# Glucose persistence on high-mannose oligosaccharides selectively inhibits the macroautophagic sequestration of N-linked glycoproteins

Eric OGIER-DENIS*, Chantal BAUVY*, Françoise CLUZEAUD†, Alain VANDEWALLE† and Patrice CODOGNO*[1]

*Institut National de la Santé et de la Recherche Médicale (INSERM) U504 Glycobiologie et Signalisation Cellulaire, 16 Avenue Paul-Vaillant-Couturier, 94807 Villejuif Cedex, France, and †INSERM U478 Hormones Corticostéroïdes et Protéines de Transport Ionique dans les Cellules Epithéliales, Faculté de Médecine Xavier Bichat, BP416, 75870 Paris Cedex 18, France

The macroautophagic–lysosomal pathway is a bulk degradative process for cytosolic proteins and organelles including the endoplasmic reticulum (ER). We have previously shown that the human colonic carcinoma HT-29 cell population is characterized by a high rate of autophagic degradation of N-linked glycoproteins substituted with ER-type glycans. In the present work we demonstrate that glucosidase inhibitors [castanospermine (CST) and deoxynojirimycin] have a stabilizing effect on newly synthesized glucosylated N-linked glycoproteins and impaired their lysosomal delivery as shown by subcellular fractionation on Percoll gradients. The inhibition of macroautophagy was restricted to N-linked glycoproteins because macroautophagic parameters such as the rate of sequestration of cytosolic markers and the fractional volume occupied by autophagic vacuoles were not affected in CST-treated cells. The protection of glucosylated glycoproteins from autophagic sequestration was also observed in inhibitor-treated Chinese hamster ovary (CHO) cells and in Lec23 cells (a CHO mutant deficient in glucosidase I activity). The interaction of glucosylated glycoproteins with the ER chaperone binding protein (BiP) was prolonged in inhibitor-treated cells in comparison with untreated CHO cells. These results show that the removal of glucose from N-glycans of glycoproteins is a key event for their delivery to the autophagic pathway and that interaction with BiP could prevent or delay newly synthesized glucosylated N-linked glycoproteins from being sequestered by the autophagic pathway.

Key words: autophagy, castanospermine, endoplasmic reticulum, lysosome, HT-29 cells.

## INTRODUCTION

Macroautophagy is one of the most dynamic and complex cellular catabolic routes. It is responsible for the bulk degradation of cytoplasmic macromolecules and organelles in lysosomes [1,2]. Macroautophagy is stimulated and becomes the principal degradative process of proteins in response to changes in the nutrient and hormonal environment [3].

Although the wrapping membrane that forms the nascent autophagic vacuole might have different origins [4], the role of ribosome-free regions of the rough endoplasmic reticulum (ER) [5,6] or that of a possible related structure called the phagophore [7] in the sequestration of material to form nascent autophagosomes has been documented. These vacuoles acquire lysosomal enzymes by direct fusion with either lysosomes or late endosomes/prelysosomes [8,9].

The non-selective sequestration of material is reflected by the fact that the organelle content of autophagic vacuoles has a composition close to that of the cytoplasm. The ER is the most abundantly sequestered material in hepatocytes, whereas mitochondria, peroxisomes and free ribosomes are less well represented [10]. This bulk sequestration is also demonstrated by the non-selective sequestration of cytosolic proteins [11]. However, selective autophagic sequestration towards rough ER, smooth ER, peroxisomes, mitochondria and proteins in comparison with RNA [12–16] might also occur under certain circumstances.

We have shown that human colonic carcinoma HT-29 cells are a suitable cell model for investigating the molecular control of macroautophagy and its effect on glycoconjugate metabolism [17–19]. In these cells, a pool of newly synthesized N-linked glycoproteins bearing ER-type high-mannose glycans ($Man_{8-9}GlcNAc_2$) is degraded along the lysosomal–autophagic pathway [18,20] by a trimeric $G_{i3}$ protein-dependent mechanism [19].

Here we show that the glucosylated N-linked glycoproteins that accumulate in the ER are insensitive to autophagic sequestration, despite continued autophagy in glucosidase-inhibited cells [21,22]. Similar results were observed in castanospermine (CST)-treated Chinese hamster ovary (CHO) cells and in the glucosidase I-deficient CHO cell line Lec23 [23]. Glucosylated N-linked glycoproteins that accumulated in CST-treated cells were shown to have a prolonged interaction time with the ER chaperone binding protein (BiP) in comparison with that observed in untreated HT-29 cells. These results therefore suggest that interaction with BiP prevents or delays newly synthesized glucosylated N-linked glycoproteins from entering the autophagic pathway.

