

Targeting of the FYVE domain to endosomal membranes is regulated by a histidine switch

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Specific recognition of phosphatidylinositol 3-phosphate [PtdIns(3)P] by the FYVE domain targets cytosolic proteins to endosomal membranes during key signaling and trafficking events within eukaryotic cells. Here, we show that this membrane targeting is regulated by the acidic cellular environment. Lowering the cytosolic pH enhances PtdIns(3)P affinity of the FYVE domain, reinforcing the anchoring of early endosome antigen 1 (EEA1) to endosomal membranes. Reversibly, increasing the pH disrupts phosphoinositide binding and leads to cytoplasmic redistribution of EEA1. pH dependency is due to a pair of conserved His residues, the successive protonation of which is required for PtdIns(3)P head group recognition as revealed by NMR. Substitution of the His residues abolishes PtdIns(3)P binding by the FYVE domain *in vitro* and *in vivo*. Another PtdIns(3)P-binding module, the PX domain of Vam7 and p40^{phox} is shown to be pH-independent. This provides the fundamental functional distinction between the two phosphoinositide-recognizing domains. The presented mode of FYVE regulation establishes the unique function of FYVE proteins as low pH sensors of PtdIns(3)P and reveals the critical role of the histidine switch in targeting of these proteins to endosomal membranes.

phosphoinositide | early endosome antigen 1

The specific recruitment of FYVE proteins to endosomes, multivesicular bodies, and phagosomes is primarily mediated by FYVE domain binding to membrane-embedded phosphatidylinositol 3-phosphate [PtdIns(3)P] (1–3). Additional membrane anchoring is provided by hydrophobic insertion into the bilayer, electrostatic interactions with acidic lipids (4, 5), and dimerization (6, 7). These synergistic factors are thought to be largely responsible for directing FYVE proteins to PtdIns(3)P-enriched membranes. Yet, the current model of FYVE domain function has several limitations and is not predictive. Some FYVE proteins localize inexplicably to sites that contain little PtdIns(3)P, such as the Golgi and endoplasmic reticulum (8, 9). On the other hand, places with significant PtdIns(3)P concentrations, such as the nucleus and mitochondria (10), do not appear to attract FYVE proteins. All canonical FYVE domains have been found to target PtdIns(3)P (refs. 6, 10–16); however, their binding properties were investigated at pH values of 6.5–8.0, leading to a discrepancy in estimated affinities despite the highly conserved sequences and structures of the FYVE proteins.

The FYVE domain is defined by the three conserved elements: the N-terminal WxxD, the central RR/KHHCR, and the C-terminal RVC motifs that form the PtdIns(3)P binding site seen in the solution and crystal structures of early endosome antigen 1 (EEA1), Hrs, and Vps27p FYVE (6, 13, 17, 18). The PtdIns(3)P headgroup is coordinated by a cluster of four Arg/Lys and two His residues and the N-terminal Asp. Among them, the two adjacent His residues are the most conserved in the FYVE domain sequences. In this work, we demonstrate that anchoring of the FYVE domain to PtdIns(3)P-enriched membranes is strongly pH-dependent, being enhanced by the acidic cytosolic environment, and is regulated by a histidine switch. The presented mode of FYVE

regulation differentiates this module from other phosphoinositide-binding domains and establishes its unique function as low pH sensor of PtdIns(3)P.

Materials and Methods

Mutagenesis and Protein Expression and Purification. See *Supporting Text*, which is published as supporting information on the PNAS web site.

NMR Spectroscopy and pH Titration. NMR spectra were recorded at 25°C on Varian INOVA 500 and 600 MHz spectrometers. The ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra of 0.1–0.2 mM uniformly ¹⁵N-labeled wild-type or mutant EEA1 FYVE domain or Vam7 PX domain containing 0–5 mM C₄-PtdIns(3)P (Echelon, Salt Lake City) were collected while pH of the samples was varied between 5.5 and 8.5. The normalized chemical-shift change was defined as $[(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2]^{0.5}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are ¹H and ¹⁵N chemical shift changes in parts per million (19).

Calculations of pK_a Values, Estimation of the Lipid-Binding Affinities, and Liposome Binding. See *Supporting Text* for details.

