REVIEW ARTICLE

Cellular functions of phosphatidylinositol 3-phosphate and FYVE domain proteins

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PtdIns3P is a phosphoinositide 3-kinase product that has been strongly implicated in regulating membrane trafficking in both mammalian and yeast cells. PtdIns3P has been shown to be specifically located on membranes associated with the endocytic pathway. Proteins that contain FYVE zinc-finger domains are recruited to PtdIns3P-containing membranes. Structural information is now available concerning the interaction between FYVE domains and PtdIns3P. A number of proteins have been

identified which contain a FYVE domain, and in this review we discuss the functions of PtdIns3P and its FYVE-domain-containing effector proteins in membrane trafficking, cytoskeletal regulation and receptor signalling.

Key words: membrane traffic, phosphoinositide, phosphoinositide 3-kinase, protein domains, recruitment.

INTRODUCTION

Phosphorylated derivatives of PtdIns, known as phosphoinositides, play a key role in the membrane recruitment and/or activation of proteins [1]. In this way they provide a means for the temporal and spatial regulation of complex cellular processes.

The products of phosphoinositide 3-kinases (PI 3-kinases) have been the subject of much investigation and have been shown to play a role in signal transduction, membrane trafficking, cytoskeletal regulation and apoptosis [2,3]. PI 3-kinases produce phosphoinositides phosphorylated at the D-3 position of the inositol ring [4] to form PtdIns3P (Figure 1), PtdIns(3,4)₂ or

PtdIns(3,4,5)₃. PtdIns(3,4,5)₃ is produced upon agonist stimulation in mammalian cells and interacts with a number of proteins containing pleckstrin homology (PH) domains [3,5]. Cellular PtdIns(3,4)₂ is probably mainly produced from a dephosphorylation of PtdIns(3,4,5)₃, since the kinetics of its production often immediately follow on from a transient PtdIns(3,4,5)₃ production [6]. There is evidence, however, that it can be produced independently of PtdIns(3,4,5)₃ production upon agonist stimulation and interacts with proteins containing PH domains [7,8]. PtdIns(3,5)₂ is produced from PtdIns3P by PtdIns3P 5-kinases, and cellular levels of this lipid are regulated by osmotic-strength changes [9,10]. In contrast with PtdIns(3,4,5)₃, PtdIns(3,4)₂ and PtdIns(3,5)₂, PtdIns3P is produced constitutively in both yeast and mammalian cells. Here we discuss how this phosphoinositide regulates membrane trafficking and other cellular processes through its interactions with proteins containing FYVE domains.

THE FYVE ZINC-FINGER DOMAIN

The FYVE domain was named after the first letter of the first four proteins in which it was found {Fab1p, YOTB, Vac1p and EEA1 (early endosomal antigen 1); [11]}. It is a cysteine-rich domain which binds two zinc ions, and the first evidence for a function of the FYVE domain arose with the finding that it was required for the endosomal localization of EEA1 [11]. FYVE domains were subsequently identified as PtdIns3P-binding domains [12–14]. It was shown that the FYVE domains of EEA1, hepatocyte-growth-factor-regulated tyrosine kinase substrate (Hrs), and the yeast proteins Vac1p, Vps27p, Fab1p and Pib1p could all bind PtdIns3P. In contrast with the phosphoinositide binding observed with PH domains, binding of FYVE domains to PtdIns3P was found to be very specific, with no

Figure 1 PtdIns3P

Abbreviations used: EEA1, early endosomal antigen 1; Hrs, hepatocyte-growth-factor-regulated tyrosine kinase substrate; PI 3-kinase, phosphoinositide 3-kinase; SARA, Smad anchor for receptor activation; PH, pleckstrin homology; EM, electron-microscopic; Rab5:GTP, GTP-bound form of the endosomal GTPase Rab5; TGF- β , transforming growth factor- β ; MTM1, myotubularin; MTMR2, MTM1-related protein-2; DSP, dual-specificity protein phosphatase; DFCP1, double FYVE-containing protein 1; PEPP1, phosphatidylinositol-three-phosphate-binding PH-domain protein-1.

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detectable binding to any other phosphoinositides. These findings identified for the first time a set of targets for the PI 3-kinase activity previously shown to be necessary for constitutive membrane trafficking.

STRUCTURAL BASIS FOR PtdIns3P BINDING BY THE FYVE DOMAIN

FYVE domains contain eight conserved cysteine residues that co-ordinate two Zn^{2+} ions in a 'cross-braced' topology [11,15]. In addition, surrounding the third and fourth cysteine residues they contain a highly characteristic R(R/K)HHCRXCG motif (Figure 2). Several hydrophobic residues are also conserved among the FYVE domains, as is an arginine residue near the C-terminus (Figure 2, position 106).

