ROLE OF CONSERVED RESIDUES

FYVE zinc finger domains, which are conserved in multiple proteins from yeast to man, interact specifically with the membrane lipid phosphatidylinositol 3-phosphate (PtdIns(3)P). Here we have investigated the structural requirements for the interaction of the FYVE finger of the early endosome antigen EEA1 with PtdIns(3)P and early endosomes. The binding of the FYVE finger to PtdIns(3)P is Zn²⁺-dependent, and Zn²⁺ could not be replaced by any other bivalent cations tested. By surface plasmon resonance, the wild-type FYVE finger was found to bind to PtdIns(3)P with an apparent Kₐ of about 50 nM and a 1:1 stoichiometry. Mutagenesis of cysteines involved in Zn²⁺ coordination, basic residues thought to be directly involved in ligand binding and other conserved residues, resulted in a 6- to >100-fold decreased affinity for PtdIns(3)P. A mutation in the putative PtdIns(3)P-binding pocket, R1375A, may prove particularly informative, because it led to a strongly decreased affinity for PtdIns(3)P without affecting the FYVE three-dimensional structure, as measured by fluorescence spectroscopy. Whereas the C terminus of EEA1 localizes to early endosomes when expressed in mammalian cells, all the FYVE mutants with reduced affinity for PtdIns(3)P were found to be largely cytosolic. Furthermore, whereas expression of the wild-type EEA1 C terminus interferes with early endosome morphology, the point mutants were without detectable effect. These results support recently proposed models for the ligand binding of the FYVE domain and indicate that PtdIns(3)P binding is crucial for the localization and function of EEA1.

Phosphatidylinositol 3-kinases are important regulators of vital cellular processes, including endocytic membrane trafficking, signal transduction, apoptosis, and cytoskeletal organization (1–3). The recent discovery of the conserved FYVE zinc finger (for Fab1p, YOTB, Vac1p, EEA1) (4) as a phosphatidylinositol 3-phosphate (PtdIns(3)P)-specific domain has shed light on the function of this phosphatidylinositol 3-kinase product in cellular processes (5–8). FYVE finger proteins include the membrane trafficking regulators EEA1, Hrs, Vac1p, Vps27p, and Fab1p, the signal transducer SARA, the putative cytoskeletal regulator Fgdi1, as well as a number of proteins with unknown function (5, 9–15).

The determination of the crystal structure of the ligand-free FYVE domain of the yeast vacuolar sorting protein Vps27p at high resolution has enabled the modeling of the FYVE-PtdIns(3)P interaction (16). According to this model, the basic residues present in the β1 strand (Fig. 1) together with a basic residue in the β4 strand form a basic pocket that may bind the 3-phosphate group, whereas the 1-phosphate may form a salt bridge with a positive side chain preceding the β1 strand. An alternative model has been proposed upon the solution of the x-ray structure of the FYVE domain of Hrs (the putative mammalian homologue of Vps27p) complexed to citrate (17). Even though the structure of Hrs is highly similar to that of Vps27p, the proposed model for PtdIns(3)P binding (based on the idea that citrate mimics PtdIns(3)P) differs in two important respects: the location of the 1-phosphate of PtdIns(3)P is different, leading to a different orientation of the PtdIns(3)P headgroup, and the FYVE domain of Hrs is suggested to bind to two molecules of PtdIns(3)P as a dimer. So far, the two models of PtdIns(3)P binding have not been compared experimentally. However, an NMR study of the FYVE domain of the early endosomal protein EEA1 (18), a regulator of endocytic membrane docking and fusion (9), has provided further insight into the FYVE-PtdIns(3)P interaction (19). Twenty of the residues of EEA1 FYVE display large chemical shift changes in the presence of a soluble PtdIns(3)P analog, suggesting that the FYVE finger may undergo a conformational change upon PtdIns(3)P binding. Six basic residues (Lys₁₁₃₉, Arg₁₁₄₈, Arg₁₁₇₇, His₁₁₃₇₂, Arg₁₁₇₇₅, and Arg₁₄₀₅) are particularly sensitive and have been proposed to interact directly with the phosphates of PtdIns(3)P, in accordance with the models based on the crystal structures of Vps27p and Hrs. Although the crystal structures of the FYVE domains of Vps27p and Hrs and the NMR analysis of EEA1 FYVE have yielded invaluable structural information, the role of individual residues has not been studied in direct lipid binding assays or in intact mammalian cells. In this study we have mutated conserved residues of EEA1 FYVE to serine or alanine and studied the effects of the mutations with respect to PtdIns(3)P binding in vitro and intracellular localization and function in fibroblasts.