***In Vivo* Localization of EEA1 FYVE and p40^{phox} PX Domains Fused with Enhanced Cyan Fluorescent Protein (ECFP) and EGFP.** The ECFP-EEA1 FYVE domain-containing mammalian plasmid was generated by using pEYFP-FYVE as a template (provided by A. Sorkin, University of Colorado Health Sciences Center). The His1371Asn mutation in EGFP-FYVE construct (pEGFP-FYVE-His1371Asn) was introduced by using a QuikChange kit (Stratagene). The pEGFP p40^{phox} PX construct was provided by W. Cho, Univ. of Illinois, Chicago. The HeLa cells were grown in DMEM (Invitrogen) supplied with 10% FBS (Sigma). The cells were transfected with pECFP-FYVE, pEGFP-FYVE-His1371Asn, or pEGFP-p40^{phox} PX by using Effectene reagent (Qiagen, Valencia, CA) 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 PBS followed by addition of buffered solutions (pH 6.0–8.5) containing 10 μg/ml nigericin (Sigma) and 10 μg/ml monensin (Sigma). The buffered solutions consisted of 120 mM KCl, 20 mM NaCl, 0.5 mM CaCl₂, 0.5 mM MgSO₄, and 25 mM one of the reagents such as Mes, 4-morpholinepropanesulfonic acid, citrate, or Hepes (Fisher Scientific). After pH equilibration for 15 min, living cells were visualized on an Axiovert 200M microscope (Zeiss) with standard ECFP and FITC filter sets (Chroma Technology, Brattleboro, VT). For apoptosis experiments, images of HeLa cells transfected with

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Abbreviations: PtdIns(3)P, phosphatidylinositol 3-phosphate; EEA1, early endosome antigen 1; HSQC, heteronuclear single quantum coherence; PtdSer, phosphatidylserine; ECFP, enhanced cyan fluorescent protein; STS, staurosporin.

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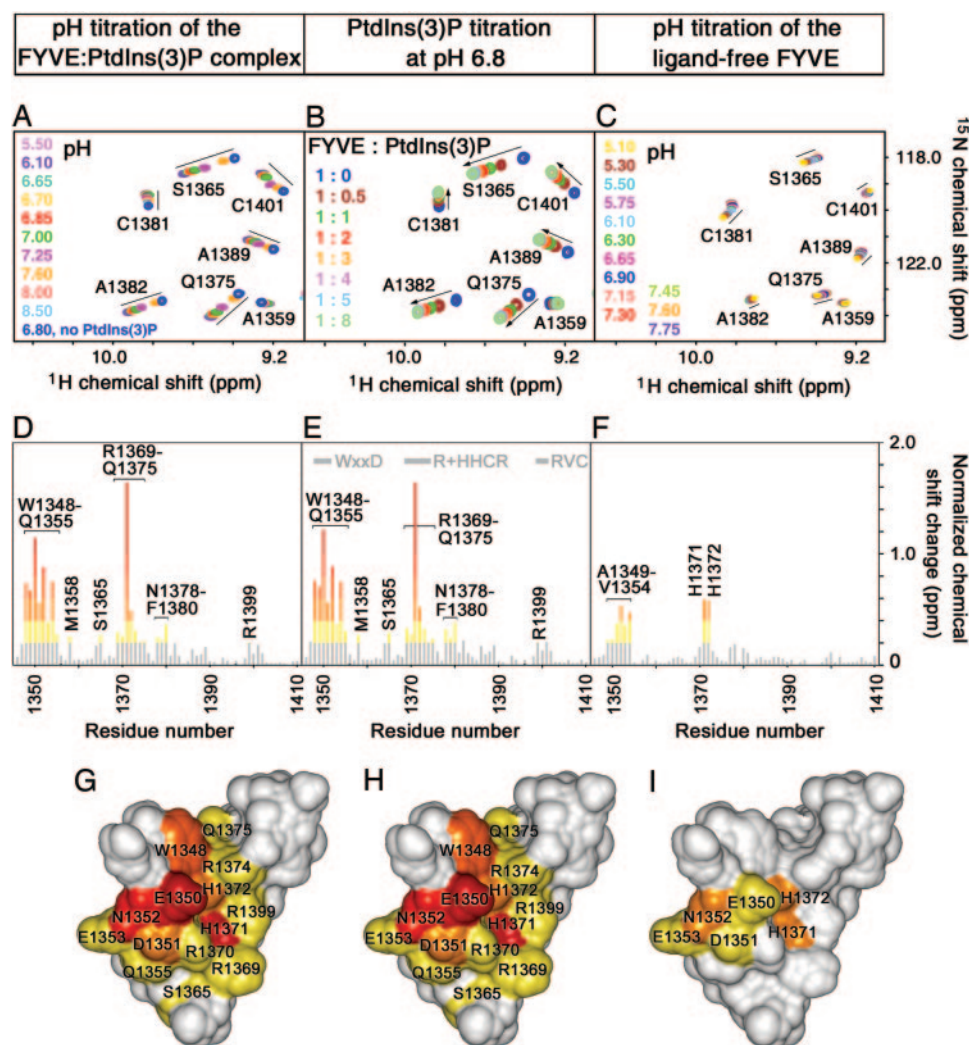


