Alfy, a novel FYVE-domain-containing protein associated with protein granules and autophagic membranes

Anne Simonsen[1,§], Hanne C. G. Birkeland[1,§], David J. Gillooly[1], Noboru Mizushima[2,3,*], Akiko Kuma[2,3], Tamotsu Yoshimori[4,5], Thomas Slagsvold[1], Andreas Brech[1] and Harald Stenmark[1,‡]

[1]Department of Biochemistry, The Norwegian Radium Hospital, Montebello, Oslo, 0310, Norway
[2]Department of Cell Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaijicho, Okazaki 444-8585, Japan
[3]PRESTO and [4]CREST, Japan Science and Technology Agency, Kawaguchi Center Building, 4-1-8, Honcho, Kawaguchi-shi, Saitama 332-0012, Japan
[5]Department of Cell Genetics, National Institute of Genetics, Yata 1111 Mishima, Shizuoka-ken, 411-8540, Japan
*Department of Bioregulation and Metabolism, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan
§These authors contributed equally to this work
‡Author for correspondence (e-mail: stenmark@ulrik.uio.no)

Accepted 22 April 2004
Journal of Cell Science 117, 4239-4251 Published by The Company of Biologists 2004
doi:10.1242/jcs.01287

Summary

Phosphatidylinositol-3-phosphate [PtdIns(3)*P*] regulates endocytic and autophagic membrane traffic. In order to understand the downstream effects of PtdIns(3)*P* in these processes, it is important to identify PtdIns(3)*P*-binding proteins, many of which contain FYVE zinc-finger domains. Here, we describe a novel giant FYVE-domain-containing protein, named autophagy-linked FYVE protein (Alfy). Alfy is ubiquitously expressed, shares sequence similarity with the Chediak-Higashi-syndrome protein and has putative homologues in flies, nematodes and fission yeast. Alfy binds PtdIns(3)*P* in vitro and partially colocalizes with PtdIns(3)*P* in vivo. Unlike most other FYVE-domain proteins, Alfy is not found on endosomes but instead localizes mainly to the nuclear envelope. When HeLa cells are starved or treated with a proteasome inhibitor, Alfy relocalizes to characteristic filamentous cytoplasmic structures located close to autophagic membranes and ubiquitin-containing protein aggregates. By electron microscopy, similar structures can be found within autophagosomes. We propose that Alfy might target cytosolic protein aggregates for autophagic degradation.

Key words: Aggresome, Atg5, Autophagy, BEACH domain, FYVE domain, PI-3-kinase

Introduction

Phosphoinositides, phosphorylated derivatives of the membrane lipid phosphatidylinositol, regulate cytoskeleton function, membrane trafficking and receptor signalling through reversible recruitment of protein complexes to specific membranes (Yin and Janmey, 2003; Toker, 2002; Simonsen et al., 2001). Phosphatidylinositol-3-phosphate [PtdIns(3)*P*] is formed by the phosphorylation of phosphatidylinositol by (mainly) class III phosphoinositide 3-kinases (PI-3-kinases) (Vanhaesebroeck et al., 2001). This lipid is crucial for endocytic and autophagic membrane traffic (Simonsen et al., 2001) and we are beginning to learn some of the underlying molecular mechanisms.

The identification of two conserved protein domains that bind PtdIns(3)*P* with high specificity has facilitated the analysis of the downstream functions of this lipid. The FYVE domain is a ~70-residue zinc finger found in 27 human proteins (Stenmark et al., 2002). All FYVE domains tested to date have been found to bind to PtdIns(3)*P*, although a few examples that lack one or more consensus residues bind with lower affinity and also show some affinity for the structurally related phosphoinositide PtdIns(5)*P* (Sankaran et al., 2001). The Phox-homology (PX) domain is a ~100-residue β-sandwich found in about 40 human proteins (Ellson et al., 2002). Most PX domains bind specifically to PtdIns(3)*P*, although some bind to other phosphoinositides. In order to learn more about how PtdIns(3)*P* regulates membrane trafficking, it is important to study the functions of various proteins that contain FYVE or PX domains, and several mammalian PtdIns(3)*P* effectors in endocytic membrane trafficking have already been identified (Simonsen et al., 1998; Raiborg et al., 2002; Nielsen et al., 2000; Ikonomov et al., 2003). Although three PtdIns(3)*P* effectors have been identified for the autophagy-related cytoplasm-to-vacuole targeting (Cvt) pathway in yeast (Wurmser and Emr, 2002; Nice et al., 2002), no mammalian PtdIns(3)*P* effectors in autophagy have been identified so far. We find it likely that such effectors exist among the many FYVE- and PX-domain-containing proteins with uncharacterized functions.

In this paper, we describe a novel 400-kDa FYVE-domain-containing protein, named autophagy-linked FYVE protein (Alfy). We show that Alfy binds to and partially colocalizes with PtdIns(3)*P*, and that it colocalizes with autophagic but not endocytic markers. We present evidence that Alfy might serve as a link between protein aggregates and the autophagic machinery.

Materials and Methods

cDNA cloning

The sequence KIAA0993 [1096 nucleotides (nt)] found in the Human Unidentified Gene-Encoded Large Proteins (HUGE) database was used to design a primer (KIA-NR: 5′-GCTCCTACCTGTGAACGT-GTTGACACTCA-3′) to do a 5′ rapid amplification of cDNA ends (5′-RACE) reaction with the AP-1 primer and human brain Marathon Ready cDNA as described by the manufacturer (Clontech, Palo Alto, CA), resulting in the amplification of nt 8662-9638 of Alfy. To obtain full-length Alfy cDNA (10,581 nt) several 5′-RACE reactions were performed using primers KIA315-NR (5′-CTCACGATGGACT-CTGATGAATTCTCC-3′) (nt 7796-8689), KIAcomp-NR (5′-TGCT-GCATGTTCTCTTGAGTTGACTA-3′) (nt 6597-7826), KIA-5R (5′-GTATCAGTTCAGTCCAAACTCTGTTGAC-3′) (nt 5230-6626), KIA-6 (5′-TGACAAGCATCTCGGTTAATCTCCC-3′) (nt 4314-5255), KIA-7 (5′-TCTTTGCTGGCTAGTGGGTTACTCTT-3′) (nt 3573-4340). The homologous *Drosophila* (AAF52302) and *Caenorhabditis elegans* (CAB16307,T26022,T25148) sequences were used to search The National Center for Biotechnology Information (NCBI) human genome database, and the human chromosome 4 contig RP11-147K21 (gi 8705090) was found to encompass the Alfy sequence. Homologous regions were used to design primers for PCR to generate more 5′ sequence: KIA-11 (5′-GAGAAACGTCCAGGCCTTTGCAGTT-3′) + KIA-8 (5′-ATTAC-CAGGACCAAATGATGCCACTG-3′) (nt 1135–3600) and KIA-12F (5′-CAGTTTTGTTTCCCCTGCGGAGGAGC-3′) + KIA-10R (5′-TTTCTCGAAGGACCGACAGGAGGGC-3′) (nt 593-1912). Nucleotides 1-656 were obtained by a 5′-RACE reaction with KIA-12R2 (5′-GCACTGAATAGAAGCTGGAGATCATC-3′). The KIA-12R2 RACE sequence contains a stop codon in frame, which was later confirmed by sequencing of polymerase chain reaction (PCR) products using a primer 5′ to the coding region. The 5′-RACE products were cloned into the pGEM-T-easy vector (Promega, Madison, WI) and sequenced (GATC, Konstanz, Germany) using the SP6 and PCR1 primers.

Plasmid constructs

We were unable to subclone Alfy in one piece into a mammalian expression vector, probably because the large size of the cDNA makes it prone to recombination. We have therefore based all our immunofluorescence and electron microscopy (EM) experiments on endogenous Alfy detected using a polyclonal rabbit anti-Alfy antibody. pMAL-Alfy$_{CT639}$ was generated by subcloning the C-terminal 1917 nt from Alfy into pMAL-c2 (New England Biolabs). pcDNA3-myc-Alfy2587-3527 was prepared by amplifying the relevant part of Alfy and subcloning it behind the Myc epitope of pcDNA3-myc (Raiborg et al., 2001). Human *Atg5* cDNA [we here use the nomenclature by Klionsky et al. for components of the autophagic machinery (Klionsky et al., 2003)] was amplified by PCR from the human brain Marathon Ready cDNA and subcloned into pEGFP-c1 (Clontech). Human LC3-B cDNA was amplified by PCR from the human brain Marathon Ready cDNA and subcloned into pEGFP-C-1 (Clontech). pGEX-Alfy-FYVE was prepared by PCR amplifying the region corresponding to the FYVE domain (residues 3451-3516) of Alfy and subcloning into pGEX-5X-3 (Amersham Pharmacia). For expression of maltose-binding protein (MBP) or glutathione-*S*-transferase (GST) fusion proteins in *Escherichia coli*, the relevant pMAL or pGEX constructs were transformed into *E. coli* BL-21 (DE3) cells, and the proteins were purified according to the manufacturers' instructions.

Cell culture and transfections

Media and reagents for cell culture were purchased from Gibco. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), 5 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. For serum and amino acid starvation conditions, cells were washed three times in Hanks' balanced salt solution (HBSS) and thereafter incubated in the same medium for the indicated time period. Cells were transfected with the above expression plasmids using the FuGENE-6 reagent (Roche Molecular Biochemicals) as described by the manufacturer.

Antibodies and fluorescent probes

Two peptides corresponding to amino acids 2969-2984 (FKKPHPPKRVRSRLNG) and 3302-3316 (SQPSSTSHRPRAASC) of Alfy were synthesized, coupled together to keyhole limpet haemocyanin and used for immunization of two rabbits to raise antisera (Eurogentech, Herstal, Belgium). A BLAST search of the human genome showed that these peptides are only found in Alfy. Sera were affinity purified against a recombinant MBP-Alfy$_{CT639}$ fusion protein coupled to Affi-gel 15 (Bio-Rad, Hercules, CA) as described by the manufacturer. Mouse monoclonal antibodies against the c-Myc epitope was from the 9E10 hybridoma (Evan et al., 1985). Human anti-EEA1 serum was a gift from B.-H. Toh (Monash University, Melbourne, Australia). Mouse anti-human LAMP-1 was from Developmental Studies Hybridoma Bank (The University of Iowa, IA, USA). Mouse anti-ubiquitin antibody (FK2) was from Affiniti (Exeter, UK). Rabbit anti-rat LC3 antibody has been described before (Kabeya et al., 2000). Horseradish-peroxidase (HRP)-labelled goat anti-rabbit antibody, HRP-labelled goat anti-mouse antibody, Cy3-labelled goat anti-rabbit antibody, Cy2-labelled goat anti-mouse antibody and FITC-labelled goat anti-human antibody were all purchased from Jackson Immunoresearch (West Grove, PE, USA). A fusion between GST and a tandem FYVE domain from hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) (GST-2×FYVE) was purified as described previously (Gillooly et al., 2000) and labelled with Alexa-488 (Molecular Probes, Oregon, USA) according to the manufacturer's instructions.

