FYVE zinc-finger proteins in the plant model *Arabidopsis thaliana*: identification of PtdIns3*P*-binding residues by comparison of classic and variant FYVE domains

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Classic FYVE zinc-finger domains recognize the phosphoinositide signal PtdIns3*P* and share the basic (R/K)^1(R/K)HHCR^6 (single-letter amino acid codes) consensus sequence. This domain is present in predicted PtdIns3*P* 5-kinases and lipases from *Arabidopsis thaliana*. Other *Arabidopsis* proteins, named PRAF, consist of a pleckstrin homology (PH) domain, a regulator of chromosome condensation (RCC1) guanine nucleotide exchange factor repeat domain, and a variant FYVE domain containing an Asn residue and a Tyr residue at positions corresponding to the PtdIns3*P*-interacting His^4 and Arg^6 of the basic motif. Dot-blot and liposome-binding assays were used *in vitro* to examine the phospholipid-binding ability of isolated PRAF domains. Whereas the PH domain preferentially bound PtdIns(4,5)P_2, the variant FYVE domain showed a weaker charge-dependent binding of phosphoinositides. In contrast, specificity for PtdIns3*P* was obtained by mutagenic conversion of the variant into a classic FYVE domain (Asn^4,Tyr^6 → His^4,Arg^6). Separate substitutions of the variant residues were not sufficient to impose preferential binding of PtdIns3*P*, suggesting a co-operative effect of these residues in binding. A biochemical function for PRAF was indicated by its ability to catalyse guanine nucleotide exchange on some of the small GTPases of the Rab family, permitting a discussion of the biological roles of plant FYVE proteins and their regulation by phosphoinositides.

Key words: PH domain, phosphoinositide, phospholipid.

INTRODUCTION

The differently phosphorylated forms of the lipid PtdIns are important in membrane trafficking and signal transduction through the binding of modular proteins to or at specific membranes. They are generated by kinase-dependent phosphorylation at specific positions of the *myo*-inositol headgroup of inositol-containing lipids [1]. The stereoisomeric headgroups are recognized by protein domains such as the pleckstrin homology (PH), PTB, C2, Src homology and FYVE zinc-finger domains [2]. These domains vary, also within the same domain type, in their affinities and specificities towards their phosphoinositide targets.

The FYVE domains, named from the parent proteins Fab1, YGLO23, VPS27 and EEA1 [3], are approx. 80-residue zinc-fingers stabilized by the co-ordination of two zinc atoms. A FYVE domain from several otherwise unrelated proteins, including the well-studied mammalian proteins EEA1 and Hrs and the yeast proteins Vps27p, Vac1p and Fab1p, have been shown to interact specifically with phospholipid liposomes containing PtdIns3*P* [4–6]. The physiological relevance of this interaction is debated. The FYVE domain in EEA1 and Hrs1 has been implicated in the localization of the proteins to their intracellular target, the endosomes [3,4,7], by binding to either PtdIns3*P* or a protein partner such as Rab5 for EEA1 [8,9]. Other studies suggest that the endosomal localization of Hrs is dependent on a region different from the FYVE domain [10]. Whereas most of the FYVE proteins studied so far have been suggested to regulate endocytic membrane trafficking, FYVE proteins might also have a role in signal transduction or cytoskeletal regulation [11,12].

The conserved basic motif (R/K)^1(R/K)HHCR^6 (single-letter amino acid codes) surrounding the third zinc-co-ordinating cysteine residue distinguish the FYVE finger family from other structurally related zinc-fingers such as the RING domain [13] and is essential for the binding of PtdIns3*P* [6]. Structural studies of a FYVE domain from Vps27 [13], EEA1 [14] and Hrs1 [15] revealed similar structures consisting of two double-stranded β-sheets and a C-terminal α-helix. Despite this, different models for PtdIns3*P* binding were proposed on the basis of the two crystal structures. Two residues, His^4 and Arg^6 of the basic motif, and a small binding pocket were predicted from modelling to determine the specificity for binding of PtdIns3*P* to the monomeric Vps27 FYVE domain [13]. In contrast, the ability of the FYVE domains from EEA1 and Hrs to form homodimers [14,15] led to the suggestion of two neighbouring identical pockets formed by dimers [15]. The recent determination of the solution structure of both the free and the PtdIns3*P*-bound form of the EEA1 FYVE domain showed pronounced conformational changes resulting from ligand binding. The NMR data further revealed a binding pocket consisting of two basic clusters and hydrogen bonds between the 3-phosphate group of PtdIns3*P* and Arg^6 [16] in a binding orientation similar to that suggested for Vps27 [13,17].

In the present study we have surveyed the proliferation of FYVE domains in the model plant *Arabidopsis*. We report the binding properties of the PH domain and the variant FYVE

Abbreviations used: CFY, classical FYVE finger; EST, expressed sequence tag; GapC, glyceraldehyde-3-phosphate dehydrogenase C subunit; GST, glutathione S-transferase; PH, pleckstrin homology; PRAF1, 'PH, RCC1 and FYVE'; PtdCho, phosphatidylcholine; RCC1, regulator of chromosome condensation; RT–PCR, reverse-transcriptase-mediated PCR; VFY, variant FYVE finger.

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domain in the *Arabidopsis* PRAF1 protein (for 'PH, RCC1 and FYVE'), which structurally and biochemically expands the group of FYVE proteins. The gain of PtdIns3*P*-binding preference by mutagenic conversion of the variant motif into a classic basic motif demonstrates the importance of specific residues for this preference.

MATERIALS AND METHODS

Identification and analysis of FYVE domain sequences and predicted proteins

Searches in the non-redundant ('NR') and expressed sequence tag (EST) databases were performed by using BLAST2 [18]. Proteins predicted only from genomic data were named by their accession number. EST cDNA clone 160B1XP 3′ was acquired from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH, U.S.A.). Sequencing was done on a ABI Model 373A DNA sequencer (Perkin Elmer). Sequence context analyses [19] and TATA box prediction were used to help in defining translation initiation sites. Predicted protein sequences were examined for similarity to known protein sequences by BLASTP and SMART [20] and for sorting signals and membrane-spanning regions by PSORT [21].

