmon, 2008). With the determination of the MyoX split PH domain structure in this study, the atomic structures of all six known split PH domains have been solved (i.e., those in phospholipase Cγ, syntrophins, Rho kinases, the Vps36 subunit of the yeast ESCRT-II protein sorting complex, the neuronal GTPase PI3 kinase enhancer [PIKE], and MyoX; Yan et al., 2005, 2008; Alam et al., 2006, 2008). Several features are common to all split PH domains. First, PH domain splitting invariably occurs at the flexible loops connecting two consecutive β-strands of the PH domain β-barrel. As a result, the insertion of one or more additional domains does not alter the overall fold of the split PH domains (Supplemental Figure S5). Second, the splitting site varies in different split PH domains. The PH domains of PLCγ, syntrophin, and MyoX are all split into two halves at their respective β3/β4 loops. The two zinc fingers of Vps36 and the Cys-rich C1 domain of Rh kinase insert into the β6/β7 loop of their PH domains, respectively. The nuclear localization sequence of PIKE is located at the β5/β6 loop of the PH domain (Supplemental Figure S5). Third, although different split PH domains contain different insertions, split PH domains invariably function together with their insertions to form multidomain
supramodules with functions distinct from the simple sum of the functions of the individual domains. For example, in PLCγ, the split PH domain and the inserted SH2-SH2-SH3 domains play an autoinhibitory role in regulating the lipase activity of PLCγ (DeBell et al., 2007). The split PH domain of Vps36 and its two inserted zinc fingers function together to tether the ESCRT-II complex to the ESCRT-I complex via zinc finger 2 to ubiquitinated cargos on the endosome membranes (Alam et al., 2006; Hirano et al., 2006; Teo et al., 2006). The split PH domains of syntrophins, PIKE, and Rho kinase function with their insertions sequences to enhance lipid membrane-binding avidities (Yan et al., 2005, 2008; Wen et al., 2008). In addition, it should be noted that the split PH domain family does not represent the only case in which intact domains are split into halves by the insertion of other domains. Statistical analysis shows that ~9% of nonredundant proteins structures deposited at the Protein Data Bank (www.rcsb.org/pdb) contain domain insertions (Selvam and Sasidharan, 2004). We believe that domain splitting is likely to be a general strategy for multiple protein domains to assemble into functional supramodules with functions distinct from the individual or simple sum of individual domains.

The split PH domain of MyoX is unique in two ways. First, its PH1 domain is split by another PH domain (PH2), forming a PH1N-PH2-PH1C tandem. Second, the two PH domains in the PH1N-PH2-PH1C tandem interact with each other to form a structurally rigid supramodule. The relative orientation of the two lipid-binding pockets allows the MyoX PH repeats to bind to PI(3,4,5)P3-containing membranes with high specificity and cooperativity (Figure 1). We demonstrate in this study that both PH1 and PH2 are required for the proper cellular localization as well as filopodial induction activities of MyoX, although PH2-mediated selective binding to PI(3,4,5)P3 plays a more prominent role in both processes (Figures 4 and 5).

The cooperative nature of the PH1N-PH2-PH1C/PI(3,4,5)P3 interaction allows the supramodule to respond sharply to relatively small changes in PI(3,4,5)P3 concentration at a specific threshold, making it an ideal sensor for PI3 kinase activity. This is reflected in the sigmoidal shape of the PH1N-PH2-PH1C/PI(3,4,5)P3 binding curve; the slope is steepest at ~3.0 ± 0.5% PI(3,4,5)P3, meaning that the bound fraction of PH1N-PH2-PH1C is most sensitive to concentration changes about this point (Figure 1D). In contrast, PI(3,4,5)P3-specific PHs domains that possess high binding affinities but lack cooperativity are unable to respond as sharply to concentration changes at a specific threshold due to the hyperbolic shape of the general, noncooperative ligand-binding curve.

It is likely that the cooperative PI(3,4,5)P3 binding of its PH repeats plays a critical role in determining the localization of MyoX at the tips of filopodia (Sousa and Cheney, 2005). We envision that the activation of PI3 kinase at the tips of filopodia leads to local and sustained elevation of PI(3,4,5)P3, consequently inducing PH repeat-mediated MyoX enrichment at filopodial tips (Paolo and De Camilli, 2006; Kolsch et al., 2008; Lemmon, 2008). Decreases in PI(3,4,5)P3 concentration at membranes distal from the filopodial tips, by simple diffusion-mediated dilution and/or by metabolic turnover, would lead to sharp decreases in MyoX association to these plasma membranes owing to the sigmoidal PI(3,4,5)P3 membrane-binding curve of the MyoX PH repeats. It is possible that the PH repeats and the MyTH4-ERM tandem of the MyoX tail might function synergistically in interpreting PI3 kinase activation signals and signals for other transmembrane receptors (e.g., netrin receptor DCC, integrin) in regulating the cellular localization and activities of the motor (Zhang et al., 2004; Zhu et al., 2007; Hirano et al., 2011; Plantard et al., 2010; Wei et al., 2011). A recent study showed that binding of PI(3,4,5)P3 to the PH domains directly regulates the motor activity by releasing the PH repeat-mediated autoinhibition of MyoX (Umeki et al., 2011). Elucidating the detailed molecular mechanism governing the interaction between the PH repeats and the motor head of MyoX will certainly be highly valuable in understanding this unusual PH repeat-mediated motor activity regulation.

