Subcellular localization of the $G_{\alpha 13}$ protein and G alpha interacting protein, two proteins involved in the control of macroautophagy in human colon cancer HT-29 cells

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Autophagic sequestration is controlled by the $G_{\alpha 13}$ protein in human colon cancer HT-29 cells. Immunofluorescence and subcellular fractionation studies showed that the $G_{\alpha 13}$ protein is preferentially associated with Golgi membranes but co-localization was also observed with the endoplasmic reticulum (ER) membrane. The $G_{\alpha 12}$ protein, which is not involved in the control of autophagic sequestration, is associated with the plasma membrane. Transfection of chimaeric G_{α} proteins ($G_{\alpha 13/2}$, $G_{\alpha 12/3}$) containing the N- and C-terminal parts of the relevant G_{α} demonstrated that the C-terminal part of the $G_{\alpha 13}$ protein, by governing its membrane localization [de Almeida, Holtzman, Peters, Ercolani, Ausiello and Stow (1994) *J. Cell Sci.* **107**, 507–515], is important in the control of macroautophagic seques-

tration. G alpha interacting protein (GAIP), which stimulates the GTPase activity of the $G_{\alpha 13}$ protein and favours macroautophagic sequestration in HT-29 cells, was shown, by immunofluorescence studies using confocal microscopy, to be confined to the cytoplasm. The cytoplasmic distribution of GAIP only partially overlaps with that of the $G_{\alpha 13}$ protein. However, the presence of the two proteins on Golgi and ER membranes was confirmed by subcellular fractionation. These results point to the importance of the cytoplasmic localization of the $G_{\alpha 13}$ protein and GAIP in controlling autophagic sequestration in HT-29 cells.

Key words: degradation, endoplasmic reticulum, GTPase, lysosome, regulator of G-protein signalling.

INTRODUCTION

Macroautophagy, an ubiquitous catabolic pathway terminating in the lysosomal compartment, contributes to cellular homeostasis by regulating the equilibrium between amino acids and proteins [1–3]. The physiological importance of macroautophagy has been clearly identified under stress and nutrient-deprivation conditions. In starved rats, macroautophagy is activated in liver, intestine and kidney to provide amino acids for protein synthesis. Morphological and biochemical studies have shown that macroautophagy is a multistep process. The first step is the sequestration of various organelles (including mitochondria, peroxisomes, smooth and rough membranes and cytosolic constituents: proteins, glycogen, neutral lipids, RNA and ribosomes) by a membrane to form an autophagosome. The origin of the wrapping membrane is still a matter of debate. Although post-Golgi and/or Golgi membranes have been reported to be involved in the sequestration step [4,5], ribosome-free regions of the rough endoplasmic reticulum (ER) [6] and a separate organelle called the phagophore (see [7] and references therein) constitute a major source of the sequestering membrane in rat hepatocytes. Whatever the origin of the wrapping membrane, nascent autophagosomes are usually delimited by a double smooth membrane. Lysosomal H^+ -ATPase and lysosomal membrane glycoproteins contained in the *trans*-Golgi-network-derived vesicles are delivered to autophagosomes to form late acidic autophagosomes [8]. These acidic vacuoles acquire acidic hydrolases by fusion with pre-existing lysosomes [9,10]. However, the acquisition of acidic hydrolases by the fusion of autophagic vacuoles with late endosomes/pre-lysosomes has been reported in the exocrine pancreas [11] and in cultured fibroblasts [12]. Furthermore, input

from the endocytic pathway can converge at different steps of the autophagic pathway [13,14].