RNA purification, Northern analyses and reverse-transcriptase-mediated PCR (RT–PCR)

Total RNA was purified from *Arabidopsis* tissues, untreated or treated as shown in Figure 4, by extraction with phenol/chloroform and precipitation with LiCl [22]. For evaluation of the quality of the RNA, 10 μg of each preparation was analysed on an agarose gel. For the RNA Northern analyses, 10 μg (for the slot-blot) or 20 μg of denatured RNA was applied to a Hybond N nylon membrane (Amersham Pharmacia Biotech) by using a vacuum slot-blot manifold (for the slot-blot) (Schleicher and Schuell) or by capillary blotting. A ^{32}P-labelled probe of a specific DNA fragment of PRAF1 or the C subunit of glyceraldehyde-3-phosphate dehydrogenase (GapC; EST clone 84E1T7) was prepared with a High Prime Labelling Kit (Boehringer Mannheim). Incubation and washing procedures were as described by the manufacturer. RT–PCR was performed as described previously [22] with the intron-spanning primers 5′-GAGGCAATCAAGTCGCTC-3′ and 5′-GGAGACTGACTACTAGTG-3′.

Cloning of the CAB36798 FYVE domain

Because no cDNA clone corresponding to the predicted CAB36798 protein was initially present in the EST database, the region encoding the FYVE domain was PCR-amplified from the λ-PRL2 library obtained from the *Arabidopsis* Biological Resource Center. The upstream *Eco*R1-linker primer 5′-GCCGGCAGAATTCATGGACTCACAAGATCACAAAG-3′ and the downstream *Xho*1-linker primer 5′-CGCCGCCACTCGAGTTAGCTTCTAGCAGAAGGCGATG-3′ were used in the following amplification cycle: 2 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C and a final extension time of 10 min at 72 °C. The amplification product was cloned into the expression vector pGEX-4T-1 (Amersham Pharmacia Biotech) as described below.

Construction of expression vectors and expression and purification of recombinant proteins

For cloning of PRAF1 domains, restriction sites were introduced into the sequences by standard PCR with the primer set 5′-GCCGGCAGAATTCGTGGAAGCTTTGAAGGATAG-3′ and 5′-GGGGATCCTCGAGTTATTAGTTAGTATCGTTTATCTCAGAC-3′ for the FYVE domain (PRAF1 residues 603–703), 5′-GCCGGCATATGGAATTCATGGCAGATCTTGTGACC-3′ and 5′-GGGGATCCTCGAGTTAGCCACTCCAGCCATCGAT-3′ for the PH domain (PRAF1 residues 1–138), and 5′-CGAGCCGAGTCGACTAGCAGATGACTCAGATGC-3′ and 5′-GAGCCGAGTCGACTTATTTGTGAAGACATATGGCTGC-3′ for the regulator of chromosome condensation (RCC1) domain (PRAF1 residues 231–629). These products and the PCR product of the CAB36798 FYVE domain (CAB36798 residues 1–127) were cloned in frame with the glutathione S-transferase (GST) gene in pGEX-4T-1. The resulting recombinant proteins were named GST–VFY (where VFY stands for variant FYVE finger), GST–PH, GST–RCC1 and GST–CFY (where CFY stands for classical FYVE finger). Single and double mutants of the PRAF1 FYVE domain were obtained by strand-overlap-extension PCR with the primers shown above, together with the internal primers 5′-GTCCACAATTGCGACAGTTATGTCTTTTCC-3′ and 5′-GGAAAAGACATAACTGTCGCAATTGTGGAC-3′ (Tyr^{660} → Arg), 5′-GGAAAAGACATCACTGTTACAATTGTGGAC-3′ and 5′-GTCCACAATTGTAACAGTGATGTCTTTTCC-3′ (Asn^{658} → His) and 5′-GGAAAAGACATCACTGTCGCAATTGTGGACTTG-3′ and 5′-CAAGTCCACAATTGCGACAGTGATGTCTTTTCC-3′ (Asn^{658} → His,Tyr^{660} → Arg). GST and GST fusion proteins were expressed in *Escherichia coli* strain sBL21(DE3) at 37 or 15 °C and purified by glutathione-affinity chromatography. GST–VFY1 fusion proteins were purified further by cation-exchange chromatography before use.

Rab and Arf proteins for the guanine nucleotide exchange assays were produced as poly-His recombinant proteins from the pET-30a vector (Novagen), because both an Arf1 deletion protein and Rab proteins have been shown to be substrates for guanine nucleotide exchange factor when fused to an N-terminal tag [23,24]. Δ17Arf1 (residues 18–181 of Arf1; accession no. P36397), Δ17Arl1 (residues 18–182 of Arl1; previously Arf3 [25]; accession no. X77382) and full-length Rab8a (previously Ara3; T04827), Rab18a (previously Rab1α; N96030), Rab2c (previously gb2; R89928), Rab11c (Z30738); Rab11g (previously gb3; T42205) and Rab5a (previously Rha1; H77002) were amplified with the following primer set: 5′-CGAGCCCCATGGGTATTCTGATGGTTGG-3′ and 5′-TATTATGGATCCTTATGCCTTGCTTGCGATGTT-3′ (Arf1), 5′-CGAGCCCCATGGGTATTCTGATGGTTGG-3′ and 5′-CGAGCCGGATCCTTATTAGCCACTTCCCGACTTC-3′ (Arl1), 5′-CGAGCCCCATGGCTGCTCCACCTGCTAG-3′ and 5′-CGAGCCGGATCCTTATTAAGTTCCACAGCATGCAG-3′ (Rab8a), 5′-CGAGCCCCATGGGATCTTCTTCTGGGC-3′ and 5′-CGAGCCGGATCCTTACTAGCTGCAACAGCCGG-3′ (Rab18a), 5′-CGAGCCCCATGGCTTACGATTATCTCTTC-3′ and 5′-CGAGCCCCATGGCTTACGATTATCTCTTC-3′ (Rab2c), 5′-CGAGCCCCATGGATGGCGAGAAGACCGGACG-3′ and 5′-CGAGCCGGATCCTTATCAAGACGATGAGCAACAAGG-3′ (Rab11c), 5′-CGAGCCCCATGGCCGGAGGAGGCGGATACG-3′ and 5′-CGAGCCGGATCCTTATCAAGAAGAAGTACAACAAG-3′ (Rab11g), and 5′-CGAGCCCCATGGCTAGCTCTGGAAACAAG-3′ and 5′-CGAGCCGGATCCTTACTAAGCACAACACGATGAACTC-3′ (Rab5a). The resulting proteins were purified by metal-chelation chromatography on HiTrap chelating beads. Ran1 (248H22T7) was cloned into pET-30a with the upstream primer 5′-ATATATATGGATCCATGGCTCTACCTAACCAGC-3′ and the downstream primer 5′-GAGGAGAGGATCCTTACTCAAAGATATCATCATCG-3′; the protein was purified by

anion-exchange chromatography. The sizes of the purified recombinant proteins were verified by matrix-assisted laser desorption ionization time-of-flight MS.