EXPERIMENTAL PROCEDURES

Polymersase Chain Reaction Mutagenesis—Site-directed mutagenesis of pGEM-Myc-EEA1₁₂₅₇–₁₄₁₁ (4) was done with the QuikChange kit from Stratagene (La Jolla, CA) according to the instructions from the manufacturer. The sequences of the various mutants were confirmed by sequencing. All numbering of mutant constructs refers to the published sequence of EEA1 (18).
Recombinant GST Fusion Proteins—The mutagenized EEA1 sequences were subcloned from the respective pGEM-Myc constructs into pGEX-EEA1<sub>1307–1411</sub> (5). pGEX-EEA1<sub>1125–1411</sub> has been described before (5), and pGEX-EEA1<sub>1136–1411</sub> was obtained by polymerase chain reaction. For expression as fusion proteins with glutathione S-transferase (GST) in <em>Escherichia coli</em>, BL-21 (DE3) were transformed with the respective pGEX plasmids, and protein expression and purification on glutathione-Sepharose (Amersham Pharmacia Biotech) was done according to the manufacturer’s instructions. Maltose-binding protein (GST)-EEA1<sub>1125–1411</sub> was expressed and purified as described (4). Finally, the fusion proteins were dialyzed overnight in a buffer containing 20 mM dithiothreitol, 20% phosphatidyl serine, 15% phosphatidyl ethanolamine, and 1.8 M CHAPS, and a fresh liposome suspension could subsequently be coated onto the same L1 sensorchip.

Fluorescence Spectroscopy—The fluorescence measurements (20) were recorded on a Perkin-Elmer LS50B spectrophotometer. The proteins were dissolved at 0.2 μM in 20 mM Tris, 60 mM NaCl, pH 7.6, at room temperature in a volume of 1 ml. The solubilized proteins were excited at 290 nm, and the fluorescence was recorded from 300 to 400 nm with a bandwidth of 10 nm at both excitation and emission.

Liposome Binding Assay—Phosphatidylcholine, phosphatidylserine, and phosphatidyl-ethanolamine were from Sigma. [3H]Phosphatidylcholine was from Amersham Pharmacia Biotech, whereas dipalmitoyl phosphatidylglycerol, dipalmitoylphosphatidylinositol-3-phosphate, dibutanol phosphoinositides, and inositol (1,3,5)trisphosphate were from Echelon (Salt Lake City, UT). The binding assays of liposomes (0.35 mg ml<sup>−1</sup>) containing 63% phosphatidyl choline, 20% phosphatidyl serine, 15% phosphatidyl ethanolamine, and 2% PtdIns (3)P were done according to the manufacturer’s instructions. Maltose-binding protein (GST)-EEA1<sub>1125–1411</sub> was expressed and purified as described (4). Finally, the fusion proteins were dialyzed overnight in a buffer containing 20 mM Tris, pH 7.6, 60 mM NaCl, 10 μM ZnCl<sub>2</sub>.

Zinc-dependent Binding of EEA1 FYVE to PtdIns (3)P—We have previously measured that the FYVE finger binds two zinc ions (4), and this has been confirmed by the determination of the crystal structure (16). To test if zinc removal causes an irreversible effect on EEA1 FYVE function, we treated a fusion protein between GST and EEA1<sub>1307–1411</sub> with EDTA in order to remove bound Zn<sup>2+</sup>. This caused a significant but not complete loss of PtdIns (3)P binding (5), as measured as the ability of the immobilized protein to bind <sup>3</sup>H-labeled liposomes containing 3.7 μM PtdIns (3)P was measured as described under “Experimental Procedures.”
Even though it is known that the FYVE finger binds to the FYVE finger are thus R1371A and R1375A. GST EEA11307–1411 and its point mutants were expressed in E. coli, then purified, and analyzed by 10% SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining of the gel. The lower bands represent degradation products. Lower panel, BHK cells transfected with Myc-EEA11307–1411 and its point mutants were analyzed by 12% SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-Myc antibodies and chemiluminescence detection.

