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Membrane insertion of the FYVE domain is modulated by pH

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Abstract

The FYVE domain associates with phosphatidylinositol 3-phosphate [PtdIns(3)P] in membranes of early endosomes and penetrates bilayers. Here, we detail principles of membrane anchoring and show that the FYVE domain insertion into PtdIns(3)P-enriched membranes and membrane-mimetics is substantially increased in acidic conditions. The EEA1 FYVE domain binds to POPC/POPE/PtdIns(3)P vesicles with a Kd of 49 nM at pH 6.0, however associates ~24 fold weaker at pH 8.0. The decrease in the affinity is primarily due to much faster dissociation of the protein from the bilayers in basic media. Lowering the pH enhances the interaction of the Hrs, RUFY1, Vps27p and WDFY1 FYVE domains with PtdIns(3)P-containing membranes *in vitro* and *in vivo*, indicating that pH-dependency is a general function of the FYVE finger family. The PtdIns(3)P binding and membrane insertion of the FYVE domain is modulated by the two adjacent His residues of the R(R/K)HHCRXCG signature motif. Mutation of either His residue abolishes the pH-sensitivity. Both protonation of the His residues and nonspecific electrostatic contacts stabilize the FYVE domain in the lipid-bound form, promoting its penetration and increasing the membrane residence time.

Keywords

FYVE; membrane; phosphoinositide; pH dependence; mechanism

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INTRODUCTION

Phosphoinositide (PI) 3-kinases regulate membrane trafficking, protein sorting and signaling by generating phosphatidylinositol (PtdIns) derivatives phosphorylated at the third position of the inositol ring.[1,2] Of the four known products of PI 3-kinases, PtdIns 3-phosphate [PtdIns(3)P] is the most abundant and is constitutively produced in the cytosolic leaflet of membranes of early endosomes. In contrast to the multiply phosphorylated PtdIns's, PtdIns(3)P does not undergo hydrolysis into the secondary messengers, but rather functions as a signaling molecule itself and is specifically recognized by FYVE domains.[3–5] The FYVE finger is a small ~70 residue module discovered in 1997 and named after the four proteins, *F*ab1, *Y*OTB, *V*ac1, and *E*EA1 (reviewed in Refs. [6] and [7]). The FYVE domain fold consists of two double-stranded antiparallel β-sheets and a carboxy-terminal α-helix and is stabilized by two zinc binding clusters. The three highly conserved regions, the N-terminal WxxD sequence, the basic 'signature' motif [R(R/K)HHCRXCG], and the C-terminal RVC region form the PtdIns(3)P binding site and distinguish the FYVE subfamily from other structurally related RING fingers. The majority of FYVE finger-containing proteins are involved in endo- and exocytosis, intracellular signaling and cytoskeletal tethering and their targeting to PtdIns(3)P-enriched membranes is mediated by the FYVE domains.

Human EEA1 (early endosome antigen 1), Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), RUFY1 (RUN and FYVE domains-containing 1), WDFY1 (WD repeat and FYVE domain containing 1), and yeast Vps27p are key downstream effectors of PI 3-kinases. EEA1 functions as a docking/tethering protein and is required for homo- and heterotypic fusion of endosomes.[8,9] Owing to their robust association with early endosomes, EEA1 and WDFY1/FENS1 (Ref. [10]) are commonly used as markers of these organelles. Hrs[11,12] is involved in signal transduction and, as its yeast homologue Vps27p,[13] in endocytic protein trafficking. RUFY1 is a human homologue of Rabip4, an effector of Rab4 GTPase that regulates recycling of endocytosed cargo.[14,15] Recognition of PtdIns(3)P by the FYVE domains results in efficient translocation of these normally cytoplasmic proteins to endosomal, lysosomal, or vacuolar membranes. We have recently found that the interaction of the EEA1 FYVE domain with PtdIns(3)P is modulated by a histidine switch and is increased by lowering pH.[16] Additional membrane anchoring is provided by the insertion of a hydrophobic loop of the FYVE domain into bilayers and by nonspecific electrostatic interactions with negatively charged membrane surfaces.[17–19] However, it remains unclear if pH-sensitivity is a common function of the FYVE domain family and whether the pH fluctuations and nonspecific interactions have an effect on the docking, insertion and membrane affinity of this module. Here, we detail principles of the membrane targeting by the FYVE domain, show that the extent of the membrane penetration is increased in acidic media, and demonstrate that the pH-dependency is a general mechanism of FYVE domain regulation.

MATERIALS AND METHODS

Expression and purification of proteins

The wild type FYVE domains of EEA1, Hrs, RUFY1, Vps27p and WDFY1, and H1371A, H1372A and H1371A/H1372A mutants of EEA1 FYVE domain were expressed in *E. Coli* BL21 cells. Two liters of LB or $^{15}NH_4Cl$-supplemented minimal media containing 100 μg/mL ampicillin were inoculated with an overnight culture of BL21 cells harboring each construct and grown at 37°C until absorbance at 600 nm reached 0.6. After induction with 0.2 m*M* of isopropyl-1-thio-β-D-galactopyrano-side and incubation at 25°C for 16 h, cells were harvested for 10 min at 6000*g*, and the resulting pellet was resuspended in 10 mL of 20 m*M* Tris pH 8.0 buffer, containing 160 m*M* NaCl, 1 m*M* dithiothreitol (DTT) and 1.0% Triton X-100. The solution was then sonicated for 8 min using a 30 s sonication on ice

followed by 30 s incubation on ice. The homogenate was centrifuged at 50,000*g*, and the supernatant was filtered into a 50 mL tube. The unlabeled and uniformly ^{15}N-labeled GST-fusion proteins were purified using GST binding resin (EMD Biosciences, San Diego, CA) or a glutathione sepharose 4B column (Amersham). In case of EEA1 and RUFY1, the GST tag was cleaved with Thrombin (Sigma). The proteins were concentrated in millipore concentrators (Millipore) and purified by FPLC. The purity of the proteins was verified by SDS-PAGE and ^{1}H NMR. Protein concentrations were determined using the BCA method (Pierce).