Immunoblotting

To determine the tissue-specific distribution of Alfy, various cell lines and mouse tissue were homogenized in homogenization buffer (20 mM Hepes pH 7.2, 100 mM KCl, 2 mM $MgCl_2$ and a protease inhibitor cocktail) by six passages through a G22 syringe. After centrifugation for 5 minutes at 6000 ***g***, 60 μg of protein of each post-nuclear supernatant (PNS) was subjected to sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) (6% polyacrylamide) and transferred onto Immobilon-P membrane (Millipore, Bedford, MA) for immunoblotting with affinity-purified rabbit anti-Alfy antibody, followed by a goat anti-rabbit HRP-labelled antibody. The blot was developed with the SuperSignal® West Pico Chemiluminescent substrate kit (Pierce, Rockford, IL). To analyse the intracellular distribution of Alfy, PNS from HeLa cells (prepared as described above) were centrifuged for 30 minutes at 100,000 ***g*** to obtain cytosol and membrane fractions. The nuclear fraction was obtained by washing the nuclear material twice in a lysis buffer (1% Triton X-100, 10 mM Na_2HPO_4, 0.1 M NaCl, protease inhibitor cocktail) containing 0.4 M sucrose. The nuclei were then centrifuged (1500 ***g***) through 0.7 M sucrose in lysis buffer before the pellet was subjected to SDS-PAGE (6% polyacrylamide) and immunoblotting as described above. To investigate whether Alfy was a peripheral membrane protein, PNS from HeLa cells was incubated with homogenization buffer alone or buffer containing 1 M NaCl or 0.1 M Na_2CO_3 pH 11 for 30 minutes on ice before preparation of cytosol and membrane fractions, SDS-PAGE and immunoblotting as described above.

Liposome-binding assay

Liposome-binding experiments were carried out as described (Patki

et al., 1997). Liposomes were prepared using an equal mixture of phosphatidylserine and phosphatidylinositol, with 5% PtdIns(3)*P* included at the expense of phosphatidylinositol. Lipids were dissolved under nitrogen and resuspended in 50 mM Hepes pH 7.4, 100 mM NaCl, 0.5 mM EDTA. Resuspended lipids were sonicated and liposomes collected by centrifugation at 16,000 ***g*** for 10 minutes. Liposomes were resuspended at a concentration of 2 mg ml^{-1} in Hepes buffer supplemented with phosphatase inhibitor cocktail II (Sigma). Aliquots of liposomes (typically 300 μl) were incubated with HeLa cytosol (typically 200 μl of 2.5 mg ml^{-1} cytosol) for 15 minutes at room temperature with rolling. Liposomes were collected by centrifugation, the cytosolic supernatant (S) removed and the liposome pellet (P) resuspended in 25 μl Hepes buffer. Samples were analysed for the presence of Alfy by 7.5% SDS-PAGE and immunoblotting using the anti-Alfy antibody.

Protein-lipid overlay assay

Nitrocellulose membranes containing spotted phosphoinositides and other lipids (PIP strips) were purchased from Echelon Research Laboratories (Salt Lake City, Utah). Membranes were blocked with 3% fatty-acid-free bovine serum albumin (BSA) (Sigma Aldrich) in TBS-T (10 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% Tween-20) for 1 hour at room temperature. Blocked membranes were incubated overnight at 4°C with 1 μg ml^{-1} GST fusion proteins in TBS-T. The membranes were then washed four times for 10 minutes in TBS-T. After washing, membranes were incubated with monoclonal anti-GST antibody (1/1000 dilution; Sigma Aldrich) for 2 hours at room temperature followed by additional washing and incubation with HRP-conjugated antibody (Jackson ImmunoResearch Laboratories). After final washing, lipid-bound GST fusion proteins were detected by chemiluminescence.

Confocal fluorescence microscopy

All immunofluorescence microscopy was performed with HeLa cells grown on coverslips. The cells were transfected, treated with the indicated inhibitors, starved in HBSS or not (as indicated) and thereafter permeabilized with 0.05% saponin for 5 minutes and fixed with 3% paraformaldehyde on ice for 15 minutes. Finally, the fixed cells were stained with the antibodies indicated. For PtdIns(3)*P* detection, HeLa cells on coverslips were starved in HBSS for the indicated times, permeabilized and fixed. The cells were thereafter stained with GST-2×FYVE-Alexa-488 (20 μg ml^{-1}) and anti-Alfy antibodies, followed by the required secondary antibodies. The coverslips were examined using a Leica TCS NT confocal microscope equipped with a Kr/Ar laser and a PL Fluotar 100×/1.30 oil-immersion objective.

Electron microscopy

Correlative immunofluorescence/EM (IF/EM) was performed on HeLa cells grown on gridded coverslips (Eppendorf) and starved in HBSS for 45 minutes to maximize the number of cells with Alfy-positive structures, before fixation with 3% paraformaldehyde in 0.1 M PBS for 30 minutes. They were thereafter permeabilized with 0.05% saponin (4 minutes) and subsequently labelled with rabbit anti-Alfy and goat anti-rabbit-Cy3 antibodies. The cells were then observed in a Leica confocal microscope and the localization of cells with Alfy-positive structure were noted for each coverslip. For EM observation, the same coverslips were then fixed with 2% glutaraldehyde in 0.1 M phosphate buffer and postfixed with 2% OsO_4, 1.5% KFeCN in 0.1 M cacodylate buffer. Thereafter, the coverslips were stained en bloc with uranyl acetate, dehydrated in ethanol and embedded in Epon. After polymerization, the coverslips were removed with 40% hydrofluoric acid and the flat specimens mounted on Epon stubs. The block was thereafter trimmed down to the regions observed on the gridded coverslips in the fluorescence microscope and sectioned parallel to the substratum at 50-70 nm section thickness. The sections were then poststained with lead citrate and observed in a Philips CM 10 microscope. For EM on cells treated with the proteasome inhibitor PSI [N-benzyloxycarbonyl-Ile-Glu(O-t-butyl)-Ala-leucinal] without prior immunolabelling, we prepared cells grown on coverslips essentially as described above. Cells were fixed in glutaraldehyde, postfixed with osmium tetroxide, stained en bloc and embedded in Epon after ethanol dehydration.. The coverslips were removed by incubation in hydrofluoric acid and the flat specimens sectioned parallel to the substratum at 50-70 nm section thickness.

RNA interference studies

Small double-stranded RNA oligonucleotides [small interfering RNAs (siRNAs)] against Alfy was ordered from Dharmacon Research (Lafayette, CO, USA) and had the sequence 5′-GUCGGG-CCAUAGUUCAGUUdTdT-3′ of the sense strand, corresponding to coding nt 314-332 in the Alfy sequence plus two 3′ (2′-deoxy)thymidine overhangs. The antisense strand (5′-AACU-GAACUAUGGCCCGACdTdT-3′) and the sense strand were delivered as a preannealed siRNA duplex. The scrambled siRNA oligonucleotides used as a control consisted of the same nucleotides as the Alfy siRNA oligonucleotides, only in a random order. The sequence of both oligonucleotides were checked against human expressed sequence tag (EST) sequences to make sure that they did not recognize any other known human sequences. The siRNA experiments were performed on HeLa cells that were seeded out in 10 cm dishes 1 day before the transfection in DMEM without penicillin and streptomycin. The next day, they were transfected with a mixture of oligofectamine (Invitrogen, Carlsbad, CA, USA) and siRNA duplex (Alfy siRNA or scrambled siRNA) or oligofectamine alone, in DMEM without serum, penicillin or streptomycin, with a final siRNA concentration of 0.2 μM. 4 hours after transfection, reagents were added to final concentrations of 10% FCS, 5 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. 2 days after transfection, the cells were split equally between three 10 cm dishes without penicillin and streptomycin, and thereafter transfected again the next day. 3 days later, the cells were washed three times in HBSS and starved for the indicated amount of time, followed by lysis in potassium acetate lysis buffer (125 mM KAc, 25 mM Hepes pH 7.1, 2.5 mM $Mg(Ac)_2$, 5 mM EGTA, 1% NP40, 1 mM dithiothreitol). After centrifugation for 10 minutes at 6000 ***g***, 60 μg of protein from each PNS was subjected to SDS-PAGE (6% polyacrylamide), for detection of Alfy by western blotting.

