Characterization of a novel phosphatidylinositol 3-phosphate-binding protein containing two FYVE fingers in tandem that is targeted to the Golgi

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We have identified a novel protein of predicted molecular mass 40 kDa that contains two FYVE domains in tandem and has therefore been named TAFF1 (**TA**ndem **F**YVE **F**ingers-**1**). The protein is expressed predominantly in heart and binds to PtdIns3*P* specifically, even though the FYVE domains in TAFF1 lacks the first Arg of the consensus sequence R(K/R)HHCR, critical for the PtdIns3*P* binding of other FYVE domains identified so far. The first Arg is replaced by a Thr and Ser in the N-terminal and C-terminal FYVE domains of TAFF1 respectively. Mutational analysis indicates that both FYVE domains are required for high affinity binding to PtdIns3*P*. Cell localization studies using a green fluorescent protein fusion show that TAFF1 is localized to the Golgi, and that the Golgi targeting sequence is located within the N-terminal 187 residues and not in either FYVE domain.

Key words: TAFF1, EEA1, DFCP1, inositol phospholipid.

INTRODUCTION

FYVE finger domains were first identified by Stenmark et al. [1] as novel zinc fingers that are present in a variety of proteins implicated in vesicular trafficking. These 70 residue domains are highly conserved between species and are named after the first four proteins (**F**ab1p, **Y**OTB, **V**ac1p and **E**EA1) shown to contain them [1]. Although first identified in proteins involved in vesicular trafficking, their functions are not restricted to this process, having been identified in proteins that participate in signal transduction. These include a protein that interacts with the transforming-growth-factor-β receptor [2], a cAMP-dependent-protein-kinase anchoring protein [3] and a protein phosphatase [4]. Although the roles played by the FYVE domains of all these proteins is unclear, in the prototypical protein EEA1 they appear to be required for membrane association with endosomes [1]. Moreover, in EEA1 and Vps27p, the FYVE domains have been shown to bind to PtdIns3*P* specifically [5–7]. Recently, the three-dimensional structures of the FYVE domains of Vps27p and Hrs have been solved and the residues predicted to bind PtdIns3*P* have been reported [8,9].

The FYVE domains have been thought of as membrane-targeting domains, much like the pleckstrin homology (PH) domains that also bind to inositol phospholipids [10–12]. However, in contrast with PH domains that bind to a variety of inositol phospholipids, PtdIns3*P* is the only inositol phospholipid that has so far been shown to bind to a FYVE domain. There has been speculation as to whether other inositol phospholipids are able to bind to a small number of FYVE domains which deviate from the consensus sequence of basic residues. Here we identify a PtdIns3*P*-binding protein that contains two FYVE domains in tandem and has therefore been termed TAFF1 (**TA**ndem **F**YVE **F**ingers-**1**). TAFF1 is targeted to the Golgi cisternal stacks but, surprisingly, the FYVE domains are not involved in association with the Golgi membranes.

MATERIALS AND METHODS

Materials

A glutathione S-transferase (GST)–EEA1 plasmid construct encoding residues 1257–1411 of EEA1 was generously provided by Dr Chris Burd (Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.). A green fluorescent protein (GFP)–EEA1 construct encoding residues 1257–1411 of EEA1 was a gift from Dr G. S. Kular (Inositol Lipid Signalling Laboratory, University of Dundee, Dundee, Scotland, U.K.). DNA mutagenesis was carried out using the Quikchange kit (Stratagene, La Jolla, CA, U.S.A.), and all plasmids sequenced to confirm the integrity of the DNA. Precast SDS/polyacrylamide gels were purchased from Invitrogen (Groningen, The Netherlands). *Pfu* Turbo DNA polymerase used for all PCR reactions was from Stratagene. Restriction enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). Anti-TGN46, anti-β1,4-galactosyltransferase (anti-GalT) and anti-(transferrin receptor) antibodies raised in rabbits were a gift from Dr Vas Ponnambalam (Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K.) (TGN46 is a *trans*-Golgi network protein of molecular mass 46 kDa).

Abbreviations used: TAFF1, **TA**ndem **F**YVE **F**ingers-**1** (FYVE is explained in the text); NCBI, National Center for Biotechnology Information; EST, expressed sequence tag; IMAGE, integrated molecular analysis of genomes and their expression; GalT, β1,4-galactosyltransferase; GFP, green fluorescent protein; GST glutathione S-transferase; PH, pleckstrin homology; PDK1, 3-phosphoinositide-dependent protein kinase-1; TMD, transmembrane domain; DFCP1, double FYVE containing protein 1; TGN46, *trans*-Golgi network protein of molecular mass 46 kDa.

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The sequence of TAFF1 has been deposited with the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under accession number AF311602.

Two-hybrid screen

A two-hybrid screen was carried out using 3-phosphoinositide-dependent protein kinase-1 (PDK1) as bait. Full-length PDK1 was subcloned into the *Eco*RI and *Bam*HI sites of pAS2-1 (Clontech, Palo Alto, CA, U.S.A.) and in-frame with the GAL4 transcriptional-activator DNA-binding domain. The plasmid pAS2-1 was transformed into Y190 yeasts with a human brain cDNA library (Clontech), subcloned into the pACT2 vector and expressed as GAL4 activation domain fusions. The transformed yeasts were grown on Synthetic Dropout medium supplemented with 25 mM 3-aminotriazole and 2 % (w/v) glucose, but lacking histidine, leucine and tryptophan. The plates were incubated for 10 days at 30 °C, and colonies which grew in the absence of histidine were assayed for β-galactosidase activity and taken for further analysis.

Plasmids

The full-length cDNA encoding human TAFF1 was obtained from the integrated molecular analysis of genomes and their expression (IMAGE) consortium (HGMP, Hinxton Hall, Cambridge, U.K.) as the IMAGE clone 729901 and sequenced in its entirety. Various constructs of TAFF1 were amplified by PCR using IMAGE clone 729901 as template and subcloned into the *Eco*RI/*Bam*H1 site of pEYFP-C1 (Clontech). TAFF1 constructs in pGEX-4T-2 vector (Amersham Pharmacia Biotech, Amersham, U.K.) were made by excision of the corresponding constructs from pEYFP-C1 with *Bgl*II/*Bam*HI and subcloned into the *Bam*HI site of pGEX-4T-2. Orientation of the inserts was confirmed by restriction analysis.

Northern blotting

A human multiple tissue Northern blot (Clontech) was probed with cDNA encoding full-length TAFF1. The 1.2 kb cDNA fragment was excised from the IMAGE clone 729901 by restriction digestion with *Eco*RI and *Not*I. The Northern blot was hybridized overnight at 55 °C with the cDNA probe randomly labelled with [α-^{32}P]ATP in ExpressHyb (Clontech). The blot was washed for 40 min in 2 × SSC/0.05 % SDS at room temperature, followed by a 40 min wash at 50 °C in 0.1 × SSC/0.1 % SDS (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). The blot was exposed for 5 days at −70 °C.