Monolayer measurements

Insertion of the wild type and mutant EEA1 FYVE domains into a phospholipid monolayer was investigated by measuring the change in surface pressure (π) of invariable surface area during gradual addition of the proteins. The experiments were performed using a 1 mL circular Teflon trough and wire probe connected to a Kibron Micro-Trough X (Kibron, Helsinki) as described previously.[20] A monolayer containing various combinations of phospholipids was spread onto the subphase composed of either 10 m*M* KH_2PO_4/160 m*M* KCl (pH 6.0), 10 m*M* HEPES/160 m*M* KCl (pH 7.4), or 10 m*M* HEPES/160 m*M* KCl (pH 8.0) until the desired initial surface pressure (π_0) was reached. After stabilization of the signal (~5 min), 15 μg of protein was injected into the subphase through a hole in the wall of the trough. The surface pressure change ($\Delta\pi$) was monitored for 45 min. The ($\Delta\pi$) value reached a maximum after 30 min in all experiments.

The *in vivo* localization of EGFP-fusion EEA1 FYVE domain in yeast cells

The EGFP-EEA1-FYVE domain yeast expression plasmid was generated as described.[3] The plasmid was transformed into a wild type yeast strain (SEY6210: MATα, *his3*-Δ200, *ura3-52, trp1-Δ901, lys2-801, suc2-Δ9, leu2-3112*). For microscopy, yeast strains were grown in selective media overnight and the following morning a fresh culture was inoculated and grown at 26°C until an OD_{600} of 0.5. The cells were visualized by fluorescence microscopy as described,[21] using a Nikon Eclipse E800 microscope fitted with a cooled, high-resolution charge-coupled device camera (Hammamatsu Photogenics). Images were acquired using Phase 3 Imaging software (Phase 3 Imaging Systems).

SPR measurements

All surface plasmon resonance (SPR) measurements were performed at 25°C as described previously.[20] Equilibrium SPR measurements were carried out at the flow rate of 2 μL/min. After injecting 85 μL of the FYVE domain, the protein-lipid association and dissociation were monitored for 1020 and 500 s, respectively. The sensor-grams were obtained using five or more different concentrations of the protein (within a 10-fold range of K_d) for each condition tested. The sensorgrams were corrected for refractive index change by subtracting the control surface response. A total of three datasets were generated to obtain a standard deviation. The maximal response (R_{eq}, saturation value) was determined from the plots and plotted versus protein concentrations (C). The K_d values, determined by a nonlinear least squares analysis of the binding isotherm using the equation $R_{eq} = R_{max}/(1 + K_d/C)$,[18] are summarized in Table I. Kinetic SPR measurements were performed at a flow rate of 30 μL/min. Ninety microliters of the wild type or mutant FYVE domain was injected, and the protein-lipid association and dissociation were monitored for 3 min and over 6 min, respectively.

Liposome binding

The liposome binding assays were performed as described in.[16] Briefly, solutions of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS)

(Avanti), C_{16}-PtdIns(3)P (Echelon Biosciences) dissolved in $CHCl_3$:MeOH:H_2O (65:25:4) were mixed and dried down under vacuum. The lipids were resuspended in 50 m*M* MOPS, pH 7.0, 150 m*M* KCl, and 1 m*M* DTT, and incubated at 65°C for 1 h. The liposomes were then frozen in liquid nitrogen and thawed at 37°C for three cycles. The liposome solution was passed through an Avanti extruder to make 1.0 μm liposomes. Liposomes were collected by centrifugation at 25,000*g* for 20 min and resuspended to a final concentration of 4 m*M* total lipids in 100 μL of binding buffers, containing 150 m*M* NaCl, 1 m*M* DTT, and 50 m*M* either MES pH 5.5, BisTris pH 6.5, HEPES pH 7.5, Tris pH 8.0, or Bicine pH 8.5. Liposomes were incubated with GST-fusion Hrs, Vps27p and WDFY1 FYVE domains, and untagged RUFY1 FYVE domain (200–250 μg/mL final protein concentration) for 30 min at room temperature and then collected again by centrifugation. The liposome pellets were resuspended in 100 μL of buffer and analyzed using SDS-PAGE and Coomassie brilliant blue staining.

The *in vivo* localization of EGFP-fusion RUFY1, Vps27p, and WDFY1 FYVE domains in HeLa cells

The N-terminal-tagged EGFP-fusion FYVE domains of RUFY1, Vps27p, and WDFY1 were generated using corresponding pGEX-FYVE domains as templates and Bam-HII and EcoRI restriction sites. The HeLa cells were grown in Dulbecco modified essential medium (DMEM) (Mediatech) supplied with 10% fetal bovine serum (FBS) (Mediatech). The cells were transfected with mammalian plasmids encoding EGFP-RUFY1, Vps27p or WDFY1 FYVE fusion proteins using Effectene reagent (Qiagen) and 24 h later were replated onto 25-mm glass coverslips. Microscopic imaging of cells was performed 72 h after transfection. Before imaging, cells were washed three times with phosphate buffered saline (PBS) followed by addition of buffered solutions (pH = 6.0–8.5) containing 10 mg/mL nigericin (Sigma) and 10 mg/mL monensin (Sigma). The buffered solutions consisted of 120 m*M* KCl, 20 m*M* NaCl, 0.5 m*M* $CaCl_2$, 0.5 m*M* $MgSO_4$, and Hydrion Chemvelope pH buffer powder (Micro Essential Laboratory) or 25 m*M* of either Mops (Sigma), citrate (Acros), or Tris (Fisher Scientific). After pH equilibration for 15 min, the living cells were visualized at 33°C using Olympus IX81 inverted microscope equipped with FITC filter set (Chroma).

NMR spectroscopy

NMR experiments were performed at 298 K on a Varian INOVA 500 MHz spectrometer as described.[16] The ^1H,^{15}N HSQC spectra of 0.2 m*M* H1371A and H1372A EEA1 FYVE domains were recorded as C_4-PtdIns(3)P (up to 1.0 m*M*), and imidazole (up to 50 m*M*) were added stepwise.