Fig. 1. pH modulates PtdIns(3)P binding. Superimposed ^1H - ^{15}N HSQC spectra of the PtdIns(3)P-bound (A) and ligand-free (C) FYVE domains collected while pH of the samples was adjusted to values shown (Insets). (B) Superimposed ^1H - ^{15}N HSQC spectra of PtdIns(3)P titration into the FYVE domain sample at a constant pH of 6.8. (D–F) Histograms show normalized (20) ^{15}N , ^1H chemical-shift changes in the FYVE domain backbone amides seen in the corresponding (A–C) spectra. The conserved sequences of the FYVE domain involved in the coordination of PtdIns(3)P are indicated by gray lines in E. (G–I) Residues that exhibit significant resonance perturbations in D–F are labeled and colored in shades of yellow, orange, and red on the FYVE domain surface.

pECFP-FYVE were acquired in DMEM-containing FBS while incubated with or without 1 μM staurosporin (STS) (Sigma) for 1 h at 33°C. Quantification of fluorescence intensities was performed with SLIDEBOOK software (Intelligent Imaging Innovations, Santa Monica, CA).

For *in vivo* localization of EGFP-fusion EEA1 FYVE proteins (wild-type, His1371Asn mutant, and His1372Asn mutant) in yeast cells, see *Supporting Text*.

Results

PtdIns(3)P Binding by the FYVE Domain Is pH-Dependent. Substantial changes in the NMR spectra of PtdIns(3)P-bound FYVE domain were induced by varying the solution pH. The most pronounced perturbations of ^1H and ^{15}N resonances were observed in the pH range of 6.0–8.0 (Fig. 1A). Furthermore, these perturbations mirrored chemical shift changes seen in the ligand-free FYVE domain as soluble C₄-PtdIns(3)P lipid was titrated in at a constant pH value of 6.8 (Fig. 1B). As the pH of the PtdIns(3)P-bound FYVE domain sample was progressively increased, all ^1H - ^{15}N crosspeaks shifted toward their positions in the ligand-free protein. At pH values >8.0, the spectrum became identical to that of the unbound FYVE domain. On the other hand, lowering the pH resulted in chemical

shift changes that converged on those of the ligand-saturated form of the protein (Fig. 1A and B; and Fig. 6, which is published as supporting information on the PNAS web site). Consequently, the ligand-bound and free states of the FYVE domain appear to be stabilized by lowering and raising the pH, respectively.

The structural effects of pH modulation on the FYVE domain were evident by mapping the chemical shift changes onto the protein's surface. The amide resonances of the WxxD, RRHHCR, and RVC motifs involved in PtdIns(3)P coordination were substantially affected by the pH adjustments (Fig. 1D). These perturbations were similar in direction and magnitude to the changes observed during PtdIns(3)P titration, where amide resonances were perturbed due to progressive increase in ligand occupancy (Fig. 1D and E).

The midpoint of pH dependency was estimated from titration curves corresponding to the amide ^1H and ^{15}N resonance changes in the complex upon varying the pH. The curves revealed a common inflection point at pH 7.4 (Fig. 7A, which is published as supporting information on the PNAS web site). This is indicative of a two-state process in which the free and PtdIns(3)P-bound populations of the FYVE domain are in balance at the average cytosolic

