The Molecular Basis of the Differential Subcellular Localization of FYVE Domains*

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This study systematically analyzed the structural and mechanistic basis of the regulation of subcellular membrane targeting using FYVE domains as a model. FYVE domains, which mediate the recruitment of signaling and membrane-trafficking proteins to phosphatidylinositol 3-phosphate-containing endosomes, exhibit distinct subcellular localization despite minor structural variations within the family. Biophysical measurements, cellular imaging, and computational analysis of various FYVE domains showed that the introduction of a single cationic residue and a hydrophobic loop into the membrane binding region of the FYVE domains dramatically enhanced their membrane interactions. The results indicated that there is a threshold affinity for endosomal localization and that endosomal targeting of FYVE domains is sensitive to small changes in membrane affinity about this threshold. Collectively these studies provide new insight into how subcellular localization of FYVE domains and other membrane targeting domains can be regulated by minimal structural and environmental changes.

Numerous cellular processes such as signal transduction, vesicle trafficking, and cytoskeletal rearrangement require the exquisite targeting of peripheral proteins to various subcellular membranes. A large portion of this cellular membrane targeting is achieved by specific recognition of particular membrane lipids by proteins. A diverse group of membrane-targeting domains that specifically recognize different types of membrane lipids have been identified in the past decade. They include Bin Amphiphysin FYVE (BAR) (1), protein kinase C Conserved 1 (C1) (2, 3), protein kinase C Conserved 2 (C2) (4), Epsin Amino-Terminal Homology (ENTH) (5), Band 4.1/Ezrin/Radixin/Moesin (FERM) (6), Fab1/YOTB/Vac1/EEA1 (FYVE) (7–11), Postsynaptic density-95/Discs large/ZO-1 (PDZ) (12), Pleckstrin Homology (PH) (13), Phosphotyrosine Binding (PTB) (14), Phox (PX) (15), Src homology 2 (SH2) (16), and tubby domains (17). Except for the C1 domain that binds diacylglycerol, these domains, which mediate the recruitment of signaling and membrane-trafficking proteins to phosphatidylinositol 3-phosphate-containing endosomes, exhibit distinct subcellular localization despite minor structural variations within the family. Biophysical measurements, cellular imaging, and computational analysis of various FYVE domains showed that the introduction of a single cationic residue and a hydrophobic loop into the membrane binding region of the FYVE domains dramatically enhanced their membrane interactions. The results indicated that there is a threshold affinity for endosomal localization and that endosomal targeting of FYVE domains is sensitive to small changes in membrane affinity about this threshold. Collectively these studies provide new insight into how subcellular localization of FYVE domains and other membrane targeting domains can be regulated by minimal structural and environmental changes.

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† The abbreviations used are: PtdIns(3)P, phosphatidylinositol 3-phosphate; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; Ins(1,3)P 2, inositol 1,3-bisphosphate; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-sn-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol, collectively known as phosphoinositides. Although much is known about the structural basis of stereospecific lipid head group recognition by these domains, less is known about the mechanisms by which they achieve efficient and reversible binding to the cell membranes containing their lipid ligands. In particular, there is much to learn about the structural and mechanistic basis of the regulation of the targeting of lipid-interacting domains to different intracellular membranes. Among various membrane-targeting domains, the FYVE domain serves as an excellent model to address these questions. This is because FYVE domains from different proteins exhibit drastically different subcellular localization behaviors (18–22) despite the fact almost all FYVE domains show high specificity and affinity for phosphatidylinositol 3-phosphate (PtdIns(3)P).

FYVE domains are zinc-containing modules of 60–80 amino acid residues (7–11). As expected from the endosomal localization of PtdIns(3)P and its role in vesicle trafficking, a large number of FYVE domain-containing proteins, including EEA1, Hrs, and FENS-1, are involved in endocytic vesicle trafficking. Some FYVE domain-containing proteins also function in cytoskeletal regulation (facio-genital dysplasia 1) (23) and growth factor signaling (SARA (22) and endofin (19)). Sequence alignment of FYVE domains (see Fig. 1) reveals several consensus motifs. High resolution structures of three different FYVE domains have illustrated how some of these conserved residues are involved in specific recognition of the PtdIns(3)P head group (24–26). Structural and biophysical studies have indicated that several factors beside PtdIns(3)P binding contribute to the membrane affinity of FYVE domains. They include non-specific electrostatic interactions between basic protein residues and the anionic membrane surface (27–29), hydrophobic interactions achieved by the partial membrane insertion of the residues located in the loop (so-called turret loop, see Fig. 1) near the PtdIns(3)P-binding pocket (24, 25, 27, 29, 30), and FYVE domain dimerization (21, 25, 26). Mutational studies of many FYVE domain-containing proteins have indicated that FYVE domains play an important role in their binding to endosomes in the cell (21, 31, 32). Interestingly, however, among many FYVE domains identified so far, only FENS-1...
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(20), endoﬁn (19), and SARA (22) FYVE domains have been reported to autonomously translocate to endosomal membranes when expressed ectopically. It is therefore unclear as to how these three isolated domains achieve unique endosomal targeting properties and how FYVE domains, in general, contribute to the membrane targeting of their host proteins in different ways.

