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Evidence for a Dual Control of Macroautophagic Sequestration and Intracellular Trafficking of N-Linked Glycoproteins by the Trimeric G_{i3} Protein in HT-29 Cells

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The trimeric G_{i3} protein-dependent lysosomal-autophagic pathway is responsible for the degradation of a pool of N-linked glycoproteins in the human colon cancer HT-29 cell line. Here we have followed the fate of N-glycans using HT-29 cells either overexpressing the wild-type $G\alpha_{i3}$ protein or transfected with different mutants of the $G\alpha_{i3}$ protein. The stabilization of N-glycans was dependent upon the inhibition of autophagic sequestration by either 3-methyladenine (3-MA) or pertussis toxin (PTX). However, PTX allowed the processing of high-mannose glycans whereas 3-MA did not. The destabilization of the Golgi apparatus by brefeldin A, which interrupts the intracellular trafficking of N-linked glycoproteins along the secretory pathway, did not interfere with the macroautophagic pathway. These results suggest that the lysosomal-autophagic pathway is not dependent upon the integrity of the Golgi apparatus and points to differences between the molecular properties of two membrane flow processes (macroautophagy, exocytic pathway) controlled by the trimeric G_{i3} protein.

Recently we have shown that an intracellularly bound trimeric G_{i3} protein controls macroautophagic sequestration in the human colon cancer HT-29 cells (1,2). In this cell line the macroautophagic pathway is responsible for the lysosomal degradation of a pool of newly synthesized N-linked glycoproteins substituted with typical endoplasmic reticulum high-mannose glycans (3,4). On the other hand it has been shown in different cell types that the trimeric G_{i3} protein is involved in the control of the transport of glycoconjugates along the exocytic pathway (5,6). In the present work we show that the trimeric G_{i3} protein controls both the macroautophagic sequestration and the intracellular trafficking of N-linked glycoproteins in HT-29 cells. However the inhibition of the macroautophagic sequestration by 3-MA (7) which interrupts the degradation of N-linked glycoproteins is not sufficient to restore their intracellular trafficking. This was achieved either by treating cells by PTX or in cells expressing a GTPase deficient form of the $G_{\alpha i3}$ protein: two conditions known to inhibit the macroautophagic sequestration in HT-29 cells (1,2). Using the fungal metabolite brefeldin A (BFA) which inhibits the intracellular trafficking along the secretory pathway (8), we have demonstrated that the G_{i3}-dependent macroautophagic pathway was not altered whereas the G_{i3}-dependent exocytic pathway was dramatically reduced. These results point to different molecular mechanisms responsible for the two G_{i3}-mediated membrane flow processes and to the independence of the macroautophagic pathway from the integrity of the Golgi apparatus.

MATERIALS AND METHODS

Cells and Culture Conditions

HT-29 cells and their different established clones were cultured as previously described (2).

Cell Labeling and N-Glycan Analysis

Cells were radiolabeled with 400 μCi/ml D-[2-^{3}H]mannose (20 Ci/mmol, Amersham, Bucks, UK) for 10 min and then chased for the indicated time in the presence of 10 mM mannose and 2 mM fucose (9). PTX (200 ng/ml) was added 18 h before the labeling period and was present throughout the pulse-chase experiment. 3-MA (10 mM) was added at the beginning of the chase period. N-linked glycoproteins were isolated from delipidated cell homogenates and N-glycans were analyzed after pronase digestion by chromatography on a Bio-Gel P6 column before and after endo-β-N-acetylglucosaminidase H treatment as described (9). PTX does not affect either the synthesis

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of lipid linked oligosaccharides or their transfer en bloc to polypeptides (1).