Results

Cloning of Alfy, an autophagy-linked FYVE protein

To date, most FYVE-domain proteins have been found to localize to early endosomes (Stenmark and Aasland, 1999), in line with the finding that PtdIns(3)*P* is highly enriched in early endosomal membranes (Gillooly et al., 2000). Among the FYVE-domain-containing proteins with unknown functions, several have structures resembling endosomal FYVE proteins (Stenmark et al., 2002) and, in order to identify a possible FYVE domain protein with a non-endosomal function, we therefore sought a protein with an unknown function whose structure was different from previously characterized FYVE-domain proteins. One of the FYVE-domain-containing sequences in the human genome was present only as a partial sequence, KIAA0993 (accession number AB023210, 1097 bp), cloned from human adult brain by the HUGE project. We noted that KIAA0993 has high sequence similarity to T23B5.2 (33% amino acid identity, alignment not shown), a hypothetical *C.*

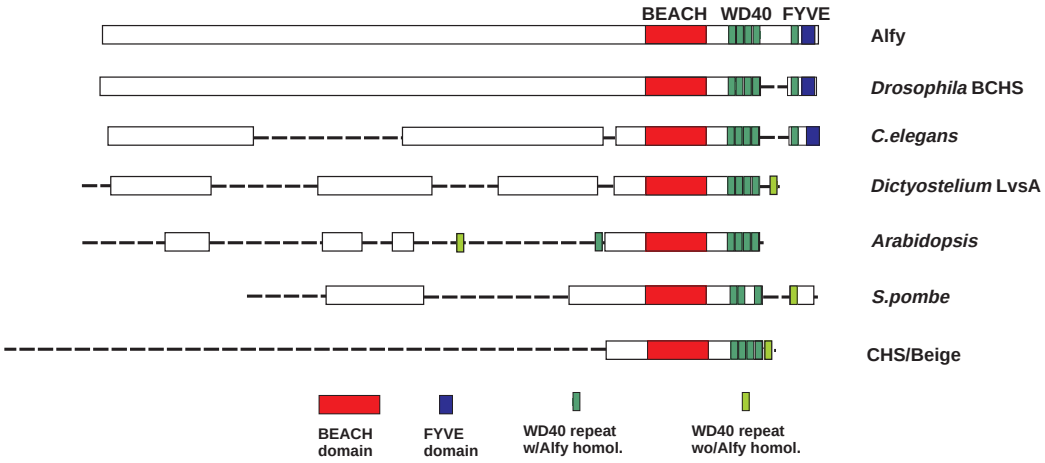

Fig. 1. Alfy, a novel human FYVE-domain protein with putative homologues in several species. The presence of known domains in human Alfy and putative Alfy homologues from different species are indicated. Boxes represent conserved regions, whereas dashed lines represent non-conserved regions. The Alfy sequence has been deposited in the EMBL database with accession number AF538685. Putative homologues of Alfy can be found in *Drosophila melanogaster* (BCHS, AE003611), *Caenorhabditis elegans* (contig of Z99169+AL032675+Z93390), *Dictyostelium discoideum* (LvsA, AF088979), *Arabidopsis thaliana* (AC002330) and *Schizosaccharomyces pombe* (CAA20312). The mammalian LYST/CHS/Beige protein is also indicated.

elegans protein that contains a BEACH domain. The function of the BEACH domain is unknown, although its structure has been solved. The BEACH domain has an unusual polypeptide backbone fold, with the peptide segments in its core not assuming regular secondary structures (Jogl et al., 2002). We regarded this as an interesting domain because it is found in LYST (Nagle et al., 1996), the gene product of the Chediak-Higashi-syndrome locus, a genetic disease characterized by the occurrence of abnormally large lysosomes (Ward et al., 2000). Because autophagosomes fuse with lysosomes, we regarded the full-length KIAA0993 protein as a possible candidate for a PtdIns(3)*P*-binding regulator of autophagy.

To study this FYVE-domain-containing protein further, we extended the KIAA0993 sequence by several rounds of 5′-RACE. Using this approach, we obtained a large full-length cDNA sequence containing 10,581 nt of open reading frame from a human brain library. The putative start codon is preceded by 398 nt of GC-rich 5′ untranslated sequence containing several in-frame stop codons, as found by sequencing of 5′-RACE products and by searching the NCBI human EST database. The cDNA is composed of 66 contigs located at chromosome 4q21 (NCBI Human Gene Map). It encodes a polypeptide of 3527 amino acids with a predicted M_r of 395 kDa and a theoretical p*I* of 6.56. For reasons described below, we term this new protein autophagy-linked FYVE protein (Alfy). Several forms of Alfy probably exist, because sequence heterogeneity, presumably caused by differential mRNA splicing, was observed at several sites.

The C-terminal region of Alfy contains a typical FYVE domain (Stenmark et al., 2002), five WD40 repeats (Smith et al., 1999) and a BEACH domain (Jogl et al., 2002). Alfy also contains a possible TBC domain in the N-terminal part (residues 248-497), a ~200 amino acid conserved domain that has been found in a wide range of different proteins including GTPase-activating proteins (Neuwald, 1997) (Fig. 1). The central part of Alfy (residues 1421-1743) is 56% similar and 20% identical to Atg9, a protein that is essential for autophagy in yeast and that localizes to punctate preautophagosomal structures (Noda et al., 2000). Even though this structural analysis predicts that Alfy might be involved in both lipid and protein interactions, it gives no further clue about the function(s) of Alfy.

Alfy is evolutionarily conserved

Putative homologues of Alfy can be found in *Drosophila melanogaster* [Blue Cheese (BCHS), AE003611], *C. elegans* (contig of Z99169+AL032675+Z93390), *Dictyostelium discoideum* (LvsA, AF088979), *Arabidopsis thaliana* (AC002330) and *Schizosaccharomyces pombe* (CAA20312) (Fig. 1). It is worth noting that the Alfy-like proteins in *D. discoideum* and *A. thaliana* lack a FYVE domain at their C-

Table 1. Comparison of sequence identity between full-length human Alfy and its putative homologues in *Drosophila* and *C. elegans*

A

Human vs *Drosophila*	Human vs *C. elegans*	*Drosophila* vs *C. elegans*
46.6%	31.0%	31.8%

B

Full-length	Residues 6676-10581	BEACH domain	WD40 repeat 1	WD40 repeat 2	WD40 repeat 3	WD40 repeat 4	WD40 repeat 5	FYVE domain
23.0%	32.5%	69.9%	11.4%	33.3%	36.8%	30.6%	43.2%	51.5%

termini. The BEACH domain and the following four WD40 repeats are, however, highly similar, suggesting that this part is conserved as a module. The putative Alfy homologues in *D. melanogaster* and *C. elegans* are more than 30% identical to human Alfy, and the version expressed in *D. melanogaster* has the highest degree of identity (46.6%) (Table 1). The BEACH domains found in human, *D. melanogaster* and *C. elegans* Alfy are approximately 70% identical, whereas the similarity in the WD40 region is less pronounced (Table 1). *D. discoideum* LvsA and *D. melanogaster* BCHS are the only Alfy-like proteins about which any functional information is available. The former protein was found to be involved in the plasma-membrane dynamics of *D. discoideum* cell division (Kwak et al., 1999), whereas *D. melanogaster bchs* mutants have reduced lifespan and accumulate protein granules in their central nervous system (see Discussion).

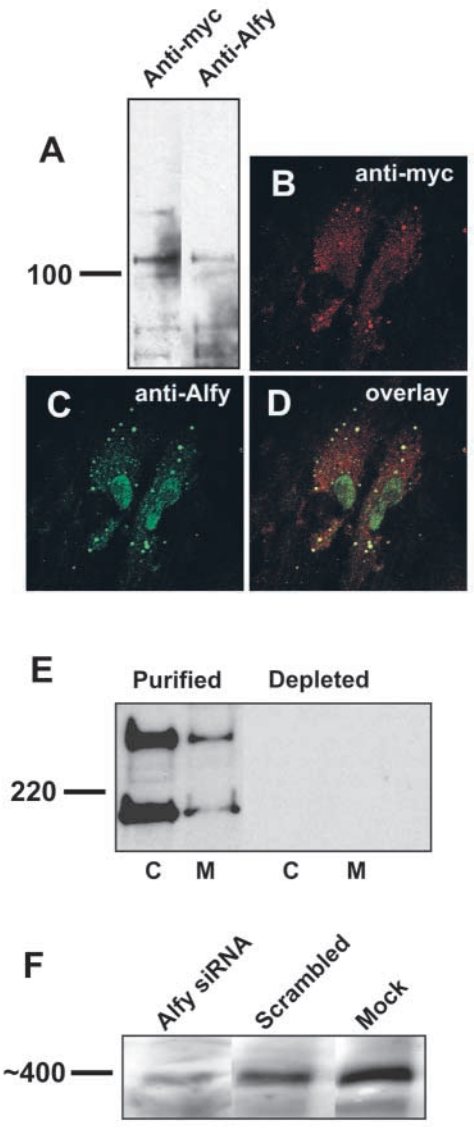

Fig. 2. Characterization of anti-Alfy antibodies. (A-D) HeLa cells were transfected with Myc-Alfy$_{2587-3527}$ and analysed by western blotting (A) or immunofluorescence microscopy with anti-Myc (A,B) and anti-Alfy (A,C) antibodies. (E) The specificity of the anti-Alfy antibody was analysed using affinity-purified anti-Alfy serum or serum depleted of anti-Alfy antibody for immunoblotting of a gel containing cytosol (C) or membrane (M) fractions from HeLa cells. (F) HeLa cells were incubated with an Alfy-specific siRNA, a scrambled siRNA or transfection reagent alone. Equal amounts (60 μg protein) of cell lysates were analysed by western blotting with affinity-purified anti-Alfy antibodies.

Preparation and characterization of antibodies against Alfy

To study the intracellular localization of Alfy, we raised a rabbit antiserum against two Alfy-specific peptides located in its C-terminal part. The antibody was affinity purified on beads containing the immobilized peptides and we performed several tests to check its specificity. First, we transfected cells with a Myc-tagged C-terminal fragment of Alfy (residues 2587-3527) and analysed the cell lysate by western blotting using anti-Alfy and anti-Myc antibodies. Both antibodies stained the same major band of about 110 kDa (Fig. 2A), indicating that anti-Alfy does recognize transfected Alfy by western blotting. To test whether anti-Alfy recognizes Alfy by immunofluorescence microscopy, we stained the transfected cells with antibodies against Alfy and the Myc epitope. Anti-Myc stained punctate structures in the cytosol (Fig. 2B), and these colocalized strongly with structures stained with anti-Alfy (Fig. 2C, overlay in Fig. 2D). In order to investigate whether anti-Alfy recognizes the endogenous protein, we analysed membrane and cytosol fractions from untransfected HeLa cells by western blotting with anti-Alfy, using a longer exposure time than we used for transfected cells. In both fractions, we detected a band of about 400 kDa, which is close to the calculated size of Alfy, as well as a lower band of about 150 kDa, possibly representing a splice variant or a degradation product. Both bands were abolished when the antibody was preadsorbed against beads containing the two peptides that were used for immunization (Fig. 2E). Finally, we tried to deplete cells for Alfy using a specific siRNA complementary to a sequence in the N-terminus of Alfy. Treatment of cells with this siRNA caused a significant reduction of the ~400 kDa Alfy-reactive band compared with mock-treated cells or cells treated with a scrambled RNA duplex (Fig. 2F). Taken together, these results indicate that the antibody specifically recognizes Alfy by western blotting and immunofluorescence microscopy.