The pK_a values of His1371 and His1372 of the EEA1 FYVE domain were calculated by monitoring changes in the chemical shifts of δ2H and ε1H of both His residues upon varying pH. The 1D ^1H NMR spectra of 0.5 m*M* FYVE domain were recorded in the presence of 120 m*M* deuterated d_{38}-dodecylphosphocholine (DPC) (Cambridge Isotopes) containing 10% w/w PS in ^2H_2O as the pH of the samples was adjusted stepwise from 4.7 to 9.4. Proton chemical shifts were calibrated and referenced to the internal standard, 2,2-dimethyl-2-silapentane-5-sulfonate (Sigma). The pK_a values of the His residues were obtained by a nonlinear least squares fitting of the chemical shifts observed in 1D spectra as a function of pH using a modified Henderson-Hasselbalch equation: $\delta_{obs} = [\delta_{acid} + \delta_{base}10^{(pH - pKa)}]/[1 + 10^{(pH - pKa)}]$, where the δ_{obs} value is chemical shift at a given pH, and δ_{acid} and δ_{base} are chemical shifts at the low and high extremes of pH, respectively.

RESULTS AND DISCUSSION

Membrane insertion of the EEA1 FYVE domain is increased in acidic pH

Recent studies have shown that the FYVE domain inserts an exposed hydrophobic loop into membranes and membrane-mimetics.[6,17–19,22,23] To test whether changes in pH influence the extent of the membrane insertion, we examined the lipid monolayer penetrating ability of the EEA1 FYVE domain in acidic, neutral, and basic conditions. The monolayers containing POPC/POPE (80:20) or POPC/POPE/PtdIns(3)P (78:20:2) were spread over an invariable area with initial surface pressure π, and after adding the FYVE domain into the subphase, the change in surface pressure (Δπ) was analyzed. Extrapolating Δπ versus π, one can obtain the critical surface pressure π_c, which defines an upper limit of the monolayer pressure that a protein can insert into.[24] Given that π of cell membranes and large unilamellar vesicles is in the range of 30–35 dyne/cm,[25,26] for the FYVE domain to be able to penetrate these bilayers, the π_c value should be above 30 dyne/cm.

Figure 1(a) shows that the EEA1 FYVE domain has no intrinsic POPC/POPE penetrating ability, and π_c (~20 dyne/cm) remains essentially unchanged at pH 6.0, 7.4, and 8.0. Thus, in the absence of PtdIns(3)P, the EEA1 FYVE domain does not penetrate POPC/POPE membranes nor does the pH influence the insertion. However, when PtdIns(3)P was incorporated in the monolayer, the π_c value increased to 31 dyne/cm at pH 7.4, indicating a moderate insertion. A similar augmentation in penetrating power due to PtdIns(3)P was observed for the FYVE domains of Hrs, Vps27p and FENS-1, where π_c raised to ~30–34 dyne/cm.[18,19] We next examined the ability of the EEA1 FYVE domain to penetrate PtdIns(3)P-containing monolayers in variable pH buffers. We found that π_c increases from 31 dyne/cm at pH 7.4 to 35 dyne/cm at pH 6.0 but decreases to 27 dyne/cm at pH 8.0. Thus, insertion of the EEA1 FYVE domain into PtdIns(3)P-enriched membranes is substantially enhanced in acidic environment.

The robust increase of membrane penetration *in vitro* upon lowering pH paralleled changes in localization of the enhanced green fluorescent protein (EGFP)-fusion EEA1 FYVE domain *in vivo* [Fig. 1(b)]. The EGFP-EEA1 FYVE domain was expressed in yeast SEY6210 cells, and after brief incubation of the cells in buffers of pH 6.0, 7.0, 8.0, and 9.0, the intracellular distribution of the protein was examined by fluorescence microscopy. Although the EGFP-FYVE domain was strongly anchored to PtdIns(3) P-containing vacuolar and endosomal membranes in acidic conditions (pH < 7.0), the protein was dispersed in the cytosol at pH 8.0 or higher. Furthermore, *in vivo* association with PtdIns(3)P-enriched membranes, particularly in the physiological pH range of 6.0–8.0, had the trend fully consistent with the trend seen for *in vitro* binding of the EEA1 FYVE domain to PtdIns(3)P-containing membrane-mimetics [Fig. 1(a)].

Quantitative analysis of the EEA1 FYVE domain recruitment to PtdIns(3)P-containing membranes

To fully evaluate the effect of pH, we investigated association of the EEA1 FYVE domain with PtdIns(3)P-containing vesicles by SPR. The equilibrium binding constants were measured at near physiological pH levels of 6.0, 7.4, and 8.0 using POPC/POPE/PtdIns(3)P (78:20:2) vesicles and various protein concentrations [Fig. 2(a) and Materials and Methods]. We found that the EEA1 FYVE domain binds POPC/POPE/PtdIns(3)P vesicles with a K_d of 49 nM at pH 6.0 (Table I). However, its association with vesicles became considerably weaker as the pH of the buffer was raised from 6.0 to 8.0. The binding affinity of the EEA1 FYVE domain dropped by 10- and 24-fold upon alkalinization of the media to pH 7.4 and 8.0, respectively (Table I). These data demonstrate that acidity mediates recruitment of the EEA1 FYVE domain to PtdIns(3)P-containing bilayers, amplifying the binding affinity by

10 fold per one pH unit in the pH range of 6.0 to 8.0. The dependence of membrane anchoring on pH is summarized in Table I, which reveals a significant increase of the EEA1 FYVE domain activity in acidic conditions and a decrease in basic conditions.