Bacterial expression of GST–TAFF1 and GST–EEA1

Plasmids encoding various constructs of TAFF1 and EEA1 were transformed into *Escherichia coli* strain BL21 and the cultures grown at 37 °C in Luria–Bertani broth containing 100 μg/ml ampicillin. When the absorbance at 600 nm reached 0.6, the culture was induced with 30 μM isopropyl β-D-thiogalactoside, supplemented with 50 μM $ZnSO_4$ and grown overnight at 26 °C. The bacterial pellet was resuspended in 50 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1 % (v/v) 2-mercaptoethanol/0.03 % (w/v) Brij 35/5 % (v/v) glycerol and subjected to one freeze–thaw cycle. The bacterial suspension was sonicated on ice at 15 W for 3 min with a Vibra Cell ultrasonic processor (Sonics and Materials Inc, Danbury, CT, U.S.A.). Triton X-100 was added to a final concentration of 1 % (v/v) and the mixture was incubated for 30 min on ice. The lysate was clarified by centrifugation for 30 min at 30000 ***g***, and the expressed fusion protein isolated from the supernatant by affinity chromatography on GSH–Sepharose 4B (Amersham Pharmacia Biotech) at 4 °C. The resin was washed with 20 bed vol. of PBS containing 0.5 M NaCl, 0.27 M sucrose and 10 mM β-mercaptoethanol, then with 1 bed vol. of PBS containing 0.27 M sucrose and 10 mM β-mercaptoethanol. Finally, the bound GST fusion protein was eluted with 20 mM GSH and stored in aliquots at −80 °C.

Lipid-binding studies

A 1 μl portion of the indicated concentrations of lipids (Cell Signals, Lexington, KY, U.S.A.) dissolved in chloroform/methanol/water (1:2:0.8, by vol.) were spotted on to Hybond-C extra nitrocellulose membrane (Amersham Pharmacia Biotech) and air-dried for 1 h [13,14]. The membrane was blocked by incubation for 1 h at room temperature with 3 % (w/v) BSA in TBS-T [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1 % Tween 20]. The membrane was incubated for 3 h at 4 °C with 1.5 μg/ml bacterially expressed GST fusion protein diluted in the same buffer and then washed extensively with TBS-T. GST fusion protein bound to the immobilized lipids on the membrane was then probed with mouse monoclonal GST-specific antibody (Sigma) diluted 1:2000 in 5 % (w/v) dried fat-free milk in TBS-T for 1 h. The primary antibody was detected by rabbit anti-mouse antibody conjugated to horseradish peroxidase (Pierce) diluted 1:5000 in 5 % (w/v) dried fat-free milk in TBS-T for 1 h. The blot was developed by enhanced bioluminescence (ECL®; Amersham Pharmacia Biotech).

Confocal microscopy

HeLa (human cervical carcinoma) cells grown on coverslips in 10-cm-diameter dishes were transiently transfected with 0.2 μg/ml of DNA/plate using the Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Transfection efficiencies of 30–50 % were obtained as assessed by fluorescence microscopy. Following transfection, cells were maintained for 24 h in Dulbecco's modified Eagle's medium to allow expression of the constructs. The coverslips containing the transfected cells were washed with PBS and fixed for 10 min with 3.7 % (w/v) paraformaldehyde in PBS. In order to immunostain for TGN46, GalT or the transferrin receptor, the cells were washed several times with PBS and further permeabilized for 10 min with PBS containing 1 % (v/v) Triton X-100. After further washing with PBS, the cells were blocked for 10 min in PBS containing 0.1 % (w/v) Tween-20, 3 % (w/v) BSA and 1 % (v/v) donkey serum. After 1 h incubation with primary antibodies that recognize TGN46, GalT or the transferrin receptor [15], the cells were washed extensively in PBS before being exposed to Texas Red-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) for 1 h. After staining, cells were mounted in Mowiol 40-88 (Aldrich, Gillingham, Dorset, U.K.)/1,4-diazadicyclo[2.2.2]octane ('DABCO') and allowed to dry before examination on a Zeiss LSM 410 confocal laser scanning microscope with excitation wavelengths of 488 nm (enhanced yellow fluorescent protein) and 543 nm (Texas Red).

Determination of the localization of TAFF1 using immunoelectron microscopy

HEK-293 cells were transiently transfected with the pEYFP-C1–GFP vector (Clontech) containing wild-type TAFF1. Briefly, cells grown in 10-cm-diameter dishes were transfected using a modified calcium phosphate-mediated procedure with 0.5 μg/ml DNA/plate [16]. At 30 h post-transfection, the medium was removed from the cells, which were then fixed for 30 min at room temperature in 8 % (w/v) paraformaldehyde in 0.2 M Pipes buffer, pH 7.2, and kept for at least 2 days at 4 °C. Cells were then scraped from the dish with a rubber policeman and pelleted in fixative for 30 min at 10000 ***g***. After cryo-

protection in 2.1 M sucrose in 4.3 mM Na_2HPO_4/1.4 mM KH_2PO_4/2.7 mM KCl/137 mM NaCl, pH 7.2 (PBS), ultra-thin sections were cut at −100 °C in a Leica ultracryomicrotome and mounted on Formvar/carbon-coated grids. Sections were labelled at room temperature by placing grids on drops of 0.5% fish-skin gelatin in PBS for 10 min and transferred to drops of rabbit anti-GFP antibody (a gift from Dr. Ken Sawin of the Wellcome Trust Centre for Cell Biology, Institute of Cell and Molecular Biology, Edinburgh, Scotland, U.K.) for 30 min. Following washes in PBS (3 × 5 min), the grids were incubated for 30 min on Protein A–gold (8 nm particle size), washed first in PBS (3 × 5 min) and then in distilled water (4 × 1 min). The sections were contrasted in methylcellulose/uranyl acetate as described [17] and observations and photographs taken on a JEOL 1200EX electron microscope.

RESULTS

Two-hybrid screen with PDK1

A two-hybrid screen of a human brain library was carried out using the protein kinase PDK1 as bait in order to identify interacting proteins [18]. In addition to a number of protein kinases that are activated by PDK1 [19], two other clones were identified that encoded overlapping fragments of the same protein. Although these two clones were able to activate transcription of the *HIS3* and *lacZ* reporter genes in the two-hybrid screen, we were unable to confirm that they were genuine interactors with PDK1 through additional experiments (P. C. F. Cheung, unpublished work).

