Supplementary Materials and Methods

DNA constructs

Fluorescent protein tagging of the open reading frames is described elsewhere (Simpson et al., 2000). Mutagenesis of the three putative GAE binding motifs was performed using the QuikChange Site-directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. All clones were verified by DNA sequencing. GST expression clones of the various γ1-adaptin, GGA1 (Hirst et al., 2000) and EpsinR (Hirst et al., 2003) proteins were provided by M. Robinson.

Cell culture and transfections

Vero (ATCC CCL-81) and HeLa cells (ATCC CCL-2) were routinely cultured in MEM and DMEM respectively supplemented with 10% FCS and transfected using FuGENE6 (Roche) as described previously (Simpson et al., 2000). Quantification of the amount of 2c18 expressed in transfected to nontransfected cells demonstrated that on average transfected cells expressed 12±4 times more 2c18 compared to nontransfected cells. Mouse fibroblasts were maintained as described previously (Meyer et al., 2000).

Antibodies and immunostaining

Fixation of cells and immunofluorescence staining were performed as described earlier (Pepperkok et al., 1993). The following primary antibodies were used: Mouse anti-γ-adaptin (clone 88, BD Biosciences) and (clone 100/3, Sigma), rabbit anti-MPR300 (Kasper et al., 1996), rabbit anti-β' COP (BSTR, Pepperkok et al., 1998), rabbit anti-GGA1 (a gift of M. Robinson), rabbit anti-GGA3 (a gift of J. Bonifacino), goat anti-GST (Amersham Biosciences), rabbit anti-cathepsin D (Kasper et al., 1996), mouse anti-α-tubulin (clone DM1A, Neomarkers), mouse anti-EEA1 (clone 14), mouse anti-GM130 (clone 35), mouse anti-p230 (clone 15) (all BD Biosciences),

sheep anti-TGN46 (Serotec) and anti-VSV-G (P5D4, Kreis, 1986). Alexa 488-, Alexa 568- and Alexa 647-conjugated secondary antibodies were from Molecular Probes. For quantification of co-localizing structures corresponding images were loaded into two distinct channels of an RGB image using Adobe Photoshop and the number of co-localizing structures determined by alternating between the channels. At least ten different cells were analyzed in each case. For the quantification of 2c18 expression in single cells respective images were loaded into ImageJ. After background subtraction the boundaries of labeled cells were drawn manually and the average intensity per cell determined.

Cell fractionation

Extracts from HeLa cells (either untreated, transfected with the various GFP plasmids, mock- or 2c18-siRNA-transfected) were prepared, and membrane and cytosol fractions isolated as described in Poussu et al. (2000). Membrane pellets were resuspended in RIPA buffer. Three times more of the cytosol extracts versus the membrane extracts were subjected to SDS PAGE and Western blotting. Band intensities were determined using Scion Image, and calculation of the abundance of γ1-adaptin in the fractions was made after normalizing the loading ratios to 1:1.

Yeast two-hybrid analysis

The complete open reading frame of 2c18 and three deletion mutants (corresponding to amino acid residues 1-151, 78-302 and 152-302) were amplified by PCR and cloned into pDONR201 using the Gateway (Invitrogen) cloning system. Sequence verified inserts were shuttled into pDEST32 containing the Gal4 DNA binding domain and pDEST22 containing the Gal4 activation domain (all Invitrogen). The pDEST32 constructs were co-transformed with empty pPC86 vector into AH109 yeast cells (Clontech) and plated on selection medium lacking His, Leu, Trp to test for

self activation. The 2c18 fragments containing amino acids 152-302 were autoactivating, and were therefore excluded from further screening. The N-terminal 2c18 construct (residues 1-151) was chosen for further analysis. For the library screen AH109 yeast cells were sequentially co-transformed with the pDEST32-2c18 (aa1-151) and a human fetal brain library in pPC86 fused to a Gal4 activation-domain (Invitrogen), then plated on synthetic medium lacking His, Leu, Trp, according to the manufacturer's instructions. Approximately 1.5x106 transformants were screened, resulting in the isolation of 74 His auxotroph and β-galactosidase-positive clones. These were sequenced and retransformed with pDEST32-2c18 (1-151) or the empty pDEST32 vector. To obtain the complete coding sequence of γ1-adaptin (AP1G1), human fetal brain cDNA (Wiemann et al., 2001) was used as a template for nested PCR. The amplified product was rendered Gateway-compatible, recombined into pDONR201, sequence verified, then shuttled into pDEST32 and pDEST22. To prove the interaction of the 2c18 fragment with full length AP1G1, pDEST32-2c18 (1-151) and pDEST22-AP1G1 as well as pDEST32-AP1G1 and pDEST22-2c18 (1-151) were co-transformed in AH109 yeast cells and grown on synthetic medium lacking His, Leu, Trp. Strengths of protein-protein interaction were assayed using the Matchmaker kit according to the manufacturer's instructions (Clontech).