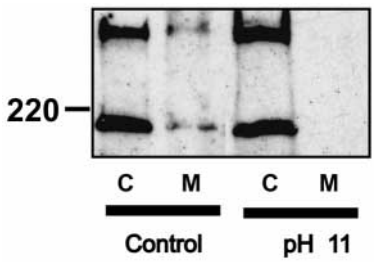

Fig. 3. In order to investigate whether Alfy is a peripheral-membrane protein, PNS from HeLa cells was treated with 0.1 M Na_2CO_3, pH 11, and the resulting cytosol (C) and membrane (M) fractions were subjected to 6% SDS-PAGE before immunoblotting using anti-Alfy antibodies.

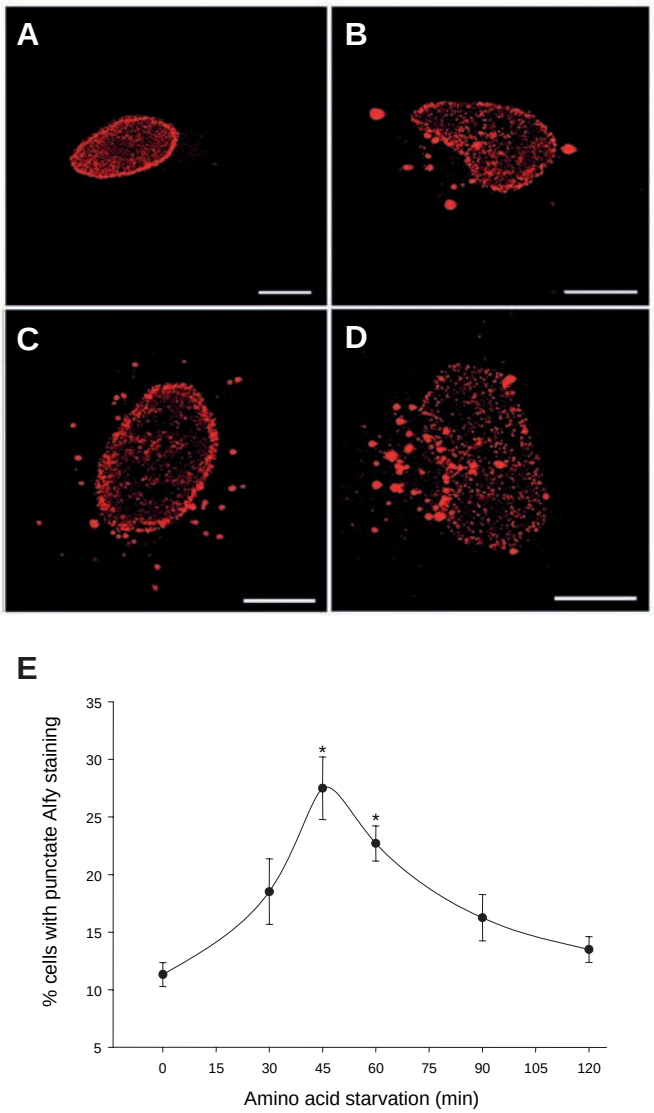

Fig. 4. Alfy is recruited to cytosolic structures upon starvation. HeLa cells were starved for 0 minutes (A) or 45 minutes (B-D) and thereafter stained with a rabbit anti-Alfy antibody. Scale bar, 10 μm. (E) HeLa cells were starved for different amounts of time in HBSS and the proportion of cells with Alfy-positive structure was calculated based on confocal immunofluorescence using anti-Alfy antibody. Each point represents the mean of three independent experiments. The error bars show s.e.m. $P^*<0.03$ as determined with the χ^2 test using the SPSS program version 11 (SPSS, Chicago, IL).

Alfy accumulates on cytoplasmic structures upon amino acid starvation

Alfy was found to be partially associated with membranes (Fig. 2E) and so we investigated whether it is an integral or a peripheral membrane protein. To this end, we incubated membranes with a high-pH buffer that should extract peripheral membrane proteins. Analysis of membrane and cytosol fractions by western blotting with anti-Alfy antibodies showed that Alfy dissociates from membranes at high pH (Fig. 3), indicating that it is a peripheral membrane protein. This is in agreement with its primary structure, which is not predicted to contain any transmembrane segments.

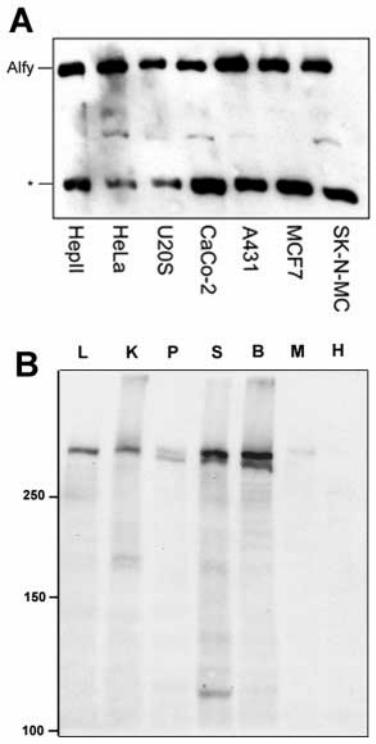

Fig. 5. Alfy is ubiquitously expressed. Extracts from cell lines (A) or mouse tissues (B) were subjected to 6% SDS-PAGE and immunoblotted using rabbit anti-Alfy antibodies. The asterisk in (A) indicates an unidentified protein in cell lines recognized by the anti-Alfy antibody. Abbreviations: B, brain; H, heart; K, kidney; L, lung; M, skeletal muscle; P, pancreas; S, spleen.

The intracellular localization of the endogenous protein was studied in more detail by confocal immunofluorescence microscopy. HeLa cells stained with anti-Alfy showed nuclear localization in essentially all cells, in particular along the nuclear envelope (Fig. 4A). Intriguingly, a small proportion (11%) of the cells had a distinct punctate staining pattern in the cytoplasm, reminiscent of that observed upon expression of the C-terminal part of Alfy. To investigate whether these structures could be related to autophagic vesicles, the cells were starved for amino acids and serum for different time periods, because this treatment has been reported to induce autophagy (Mitchener et al., 1976). The results showed that the proportion of cells with punctate cytosolic staining increased to a maximum of 28% after 45 minutes of starvation and thereafter decreased again to the unstarved level after 2 hours (Fig. 4E). The starved cells showed a much greater variety in the size and number of immunoreactive cytosolic structures. Small punctate structures were detected in many cells (Fig. 4C,D), whereas a few cells contained one to three large structures (Fig. 4B). These results suggested that induction of autophagy causes a relocalization of the Alfy protein to cytoplasmic structures.

Alfy is ubiquitously expressed

By analysis of the NCBI human EST database, we could identify ESTs representing Alfy in a wide range of tissues (liver, kidney, testis, uterus, brain, breast, prostate, intestine,

lung, pharynx, nervous, placenta, ovary, marrow, head/neck, colon, hypothalamus, stomach), indicating that Alfy is ubiquitously expressed. To determine the expression of Alfy at the protein level, equal amounts (60 μg) of PNS from different human cell lines (HeLa, cervix; HepII, larynx; A431, epidermal; MCF-7, breast; U20S, osteogenic sarcoma; CaCo-2, colon; SK-N-MC, neuronal) were analysed by SDS-PAGE and immunoblotting with affinity-purified rabbit anti-Alfy. As in HeLa cells, two main immunoreactive proteins (~400 kDa and ~150 kDa) were detected in all human cell lines at similar levels (Fig. 5A).

We also analysed the distribution of Alfy in various mouse tissues, in which we only detected a large (~400 kDa) doublet immunoreactive band. The expression levels in different tissues varied (Fig. 5B), with the highest level in brain but also detectable expression in liver, kidney, pancreas, spleen and skeletal muscle. There was also a weak expression in heart (visible upon higher exposure of blots, not shown). A similar analysis of rat tissues (not shown) indicated in addition that Alfy is highly expressed in lung. Alfy was found to be equally abundant in wild-type and Atg5-deficient (Mizushima et al., 2003) mouse embryonic stem cells (not shown), indicating that its expression is independent of an intact autophagic machinery. Taken together, the above data indicate that Alfy is ubiquitously expressed.