Autophagic Sequestration of [^{3}H]Raffinose

[^{3}H]Raffinose sequestration was monitored using the method of Kopitz et al. (10) as modified by Houri et al. (3). Briefly, the cells were suspended at a density of 5×10^6/500 μl with 2 μCi of [^{3}H]raffinose (5-15 Ci/mmol, Amersham), after which they were incubated for 15 min at 37°C and submitted to electroinjection by a single voltage pulse (330 V, 1000 mF). When required BFA (from 0.010 to 10 μg/ml) and PTX (200 ng/ml) were added 1h and 18h before the experiment, respectively.

Assay of the Secretion of Proteins and Glycoproteins

Secretion of glycoproteins. Cells were radiolabeled with 50 μCi/ml Tran[^{35}S]label (>1000 Ci/mmol, Amersham) for 20 min at 37°C, washed twice with prewarmed PBS (20°C), pH 7.4 and then chased with 10 mM unlabeled methionine and cysteine at 20°C for 2 h. This procedure allowed the labeled pool of proteins to pass beyond the medial Golgi and to accumulate in the late Golgi/TGN (11). After the 20°C blockade, the chase was extended for 4 h at 37°C. When used, 200 ng/ml PTX was added to cells for 16 h before and during the metabolic labeling. PTX was present during all chase periods. At the end of the 37°C chase, media were incubated overnight at 4°C with WGA-agarose, preequilibrated in buffer A (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% BSA, 100 μg/ml leupeptin, 100 μg/ml aprotinin, 100 μM PMSF). Immobilized lectin was rinsed once with buffer A and then three times with buffer B (25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 100 mM NaCl). Elution of WGA-bound glycoproteins was performed for 1 h at 4°C in buffer B containing 5 mM GlcNAc.

Secretion of protein. Cells were radiolabeled as described above and chased for 1 h at 37°C in a medium containing 10 mM unlabeled methionine and cysteine. At the end of the chase period the medium was collected and 10% trichloroacetic acid(TCA)/1% phosphotungstic acid (PTA) (v/v) precipitable radioactivity was measured by scintillation counting. When required BFA at the indicated concentration was added 1 h before the labelling period and present throughout the pulse-chase period.

Measurement of the Degradation of Long-Lived [^{14}C]Valine-Labeled Proteins and [^{3}H]Mannose-Labeled N-Linked Glycoproteins

The degradation of [^{14}C]valine-labeled proteins and [^{3}H]mannose-labeled N-linked glycoproteins was analyzed as previously described (2,12). Briefly HT-29 cells were incubated for 18 hours at 37°C with either 0.2 μCi/ml of L-[U-^{14}C]valine (288.5 mCi/mmol, Amersham) or 20 μCi/ml of [^{3}H]mannose. 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) plus 0.1% of BSA. The medium was supplemented with either 10 mM cold valine or 10 mM cold mannose. When required 10 mM 3-MA was added throughout 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 hours. Cells were scraped into 0.5 ml of PBS and the radiolabeled proteins in the 4h media and cells precipitated in 10% TCA- 1% PTA (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 Soluene 350. The rate of (glyco)protein degradation was calculated as acid-soluble radioactivity recovered from both cells and media. When required, BFA at the indicated concentrations, was added 1 h before the labelling period and present throughout the pulse-chase period. 1-Deoxymannojirimycin (5 mM) was added during the pulse-chase experiment using [^{3}H]mannose to avoid loss of radioactivity due to N-glycan processing (12).

RESULTS

Effect of the Inhibition of Autophagic Sequestration on the Processing of High-Mannose Glycans into Complex-type Glycans

We have previously shown that the lysosomal-autophagic pathway is responsible for the degradation of a pool of newly synthesized N-linked glycoproteins (3) and impairment of the biosynthesis of glycosphingolipids (13) in HT-29 cells. Autophagic sequestration was further shown to be dependent upon the expression and the activity of an intracellular bound trimeric G_{i3} protein (1,2). Wild-type $G_{\alpha i3}$ overexpression increases the rate of autophagic sequestration in HT-29 cells (1). Similarly cells expressing either a mutant of the $G_{\alpha i3}$ protein which has an increased guanine nucleotide exchange rate and increased preference for GDP over GTP (S47N) or a PTX-insensitive mutant of the $G_{\alpha i3}$ protein (C351S) reveal an elevated percentage of autophagic sequestration (2). In contrast the autophagic sequestration was very low in cells expressing a $G_{\alpha i3}$ protein with no GTPase activity (Q204L) (2).