In this study, we performed in vitro membrane binding measurements, subcellular localization measurements, and computational analyses of the FYVE domains of FENS-1, endoﬁn, Hrs, Vps27p, and their respective mutants to elucidate the structural and mechanistic basis of unique endosomal targeting behaviors of the FENS-1 and endoﬁn FYVE domains. These studies provide new insight into how distinctively different subcellular localization behaviors of FYVE domains are caused by minor structural variations in the membrane binding region and how subcellular localization of these membrane targeting domains can be regulated by minimal structural and environmental changes.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), -phosphatidylserine (POPS), and -phosphatidylethanolamine (POPE) were purchased from Avanti Polar Lid. Inositol 1,3-bisphosphate (Ins(1,3)P2) was from A. G. Scientiﬁc. PtdIns(3)P, phosphatidylinositol 4-phosphate (PtdIns(4)P), and phosphatidylinositol 5-phosphate (PtdIns(5)P) were synthesized as described previously (33). The Pioneer L1 sensor chip was from Biacore AB. The human embryonic kidney 293 cell line, Zeocin, and pANPLA 1.0 were from Invitrogen.

Mutagenesis and Protein Expression—Mutations of FYVE domains were performed by the overlap extension polymerase chain reaction method (34). Each construct was subcloned into the pGEX-4T-1 vector containing an N-terminal glutathione S-transferase fusion and transformed into Escherichia coli BL21 cells for protein expression. Recombinant FYVE domains were expressed and puriﬁed as described previously (27). The genes for FYVE domains and mutants were also subcloned into a modiﬁed pIND vector to contain a C-terminal enhanced green ﬂuorescent protein (EGFP) construct as described previously (35).

Fluorimetric Binding Measurements—The kinetic SPR measurements were performed at 23 °C using a mid-coated L1 chip in the BIACORE X system as described previously (36). All data were analyzed using Biacore Evaluation 3.0 software (Beckman). Samples were loaded into six-channel Epon charcoal-ﬁlled centerpieces using quartz windows. Measurements were done at 25 °C using four different speeds (16,000, 18,800, 23,500, and 31,000 rpm), detecting at 280 nm, with sufﬁcient time for equilibrium to be reached in all speed increases. All FYVE domains (10, 20, and 30 μM) were performed in 20 mM HEPES, pH 7.4, 0.16 M KCl, which was also used as the reference buffer. Solvent density was calculated to be 0.9892 g/ml. From the amino acid composition of each FYVE domain, the partial speciﬁc volume was estimated to be 0.719 (endoﬁn-FYVE), 0.715 (FENS-1-FYVE), and 0.712 (Hrs-FYVE) mg/l. Data were analyzed with the program BioSensorScan 6.2 software (Beckman) to determine the maximum binding (max) and dissociation constants (Kd) as described previously (27).

Structural Modeling—The Drosophila Hrs FYVE domain (25) was used as the structural template for building homology models for both endoﬁn and FENS-1 FYVE domains. The alignment between the endoﬁn and Hrs sequences and the homology model were both constructed with the automated homology model server 3D-Jigview (39). The sequence identity between the endoﬁn and Hrs FYVE domains is 42%. The alignment between the FENS-1 and Hrs FYVE domains was extracted from a multiple alignment of FYVE domain sequences constructed with ClustalW (40); the sequence identity between the two FYVE domains is 36%. According to the alignment, the FENS-1 FYVE domain has an 11-residue insertion in the vicinity of the hydrophobic motif, which is conserved across FYVE domains and occurs immediately before the first β-strand observed in the FYVE domain structure (22). The alignment between the FENS-1 and Hrs FYVE domains was used to construct models for the FENS-1 FYVE domain based on the alignment extracted from the multiple sequence alignment. We implemented the loop prediction modules that are offered by both programs (45, 46). Modeler6 predicted a fairly extended loop, while Nest predicted a more compact loop (Fig. 1, a representative example of which is given in Fig. 2). The insert predicted by Nest scored better according to the structure evaluation tool Verify3D (47). Overall, however, the analysis suggests that the insert is disordered, and its conformation may be affected by its interaction with the membrane surface. Electrostatic potential calculations were performed as described previously (27).