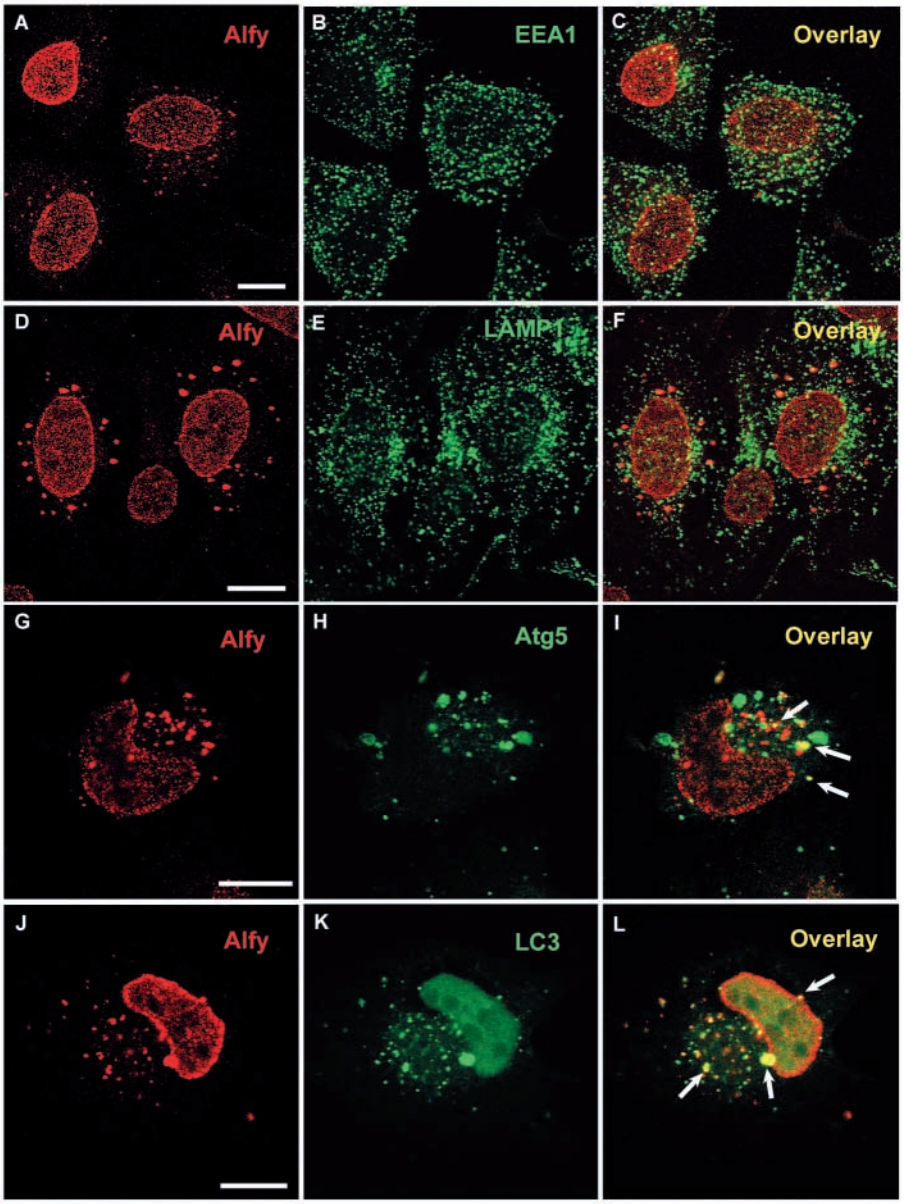

Fig. 6. Alfy colocalizes with hAtg5 and LC3 by confocal microscopy. HeLa cells grown on coverslips were starved for 45 minutes in HBSS, permeabilized, fixed and double stained against endogenous Alfy (A,D,G,J) and endogenous EEA1 (B), endogenous LAMP-1 (E), overexpressed GFP-hAtg5 (H) or GFP-LC3 (K). Yellow colour in the merged images (C,F,I,L) indicates colocalization. Colocalization between Alfy and autophagic markers is indicated by arrows. Scale bar, 10 μm.

Alfy colocalizes with autophagic marker proteins by confocal microscopy

Alfy-positive, cytosolic, vesicle-like structures were induced by starvation, so we next asked whether Alfy is localized to the autophagic pathway. The localization of the Alfy-positive structures was analysed by double-immunofluorescence microscopy of serum- and amino-acid-starved HeLa cells using various marker antibodies. There was essentially no colocalization between Alfy-positive structures (Fig. 6A,D) and a marker of early endosomes, early endosome antigen 1 (Mu et al., 1995) (Fig. 6B). Likewise, there was only a limited colocalization with the late endosome/lysosome marker lysosomal membrane protein 1 (Chen et al., 1985), although we did note colocalization of a few profiles (Fig. 6E). There was no colocalization with internalized endocytic markers (transferrin and dextran) (data not shown). The weak colocalization with endocytic markers supports the hypothesis that Alfy could be involved in a different pathway, such as autophagy.

To explore further the possibility in which Alfy might be involved in autophagy, we treated cells with drugs known to affect this process. The PI-3-kinase inhibitors wortmannin and 3-methyladenine inhibit the formation of autophagosomes (Blommaart et al., 1997; Petiot et al., 2000), whereas vinblastine, which destabilizes microtubules and thereby inhibits the fusion of autophagosomes with lysosomes, leads to an accumulation of autophagosomes and possibly also pre-autophagosomal structures (Hoyvik et al., 1986; Stephens et al., 1994). Treatment with vinblastine for 2 hours with or

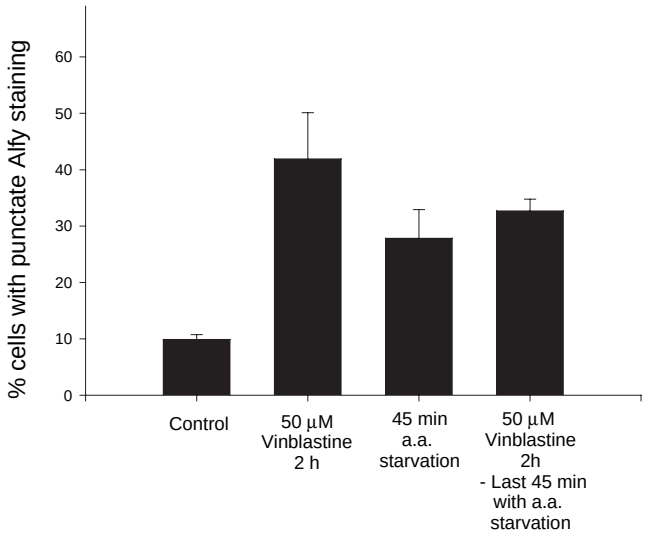

Fig. 7. The effect of vinblastine on cytoplasmic Alfy-positive vesicles. HeLa cells on coverslips were either left untreated or starved for 2 hours. When indicated, the cells were pretreated with 50 μM vinblastine. Fixed cells were stained with anti-Alfy antibodies and studied by confocal microscopy. Error bars indicate s.e.m. from five experiments.

without serum- and amino-acid-free medium for the last 45 minutes led to an increase in the proportion of cells with Alfy-positive structure (Fig. 7). This is consistent with a localization of Alfy to the autophagic pathway, which was also indicated by the fact that amino acid starvation for 45 minutes induces Alfy localization to punctate cytosolic structures (Fig. 4E). Surprisingly, addition of the PI-3-kinase inhibitors wortmannin or 3-methyladenine to HeLa cells for 45 minutes in medium without serum and amino acids gave little or no effect on the proportion of cells with punctate Alfy staining compared with control cells (results not shown). This suggests that Alfy could be recruited to cytoplasmic structures without the need for PtdIns(3)*P* production by an active PI-3-kinase, implying that the Alfy-positive structures might not represent autophagosomes.

To investigate whether Alfy colocalizes with autophagic membranes, cDNAs encoding human Atg5 and LC3 were transfected transiently into HeLa cells as green fluorescent protein fusions and used as autophagic markers. Human Atg5 is required for the initial sequestration of cytosolic material (Mizushima et al., 2003; George et al., 2000), whereas LC3 (a mammalian Atg8 homologue) localizes to the pre-autophagosomal structure, the isolation membrane and autophagosomes (Suzuki et al., 2001; Kabeya et al., 2000). Endogenous Alfy protein was found to colocalize with both Atg5 (Fig. 6G-I) and LC3 (Fig. 6J-L), although the colocalization with LC3 was more pronounced. Taken together, our results indicate that Alfy-positive structures are localized close to autophagic membranes.

Alfy binds PtdIns(3)*P* in vitro and partially colocalizes with PtdIns(3)*P* in vivo

Alfy contains a FYVE domain and so we asked whether Alfy also binds to and colocalizes with PtdIns(3)*P*. To investigate whether Alfy could bind PtdIns(3)*P*, cytosol from HeLa cells was incubated with liposomes containing an equal mixture of phosphatidylserine and phosphatidylinositol, in the presence or absence of 5% PtdIns(3)*P*, included at the expense of phosphatidylinositol. Cytosolic Alfy protein was found to bind to PtdIns(3)*P*-containing liposomes, and little or no binding was detected to liposomes lacking PtdIns(3)*P*, as determined by western blotting with an anti-Alfy antibody (Fig. 8A). This indicates that cytosolic Alfy can bind to PtdIns(3)*P*-containing membranes.

To investigate whether the FYVE domain of Alfy can bind directly to PtdIns(3)*P* and whether this binding is specific, we purified the recombinant FYVE domain of Alfy as a GST fusion protein and measured its binding to various lipids immobilized on nitrocellulose. As a negative control we used GST alone and, as a positive control, we used a tandem FYVE domain from Hrs (2×FYVE), which has a well-documented ability to bind specifically to PtdIns(3)*P* (Gillooly et al., 2000). As shown in Fig. 8B, GST-2×FYVE bound specifically to PtdIns(3)*P*, as expected, whereas no significant lipid binding was detected with GST alone. The GST-tagged FYVE domain of Alfy bound strongly to PtdIns(3)*P*, whereas a weaker binding to PtdIns(3,5)P_2 could also be detected. Upon higher exposure of the blot, we could also detect some binding to PtdIns(4)*P* and PtdIns(5)*P* (not shown). However, because binding of GST-2×FYVE to PtdIns(4)*P*, PtdIns(5)*P* and PtdIns(3,5)P_2 could also be detected at higher exposure (not shown), we consider this binding to be non-specific. We conclude that the FYVE domain of Alfy, similar to other FYVE domains tested, binds preferentially to PtdIns(3)*P* in vitro.

Next, we investigated whether the Alfy-positive structure would also colocalize with PtdIns(3)*P*. HeLa cells were starved for 45 minutes and then co-stained with an Alexa-488-labelled 2×FYVE probe and anti-Alfy antibody. Confocal immunofluorescence showed that some of the Alfy-positive structures colocalized partially with PtdIns(3)*P*-containing areas in the cells (Fig. 8C). Thus, although PtdIns(3)*P* formation does not seem to be necessary for the starvation-induced formation of these structures (see above), they might transiently contain PtdIns(3)*P* or be localized very close to PtdIns(3)*P*-containing structures. The reason why Alfy is not recruited to endosomes in vivo through PtdIns(3)*P* binding is not clear. However, most FYVE proteins need either dimerization or additional binding to endosomal proteins in order to be targeted to endosomes (Stenmark et al., 2002).

Alfy localizes to characteristic filamentous structures in the cytoplasm

Our immunofluorescence colocalization experiments suggested that cytoplasmic Alfy localizes close to autophagic membranes. In order to determine the ultrastructure of the Alfy-positive structures and how they relate to the autophagic pathway, we sought to visualize them by EM. Because our anti-Alfy antibody gave an unsatisfactory labelling of cryosections (not shown), we attempted to visualize Alfy by correlative IF/EM of starved HeLa cells grown on gridded coverslips. The cells were first labelled with anti-Alfy antibody and observed by immunofluorescence microscopy (Fig. 9A). Cells with Alfy-positive structures were then localized on the coverslip (Fig. 9A, arrowheads indicate Alfy-positive structures) and the

same cells observed further by EM (Fig. 9B). Our results indicate that most starvation-induced Alfy-positive structures consist of a filamentous protein meshwork without any detectable limiting membrane (Fig. 9C-F). We noted that rough endoplasmic reticulum (ER) often seemed to be in close contact with the Alfy-positive structures (Fig. 9C-D, arrowheads). On a few occasions, we found autophagic structures resembling isolation membranes or autophagosomes attached to the Alfy-positive structures (Fig. 9E, double arrow).