For further studies, interestingly, all cysteine mutants migrated as a single band on acrylamide gel, whereas the other point mutants as well as the wild type protein migrated as a doublet, with the faint upper band at the same position as that of the cysteine mutants. Expression of the Myc epitope-tagged proteins in mammalian cells also demonstrated the different migration of the cysteine mutants as compared with the wild-type and other mutants. In this case, all proteins migrated as single bands (Fig. 3, lower panel). Because the C1358S and C1405S mutants contain only one bound Zn$^{2+}$ ion (4), this suggests that the presence of two zinc ions keeps the wild-type FYVE domain tightly folded, even under the conditions of SDS-polyacrylamide gel electrophoresis. This also suggests that the other mutants contained two Zn$^{2+}$ ions.

Fluorescence Spectroscopy of Wild-type and Mutant FYVE Domains—To obtain information about the effects of mutations on the three-dimensional structure of the FYVE finger, we analyzed wild-type and mutant EEA11307–1411 by fluorescence spectroscopy. The presence of the conserved Trp$^{1349}$ in the EEA1 FYVE domain allowed us to record the fluorescence spectrum emitted by the protein upon excitation at 290 nm (Fig. 4A). The emission spectrum was typical for tryptophan, with a maximum fluorescence at 345 nm. Tryptophan residues present in the GST tag contributed little to the GST-FYVE fluorescence, because GST alone generated only a weak emission. The same weak emission was obtained with the W1349A mutant (used as a control), as well as the F1365A, H1373A, and G1378A mutants. Whereas the R1371A and R1375A mutants exhibited the same fluorescence as the wild type, all the cysteine mutants as well as the R1400A mutant showed a 40–50% reduction in fluorescence intensity. The addition of EDTA significantly quenched the fluorescence of the wild-type FYVE domain, yielding a fluorescence emission comparable to that of the cysteine mutants (Fig. 4B). These results show that the environment of Trp$^{1349}$ is sensitive to mutations not only of residues in direct contact with Trp$^{1349}$ (such as His$^{1373}$), but also of distal conserved residues. The two mutations that are the least likely ones to affect the three-dimensional structure of the FYVE finger are thus R1371A and R1375A.

Binding of Wild-type and Mutant EEA1 FYVE to PtdIns (3)P—Even though it is known that the FYVE finger binds to PtdIns (3)P, a $K_D$ value for this interaction has not been determined. To measure the association and dissociation kinetics of the EEA1 FYVE finger with PtdIns (3)P, we employed surface plasmon resonance, which has been used successfully for measuring the binding of GST- and green fluorescent protein-tagged pleckstrin homology domains to phosphoinositides (26, 27). A lipid mixture containing 2% PtdIns (3)P was immobilized onto a sensor surface, and sensorgrams were recorded upon the injection of EEA1 FYVE at different concentrations (Fig. 5A). The association and dissociation kinetics followed a profile typical of a 1:1 interaction model (Langmuir).