To examine the effect of pH on the on- and off-rates, we analyzed kinetics of the EEA1 FYVE domain's interaction with POPC/POPE/PtdIns(3)P vesicles at pH 6.0, 7.4, and 8.0. As shown in Figure 2(b), the dissociation rate is substantially slower in acidic (pH 6.0) conditions than in neutral (pH 7.4) or basic (pH 8.0) conditions. The association rate, however, remains largely unchanged at all pHs. Thus, enhancement of the EEA1 FYVE domain binding to PtdIns(3)P-containing membranes in low pH buffers is primarily due to a much slower dissociation rate of the protein from bilayers.

pH-dependent membrane docking is a general feature of FYVE domains

To determine whether the pH-sensitive membrane anchoring is a function of the FYVE family, FYVE domains of Hrs, RUFY1, Vps27p and WDFY1 were investigated by liposome binding assays and fluorescence microscopy. The GST-fusion Hrs, Vps27p and WDFY1 FYVE modules and untagged RUFY1 FYVE domain were incubated with small unilamellar vesicles (SUVs) composed of PC, PE, PS and PtdIns(3)P at pH 5.5, 6.5, 7.5, 8.0 or 8.5. The distribution of the proteins between supernatant and SUVs was examined by centrifugation [Fig. 3(a)]. Densitometry analysis of the gel bands revealed that 88% of the Hrs1 FYVE domain, 85% of the RUFY1 FYVE domain, 78% of the WDFY1 FYVE domain, and 58% of the Vps27p FYVE domain were retained in the pelleted liposome fraction at a low pH of 5.5. However, FYVE domains of all proteins were increasingly redistributed to the supernatant as the pH of the buffer was progressively increased. Particularly, binding of the Hrs1, RUFY1, and Vps27p FYVE domains to PtdIns(3)P-enriched vesicles was reduced to ~20% as a result of alkalinization of the buffer from pH 6.0 to 8.5. Although the WDFY1 FYVE domain clearly displayed the pH dependence toward PtdIns(3)P, almost a half of this protein (47%) was still bound to the SUVs at pH 8.5. This is consistent with the fact that the WDFY1 FYVE domain has a 10-residue extension in the membrane insertion loop and associates with membranes particularly tightly.[10]

A similar pH-dependency was observed for the *in vivo* localization of the EGFP-fusion RUFY1, Vps27p, and WDFY1 FYVE domains to endosomal membranes in human HeLa cells [Fig. 3(b)]. Following acidification of the cytosol, most of the FYVE domains were found to co-localize with PtdIns(3)P-enriched early endosomes. However, when the cytosolic pH was gradually increased, the EGFP-FYVE domains became more soluble, and at pH > 7.5 were entirely cytoplasmic. Thus, FYVE domains of RUFY1, Vps27p, and WDFY1 translocate to PtdIns(3)P-containing membranes at low cytosolic pH, demonstrating that the pH-sensitivity represents a general characteristic of the FYVE module.

The tandem His residues in the PtdIns(3)P binding pocket are necessary for deep penetration into the bilayer

Of the all residues in the R(R/K)HHCRXCG "signature" motif, the two histidine residues (His1371 and His1372 of EEA1) are absolutely conserved among all FYVE domains (see Fig. 3(c, d)). They form hydrogen bonds to the 3-phosphate and the 4- and 5-hydroxyl groups of PtdIns(3)P (Ref. [27]) and are necessary for the lipid recognition.[16] To determine if the membrane insertion of the EEA1 FYVE domain requires both histidine residues, H1371 and H1372 were substituted with Ala, and the mutant proteins were probed by monolayer penetration experiments [Fig. 4(a, b)]. In acidic conditions (pH 6.0), in which the wild type EEA1 FYVE domain inserts furthest, penetration of the H1371A and H1372A mutants into a POPC/POPE/PtdIns(3)P monolayer was significantly compromised. The π_c value was

decreased by 3 dyne/cm for H1371A and by 2.5 dyne/cm for H1372A. The double H1371A/H1372A mutation caused a further drop in penetrating power, with the surface pressure decreasing to 30 dyne/cm. Thus, both His residues are necessary for the substantial penetration of the EEA1 FYVE domain into PtdIns(3)P-containing membranes. Furthermore, deprotonation of the His residues abolished insertion. The π_c value was decreased to 30 dyne/cm at pH 7.4 and to 27 dyne/cm at pH 8.0 for the single mutants. At pH 8.0, both His residues became fully deprotonated based on their pK_a values determined by chemical shift changes in imidazole's δ2H and ε1H upon varying pH [Fig. 4(c)]. Consequently, in a basic environment, neither mutants nor wild type protein were able to insert into the monolayer, suggesting that the two additional positive charges in the PtdIns(3)P binding site are essential for the robust interaction and membrane penetration.

The critical role of His residues in pH-sensitivity was underscored by measuring the monolayer penetration of PtdIns(3)P-binding p40^{phox} PX domain. Unlike FYVE domain, the p40^{phox} PX module contains no His residues in the PtdIns(3)P binding site. As expected, its penetrating ability remained unchanged in all pH buffers [Fig. 4(d)].

The tandem His residues are required for the strong membrane anchoring

To determine the contribution of individual His1371 and His1372 residues in the association of the EEA1 FYVE domain with bilayers, binding of the mutants to POPC/POPE/PtdIns(3)P vesicles was tested by SPR. We found that the H1371A mutant associates with vesicles ~13-fold weaker than the wild type protein at pH 6.0, 9-fold weaker at pH 7.4 and at least 6-fold weaker at pH 8.0 (Table I). Overall, the H1371A mutation caused at least a 10-fold drop in affinity when the pH of the buffer was changed from 6.0 to 8.0. Likewise, the H1372A mutant showed a steady decrease in binding upon alkalinization of the media. Together, these results reveal that protonation of both His residues is required for the membrane recruitment. The H1371A/H1372A double mutation abolished the pH sensitivity of the FYVE domain ($K_d > 5$ μ*M* in all conditions tested), indicating that the two His residues play a major role in the pH dependent behavior of the FYVE domain. The tandem histidines occupy strikingly similar positions in the binding pockets of FYVE modules [Fig. 3(d)], suggesting that they may regulate general function of the FYVE proteins.