Proteasome inhibitor increases the number of Alfy-positive structures and reveals the presence of similar structures within autophagosomes

Degradation of cytosolic proteins occurs both by proteasomes and by autophagy (Ravikumar et al., 2002; Qin et al., 2003). Inhibition of proteasomal function might upregulate autophagic protein degradation in order to maintain cellular amino acid homeostasis. Therefore, if Alfy plays a role in autophagic protein degradation, its activity might be upregulated when proteasomal proteolysis is inhibited. To investigate this, we treated cells with the proteasome inhibitor PSI (Traenckner et al., 1994; Wojcik et al., 1996) and stained them for confocal immunofluorescence microscopy with anti-Alfy antibodies. Strikingly, whereas cytoplasmic Alfy-positive structures were only observable in a small proportion of the untreated cells (Fig. 10A, see also Fig. 4E), more than 95% of the PSI-treated cells showed many Alfy-positive structures in their cytoplasm (Fig. 10C). This is consistent with the idea that Alfy could be involved in turnover of cytosolic proteins.

Because intracellular protein aggregates often contain ubiquitin (Johnston et al., 1998; Kopito, 2000), we co-stained PSI-treated cells using an antibody (FK2) that recognizes conjugated mono- and polyubiquitin. As shown in Fig. 10A-D, Alfy-positive structures in untreated and PSI-treated cells colocalized with ubiquitin. Upon higher magnification of the confocal images, it appeared that this colocalization was not absolute – it rather appeared as though an Alfy meshwork was interwoven in the ubiquitin-containing protein aggregates (Fig. 10E). This indicates that Alfy-positive structures are not identical to ubiquitin-containing protein aggregates even though they tend to colocalize with such structures.

To investigate the morphology of Alfy structures and surrounding membranes in ultrastructurally better preserved tissue than the samples obtained from the correlative IF/EM experiments, we prepared PSI-treated cells for conventional plastic embedding. Interestingly, in PSI-treated cells, we often

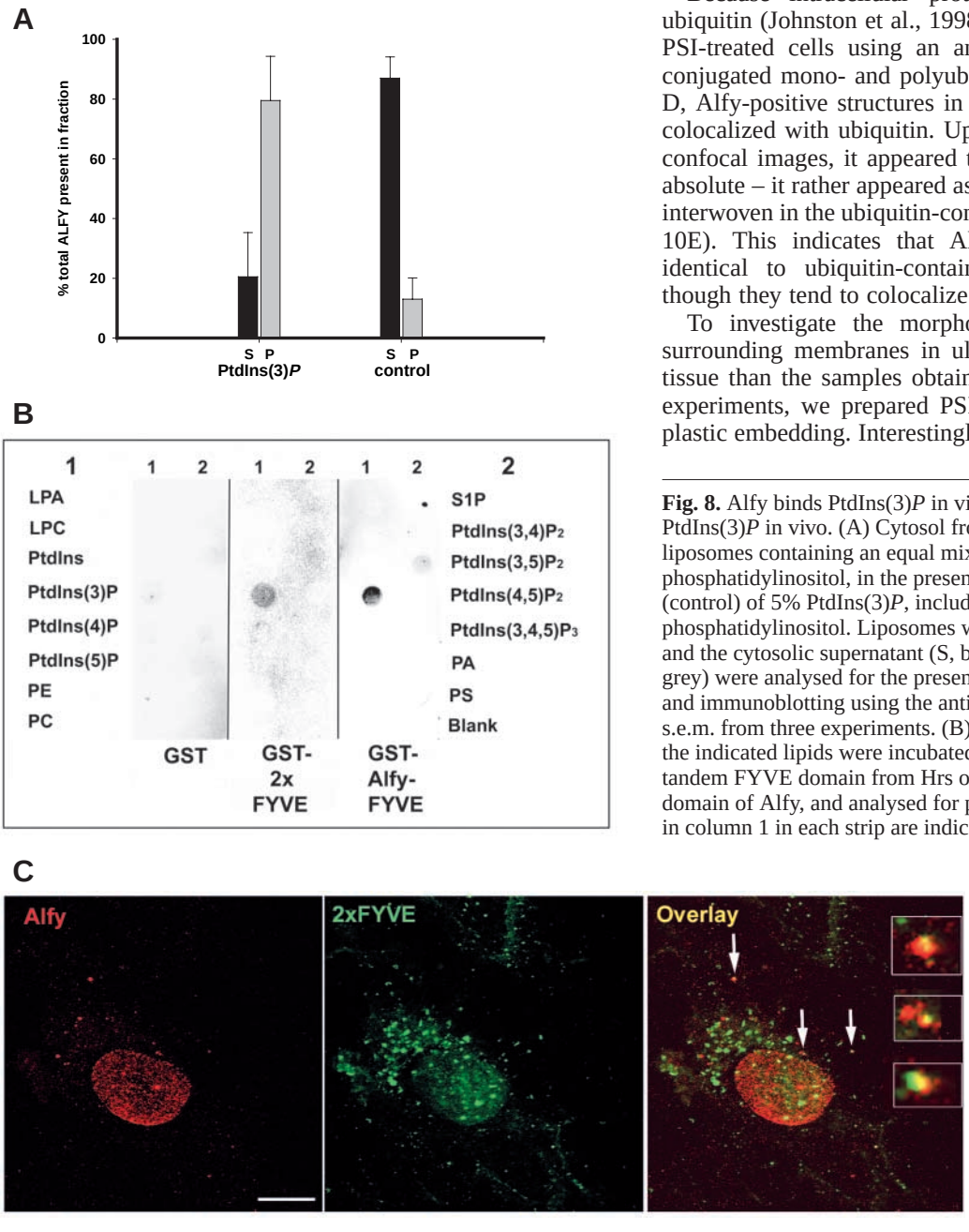

Fig. 8. Alfy binds PtdIns(3)*P* in vitro and colocalizes partially with PtdIns(3)*P* in vivo. (A) Cytosol from HeLa cells was incubated with liposomes containing an equal mixture of phosphatidylserine and phosphatidylinositol, in the presence [PtdIns(3)*P*] or absence (control) of 5% PtdIns(3)*P*, included at the expense of phosphatidylinositol. Liposomes were collected by centrifugation and the cytosolic supernatant (S, black) and the liposome pellet (P, grey) were analysed for the presence of Alfy by SDS-PAGE (7.5%) and immunoblotting using the anti-Alfy antibody. Error bars denote s.e.m. from three experiments. (B) Nitrocellulose strips containing the indicated lipids were incubated with GST, GST fused with a tandem FYVE domain from Hrs or GST fused with the FYVE domain of Alfy, and analysed for protein binding. The lipids spotted in column 1 in each strip are indicated to the left, whereas lipids spotted in column 2 are indicated to the right. (C) HeLa cells grown on coverslips were starved for 45 minutes in HBSS, permeabilized, fixed and stained against endogenous Alfy (left), and the presence of PtdIns(3)*P* detected with GST-2×FYVE-Alexa 488 (middle). The merged image (right) shows that some of the Alfy-positive structures (arrows and inset) colocalized partially with PtdIns(3)*P*-containing areas in the cells. Scale bar, 10 μm.

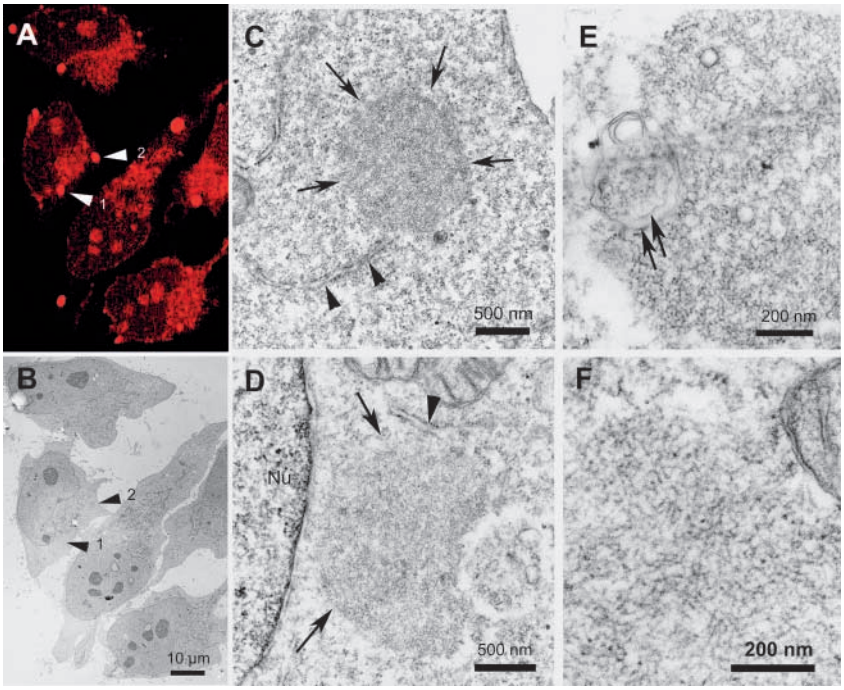

Fig. 9. Alfy localizes to a novel filamentous structure. (A) HeLa cells grown on gridded coverslips were starved in HBSS for 45 minutes, fixed and labelled with anti-Alfy to detect cells with Alfy-positive structures (A,B, arrowheads). The same cells were thereafter localized on EM using the grid on the coverslip (B). The Alfy-positive structures detected by immunofluorescence (A) could also be detected at the EM level (B), making it possible to study these structures at a higher resolution (D corresponds to structure 2 and C corresponds to structure 1). This showed that Alfy localizes to a novel filamentous structure (C,D, arrows) that, in some cases, localized close to rough ER membranes (C,D, arrowheads). Occasionally, we observed double-membrane structures embedded within the Alfy-positive structures (E, arrows). The filamentous nature of Alfy structures becomes more evident at a higher magnification (F). Abbreviation: Nu, nucleus.

observed Alfy-like structures enclosed by double membranes (Fig. 11A,B) and in autophagosome- (Fig. 11C) or autolysosome-like structures (Fig. 11D). This suggests that Alfy-positive structures are targeted to autophagosomes and is consistent with the idea that Alfy might function as a scaffold protein for autophagic degradation of protein aggregates.