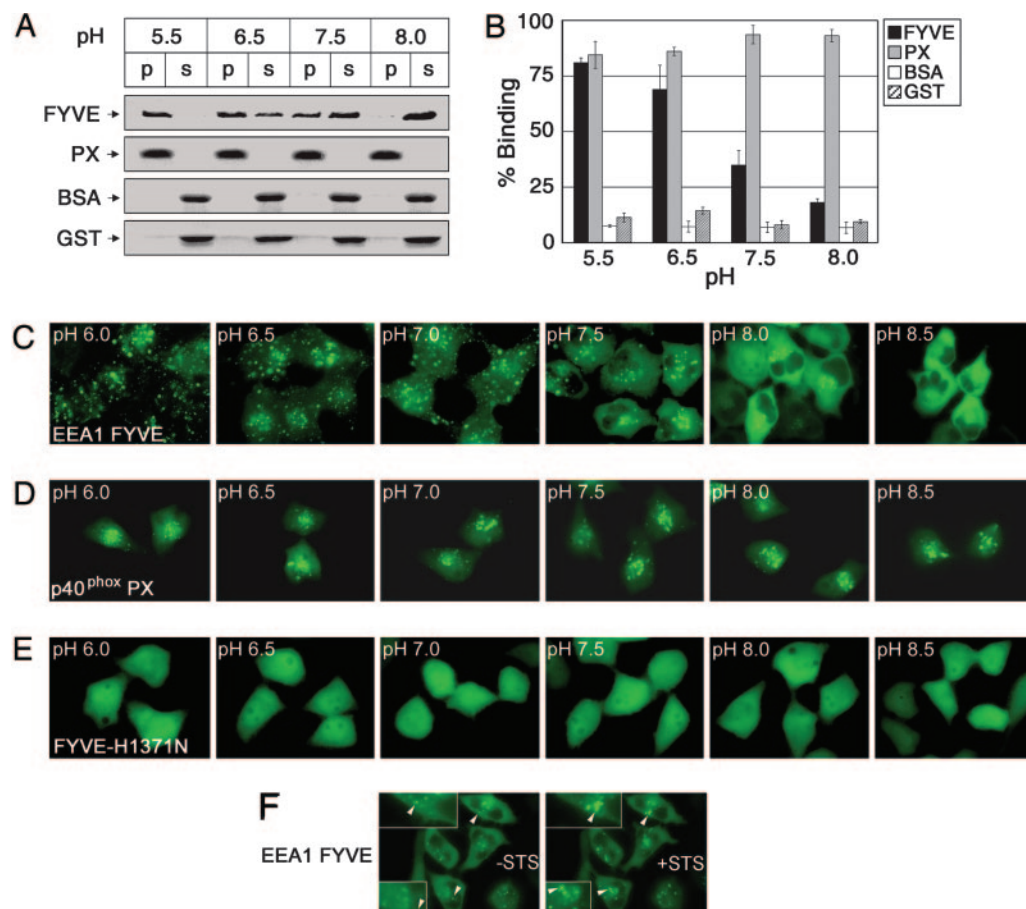


Fig. 2. pH-dependent membrane targeting by the FYVE domain. The SDS/PAGE gels (A) and histogram (B) show the partition of EEA1 FYVE, Vam7 PX, BSA, and GST between the supernatant (S) and PtdIns(3)P-enriched liposome pellet (P) at different pHs. The experimental points were averaged over three measurements. Changes in localization of the ECFP-FYVE domain (C), EGFP-p40^{phox} PX domain (D), and EGFP-FYVE-His1371Asn mutant (E) in HeLa cells upon varying the cytosolic pH. Cultures were incubated in solutions buffered to the indicated pH for 15 min. The cells were visualized by fluorescence microscopy. (F) STS-induced apoptosis and acidification of the cytosol. HeLa cells transfected with pECFP-FYVE were incubated for 1 h with or without 1 μ M STS.

pH levels. Thus, the FYVE domain is fully PtdIns(3)P-bound at pH values of 6.1 or less and is fully unligated when the pH >8.0.

To determine the intrinsic effect of pH on the FYVE domain, the ligand-free protein was examined at different pH values. Only small perturbations in ^1H and ^{15}N resonances of the unbound FYVE domain were observed as the pH was varied (Fig. 1 C and F), and the patterns of crosspeak shifts did not match those of the bound state (Fig. 1 A and C). Furthermore, pH titration of the ligand-free FYVE domain yielded widespread inflection points, conceivably corresponding to the changes in protonation states of individual residues (Fig. 7B). Most of the inflection points were clustered in the pH range of 6.5–7.0, whereas that of His1371 was depressed at 6.0. Taken together, these data suggest that varying the pH alters the protonation state of the FYVE domain residues but does not by itself induce the significant chemical shift perturbations observed in the spectra of the ligand-bound protein. We conclude that the major effect of pH on the lipid-bound FYVE domain is the modulation of PtdIns(3)P interaction.