The kinetic studies of the interaction involving H1371A or H1372A EEA1 FYVE domain and PtdIns(3) P-containing vesicles demonstrate that the loss of binding activity by the mutants was primarily due to a much faster off-rate when compared with the off-rate of the wild type protein [Fig. 2(c)]. This underscores the importance of the His residues and their protonation in stabilization of the FYVE-PtdIns(3)P complex at membranes. Addition of imidazole to either His mutant did not restore their binding activity based on the lack of resonance perturbations in $^{1}H,^{15}N$ heteronuclear single quantum coherence (HSQC) spectra of the mutants [Fig. 4(e) and data not shown], demonstrating that the intact side chains of His residues are necessary for the proper coordination of PtdIns(3)P.

It has been shown that membrane association of the FYVE domain is facilitated by nonspecific electrostatic interactions, which involve basic residues of the protein and anionic phospholipids in the bilayer.[18,28] A strong positive potential surrounding the insertion loop and PtdIns(3)P binding pocket may drive initial association with the membrane and facilitate PtdIns(3)P recognition.[18,28] Protonated His1371 and His1372 residues may contribute to the electrostatic component because their pK_a values were increased by 0.2–0.3 pH units in the presence of PS in DPC micelles [Fig. 4(c)]. However, their contribution to the association appears to be small because the on-rate of the EEA1 FYVE domain was not significantly affected by the changes in pH [Fig. 2(b)]. We concluded that Arg and/or Lys residues surrounding the insertion loop and PtdIns(3)P binding site are primarily involved in the electrostatic interactions, whereas protonation of His residues strengthens the coordination

of PtdIns(3)P and promotes membrane penetration. This is consistent with the notion that PI-coordination and close range interactions mediate the dissociation of other peripheral proteins from membranes.[7,29]

In conclusion, the data presented here provide new insight into the mulivalent mechanism of docking and penetration into PtdIns(3)P-enriched membranes and membrane-mimetics by the FYVE domain. Our results reveal that the membrane insertion is enhanced in acidic media and that the pH-dependency represents a general mechanism for regulation of the FYVE domain family.

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References

1. Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD. Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem 2001;70:535–602. [PubMed: 11395417]
2. Stenmark H, Gillooly DJ. Intracellular trafficking and turnover of phosphatidylinositol 3-phosphate. Cell Dev Biol 2001;12:193–199.
3. Burd CG, Emr SD. Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. Mol Cell 1998;2:157–162. [PubMed: 9702203]
4. Gaullier JM, Simonsen A, D'Arrigo A, Bremnes B, Stenmark H, Aasland R. FYVE fingers bind PtdIns(3)P [letter; comment]. Nature 1998;394:432–433. [PubMed: 9697764]
5. Patki V, Lawe DC, Corvera S, Virbasius JV, Chawla A. A functional PtdIns(3)P-binding motif [letter] [see comments]. Nature 1998;394:433–434. [PubMed: 9697765]
6. Kutateladze TG. Phosphatidylinositol 3-phosphate recognition and membrane docking by the FYVE domain. Biochim Biophys Acta 2006;1761:868–877. [PubMed: 16644267]
7. Cho W, Stahelin RV. Membrane-protein interactions in cell signaling and membrane trafficking. Annu Rev Biophys Biomol Struct 2005;34:119–151. [PubMed: 15869386]
8. Simonsen A, Lippe R, Christoforidis S, Gaullier JM, Brech A, Callaghan J, Toh BH, Murphy C, Zerial M, Stenmark H. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion [see comments]. Nature 1998;394:494–498. [PubMed: 9697774]
9. Mills IG, Jones AT, Clague MJ. Involvement of the endosomal auto-antigen EEA1 in homotypic fusion of early endosomes. Curr Biol 1998;8:881–884. [PubMed: 9705936]
10. Ridley SH, Ktistakis N, Davidson K, Anderson KE, Manifava M, Ellson CD, Lipp P, Bootman M, Coadwell J, Nazarian A, Erdjument-Bromage H, Tempst P, Cooper MA, Thuring JW, Lim ZY, Holmes AB, Stephens LR, Hawkins PT. FENS-1 and DFCP1 are FYVE domain-containing proteins with distinct functions in the endosomal and Golgi compartments. J Cell Sci 2001;114 (Pt 22):3991–4000. [PubMed: 11739631]
11. Raiborg C, Bache KG, Mehlum A, Stang E, Stenmark H. Hrs recruits clathrin to early endosomes. EMBO J 2001;20:5008–5021. [PubMed: 11532964]
12. Komada M, Soriano P. Hrs, a FYVE finger protein localized to early endosomes, is implicated in vesicular traffic and required for ventral folding morphogenesis. Genes Dev 1999;13:1475–1485. [PubMed: 10364163]
13. Piper RC, Cooper AA, Yang H, Stevens TH. VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae*. J Cell Biol 1995;131:603–617. [PubMed: 7593183]