Cell Culture and Microscopy—A stable human embryonic kidney 293 cell line expressing the ecdysone receptor (Invitrogen) was used for all experiments as described previously (35). Microscopy data were collected on a Zeiss LSM 510 laser scanning confocal microscope. A 40×, 1.2 numerical aperture immersion objective was used for all experiments. Transfected cells were washed twice with 1 mM HEPES, buffer pH 7.4, containing 2.5 mM MgCl2, 140 mM NaCl, 5 mM KCl, and 6 mM sucrose. After washing, cells were overlaid with 150 μl of the same buffer. Suitable cells were selected for imaging, and a single image was taken. In control experiments, cells were treated with 100 nM wortmannin for 30 min to determine the effects of phosphatidylinositol 3-kinase inhibition on FYVE domain subcellular localization. For colocalization experiments, transfected cells expressing EGFP-FYVE domain constructs were incubated with 25 μg/ml human transferrin conjugated to Alexa Fluor® 633 (Molecular Probes) for 30 min at 37 °C. Cells were then washed three times with the above buffer to remove excess dye and subjected to dual channel imaging. EGFP and Alexa Fluor 633 were excited optimally at 488 and 633 nm, respectively, and emission was measured using a band pass 505–530 nm filter for EGFP and a line pass 650 nm filter for Alexa Fluor 633 on separate channels.

RESULTS

Structural Properties of FYVE Domains—When compared with other FYVE domains, FENS-1 (20), endoﬁn (19), and SARA (22) FYVE domains have some unique structural features (see Fig. 1). SARA-FYVE and endoﬁn-FYVE have highly

| Vps27p | 163 | DSRVPADMD-DGDA-COHGKX------ | 3LLK | RXXCR 193 |
| Hrs | 154 | WAPAAA-DGSH-CHRKEYY------- | 4TPF | RXXCR 181 |
| Endoﬁn-1 | 126 | HDABQAV-EEAFLQGKX--------- | 267L | RXXCR 856 |
| Endoﬁn | 739 | DRQKWTPNVEPAHPCMKQX------- | 7TPK | RXXCR 770 |
| SARA | 1374 | GMLQWAFEDRTDPSGNMEG------- | 15UNF | RXXCR 656 |

Fig. 1. Amino acid sequence alignment of FYVE domains. The loop turn is shown in a box, and the putative dimer interface region of SARA and EEA1 FYVE domains is underlined. Residues of FENS-1 and endoﬁn FYVE domains are shown in boldface characters.
homologous primary structures, and both contain a unique Lys residue in the turret loop, which is a Val in EEA1 and an Asn in Vps27p and Drosophila Hrs. FENS-1-FYVE has an 11-amino acid insertion next to the turret loop region. To understand how these unique structural properties of the three FYVE domains affect their membrane binding, we first performed structural modeling of the FYVE domains of endofin and FENS-1 based on the sequence homology to other FYVE domains with known tertiary structure (i.e. Vps27p, Hrs, and EEA1). We also calculated the electrostatic potentials for four FYVE domains used in this study. Since endofin-FYVE and SARA-FYVE are structurally similar, we characterized only endofin-FYVE in this study.

Structural modeling suggests that endofin-FYVE (Fig. 2, first row) should have essentially the same structure as the Hrs and Vps27p FYVE domains (Fig. 2, third and fourth rows). However, it has a dramatically stronger positive potential than the other two FYVE domains with an overall charge of +13 compared with that of +7 for Vps27p and +10 for Hrs (Fig. 2, compare row 1 with rows 3 and 4). Thus, this FYVE domain may achieve high affinity for the membrane through enhanced nonspecific electrostatic interactions with anionic lipids in the membrane in addition to its specific interaction with PtdIns(3)P. The FYVE domain sequence alignment (Fig. 1) as well as homology models (Fig. 2, second row) suggest that FENS-1-FYVE has an extended turret loop due to the 11-amino acid extension, which is rich in aromatic residues (Fig. 2, second row, green). It has been shown that aromatic residues, such as two Trp residues in FENS-1-FYVE turret loop, play an important role in membrane-protein interactions (36) and that the turret loop of the Hrs and Vps27p FYVE domains partially penetrates PtdIns(3)P-containing membranes and thereby increases membrane affinity (27). Thus, it is expected that FENS-1-FYVE will have an exceptional ability to penetrate the membrane and exhibit high membrane affinity. A model structure of FENS-1-FYVE in Fig. 2 has a positive electrostatic potential profile in the membrane binding region that is comparable to those of the Hrs and Vps27p FYVE domains (Fig. 2, compare row 2 with rows 3 and 4), suggesting that its nonspecific electrostatic interactions with the membrane would be similar to those of the other two FYVE domains.