Recently, studies have shed light on the intracellular mechanisms that control membrane flow along the autophagic/lysosomal pathway (for reviews, see [15,16]). Our laboratory and others have shown that GTPases play a key role during the sequestration step [17,18]. We have shown that autophagic sequestration is controlled by the cytoplasmic trimeric $G_{\alpha 13}$ protein in cultured human colonic carcinoma HT-29 cells [18]. Using site-directed mutagenesis and stable cell transfections we demonstrated that autophagic sequestration is inhibited when the $G_{\alpha 13}$ protein is bound to GTP [19]. These results now add to the mounting evidence showing that cytoplasmic heterotrimeric G-proteins play a role in controlling intracellular trafficking along the exo/endocytotic pathway (for reviews, see [20,21]) and the structural organization of the Golgi apparatus [22,23]. From the data reported above, it appears that a better understanding of macroautophagy is dependent upon the identification and localization of cytoplasmic molecular motors that control the formation of autophagic vacuoles.

In the present study we show that the C-terminal portion of the $G_{\alpha 13}$ protein dictates its association with ER and Golgi complex membranes and its function in controlling autophagic sequestration. When the N-terminal part of the $G_{\alpha 13}$ protein was associated with the C-terminal part of the $G_{\alpha 12}$ protein ($G_{\alpha 13/2}$), the chimaeric protein was no longer operative in controlling autophagy and was expressed at the cell surface similarly to the genuine $G_{\alpha 12}$ protein. Moreover, G alpha interacting protein (GAIP) [24], a member of the regulators of G-protein signalling (RGS) family [25,26], which stimulates the GTPase activity of the $G_{\alpha 13}$ protein [27] and regulates autophagic sequestration [28],

Abbreviations used: CLSM, confocal laser scanning microscopy; ER, endoplasmic reticulum; GAIP, G alpha interacting protein; Man II, α -mannosidase II; 3-MA, 3-methyladenine; PDI, protein disulphide-isomerase; RGS, regulator of G-protein signalling.

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is confined to the cytoplasm. A partial overlap between the distribution of GAIP and that of the $G_{\alpha 13}$ protein was observed. The association of GAIP with vesicular structures suggests that it can cycle between different cytoplasmic compartments.

MATERIALS AND METHODS

Cells and materials

HT-29 cells were cultured as previously described [18,29]. Cells were maintained at 37 °C under a 10% CO_2 /air atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal-calf serum/1% penicillin/streptomycin (100 units/ml). 3-Methyladenine (3-MA) and other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Cell-culture reagents, lipofectamine and Geneticin (G418) were from Life Technologies Inc. (Eragny, France). Nitrocellulose membranes were from Schleicher et Schuell (Dassel, Germany). The bicinchoninic acid (BCA) kit was from Pierce (Rockford, IL, U.S.A.). Rat cDNA encoding the α_{12} and α_{13} subunits were kindly provided by Dr. R. Reed (John Hopkins University, Baltimore, MD, U.S.A.). Enzymes, materials for sequencing, and synthetic oligonucleotides were from Life Technologies, and the eukaryotic expression vectors pcDNA3 and PBK/CMV were from InVitrogen (San Diego, CA, U.S.A.) and Stratagene (La Jolla, CA, U.S.A.) respectively. The radioisotopes [α - ^{35}S]ATP (specific radioactivity 1000 Ci/mmol), [^3H]raffinose (specific radioactivity 5–15 Ci/mmol) and L-[U- ^{14}C]valine (specific radioactivity 28805 mCi/mmol) were from Amersham (Les Ulis, France).

Construction of cDNA chimaeras

cDNA chimaeras $G_{\alpha 12/3}$ and $G_{\alpha 13/2}$ were constructed as described previously [30]. Briefly, the *Bam*HI restriction sites at positions 785 and 716 in $G_{\alpha 12}$ and $G_{\alpha 13}$ cDNA, respectively, were used to cleave the coding regions and obtain the N- and C-terminal coding regions of both G_{α} proteins. For construction of $G_{\alpha 12/3}$ cDNA the 785 bp $G_{\alpha 12}$ fragment was ligated to the 2356 bp of the $G_{\alpha 13}$ fragment, and the 716 bp of $G_{\alpha 13}$ was ligated to the 963 bp of the $G_{\alpha 12}$ fragment to obtain $G_{\alpha 13/2}$ cDNA. Each cDNA was then inserted into the *Eco*RI/*Hind*III restriction sites of the PBK/CMV plasmid. Before transfection, both cDNA constructions were analysed by sequencing.