FYVE Domain Targeting to PtdIns(3)P-Containing Membranes Is pH-Dependent. To investigate whether pH dependence is preserved for PtdIns(3)P embedded in bilayers, liposome association of the FYVE domain was assayed. The FYVE domain was incubated with small unilamellar vesicles composed of lipids normally found in endosomal membranes including phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), PtdIns, and PtdIns(3)P. After incubation at pH values of 5.5, 6.5, 7.5, or 8.0, partitioning of the FYVE domain between the supernatant and the pellet was examined by centrifugation. Only at pH 5.5 was most of the protein retained in the pelleted liposome fraction. At each progressively higher pH, the FYVE domain was increasingly distributed to the supernatant (Fig. 2 A and B). Such

pH-dependent recruitment to bilayers is fully consistent with the pH dependency of PtdIns(3)P binding, with only 35–40% of the protein being active at pH 7.5, and redefines the FYVE domain as a pH sensor that targets PtdIns(3)P-containing bilayers.

pH-Independent Recruitment of the Vam7 PX Domain. To establish whether the FYVE domain is unique in its pH sensitivity, we compared it with the only other module known to have canonical PtdIns(3)P-binding ability, the PX domain (20, 21). Unlike the FYVE domain, the Vam7 PX module localized to PtdIns(3)P-containing liposomes at all pH values tested (Fig. 2 A and B). This lack of pH-dependent targeting was supported by the absence of any significant chemical shift changes in the NMR spectra of Vam7 PX recorded at pH 6.0, 7.0, or 8.0 (Fig. 8, which is published as supporting information on the PNAS web site). Comparing the FYVE domain sequence with that of other phosphoinositide-binding proteins reveals that it is the only module with a conserved bis-histidine sequence (see below) or known pH sensitivity, suggesting a unique pH-dependent function.

Acidic Environment Enhances FYVE Domain Affinity. To determine the effect of the physiological pH range on the FYVE domain function, PtdIns(3)P binding was investigated at pH values of 6.0, 6.8, 7.4, and 8.0. The soluble C_4 -PtdIns(3)P lipid was gradually added to the FYVE domain at a constant pH, and ^1H - ^{15}N HSQC spectra were recorded and superimposed (Figs. 3 A and 1B). Large progressive changes in the amide resonances of the FYVE domain were detected at pH 6.0 or 6.8, indicating a relatively strong interaction in the fast exchange regime on the NMR timescale. However, at pH 8, an excess of PtdIns(3)P induced barely any chemical shift perturbations, implying that ligand affinity is very weak under basic conditions (Figs. 3 A and 1B).