Discussion

In this work, we have identified Alfy, a novel PtdIns(3)*P*-binding protein that colocalizes with protein granules and autophagic membranes. Because Alfy is ubiquitously expressed, it is likely to be involved in a process that takes place in most tissues. The following findings implicate Alfy as a possible link between protein granules and the autophagic pathway. First, proteinaceous Alfy-positive structures accumulate in the cytoplasm upon serum and amino acid starvation, a condition that induces autophagy. Second, these structures partially colocalize with autophagic markers by confocal immunofluorescence microscopy. Third, inhibition of proteasomal protein degradation causes a strong increase in the number of cytoplasmic Alfy-positive structures. Fourth, similar structures can be detected inside autophagosomes by EM. We propose that Alfy might recognize protein aggregates and act as a scaffold for components of the autophagic machinery.

Correlative IF/EM showed that cytoplasmic Alfy mostly localized to a filamentous protein meshwork without limiting membranes, which was occasionally associated with typical

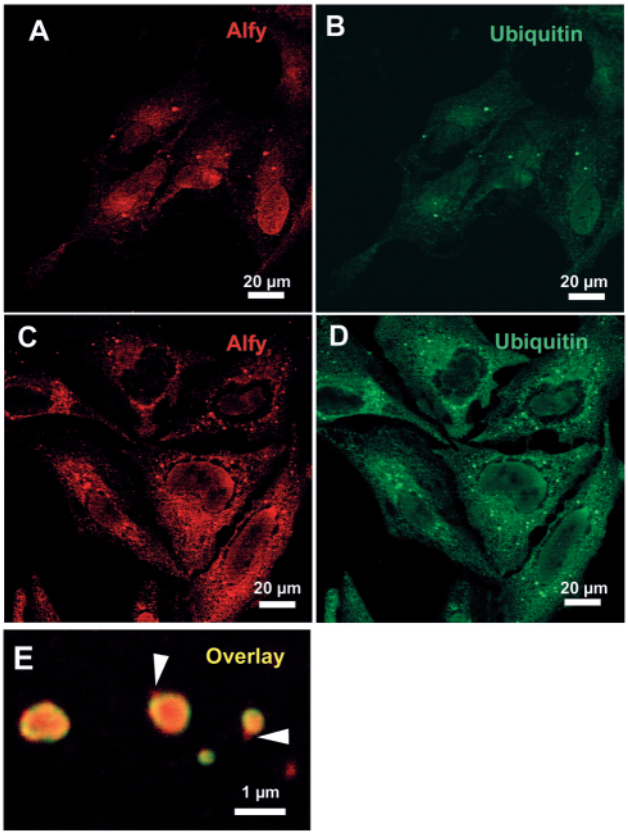

Fig. 10. Cytoplasmic Alfy-positive structures partially colocalize with ubiquitin-containing profiles and are induced by a proteasome inhibitor. HeLa cells were treated with 50 μM PSI or left alone for 5 hours and then further chased for 18 hours without PSI in the medium. Cells were then double labelled against Alfy (red) and ubiquitin (antibody against mono- and polyubiquitin, FK2; green). In both control cells (A,B) and treated cells (C,D), we observed an apparent colocalization between Alfy and ubiquitin. The number of labelled structures had dramatically increased in the PSI-treated cells (C,D). A closer observation of Alfy-positive structures at high magnification revealed that the markers did not completely colocalize. It was especially evident that some Alfy labelling seemed to extend beyond the ubiquitin-labelled area (E, arrowheads).

autophagic membranous structures such as isolation membranes and autophagosomes. Recently, a novel pre-autophagosomal structure containing both Atg5 and LC3 has been identified in yeast (Kirisako et al., 2000; Suzuki et al., 2001). EM studies of this structure, like our studies of the Alfy-positive structure, have failed to show a definite membrane corresponding to the structures observed using immunofluorescence microscopy (Suzuki et al., 2001). Because we found that the Alfy-positive structure also contains both human Atg5 and LC3, it is possible that the starvation-induced cytoplasmic Alfy-positive structure are the mammalian equivalent of the pre-autophagosomal structure identified in yeast. However, because the formation of pre-autophagosomal structures (Suzuki et al., 2001), but not of Alfy-positive structures, requires PI-3-kinase activity, we find it more likely that Alfy-positive structures are distinct from pre-autophagosomal structures. In any case, Alfy possibly has an early involvement in autophagy, because the proportion of Alfy-positive structure-positive cells is maximal after only 45 minutes of starvation and is decreased to the unstarved level after 2 hours. By contrast, the amount of autophagosomes has previously been shown to be maximal after 3 hours (Mitchener et al., 1976). The localization of Alfy to the early autophagic pathway opens the possibility that Alfy could play a role in the formation of the membranes that become the isolation membrane. The FYVE domain might be involved in recruiting PtdIns(3)*P*-containing membranes formed elsewhere to the Alfy-positive structure, where they would acquire autophagic marker proteins like Atg5 and LC3, and thereafter form elongation membranes.

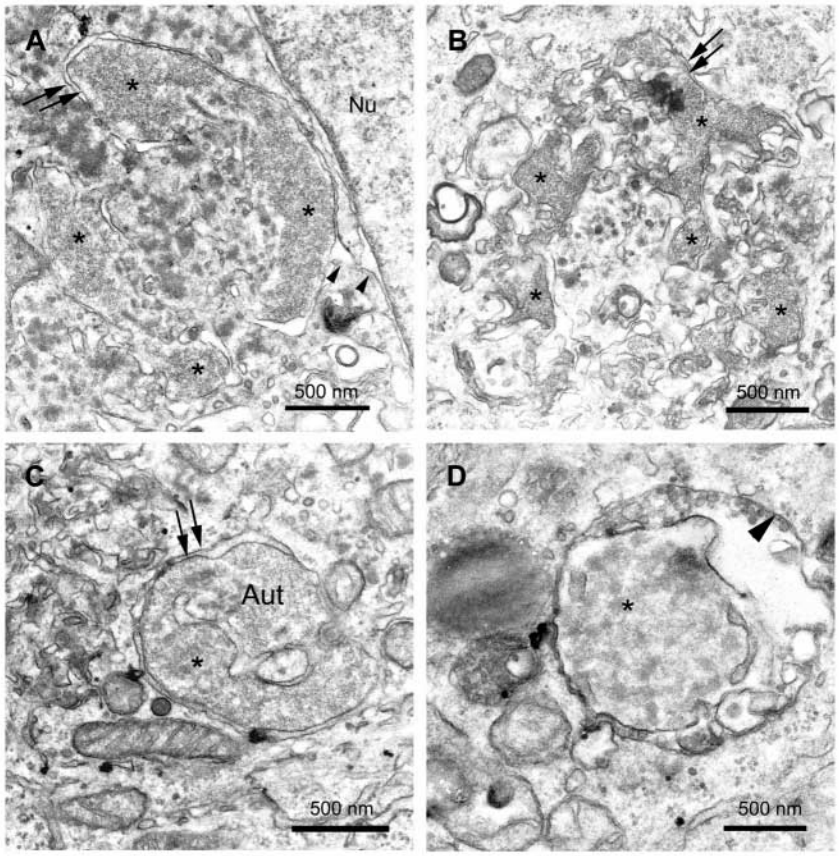

Fig. 11. Alfy-like structures are found inside autophagic structures. In order to understand the morphology of Alfy structures more clearly, we treated cells with 50 μM PSI (5-hour pulse followed by 18-hour chase) and prepared them by conventional plastic embedding for EM. We observed filamentous meshworks resembling Alfy-positive structures (asterisk in all micrographs) close to (A, double arrow) or seemingly engulfed by double-membrane structures (B, double arrow). These membranes probably represent ER membranes, as indicated by their continuity with the nuclear envelope (A, arrowheads). The double membranes could form structures resembling autophagosomes (C, double arrow). A more autolysosome-like vesicle with only one limiting membrane (arrowhead) is seen in (D). Abbreviations: Aut, autophagosome; Nu, nucleus.

Degradation of cytoplasmic proteins can occur by proteasomal proteolysis or by autophagy (Webb et al., 2003). Autophagy appears to be important in order to prevent the formation of protein deposits in the central nervous system, possibly because large protein aggregates are inefficiently degraded by proteasomes (Ravikumar et al., 2002). This raises the interesting possibility that autophagy in neurons might counteract neurodegenerative diseases. Treatment of cells with the proteasome inhibitor PSI caused a strong increase in the number of cells with cytoplasmic Alfy-positive structures. Although this could mean that Alfy-positive structures are normally degraded by proteasomes, we favour an alternative explanation. A block in proteasomal degradation causes a strong increase in cytoplasmic protein aggregates that localize close to Alfy-positive structures. The presence of similar structures within autophagosomes leads us to speculate that the PSI-induced Alfy labelling could represent an increased cytoplasmic recruitment of Alfy, and a role in autophagy, rather than a decreased degradation.

If Alfy is indeed associated with autophagy, what might its role be? Despite several attempts, we have only obtained a limited knockdown of the expression of Alfy by RNA interference (Fig. 2F), so it might take some time before a detailed functional analysis becomes feasible. However, a recent paper describes the putative *Drosophila* homologue of Alfy, Blue Cheese (BCHS), and highlights its physiological importance (Finley et al., 2003). Interestingly, *bchs* mutant flies are viable but have a reduced lifespan owing to age-dependent formation of ubiquitin-positive protein aggregates in their central nervous system. The fact that *bchs* mutant flies accumulate protein aggregates is consistent with the idea that Alfy is a regulator of a degradative pathway. Because BCHS/Alfy is not essential for *Drosophila* development and no overt Alfy homologue can be identified in *S. cerevisiae*, Alfy is probably not part of the basal machinery for autophagy. We speculate that Alfy might be a regulator of selective autophagy for certain cytoplasmic protein aggregates that are inefficiently degraded by proteasomes.