## MATERIALS AND METHODS

### Reagents

CST and 1-deoxymannojirimycin (dMM) were from Cambridge Research Biochemicals (Cambridge, Cambs., U.K.) and Toronto Research Chemicals (Toronto, Canada) respectively. 1-Deoxynojirimycin (dNM), apyrase and all other chemicals were purchased from Sigma (Saint Louis, MO, U.S.A.). 3-Methyladenine (3-MA) was from Fluka (Saint-Quentin Fallavier, France). Cell culture reagents and Geneticin (G418) were from Life Technologies (Eragny, France). Nitrocellulose membrane was from Schleicher & Schuell (Dassel, Germany). Bicinchoninic acid kit was from Pierce. Pansorbin and pronase grade CB were from

Abbreviations used: BiP, binding protein; CHO, Chinese hamster ovary; CST, castanospermine; ConA, concanavalin A; dMM, 1-deoxymannojirimycin, dNM, 1-deoxynojirimycin; endo H, endoglycosidase H; ER, endoplasmic reticulum; 3-MA, 3-methyladenine.

[1] To whom correspondence should be addressed (e-mail codogno@vjf.inserm.fr).

Calbiochem (Meudon, France) and endoglycosidase H (endo H) was from Genzyme (Cambridge, MA, U.S.A.). AG 1-X2 (acetate form) and AG 50W-X2 ($H^+$ form) resins and horseradish peroxidase anti-rabbit, anti-mouse IgG were from Bio-Rad (Hercules, CA, U.S.A.). Plastic TLC plates coated with silica were from Merck (Darmstadt, Germany). Concanavalin A (ConA)–Sepharose and Protein A–Sepharose were from Pharmacia Biotech (Uppsala, Sweden). The radioisotopes UDP-[$^{14}$C]galactose (300 mCi/mmol), L-[$^{35}$S]methionine/cysteine cell labelling mix (more than 1000 Ci/mmol), L-[$^{14}$C]valine (288.5 mCi/mmol) and the ECL™ (enhanced chemiluminescence) Western blotting detection kit were purchased from Amersham (Little Chalfont, Bucks., U.K.). D-[2-$^3$H]Mannose (20–30 Ci/mmol) and [$^3$H]raffinose (5–15 Ci/mmol) were from NEN Dupont de Nemours (Les Ulis, France). Rabbit polyclonal anti-BiP and mouse monoclonal anti-BiP (10C3) antibodies were from StressGen Biotechnologies Corp. (Victoria, BC, Canada).

### Cultured cells

HT-29 cells and stable clones of HT-29 ($G_{\alpha i3}$-overexpressing cells and Q204L-expressing cells) were selected and cultured as described previously [19,24]. Parental CHO cells were cultured in Ham's F12 medium supplemented with 2 mM glutamine and 10 % (v/v) heat-inactivated fetal calf serum at 37 °C under air/$CO_2$ (9:1). The Lec23 mutant cells (provided by Dr. P. Stanley, Albert Einstein College of Medicine, New York, NY, U.S.A.) were cultured in minimum essential alpha medium supplemented with RNA and DNA precursors and 10 % (v/v) heat-inactivated fetal calf serum.

### Cell labelling and N-glycan analysis

HT-29 cells were radiolabelled with D-[2-$^3$H]mannose (400 $\mu$Ci/ml) for 10 min and then chased for the indicated durations in the presence of 10 mM mannose and 2 mM fucose [25]. Glycosidase inhibitors (2 mM dMM or 2 mM CST) were added 6 h before the labelling period and were kept throughout the time course of pulse–chase experiments. When used, 3-MA (10 mM) was present throughout the chase period. N-linked glycoproteins were isolated from delipidated cell homogenates. After treatment with pronase, glycopeptides were analysed by chromatography on a column of Bio-Gel P6 equilibrated with 0.1 M pyridine acetate, pH 5.0, before and after treatment with endo H as described [25].