MATERIALS AND METHODS

Constructs and protein expression

The coding sequence of MyoX PH1N-PH2-PH1C (XP_226874, residues 1170–1317) and PH123 (XP_226874, residues 1170–1500) were PCR amplified from rat Myo10 and cloned into a pET32a vector. The GFP-tagged full-length MyoX expression plasmid (NP_036466.2, 1–2058), was a gift from Wencheng Xiong. Point mutations, including PH12_1M (K1195A/R1197A), PH12_2M (K1237A/R1239A), PH12_3M (K1417A/R1419A), PH12_3KN (K1187N/K1224N/K1225N), PH12_D1254R, PH12_3M (K1195A/R1219A), PH12_3M (K1417A/R1419A), FL_1M (K1193A/R1195A), FL_2M (K1235A/R1237A), and FL_3M (K1415A/R1417A), were created by PCR-based mutagenesis. For truncation mutant FL_5APH123, the coding sequence of PH123 (NP_036466.2, residues 1168x1498) was deleted from the full-length MyoX. Protein was expressed in BL21(DE3) Escherichia coli cells. The His6-tagged proteins were purified using Ni2+ nitrilotriacetic acid–agarose column follow by a size-exclusion chromatography.

Lipid-binding assays

Defined liposomes were reconstituted from synthetic PC and PS (Avanti Polar Lipids, Alabaster, AL) with or without certain concentrations of PIPs (Echelon Biosciences, Salt Lake City, UT). Lipids dissolved in chloroform were mixed in a glass tube at an appropriate ratio, and the solvent was evaporated under a stream of N2 gas at 4°C. An appropriate amount of buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 100 mM NaCl, 1 mM dithiothreitol [DTT]) was added to bring the final lipid concentration to 5 mg/ml. The lipid solution was rigorously vortexed for 5 min, and then the mixture was hydrated by 10 cycles of freeze and thaw with liquid N2. Brain lipid extracts (Folch fraction I, B1502, Sigma-Aldrich, St. Louis, MO) were resuspended in a buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, and 1 mM DTT. The protein sample (5 μM final concentration) was incubated with liposomes in 50 μl of buffer for 15 min at room temperature and then spun at 80,000 × g for 60 min at 4°C in a Beckman TLA100.1 rotor. The supernatants were removed for determination of proteins not bound to liposomes. The pellets were washed twice with the same buffer and brought up to the same volume as the supernatant. The supernatant and the pellet proteins were subjected to SDS–PAGE and visualized by Coomassie blue staining.

Crystalization, data collection and process

Crystals of PH12_3KN mutant (10.0 mg/ml in 50 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0) were grown using the hanging-drop method by mixing 1 μl of protein sample with equal volume of 20% (w/v) PEG8000 and 0.05 M potassium phosphate monobasic at 16°C. Crystals were transferred to the reservoir solution containing 15% (v/v) glycerol as the cryoprotectant and flash cooled with liquid nitrogen. Diffraction data were collected at 110 K on a RAXIS IV++ imaging-plate system with a MicroMax-007 copper rotating-anode generator (Rigaku, The Woodlands, TX). The diffraction data were processed and scaled using MOSFLM and SCALA in the CCP4 suite (CCP4, 1994).
Structure determination and refinement

The PH12_3KN mutant structure was solved by the molecular replacement method with the program Phaser (McCoy et al., 2007) using the structure of DAPPT1 PH domain (Protein Data Bank code 1FAO) as the search model. Structures were fitted and rebuilt with the program COOT (Emsley and Cowtan, 2004) and refined with REFMAC5 (Vagin et al., 2004) and CNS (Brünger et al., 1998). The overall qualities of the structural models were assessed using PROCHECK (Laskowski et al., 1993). The coordinates of PH12_3KN were deposited in the Protein Data Bank (www.rcsb.org/pdb) under the access code of 3TFM. Data collection and refinement statistics of the PH12_3KN structures are summarized in Table 1.

Cellular localization and filopodia quantification

HEK293T cells were transiently transfected with 0.5 μg of each plasmid per well using a Lipofectamine PLUS Kit (Invitrogen, Carlsbad, CA) in 12-well plates, and cells were cultured 24 h in DMEM containing 10% fetal bovine serum (FBS) in 10% CO2 before fixation. MDCK cells were transfected using a nucleo-transfection kit (Amaxa, Köln, Germany) by electroporation, and cells were cultured 48 h in MEM containing 10% FBS in 10% CO2 before fixation. Cells were imaged with an LSM 510 META laser scanning confocal microscope (Zeiss, Wetzlar, Germany) or a TE2000E inverted fluorescence microscope (Nikon, Melville, NY). Images from the Nikon microscope were used for quantification. Anti–ZO-1 antibody was used as the marker for tight junctions. Fluorescence intensities of plasma membrane and cytosolic region were quantitatively measured by MetaMorph software (Molecular Devices, Sunnyvale, CA). For HEK293T cells, actin was stained by rhodamine-conjugated phalloidin (Nikon, Melville, NY). Images from the Nikon microscope were used for quantification. Anti–ZO-1 antibody was used as the marker for tight junctions. Fluorescence intensities of plasma membrane and cytosolic region were quantitatively measured by MetaMorph image processing software (Molecular Devices, Sunnyvale, CA).

REFERENCES


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