TAFF1 cDNA encodes a 40 kDa protein and contains two FYVE domains

The partial cDNA sequences were used to interrogate the expressed sequence tag (EST) database at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the BLAST program [20]. Interrogation of the human genome sequences at the NCBI show that the gene sequence resides on chromosome 14. A matching EST sequence (joint-database accession number AA399630) from a human testis library was found. The corresponding clone (ID 729901) harbouring the full-length sequence in the pT7T3D-Pac vector was obtained from the IMAGE consortium [21] and sequenced in its entirety. A further search of the EST database has revealed that homologues are present in a number of other mammals, including mouse (accession numbers AA144972 and AA497799) and pig (accession AW424878). The TAFF1 open reading frame is 1.1 kb in length and encodes a protein with a predicted molecular mass of 40 kDa (Figure 1). Two in-frame stop codons are present 48 and 93 nt upstream of the likely initiating codon ATG. An in-frame stop codon 48 nt upstream of the putative initiating codon ATG is also found in the two mouse EST sequences (accession numbers AA144972 and AA497799). Although the sequences flanking the putative initiating codon do not conform fully to the Kozak sequence [22], the presence of in-frame upstream stop codons in three independent clones from two different species indicates that it is the initiating codon ATG. A poly(A) tail is found immediately after the stop codon in both the human and mouse full-length clones (IMAGE ID 729901 and 917790 respectively). This is consistent with the presence of the canonical AATAAA polyadenylation consensus sequence 14 nt upstream of the stop codon.

Analysis of the predicted amino acid sequence shows that it contains two FYVE domains in tandem at the C-terminus. However, database searching with the N-terminus of TAFF1

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    cctttctcttctagctttgggatctcaggaggaggagaacccaagaaactgtggaatgat 60
    gtgaggcactaagtgaccgcttcagcggtgagatccccgatgaccagatggcgcacagct 120
1                                                    M  A  H  S  S

    ccttttttccagatgagtatttcacctgctcctccttgtgcctcagctgtggggttggat 180
6     F  F  P  D  E  Y  F  T  C  S  S  L  C  L  S  C  G  V  G  C

    gtaagaaaagcatgaatcatgggaaggaaggagtgcctcatgaagccaagagccgctgca 240
26    K  K  S  M  N  H  G  K  E  G  V  P  H  E  A  K  S  R  C  R

    gatactcccaccagtatgacaaccgagtgtatacctgcaaggcctgctatgagagaggcg 300
46    Y  S  H  Q  Y  D  N  R  V  Y  T  C  K  A  C  Y  E  R  G  E

    aggaagtcagtgtagtgcccaaaacatctgcttccactgactccccctggatgggtctcg 360
66    E  V  S  V  V  P  K  T  S  A  S  T  D  S  P  W  M  G  L  A

    caaaatatgcctggtctgggtatgtgatcgaatgtcctaactgtggcgtggtctatcgta 420
86    K  Y  A  W  S  G  Y  V  I  E  C  P  N  C  G  V  V  Y  R  S

    gtcggcagtactggtttggaaaccaagatcctgtggatacggtggtgcggacagagattg 480
106   R  Q  Y  W  F  G  N  Q  D  P  V  D  T  V  V  R  T  E  I  V

    tgcatgtgtggcctggaactgatgggtttctgaaggacaacaacaatgctgcccagcgcc 540
126   H  V  W  P  G  T  D  G  F  L  K  D  N  N  N  A  A  Q  R  L

    tgttggacgggatgaacttcatggctcagtcggtgtccgagcttagccttggacccacca 600
146   L  D  G  M  N  F  M  A  Q  S  V  S  E  L  S  L  G  P  T  K

    aggctgtgacttcctggctgacagaccagatcgcccctgcctactggaggcccaactccc 660
166   A  V  T  S  W  L  T  D  Q  I  A  P  A  Y  W  R  P  N  S  Q

    agattctgagctgcaacaagtgtgcgacgtcctttaaagataacgacactaagcatcact 720
186   I  L  S  C  N  K  C  A  T  S  F  K  D  N  D  T  K  H  H  C

    gccgagcctgtggggagggcttctgtgacagctgttcatcaaagactcggccagtgcctg 780
206   R  A  C  G  E  G  F  C  D  S  C  S  S  K  T  R  P  V  P  E

    agcggggctgggccctgcgccagtgcgggtctgtgacaactgctacgaagccaggaacg 840
226   R  G  W  G  P  A  P  V  R  V  C  D  N  C  Y  E  A  R  N  V

    tccagttagctgttaccgaggcacaagtggacgatgaaggtggaacactcattgctcgga 900
246   Q  L  A  V  T  E  A  Q  V  D  D  E  G  G  T  L  I  A  R  K

    aggtgggcgaggccgtgcagaacactctgggagccgtggtgacagccattgacataccac 960
266   V  G  E  A  V  Q  N  T  L  G  A  V  V  T  A  I  D  I  P  L

    taggtctggtaaaggacgcggccaggcctgcgtactgggtgcctgaccacgaaatcctcc 1020
286   G  L  V  K  D  A  A  R  P  A  Y  W  V  P  D  H  E  I  L  H

    actgccacaactgccggaaggagttcagcatcaagctctccaagcaccactgccgggcct 1080
306   C  H  N  C  R  K  E  F  S  I  K  L  S  K  H  H  C  R  A  C

    gcggacagggcttctgtgatgagtgctcccatgaccgccgggctgttccttctcgtggct 1140
326   G  Q  G  F  C  D  E  C  S  H  D  R  R  A  V  P  S  R  G  W

    gggaccatcccgtccgagtctgcttcaactgcaataaaaagcccggtgaccttttaa     1196
346   D  H  P  V  R  V  C  F  N  C  N  K  K  P  G  D  L  *
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Figure 1 Nucleotide and predicted amino acid sequence of TAFF1

The initiating codon and stop codon and two in-frame stop codons upstream of the initiating methionine are shown in **boldface** type. The two FYVE domains are underlined. Nucleotides are numbered on the right and amino acid residues on the left. The two partial cDNAs identified in the original two-hybrid screen with PDK1 encode residues 57–362 and 116–362.

using the BLAST program did not reveal sequence similarity with any other protein in the database. Sequence alignment of TAFF1 shows that both FYVE domains contain the eight conserved cysteine residues essential for chelating Zn^{2+} and the 'basic patch' found in all FYVE-domain proteins (Figure 2). The basic patch contains the consensus sequence R(K/R)HHCR thought to be responsible for binding of the PtdIns3*P*, mutagenesis of which abolishes lipid binding [5]. However, an unusual feature of both FYVE domains of TAFF1 is the replacement of the first arginine residue by threonine (Thr^{201}) and serine residues (Ser^{318}). These amino acid replacements led us to examine the lipid-binding specificity of TAFF1. Interestingly, apart from the eight pairs of cysteine residues present in the FYVE domains of TAFF1 thought to be required for chelating Zn^{2+}, there are three pairs of cysteine residues in the N-terminal region. These three cysteine pairs are separated by 35 residues exactly, and the significance of this feature is unknown at the present time.