Structures have been determined by X-ray crystallography for the FYVE domains of Hrs and its putative yeast homologue Vps27p. Misra and Hurley [15] solved the ligand-free structure of the FYVE domain from Vps27p at high resolution. The structure consists of two two-stranded β -sheets stabilized by the two zinc ions and a C-terminal α -helix (Figure 3a). The basic conserved patch is localized to the β 1-strand and the following β -turn, and, together with the conserved arginine residue (position 106 in Figure 2), it forms a basic pocket. The inositol ring of PtdIns3P

fits into a pocket created by the backbone of β 1, whereas the 3-phosphate group of PtdIns3P could contact the side-groups of a histidine residue and an arginine residue from the RRHHCR motif (Figure 3a). PtdIns3P lies sideways or parallel with respect to the β 1 strand with its 1-phosphate group directed toward the apical end of the FYVE domain.

A similar structure, but a quite different model, was proposed for the FYVE domain of Hrs, which was crystallized in the presence of citrate (a substrate substitute) by Mao et al. [16]. The antiparallel association of Hrs FYVE domains leads to the formation of a homodimer with two ligand-binding pockets (Figure 3b). Mainly residues from the β 1 strand of one FYVE domain, which contains the basic RKHHCR motif, and the β 4 strand of the symmetry-related FYVE domain, which contains hydrophobic residues, line each of these pockets. The 1- and 3-phosphate groups of PtdIns3P are orientated such that they occupy the opening and bottom of the pocket respectively. The orientation of PtdIns3P is normal to the β 1 strand, and the dimeric FYVE domain lies consequently horizontally with respect to the membrane. This model for PtdIns3P interaction is thus radically different from the one presented by Misra and Hurley [15], which proposes that the FYVE domain lies in a vertical orientation with respect to the membrane (Figure 3a). In order to resolve this controversy, it will be necessary to solve the FYVE

Figure 2 Multiple alignment of various FYVE-domain-containing proteins

Sequences were aligned using the ClustalW program [74] and edited using CINEMA (Colour INteractive Editor for Multiple Alignments; [75]). Amino acids (which are given below in the one-letter notation) are coloured as follows: polar positive (H, K and R), blue; polar negative (D and E), red; polar neutral (S, T, N and Q), green; non-polar aliphatic (A, V, L, I and M), white; non-polar aromatic (F, Y and W), purple; P and G, brown; C, yellow. The sequences shown correspond to the following amino acids residues from each protein: EEA1, 1345–1410; Rabenosyn-5, 150–260; Hrs, 156–220; Vac1, 208–297; Vps27p, 166–230; Fab1p, 233–299; DSP1c, 1112–1180; DFCP1, 708–775; Fgd1, 724–790; Fgd3, 521–584; SARA, 590–656 (for a more complete alignment of FYVE domains, see [76]).

domain structure in the presence of a PtdIns3P-containing membrane.

NMR studies on the FYVE domain of EEA1 implicated a number of residues as being involved in PtdIns3P binding [17]. In particular, the residues of the basic RRHHCRQCGNIF motif were shown to participate in the binding and also to play a role in ensuring the specificity of the FYVE domain for PtdIns3P as opposed to PtdIns5P, the structurally most similar phosphoinositide to PtdIns3P. The three basic residues Lys⁴, Arg²⁷ and Arg²⁸ (Figure 2) were shown to be much more influenced by PtdIns3P binding than they were by PtdIns5P binding, and so were proposed to directly contact the 3-, but not the 5-, phosphate group, thus determining the specificity for PtdIns3P over PtdIns5P. The dimer model of FYVE domain structure also found support with some of the NMR data. Homodimeric dimerization of EEA1 had been previously shown [18], but NMR studies also suggested that the FYVE domain itself can form dimers and that dimerization increased the FYVE domain's affinity for PtdIns3P.

The structural information provided by the X-ray-crystallographic and NMR studies has been complemented by site-directed mutagenesis of individual residues in the FYVE domain of EEA1 [19]. This domain was found to be very sensitive to mutation. All point mutations of conserved FYVE domain residues were found to lower the affinity for PtdIns3P by 6- to more than 100-fold and to ablate the membrane-binding ability of the FYVE domain, thus indicating that the functional consequence of PtdIns3P binding by the FYVE domain of EEA1 is an endosomal localization of the protein. Both of the two coordinated zinc ions were found to be necessary for stabilizing the FYVE domain structure, and these zinc ions could not be replaced by other bivalent cations. All of the residues in the basic patch RRHHCR in the β 1 strand were shown to play some role, either directly or indirectly, in PtdIns3P binding by the FYVE domain. It is very likely that the final arginine residue in the basic patch motif (Arg³², Figure 2) forms a direct interaction with PtdIns3P, since mutation of this residue reduced the affinity of the FYVE finger by more than 100-fold without altering the protein structure. In the two alternative structure-based models [15,16], this residue was proposed to interact with the 3-phosphate group of PtdIns3P or with inositol-ring hydroxy groups. Both models thus agree that there is a direct interaction of this residue with PtdIns3P.