14. Cormont M, Mari M, Galmiche A, Hofman P, Le Marchand-Brustel Y. A FYVE-finger-containing protein. Rabip4, is a Rab4 effector involved in early endosomal traffic. Proc Natl Acad Sci USA 2001;98:1637–1642. [PubMed: 11172003]
15. Yang J, Qiu Y. Interaction between tyrosine kinase Etk and a RUN domain- and FYVE domain-containing protein RUFY1. A possible role of ETK in regulation of vesicle trafficking. J Biol Chem 2002;277:30066–30071. [PubMed: 12023958]
16. Lee SA, Eyeson R, Cheever ML, Geng J, Verkhusha VV, Burd C, Overduin M, Kutateladze TG. Targeting of the FYVE domain to endosomal membranes is regulated by a histidine switch. Proc Natl Acad Sci USA 2005;102:13052–13057. [PubMed: 16141328]
17. Kutateladze TG, Capelluto DGS, Ferguson CG, Cheever ML, Kutateladze AG, Prestwich GD, Overduin M. Multivalent mechanism of membrane insertion by the FYVE domain. J Biol Chem 2004;279:3050–3057. [PubMed: 14578346]
18. Stahelin RV, Long F, Diraviyam K, Bruzik KS, Murray D, Cho W. Phosphatidylinositol 3-phosphate induces the membrane penetration of the FYVE domains of Vps27p and Hrs. J Biol Chem 2002;277:26379–26388. [PubMed: 12006563]
19. Blatner NR, Stahelin RV, Diraviyam K, Hawkins PT, Hong W, Murray D, Cho W. The molecular basis of the differential subcellular localization of FYVE domains. J Biol Chem 2004;279:53818–53827. [PubMed: 15452113]
20. Hom RA, Vora M, Regner M, Subach OM, Cho W, Verkhusha VV, Stahelin RV, Kutateladze TG. pH-dependent binding of the Epsin ENTH domain and the AP180 ANTH domain to PI(4,5)P2-containing bilayers. J Mol Biol 2007;373:412–423. [PubMed: 17825837]
21. Kutateladze TG, Ogburn KD, Watson WT, de Beer T, Emr SD, Burd CG, Overduin M. Phosphatidylinositol 3-phosphate recognition by the FYVE domain. Mol Cell 1999;3:805–811. [PubMed: 10394369]
22. Misra S, Hurley JH. Crystal structure of a phosphatidylinositol 3-phosphate-specific membrane-targeting motif, the FYVE domain of Vps27p. Cell 1999;97:657–666. [PubMed: 10367894]
23. Kutateladze TG, Overduin M. Structural mechanism of endosome docking by the FYVE domain. Science 2001;291:1793–1796. [PubMed: 11230696]
24. Cho W, Bittova L, Stahelin RV. Membrane binding assays for peripheral proteins. Anal Biochem 2001;296:153–161. [PubMed: 11554709]
25. Blume A. A comparative study of the phase transitions of phospholipid bilayers and monolayers. Biochim Biophys Acta 1979;557:32–44. [PubMed: 549642]
26. Marsh D. Lateral pressure in membranes. Biochim Biophys Acta 1996;1286:183–223. [PubMed: 8982283]
27. Dumas JJ, Merithew E, Sudharshan E, Rajamani D, Hayes S, Lawe D, Corvera S, Lambright D. Multivalent endosome targeting by homodimeric EEA1. Mol Cell 2001;8:947–958. [PubMed: 11741531]
28. Diraviyam K, Stahelin RV, Cho W, Murray D. Computer modeling of the membrane interaction of FYVE domains. J Mol Biol 2003;328:721–736. [PubMed: 12706728]
29. Stahelin RV, Cho W. Differential role of ionic, aliphatic, and aromatic residues in membrane-protein interactions: a surface plasmon resonance study on phospholipases A2. Biochemistry 2001;40:4672–4678. [PubMed: 11294634]

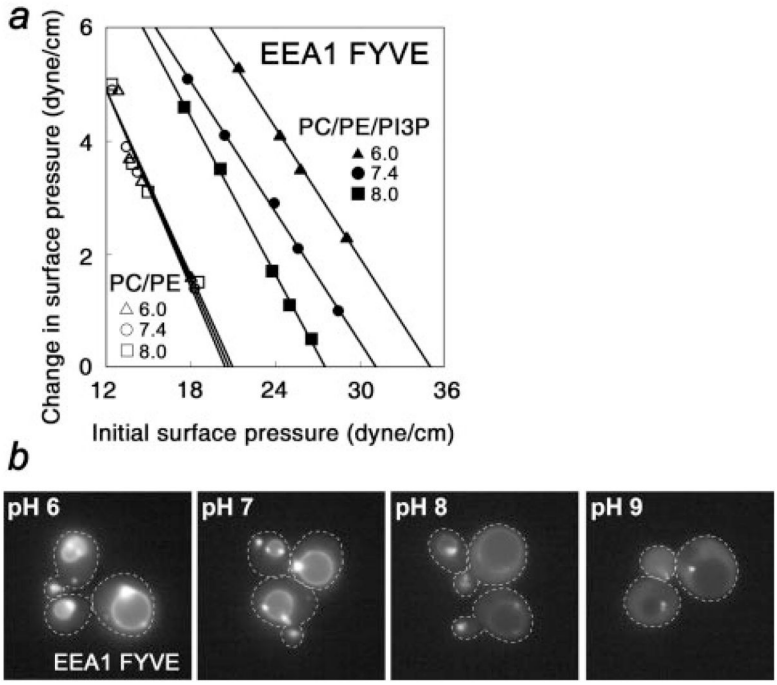

Figure 1.

Association of the EEA1 FYVE domain with PtdIns(3)P (PI3P)-containing monolayers and membranes is pH dependent. (**a**) Insertion of the EEA1 FYVE domain into a POPC/POPE (80:20) monolayer (open symbols) and a POPC/POPE/PtdIns(3)P (77:20:3) monolayer (filled symbols) at pH 6.0 (triangle), 7.4 (circle) and 8.0 (square) monitored as a function of π. (**b**) The distribution of the EGFP-EEA1 FYVE domain between the cytosol and PtdIns(3)P-enriched vacuolar and endosomal membranes in yeast SEY6210 cells is altered by varying cytosolic pH.

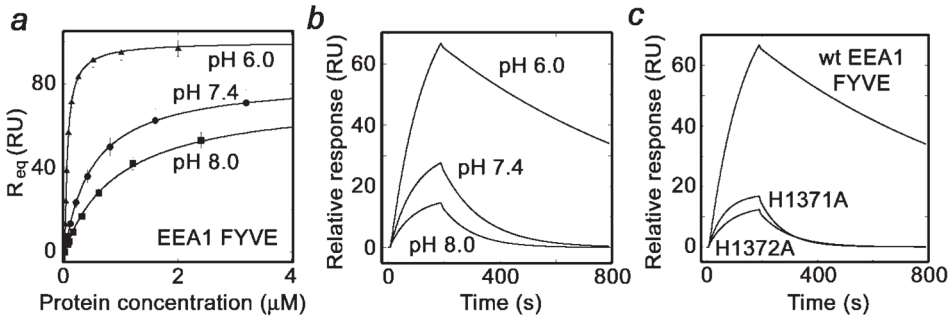

Figure 2.