Northern blotting

A multiple-tissue Northern blot was probed with cDNA encoding full-length TAFF1. A 4.5 kb mRNA transcript was detected in

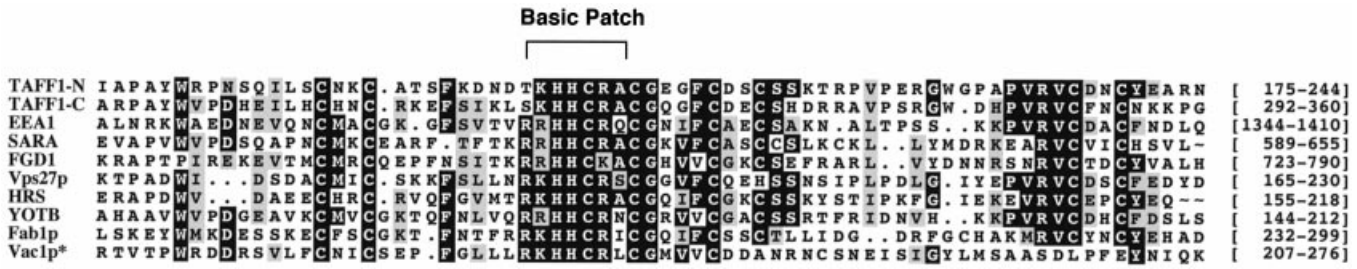

Figure 2 Sequence alignment of the FYVE domains of TAFF1 with some other FYVE domain proteins

The sequences were aligned using the Pileup program of the Wisconsin Package Version 10.0 with a gap weight of 8 and gap length weight of 2. Dots indicate gaps introduced to maximize the alignment. Identities between the sequences are shaded in black, while similar residues are shaded in grey. The basic patch with the consensus sequence R(R/K)HHCR predicted to bind phosphoinositides and the residue numbers are indicated. Sequences of the other FYVE domain proteins are published in Stenmark and Aasland [30]. TAFF1-N and TAFF1-C refer to the N-terminal and C-terminal FYVE domains of TAFF1 respectively. *C-terminal FYVE domain.

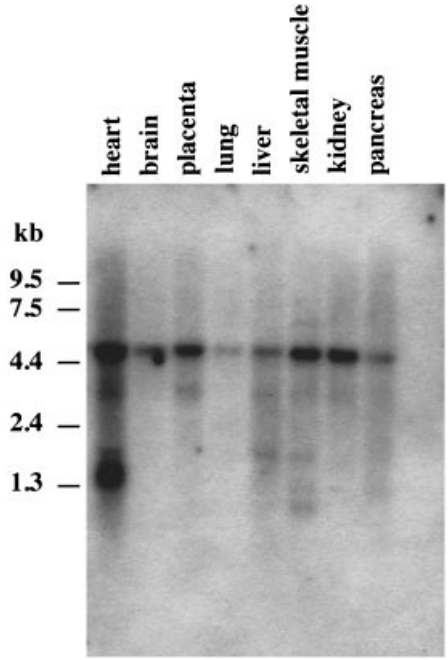

Figure 3 Tissue distribution of TAFF1 mRNA

A human multiple tissue Northern blot was probed with a cDNA fragment encoding full-length TAFF1 and labelled with [α-^{32}P]ATP. Hybridization was carried out at 55 °C overnight and the blot washed at room temperature with 2 × SSC/0.5% SDS and at 50 °C with 0.1 × SSC/0.1% SDS. The RNA standards are marked.

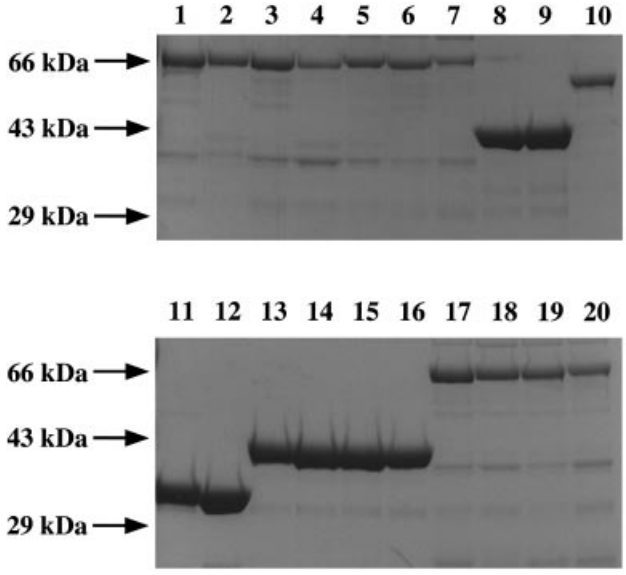

Figure 4 SDS/polyacrylamide gel of GST–TAFF1 and GST–EEA1 fusion proteins after their expression in *E. coli*

Electrophoresis was carried out on 10% gels, and 8 μg of protein was applied to each lane. The position of the molecular-mass markers BSA (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa) are marked. Lane 1, full-length GST–TAFF1; lane 2, GST–TAFF1(K202A, H204A, R206A) triple mutant; lane 3, GST–TAFF1(K319A, H321A, R323A) triple mutant; lane 4, GST–TAFF1 mutant in which the three residues mutated in lane 2 and the three residues in lane 3 were all changed to alanine; lane 5, GST–TAFF1(C239S); lane 6, GST–TAFF1(C355S); lane 7, GST–TAFF1(C239S, C355S) double mutant; lane 8, GST–TAFF1(166–362); lane 9, GST–TAFF1(166–362) (T201R, S318R) double mutant; lane 10, GST–TAFF1(56–362); lane 11, GST–TAFF1(166–269); lane 12, GST–TAFF1(270–362); lane 13, EEA1(1257–1411); lane 14, EEA1(1257–1411)(R1369T); lane 15, EEA1(1257–1411) (R1369S); lane 16, EEA1(1257–1411) (R1369A); lane 17, full-length GST–TAFF1; lane 18, GST–TAFF1(T201R); lane 19, GST–TAFF1(S318R); lane 20, GST–TAFF1(T201R, S318R) double mutant. In the mutant designations, amino acids are given in the one-letter code, that is, K202A means $Lys^{202} \rightarrow$ alanine etc.

all the tissues examined (Figure 3). However, a shorter transcript at 1.3 kb was also detected and was particularly prominent in heart, suggesting alternative splicing of the transcript.

Lipid-binding studies

Constructs expressing full-length and a variety of truncated and mutant versions of TAFF1 and EEA1 were expressed in *E. coli* as GST fusion proteins, subjected to affinity chromatography on GSH–Sepharose and the purity examined by SDS/PAGE (Figure 4).