LOCALIZATION AND TRAFFICKING OF PtdIns3P

In order to understand the functions of phosphoinositide-binding proteins it is of great importance to know the cellular localization of the phosphoinositides that they interact with. Therefore, to understand the functions of FYVE-finger-containing proteins, it is important to know the localization of PtdIns3P. For this purpose, a novel probe for the phospholipid was developed, consisting of two FYVE domains in tandem [20]. The 2 $\times$ FYVE probe was found to bind PtdIns3P specifically and with high affinity both *in vitro* and *in vivo*. When the tagged probe was expressed in fibroblasts, it was found by confocal immunofluorescence microscopy to be localized on early endosomes. In order to localize PtdIns3P at the ultrastructural level, an '*in situ*' electron-microscopic (EM)-labelling approach was taken in which cell sections were incubated with recombinant 2 $\times$ FYVE, followed by an antibody and then Protein A-gold. This EM approach, when used on untransfected fibroblasts, showed that PtdIns3P is highly enriched on early endosomes and in the

internal vesicles of multivesicular endosomes. PtdIns3P was also found to be present in the nucleolus, although the significance of this is unknown. PtdIns3P was not found on the plasma membrane, Golgi apparatus or the membranes of other cellular compartments.

PtdIns3P was found on intraluminal vesicles of endosomes and vacuoles of yeast, whereas cells devoid of PI 3-kinase activity were not labelled. Yeast cells with impaired endosome-to-vacuole trafficking showed a decreased vacuolar labelling and an increased endosomal labelling. These results led to a model for the role of PtdIns3P in endosomal dynamics (Figure 4). PtdIns3P is proposed to be generated on the early endosomes or incoming endocytic vesicles via recruitment of the PI 3-kinase hVps34p by the GTP-bound form of the endosomal GTPase Rab5 (Rab5:GTP) [21]. The PtdIns3P subsequently produced would facilitate the recruitment of EEA1 to the limiting membrane of the early endosome, in conjunction with binding to Rab5:GTP. Inward budding of the limiting membrane of the early endosome would then remove PtdIns3P from the cytoplasmic face of the endosome while generating the multivesicular region. This would then physically prevent the binding of EEA1 and other FYVE finger proteins to PtdIns3P. Inward invagination may be triggered by Rab5:GTP hydrolysis, which may cause EEA1 to dissociate from the membrane. A relative depletion of PtdIns3P from late endosomes suggests that internal vesicles containing PtdIns3P are degraded or that the PtdIns3P is further modified by kinases, phosphatases or lipases. PtdIns3P may actually be specifically required for the inward invagination of the membranes, and it is noteworthy that the PI 3-kinase inhibitor wortmannin blocks multivesicular body formation [22]. Thus it seems as though PtdIns3P follows a conserved intraluminal degradation pathway. These findings are in agreement with yeast genetic studies that have indicated that PtdIns3P is transported from endosomes to the vacuole where it is degraded by a process that requires luminal vacuolar hydrolases or is modified by the PtdIns3P 5-kinase Fab1p [23,24]. The 2 $\times$ FYVE probe could be of potential use when studying endocytic traffic and the roles of PtdIns3P in other cell types such as neurons and antigen-presenting cells and also in other eukaryotic systems.

It has been previously proposed that PtdIns3P is present on the Golgi apparatus [25,26]. A PtdIns3P-producing PI 3-kinase has been proposed to be recruited to the Golgi, where it is involved in the formation of constitutive transport vesicles from the *trans*-Golgi network [25], and Vps34p has been detected in endosome- and Golgi-enriched membrane fractions in yeast [27]. There is also some evidence of a Golgi-located inositol-polyphosphate 5-phosphatase, capable of dephosphorylating PtdIns(3,5)P₂ and PtdIns(3,4,5)P₃ [28]. However, 2 $\times$ FYVE labelling revealed no evidence of PtdIns3P being present in the mammalian Golgi. It remains a possibility that the binding of 2 $\times$ FYVE to PtdIns3P is prevented by competition for PtdIns3P binding with a Golgi protein that has a much higher affinity for PtdIns3P, although when transfected 2 $\times$ FYVE was expressed at levels capable of displacing FYVE finger proteins from early endosomes PtdIns3P was still not detected on the Golgi [20].

The detailed knowledge now gained as to the cellular localization of PtdIns3P has interesting implications when considering the functions of FYVE domain proteins. If the function of FYVE domains is to bind PtdIns3P, then all FYVE domain proteins should have contact at some stage in their cellular functions with the membranes of the endosomal trafficking system. This information may also be of use when studying PtdIns3P-generating, -degrading and -modifying enzymes with regards to the proteins with which they interact or by which they are regulated.

Figure 3 Alternative models for FYVE–PtdIns3P interactions

(a) Two views of a model of the interaction of the Vps27p FYVE domain with PtdIns3P. The protein backbone is shown in blue, with the zinc atoms in cyan and the modelled PtdIns3P ('PI3P') in white. The basic amino acid residues of the conserved RKHCR motif, as well as two leucine residues at the membrane interface, are highlighted in yellow, and the 3-phosphate group of the