The effect of the autophagic sequestration on the stability of newly synthesized N-linked glycoproteins was studied during pulse-chase experiments using D-[2-^{3}H]mannose as previously described (9) (Figure 1). After a 10 min pulse, a similar amount of radioactivity was incorporated in dolichol-P-P-oligosaccharides in all the cell populations considered (data not shown). In addition oligosacharides linked to dolichol have the same structure $Glc_{1\text{-}3}Man_9GlcNAc_2$ (data not shown). In Q204L-expressing cells we observed a classical pattern of N-glycan metabolism, i.e., a quantitative processing of high-mannose glycans to complex-type glycans (Figure 1). This processing is identical to that we have previously shown to occur in enterocytic differentiated HT-29 cells (4,9) [a cell population with low autophagic capacities, see ref. 3]. Whatever the other cell populations considered (wild-type $G_{\alpha i3}$ overexpressing cells, S47N-, C351S-expressing cells) the decrease of the radioactivity associated with high-mannose glycans was not correlated with a quantitative recovery of radioactivity in complex-type glycans (Figure 1). These results are in line with our previous data demonstrating that autophagy is responsible for a marked degradation of N-linked glycoproteins substituted with high-mannose glycans (1,3). The inhibition of the autophagic sequestration in these cells by 3-MA treatment did not allow the processing of high-mannose glycans to complex-type glycans. This drug only induced the stabilization of high-mannose glycans (Figure 1 insets). This defect in N-glycan processing in the presence of 3-MA is not due to an inhibition of Golgi enzymes as 3-MA