### TLC

Released high-mannose oligosaccharides were desalted on combined columns of AG 1-X2 (acetate form) and AG 50W-X2 ($H^+$ form). Oligosaccharides were resolved on plastic TLC plates coated with silica (Merck, Darmstadt, Germany), which were developed in acetic acid/propan-1-ol/water (3:3:2, by vol.) for 12 h [26]. Resolved components were detected on Hyperfilm-MP (Amersham) by fluorography. Standard oligosaccharides were prepared from HepG2 cells as described [26].

### Macroautophagic parameters

#### Autophagic sequestration of [$^3$H]raffinose and lactate dehydrogenase

[$^3$H]Raffinose and lactate dehydrogenase sequestrations were monitored exactly as reported previously [18,24].

#### Electron microscopy and morphometry analysis

Confluent HT-29 cells were cultured for 3 h in a nutrient-free medium to stimulate macroautophagy before being fixed for 2 h with 2.5 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer, embedded in Epon and processed for transmission electron microscopy by standard procedures. For morphometric analysis, 10–14 electron micrographs (magnification × 34000) were used for each condition. The fractional volume represented by autophagic vacuoles was quantified by the method of Weibel et al. [27], assuming a spherical shape for the calculation. Morphological criteria were used to identify autophagic vacuoles [8].

#### Measurement of the degradation of [$^3$H]mannose- and [$^{14}$C]valine-labelled glycoproteins

Cells were incubated in complete medium with D-[2-$^3$H]mannose (20 $\mu$Ci/ml) plus dMM (2 mM) or L-[14C]valine (0.2 $\mu$Ci/ml) for 18 h at 37 °C. After three rinses with PBS, cells were incubated in serum-free, amino acid-free medium: Hank's balanced salt solution (HBSS) containing BSA (0.1 %) supplemented with either 10 mM mannose/2 mM fucose or 10 mM valine. When required, 2 mM CST or 10 mM dNM was added throughout the pulse–chase experiment and 10 mM 3-MA was added throughout the chase period. After the first 1 h of the chase period, at which time short-lived glycoproteins were being degraded, the medium was replaced by the appropriate fresh medium and the incubation was prolonged for an additional 4 h [24]. Wash-out experiments were performed after radiolabelling with D-[2-$^3$H]mannose (20 $\mu$Ci/ml) in the presence of 2 mM dMM and 2 mM CST for 18 h. Cells were then rinsed three times with PBS and chased as described above in the appropriate medium without CST. At the end of the chase period, the radiolabelled glycoproteins were precipitated with 10 % (w/v) trichloroacetic acid/1 % (v/v) phosphotungstic acid at 4 °C, as described previously [24].

### Subcellular fractionation

$G_{\alpha i3}$-overexpressing HT-29 cells pulse-chased (24 h) with D-[2-$^3$H]mannose in the presence of 2 mM dMM and 100 $\mu$g/ml leupeptin were submitted to subcellular fractionation on Percoll gradients, as reported previously [18].

### Enzymic and protein assays

Glucose-6-phosphatase (EC 3.1.3.9), galactosyltransferase (EC 2.4.1.38) and $\beta$-hexosaminidase (EC 3.2.1.52) were used as specific ER/Golgi and lysosomal markers respectively. Enzymic activities were measured as described previously [18].

### Interaction of N-linked glycoproteins with BiP

CHO cells were pulse-labelled with [$^{35}$S]methionine/cysteine mix (0.2 mCi/ml) for 30 min and then chased at the indicated times in the presence of unlabelled methionine and cysteine (each at 5 mM). When used, 2 mM CST was added 6 h before labelling and throughout the experiment. Cells were lysed in a buffer containing 2 % (w/v) CHAPS, 200 mM NaCl, 50 mM Hepes, pH 7.6, chymostatin, leupeptin, antipapain, pepstatin (each at 10 $\mu$g/ml) and apyrase (30 m-units/ml) or ATP (10 mM) [28]. Nuclei were pelleted by centrifugation at 15000 ***g*** for 5 min. Lysates were precleared at 4 °C with pansorbin and then incubated with the polyclonal anti-BiP antibody (1:100 dilution) and Protein A–Sepharose beads for 3 h. After three washes in a buffer containing 0.5 % (w/v) CHAPS, 200 mM NaCl and 50 mM Hepes, pH 7.6, bound glycoproteins were removed by being heated in PBS containing 0.2 % SDS and 0.5 % (w/v) CHAPS and then divided into two equal parts. A first aliquot of immunoprecipitated proteins was loaded on to a ConA–Sepharose column equilibrated in 0.025 M Tris/HCl, pH 7.5, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.1 M NaCl. After washing of the column, glycoproteins bearing

high-mannose type chains were eluted by the same buffer supplemented with 0.3 M $\alpha$-methylmannopyrannoside and the radioactivity was determined. A second portion of the immunoprecipitated proteins was analysed by Western blotting with the monoclonal anti-BIP antibody (1:100 dilution).