Figure 4 FYVE-domain proteins in membrane trafficking and signal transduction

PtdIns3P ('PI3P') has been localized in patches at the early endosome (EE) and in internal vesicles of the multivesicular body (MVB), with low amounts in late endosomes (LE). Several FYVE-domain-containing proteins (yeast homologues indicated in parentheses) have been localized to the EE. Functions have been proposed for several FYVE-domain-containing proteins: EEA1 is involved in tethering Rab5 positive vesicles; SARA, mediating signalling from the TGF- β receptor by the recruitment of Smads 2 and 3; Hrs, regulating formation of MVBs and is also involved in signal transduction; Rabenosyn-5, regulating fusion of endocytic vesicles and *trans*-Golgi-network (TGN)-derived vesicles with the EE; PIKfyve, a PtdIns3P 5-kinase is involved in the formation of MVBs and/or recycling from the vacuole; The Fgd1 family, function as guanine-nucleotide exchange factors for Rho-like GTPases, thereby regulating the actin cytoskeleton; FYVE-DSP1 has PtdIns3P phosphatase activity, but has so far not been localized to the EE; DFCP1 contains two FYVE domains and has been localized to the endoplasmic reticulum, the Golgi and cytosolic vesicles. Other abbreviations: PI, PtdIns; PI(3,5)P₂, PtdIns(3,5)P₂.

FYVE-DOMAIN-CONTAINING PROTEINS HAVE DIVERSE FUNCTIONS

Mammalian cells express more than 25 different FYVE-domain-containing proteins. Relatively little is known as yet about the exact functions of the majority of these proteins. It seems apparent from the available information about these proteins that they comprise a group with a wide range of different structures and functions (Figure 4; Table 1).

FYVE domain proteins involved in membrane trafficking

The most studied FYVE domain protein is EEA1, a large coiled-coiled protein with a C-terminal FYVE domain [29]. This protein also contains two binding domains for the early-endosomal GTP:Rab5 [30]. One of these domains is N-terminal, whereas the other is adjacent to the FYVE domain. Slightly conflicting reports have been published regarding the exact structural regions responsible for Rab5 binding by EEA1. By means of the yeast two-hybrid technique the C-terminal Rab5-binding region of EEA1 was mapped to residues 1277–1348, a region immediately N-terminal of the FYVE domain [30]. However, another study found that a 52-amino-acid region immediately upstream of the FYVE domain (residues 1306–1357) and also a structurally intact FYVE domain were required for Rab5 binding *in vitro*, leading to the conclusion that the FYVE finger is involved in Rab5 binding [31]. It still remains a possibility that the FYVE domain is not directly involved in the C-terminal Rab5 binding of EEA1. For instance, Rabphilin-3A contains a FYVE-like domain (it lacks the stretch of basic residues), and data have shown that this domain is necessary for Rab3A binding [32]. However, the X-ray crystal structure of the Rabphilin–Rab3A complex showed that the FYVE-like domain and Rab3A do not interact directly [33]. Instead, the FYVE-like domain plays a role in stabilizing the three-dimensional structure of the actual Rab3A-binding region.

Endosomal targeting of EEA1 requires both PtdIns3P binding by the FYVE domain and binding to Rab5:GTP. It has been proposed that this dual binding requirement enables the very specific and regulated recruitment of EEA1 to early endosomes [30,31,34], with only membranes positive for both PtdIns3P and Rab5:GTP able to bind EEA1.

EEA1 plays a role in endosome fusion, since depletion of EEA1 inhibits, and excess EEA1 stimulates, this process *in vitro* [30,35]. Endosome fusion also requires PI 3-kinase activity and Rab5:GTP, with Rab5 required on both membranes for fusion to take place [36]. Thus it seems as though EEA1 acts as a tether for two Rab5-positive membranes. This tethering is thought to facilitate the pairing of SNARE proteins [37], the process that drives membrane fusion. The role of the FYVE domain–PtdIns3P interaction is probably to concentrate EEA1 at the correct location to participate in this process.

Rabenosyn-5 is a recently identified FYVE domain protein which has also been shown to play a role in endosome fusion and has been proposed to be a mammalian homologue of Vac1p (see below) [38]. This protein contains an internal FYVE domain, an N-terminal C₂H₂-type zinc finger and a C-terminal Asn-Pro-Phe-containing motif which potentially could bind epsin-homology-domain-containing proteins. As with EEA1, Rabenosyn-5 is an effector of Rab5:GTP, is localized to early endosomes in a PtdIns3P-dependent manner and is required for endosome fusion. Unlike EEA1, Rabenosyn-5 directly interacts with the Sec-1 like protein hVPS45, and the overexpression of its NPF-motif containing C-terminus inhibits cathepsin D processing. This suggests that EEA1 and Rabenosyn-5 play distinct roles in endosomal trafficking, possibly by facilitating the interaction of different SNARE molecules.

inositol ring is red. The Figure is based on the structure solved by Misra and Hurley [15] and is reprinted from Driscoll, P. C. and Vuidepot, A. L. {© (1999) Peripheral membrane proteins: FYVE sticky fingers. *Curr. Biol.* **9**, R857–R860 [77], with permission from Elsevier Sciences and the authors}. (B) Model of the interaction of the dimeric VHS ('conserved in Vps27p, Hrs and STAM') and FYVE domains of Hrs with the membrane. The antiparallel association of FYVE domains lead to the formation of a homodimer with two ligand-binding pockets, each of which bind one PtdIns3P molecule. FYVE domains are shown in green whereas VHS domains are shown in red. PtdIns3P ('PI3P') is shown in purple and red with the phosphate groups in red/yellow. The Figure is reprinted from Mao, Y., Nickitenko, A., Duan, X., Lloyd, T. E., Wu, M. N., Bellen, H. and Quijcho, F. A. {© (2000) Crystal structure of the VHS and FYVE tandem domains of Hrs, a protein involved in membrane trafficking and signal transduction. *Cell* **100**, 447–456 [16], with permission from Elsevier Science and the authors}.