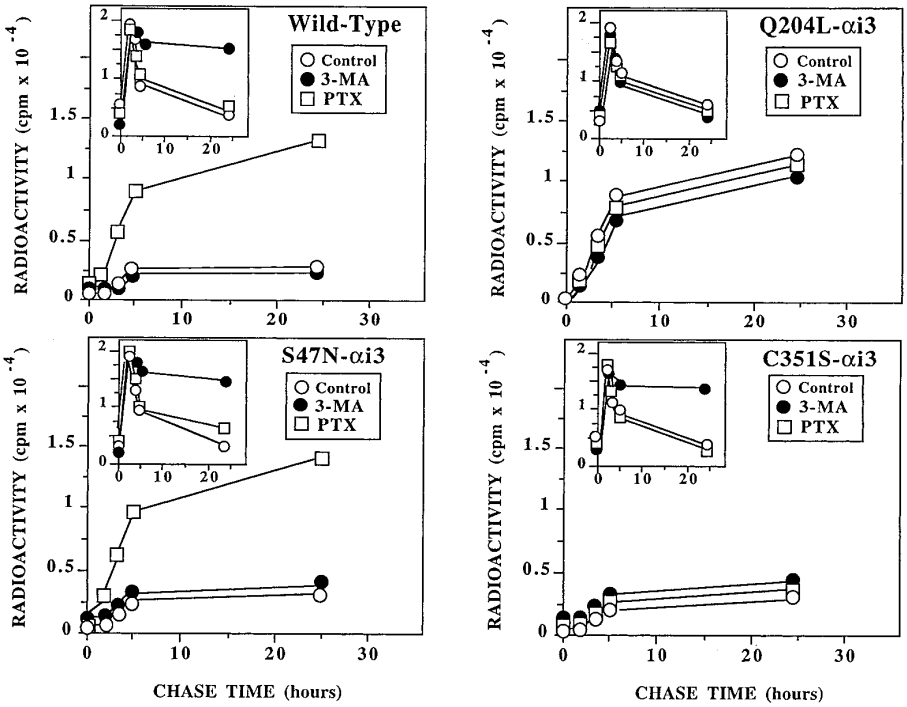

FIG. 1. Effect of 3-MA and PTX on the processing of N-glycans in HT-29 cells expressing either the wild-type or mutants of the $G_{\alpha i3}$ proteins. Cells were radiolabeled for 10 min with 400 μCi/ml D-[2-^{3}H]mannose and then chased for the indicated times. When used, PTX (200 ng/ml) was added 18 h before cell labeling and 3-MA (10 mM) was added at the beginning of the chase. High-mannose and complex-type glycans were analyzed as indicated in Materials and Methods. In each panel main curves and insets represent the radioactivity associated with complex-type glycans and high-mannose glycans, respectively.

did not impair the N-glycan processing in Q204L-expressing cells. In contrast PTX treatment, which stopped autophagic sequestration (1), restored the biosynthesis of complex-type glycans in wild-type $G_{\alpha i3}$ overexpressing cells and S47N-expressing cells. The amount of complex-type glycans produced in these cells is similar to that observed in Q204L-expressing cells. However PTX did not modify the processing of N-glycan in C351S-expressing cells demonstrating that the restoration of N-glycan processing in autophagic cells is dependent upon the action of the toxin on the $G_{\alpha i3}$ protein (Figure 1). These results suggested that the $G_{\alpha i3}$ protein has a dual effect in HT-29 cells in controlling autophagic sequestration (1) and the intracellular trafficking along the exocytic pathway as previously shown in other cell lines (5,6). Thus we wanted next to investigate whether or not autophagic sequestration and intracellular trafficking of N-linked glycoproteins are connected events.

The $G_{\alpha i3}$ Protein Controls the Exocytic Pathway in HT-29 Cells

The lectin Wheat Germ Agglutinin (WGA) is known to recognize sialic acid linked at the non-reducing ends of oligosaccharide chains (14). This sugar is added to oligosaccharides in the late Golgi/TGN (15). In order to show that the G_{i3} protein is directly involved in the control of the transport of N-linked glycoproteins along the exocytic route, radiolabeled WGA-positive glycoproteins were accumulated in the late Golgi/TGN of $G_{\alpha i3}$ overexpressing and C351S expressing cells by lowering the temperature to 20°C (11) and thereafter their secretion was measured in the presence or absence of PTX. As shown in Figure 2, PTX stimulated the secretion of glycoproteins in $G_{\alpha i3}$ overexpressing cells. This result is in line with those reported previously (5,16,17) on the effect of PTX on exocytosis in other cell lines. In contrast PTX did not increase the secretion of glycoproteins in C351S expressing cells. This result demonstrates that the $G_{\alpha i3}$ protein is directly involved in the control of the exocytic pathway in HT-29 cells.

The Integrity of the Golgi Apparatus Is Not Required for Autophagic Sequestration

In most of the cell lines studied BFA impairs the intracellular trafficking of N-linked glycoproteins along the secretory pathway as aconsequence of its ability to destabilize the Golgi apparatus (see for a review (8)). Recently it has been shown that BFA disorganizes the Golgi apparatus in HT-29 cells (18). In a first series of experiments we have studied the effect of BFA on the autophagic sequestration of cytosolic material in wild-