Electron microscopy

SA:48 cells (HeLa cells stably transfected with sialyltransferase tagged with the VSVG P5D4 epitope) were maintained as described previously (Rabouille et al., 1995) and fixed with 3.5% PFA, 0.1% glutaraldehyde for 1 h, followed by fixation in 3.5% PFA overnight. The 5 nm gold-BSA was internalized for 10 min at 37°C, the cells washed, then chased for 20 min at 37°C, before fixing at 37°C as described above. Cell pellets were embedded in agarose, infiltrated with 2.3 M sucrose

overnight and then frozen in liquid nitrogen. Rat pancreatic tissue was prepared by perfusion with 2% formaldehyde and 0.2% glutaraldehyde in PBS. The fixed tissue was stored in 1% formaldehyde in PBS at 4°C. For cryosectioning small blocks of tissue were cryoprotected by infiltration with 2.3 M sucrose in PBS and frozen in liquid nitrogen. After cryosectioning the frozen sections were picked up with a 1:1 mixture of 2% methycellulose and 2.3 M sucrose according to Liou et al. (1996). Thawed sections were labeled with either rabbit anti-2c18 and mouse anti-P5D4 antibodies or rabbit anti-2c18 and mouse anti-γ1-adaptin antibodies. Rabbit antibodies were detected with 10 or 15 nm gold-protein A, and mouse antibodies with anti-mouse IgGs (Jackson Immunoresearch Labs) coupled to 12 nm gold. Images were acquired on a Phillips CM120 BioTwin EM. For quantification of the relative distribution of 2c18 in HeLa SA:48 cells, more than 2000 randomly selected gold particles were counted from ten grids at a magnification of 13,500 and each of these assigned to a compartment. Standard deviation was determined between the ten grids using Microsoft Excel. To determine the labeling frequency of each organelle of interest by 2c18, and thereby take into account organelles of low abundance (Rabouille, 1999), the total number of organelles from ten grids were counted (26 Golgi complexes, 631 endosomes, 253 plasma membrane occurrences), and the presence or absence of 2c18 labeling was noted. Standard deviation was determined between the ten grids using Microsoft Excel. To determine the degree of co-localization of 2c18 with γ1-adaptin only labeled structures (single or double) were counted. A total of 46 TGN areas were identified from three different grids. Labeled structures were assigned as either being single or double labeled for these markers. Standard deviation was determined between the three grids using Microsoft Excel. Since antibody dilutions in the co-localization experiments were optimized for high

labeling specificity only a fraction of each of the antigens may be detected. Therefore, the degree of co-localization determined and summarized in Table2 is most likely an underestimation.

2c18 protein purification

The coding sequence of 2c18, its shorter splice variant and the coiled coil region (2c18 aa 78-138) were inserted into the vector pETM11 (EMBLHeidelberg) to generate His-tagged versions. Following transformation and growth of E. coli BL21 (DE3) to log phase, protein expression was induced with 100 μM IPTG. The cells were grown for an additional 20 h, pelleted, then sonicated in lysis buffer (20 mM Tris pH 8, 0.05% Triton X-100, 1 μg/ml lysozyme, 1 mM PMSF, 1 μg/ml DNAse). A HiTrap chelating Sepharose column (Amersham Biosciences) was prepared by charging with 100 mM CoCl2 followed by washing with 40 mM Tris pH 8, 20 mM imidazole, 250 mM NaCl. After removal of cell debris the supernatant was loaded on to the column. The protein was eluted with 40 mM Tris pH 8, 250 mM NaCl, 500 mM imidazole and elution fractions were collected.