## RESULTS

### Autophagic degradation of N-linked glycoproteins is inhibited in glucosidase-inhibitor-treated HT-29 cells

We have previously shown that the lysosomal–autophagic pathway is responsible for the degradation of a pool of newly synthesized N-linked glycoproteins in HT-29 cells [18]. Autophagic sequestration was shown to be dependent on the expression and activity of an intracellular heterotrimeric $G_{i3}$ protein ($\alpha i3\beta\gamma$). Wild-type $G_{\alpha i3}$ overexpression increases the rate of autophagic sequestration in HT-29 cells [19]. In contrast, autophagic sequestration was very low in cells expressing a $G_{\alpha i3}$ protein with no GTPase activity (Q204L) [24].

Macroautophagy is known to be the major catabolic pathway for long-lived proteins (reviewed in [1,2]). The degradation of long-lived N-linked glycoproteins was followed after cell labelling with [$^3$H]mannose for 16 h and then chasing the cells for 5 h in a serum-free, amino acid-free medium to stimulate the macroautophagic pathway. The first 1 h of the 5 h chase period was not considered in the estimation of the rate of degradation because short-lived glycoproteins are not degraded by the autophagic pathway [24]. At the end of the 4 h chase period, radiolabelled glycoproteins were precipitated with 10 % (w/v) trichloroacetic acid/1 % (v/v) phosphotungstic acid. In all the experiments reported in Figure 1, cells were treated with 2 mM dMM, an inhibitor of mannosidase I, to avoid the loss of radioactivity due to N-glycan trimming during the pulse–chase period. From results shown in Figure 1, the rate of degradation of [$^3$H]mannose-labelled glycoprotein was 5.6 %/h in the absence of 3-MA, an inhibitor of autophagic sequestration [29], and 2.5 %/h in the presence of the drug in the parental untransfected HT-29 cells (Figure 1A). Thus the calculated rate of $^3$H-labelled N-linked glycoprotein degradation along the autophagic pathway was 3.1 %/h in this cell population. In $G_{\alpha i3}$-overexpressing cells the rate of autophagic degradation was 6.0 %/h. In contrast, the rate of glycoprotein degradation along the autophagic pathway in Q204L-expressing cells (0.36 %/h) was approx. 1/10 of that in parental HT-29 cells (Figure 1A). These rates of [$^3$H]mannose-labelled glycoprotein degradation are in good agreement with the rate of [$^{14}$C]valine-labelled long-lived protein degradation measured in HT-29 cells, $G_{\alpha i3}$-overexpressing cells HT-29 and Q204L-expressing HT-29 cells respectively [24].

After treatment of cells with CST we observed a marked decrease in the rate of N-linked glycoprotein degradation in both parental and $G_{\alpha i3}$-overexpressing cells. Furthermore, 3-MA did not greatly increase the inhibition of N-linked glycoprotein degradation in CST-treated cells (Figure 1A). These results strongly suggested that CST was a potent inhibitor of the macroautophagic degradation of N-linked glycoproteins in HT-29 cells. This conclusion was supported by the fact that CST had no significant effect on the rate of N-linked glycoprotein degradation in Q204L-expressing cells (Figure 1A), which have a low rate of autophagy. In addition, CST did not significantly modify the lactacystin-sensitive degradation of [$^3$H]mannose- or [$^{14}$C]-valine-labelled (glyco)proteins (results not shown). This result suggests that the cytosolic proteasome-dependent degradative