Antibodies and transfection

Rabbit antisera against $G_{\alpha 12}$ (AS-7) or against $G_{\alpha 13}$ (EC-2) subunits were from NEN Dupont (Les Ulis, France). Rabbit antiserum against a synthetic peptide corresponding to the C-terminal amino acid sequence (residues 202–217) of GAIP was produced as previously described [31]. All injections and bleeds were done by Neo-system (Strasbourg, France). The polyclonal antibody directed against α -mannosidase II (Man II) was a gift from Dr. K. Moremen (CCRC, University of Georgia, GA, U.S.A.). Monoclonal antibodies against 5'-nucleotidase and protein disulphide-isomerase (PDI) were gifts from Dr. H. G. Mannherz (University of Bochum, Germany) and Dr. S. Fuller (EMBL, Heidelberg, Germany), respectively. ^{125}I -labelled donkey anti-rabbit IgF(ab') fragment (specific radioactivity 500 Ci/mmol) and ^{125}I -labelled sheep anti-mouse IgF(ab') fragment (specific radioactivity 500 Ci/mmol) were from Amersham and fluorescent secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

Plasmids (1 $\mu\text{g}/\mu\text{l}$) containing the cDNA coding for either $G_{\alpha 13/2}$ or $G_{\alpha 12/3}$ were introduced into exponentially growing cells, by the lipofectamine method. Following transfection (24 h), cells

were grown in selection medium containing G418 (400 $\mu\text{g}/\text{ml}$) for at least 3 weeks. Resistant cells were then cloned by limiting dilution. Clones were selected according to Western blotting using either anti- $G_{\alpha 13}$ for cells expressing the $G_{\alpha 12/3}$ protein or anti- $G_{\alpha 12}$ for cells expressing the $G_{\alpha 13/2}$ protein. Selected clones had a 2.5–3-fold increased signal when compared with untransfected cells.

Subcellular fractionation

Cells were scraped into 10 mM Hepes (pH 7.4)/0.25 M sucrose and disrupted by multiple passages in a Potter homogenizer until greater than 90% of the cells had been broken. Post-nuclear supernatants (1 ml), prepared by centrifugation (450 g for 15 min) of the homogenates, were layered on top of preformed 10–50% sucrose in $^2\text{H}_2\text{O}$ gradients as described by Lodish et al. [32]. Centrifugation was carried out at 4 °C for 3 h at 164 000 g in a Beckman SW 41 Ti rotor. The gradients were then separated into 24 fractions of 500 μl each. Starting from the bottom, fractions 1–6 contained ER membranes and fractions 7–16 contained Golgi membranes. The second fractionation was performed as previously described [33]. Cells from one confluent 25 cm^2 flask were scraped into 1 ml of ice-cold PBS containing PMSF (1 mM) and aprotinin (100 units/ml). All the following steps were performed at 4 °C. The cells were homogenized in a Potter homogenizer until more than 90% of the cells had been broken, and a post-nuclear supernatant was prepared by centrifugation for 10 min at 5000 g . Of the supernatant, 500 μl was layered on to a discontinuous sucrose gradient (0.2, 0.4, 0.6, 1.0, 1.4 and 1.8 M; 750 μl each) and centrifuged for 2 h at 70 000 g (Beckman SW 41 Ti rotor). Twelve fractions (400 μl) were collected from the top and centrifuged for 1 h at 100 000 g . The resultant pellets were solubilized in Laemmli's sample buffer, and the proteins were separated by SDS/PAGE. The amount of $G_{\alpha 13}$ protein and GAIP in cell fractions was quantified by densitometry using the Scan Analysis program.