Table 1 Proposed functions of human and yeast FYVE domain proteins

Human	<i>Saccharomyces cerevisiae</i>	Putative function
EEA1		Tethering of Rab5-positive endocytic vesicles and early endosomes (both homo- and hetero-typic fusion) via an N-terminal Rab5-binding C ₂ H ₂ finger and a C-terminal Rab5-binding domain
Rabenosyn-5	Vac1p/Vps19p	Rab-5 effector molecule required for homo- and hetero-typic endosome fusion and Golgi to endosome trafficking; regulation of SNARE-complex formation through interaction with Vps45
Hrs	Vps27p	Involved in trafficking from the endosome to the lysosome/vacuole and the formation of multivesicular endosomes; involved in signal transduction through interaction with STAM and Smad2
SARA		Mediator of TGF- β signalling through recruitment of Smad2 and Smad3 to the TGF- β receptor
PIKfyve	Fab1p	PI 5-kinase that produces PtdIns(3,5)P ₂ ; involved in the formation of multivesicular endosomes or in recycling from the vacuole/lysosome
Fgd1, Fgd2, Fgd3 Frabin		Guanine-nucleotide exchange factors for Cdc42, regulation of actin cytoskeleton, induce the formation of filopodia. Frabin also activates Rac and can induce lamellipodia formation
DFCP1		Unknown; trafficking between the biosynthetic and the endocytic pathway?
FYVE-DSP1/MTMR3		PtdIns3P phosphatase that regulates PtdIns3P levels

Yeast cells contain only one PI 3-kinase, Vps34p, and this enzyme only produces a single phosphoinositide, namely PtdIns3P. Vps34p was discovered in a screen for mutants in which vacuolar protein sorting is defective (*vps* mutants). The inactivation of this PI 3-kinase led to a mis-sorting of a subset of hydrolases from the Golgi apparatus to the vacuole, which is the yeast equivalent of the lysosome [39]. This was the only effect of the *vps34* mutation, thus indicating that PtdIns3P is required exclusively in this pathway and also suggesting that all of the FYVE domain proteins in yeast are involved in this pathway. Five FYVE domain proteins have been identified in yeast, and indeed the three of them for which a function has been proposed (Vac1p, Vps27p and Fab1p) are all involved in endocytic/vacuolar trafficking.

Vac1p has been proposed to be a homologue of EEA1 [40], but recently it has been proposed that in fact the newly identified Rabenosyn-5 is the real homologue of Vac1p and not EEA1 [38]. Yeast *vac1* mutants accumulate vesicles destined for the endosome, suggesting that Vac1p is involved in endocytic docking and fusion [39]. Besides a PtdIns3P-binding FYVE domain, Vac1p also contains an N-terminal C₂H₂ zinc finger and binds to the yeast homologue of Rab5 [40]. Rabenosyn-5 also interacts with a Vps45p homologue, whereas EEA1 does not. Vac1p has been shown to interact with Vps45p, although the exact role that this interaction plays in endosome fusion is not fully understood [40]. It is noteworthy that Vac1p has a second FYVE domain N-terminal to the one studied in regard to PtdIns3P binding. However, this FYVE domain lacks most of the conserved basic residues of typical FYVE domains and its role in PtdIns3P binding is unclear.

Vps27p has been proposed to be the yeast homologue of Hrs (below). Its role in endocytic fusion is thought to be at a later stage than that of Vac1p. This is because *vps27* mutants contain a decreased number of intraluminal vesicles in endosomes and vacuoles and the occurrence of what is known as the 'class E compartment' [41,42], a cup-shaped multilamellar organelle that distinguishes the class E subset of *vps* mutants. This structure is thought to represent an aberrant late-endocytic structure. The FYVE domain of Vps27p has been shown to be essential to its function, since Vps27p with mutations in the FYVE domain are unable to complement the phenotype of the *vps27* mutation, although these FYVE domain mutants are still able to associate with membranes [41]. It therefore appears that PtdIns3P binding might not be essential to the membrane localization of Vps27p, although this binding appears to be essential to its function.

The third yeast FYVE-domain-containing protein that has been implicated in vesicular trafficking is Fab1p. This protein is a PtdIns3P 5-kinase, i.e. it phosphorylates PtdIns3P to form PtdIns(3,5)P₂ [9], a phosphoinositide that has only recently been discovered [43] and for which there are at present no known effectors. *fab1* mutants have morphological defects in the form of an expanded vacuole, indicating a role for PtdIns(3,5)P₂ in vacuolar homeostasis [42]. A mammalian orthologue of Fab1p, termed PIKfyve (p235), has been identified [44]. This protein is able to produce both PtdIns5P and PtdIns(3,5)P₂ *in vitro* and recruits PtdIns3P-producing PI 3-kinases upon insulin stimulation. It remains to be seen whether this protein functions in membrane-trafficking events or is membrane-localized.