Alfy could bind to these structures and, by recruiting components of the autophagic machinery, mark protein aggregates for autophagic degradation. Further work is required in order to determine whether ubiquitination of protein aggregates plays a role in their recruitment of Alfy or whether Alfy recognizes other determinants. It will also be interesting to study whether the protein aggregates exist permanently or are induced by starvation. Because the phenotype of *bchs* mutant *Drosophila* is reminiscent to human neurodegenerative diseases (Finley et al., 2003), future studies of Alfy might shed light on the molecular mechanisms of such diseases.

We thank E. Rønning and Y. Kabeya for excellent technical assistance, T. Nagase (Kazusa DNA Research Institute, Chiba, Japan) for providing the KIAA0993 cDNA, and K. Finley, P. Seglen and P. E. Strømhaug for helpful discussions. This work was supported by the Top Research Programme, the Research Council of Norway, the Norwegian Cancer Society, the Novo-Nordisk Foundation and the Anders Jahre's Foundation for the Promotion of Science.

References

Blommaart, E. F. C., Krause, U., Schellens, J. P. M., Vreeling-Sindelárová, H. and Meijer, A. J. (1997). The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *Eur. J. Biochem.* **243**, 240-246.

Chen, J. W., Murphy, T. L., Willingham, M. C., Pastan, I. and August, J. T. (1985). Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* **101**, 85-95.

Ellson, C. D., Andrews, S., Stephens, L. R. and Hawkins, P. T. (2002). The PX domain: a new phosphoinositide-binding module. *J. Cell Sci.* **115**, 1099-1105.

Evan, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-Myc proto-oncogene product. *Mol. Cell. Biol.* **5**, 3610-3616.

Finley, K. D., Edeen, P. T., Cumming, R. C., Mardahl-Dumesnil, M. D., Taylor, B. J., Rodriguez, M. H., Hwang, C. E., Benedetti, M. and McKeown, M. (2003). *Blue cheese* mutations define a novel, conserved gene involved in progressive neural degeneration. *J. Neurosci.* **23**, 1254-1264.

George, M. D., Baba, M., Scott, S. V., Mizushima, N., Garrison, B. S., Ohsumi, Y. and Klionsky, D. J. (2000). Apg5p functions in the sequestration step in the cytoplasm-to-vacuole targeting and macroautophagy pathways. *Mol. Biol. Cell* **11**, 969-982.

Gillooly, D. J., Morrow, I. C., Lindsay, M., Gould, R., Bryant, N. J., Gaullier, J. M., Parton, R. G. and Stenmark, H. (2000). Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J.* **19**, 4577-4588.

Hoyvik, H., Gordon, P. B. and Seglen, P. O. (1986). Use of a hydrolysable probe, [^{14}C]lactose, to distinguish between pre-lysosomal and lysosomal steps in the autophagic pathway. *Exp. Cell Res.* **166**, 1-14.

Ikonomov, O. C., Sbrissa, D., Foti, M., Carpentier, J. L. and Shisheva, A. (2003). PIKfyve controls fluid phase endocytosis but not recycling/degradation of endocytosed receptors or sorting of procathepsin D by regulating multivesicular body morphogenesis. *Mol. Biol. Cell* **14**, 4581-4591.

Jogl, G., Shen, Y., Gebauer, D., Li, J., Wiegmann, K., Kashkar, H., Kronke, M. and Tong, L. (2002). Crystal structure of the BEACH domain reveals an unusual fold and extensive association with a novel PH domain. *EMBO J.* **21**, 4785-4795.

Johnston, J.A., Ward, C.L. and Kopito, R.R. (1998). Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* **143**, 1883-1898

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y. and Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* **19**, 5720-5728.

Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T. and Ohsumi, Y. (2000). The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J. Cell Biol.* **151**, 263-276.

Klionsky, D. J., Cregg, J. M., Dunn, W. A., Jr, Emr, S. D., Sakai, Y., Sandoval, I. V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M. et al. (2003). A unified nomenclature for yeast autophagy-related genes. *Dev. Cell* **5**, 539-545.

Kopito, R.R. (2000). Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol.* **10**, 524-530

Kwak, E., Gerald, N., Larochelle, D. A., Vithalani, K. K., Niswonger, M. L., Maready, M. and de Lozanne, A. (1999). LvsA, a protein related to the mouse beige protein, is required for cytokinesis in *Dictyostelium*. *Mol. Biol. Cell* **10**, 4429-4439.

Mitchener, J. S., Shelburne, J. D., Bradford, W. D. and Hawkins, H. K. (1976). Cellular autophagocytosis induced by deprivation of serum and amino acids in HeLa cells. *Am. J. Pathol.* **83**, 485-491.

Mizushima, N., Yoshimori, T. and Ohsumi, Y. (2003). Role of the Apg12 conjugation system in mammalian autophagy. *Int. J. Biochem. Cell Biol.* **35**, 553-561.

Mu, F. T., Callaghan, J. M., Steele-Mortimer, O., Stenmark, H., Parton, R. G., Campbell, P. L., McCluskey, J., Yeo, J. P., Tock, E. P. and Toh, B. H. (1995). EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine 'fingers' and contains a calmodulin-binding IQ motif. *J. Biol. Chem.* **270**, 13503-13511.

Nagle, D. L., Karim, M. A., Woolf, E. A., Holmgren, L., Bork, P., Misumi, D. J., McGrail, S. H., Dussault, B. J., Jr, Perou, C. M., Boissy, R. E. et al. (1996). Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. *Nat. Genet.* **14**, 307-311.

Neuwald, A. F. (1997). A shared domain between a spindle assembly checkpoint protein and Ypt/Rab-specific GTPase-activators. *Trends Biochem. Sci.* **22**, 243-244.

Nice, D. C., Sato, T. K., Stromhaug, P. E., Emr, S. D. and Klionsky, D. J. (2002). Cooperative binding of the cytoplasm to vacuole targeting pathway proteins, Cvt13 and Cvt20, to phosphatidylinositol 3-phosphate at the pre-autophagosomal structure is required for selective autophagy. *J. Biol. Chem.* **277**, 30198-30207.

Nielsen, E., Christoforidis, S., Uttenweiler-Joseph, S., Miaczynska, M., Dewitte, F., Wilm, M., Hoflack, B. and Zerial, M. (2000). Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. *J. Cell Biol.* **151**, 601-612.

Noda, T., Kim, J., Huang, W. P., Baba, M., Tokunaga, C., Ohsumi, Y. and Klionsky, D. J. (2000). Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. *J. Cell Biol.* **148**, 465-480.

Patki, V., Virbasius, J., Lane, W. S., Toh, B. H., Shpetner, H. S. and Corvera, S. (1997). Identification of an early endosomal protein regulated by phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* **94**, 7326-7330.

Petiot, A., Ogier-Denis, E., Blommaart, E. F. C., Meijer, A. J. and Codogno, P. (2000). Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J. Biol. Chem.* **275**, 992-998.

Qin, Z. H., Wang, Y., Kegel, K. B., Kazantsev, A., Apostol, B. L., Thompson, L. M., Yoder, J., Aronin, N. and DiFiglia, M. (2003). Autophagy regulates the processing of amino terminal huntingtin fragments. *Hum. Mol. Genet.* **15**, 3231-3244.

Raiborg, C., Bremnes, B., Mehlum, A., Gillooly, D. J., Stang, E. and Stenmark, H. (2001). FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes. *J. Cell Sci.* **114**, 2255-2263.

Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E. and Stenmark, H. (2002). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. *Nat. Cell Biol.* **4**, 394-398.

Ravikumar, B., Duden, R. and Rubinsztein, D. C. (2002). Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet.* **11**, 1107-1117.

Sankaran, V. G., Klein, D. E., Sachdeva, M. M. and Lemmon, M. A. (2001). High-affinity binding of a FYVE domain to phosphatidylinositol 3-phosphate requires intact phospholipid but not FYVE domain oligomerization. *Biochemistry* **40**, 8581-8587.

Simonsen, A., Lippé, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M. and Stenmark, H. (1998). EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* **394**, 494-498.

Simonsen, A., Wurmser, A. E., Emr, S. D. and Stenmark, H. (2001). The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* **13**, 485-492.

Smith, T. F., Gaitatzes, C., Saxena, K. and Neer, E. J. (1999). The WD repeat: a common architecture for diverse functions. *Trends Biochem. Sci.* **24**, 181-185.

Stenmark, H. and Aasland, R. (1999). FYVE-finger proteins – effectors of an inositol lipid. *J. Cell Sci.* **112**, 4175-4183.

Stenmark, H., Aasland, R. and Driscoll, P. C. (2002). The phosphatidylinositol 3-phosphate-binding FYVE finger. *FEBS Lett.* **513**, 77-84.

Stephens, L., Cooke, F. T., Walters, R., Jackson, T., Volinia, S., Gout, I., Waterfield, M. D. and Hawkins, P. T. (1994). Characterization of a phosphatidylinositol-specific phosphoinositide 3-kinase from mammalian cells. *Curr. Biol.* **4**, 203-214.

Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T. and Ohsumi, Y. (2001). The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J.* **20**, 5971-5981.

Toker, A. (2002). Phosphoinositides and signal transduction. *Cell Mol. Life Sci.* **59**, 761-779.

Traenckner, E. B., Wilk, S. and Baeuerle, P. A. (1994). A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B. *EMBO J.* **13**, 5433-5441.

Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J. and Waterfield, M. D. (2001). Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70**, 535-602.

Ward, D. M., Griffiths, G. M., Stinchcombe, J. C. and Kaplan, J. (2000). Analysis of the lysosomal storage disease Chediak-Higashi syndrome. *Traffic* **1**, 816-822.

Webb, J. L., Ravikumar, B., Atkins, J., Skepper, J. N. and Rubinsztein, D. C. (2003). Alpha-Synuclein is degraded by both autophagy and the proteasome. *J. Biol. Chem.* **278**, 25009-25013.

Wojcik, C., Schroeter, D., Wilk, S., Lamprecht, J. and Paweletz, N. (1996). Ubiquitin-mediated proteolysis centers in HeLa cells: indication from studies of an inhibitor of the chymotrypsin-like activity of the proteasome. *Eur. J. Cell Biol.* **71**, 311-318.

Wurmser, A. E. and Emr, S. D. (2002). Novel PtdIns(3)*P*-binding protein Etf1 functions as an effector of the Vps34 PtdIns 3-kinase in autophagy. *J. Cell Biol.* **158**, 761-772.

Yin, H. L. and Janmey, P. A. (2003). Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol* **65**, 761-789.