The full-length wild-type GST-TAFF1 was found to bind PtdIns3*P* specifically in a lipid binding overlay assay; binding to other inositol phospholipids being minimal (Figure 5). In order to determine which TAFF1 domain was involved in binding PtdIns3*P*, we mutated three of the basic residues present in each TAFF1 domain that are known to be critical for the binding of PtdIns3*P* to the FYVE domain of EEA1 (Figure 5, constructs 2 and 3). Surprisingly, we found that the mutation of these residues to alanine in either the N-terminal or C-terminal FYVE domains

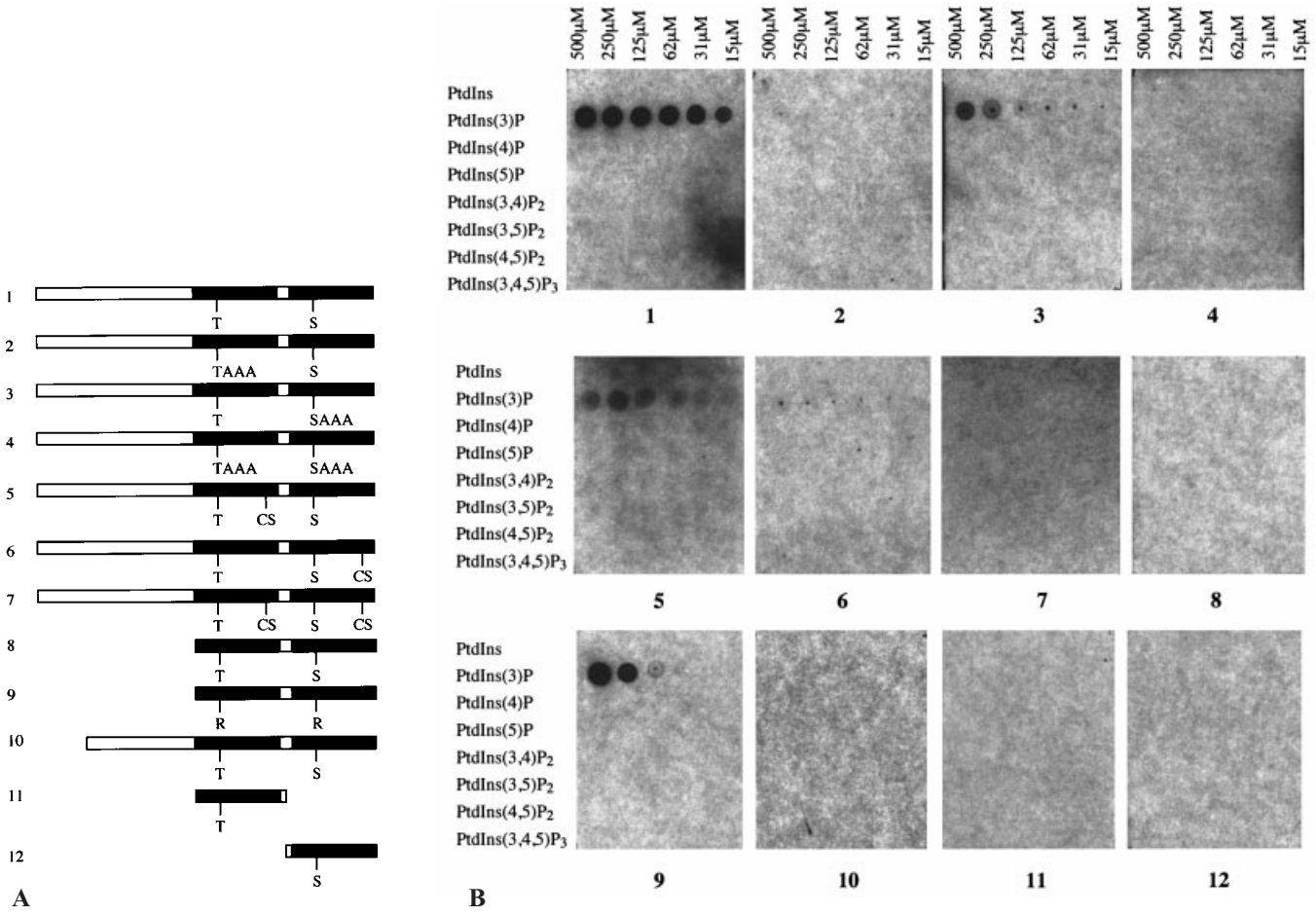

Figure 5 Binding of wild-type and mutant TAFF1 proteins to inositol phospholipids

Each phosphoinositide (1 μl) was spotted on to a nitrocellulose membrane at the concentrations indicated and incubated for 3 h with 1.5 μg/ml of the indicated GST fusion proteins shown in (**A**). Solid black boxes represent FYVE domains of TAFF1. The constructs correspond to the proteins shown in lanes 1–12 of Figure 4. After extensive washing as described in the Materials and methods section, the membranes were probed with an anti-GST antibody followed by horseradish peroxidase-conjugated sheep anti-mouse secondary antibody. The GST fusion proteins bound to the lipids were detected by enhanced chemiluminescence (**B**). The results shown are representative of three independent experiments.

of TAFF1 drastically reduced PtdIns3*P* binding, suggesting that both FYVE domains might be required for high-affinity binding to PtdIns3*P*. Binding was also abolished when both FYVE domains were mutated in this way (Figure 5, construct 4). Similar results were obtained when either FYVE domain was disabled in a different way by mutation of a cysteine residue to serine that in other FYVE domain proteins is known to be essential for the binding of zinc, and hence the correct folding of the domain (Figure 5, constructs 5 and 6). The binding of PtdIns3*P* was abolished when the essential cysteine residues in both FYVE domains were mutated to serine (Figure 5, construct 7).

The isolated FYVE domains themselves lacking the N-terminal 165 residues did not bind PtdIns3*P*, suggesting that a region of the N-terminus is also required to facilitate binding of this inositol lipid to the FYVE domains (Figure 5, construct 8) or that the deletion of the N-terminus causes structural instability leading to the loss of PtdIns3*P* binding. However, binding of PtdIns3*P* to the isolated FYVE domains was partially restored when Thr^{201} and Ser^{318} were both mutated to arginine (Figure 5, construct 9). The removal of only the first 56 residues was also sufficient to destroy the ability to bind to PtdIns3*P* (Figure 5, construct 10). The two FYVE domains of TAFF1 expressed separately, TAFF1(166–269) and TAFF1(270–362) also did not bind to any inositol phospholipid tested, as expected (Figure 5, constructs 11 and 12) in contrast with the FYVE domain of EEA1 in which residues 1325–1411 bound strongly to PtdIns3*P* [23].