Phospholipid dot-blot assay

Initial screening for phospholipid binding was performed as described recently [26]. The indicated amount in 5 μl of the lipids (from Avanti Polar Lipids, Sigma or Matreya) were spotted on nitrocellulose (NitroBind, 0.45 μm pore size; MSI) and the membrane was dried before being blocked for 1 h by 3 % (w/v) fatty-acid-free BSA (Sigma) in TBST [10 mM Tris/HCl (pH 8.0)/150 mM NaCl/0.1 % (v/v) Tween 20]. Incubation with 2 μg/ml protein was performed overnight at 4 °C, with shaking. After being washed, the filter was incubated for 2 h at room temperature with polyclonal goat antibodies against GST (Amersham Pharmacia Biotech) diluted 1:2000 in TBST. After a further washing, goat anti-IgG horseradish peroxidase conjugate (Dako) was used as the secondary antibody and reactions were detected by chemiluminescence.

Liposome binding assays

Quantitative measurements of phospholipid binding were based on a frequently used strategy [27], with minor modifications. Phosphatidylcholine (PtdCho) alone or mixed with 4 % or 1 % (mol/mol) of the indicated phosphoinositide and a trace of ^{3}H-labelled PtdCho (1.0 μCi/ml; Amersham Pharmacia Biotech) were dried under nitrogen, resuspended in 50 mM Hepes/NaOH (pH 7.4)/100 mM NaCl by vortex-mixing, then sonicated for 2 min. For each assay, 0.6 nmol of protein, determined by amino acid analysis and A_{280}, was preincubated with 20 μl of glutathione–Sepharose 4B before the addition of 20 μg of phospholipid and incubation at room temperature for 20 min, with shaking. After incubation, the beads were washed three times and lipid binding was quantified by liquid-scintillation counting.

Guanine nucleotide exchange assays

The ability of GST–RCC1 to catalyse guanine nucleotide exchange was examined by measuring the dissociation of [^{3}H]GDP from the GTPase in accordance with procedures described previously for Δ17ARF1 [23] and different Rab proteins [28,29]. GTPase (1 μM) was incubated for 30 min at 30 °C with 10 μM [^{3}H]GDP (Amersham Pharmacia Biotech) in 50 mM Tris/HCl (pH 8.0)/100 mM NaCl/0.5 mM dithiothreitol/0.12 % CHAPS (Rabs and Ran1) or in 50 mM Tris/HCl (pH 8.0)/1 mM $MgCl_2$/100 mM KCl/1.0 mM dithiothreitol (Δ17ARFs). The [^{3}H]GDP-bound GTPase was incubated at 0.2 μM for 20 min at 30 °C in 25 μl of a reaction mixture containing 50 mM Tris/HCl, pH 8.0, 12 mM $MgCl_2$, 2 mM EDTA, 100 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM guanosine 5′-[γ-thio]triphosphate, 0.06 % CHAPS and 1.0 μM GST–RCC1. After incubation the sample was filtered through a nitrocellulose membrane (BA85, 0.45 μm pore size; Schleicher and Schuell), washed with an ice-cold solution of 20 mM Tris/HCl (pH 7.5)/100 mM NaCl/10 mM $MgCl_2$; the radioactivity associated with the filter was quantified.

Visualization of three-dimensional structure

WebLab ViewerLite 3.7 (Molecular Simulations Inc.) was used to display the molecular surface of the FYVE domain of Vps27p (Protein Data Bank entry 1VFY) and a substituted domain generated in Swiss-Pdb Viewer OpenGL 3.51 (Glaxo Wellcome Experimental Research).

RESULTS

Identification and domain structure of *Arabidopsis* FYVE domain proteins

Although specific phosphoinositides, including PtdIns3*P*, have been identified in plants, their target proteins and roles remain mainly unknown [30,31]. To examine the diversity of plant FYVE proteins we searched the databases for sequences encoding FYVE domains in *Arabidopsis*, a plant model organism with a completed genome sequence. BLAST2 analyses with well-defined FYVE domains as search strings identified nine *Arabidopsis* genes encoding FYVE domains. On the basis of domain composition and global sequence similarity the predicted proteins belong to four groups, represented by PRAF1, CAB36798, AAF79901 and CAB89044, three of which have additional recognizable domains (Figures 1A and 2). Proteins from a larger group, represented by AAD10163, contain FYVE-related domains that conform to the FYVE consensus sequence with regard to Zn^{2+}-chelating residues but diverging from it at additional conserved positions.

The CAB36798 and AAF79901 proteins both contain a classical basic FYVE motif. Analysis of the predicted CAB36798 protein (1757 residues) revealed an N-terminal FYVE finger, a coiled-coil region and a C-terminal PtdIns kinase domain. Because most of the CAB36798 sequence showed a pronounced similarity to the yeast protein Fab1p, which possesses PtdIns3*P* 5-kinase activity [32], the predicted *Arabidopsis* protein might be a plant Fab1p orthologue (Figure 2). In contrast, the predicted AAF79901 protein (967 residues) has a novel domain structure consisting of the FYVE finger, several proline-rich regions and a Gly-Asp-Ser-Leu lipase domain [33]. This domain composition is known from anther-specific *Arabidopsis* and *Brassica* proteins; however, these lack the FYVE domain [34].

Variant FYVE domain in the PRAF proteins

PRAF1, together with five additional predicted proteins, contain the same novel PH-RCC1-FYVE domain context (Figures 2 and 3). PRAF1 is represented in the EST database by only two entries, and one of the clones was sequenced. Comparison of the encoded sequence and that annotated for the gene (accession no. AAC00618) suggested the faulty prediction of an intron splice site at the 5′ end of the gene. In this case the five proteins would have similar N-termini and would be of approximately the same size (120 kDa), with PRAF1 consisting of 1103 residues.

The FYVE domain in the PRAF proteins is a variant of the classical FYVE domain and contains an Asn residue and a Tyr residue at positions corresponding to His^4 and Arg^6 of the basic motif (Figure 1). On the basis of the PtdIns3*P*-bound EEA1 FYVE domain structure, His^4 is close to the 4′ and 5′ positions of the inositol ring, and Arg^6 forms hydrogen bonds to the 3-phosphate group of the inositol ring [16]. The additional basic residues forming the two essential basic binding clusters are conserved in the variant FYVE domain. Conservation of Zn^{2+}-chelating and domain-stabilizing hydrophobic residues in the variant domain suggests that it has the same folding as the classic FYVE domain. This makes the variant FYVE domain an obvious model for structure–function studies.