In Vitro Membrane Binding Properties of FYVE Domains—To test whether the FYVE domains of endofin and FENS-1 have the predicted membrane binding properties, we characterized their in vitro membrane binding by two independent methods. First, we measured the interaction of differ-
ent FYVE domains with phospholipid monolayers at the air-water interfaces that serve as a highly sensitive probe of the membrane penetrating capability of proteins (37, 48). In this study, a monolayer composed of POPC/POPE/POPS/PtdIns(3)P (63:20:15:2), which simulates the lipid composition of early endosomes, of a given surface pressure (π0) was spread at constant area, and the change in surface pressure (∆π) was monitored after the injection of FYVE domains into the subphase. In general, ∆π is inversely proportional to π0 of the phospholipid monolayer, and an extrapolation of ∆π versus π0 yields πe, which specifies an upper limit to the value of π0 of a monolayer into which a protein can penetrate (37, 48).

Fig. 3A shows that FENS-1-FYVE has significantly higher monolayer penetrating power (πe ~ 34 dynes/cm) than Vps27p and Hrs FYVE domains (πe ~ 28–29 dynes/cm). Intriguingly endofin-FYVE also exhibited high monolayer penetration (πe ~ 34 dynes/cm) (Fig. 3B), which is comparable to that of FENS-1-FYVE. Since the surface pressures of biological membranes have been estimated to be in the range of 31–35 dynes/cm (49–51), the observed differences in πe suggest that, if endosomal membranes contain 2 mol % PtdIns(3)P, FENS-1-FYVE and endofin-FYVE significantly penetrate endosomal membranes, while Vps27p and Hrs FYVE domains do not (27). The effect of PtdIns(3)P was specific because 2 mol % PtdIns(4)P or PtdIns(5)P had no significant effect on the monolayer penetration of all these proteins. The subphase contained 20 mM Tris buffer, pH 7.4 containing 0.16 M KCl.

**Fig. 3.** Monolayer penetration of FYVE domains and mutants at different initial surface pressure. A, the FENS-1 FYVE domain (●), Δ295–306 (∆), and F295A/W296A/W302A (■) were allowed to interact with the POPC/POPE/POPS/PtdIns(3)P (63:20:15:2) monolayer. B, the endofin FYVE domain (○), K759A (□), F762A (■), K764A (▲), and R783A/K784A/K786A (△) were allowed to interact with the POPC/POPE/POPS/PtdIns(3)P (63:20:15:2) monolayer. C, the Hrs FYVE domain (○), N175K (△), and the 11-amino acid insertion mutant (□) were allowed to interact with the POPC/POPE/POPS/PtdIns(3)P (63:20:15:2) monolayer. The Vps27p FYVE domain (●), N187K (∆), and the 11-amino acid insertion mutant (■) were also used. 2 mol % PtdIns(4)P or PtdIns(5)P had a negligible effect on the monolayer penetration of all these proteins. The subphase contained 20 mM Tris buffer, pH 7.4 containing 0.16 M KCl.

**Fig. 4.** SPR binding analysis of FENS-1-FYVE Δ295–306. A, sensorgrams from kinetic measurements. FENS-1-FYVE Δ295–306 of varying concentrations (6, 15, 30, 60, and 180 nM) was injected at 30 μl/min, and the subsequent association to and dissociation from the POPC/POPE/POPS/PtdIns(3)P (63:20:15:2) monolayer. B, the FENS-1 FYVE domain (○), K759A (□), F762A (■), K764A (▲), and R783A/K784A/K786A (△) were allowed to interact with the POPC/POPE/POPS/PtdIns(3)P (63:20:15:2) monolayer. The Vps27p FYVE domain (●), N187K (∆), and the 11-amino acid insertion mutant (■) were also used. 2 mol % PtdIns(4)P or PtdIns(5)P had a negligible effect on the monolayer penetration of all these proteins. The subphase contained 20 mM Tris buffer, pH 7.4 containing 0.16 M KCl.
membrane dissociation of the FYVE domains of FENS-1 and endofin than that of the Vps27p FYVE domain is also consistent with their greater membrane penetrating power.

To determine whether the higher affinity of FENS-1-FYVE and endofin-FYVE for PtdIns(3)P-containing membranes could be due to a higher intrinsic affinity for PtdIns(3)P or not, we measured the affinity of the FYVE domains of Vps27p, FENS-1, and endofin for a water-soluble derivative of PtdIns(3)P, Ins(1,3)P$_2$. As shown in Fig. 5, all three FYVE domains have comparable affinity for Ins(1,3)P$_2$. Therefore, the higher membrane affinity of FENS-1-FYVE and endofin-FYVE derives not from their higher affinity for PtdIns(3)P per se but from their enhanced ability to interact with PtdIns(3)P-containing membranes.