FYVE domain proteins involved in signal transduction

An example of a FYVE finger protein that is involved in a signalling cascade is SARA (Smad anchor for receptor activation). This protein is responsible for the recruitment of Smad2 and Smad3 to the transforming growth factor (TGF)- β receptor upon receptor stimulation [45]. These Smads are then phosphorylated by the activated receptor kinases. The phosphorylation of Smad2 and Smad3 enables them to bind Smad4, with the resulting heteromeric complex subsequently translocated to the nucleus, where it controls the transcription of target genes. SARA has been shown to be located on vesicular structures. These structures probably represent early endosomes. It therefore seems that the role SARA plays in the TGF- β signalling cascade is to recruit molecules to early endosomes, to which the receptor-ligand complex localizes upon endocytosis. The endosomal localization of SARA is most likely due to an interaction of its FYVE domain with PtdIns3P. SARA thus provides a potential example of signalling not from the plasma membrane but from the endosomal membrane, a compartment not classically associated with this function [46]. It also represents an example of a role for a PI 3-kinase product in a cell-signalling cascade that is not that of a second messenger.

Hrs [47] is a mammalian FYVE domain protein that has been implicated in both membrane-trafficking and signal-transduction events. A mouse knockout study indicated that Hrs is necessary for ventral folding morphogenesis [48]. The findings that Hrs interacts with the SNARE protein SNAP-25 [49] and is a likely homologue of the yeast vacuolar sorting protein Vps27p suggest that it regulates membrane-trafficking events. Hrs is conserved

from flies to humans, and rats also contain a very closely related protein, Hrs-2 [49], which has a distinct C-terminus, although its DNA sequence is almost identical with that of Hrs. Hrs has an endosomal localization, although conflicting data have been presented as to whether this localization depends upon PtdIns3P binding. It has been shown that PI 3-kinase activity and PtdIns3P binding by the FYVE domain of Hrs are essential for the endosomal localization of Hrs, which is in turn necessary for the phosphorylation of Hrs upon growth-factor-receptor stimulation [50]. Treatment of cells with the PI 3-kinase inhibitor wortmannin has also been reported to cause a displacement of Hrs from endosomal membranes [48]. However, another study reported that the endosomal localization of Hrs was due to a C-terminal proline- and glutamine-rich region of Hrs with neither PtdIns3P binding nor PI 3-kinase activity necessary for membrane association [51]. Data presented also indicated that full-length Hrs from cytosol does not interact with PtdIns3P at all. This suggests that additional proteins may regulate the association of Hrs with PtdIns3P, perhaps by regulating the exposure of the FYVE domains. Our recent data support the idea that the PtdIns3P–FYVE interaction is necessary for the endosomal localization of Hrs (C. Raiborg, B. Bremnes, A. Mehlum, D. J. Gilooley, A. D'Arrigo, E. Stang and H. Stenmark, unpublished work). We found that both the FYVE domain and a coiled-coiled domain were necessary for Hrs to bind to membranes. It is clear that more work is required to clarify the membrane targeting in order to understand the function of Hrs. Hrs has been shown to bind to multiple proteins, including the signal-transducing adaptor molecule STAM [52] and Smad2 [53]. This indicates that Hrs is involved in signalling pathways, and there is evidence that it participates in activin-receptor-mediated signalling in co-operation with SARA [53]. Hrs could therefore provide a link between membrane trafficking and signal transduction.

PtdIns3P phosphatases

An interesting family of proteins that has come to light recently, and of which several have FYVE domains, is the myotubularin (MTM1) family of proteins. MTM1 is a protein that is mutated in the X-chromosome-linked congenital disorder myotubular myopathy, a disease in which muscle differentiation is severely affected. MTM1 family members belong to the superfamily of protein phosphatases containing the Cys-Xaa₅-Arg active-site motif and exhibit dual-specificity protein phosphatase (DSP) activity *in vitro* [54,55]. This means that they can dephosphorylate serine/threonine residues as well as tyrosine residues. However, recent findings have shown that MTM1 is in fact a potent phosphoinositide phosphatase *in vitro* and *in vivo* and that this activity is specific for PtdIns3P [56,57]. Another member of this protein family is MTM1-related protein-2 (MTMR2), a protein that is mutated in the demyelinating neuropathy Charcot-Marie-Tooth disease [58]. MTMR2 contains the characteristic DSP motif but it has not been shown whether or not it has phosphoinositide phosphatase activity. It would be interesting to investigate whether these two similar proteins have differing functions, since mutations in each of them result in different diseases.