From the association and dissociation curves, we calculated a $K_D$ value of 45 nM for the interaction of GST-FYVE with PtdIns (3)P ($k_a$ of $6.9 	imes 10^4$ s$^{-1}$ and $k_d$ of $3.1 	imes 10^{-3}$ ms$^{-1}$). Similarly, we calculated the corresponding $K_D$ value for EEA1 fused to maltose-binding protein to be 50 nM (data not shown), indicating that the GST tag does not significantly affect the binding of the FYVE domain to PtdIns (3)P. The $K_D$ value of EEA1 FYVE with PtdIns (3)P is about 30-fold higher than that of the PDK1 pleckstrin homology domain with phosphatidylinositol-3,4,5-trisphosphate but lower than the $K_D$ value of PDK1 pleckstrin homology domain with phosphatidylinositol-3,4,5-trisphosphate to be 50 nM (data not shown), indicating that the GST tag does not significantly affect the binding of the FYVE domain to PtdIns (3)P. The $K_D$ value of EEA1 FYVE with PtdIns (3)P is about 30-fold higher than that of the PDK1 pleckstrin homology domain with phosphatidylinositol-3,4,5-trisphosphate but lower than the $K_D$ value of PDK1 pleckstrin homology domain with phosphatidylinositol-3,4,5-trisphosphate.
domain for Ins(1,3)P$_2$ of about 65 µM. In contrast, dibutanoyl PtdIns (3)P did not compete (not shown), possibly because this molecule may partially associate with the lipid membrane. These results indicate that the FYVE domain interacts with membrane-associated PtdIns (3)P with an intermediate affinity and that the headgroup of PtdIns (3)P is necessary but not sufficient for the stable FYVE-membrane interaction. The latter is consistent with the view that hydrophobic residues preceding the β1 strand of the FYVE domain may contribute to membrane binding by interacting with bilayer acyl chains (16, 19).

To study how mutations of the FYVE domain affect its ability to bind PtdIns (3)P, we recorded sensorgrams at different concentrations of the various point mutants and calculated their $K_D$ values for PtdIns (3)P (Fig. 5C and Table I). Interestingly, all mutations affected PtdIns (3)P binding. Smallest effects were observed with the F1365A and R1400A mutations, which led to a ~6-fold reduction in affinity. Other mutations resulted in stronger effects. For instance, all mutations of cysteines caused a >20-fold loss of affinity for PtdIns (3)P. Among the conserved histidine and arginine residues, all mutations, except R1400A, had profound effects on PtdIns (3)P binding. The potentially most interesting mutant for further functional studies is R1375A, because this mutant could be obtained in good yields and showed a fluorescence emission similar to that of the wild-type FYVE finger, and yet it had a >100-fold higher $K_D$ for PtdIns (3)P (Fig. 5C and Table I). Altogether, the surface plasmon resonance analyses indicate that the FYVE finger of EEA1 is highly sensitive to mutations and that all the conserved residues play an important role, either in the direct binding to PtdIns (3)P or in the stabilization of the correct FYVE structure.

Intracellular Localization of FYVE Mutants—Whereas the isolated FYVE finger of EEA1 binds poorly to endosome membranes, EEA1$_{1257-1411}$, which contains an additional domain that binds to the GTP-bound form of the GTPase Rab5, binds strongly to early endosomes when expressed in mammalian cells (4, 9). We have previously found that the mutagenesis of two of the zinc-coordinating residues, Cys$^{1358}$ and Cys$^{1405}$, abolishes the early endosome binding of EEA1$_{1257-1411}$ (4). To study if this is true also for other mutations that interfere with PtdIns (3)P binding, we expressed Myc epitope-tagged point mutants of EEA1$_{1257-1411}$ in BHK cells and examined their intracellular localization by confocal immunofluorescence microscopy. The cells were permeabilized with saponin prior to fixation to remove cytosolic protein, and the fixed cells were stained with anti-Myc antibodies (24). As shown in Fig. 6, the wild-type Myc-EEA1$_{1257-1411}$ construct localized extensively to vesicular structures (a), whereas no membrane staining was observed with the mutants W1349A (b), R1371A (c), and R1400A (d). Similar results were obtained with all the other PtdIns (3)P-binding-deficient mutants, indicating that the mutant proteins localized mainly to the cytosol (Table I). These results show that PtdIns (3)P binding is a prerequisite for the localization of EEA1$_{1257-1411}$ to early endosomes.