The variant FYVE domain is fused at its N-terminus to a large region (residues 238–620) exhibiting similarity to RCC1 [35] (Figure 3). RCC1 consists of a motif of 45–73 residues repeated seven times; it functions as a guanine nucleotide exchange factor for the GTP-binding protein Ran [36]. The repeats in human RCC1 form a seven-bladed propeller structure [37]. The sequence repeats in PRAF1 align with those in RCC1 (Figure 3) but

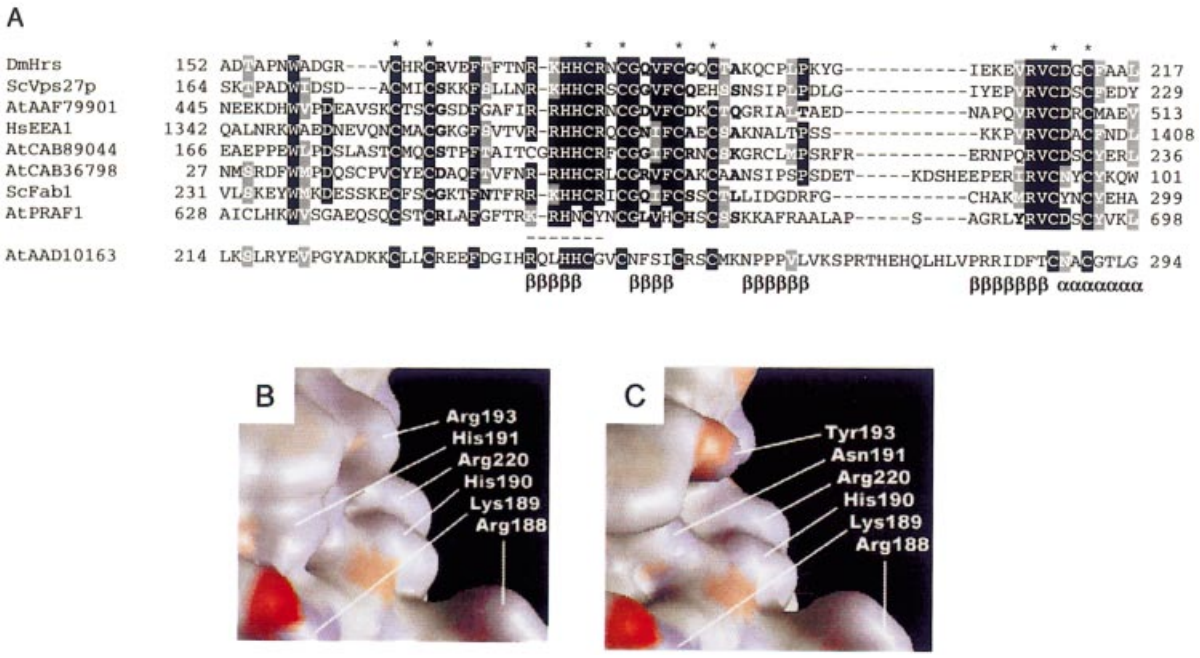

Figure 1 FYVE domain characteristics

(**A**) Alignment of nine FYVE or FYVE-related (AtAAD10163) domains representing five families of *Arabidopsis* proteins (AtAAF79901, AtCAB89044, AtCAB36798, AtPRAF1 and AtAAD10163), human early endosome antigen 1 (HsEEA1, accession no. X78998), yeast PtdIns3*P* 5-kinase Fab1 (ScFab1, P34756), *Drosophila* Hrs (DmHRS, AAF51221), and yeast vacuolar sorting protein (Vps27p, P40343). Asterisks identify Zn^{2+}-co-ordinating residues; dashes above the bottom sequence indicate the basic motif. Residues conserved or chemically similar in 50% or more of the sequences are highlighted in black and grey respectively. The positions of α-helix and β-strands in the three-dimensional structure of the Vps27p FYVE domain [13] are shown below the alignment. (**B**, **C**) Molecular surface of the proposed PtdIns3*P*-binding pocket of Vps27p (PDB entry 1VFY) (**B**) and of the same Vps27p pocket containing the $His^{191} \rightarrow Asn$, $Arg^{193} \rightarrow Tyr$ double mutations (**C**) present in the variant FYVE domain of PRAF1. Surfaces were drawn and coloured in accordance with charge, by using WebLab ViewerLite.

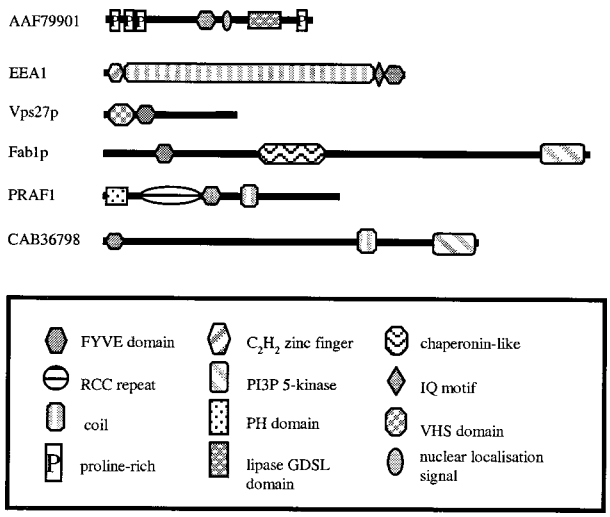

Figure 2 Structural outline of selected FYVE domain proteins

The domain structures of PRAF1, CAB36798 and AAF79901 were assigned in this study; the domain structures of EEA1, Vps27p and Fab1p were adapted from information in [3] and [13]. Abbreviation: PI3P, PtdIns3*P*.

PRAF1 lacks a basic stretch similar to the chromatin-binding region in RCC1. These characteristics suggest that PRAF1 is a guanine nucleotide exchange factor for a small GTPase, although it is not possible to predict its substrate.

PRAF1 contains a PH domain at its N-terminus (Figures 2 and 3). Whereas some PH domains show high affinity for a specific phosphoinositide, others are promiscuous via their low-affinity binding [38]. Conservation of specific residues, and the presence of basic residues in the predicted $\beta 1/\beta 2$ loop region, suggest that PRAF1 binds phosphoinositides despite the small size of this region [39].

Accumulation of PRAF1 mRNA

The accumulation of PRAF1 mRNA *in vivo* was examined in a Northern slot-blot assay with RNA representing various *Arabidopsis* tissues and treatments with a *PRAF1*-specific probe. A duplicate blot analysis was performed for the *GapC* gene, which is widely expressed at different levels in different tissues [40]. A low level of expression of *PRAF1* was detected for all tissues tested (several in addition to those shown in Figure 4A; results not shown); the highest level was observed in flower tissue. The size of the RNA hybridizing to the *PRAF1* probe was as expected (approx. 4 kb) when determined in a gel Northern blot analysis (Figure 4B). The accumulation of the *PRAF1* gene was also examined by RT–PCR. Again, a low level of *PRAF1* accumulation was indicated, in this case by comparison with the level detected for the constitutively expressed *EF-1α* gene (Figure 4C).