Immunoblotting

Proteins (100 μg) from the pooled ER and Golgi fractions were resolved by electrophoresis on a 10% SDS/PAGE gel and electrotransferred on to a nitrocellulose membrane. Membranes were then incubated in blocking buffer containing PBS (pH 7.4), 5% BSA and 0.02% NaN_3 , and then with anti- α_{13} (1:1000), anti-PDI (1:200), anti-Man II (1:500) or anti-GAIP (1:200) antibodies diluted in PBS (pH 7.4) supplemented with 2.5% BSA for 1 h at room temperature. After washing with PBS (pH 7.4) supplemented with 2.5% BSA and 0.1% Triton X-100, membranes were incubated with the corresponding ^{125}I -labelled secondary antibodies for 1 h and exposed to Kodak X-Omat AR film overnight.

Autophagic sequestration of [^3H]raffinose

[^3H]Raffinose sequestration was monitored as previously reported [34]. Briefly, cells ($5 \times 10^6/500 \mu\text{l}$) were incubated for 15 min at 37 °C with 2 μCi of [^3H]raffinose, subjected to electroporation (at room temperature), and incubated at 4 °C for 30 min, then at 37 °C for 15 min. Afterwards, cells were rinsed twice with PBS and dispersed in complete medium with 5 mM 3-MA. Subsequently, at different times, the cells were washed twice with 10% sucrose at 4 °C, resuspended in 0.5 ml of 10% sucrose and homogenized by five strokes in a glass/Teflon homogenizer on ice. Immediately after homogenization, 0.5 ml ice-cold phosphate buffer [100 mM potassium phosphate/2 mM EDTA/2 mM dithiothreitol/100 $\mu\text{g}/\text{ml}$ BSA/0.01% Tween 20 (pH 7.5)]

Figure 1 Localization of G_{αi2} and G_{αi3} proteins in HT-29 cells by CLSM

Cells were fixed with paraformaldehyde and permeabilized with Triton X-100 and double labelled with antibodies to G_{αi3} protein and ER (PDI, upper row), the G_{αi3} protein and Golgi complex (Man II, middle row) and the G_{αi2} protein and 5'-nucleotidase, a plasma-membrane protein (5'-Nu, lower row). The third column (Merge) represents the overlay image of G_{αi} proteins and each of the organelle markers used. Bars, 10 μm.

was added, and 1 ml of the cell homogenate was layered on the top of a 4 ml cushion of buffered metrizamide/sucrose [10 % sucrose/8 % metrizamide/1 mM EDTA/100 μg/ml BSA/0.01 % Tween 20 (pH 7.5)] and centrifuged at 7000 *g* for 60 min. The radioactivity associated with the pellet and total homogenate was measured by liquid-scintillation counting.

Measurement of the degradation of long-lived proteins

Protein degradation was determined as previously reported [19]. HT-29 cells were incubated for 18 h at 37 °C with 0.2 μCi/ml of L-[¹⁴C]valine. Unincorporated radioisotope was removed by three rinses with PBS (pH 7.4). Cells were then incubated in nutrient-free medium (without amino acids and in absence of fetal calf serum) supplemented with 0.1 % BSA/10 mM unlabelled valine. When required, 200 ng/ml pertussis toxin was added during the labelling period and was present throughout the chase period; 3-MA (10 mM) was added at the beginning of the chase period. After the first hour of incubation, at which time short-lived proteins were being degraded, the medium was replaced with the appropriate fresh medium and the incubation continued for an additional 4 h. Cells were scraped into 0.5 ml of PBS, and then the radiolabelled proteins present in the 4 h incubation media and in cells were precipitated with 10 % trichloroacetic acid/1 % phosphotungstic acid (v/v) at 4 °C. The precipitated proteins were separated from the soluble radioactivity by centrifugation

at 600 *g* for 10 min then dissolved in 1 ml of Soluene 350 (Packard, Meriden, CT, U.S.A.). The rate of protein degradation was calculated as acid-soluble radioactivity recovered from both cells and media.