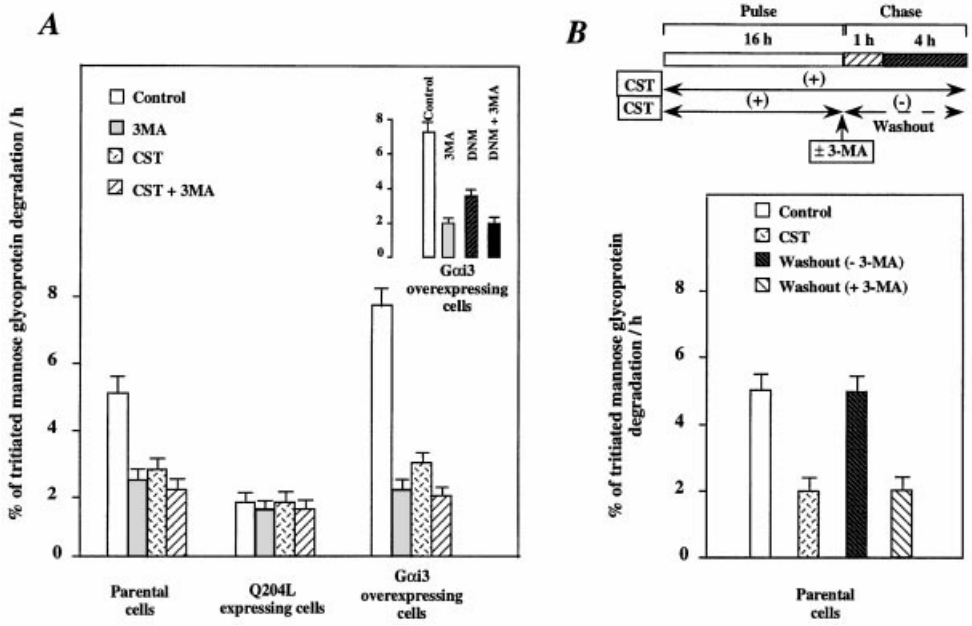


**Figure 1 Effect of glucosidase inhibitors on autophagic degradation of long-lived N-linked glycoproteins**

(**A**) HT-29 cells were radiolabelled for 18 h with [$^3$H]mannose (20 $\mu$Ci/ml) in complete medium in the presence of 2 mM dMM. When required, experiments were performed in the presence of 2 mM CST or 10 mM dNM (inset). The rate of [$^3$H]mannose-labelled long-lived glycoproteins was then measured in cells incubated in serum-free, amino acid-free medium as described in the Materials and methods section. Experiments were performed in the presence or absence of 10 mM 3-MA. Values are expressed as percentages of cellular protein degraded in 1 h. (**B**) Washout experiments were performed in the presence or absence of 10 mM 3-MA and CST. The protocol used is summarized in the upper part of the panel. After a 1 h chase, fresh medium was added and the protein degradation was measured at the end of the 4 h incubation period. Values are means $\pm$ S.E.M. for five separate experiments.