Both FYVE domains of TAFF1 are unusual in that an arginine residue known to be important for the binding of PtdIns3*P* in other FYVE domain proteins is replaced by threonine and serine respectively in the N-terminal and C-terminal FYVE domains of TAFF1. Indeed, we found that the mutation of this arginine residue to threonine or serine in EEA1 abolished its ability to bind to PtdIns3*P* (Figure 6, constructs 14 and 15). We therefore decided to examine the effect on PtdIns3*P* binding of mutating these threonine and serine residues present in wild-type TAFF1 to arginine. The mutation to arginine of Thr^{201} in the N-terminal FYVE domain or Ser^{318} in the C-terminal FYVE domain (Figure 6, constructs 18 and 19) enhanced the affinity of TAFF1 for PtdIns3*P* compared with the wild-type protein (Figure 6, construct 17), whereas the mutation of both residues to arginine (Figure 6, construct 20) greatly increased the affinity for PtdIns3*P*. In contrast with wild-type TAFF1, the removal of the N-terminal 165 residues of TAFF1 reduced significantly, but

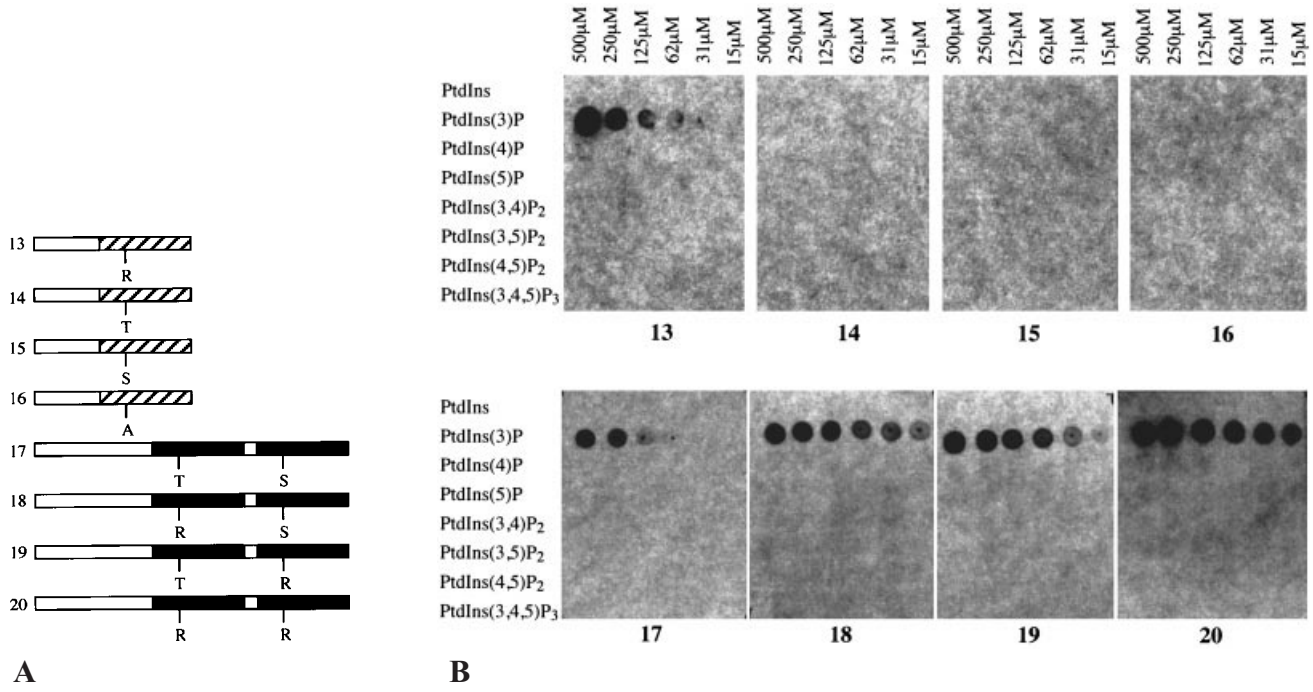

Figure 6 Binding of wild-type and mutant EEA1 and TAFF1 proteins to inositol phospholipids

The experiment described in Figure 5 was repeated with the constructs shown in (**A**). Solid black boxes represent FYVE domains of TAFF1, whereas striped boxes represent the FYVE domain of EEA1. The constructs correspond to the proteins shown in lanes 13–20 of Figure 4. The results shown are representative of three independent experiments. The blots shown in (**B**) were subjected to a shorter time of exposure compared with those of Figure 5(B). The results shown are representative of three independent experiments.

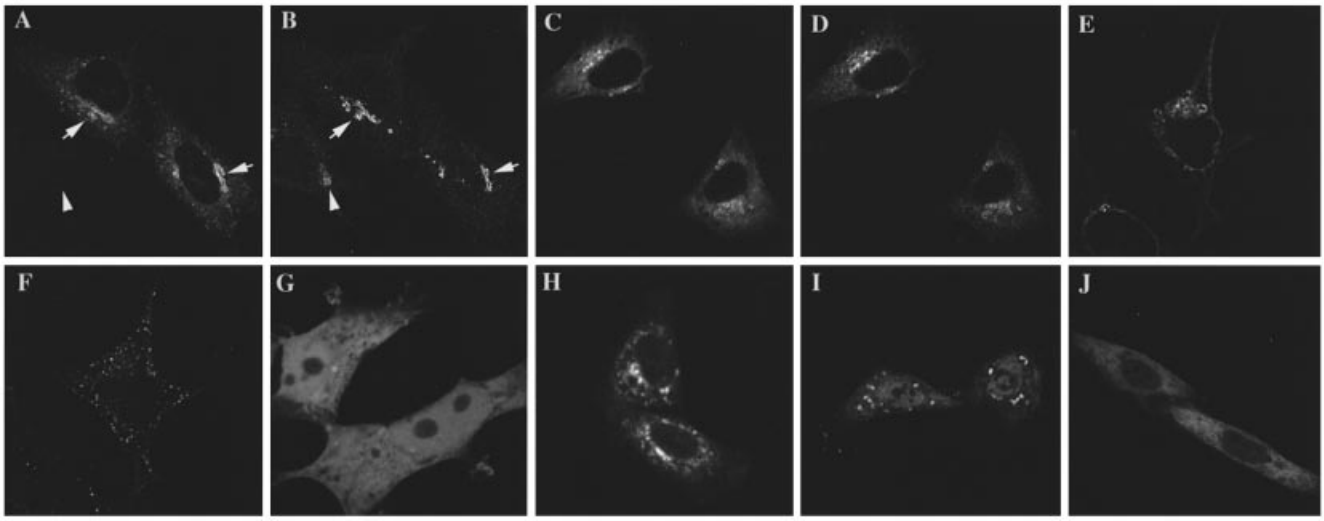

Figure 7 Localization of GFP–TAFF1 and GFP–EEA1 in HeLa cells

This was carried out by confocal microscopy in live cells as described in the Materials and methods section. (**A**) Full-length GFP–TAFF1; (**B**) TGN46; (**C**) full-length GFP–TAFF1; (**D**) GalT; (**E**) full-length TAFF1; (**F**) transferrin receptor; (**G**) GFP–TAFF1(166–362); (**H**) GFP–TAFF1(1–187); (**I**) GFP–EEA1; (**J**) GFP–EEA1 (Arg → 1369threonine mutation). The arrows in (**A**) and (**B**) show the same cell transfected with GFP–TAFF1 (**A**) or stained with anti-TGN46 antibodies (**B**). The arrowhead denotes an untransfected cell. (**C**) and (**D**) show the same cells transfected with GFP–TAFF1 (**C**) or stained with anti-GalT antibodies (**D**). (**E**) and (**F**) show the same cells transfected with GFP–TAFF1 (**E**) or stained with anti-(transferrin receptor) antibodies (**F**).

did not abolish, the binding of PtdIns3*P* to the TAFF1 mutant in which both Thr^{201} and Ser^{318} were mutated to arginine (Figure 5, construct 9).