Immunofluorescence and detection of G_{αi} proteins and GAIP

Cells grown on glass slides were processed for double immunofluorescence studies. Cells were fixed and permeabilized with 3 % paraformaldehyde for 2 h and 0.1 % Triton X-100 for 60 min at room temperature. Samples were then incubated with ammonium chloride (50 mM) for 15 min and rinsed with PBS. In all cases, samples were first incubated with a rabbit anti-G_{αi3} polyclonal antibody (1:50) or a rabbit anti-GAIP (1:200) polyclonal antibody for 30 min at room temperature. After rinsing with PBS, samples were incubated with a CY3[™]-conjugated affinity-purified goat anti-rabbit IgG (1:100) for 30 min at room temperature. Samples were then incubated with either the mouse anti-PDI monoclonal antibody (1:100) or a rabbit anti-Man II (1:1000) polyclonal antibody. In the case of double staining with the anti-Man II antibody, samples were additionally incubated with a rabbit anti-mouse F(ab')₂ IgG fragment to saturate the first anti-rabbit CY3[™] antibody. After rinsing, samples were incubated with biotinylated species-specific anti-IgG antibody, followed by streptavidin-fluorescein. As controls, no staining was observed by omitting the primary antibody. The coverslips

were mounted and examined by confocal laser scanning microscopy (CLSM; Leica) equipped with epifluorescent optics (x63, NA 1.3 oil-immersion objective). Simultaneous two-channel recording was performed with a pinhole size of 90 μm using excitation wavelengths of 488/588 nm, a 510/580 double dichroic mirror, and a 515–545 band pass FITC filter together with a 590 nm long pass filter. Double-labelled cells were analysed separately to avoid leakage from one channel to another. All the figures generated were derived from single x–y optical sections (1 μm in thickness) passing through the plan of nuclei. Images were processed using the Adobe Photoshop program and printed on a XLS 8600 PS printer (Eastman Kodak Co).

Electron microscopy and morphometry

Confluent cells grown on Petri dishes were cultured for 3 h in a nutrient-free medium to stimulate macroautophagy before being fixed for 2 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, embedded in Epon and processed for transmission electron microscopy by standard procedures. For morphometric analysis, 10–14 micrographs per condition were used. The fractional volume represented by autophagic vacuoles was quantified by using the method of Weibel et al. [35], assuming a spherical shape for the calculation. Morphological criteria were used to identify autophagic vacuoles [9].

RESULTS

Localization of the $G_{\alpha i}$ proteins in HT-29 cells

HT-29 cells express the $G_{\alpha 12}$ and $G_{\alpha 13}$ isoforms of the $G_{\alpha i}$ protein [18]. From preliminary observations we concluded that the $G_{\alpha 12}$

protein, which does not control autophagic sequestration, is associated with the plasma membrane, whereas the $G_{\alpha 13}$ protein is bound to cytoplasmic membranes [18]. We have investigated more precisely the localization of the $G_{\alpha i}$ proteins using double immunostaining experiments analysed by CLSM (Figure 1). A predominant co-localization of the $G_{\alpha 13}$ protein with the Golgi marker Man II was observed. Co-localization was also observed between the $G_{\alpha 13}$ protein and PDI, used as an ER marker. In contrast with the $G_{\alpha 13}$ protein, the $G_{\alpha 12}$ protein is confined to the cell surface where it co-localized with the plasma membrane enzyme 5'-nucleotidase (Figure 1). From these results we asked whether or not the intracellular localization of the $G_{\alpha 13}$ protein is important for the control of macroautophagic sequestration.