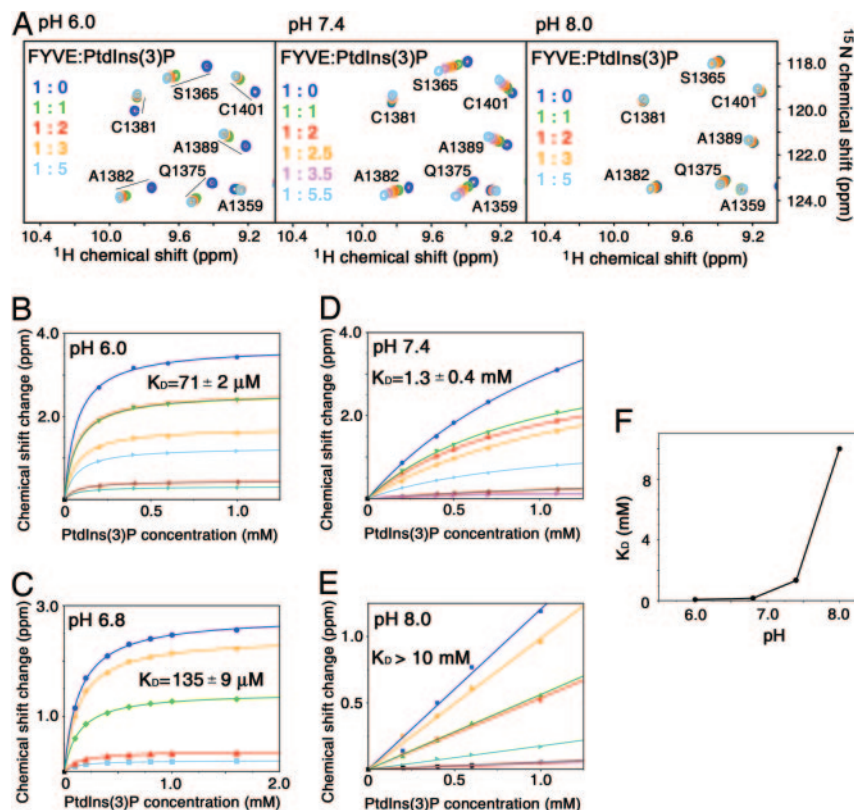


Fig. 3. Affinity for PtdIns(3)P increases in acidic media. (A) Superimposed ^1H - ^{15}N HSQC spectra of the FYVE domain recorded during addition of PtdIns(3)P at pH values 6.0, 7.4, and 8.0. The relative concentrations of the ^{15}N -labeled FYVE domain and PtdIns(3)P (Inset) are color-coded. (B–E) The binding curves used to calculate affinities for PtdIns(3)P based on changes in ^1H and ^{15}N resonances of the FYVE domain are shown and colored (B) in purple, red, green, orange, blue, brown, and cyan for ^{15}N resonances (N) of Asn1352, His1372, Ala1349, Asp1351, Arg1369, and ^1H resonances (H) of Ala1349 and Asp1351; (C) in purple, orange, green, brown, and blue for N of His1372, Asp1351, Arg1369, and H of Asp1351 and Arg1369; (D) in purple, green, red, orange, blue, cyan, brown, and magenta for N of Asn1352, Ala1349, His1372, Asp1351, Arg1369, and H of Asp1351, Ala1349, and Arg1369; and (E) in purple, orange, green, red, cyan, blue, brown, and magenta for N of His1371, Asn1352, Ala1349, His1372, and H of His1371, Ala1349, His1372, and Asn1352, respectively. (F) A graph summarizes the dependence of K_d on pH.

Binding affinities were determined by analyzing changes in amide resonances of the FYVE domain. At pH 6.0, C_4 -PtdIns(3)P was bound by the FYVE domain with a K_d of $71 \pm 2 \mu\text{M}$ (Fig. 3B). However, the same interaction was twice as weak at pH 6.8, 20 times weaker at pH 7.4, and 150 times weaker at pH 8.0, yielding affinities of $135 \pm 9 \mu\text{M}$, $1.3 \pm 0.4 \text{ mM}$, and $>10 \text{ mM}$, respectively (Fig. 3C–E). The dependence of PtdIns(3)P affinity on pH is summarized in Fig. 3F, which shows a significant increase of FYVE activity in acidic environments, whereas under basic conditions, the protein becomes inactive.

The FYVE Domain Localizes to Endosomes at Low Cytosolic pH. To examine the role of intracellular pH in the targeting of FYVE domain *in vivo*, ECFP-fusion EEA1 FYVE was expressed in HeLa cells that were briefly incubated in media buffered to various pH values (Fig. 2C). Mammalian cells maintain an intracellular pH of ≈ 7.3 (23); however, cytosolic pH can be manipulated by changing the pH of the medium (24). After incubation of the ECFP-FYVE-expressing cells at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5, protein localization was examined by fluorescence microscopy. In media buffered to pH 6.0, ECFP-FYVE was strongly anchored to endosomal membranes (Fig. 2C). When the pH of the media was progressively increased, the ECFP signal of the membrane-associated FYVE domain was substantially diminished and a concomitant increase in the diffuse cytosolic ECFP-FYVE signal was observed. In basic conditions, ECFP-FYVE appeared completely released from the membranes. The most significant changes in FYVE domain localization occurred in the physiological pH range of 6.5–7.5. In contrast, the EGFP-p40^{phox} PX domain was similarly bound to endosomal membranes at all pH values (Fig. 2D). Thus, the EEA1 FYVE domain localizes to endosomal compartments at low cytosolic pH, whereas at high pH values, the protein is dispersed in the cytosol.

To test the physiological relevance of pH sensing by the FYVE domain, the localization of ECFP-FYVE during apoptosis-induced cytosol acidification in HeLa cells was investigated. The cells were

treated with the kinase inhibitor STS, which induces apoptosis and decreases cytosolic pH by 0.4 units (25). Although in the untreated cells the ECFP-FYVE domain was equally distributed between the cytosolic and endosome-bound fractions, STS treatment substantially shifted the equilibrium toward the membrane-bound state of the protein (Fig. 2F). These *in vivo* data corroborate the *in vitro* experiments and suggest that recruitment of the FYVE domain to membranes can be regulated by intracellular pH.

The Protonated State of a Histidine Switch Is Required for Interaction. The pH value of 7.4, where the free and PtdIns(3)P-bound states of the FYVE domain are in balance, is near the pK_a range of His residues. The FYVE domain contains absolutely conserved His1371 and His1372 residues, which are located in the RRHCR motif (Fig. 4A) and are intrinsically pH-sensitive (Fig. 1F). Another residue, His1340, is not conserved and is located in a 23-residue sequence N-terminal to the domain. Consequently, we investigated whether PtdIns(3)P binding is modulated by the protonation of these His residues.