Last, we performed analytical ultracentrifugation to see whether high membrane affinity of FENS-1-FYVE and endofin-FYVE is related to their tendency to form dimers or larger aggregates in solution. When FENS-1, endofin, and Hrs FYVE domains were loaded into cells at 10, 20, and 30 nM, and for FENS1-FYVE. Similarly reduced membrane binding surface. Finally we mutated Vps27p and endofin-FYVE for a water-soluble derivative of PtdIns(3)P, Ins(1,3)P$_2$. As shown in Fig. 5, all three FYVE domains have comparable affinity for Ins(1,3)P$_2$. Therefore, the higher membrane affinity of FENS-1-FYVE and endofin-FYVE derives not from their higher affinity for PtdIns(3)P per se but from their enhanced ability to interact with PtdIns(3)P-containing membranes.

As shown in Table I, the FYVE domains of FENS-1 and endofin, respectively, showed 58- and 27-fold higher affinity than the Vps27p FYVE domain. This exceptional membrane affinity derives from both faster membrane association (i.e. larger $k_a$) and slower membrane dissociation (i.e. smaller $k_d$). Our previous SPR studies of membrane-protein interactions have shown that $k_a$ is enhanced by long range electrostatic interactions, whereas $k_d$ is reduced by short range specific interactions and hydrophobic interactions (achieved by membrane penetration) (36). It was also shown that the membrane interactions by aromatic residues, Trp in particular, increase $k_a$ with a smaller decreasing effect on $k_d$ (36), although the mechanism underlying this complex effect is not fully understood. In accordance with these findings, the Trp-rich turret loop of FENS-1-FYVE and the cationic surface of endofin-FYVE greatly accelerate their membrane association (10–16-fold increase in $k_a$). The significantly slower (~3-fold increase in $k_d$) membrane dissociation of the FYVE domains of FENS-1 and endofin than that of the Vps27p FYVE domain is also consistent with their greater membrane penetrating power.

![Fig. 5. FYVE domain-Ins(1,3)P$_2$ binding isotherms.](image)

**Membrane Targeting of FYVE Domains**

| Proteins      | $k_a$ ($M^{-1}s^{-1}$) | $k_d$ ($s^{-1}$) | $K_d$ ($M$) | Relative affinity$^a$ | $A / M_{max}$ |
|---------------|------------------------|-----------------|-------------|-----------------------|--------------|
| FENS-1 WT     | (5.6 ± 0.8) × 10$^5$   | (3.1 ± 0.2) × 10$^{-4}$ | (5.5 ± 1.0) × 10$^{-10}$ | 1         |
| FENS-1 WT-GFP | (5.3 ± 0.7) × 10$^5$   | (3.2 ± 0.3) × 10$^{-4}$ | (6.0 ± 0.8) × 10$^{-10}$ | 0.9       |
| FENS-1 A$_{295-306}$ | (3.1 ± 0.2) × 10$^4$ | (1.3 ± 0.3) × 10$^{-3}$ | (4.2 ± 1.0) × 10$^{-6}$ | 1/78        |
| FENS-1 E926A/W926A/W302A | (8.6 ± 0.9) × 10$^4$ | (9.3 ± 0.3) × 10$^{-4}$ | (1.1 ± 0.1) × 10$^{-6}$ | 1/20        |
| Endofin WT    | (3.1 ± 0.2) × 10$^5$   | (3.7 ± 0.2) × 10$^{-4}$ | (1.2 ± 0.1) × 10$^{-4}$ | 1        |
| Endofin WT-GFP| (2.9 ± 0.5) × 10$^5$   | (3.2 ± 0.4) × 10$^{-4}$ | (1.1 ± 0.2) × 10$^{-4}$ | 1.1       |
| Endofin F762A | (9.5 ± 0.6) × 10$^4$   | (7.5 ± 0.1) × 10$^{-3}$ | (8.1 ± 1.3) × 10$^{-6}$ | 1/7        |
| Endofin K764A | (6.5 ± 0.5) × 10$^4$   | (8.0 ± 0.4) × 10$^{-3}$ | (1.2 ± 0.1) × 10$^{-6}$ | 1/10       |
| Endofin K759A | (1.2 ± 0.5) × 10$^5$   | (4.1 ± 0.8) × 10$^{-4}$ | (3.4 ± 1.6) × 10$^{-6}$ | 1/3        |
| Endofin R783A/K784A/K786A | (2.1 ± 0.8) × 10$^5$ | (9.4 ± 0.7) × 10$^{-4}$ | (4.5 ± 1.7) × 10$^{-6}$ | 1/4        |
| Hrs WT        | (4.3 ± 0.2) × 10$^4$   | (1.1 ± 0.3) × 10$^{-3}$ | (2.5 ± 0.5) × 10$^{-4}$ | 1        |
| Hrs N175K     | (4.2 ± 0.5) × 10$^5$   | (2.9 ± 0.6) × 10$^{-4}$ | (6.9 ± 1.6) × 10$^{-10}$ | 36        |
| Hrs 11-aa insertion | (3.0 ± 0.9) × 10$^5$ | (6.6 ± 1.0) × 10$^{-4}$ | (2.2 ± 0.7) × 10$^{-4}$ | 11        |
| Vps27p WT     | (3.4 ± 0.5) × 10$^4$   | (1.1 ± 0.3) × 10$^{-3}$ | (3.2 ± 1.0) × 10$^{-6}$ | 1        |
| Vps27p N187K  | (2.6 ± 0.7) × 10$^5$   | (4.0 ± 0.4) × 10$^{-4}$ | (1.5 ± 0.4) × 10$^{-6}$ | 21        |
| Vps27p 11-aa insertion | (2.3 ± 0.5) × 10$^5$ | (6.8 ± 1.8) × 10$^{-4}$ | (2.9 ± 1.0) × 10$^{-6}$ | 11        |