GST protein purification and direct binding assay

GST fusion proteins with γ1-adaptin and GGA1 domains (Seaman et al., 1996) were produced in BL21 (DE3) cells. One litre of log phase cells was induced with 100 μM IPTG and then shaken at 22°C for 6 h. Cells were recovered and lysed in 20 ml of lysis buffer (20 mM Tris pH 7.5, 0.1% Triton X-100, 1 μg/ml lysozyme, 1 μg/ml DNAse), sonicated briefly, and centrifuged at 138,000 g (45 Ti rotor, Beckman) at 4°C for 20 min to remove insoluble material. The clarified lysate was then mixed by tumbling at 4°C for 10 h with glutathione-Sepharose 4B (Amersham Biosciences) preequilibrated with 20 mM Tris pH 7.5, 0.1% Triton X-100. Beads were washed four times with this buffer, followed by a single wash with 50 mM Tris pH 8.0. The

GSTfusion proteins were competitively eluted with 10 mM reduced glutathione in 50 mM Tris pH 8.0, then dialyzed overnight against PBS before use in the direct binding assay. The sizes of the GST-γ1-adaptin domains were confirmed by SDS-PAGE, and were found to be comparable to those reported previously (Doray and Kornfeld, 2001). The bacterial lysate containing histidine-tagged 2c18 was prepared as described above. Fast Flow Sepharose / CoCl2 beads (Amersham Biosciences), preequilibrated with 25 mM HEPES pH 7.5, 125 mM KOAc, 25 mM MgOAc, were incubated with the clarified lysate for 1 h at room temperature and then washed five times with this buffer. These beads were directly subjected into the binding assay. The binding of 2c18 with various GST fusion proteins was assayed in binding buffer (25 mM HEPES pH 7.2, 125 mM KOAc, 2.5 mM MgOAc, 1 mM beta-mercaptoethanol, 0.1% Triton X-100), using 50 μg of 2c18-His6 and a final concentration of 16.6 μg/ml of the purified GST fusion proteins in a total volume of 900 μl. The reactions were incubated for 1 h at 4°C with tumbling, and then the beads recovered by centrifugation at 500 g for 5 min. The supernatants were saved, and the pellets were washed four times with 1.5 ml of cold binding buffer. The pellets were resuspended in SDS sample buffer and 1/10th of each pellet and 1/60th of each supernatant were subjected to SDS-PAGE and Western blotting. Blots were probed with anti-GST antibodies, and detected using the ECL Advance detection system (Amersham Biosciences). For the competition experiment purified GST- γ1-adaptin ear was bound to immobilized 2c18. GST or GST-EpsinR was purified as described above, and a five-fold molar excess was added to the column and incubated at 4°C for 90 min. Columns were washed and processed as before, and the amount of γ1-adaptin remaining associated with 2c18 was determined.

Binding of synthetic peptides to the γ1-adaptin and GGA1 'ear' domains

Peptides CWTQCFGLLRKEAG ‘pep 1’ (2c18 aa 5-18), C-TIEFENLVESDE ‘pep 2’ (2c18 aa 42-53) and C-DDNGNSEYSGFVNPVL ‘pep 3’ (2c18 aa 270-285) and their corresponding controls with AxxA substitutions at the underlined positions were synthesized. 30 nmol of each peptide were bound to CN-Br activated Sepharose 4B beads (Amersham Biosciences). The peptide binding assay was performed as described in Mattera et al. (2003) using 1 μg GST γ1-adaptin ‘ear’ in a final volume of 0.5 ml. Finally the pellets were resuspended in SDS sample buffer and 1/20th of each pellet and 1/35th of each supernatant were subjected to SDS-PAGE and Western blotting. Blots were probed with anti-GST antibodies, and detected using the ECL Advance detection system (Amersham Biosciences).

Metabolic labeling and analysis of cathepsin D secretion

Metabolic labeling and analysis of cathepsin D secretion was performed essentially as described previously (Mauxion et al., 1996). Specifically, HeLa cells in 6 well plates were first transfected with plasmids encoding either GFP or 2c18-GFP, and allowed to express these for 24 h. For siRNA treatments, double stranded RNA oligonucleotides against 2c18 or μ1A-adaptin were used as described in Materials and Methods. Cells were starved in methionine- and cysteine-deficient MEM medium for 30 min, then pulsed with medium containing 1 mCi 35S-Promix (Amersham Biosciences) and 10 mM mannose-6-phosphate per well for 30 min. Proteins were chased in MEM supplemented with 1 mM methionine, 2.5 mM cysteine and 10 mM mannose-6-phosphate for 4 h. Immunoprecipitations of cathepsin D from both the medium and cell lysates were performed as described previously (Mauxion et al., 1996). For quantification of the secretion of cathepsin D, non-saturated autoradiograms were taken, scanned and quantified using Scion Image.

Supplementary References

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