Expression and purification of recombinant fusion proteins

The sequence characteristics of the FYVE domain and the PH domain in PRAF1 suggest that they both possess the ability to bind phosphoinositides. To examine this, these domains, and the

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   1 MADLVTYSNA DHNLEQALIT LKKGTQLLKY GRKGKPKFYP FRLSSDEKSL  PH
  51 IWISSSGEKR LKLASVSKIV PGQRTAVFQR YLRPEKDYLS FSLLYNGKKK
 101 SLDLICKDKV EAEIWIGGLK TLISTGQGGR SKIDGWSGGG LSVDASRELT
 151 SSSPSSSSAS ASRGHSSPGT PFNIDPITSP KSAEPEVPET DSEKSHVALD
 201 NKNMQTKVSG SDGFRVSVSS AQSSSSHGSA ADDSDALGLV YIWGEVICDN  RCC
 251 VVKVGIDKNA SYLTTRTDVL VPKPLESNIV LDVHQIACGV RHAAFVTRQG
 301 EIFTWGEESG GRLGHGIGKD VFHPRLVESL TATSSVDFVA CGEFHTCAVT
 351 LAGELYTWGD GTHNVGLLGH GSDISHWIPK RIAGSLEGLH VASVSCGPWH
 401 TALITSYGRL FTFGDGTFGV LGHGDKETVQ YPREVESLSG LRTIAVSCGV
 451 WHTAAVVEII VTQSNSSSVS SGKLFTWGDG DKNRLGHGDK DPRLKPTCVP
 501 ALIDYNFHKI ACGHSLTVGL TTSGQVFTMG STVYGQLGNL QTDGKLPCLV
 551 EDKLASEFVE EISCGAYHVA ALTSRNEVYT WGKGANGRLG HGDLEDRKVP
 601 TIVEALKDRH VKYIACGSNY TAAICLHKWV SGAEQSQCST CRLAFGFTRK  FYVE
 651 RHNCYNCGLV HCHSCSSKKA FRAALAPSAG RLYRVCDSCY VKLSKVSEIN
 701 DTNRRNSAVP RLSGENRDRL DKSEIRLAKF GTSNMDLIKQ LDSKAAKQGK
 751 KTDTFSLGRN SQLPSLLQLK DAVQSNIGDM RRATPKLAQA PSGISSRSVS
 801 PFSRRSSPPR SATPMPSTSG LYFPVGIADN MKKTNEILNQ EIVKLRTQVD  Coil
 851 SLTQKCEFQE VELQNSVKKT QEALALAEEE SAKSRAAKEA IKSLIAQLKD
 901 VAEKLPPGES VKLACLQNGL DQNGFHFPEE NGFHPSRSES MTSSISSVAP
 951 FDFAFANASW SNLQSPKQTP RASERNSNAY PADPRLSSSG SVISERIEPF
1001 QFQNNSDNGS SQTGVNNTNQ VEAEWIEQYE PGVYITLVAL HDGTRDLRRV
1051 RFSRRRFGEH QAETWWSENR EKVYEKYNVR VSEKSTASQT HRDRDEEEED
1101 IPH
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Figure 3 Predicted amino acid sequence of PRAF1

The PH (boxed in grey), RCC1 repeat (boxed) and the FYVE (bold italics) domains, and the predicted coiled coil region (underlined) are marked. Six out of ten residues corresponding to positions shown to be of importance for the guanine nucleotide exchange activity of RCC1 and correlating with the Ran-RCC1 structure [47,48] are (chemically) conserved in PRAF1 and are shown in bold italics. The first residue in each RCC1 sequence repeat is marked by a star. Positions of importance for the phosphoinositide-binding ability of other PH domains [49] are shown in bold italics. The regions corresponding to the $\beta1/\beta2$ loop in other PH domains and the basic motif in classical FYVE domains are underlined.

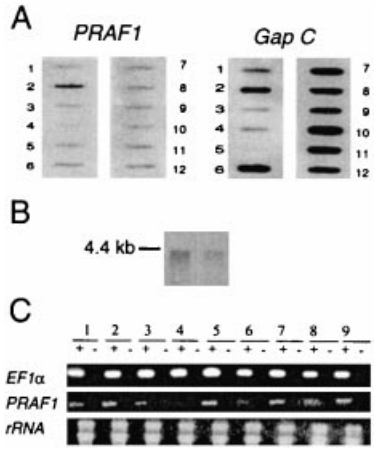

Figure 4 Accumulation of PRAF1 mRNA

(**A**) Northern slot-blot analysis of *PRAF1* and *GapC* accumulation in 20 μg of total RNA from stems (1), flowers (2), cauline leaves (3), green siliques (4), seeds (5), 7-day-old liquid culture (6), 7-day-old liquid culture treated with 100 μM auxin for 24 h (7), 7-day-old liquid culture treated with 100 μM gibberellic acid for 24 h (8), 7-day-old liquid culture treated with 100 μM abscisic acid for 24 h (9), 7-day-old liquid culture treated with 100 μM jasmonic acid for 24 h (10), 7-day-old liquid culture treated with 250 mM NaCl for 24 h (11), and 14-day-old seedlings desiccated for 8 h (12). (**B**) Northern gel blot analysis of *PRAF1* accumulation in flowers (left lane) and in 7-day-old liquid culture (right lane). (**C**) PCR analysis in the presence (+) and absence (−) of reverse transcriptase. The following total RNA templates were used: 7-day-old etiolated seedlings (1), 40-day-old light-grown plants (2), 7-day-old light-grown plants (3), stems (4), flowers (5), green siliques (6), cauline leaves (7), seeds (8) and roots (9). In the panel labelled rRNA, 10 μg of the RNA sample was stained with ethidium bromide.

classical FYVE domain from CAB36798, were produced as recombinant GST fusion proteins, with *E. coli* as host. Whereas high levels of soluble GST–PH and GST–CFY recombinant protein of the expected molecular masses were produced under standard conditions for growth and induction, GST–VFY was obtained primarily in inclusion bodies (Figure 5). However, a lower fermentation temperature (15 °C instead of 37 °C) yielded a fraction of soluble GST–VFY. Because GST–VFY purified by glutathione-affinity chromatography was contaminated with GST, it was further purified by cation-exchange chromatography, resulting in almost pure protein suitable for comparative phospholipid binding assays (Figure 5).