$^a$-Fold decrease in $K_d$ with respect to their wild types.

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Last, we performed analytical ultracentrifugation to see whether high membrane affinity of FENS-1-FYVE and endofin-FYVE is related to their tendency to form dimers or larger aggregates in solution. When FENS-1, endofin, and Hrs FYVE domains were loaded into cells at 10, 20, and 30 nM, and for Hrs FYVE domains loaded into cells at 10, 20, and 30 nM, they all migrated as single species (9068 Da for FENS-1, 7923 Da for endofin-FYVE, and 9930 Da for endofin-FYVE).
duced monolayer penetration was observed for a triple mutant F295A/W296A/W302A, which indicates the direct involvement of these aromatic residues in membrane penetration. For endo-
fin-FYVE, the F762A mutation in the turret loop greatly re-
duced the monolayer penetration (see Fig. 3B); a similar result
was obtained for the corresponding mutations in Hrs and Vps27p (27). Surprisingly K764A, which has a significantly
reduced positive electrostatic potential in the turret loop region
(Fig. 2, first row), also showed significantly reduced monolayer
penetration, indicating that this unique cationic residue in the
turret loop imparts the extraordinary membrane penetrating
power to endofin-FYVE. In contrast to this mutation, muta-
tions of other cationic residues (K759A and R783/K784/K786A)
of endofin exhibited much smaller effects on the monolayer
penetration.

We also measured the monolayer penetration properties of
the mutants of Hrs and Vps27p FYVE domains. The mutants
that contained the 11-amino acid insertion of FENS-1-FYVE
were able to interact more favorably with POPC/POPE/POPS/
PtdIns(3)P (63:20:15:2) monolayers than the wild type proteins
with \( \pi_c \) values near 34 dynes/cm, which is similar to that
observed for wild type FENS-1-FYVE (see Fig. 3, B and C).
Likewise, when a Lys was introduced into the turret loop of Hrs
and Vps27p FYVE domains in simulation of endofin-FYVE,
these mutants were more similar to endofin-FYVE than to
their respective wild type forms with respect to electrostatic
(see Fig. 2, rows 3 and 4) and monolayer binding properties (see
Fig. 3, B and C). Thus, these data corroborate the notion that
the inserted loop and the cationic residue confer exceptional
monolayer penetration capability on the FENS-1 and endofin
FYVE domains, respectively.

We then measured the binding of selected mutants to endo-
some-mimicking vesicles by the SPR analysis. In agreement
with the monolayer data, \( \Delta^{295-306} \) of FENS-1-FYVE exhibited
dramatically (i.e. 78-fold) reduced affinity, which was due to an
\(-4\)-fold larger \( k_d \) and an \(-18\)-fold smaller \( k_a \). Again this large
decrease in \( k_d \) is consistent with our previous findings on interfacial aromatic residues (see above). Similarly the triple
mutant of FENS-1-FYVE (F295/W296/W302A) had greatly (20-
fold) reduced membrane affinity due to an \(-7\)-fold drop in \( k_a \)
and a \(3\)-fold increase in \( k_d \). As expected from the monolayer
data, single mutations of endofin-FYVE had large negative
effects. K764A had \(10\)-fold lower affinity than the wild type due
to \(5\)-fold reduction in \( k_a \) and \(2\)-fold increase in \( k_d \). Similarly the
F762A mutation in the turret loop reduced the membrane
affinity of endofin-FYVE by \(7\)-fold by affecting both \( k_a \) and \( k_d \).