The mutation of the essential arginine in EEA1 to alanine also abolishes the binding of PtdIns3*P* to this protein [24] (see Figure 6, construct 16). However, the mutation to alanine of Thr^{201}

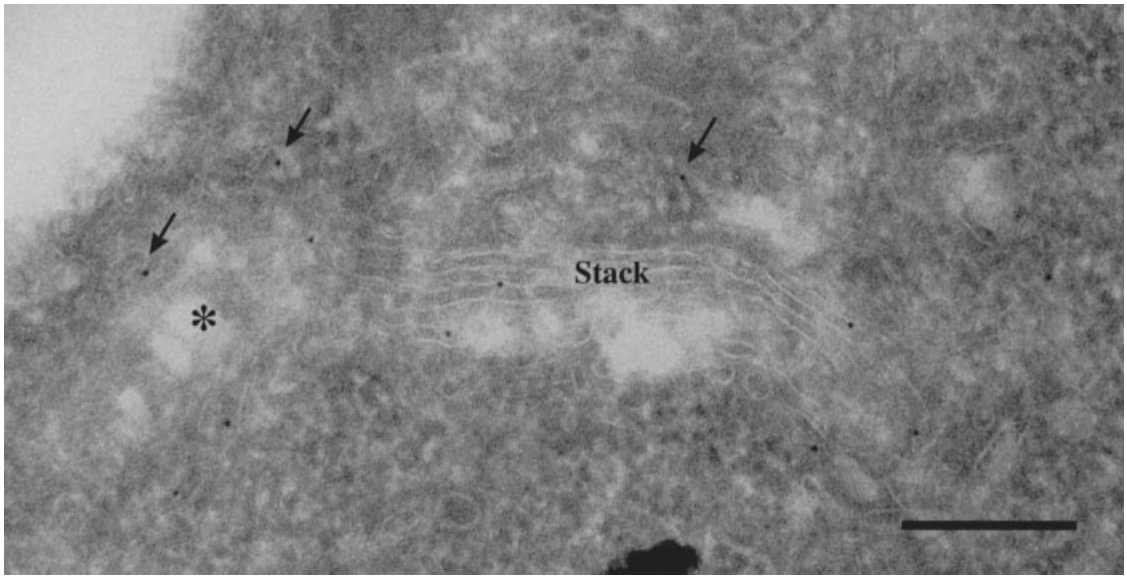

Figure 8 Localization of GFP–TAFF1 in 293 cells determined by immunogold electron microscopy

This was carried out with full-length GFP–TAFF1 as described in the Materials and methods section. Gold particles are present in the cisternal stack, including a region which sectioned tangentially (asterisk). Labelling close to the stack (arrows) is located over indistinct vesiculotubular profiles. The bar represents 200 nm.

and/or Ser^{318} only impaired PtdIns3*P* binding to TAFF1 slightly (results not shown).

Subcellular localization of TAFF1

We examined the subcellular localization of GFP–TAFF1 fusion proteins in live cells after expressing various constructs in HeLa cells. Full-length GFP–TAFF1 was found to be targeted to the Golgi apparatus (Figure 7A), but TAFF1(166–362) containing both FYVE domains showed diffuse cytosolic staining (Figure 7G), similar to expression of GFP itself (results not shown). GFP fusions of the isolated FYVE domains of TAFF1(166–269) or TAFF1(270–362) showed similar diffuse cytosolic staining (results not shown). However, a GFP fusion of TAFF1(1–187) (Figure 7H), lacking both FYVE domains, localized at the Golgi, just like full-length TAFF1. The pattern of staining was similar to, but did not overlap completely with, that of the standard Golgi marker TGN46, which is concentrated mainly in tubules of the *trans*-Golgi network (Figure 7B) [15]. A more exact pattern of co-localization was seen by staining with antibodies specific for GalT (Figure 7D), another standard Golgi marker localized mainly in cisternae of the Golgi stack [15]. The same cells expressing GFP–TAFF1 are shown in Figure 7(C). This suggests that TAFF1 resides predominantly in the cisternal stacks of the Golgi apparatus. Cells were also stained with an antibody specific for SEC13, a protein involved in the secretory pathway, which is found on vesicles budding from the endoplasmic reticulum and in the intermediate compartment between the endoplasmic reticulum and the Golgi [39]. No overlap was found between TAFF1 and SEC13, again supporting the idea that TAFF1 is restricted to the Golgi stacks (results not shown). In addition to the Golgi, vesicule-like structures containing TAFF1 were found throughout the cytoplasm in a proportion of HeLa cells. These structures are distinct from endosomes, as determined by counter-staining with an antibody to the transferrin receptor, an endosomal marker (Figure 7F). The same cell expressing GFP–TAFF1 is shown (Figure 7E). TAFF1(1–56), TAFF1(1–106), TAFF1(57–362) and TAFF1(116–362) failed to localize to the Golgi.

In a parallel study we confirmed that EEA1 is targeted to the endosome (Figure 7I) [5,25] and found that mutation to threonine of the conserved Arg^{1369} at the start of the basic patch totally disrupted its endosomal targeting (Figure 7J), and similar results were obtained if Arg^{1369} was mutated to alanine or serine (results not shown). This result is consistent with the loss of PtdIns3*P* binding caused by these mutations (Figure 6).

The mutation of both Thr^{201} and Ser^{318} to arginine in full-length TAFF1, which enhanced binding of PtdIns3*P* (Figure 6), did not affect the subcellular localization of TAFF1, which remained associated with the Golgi. Similarly, TAFF1(166–362), in which both Thr^{201} and Ser^{318} were changed to arginine continued to show diffuse cytosolic staining, despite the restoration of PtdIns3*P* binding (results not shown). Similar results were observed when these studies were repeated in HEK-293 cells (results not shown).

Electron microscopy

We also localized GFP–TAFF1 using immunogold methods applied to thawed cryosections (Figure 8). Labelling was almost exclusively over the Golgi stack and tubulovesicular structures close to it. Endosomal structures, plasma membrane and endoplasmic reticulum did not display significant labelling.

DISCUSSION

In the present study we have identified a novel protein called TAFF1 in a yeast two-hybrid screen using PDK1 as bait, but subsequent work failed to detect any interaction between these two proteins (P. Cheung, unpublished work). Nevertheless, the unusual finding of two FYVE fingers arranged in tandem led us to undertake a more detailed analysis of the properties of TAFF1. This led to the discovery that TAFF1 binds PtdIns3*P* specifically and that it is targeted to the cisternal stacks of the Golgi.