Phospholipid binding by PRAF1 domains

The ability of VFY to bind phospholipids *in vitro* was first examined using a fast qualitative dot-blot technique. The method involves the immobilization of phospholipids to a membrane and the immunological detection of protein bound to the immobilized phospholipids. These assays used serial dilutions of the 3-phosphoinositides PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, the 4-phosphoinositide PtdIns(4,5)P_2 and the plant membrane constituents PtdCho, phosphatidylethanolamine, phosphatidic acid and phosphatidylserine [41]. GST–PH and GST–VFY both showed significant binding to phosphatidic acid and to all of the phosphoinositides tested, but the binding of GST–PH to PtdIns(4,5)P_2 was significantly stronger than the binding of GST–VFY to this phosphoinositide. For GST–VFY, the strongest signal obtained was for PtdIns(3,4,5)P_3. GST alone did not bind to any of the phospholipids tested (Figure 6A).

The importance of His[4] and Arg[6] of the classical FYVE motif for specific PtdIns3*P* binding was examined by replacing the Asn and Tyr residues at the corresponding positions in the variant FYVE domain. Single mutations, present in the GST–VFY(N658H) and GST–VFY(Y660R) proteins, were not sufficient to introduce a significant preference for PtdIns3*P* (Figure 6B). In contrast, the double-mutant protein GST–VFY(N658H,Y660R) showed an increased affinity for only PtdIns3*P* and resembled GST–VFY, included as a control, in its binding pattern. This suggested that the binding specificity for PtdIns3*P* was increased by the simultaneous replacement of the two conserved residues and that both residues are important for the specific binding of PtdIns3*P*.

The phospholipid dot-blot binding assay is a qualitative and insensitive assay, which can be used only for initial screening. Furthermore, a tendency to obtain stronger signals from 3-phosphoinositides than from 4-phosphoinositides has been reported for similar assays [38]. We therefore also examined the phosphoinositide-binding ability of the GST fusion proteins in a quantitative assay [27] that measured the ability of fusion proteins immobilized on glutathione–Sepharose to retain radiolabelled phospholipid liposomes. Liposomes consisting either entirely of PtdCho or a PtdCho framework and a specific phosphoinositide at 4% (mol/mol) were examined for their ability to bind to the fusion proteins.

For GST–PH, PtdIns(4,5)P_2-containing liposomes were bound most efficiently, whereas the interaction with the rest of the phosphoinositides tested seemed dependent on the net charge of the phosphoinositide headgroup; the binding efficiency was PtdIns(4,5)P_2 > PtdIns(3,4,5)P_3 > PtdIns(3,4)P_2 > PtdIns3$P \approx$ PtdIns5$P \approx$ PtdIns4*P* (Figure 7A). Binding of liposomes to immobilized GST–VFY also seemed charge-dependent, with PtdIns(3,4,5)P_3-containing liposomes being most efficiently retained. In contrast, GST–CFY preferred PtdIns3*P*-containing liposomes, as expected. GST–VFY(N658H) and GST–VFY

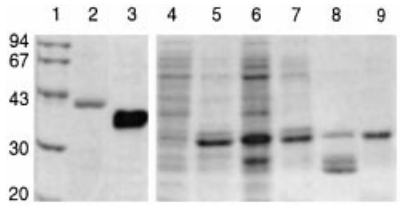

Figure 5 Production and purification of recombinant proteins for phospholipid-binding assays

SDS/PAGE and Coomassie Blue staining of molecular mass markers (molecular masses indicated in kDa at the left) (lane 1), GST–PH (lane 2), GST–CFY (lane 3), soluble (lane 4) and insoluble (lane 5) fractions from lysed cells expressing GST–VFY grown at 37 °C after induction, soluble (lane 6) and insoluble (lane 7) fractions from lysed cells expressing GST–VFY grown at 15 °C after induction, GST–VFY eluted from glutathione–Sepharose beads (lane 8), and GST–VFY eluted from a cation-exchange matrix (lane 9).

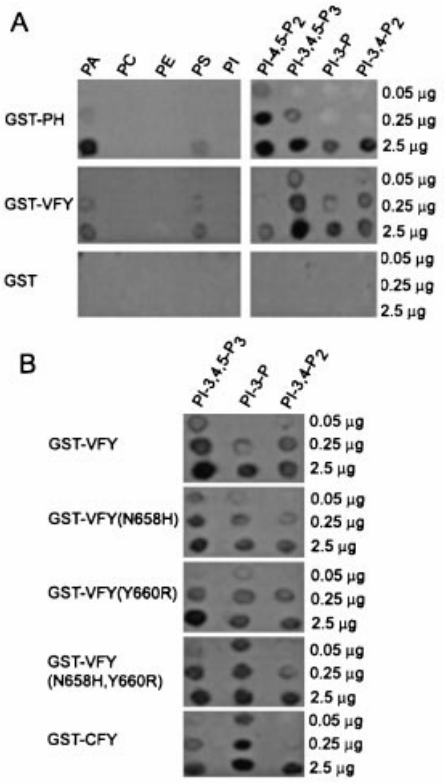

Figure 6 Phospholipid dot-blot assay with recombinant GST–PRAF1 fusion proteins

The indicated phospholipids in the amounts shown were spotted on the membrane and the membrane was incubated with GST fusion protein as indicated; binding was monitored immunologically with a polyclonal antibody against GST. (**A**) Binding of GST–PH, GST–VFY and GST to the phospholipids phosphatidic acid (PA), PtdCho (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) and to the phosphoinositides PtdIns(4,5)P_2 (PI-4,5-P_2), PtdIns(3,4,5)P_3 (PI-3,4,5-P_3), PtdIns3P (PI-3-P) and PtdIns(3,4)P_2 (PI-3,4-P_2). (**B**) Binding of GST–VFY, GST–VFY(N658H), GST–VFY(Y660R) and GST–VFY(N658H,Y660R) to the 3-phosphoinositides PtdIns(3,4,5)P_3, PtdIns3P and PtdIns(3,4)P_2. The blots shown are representative of two independent experiments with the same binding characteristics.

(Y660R) both showed significant increases in their ability to bind PtdIns3P-containing liposomes, but still preferred PtdIns(3,4,5)P_3. In contrast, GST–VFY(N658H,Y660R) preferred PtdIns3P but exhibited an affinity for PtdIns(3,4)P_2 and