When the extra loop of FENS-1-FYVE was inserted into the
intercorresponding positions of Hrs and Vps27p, the resulting
mutants had \(11\)-fold higher affinity than their respective wild

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**Membrane Targeting of FYVE Domains**

**FIG. 6. Analytical ultracentrifugation.** For the FYVE domains of FENS-1 (A), endofin (B), and Hrs (C), the logarithm of the absorbance at 280 nm (\(\text{In (Abs}_{280})\)) versus the square of the radius (\(r^2 - r_0^2\)) is plotted; \(r\) is the radial position in the sample, and \(r_0\) is the radial position of the meniscus. For a single species, the plot is linear, and the molecular mass of the species can be calculated from the slope of the line. For each FYVE domain, theoretical lines are plotted for a monomeric and a dimeric species. In all cases, experimental data show excellent agreement with the theoretical line for a monomer.
mutant with $K_d = 2.9 \pm 0.7 \text{ nM}$ was endosome-localized, whereas endofin F762A with $K_d = 8.1 \pm 1.3 \text{ nM}$ was not. To further investigate this effect, we measured the subcellular localization of K759A ($K_d = 3.4 \pm 1.6 \text{ nM}$) and R783/K784/K786A ($K_d = 4.5 \pm 1.7 \text{ nM}$). As shown in Fig. 7, M and N, these mutants displayed an endosomal localization pattern, confirming the presence of a sharp threshold value of membrane affinity (i.e. $K_d < 5–7 \text{ nM}$) that is necessary for autonomous endosomal localization of the FYVE domain. This also suggests that cellular membrane targeting can be readily switched on and off by small structural and environmental changes that have only minor impact on the strength of membrane-protein interactions.

**DISCUSSION**

Despite the explosion of studies on membrane-targeting domains in the past decade, fundamental understanding of the mechanisms by which subcellular localization of membrane-targeting domains and their host proteins is regulated is still lacking. Numerous mutational studies on membrane-targeting domains have shown that residues important for *in vitro* membrane binding are also crucial for their subcellular localization (31, 35). However, a direct and quantitative correlation between membrane affinity and subcellular localization of peripheral proteins has not been established. It is still technically challenging to quantitatively analyze the kinetics and thermodynamics of protein-membrane interactions in the living cell. However, the recent advent of SPR analysis has allowed us to directly determine kinetic and thermodynamic parameters for *in vitro* protein-membrane interactions (36, 37, 52). Our recent study of C2 domains indicated that their *in vitro* affinity for the vesicles whose lipid compositions recapitulate those of their target cell membranes is semiquantitatively correlated to the efficiency of their subcellular localization (35). The present study addressed this issue more systemically and quantitatively using FYVE domains as a model.

Our structural modeling and *in vitro* membrane binding measurements unequivocally identified the structural determinants of high membrane affinity of FENS-1-FYVE and endofin-FYVE. FENS-1-FYVE has a unique extended turret loop rich in

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**Fig. 7.** Subcellular localization of FYVE domains. A, FENS-1-FYVE wild type; B, FENS-1-FYVE Δ295–306; C, FENS-1-FYVE (green) and transferrin (red) dual imaging. Yellow puncta indicate colocalization. D, endofin-FYVE wild type; E, endofin-FYVE F762A; F, endofin-FYVE K764A; G, Hrs-FYVE wild type; H, Hrs-FYVE 11-amino acid insertion; I, Hrs-FYVE N175K; J, Vps27p-FYVE wild type; K, Vps27p-FYVE 11-amino acid insertion; L, Vps27p-FYVE N187K; M, endofin-FYVE K759A; N, endofin-FYVE R783A/K784A/R786A; O, FENS-1-FYVE with 100 nM wortmannin.
aromatic side chains, and endofin-FYVE has a strongly positively charged membrane binding surface, which greatly enhance their affinity for PtdIns(3)P-containing vesicles through aromatic residue-phospholipid interactions and nonspecific electrostatic interactions, respectively. High membrane penetrating power of FENS-1-FYVE is consistent with its unique structural feature. However, the enhanced membrane penetrating power of endofin-FYVE is somewhat unexpected since the turret loop is similar to that found in Hrs-FYVE and Vps27p-FYVE. When compared with other FYVE domains, endofin-FYVE has several extra cationic residues that contribute to the overall positive electrostatic potential of the molecule. However, it is a single cationic residue in the turret loop (Lys<sup>764</sup>) that is primarily responsible for the enhanced membrane affinity (and penetration) of this FYVE domain. This residue, which has a large contribution to the local electrostatic potential in the membrane binding region (Fig. 2), modulates both membrane association and dissociation and presumably assists the membrane penetration of neighboring Phe<sup>762</sup> either by affecting the conformation and/or membrane-bound orientation of Phe<sup>762</sup> or by reducing the electrostatic repulsion among anionic lipids that may be locally sequestered during the partial membrane insertion of Phe<sup>762</sup>. The specific nature of this remarkable effect is demonstrated by a much weaker effect observed upon mutation of another neighboring cationic residue, Lys<sup>769</sup>. Grafting unique structural features of FENS-1-FYVE and endofin-FYVE onto the FYVE domains of Hrs and Vps27p produces the domains with greatly enhanced membrane affinity, which underscores the critical roles of these residues in membrane binding. For both FENS-1 and endofin FYVE domains, the high membrane affinity observed is indeed due to the residue character of the turret loop and is ascribed neither to improved PtdIns(3)P affinity per se nor a higher tendency to form a dimer or an aggregate.