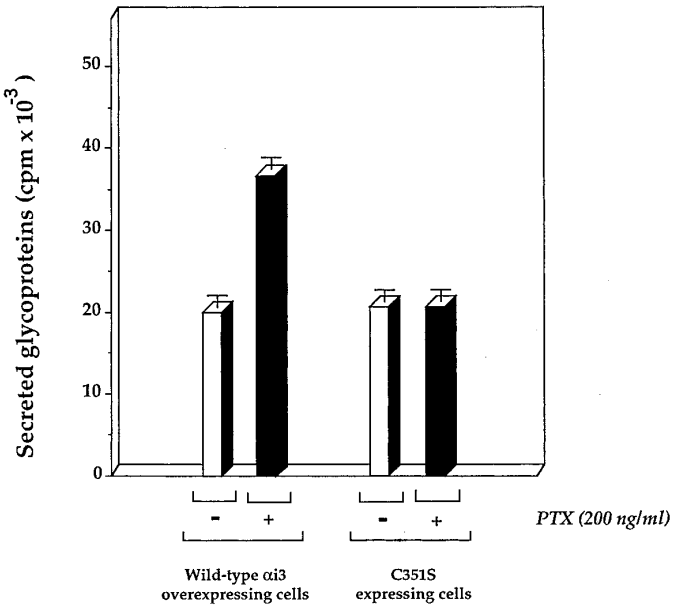

FIG. 2. Effect of PTX on the exocytic pathway in HT-29 expressing either the wild-type $G_{\alpha i3}$ protein or its PTX insensitive mutant (C351S). Cells were radiolabeled with 50 μCi/ml Tran[^{35}S]label for 20 min at 37°C and then chased with 10 mM unlabeled methionine and cysteine at 20°C for 2 h. After the 20°C blockade, the chase was extended for 4 h at 37°C. When used, 200 ng/ml PTX were added to cells for 16 h before and during the metabolic labeling. PTX was present during all chase periods. At the end of the 37°C chase, media were incubated overnight at 4°C with WGA-agarose and bound material was eluted as detailed in Materials and Methods.

type $G_{\alpha i3}$ overexpressing cells. Whatever the concentration of BFA used (up to 10 μg/ml) we observed no modification in the autophagic sequestration of the electroloaded [^{3}H]raffinose (Figure 3). At each concentration of BFA used autophagic sequestration was highly sensitive to PTX treatment. The absence of any effect of BFA on the lysosomal-autophagic pathway was confirmed by the assay of the rate of 3-MA-sensitive [^{14}C]-valine labeled long-lived protein degradation (data not shown). Similarly BFA did not inhibit the autophagic degradation of [^{3}H]mannose labeled N-linked glycoproteins. The degradation of [^{3}H]mannose labeled N-linked glycoproteins was measured in cells treated with 1-deoxymannojirimycin (an inhibitor of endoplasmic reticulum mannosidases I and II and Golgi mannosidase IA and IB reviewed in (19)) to avoid any loss of radioactivity due to the trimming of high-mannose glycans in control (12) and BFA-treated cells (20). The slight increase in 3-MA sensitive N-linked glycoprotein degradation in BFA treated cells (7.8%/h vs 6.6%/h in control cells; values are representative of 6 independent experiments) could be a consequence of the redistribution of Golgi material to the endoplasmic reticulum that increases the amount of material susceptible to sequestration. In contrast to these results BFA induced a dose-dependent inhibition of the exocytic pathway as determined by the assay of the secretion of [^{35}S]-methionine labeled proteins (Figure 3).

DISCUSSION

It is now clearly established that cytoplasmic trimeric G proteins are involved in the control of many steps along the exocytic pathway [reviewed in refs 21 and 22]. Recently we have shown that the cytoplasmic trimeric G_{i3} protein controls macroautophagic sequestration in HT-29 cells (1,2). As previously shown in other cell lines (5,6), we have further shown in the present work that the G_{i3} protein controls the intracellular trafficking of N-linked glycoproteins along the exocytic pathway. This result led us to ask if autophagic sequestration and intracellular trafficking along the exocytic pathway, two G_{i3}-mediated membrane flow processes are identically regulated in HT-29 cells. Inhibition of autophagic sequestration by 3-MA is not sufficient to restore a complete intraGolgi transport (determined by the sensitivity of N-linked glycans to endoH) in HT-29 cells whereas modulation of the activity of the G_{i3} protein either by PTX treatment or by expressing a GTPase deficient mutant of the $G_{\alpha i3}$ protein blocks the autophagic sequestration (1,2) and allows