MTM1 and MTMR2 do not contain FYVE domains, but several MTM1-like proteins contain C-terminal FYVE domains. These proteins are conserved across species from nematodes and flies to humans, so it is likely that they have a common substrate and function. One such MTM1-like protein that contains a FYVE domain is FYVE–DSP1, also known as MTM1-related protein-3 ('MTMR3'). This protein can dephosphorylate

PtdIns3P [56] and is localized in membrane fractions [59], although it has still to be ascertained as to whether this interaction is due to the FYVE-finger-binding PtdIns3P. The cellular function of the MTM1 family of proteins is not known, although it seems likely that they play a role in regulating PtdIns3P levels as a counterbalance to PI 3-kinases. It has still to be resolved as to whether PtdIns3P levels are increased in the myotubular myopathy and Charcot-Marie-Tooth disorders, but it will be interesting to determine if this is the case and why increased PtdIns3P levels have such a profound effect upon muscle and myelin development. Future developments as to how this new family of PtdIns3P phosphatases is regulated and whether or not they interact with other proteins will surely prove informative.

FYVE domain proteins involved in regulating the cytoskeleton

There is a subgroup of FYVE domain proteins that also contain two PH domains, suggesting that these proteins bind to other phosphoinositides as well as to PtdIns3P. These proteins are Fgd1 and the closely related Fgd2, Fgd3 and Frabin. Fgd1 was one of the first FYVE domain proteins identified and is the product of the faciogenital-dysplasia (Aarskog syndrome) gene [60]. This is an X-chromosome-linked developmental disease primarily associated with skeletal, facial and genital anomalies. Fgd1 is a guanine-nucleotide exchange factor that specifically activates the Rho GTPase Cdc42 [61]. In this way Fgd1 regulates the actin cytoskeleton and activates the c-Jun N-terminal kinase signalling cascade to regulate cell growth and differentiation. Fgd3 and Frabin have been shown to have very similar functions to Fgd1. Both induce the formation of filopodia and, in addition, Frabin has been shown to induce lamellipodia formation and a Cdc42-independent activation of the small GTPase Rac [62,63]. The multiple functions of Frabin have been attributed to distinct parts of the Frabin molecule. There is evidence that the FYVE domain is involved in the formation of lamellipodia, but not of filopodia. Thus it seems likely that different domains of the Frabin molecule recruit differing molecules, or facilitate the binding to different membranes via different phosphoinositides, thus enabling the molecule to elicit different cellular responses. Preliminary evidence suggests that the proteins of the Fgd1 family are not localized to the endocytic pathway. The FYVE domains of this family of proteins are slightly atypical in that they lack a tryptophan residue conserved in the other FYVE domains (residue 5, Figure 2). Considering the probable localization of these proteins, it therefore needs to be established whether or not these FYVE domains bind PtdIns3P, and, if they do not, to which ligand they do bind.

Double-FYVE-containing protein

A new protein has recently been identified from a human bone marrow cDNA library which contains two FYVE domains and has been termed 'double FYVE-containing protein 1' (DFCP1) [64]. DFCP1 contains an N-terminal cysteine- and histidine-rich region that has a loose correlation with different types of zinc fingers, a consensus ATP/GTP binding site and a unique C-terminus that contains two FYVE domains. The two FYVE domains share 53% amino acid identity with each other and 40% identity with FYVE domains from other proteins. Both of the FYVE domains contain the conserved cysteine residues necessary for zinc co-ordination, but it is noteworthy that a serine and a threonine residue replace the first arginine residue in the conserved basic RR/KHHCR motif in the two DFCP1 FYVE domains (Figure 2). It will be interesting to see whether or not these FYVE fingers can bind PtdIns3P or indeed to any other

phosphoinositides. The double-FYVE domain probe that has been used to localize PtdIns3P has been shown to form a more stable interaction with PtdIns3P than a singly expressed FYVE domain [20]. It is possible that DFCP1 has two FYVE domains to form a more stable interaction with PtdIns3P. When expressed in fibroblasts, DFCP1 was found to be localized on cytoplasmic vesicles (possibly endosomes), the endoplasmic reticulum and the Golgi apparatus. This is in contrast with the more restricted endosomal localization of EEA1, Hrs and PtdIns3P. This pattern of localization is consistent with a possible role for this protein in vesicular trafficking, but it remains to be determined whether this is the case and what role the FYVE domains play in the localization and function of this protein.