The microscopic ionization constants of the three His residues of the FYVE domain were determined by NMR. The chemical shifts of imidazole's $\delta 2\text{H}$ and $\epsilon 1\text{H}$ in the ligand-free FYVE domain were monitored while the pH of the sample was varied. The pK_a values of the His1340, His1371, and His1372 side chains were found to be 6.5 ± 0.1 , 5.9 ± 0.1 , and 6.5 ± 0.1 , respectively (Fig. 4C). However, in the presence of the ligand, the pK_a of both His1371 and His1372 increased to 7.4 ± 0.1 , whereas the pK_a of His1340, which is not a part of the FYVE domain, remained essentially unchanged at 6.3 (Fig. 4D). Thus, the new PtdIns(3)P recognition mechanism includes the protonation first of His1372 then of His1371, yielding two additional positive charges in the binding pocket. The active doubly protonated form of the FYVE domain predominates at pH values <5.9 in the absence of ligand or <7.4 when stabilized by bound ligand. These results, being in good agreement with the crystal and solution structures of the EEA1 FYVE domain (6, 13), also imply that the imidazole side chain of His1371 must be protonated to form

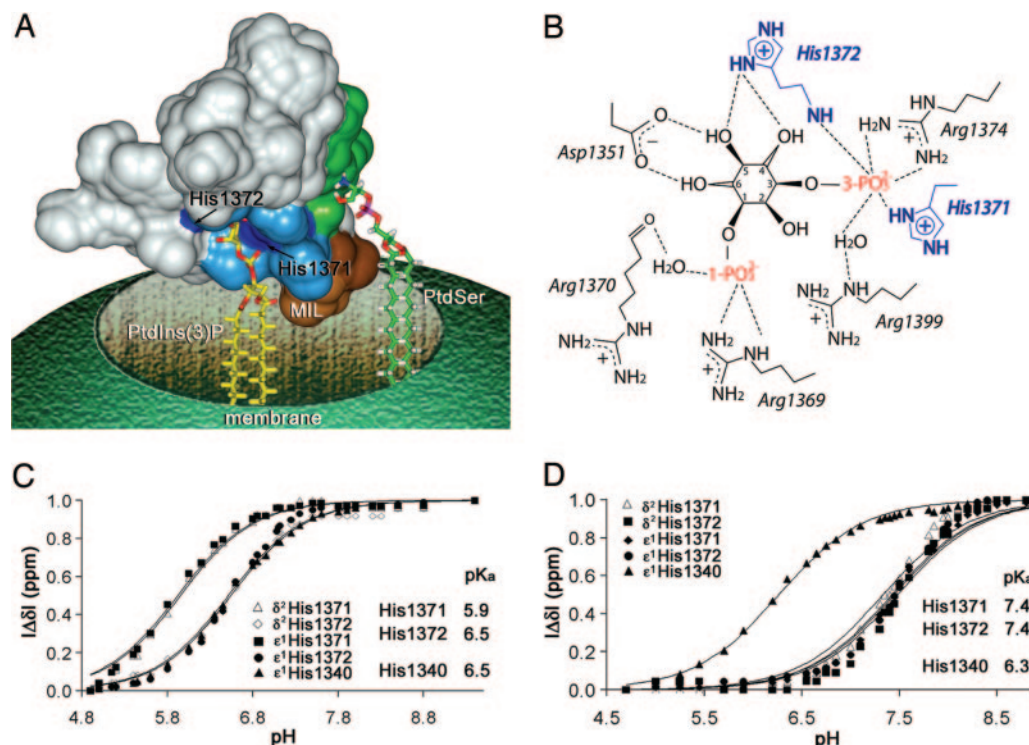


Fig. 4. Protonated His residues are required for the interactions. (A) A model of FYVE domain targeting to PtdIns(3)P-enriched membranes. The FYVE domain binds PtdIns(3)P, inserts its hydrophobic loop (MIL) into the membrane interior, and electrostatically contacts PtdSer. The conserved His and Arg residues of the PtdIns(3)P-binding pocket, as well as MIL and the PtdSer-binding site, are colored in blue, light blue, brown, and green, respectively. PtdIns(3)P and PtdSer are shown as stick models. (B) Schematic diagram showing the PtdIns(3)P head group coordination based on the crystal structure of the EEA1 FYVE domain complexed with 1,3-inositol bisphosphate (6). (C and D) Determining the microscopic ionization constants of His1340, His1371, and His1372 in the absence (C) and in presence (D) of PtdIns(3)P by monitoring perturbations of ^1H resonances in NMR spectra.

a hydrogen bond to the 3-phosphate group of PtdIns(3)P (Fig. 4B). Similarly, protonated His1372 forms a pair of stabilizing hydrogen bonds to the 4- and 5-hydroxyl groups of PtdIns(3)P (Fig. 4B). Thus, we conclude that the successive dual protonation of His1372 and 1371 is required for coordination of the PtdIns(3)P headgroup by the FYVE domain.