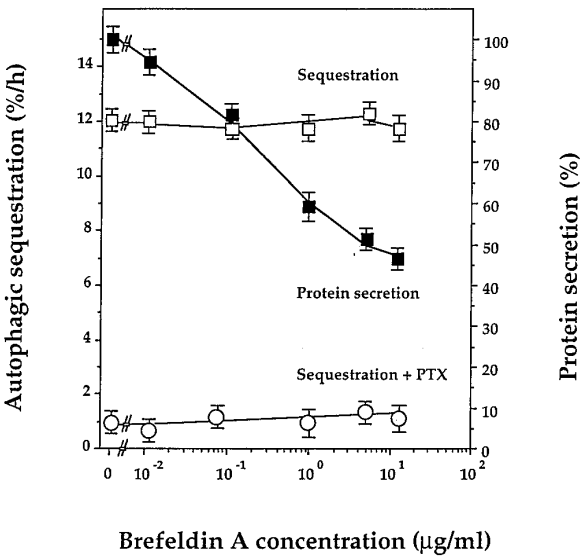

FIG. 3. Effect of BFA on macroautophagic sequestration and protein secretion. For the determination of autophagic sequestration, the cells were suspended in 2 μCi of [^{3}H]raffinose and submitted to electroinjection by a single voltage pulse (330 V, 1000 mF). When required BFA and PTX (200 ng/ml) were added 1h and 18h before the experiment, respectively. For the measurement of protein secretion, the cells were radiolabeled as described in Figure 2 and chased for 1 h at 37°C in a medium containing 10 mM unlabeled methionine and cysteine. At the end of the chase period the medium was collected and trichloroacetic acid/phosphotungstic acid precipitable radioactivity was measured. When required BFA at the indicated concentration was added 1 h before the labeling period and present throughout the pulse-chase period.

the restoration of intracellular trafficking along the secretory pathway (this study). The function of the G_{i3} protein was confirmed by the fact that in cells expressing a PTX-insensitive mutant (C351S) of the $G_{\alpha i3}$ protein the toxin neither inhibits the autophagic sequestration (2) nor restores the intracellular trafficking of N-linked glycoproteins. However in C351S-expressing cells macroautophagic sequestration is still sensitive to 3-MA treatment (2). This observation suggests that the target of 3-MA is not the G_{i3} protein. In fact a recent study has brought strong support to the notion that the target of 3-MA is the enzyme phosphatidylinositol 3-kinase (23). This would suggest that the Gi3 protein and phosphatidylinositol 3-kinase are involved in the same transduction pathway to control autophagic sequestration. In fact different studies have shown that trimeric G_i proteins act upstream of phosphatidylinositol 3-kinase (24,25). In addition the observation that 3-MA does not modify the processing of high mannose glycans whatever the cell population considered is in line with the fact that known inhibitors of phosphatidylinositol 3-kinase do not alter the vesicular transport between the endoplasmic reticulum and the early Golgi [reviewed in ref.26].

Various steps along the exocytic pathway which depend upon the activity of GTPases are sensitive to BFA (8). BFA inhibits a membrane associated factor involved in the GDP/GTP cycle on the monomeric GTPase ADP-ribosylation factor (ARF) (27,28). This GTPase is necessary to recruit coat proteins for the formation of transport vesicles from donor compartments (29). Our results show that ARF is not required along the lysosomal-autophagic pathway. However the contribution of other monomeric G proteins cannot be excluded (30,31). As BFA destabilizes the Golgi apparatus our results are in line with those demonstrating that this organelle is not directly involved in the formation of initial autophagic vacuoles (32,33). A better understanding of the dual function of the cytoplasmic G_{i3} protein in controlling autophagic sequestration and intracellular trafficking requires an investigation into its membrane localization.

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