Kinetic and equilibrium SPR analyses of the EEA1 FYVE domain. (**a**) Equilibrium binding of the EEA1 FYVE domain to the sensor chip coated with POPC/POPE/PtdIns(3)P (78:20:3) was measured at pH 6.0 (▲), 7.4 (●), and 8.0 (■). Binding isotherms were generated from the R_{eq} (average of triplicate measurements) versus the protein concentration. Solid line represents theoretical curve constructed from R_{max} and K_d values determined from nonlinear least-squares analysis using the equation $R_{eq} = R_{max}/(1 + K_d/C)$. (**b**) Sensorgrams from kinetic experiments show binding of the EEA1 FYVE domain (300 n*M*) to POPC/POPE/PtdIns(3)P (77:20:3) vesicles at indicated pH values. The flow rate was maintained at 30 μL/min for both association and dissociation phases. (**c**) Sensorgrams from kinetic experiments show binding of the wild type, and H1371A and H1372A mutants of the EEA1 FYVE domain to the POPC/POPE/PtdIns(3)P (77:20:3) vesicles at pH 6.0.

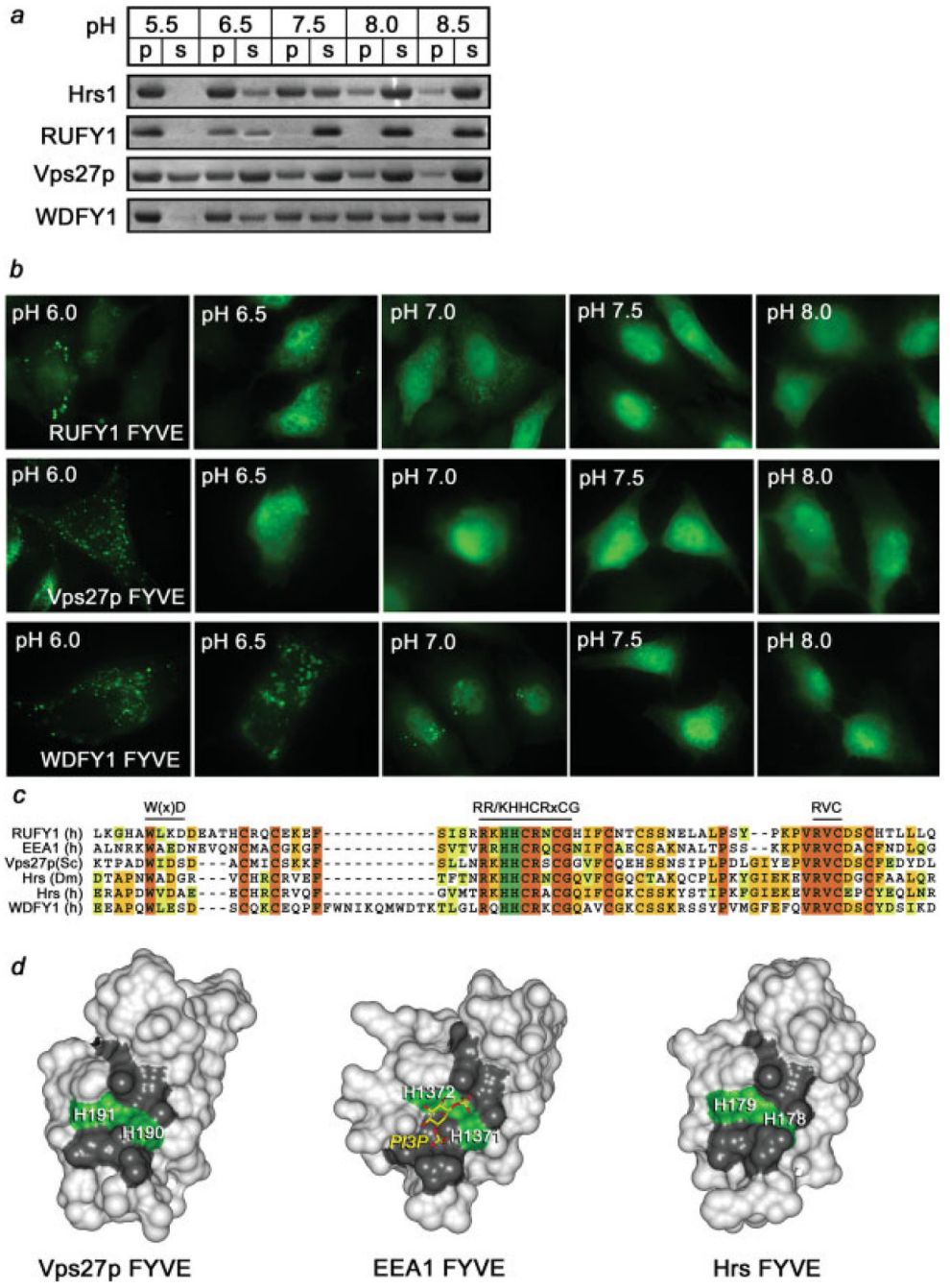

Figure 3.

The pH-sensitivity is a general feature of the FYVE domain family. (**a**) The SDS-PAGE gels show the partition of Hrs1, RUFY1, Vps27p, and WDFY1 FYVE domains between the supernatant (s) and PtdIns(3)P-enriched liposome pellet (p) at different pHs. (**b**) Changes in localization of EGFP-tagged RUFY1, Vps27p, and WDFY1 FYVE domains in HeLa cells upon varying the cytosolic pH. The cells were incubated in solutions buffered to indicated pH prior to visualizing by fluorescence microscopy. (**c**) The tandem His residues are conserved in the FYVE domain family. Alignment of the FYVE domain sequences: absolutely, moderately, and weakly conserved residues are colored brown, orange, and yellow, respectively. The His residues are in green. Three regions of the FYVE domain

involved in coordination of PtdIns(3)P are indicated by black lines above the alignment. (**d**) The PtdIns(3)P binding pocket defined from the crystal structures of Vps27p FYVE (PDB 1VFY), EEA1 FYVE (PDB 1JOC), and Hrs FYVE (PBD 1DVP). The tandem His residues and Arg/Lys residues located in the R(R/K)HHCRXCG and RVC regions are labeled and colored green and dark gray, respectively.