EEA1 was the first FYVE domain protein shown to bind PtdIns3*P* specifically, and several other FYVE domain proteins have also been shown to bind this inositol phospholipid [5–7]. Binding of EEA1 to PtdIns3*P* was abolished if the residues in the basic patch (Figure 2) were mutated to alanine [5]. The determination of the three-dimensional structure of Vps27p at high resolution identified a shallow groove at the surface of the protein formed by the basic patch (Figure 2) that was predicted to bind PtdIns3*P* [8]. The small size of this groove was consistent with the ability to bind monophosphoinositides, but not diphosphoinositides such as PtdIns(3,4)P_2 and PtdIns(4,5)P_2 or triphosphoinositides such as PtdIns(3,4,5)P_3. PtdIns5*P* is the closest structural homologue to PtdIns3*P* and was reported to induce significant chemical-shift changes in EEA1 as judged by NMR spectroscopy [26]. However the concentration required was much higher than for PtdIns3*P*, and we were unable to detect any binding of PtdIns5*P* to EEA1 in our lipid-binding studies (Figure 5). The longer distance between the 1- and 4-phosphates of PtdIns4*P* has often been cited as the reason why it is excluded from binding to the FYVE domain (e.g. [27]).

In EEA1, the first residue of the basic patch is critical for binding PtdIns3*P*, because its mutation to threonine/serine or alanine (Figure 6) [24] destroys PtdIns3*P* binding. On the basis of the three-dimensional structure of Vps27p it has been predicted that this arginine residue forms a salt bridge with the 1-phosphate of PtdIns3*P* which stabilizes the interaction of the 3-phosphate with the second histidine residue and the arginine adjacent to the cysteine in the basic patch [8]. A different model was proposed by others [9], who solved the structure of Hrs, another FYVE domain protein. They predicted that the N-terminal arginine residue of the basic patch is juxtaposed between the 1- and 3-phosphates of PtdIns3*P* and may form salt links with both phosphates. These two models predict different orientations of the PtdIns3*P* headgroup in relation to the FYVE-domain-binding pocket when bound. It is therefore surprising that TAFF1 binds PtdIns3*P*, because this residue is threonine and not arginine in the first FYVE domain of TAFF1, and serine and not arginine in the second FYVE domain of TAFF1 (Figure 5).

A clue as to why TAFF1 can bind PtdIns3*P* comes from the finding that Hrs crystallizes as a dimer – dimerization resulting from interactions between the FYVE domains [9]. Similarly, the isolated FYVE finger of EEA1 has also been reported to dimerize in solution [26]. These investigators predicted that dimerization is likely to enhance the binding of PtdIns3*P*, and Stenmark and co-workers recently observed that the binding to PtdIns3*P* was enhanced if the isolated FYVE domain of Hrs was fused to a second identical FYVE domain [28], although the mechanism of the increased affinity is not fully understood. In the present study we demonstrated that both FYVE domains are required for significant binding of PtdIns3*P* to TAFF1. It is therefore likely that the two FYVE domains located on the same polypeptide of TAFF1, whose sequences are very similar (Figure 2), interact with one another to stabilize PtdIns3*P* binding. The removal of the N-terminal 56 residues was sufficient to abolish PtdIns3*P* binding, perhaps because it caused structural instability that weakened the interaction between the two FYVE domains.

Only one other protein, yeast Vac1p, has been identified that possesses two FYVE domains in a single polypeptide. However, apart from the FYVE domains themselves, Vac1p and TAFF1 do not share any sequence similarity [29,30]. Moreover the isolated C-terminal FYVE domain of Vac1p binds PtdIns3*P*, consistent with the presence of an arginine residue at the start of the basic patch (Figure 2). The basic patch of the N-terminal FYVE domain of Vac1p starts with glycine and not arginine and also lacks several other basic residues thought to be required for PtdIns3*P* binding. A search of EST databases identified only one other FYVE domain protein in which the first residue of the basic patch is not arginine, namely the protein encoded by the *CeF01F1.6* gene of the nematode worm *Caenorhabditis elegans*, in which the first arginine residue is replaced by valine [30]. Two other basic residues are also missing in this region. It would be interesting to investigate the lipid binding properties of the N-terminal FYVE domain of Vac1p and the protein encoded by the *ceF01F1.6* gene.

TAFF1 is the first protein containing a FYVE domain that has been shown to localize to the Golgi. Proteins containing FYVE domains that have a vesicular localization include EEA1 [1], Hrs [31] and Vac1p [32]. In the case of EEA1, mutation of the cysteine residues in the FYVE domain, or treatment of cells with the phosphoinositide 3-kinase inhibitor wortmannin to prevent PtdIns3*P* formation, disrupted vesicular localization, implying that a correctly folded FYVE domain and the binding of PtdIns3*P* are both required for binding to the endosomal membrane [5,25]. However, mutation of the cysteine residues in the FYVE domains of Hrs or Vac1p did not disrupt membrane targeting [31,32]. This is similar to our results with TAFF1, in which removal of both FYVE domains did not affect the localization of the N-terminal region of the protein to the Golgi complex. It is possible that PtdIns3*P*, which is detectable in the Golgi [33], may regulate TAFF1 function via the FYVE domains.

TAFF1 could be localised to the Golgi via a transmembrane domain (TMD), by interaction with a resident Golgi protein or by covalent lipid modification and insertion into the Golgi membrane [34]. We do not believe that TAFF1 contains a TMD, as the TMPred program [35], located at the Expasy server (http://www.expasy.ch/), was unable to detect high scores indicative of a transmembrane region for the N-terminal region of TAFF1 (results not shown). This program has been used to predict transmembrane regions for putative integral Golgi membrane proteins such as Golgin-67 [36]. Visual inspection of the amino acid sequence of TAFF1 did not show the presence of known Golgi localization motifs such as GRIP domains [37]. Another possibility is that TAFF1 may be fatty-acylated, as occurs in endothelial nitric oxide synthase, glutamate decarboxylase and superior cervical ganglia protein 10 [34]. It will clearly be important to identify the role of TAFF1 in Golgi function because, to our knowledge, it is the first Golgi-associated protein shown to bind PtdIns3*P*.

While this manuscript was under revision, the cloning of a 777-residue protein termed 'double FYVE containing protein 1' (DFCP1) was reported [38]. The predicted amino acid sequence of TAFF1 is identical with residues 416–777 of DFCP1. Inspection of the genomic sequence deposited at NCBI led us to conclude that TAFF1 is encoded by the 1.3 kb mRNA transcript shown in Figure 3 and that it is an alternatively spliced variant of DFCP1, which corresponds to the 4.5 kb mRNA transcript that we detected (Figure 3).

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