The C-terminal part of the $G_{\alpha 13}$ protein is important for controlling macroautophagic sequestration

It is known that the C-terminal parts of $G_{\alpha i}$ proteins contain determinants for their membrane targeting [30,36]. In order to correlate the function of the $G_{\alpha 13}$ protein in autophagic sequestration with its subcellular localization we constructed two chimaeric $G_{\alpha i}$ proteins using the strategy developed by de Almeida et al. [30]. The first one contained the N-terminal part of the $G_{\alpha 12}$ protein and the C-terminal part of the $G_{\alpha 13}$ protein ($G_{\alpha 12/3}$) and the second contained the N-terminal part of the $G_{\alpha 13}$ protein and the C-terminal part of the $G_{\alpha 12}$ protein ($G_{\alpha 13/2}$).

As previously reported [30], the C-terminal region of $G_{\alpha i}$ dictates the subcellular localization of chimaeric proteins. Immunofluorescence examination showed that the distributions of the $G_{\alpha 12/3}$ and $G_{\alpha 13/2}$ proteins were identical with those of the $G_{\alpha 13}$ and $G_{\alpha 12}$ proteins, respectively (results not shown).

Figure 2 Biochemical parameters of macroautophagy in cells that express $G_{\alpha i}$ chimaeras

(A) Autophagic sequestration of [³H]raffinose was determined in $G_{\alpha i2/3}$ - and $G_{\alpha i3/2}$ -expressing cells and parental cells (control). In control experiments using the autophagic sequestration inhibitor 3-MA (10 mM), the rate of recovery of [³H]raffinose in sedimentable material was inhibited by 85–90% in all the cell populations studied. (B) The rate of [¹⁴C]valine-labelled long-lived protein degradation was measured in cells incubated in nutrient-free medium. Experiments were performed in the presence or absence of 10 mM 3-MA. When used, pertussis toxin (PTX) was added at a final concentration of 200 ng/ml. Data are expressed as the percentage of cellular protein degraded in 4 h. The values reported in (A) and (B) are the means $\pm$ S.D. of 5 determinations.

Figure 3 Morphology and morphometric analysis in different HT-29 cell populations

Right panels: representative illustrations of autophagic structures observed in HT-29 cells. (A) Early autophagic vacuole; (B) a multilamellar structure in an autophagic vacuole. Bars: 1 μ m. Graph: the relative volume occupied by autophagic vacuoles in six different HT-29 cell populations is expressed as a percentage of the cell volume, as described in the Materials and methods section. Untransfected cells, parental HT-29 cells; α i3 WT and α i2 WT, cells overexpressing the wild-type G_{α13} and G_{α12} proteins, respectively; α i2/ α i3, G_{α12/3}-expressing cells; α i3/ α i2, G_{α13/2}-expressing cells; α i3 GTP (Q204L), Q204L-expressing cells. Values are means $\pm$ S.E.M. of 10 analysed micrographs in each condition tested.

The rate of autophagic sequestration of electroloaded [³H]-raffinose in G_{α12/3}-expressing cells was stimulated when compared with untransfected cells (Figure 2A). Autophagic sequestration in G_{α12/3}-expressing cells was similar to that observed in G_{α13}-overexpressing cells (results not shown; see [19]). Accordingly, we observed a stimulation of the 3-MA-sensitive degradation of [¹⁴C]valine-labelled long-lived proteins (3-MA is a potent inhibitor of macroautophagic sequestration [37]) in G_{α12/3}-expressing cells when compared with untransfected cells (Figure 2B). Autophagic degradation (Figure 2B) and sequestration (results not shown) were inhibited by pertussis toxin treatment (200 ng/ml) in a similar manner to that observed in G_{α13}-overexpressing cells [18,19]. The fact that pertussis toxin, which ADP-ribosylates G_{αi} proteins in their heterotrimeric forms [38], is a very potent inhibitor of autophagic sequestration in the G_{α12/3}-expressing cells suggests that the chimaeric protein is able to interact with the $\beta\gamma$ dimer.

In contrast with the results reported above, autophagy was not modified in G_{α13/2}-expressing cells when compared with either untransfected cells (Figure 2) or G_{α12}-overexpressing cells (Figure 2B). These biochemical results were confirmed by quantitative morphological analysis. Early autophagic vacuoles surrounded by a double limiting membrane and containing recognizable cytoplasmic elements were observed at the ultrastructural level (Figure 3A). As reported for HeLa cells [39], autophagosomes containing multilamellar structures surrounded by a single limiting membrane were also frequently observed in HT-29 cells (Figure 3B).