Comparison of the in vitro membrane binding properties of FYVE domains and respective mutants with their patterns of subcellular localization to endosomes in cells provides insight into features that control endosomal localization. These proteins exhibit a wide range of affinities (K<sub>d</sub> = 0.6–43 nM) for endosome-mimicking vesicles. As illustrated in Fig. 8, as the K<sub>d</sub> value gradually increases among these FYVE domains there is a sharp division between endosomal proteins and

![Graph showing correlation between in vitro vesicle affinity of FYVE domains proteins and their endosomal translocation](image)

**Fig. 8.** Correlation between in vitro vesicle affinity of FYVE domains proteins and their endosomal translocation. The x axis shows the K<sub>d</sub> values of FYVE domains (see Table I) and the y axis illustrates whether or not they are localized to endosomes (see Fig. 7). The arrows indicate the narrow range of a threshold affinity necessary for endosomal localization. aa, amino acid.

![Graph showing dependence of membrane affinity of FYVE domains on PtdIns(3)P concentration](image)

**Fig. 9.** Dependence of membrane affinity of FYVE domains on PtdIns(3)P concentration. Relative affinity of endofin-FYVE (○), hrs-FYVE (∆), and Vps27p-FYVE (□) was plotted as a function of PtdIns(3)P in POPC/POPE/POPS/PtdIns(3)P (65:20:15:x) vesicles coated onto the L1 chip. Relative affinity was calculated using K<sub>d</sub> (34 μM) for Vps27p-FYVE at 0.3 mol % PtdIns(3)P as reference and shown in the logarithmic scale.
cytosolic proteins in the remarkably narrow range of 4–8 nM. 

Obviously our *in vitro* measurements cannot fully simulate the binding to cellular endosomal membranes, and thus the absolute values of $K_d$ may not be physiologically meaningful. It should also be noted that our $K_d$ is defined in terms of the number of binding sites (not the concentration of lipid) (27).

Based on our previous study on the Vps27p-FYVE, which showed that each FYVE domain binds about 27 lipid molecules (27), our $K_d$ range of 4–8 nM should correspond to 0.1–0.2 μM if the $K_d$ is defined in terms of lipid concentration.

Regardless of actual $K_d$ values, the relative *in vitro* vesicle affinity (i.e., $K_d$ ratios) of FYVE domains should remain unchanged. Thus, our results suggest that the endosomal localization of FYVE domains can be turned on and off by about or less than a 2-fold change in membrane affinity. This minor change in membrane affinity can be induced by many different factors, including a conformational change of protein and a change in local PtdIns(3)P concentration. The membrane affinity of FYVE domains increases with the concentration of PtdIns(3)P in the membrane (27). As shown in Fig. 9, the membrane affinity of endofin-FYVE is higher than that of Hrs-FYVE and Vps27p-FYVE by more than an order of magnitude over a wide range of PtdIns(3)P concentrations. Thus, although the threshold $K_d$ value was determined only with 2 mol % PtdIns(3)P in the vesicles in this study, it is expected that there will be discrete threshold $K_d$ values at different PtdIns(3)P concentrations. This in turn should allow for the fine tuning of the spectrum of FYVE domains that are efficiently targeted to endosomes as the PtdIns(3)P content is changed.

Another salient feature is that all FYVE domains that exhibit endosomal localization have an ability to penetrate the POPC/POPE/POPS/PtdIns(3)P (63:20:15:2) monolayers that have packing densities similar to that of biological membranes. Thus, it would seem that the ability to partially penetrate endosomal membranes at this PtdIns(3)P concentration (2 mol %) is essential for endosomal localization. We previously showed that the monolayer penetration of FYVE domains depends on PtdIns(3)P concentration (27). It is therefore possible that FYVE domains can be selectively targeted to endosomes based on their differing abilities to penetrate endosomal membranes at a given concentration of PtdIns(3)P. Although beyond the scope of this investigation, accurate determination of the PtdIns(3)P concentration and surface pressure of endosomal membranes will test the viability of this interesting hypothesis.

It was recently reported (21) that the homodimerization of the SARA FYVE domain plays an important role in its endosomal localization. This conclusion was based on the findings that the SARA FYVE domain has a high tendency to dimerize in solution and in the cell and that the induced dimerization of the frabin FYVE domain causes its endosomal localization. We are grateful to John D. Raffer for assistance in dual imaging experiments.

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