Inability of FYVE Mutants to Engage in the Endocytic Fusion Machinery—The expression of the GTPase-deficient Rab5$^{979L}$

![Table I](https://example.com/)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Localization</th>
<th>$K_D$ (µM)</th>
<th>Effect on EE morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>EE + cytosol</td>
<td>0.045 ± 0.020</td>
<td>+</td>
</tr>
<tr>
<td>W1349A</td>
<td>Cytosol</td>
<td>0.45 ± 0.18</td>
<td>–</td>
</tr>
<tr>
<td>C1358S</td>
<td>Cytosol</td>
<td>&gt;1</td>
<td>–</td>
</tr>
<tr>
<td>F1365A</td>
<td>Cytosol</td>
<td>0.26 ± 0.10</td>
<td>–</td>
</tr>
<tr>
<td>R1370A</td>
<td>Cytosol</td>
<td>&gt;1</td>
<td>–</td>
</tr>
<tr>
<td>R1371A</td>
<td>Cytosol</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td>H1372A</td>
<td>Cytosol</td>
<td>&gt;1</td>
<td>–</td>
</tr>
<tr>
<td>H1373A</td>
<td>Cytosol</td>
<td>&gt;1</td>
<td>–</td>
</tr>
<tr>
<td>R1374A</td>
<td>Cytosol</td>
<td>&gt;1</td>
<td>–</td>
</tr>
<tr>
<td>R1375A</td>
<td>Cytosol</td>
<td>&gt;5</td>
<td>–</td>
</tr>
<tr>
<td>C1377A</td>
<td>Cytosol</td>
<td>&gt;1</td>
<td>–</td>
</tr>
<tr>
<td>G1378A</td>
<td>Cytosol</td>
<td>2.1 ± 0.7</td>
<td>–</td>
</tr>
<tr>
<td>C1385A</td>
<td>Cytosol</td>
<td>&gt;1</td>
<td>–</td>
</tr>
<tr>
<td>R1400A</td>
<td>Cytosol</td>
<td>0.28 ± 0.16</td>
<td>–</td>
</tr>
<tr>
<td>C1405S</td>
<td>Cytosol</td>
<td>&gt;1</td>
<td>–</td>
</tr>
</tbody>
</table>
mutants of EEA1 1257–1411 were coexpressed with Rab5 Q79L in Rab5-dependent endosome fusion observations to study the effects of FYVE point mutants on cytic docking/fusion machinery (28). We took advantage of these presumably by sequestering essential components of the endocytic docking/fusion machinery (28). We took advantage of these observations to study the effects of FYVE point mutants on Rab5-dependent endosome fusion in vivo. Myc epitope-tagged mutants of EEA1 1257–1411 were coexpressed with Rab5Q79L in BHK cells, and their effects on the Rab5Q79L-induced giant endosomes were detected by dual label confocal immunofluorescence microscopy. To detect cytosolic protein, we fixed the cells prior to permeabilization in these experiments (Fig. 7). Whereas the wild-type protein (b) colocalized extensively with Rab5Q79L (a) and caused the formation of small, clustered endosomes (arrowheads in a), the mutants R1371A (c and d), H1373A (e and f), and R1375A (g and h) accumulated in the cytosol and were without effect on the Rab5Q79L-induced giant endosomes (arrows). Similar results were obtained with all of the other PtdIns (3)P-binding-deficient mutants (Table 1). This provides strong evidence that the binding to PtdIns (3)P is crucial for the interaction of EEA1 with the rest of the docking/fusion machinery at the endosome membrane.

**DISCUSSION**

In the present paper we have functionally characterized the FYVE domain of EEA1. We show that it functions in the presence of Zn$^{2+}$ but not other bivalent cations, and that its PtdIns (3)P binding and biological function are highly sensitive to mutations of conserved residues.

The intermediate affinity of the EEA1 FYVE domain for PtdIns (3)P (apparent $K_d$ value of 45 nM) may explain why the efficient recruitment of EEA1 to membranes requires an additional domain that binds to Rab5GTP (9). All point mutations of conserved residues lowered the affinity for PtdIns (3)P by 6-100-fold, demonstrating the important role of these residues for PtdIns (3)P binding. Interestingly, all the point mutants were largely cytosolic, and they were unable to engage in the endocytic docking apparatus. This suggests that the binding of EEA1 to endosome membranes is critically dependent on the binding to PtdIns (3)P.