Morphometric analyses were carried out in six different populations of HT-29 (Figure 3, graph). The fractional volume occupied by autophagic vacuoles was the most elevated in G_{α13}-overexpressing cells and G_{α12/3}-expressing cells. In untransfected cells, the fractional volume occupied by autophagic vacuoles was similar to that observed in G_{α12}-overexpressing cells and G_{α13/2}-expressing cells. In contrast, in Q204L-expressing cells, i.e. cells transfected with a G_{α13} protein deficient in GTPase activity, the proportion of autophagic structures was greatly reduced (Figure 3, graph). This last observation is in agreement with our previous

Figure 4 Characterization of anti-GAIP antibody

Homogenates from HT-29 cells were immunoblotted with anti-GAIP antibody, 1/200, in the absence (lanes 1 and 2) or in the presence (lane 3) of the antigenic GAIP peptide (100 ng/ml). In lane 4, the incubation was performed with preimmune serum. Protein was loaded at 100 μ g per lane, except in lane 1 (200 μ g/ml).

data showing that autophagic sequestration is impaired in Q204L-expressing cells [19].

GAIP and the G_{α13} protein have a partially overlapping membrane distribution

GAIP, a membrane-anchored protein of the RGS family [40], interacts with the G_{α13} protein [24] and stimulates its GTPase activity [27]. Recently, we have shown that GAIP is functionally linked to the G_{α13} protein in controlling macroautophagic sequestration in HT-29 cells [28]. In order to have a better understanding of the mechanism by which the G_{α13} protein controls macroautophagy, we have investigated the subcellular distribution of GAIP. To that aim a polyclonal antibody directed against a C-terminal peptide of GAIP was raised as previously reported [31]. Characterization of the antibody by Western blotting showed that it specifically recognized GAIP in a concentration-dependent manner (Figure 4).

A heterogeneous cytoplasmic-staining pattern of GAIP was observed by immunofluorescence examination. An intense punc-

Figure 5 Localization of GAIP in HT-29 cells by CLSM

Cells were fixed with paraformaldehyde and permeabilized with Triton X-100 and double labelled with antibodies to the C-terminal epitope of GAIP and the ER marker PDI (upper row) or antibodies to the C-terminal epitope GAIP and the Golgi complex marker Man II (lower row). The third column (Merge) represents the overlay image of GAIP and each of the organelle markers used. Bar, 5 μ m.

Figure 6 Distribution of GAIP and the $G_{\alpha i3}$ protein after subcellular fractionation of HT-29 cells

Subcellular fractionation was performed on a sucrose gradient as detailed in the Materials and methods section. The Golgi marker Man II was associated with fractions 3–6 whereas the ER marker PDI was detected in fractions 7–11. The $G_{\alpha i3}$ protein, like the GAIP protein, co-distributed with Man II in fractions 4–6 and with PDI in fractions 7–9. Fractions 5–6 contain 45% of the total $G_{\alpha i3}$, and 50% of the total GAIP protein. Fractions were lysed in Laemmli buffer, and proteins from each fraction were separated by SDS/PAGE, transferred on to nitrocellulose membranes, and revealed by the relevant antibody. Fractions 1 and 12 represent the top and the bottom of the gradient, respectively.

tate staining of cytoplasmic vesicular structures was detected but no labelling of the plasma membrane was observed. However, double-staining experiments revealed an overlapping staining of GAIP with the Golgi apparatus and also some labelling co-localized with the ER marker PDI (Figure 5).

These morphological observations were complemented by subcellular fractionation experiments on sucrose density gradients (Figure 6). The Golgi marker Man II was associated with fractions 3–6 whereas the ER marker PDI was detected in fractions 7–11. The $G_{\alpha i3}$ protein, like the GAIP protein, co-distributed with Man II in fractions 4–6 and with PDI in fractions 7–9.