The Presence of His1371 and His1372 Is Necessary for FYVE Domain Function. The critical role of His1371 and His1372 for PtdIns(3)P recognition was confirmed by mutagenesis. Substitution of these residues with Ala, Arg, Asn, Lys, or Tyr abolished PtdIns(3)P binding *in vitro* at any pH, as judged by NMR titrations (Fig. 9, which is published as supporting information on the PNAS web site). This indicates that both His residues are necessary for the interaction, and no residue with similar basic or aromatic character or hydrogen-bonding potential will suffice in either position. Furthermore, lowering the pH of the NMR samples of His1371 mutant proteins resulted in the collapse of the tertiary structure, whereas the fold of His1372 mutants was not disturbed (data not shown). Thus, although both conserved histidines are functionally essential, His1371, located in the center of the first β strand, is also required for protein structural stability.

To test whether His residues are necessary for the EEA1 targeting to endosomes, *in vivo* localization of ECFP- and EGFP-fusion FYVE proteins was examined by fluorescence microscopy. The wild-type fusion FYVE domain localized predominantly to endosomes in mammalian and yeast cells, whereas EGFP-fusion His1371Asn and His1372Asn mutant proteins were dispersed into the cytosol (Fig. 2E and Fig. 10, which is published as supporting information on the PNAS web site). These data reveal the importance of both histidine residues for the biological function of the FYVE domain.

Hydrophobic Insertion and PtdSer Interaction Are pH-Independent. Because the FYVE domain docking to membranes involves hydrophobic insertion into the bilayer (13) and nonspecific electrostatic contacts with acidic lipids (4), the effect of pH on these synergistic interactions was investigated (Fig. 11, which is published as supporting information on the PNAS web site). We found that the

insertion of the PtdIns(3)P-bound FYVE domain into PtdSer-containing dodecyl phosphocholine (DPC) micelles was not pH-dependent, yielding similar affinities of 0.45 ± 0.05 and 0.44 ± 0.1 mM at pH values of 6.0 and 6.8. Nonspecific binding to PtdSer-containing DPC micelles in the absence of PtdIns(3)P was also pH-insensitive, based on a similar K_d of 7.3 ± 2 and 7.0 ± 1.4 mM at pH 6.0 and 6.8, respectively. However, pH dependency of PtdIns(3)P recognition was apparent for the micelle-associated state of the FYVE domain. That is, the affinities of the DPC-saturated FYVE domain for C₄-PtdIns(3)P at pH 6 and 6.8 were estimated to be 15 ± 2 and 25 ± 3 μM , respectively. This 40% enhancement of binding was similar to the increase of the FYVE affinity for PtdIns(3)P in the absence of micelle systems.

Discussion

Our results reveal that the EEA1 FYVE domain is recruited to PtdIns(3)P-enriched membranes in a pH-dependent manner. PtdIns(3)P is present in endosomal membranes at concentrations of ≈ 200 μM (26). Based on estimated affinities and the lipid's physiological concentrations, the FYVE domain exists in a mainly bound state in a low pH environment (pH 6.0–6.6). At the cytosolic pH level of 7.3, only half of the protein would be active, whereas essentially no activity would be detected in basic conditions. Thus, we predict that the FYVE domains exist in a balance between diffuse cytosolic and membrane-anchored populations and are sensitive to changes in both PtdIns(3)P concentrations and acidity within the cell.

pH dependence can influence the functions of FYVE proteins in cells with unusual cytosolic pH levels and in normal cells during physiological processes that involve changing of pH. Intracellular pH often fluctuates in response to cell growth, development, and apoptosis, with pH values ranging from 6.3 to 7.5 (27–30), and varies in anomalous cells during ischemia (31), inflammation (32), and cancer (33). For example, cytosol acidification by as much as 0.8 pH units is associated with apoptosis (30), whereas several growth and survival factors induce alkalization of cytosol by 0.25 pH units (34). Fluctuations of cytosolic pH in abnormal cells appear to be wider. Intracellular pH during ischemia drops to 6.2, whereas the