MEMBRANE RECRUITMENT OF FYVE DOMAIN PROTEINS

The affinity of the FYVE domain–PtdIns3P interaction has been measured using surface plasmon resonance for the EEA1 and Hrs FYVE domain–PtdIns3P interactions, with the apparent K_D found to be about 50 nM [19,20]. This affinity is in the same order of magnitude as the 10–250 nM range found for interactions between PH domains and phosphoinositides [5] and is certainly a strong enough affinity to enable an *in vivo* interaction. Stephens et al. [65] estimated that the local concentrations of PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 at the inner leaflet of the plasma membrane are 5 μ M and 10–20 μ M respectively at basal levels, but upon agonist stimulation increase to 200 μ M PtdIns(3,4,5) P_3 and 100–200 μ M PtdIns(3,4) P_2 . These increases in phosphoinositide concentration are enough to result in a translocation of PH domains with affinities in the 10–250 nM range to the inner leaflet of the plasma membrane [66,67] (see [5]). PtdIns3P was estimated to be present upon membranes at a concentration of 200 μ M, so, by analogy with PH domains, a FYVE domain with an affinity for PtdIns3P of 50 nM ought to be localized on the membrane. It is therefore a little surprising that EEA1 requires Rab5:GTP binding as well as PtdIns3P binding in order to associate with endosomal membranes [30]. It is possible that dimerization of EEA1 results in a higher affinity for PtdIns3P and is induced by PtdIns3P binding [17]. High-level expression of a double-FYVE-domain-containing construct with a high affinity for PtdIns3P (2 $\times$ FYVE) leads to the displacement of EEA1 from endosomal membranes [20]. Therefore it seems as though there is a limited amount of PtdIns3P available for FYVE domain binding. EEA1 may face competition with other FYVE domain proteins for free PtdIns3P, and it is possible that Rab5 binding is necessary for EEA1 to be able to ‘overcome’ the competition from other FYVE-containing proteins. As yet there are no reports of a FYVE domain alone being sufficient to target a protein to a membrane. Reports have shown that small FYVE-domain-containing constructs of EEA1 and Rabenosyn-5 are localized to early endosomes [14,38], but neither of these constructs expressed only a FYVE domain, and in the case of the EEA1 construct, the construct contained the region responsible for Rab5 binding. It therefore seems that the FYVE domain–PtdIns3P interaction alone is not stable enough to anchor a protein on to a membrane surface. Additional interactions with the membrane, for example with membrane-associated proteins, may also be required.

PH DOMAINS AND PtdIns3P

Although FYVE-domain-containing proteins have been characterized as PtdIns3P effectors, this does not exclude proteins that do not contain a FYVE domain from being PtdIns3P effectors. In fact, several PH domains have been shown to interact with

PtdIns3P *in vitro*, although most of these PH domains also interact with other phosphoinositides, and also with proteins. None of them has convincingly been shown to be a PtdIns3P effector. The PH domain of 3-phosphoinositide-dependent protein kinase-1 (‘PDK1’) was shown, using surface plasmon resonance, to have an apparent K_D for PtdIns3P of 84 nM [68], a value which is comparable with that of FYVE domains tested [19,20]. However this PH domain had a greater affinity for PtdIns(3,4,5) P_3 , PtdIns(3,4) P_2 and PtdIns(4,5) P_2 , and *in vivo* data implicate PtdIns(3,4,5) P_3 as the true ligand for this protein [66,68]. The plasma-membrane targeting of phospholipase $C\beta_1$ following agonist stimulation has been proposed to be due to an interaction of the PH domain from this protein with PtdIns3P [69]. This could potentially be a very interesting finding, but it is clear that this PH domain has a significant affinity for other and much more abundant phosphoinositides and also interacts with $G_{\beta\gamma}$ regulatory subunits, so it remains unclear as to whether this protein is a true effector of PtdIns3P. Possibly the most likely candidate for a PH-domain-containing PtdIns3P effector is the recently discovered PEPP1 (phosphatidylinositol-three-phosphate-binding PH-domain protein-1) [70]. This protein was initially identified in a screen for PH-domain-containing proteins in expressed-sequence-tag databases. Phosphoinositide-binding studies indicate that the PH domain of PEPP1 binds specifically to PtdIns3P. Two homologues of PEPP1, namely PEPP2 and PEPP3, were also identified, although their phosphoinositide-binding abilities have yet to be investigated. As yet no function has been attributed to PEPP1, although its expression has been seen to be elevated in several melanoma cell lines.

CONCLUSIONS AND PERSPECTIVES

The identification of the FYVE finger domain has, in a relatively short space of time, significantly increased the knowledge available concerning vesicular trafficking and the functions of PtdIns3P in both yeast and mammalian cells. It has enabled the determination of the localization of PtdIns3P, the first detailed ‘mapping’ of a phosphoinositide, and advanced the knowledge of this lipid and its effectors. So far the FYVE-domain-containing proteins for which most information is known are involved in vesicular trafficking. However, it is also evident that the functions of FYVE-domain-containing proteins are not just restricted to the vesicular-trafficking process (Figure 4; Table 1). Hrs and SARA are involved in signal transduction, and the Fgd1, Fgd3 and Frabin family of proteins play a role in the regulation of the actin cytoskeleton. Two other processes worth investigating for possible roles of FYVE-domain-containing proteins are autophagy and phagocytosis. PtdIns3P has been implicated in the stimulation of autophagy [71], and PI 3-kinases and EEA1 have been implicated as having a role in phagocytosis [72,73].

Most of the evidence available presents FYVE domains as targeting membrane anchors, in that they facilitate the membrane localization of proteins at sites of PtdIns3P production. However, this may not be the case for all FYVE domain proteins, since the proteins of the Fgd1 family seem not to be located on the endosomal pathway, and their atypical FYVE domains have yet to be shown to be able to bind PtdIns3P. It remains important to determine whether all FYVE domains bind PtdIns3P or whether some FYVE domains can interact with other phospholipids or even proteins, as is the case for PH domains [5]. It will also be interesting to see whether proteins that do not contain FYVE domains can also be effectors of PtdIns3P. An interesting idea worth investigating is that PtdIns3P may be formed in microdomains on the membrane (see Figure 4). In this way it may act to influence membrane structure or as a vehicle for

specifically recruiting a set of effector proteins to restricted membrane areas where they can then perform a specific function.

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