The distribution patterns of GAIP and the $G_{\alpha i3}$ protein were confirmed by using another subcellular fractionation protein with sucrose/ $^2\text{H}_2\text{O}$ gradients (results not shown).

DISCUSSION

A well-known function of trimeric G proteins associated with plasma-membrane-bound heptahelical receptors is to transduce signals emanating from the cell surface [41]. Here we show, using construction of chimaeric $G_{\alpha i}$ proteins, the importance of the intracellular localization of the $G_{\alpha i3}$ protein in its ability to control autophagic sequestration. These results confirm earlier reports on the importance of the C-terminal part of $G_{\alpha i}$ proteins in membrane targetting [30,38], and they are to be added to mounting evidence for the function of trimeric G-proteins in intracellular membrane dynamics [20,21].

The $G_{\alpha i3}$ protein has been shown to control intra-Golgi trafficking in renal cells [42]. Recently, we have shown that the $G_{\alpha i3}$ protein also controls the autophagic pathway in HT-29 cells [18,43]. We [43] and others [44] have shown that, whereas brefeldin A interrupts transport along the secretory pathway by destabilizing the Golgi apparatus, it does not inhibit autophagic sequestration. As the integrity of the Golgi apparatus is not required for autophagic sequestration these results strengthen the hypothesis that the ER membrane is involved in the formation of autophagic vacuoles in HT-29 cells.

At least two non-mutually-exclusive mechanisms can be considered for the $G_{\alpha i3}$ protein in controlling macroautophagic

sequestration. First, the G_{α13} protein is directly involved in the maintenance of the membrane responsible for the formation of autophagic vacuoles. Recently, it has been demonstrated that trimeric G-proteins are involved in the structural organization of the Golgi complex [22,23]. Secondly, the G_{α13} protein is part of a signal-transduction pathway leading to changes in the lipid composition, which, by analogy to other membrane-remodelling events such as transport-vesicle formation [45,46], could be responsible for the formation of autophagic vacuoles. In fact, inhibition of phosphatidylinositol 3-kinase stops autophagic sequestration in rat hepatocytes [15] and in HT-29 cells (A. Petiot, E. Ogier-Denis, A. Meijer and P. Codogno, unpublished work). It has been shown that the activity of members of the phosphatidylinositol 3-kinase family act down-stream of trimeric G proteins and that elements of G proteins (α subunits or the βγ dimer) modulate the kinase activity [47,48]. As inositol phospholipids are associated with various membrane flow events [45,49], it is tempting to hypothesize that these lipids and the G_{α13} proteins act in a co-ordinated manner to control autophagic sequestration.

A better understanding of the control of macroautophagy is dependent upon the characterization of proteins interacting with the G_{α13} protein. Recently we have reported that GAIP, a GTPase-activating protein belonging to the RGS family, controls the G_{α13}-dependent autophagic sequestration in HT-29 cells. GAIP is a membrane-bound protein [40]; 90 % of the total GAIP is membrane-bound in HT-29 cells (results not shown). Although both proteins are associated with Golgi and ER membranes, GAIP is also associated with cytoplasmic vesicular structures. While this work was in progress, it was reported that GAIP is associated with clathrin-coated vesicles in pituitary and liver cells [50]. The authors suggest a model whereby GAIP comes into contact with the G_{α13} protein when vesicles fuse with GTPase-containing membranes. The heterogeneous cytoplasmic distribution of GAIP observed in the present study suggests that this protein may also cycle between different membrane-bound compartments in HT-29 cells.

However, from our results we cannot exclude the possibility that constitutive presence of the G_{α13} protein and GAIP in the same membrane exists. The interaction between the two proteins may depend on the guanine nucleotide bound to the G protein and/or on other modifications that modulate protein-protein interaction.

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