The eight conserved cysteines in the EEA1 FYVE domain are involved in Zn$^{2+}$ coordination, and the Zn$^{2+}$ binding is crucial for the stabilization of the FYVE structure, as indicated by our spectrofluorometric analysis of cysteine mutants of EEA1 FYVE. This is in agreement with the crystal structures of Vps27p FYVE and Hrs FYVE, which show that the coordination of two Zn$^{2+}$ ions stabilizes the FYVE domain, including the basic pocket implicated in PtdIns (3)P binding. Our results further indicate that both of the two Zn$^{2+}$ ions are required for PtdIns (3)P binding.

Besides the eight conserved cysteines, the most characteristic feature of the FYVE domain is the highly conserved Arg/Lys-Arg/Lys-His-His-Cys motif associated with the $\beta$1 strand (4). Molecular modeling suggests that the basic residues of this motif may be directly involved in the binding of the two phosphate groups of PtdIns (3)P (16, 17). Our finding that all of the basic residues in the Arg$^{1370}$-Arg$^{1371}$-His$^{1372}$-His$^{1373}$-Cys$^{1374}$-Arg$^{1375}$ motif are important for PtdIns (3)P binding,
supports this view. Arg$^{1375}$, which we found critical for the interaction with PtdIns (3)P, has been proposed to either interact directly with the 3-phosphate group (16) or with inositol ring hydroxyl groups (17). Even though our results do not allow us to discriminate between the two alternative models for FYVE-ligand interactions (16, 17), it is interesting to note that the association/dissociation kinetics we detected showed typical features of a 1:1 molecular interaction. If a dimeric EEA1 FYVE did bind to two molecules of PtdIns (3)P (17), we would have expected to observe more complex binding kinetics. Moreover, we have previously found that the FYVE finger of EEA1 does not efficiently dimerize at low concentrations (29). We therefore favor the idea that the EEA1 FYVE finger binds to PtdIns (3)P as a monomer (16).

Three of the highly conserved residues in the FYVE finger, Trp$^{1349}$, Phe$^{1365}$, and Gly$^{1378}$, have neither been implicated in Zn$^{2+}$ coordination nor in the direct interaction with PtdIns (3)P. The present results show that these residues are nevertheless important for the binding to PtdIns (3)P. According to the Vps27p FYVE structure, Trp$^{1349}$ would be at the edge of the highly basic patch and play a supportive role by stabilizing His$^{1373}$ and Arg$^{1375}$. When this residue is substituted by alanine, the basic pocket is thus likely to be affected. Phe$^{1365}$ is predicted to be partially buried and to be part of a hydrophobic chain that runs through the center of the FYVE domain (16). Both this residue and Gly$^{1378}$ show differences in their NMR signals in the presence of micelle-embedded, but not soluble, PtdIns (3)P, suggesting that these residues may participate in lipid interactions (19). It is unlikely, however, that their replacement with alanine would strongly affect their lipid-interacting properties, and the effects of these mutations on PtdIns (3)P binding might thus be because of a distortion of the FYVE structure. This is in agreement with our finding that the F1365A and G1378A mutations abolished the fluorescence of the FYVE domain.

What is the biological significance of the binding of FYVE proteins to PtdIns (3)P? The most obvious possibility is that this interaction may serve to recruit FYVE proteins to specific membranes. Although the FYVE domain may have other ligands in addition to PtdIns (3)P (28, 30, 31), our finding that 12 mutations that affect PtdIns (3)P binding also abolish the localization of EEA1 to early endosomes strongly argue that PtdIns (3)P binding is the critical property of the FYVE domain with respect to the localization of EEA1. The finding that overexpression of the C terminus of EEA1 but not of its PtdIns (3)P-binding-deficient mutants affects Rabs$^{170}$-induced endosome further indicates that PtdIns (3)P binding is essential for the interaction of EEA1 with other components of the endosomal docking/fusion machinery. Future studies need to clarify how the recruitment of EEA1 to early endosomes affects its interactions with endosomal protein complexes.

REFERENCES