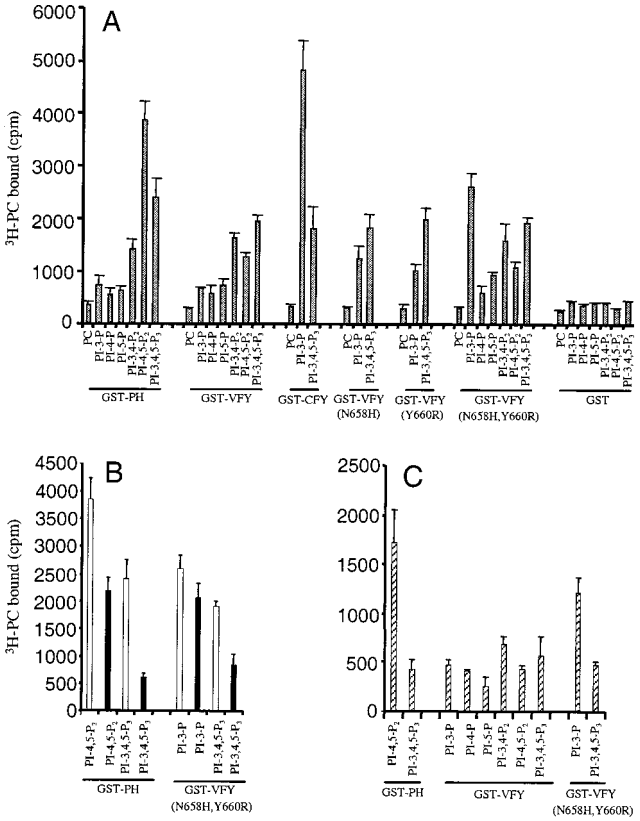

Figure 7 Identification of PtdIns3*P*-binding residues in FYVE domains

(**A**) Binding of ^{3}H-labelled phospholipid vesicles consisting of PtdCho (PC) or 96% (mol/mol) PtdCho and 4% (mol/mol) PtdIns3P (PI-3-P), PtdIns4P (PI-4-P), PtdIns5P (PI-5-P), PtdIns(3,4)P_2 (PI-3,4-P_2), PtdIns(4,5)P_2 (PI-4,5-P_2) or PtdIns(3,4,5)P_3 (PI-3,4,5-P_3) to immobilized GST–PRAF1 domain fusion proteins. (**B**) Binding of ^{3}H-labelled phospholipid vesicles consisting of 96% or 99% (mol/mol) PtdCho (white and black bars respectively) and the phosphoinositide shown at 4% or 1% (mol/mol). (**C**) Binding of ^{3}H-labelled phospholipid vesicles consisting of 96% (mol/mol) PtdCho and the indicated phosphoinositide at 4% (mol/mol) in the presence of 2 mM $MgCl_2$. Results are means ± S.E.M. for duplicate determinations.

PtdIns(3,4,5)P_3 similar to that of GST–VFY. A PtdIns3P-preferring domain was thus created by the two substitutions present in GST–VFY(N658H,Y660R).

Phosphoinositide at 4% was initially included in the liposomes to permit the measurement of weak, charge-dependent binding. However, the physiological relevant concentration of a specific phosphoinositide might be lower in target membranes. We therefore compared the ability of GST–PH and GST–VFY(N658H,Y660R) to bind their preferred phosphoinositide or PtdIns(3,4,5)P_3 present at only 1% (mol/mol) in PtdCho-based liposomes. Whereas some decrease in the ability of GST–PH to retain PtdIns(4,5)P_2-containing liposomes was monitored by decreasing the proportion of PtdIns(4,5)P_2 in the liposomes, a similar decrease in the PtdIns(3,4,5)P_3 content resulted in a large decrease in binding (Figure 7B). For GST–VFY(N658H,Y660R) no significant decrease in the binding of PtdIns3P-containing vesicles was detected by decreasing the content of PtdIns3P from 4% to 1%. In contrast, the binding of PtdIns(3,4,5)P_3-containing vesicles was decreased by 57% by a similar decrease in the PtdIns(3,4,5)P_3 content. This suggested that the PH domain in PRAF1 has a specific binding site for PtdIns(4,5)P_2 and that the binding of PtdIns(3,4,5)P_3 detected *in vitro* takes place at a less optimal site than that for PtdIns(4,5)P_2.

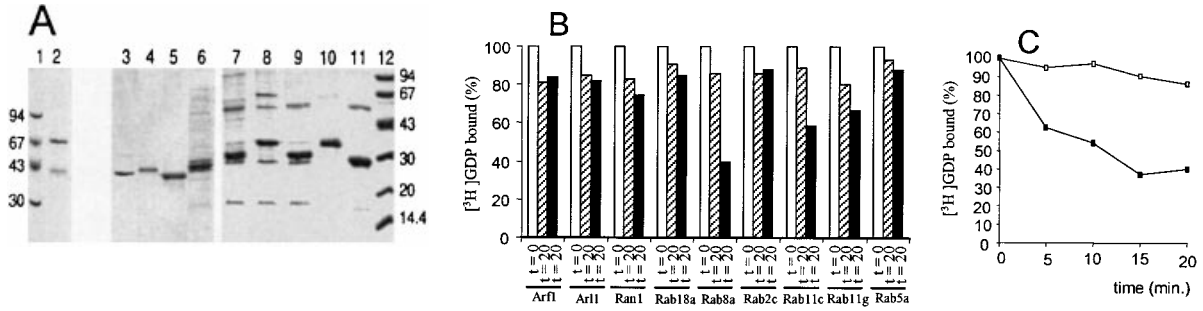

Figure 8 RCC1 domain from PRAF1 catalyses guanine nucleotide exchange on specific Rab proteins

(**A**) SDS/PAGE analysis and Coomassie Blue staining of molecular mass markers (molecular masses indicated in kDa at the left) (lane 1), purified recombinant GST–RCC1 (lane 2), Ran1 (lane 3), Arf1 (lane 4), Arl1 (lane 5), Rab18a (lane 6), Rab8a (lane 7), Rab11c (lane 8), Rab2c (lane 9), Rab11g (lane 10), Rab5a (lane 11) and molecular mass markers (molecular masses indicated in kDa at the right) (lane 12). (**B**) [^{3}H]GDP-bound GTPase (0.2 μM) was incubated for 20 min at 37 °C without (hatched bars) or with (filled bars) 1 μM recombinant GST–RCC1 and the amount of bound [^{3}H]GDP was compared with the amount bound at the start of the incubation without GST–RCC1 (100%, open bars). (**C**) Time dependence of the amount of [^{3}H]GDP bound to Rab8a in the absence (□) or presence (■) of GST–RCC1.

The doubly mutated FYVE domain also bound negatively charged phosphoinositides but apparently did so through a lower-affinity site than that created for PtdIns3*P* by the mutations.

Binding specificity was also examined in the presence of a high concentration (2 mM) of $MgCl_2$. This and other bivalent cations might decrease non-specific interactions between protein domains and phospholipids and might thereby help to reveal specific interactions [42]. Again, the altered assay conditions revealed a clear preference of GST–PH for PtdIns(4,5)P_2 over PtdIns(3,4,5)P_3 and of GST–VFY(N658H,Y660R) for PtdIns3*P* over PtdIns(3,4,5)P_3. In contrast, GST–VFY did not show differential binding to any of the phosphoinositides examined, further suggesting that the interactions observed previously were mainly non-specific (Figure 7C).