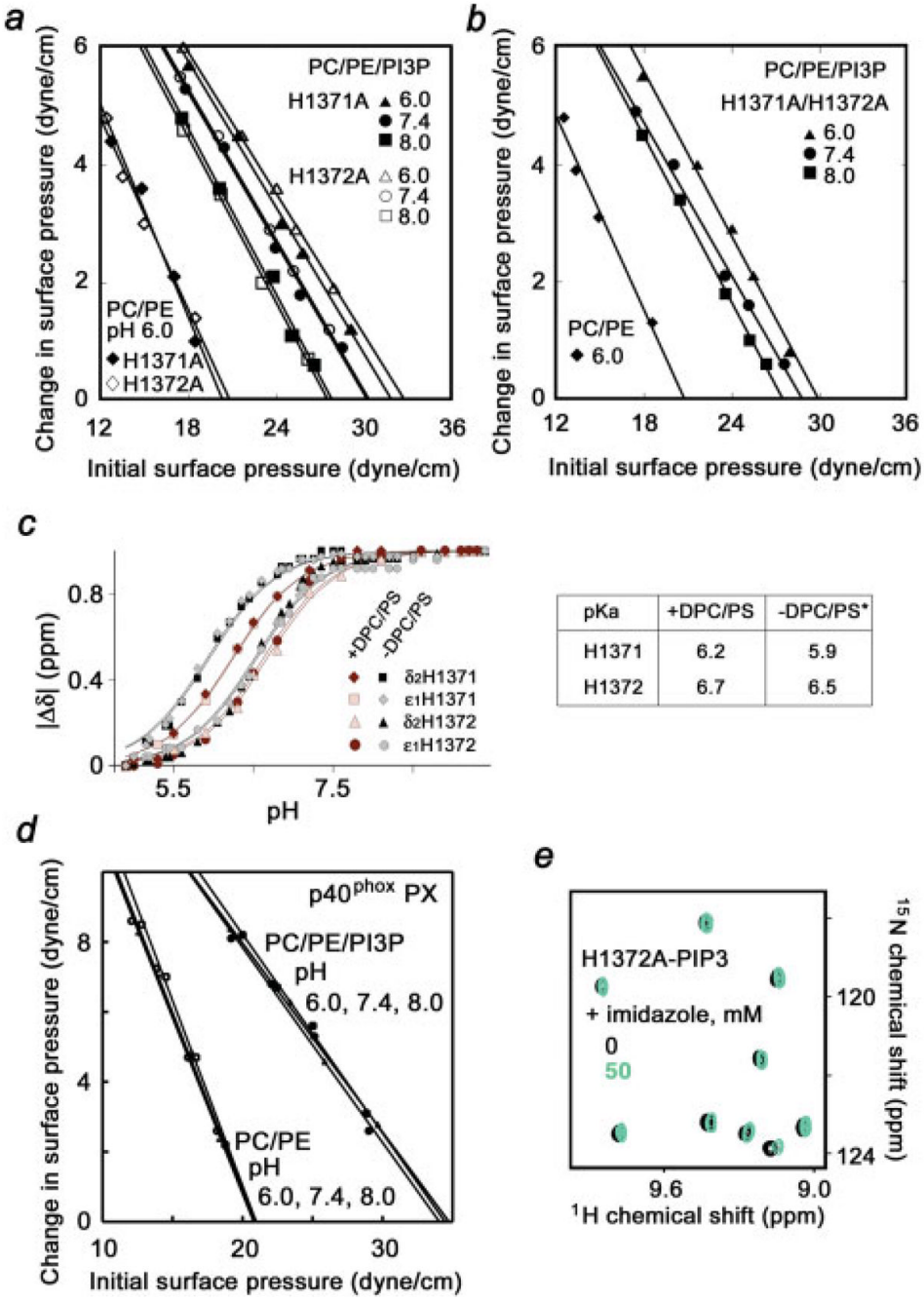

Figure 4.

The tandem His residues are essential for membrane penetration by the FYVE domain. (**a**) Insertion of the H1371A (filled symbols) and H1372A (open symbols) EEA1 FYVE domain into a POPC/POPE/PtdIns(3)P (78:20:2) monolayer at pH 6.0 (triangle), 7.4 (circle), and 8.0 (square), and into a POPC/POPE (80:20) monolayer at pH 6.0 (diamond). (**b**) Penetration of the H1371A/H1372A EEA1 FYVE domain into a POPC/POPE/PtdIns(3)P (78:20:2) monolayer at pH 6.0 (triangle), 7.4 (circle), and 8.0 (square) and into a POPC/POPE (80:20) monolayer at pH 6.0 (diamond). (**c**) pK_a values of His1371 and His1372 in the presence and absence of PS-containing DPC micelles were measured by chemical shift perturbation analysis of ^1H resonances in NMR spectra. (*) is taken from.[16] (**d**) Insertion of the $p40^{phox}$

PX domain into POPC/POPE/PtdIns(3)P (77:20:3) and POPC/POPE (80:20) monolayers at pH 6.0, 7.4, and 8.0. (**e**) Superimposed $^{1}H,^{15}N$ HSQC spectra of the H1372A EEA1 FYVE domain (0.2 m*M*) collected in the presence of 1.0 m*M* PtdIns(3)P and 0–50 m*M* imidazole.

Table I

Lipid Binding Properties of the EEA1 FYVE Domain, Wild Type (WT), and Mutants

EEA1 FYVE domain	K_d (n*M*) POPC/POPE/PtdIns(3)P (77:20:3)	Fold change relative to WT at pH 6.0
WT, pH 6.0	49 ± 6	–
H1371A, pH 6.0	660 ± 40	13
H1372A, pH 6.0	880 ± 50	18
H1371A/H1372A, pH 6.0	>5000	>100
WT, pH 7.4	510 ± 10	10
H1371A, pH 7.4	4600 ± 300	94
H1372A, pH 7.4	5200 ± 400	110
H1371A/H1372A, pH 7.4	>6000	>120
WT, pH 8.0	1200 ± 40	24
H1371A, pH 8.0	>6000	>120
H1372A, pH 8.0	>6000	>120
H1371A/H1372A, pH 8.0	>8000	>160