Guanine nucleotide exchange activity of PRAF1

To examine whether PRAF1 has guanine exchange activity, the ability of GST–RCC1 to catalyse the removal of GDP bound to different lipid-unmodified [23,28] ARF, Rab and Ran proteins was screened. ARF proteins (now named ARF1 and Arl1) and Rab proteins (now named Rab8a, Rab18a, Rab11c, Rab11g, Rab5a and Rab2c [25]) were selected to cover the structural range of ARF and Rab proteins in *Arabidopsis* and, together with Ran1, were tested as substrates for GST–RCC1 *in vitro* (Figure 8A). The recombinant proteins were purified from *E. coli*, loaded with GDP and incubated with GST–RCC1 at a high (5:1) molar ratio of GST–RCC1 to substrate because lipid-unmodified substrates were used. This suggested that GST–RCC1 can catalyse guanine exchange on specific Rab proteins when present in molar excess. In contrast, no significant activity was detected towards some of the other small GTPases tested (Figures 8B and 8C).

DISCUSSION

The FYVE domain has been referred to as the PtdIns3*P*-binding domain; so far its only known phosphoinositide ligand is PtdIns3*P*. The proliferation of the domain in *Arabidopsis* was examined to gain information about phosphoinositide-regulated processes in plants. Only nine genes encoding distinguishable FYVE domains were identified. These genes are probably expressed at low levels, as suggested by the small number of representative EST clones (e.g. two for PRAF1) and the Northern dot-blot and RT–PCR analyses of PRAF1. This suggests a narrow or well-defined function of the FYVE domain compared with, for example, the PH domain, which is widespread and interacts with a variety of reported ligands [39].

PtdIns3*P* and a PtdIns3*P*-specific PH domain have been identified in plants [30,43], making the existence of additional PtdIns3*P*-binding domains likely. Here we showed that a classical FYVE domain from *Arabidopsis* binds PtdIns3*P* specifically *in vitro*, as expected. Its parent protein, CAB36798, is based on a sequence similarity to Fab1p, a PtdIns3*P* 5-kinase generating PtdIns(3,5)P_2, which is a phosphoinositide that has also been identified in plants [44]. The study of a putative plant orthologue of Fab1p might therefore shed light on the role of both PtdIns3*P* and PtdIns(3,5)P_2 in plant cells.

In contrast, FYVE-domain-containing AAF79901 lacks global similarity to well-studied proteins from other systems. The discovery of this protein therefore expands the repertoire of FYVE protein structures [12]. It contains a Gly-Asp-Ser-Leu lipase domain [33]. This domain is present in several predicted plant proteins with differentially expressed genes, which suggests a regulatory role for the lipases in plant development [45]. The subcellular localization of AAF79901 and a related anther-specific protein lacking a FYVE domain [34] might help to explain a biological function for the proteins and contribute to the understanding of the still debated role of the classic FYVE domain in membrane targeting [8,10].

GST–VFY bound phosphoinositides in both assays used but no phosphoinositide preference was revealed. If the structure of the classic FYVE domain is taken as a model, the variant FYVE domain is also likely to possess a basic surface suitable for binding phosphoinositides (Figure 1B). The similarity of the classic and variant folds was confirmed by our ability to convert the variant domain into an isomer-specific, PtdIns3*P*-preferring domain by the replacement of two residues. The results show that both His^4 and Arg^6 of the basic motif are essential for a PtdIns3*P*-specific headgroup-binding pocket. The variant FYVE domain contains an Asn residue instead of His^4. Its geometry apparently does not permit the formation of essential interactions between this residue and PtdIns3*P*. Although the solution structure of the EEA1 FYVE domain in complex with PtdIns3*P* suggests that the 4′ and 5′ positions of the inositol ring are close to His^4, specific details of hydrogen bonds and electrostatic interactions are still lacking [16]. Not surprisingly, a Tyr residue at position 6 cannot substitute for an Arg residue, which is likely

to form a hydrogen bond with the 3-phosphate group of PtdIns3*P*. Although NMR studies indicate that several basic residues contribute to binding [14,16], the results obtained here show that two of them, His^4 and Arg^6, contribute significantly to the co-operative effect. The conservation of these positions in most of the FYVE domains [12] favours the idea that the main role of the FYVE domain is related to binding PtdIns3*P*.

The presence of two phosphoinositide-binding domains in the PRAF proteins suggests that association with a membrane is crucial for their function. The PH domain bound various phospholipids but preferred PtdIns(4,5)P_2. Some PH domains are targeted to specific cellular membranes when expressed *in vivo* [39] but many bind phospholipids promiscuously and with low affinity *in vitro* [46]. Membrane affinity and specificity might be increased by oligomerization [38] through, for example, a coiled-coil structure, which is likely to be formed by the PRAF proteins, or by the co-operation of different membrane-binding domains such as the PH and FYVE domains in PRAF1. This would permit membrane targeting by the functional protein despite some promiscuity of its isolated domains.

The recombinant RCC1 domain from PRAF1 stimulated guanine exchange with differential efficiency on several GTPases belonging to the Rab family. A 5-fold molar excess of GST–RCC1 over substrate was needed for the detection of activity. This might reflect the need for post-translational modification of the substrate, for example isoprenylation of the Rab C-termini, or co-localization of the enzyme and substrate to a membrane. Unfortunately, attempts to measure the effect of specific phosphoinositides, such as PtdIns(4,5)P_2, on the activity of the RCC1 domain have so far failed owing to our inability to produce active recombinant PH-RCC1 and RCC1-VFY proteins. Furthermore, such effects might be weakened by the high levels of Mg^{2+} in the assays used for the measurements (Figure 7C). The catalytic effect of GST–RCC1 on several different GTPases might be explained by a relatively broad specificity, as seen previously for an RCC1 domain in P532 [29]. Alternatively, its natural substrate remains to be identified.

A preference of the PRAF1 PH domain for PtdIns(4,5)P_2 is in accordance with the existence of this phosphoinositide in plants at low but varying levels [30,31]. Given its binding affinity, PRAF1 localization to a membrane is likely and is compatible with a regulatory role related to Rab proteins such as Rab8a and Rab11c, which are potential substrates of GST–RCC1 involved in the later steps of secretory pathways. Because many of the proteins that contain a FYVE domain have a role in regulated secretion, their ligand(s) might be located in exocytic vesicles or the plasma membrane. However, the intracellular localization of the PRAF1 protein needs to be confirmed *in vivo*, which might help to identify their substrates and ligands. In any event, we have here characterized the phospholipid-binding properties *in vitro* of PRAF1 containing a variant FYVE domain and have shown that the RCC1 domain functions as a guanine exchange factor.

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