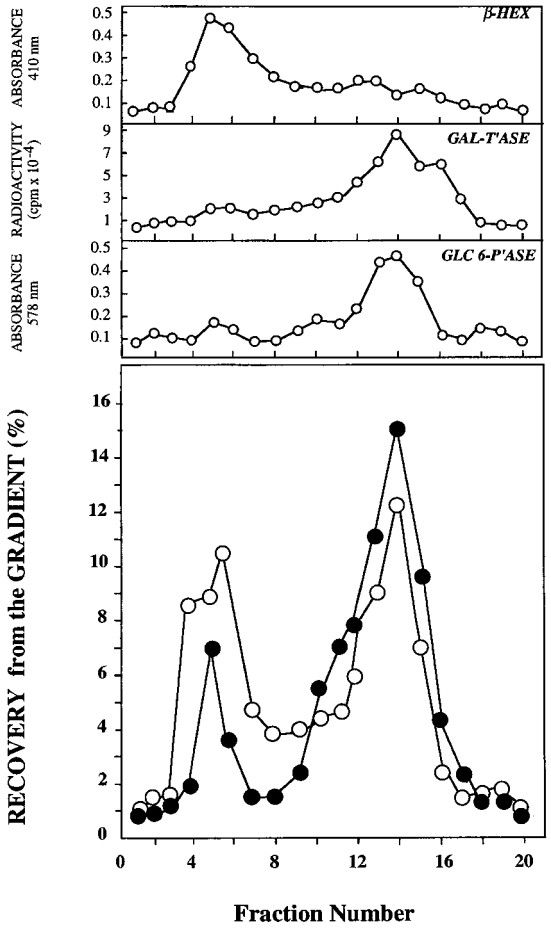


**Figure 2 Subcellular fractionation of high-mannose glycoproteins in untreated and CST-treated HT-29 cells**

Cells treated (●) or not (○) with CST were radiolabelled for 10 min with 50 $\mu$Ci/ml of [$^3$H]mannose. Subcellular fractionation was performed with a 30% (v/v) Percoll gradient after a chase period of 24 h in the presence of 2 mM dMM and 100 $\mu$g/ml leupeptin. The top three panels represent the sedimentation position of lysosomes, Golgi apparatus and ER determined by the measurements of $\beta$-hexosaminidase ($\beta$-HEX), $\beta$-1,4-galactosyltransferase (GAL-T'ASE) and glucose-6-phosphatase (GLC 6-P'ASE) activities respectively. The positions of organelle markers along the gradient were the same in untreated cells (○) and in CST-treated cells (results not shown). High-mannose glycoproteins were precipitated in each fraction with trichloroacetic acid/phosphotungstic acid and the radioactivity was counted (bottom panel). Note that a larger amount of radioactivity associated with high-mannose glycoproteins is recovered in the ER/Golgi fraction from CST-treated cells than from untreated cells. These subcellular fractionation profiles are representative of four independent experiments.

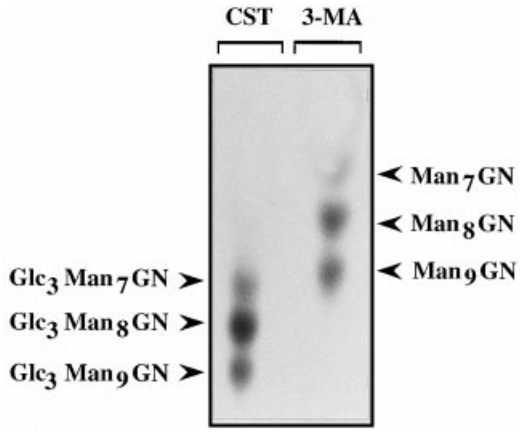


**Figure 3 Analysis of high-mannose oligosaccharides in HT-29 cells treated with CST or 3-MA**

HT-29 cells were incubated (6 h) or not with 2 mM CST before being labelled with 400 $\mu$Ci/ml D-[2-$^3$H]mannose for 10 min followed by a 24 h chase. When required, 3-MA (10 mM) was added to the chase medium. Oligosaccharides accumulated on N-linked glycoproteins were released by treatment with endo H and analysed by TLC as described in the Materials and methods section. Abbreviation: GN, GlcNAc.

**Table 1 Measurement of the degradation of [$^{14}$C]valine-labelled and [$^3$H]mannose-labelled glycoproteins along the macroautophagic pathway in CHO and Lec23 cells**

The degradation of long-lived proteins was measured in CHO and Lec23 cells incubated with nutrient-free medium, in untreated cells (control) and in 3-MA-treated or CST-treated cells as described in the Materials and methods section. The rate of protein degradation was calculated from the acid-soluble radioactivity recovered from both cells and medium. Values (means ± S.E.M. for three separate experiments) are expressed as percentages of cellular protein degraded in 4 h. Autophagic degradation was calculated as (control − 3-MA)/4. The rate of inhibition induced by CST was calculated from the expression 100 × [(control − CST)/(control − 3-MA)].

| | [$^{14}$C]Valine | | [$^3$H]Mannose | |
|---|---|---|---|---|
| | CHO | Lec23 | CHO | Lec23 |
| Degradation in 4 h (%) | | | | |
| Control | 16.3 ± 1.8 | 12.4 ± 1.3 | 13.6 ± 1.0 | 9.3 ± 1.1 |
| 3-MA (10 mM) | 8.4 ± 0.8 | 6.3 ± 0.7 | 6.5 ± 0.7 | 7.4 ± 0.5 |
| CST (2 mM) | 12.6 ± 0.8 | 11.8 ± 1.0 | 8.6 ± 0.5 | 9.2 ± 0.8 |
| Autophagic degradation (%/h) | 1.97 | 1.52 | 1.77 | 0.47 |
| Inhibition by CST (%) | 46.8 | 9.8 | 70.4 | 5.2 |

pathway is not altered by CST under the experimental conditions used.

To verify that the effect of CST on the autophagic degradation of N-linked glycoproteins was due only to its inhibitory properties on glucosidase activities, we tested the effect of the piperidine derivative dNM, another glucosidase inhibitor different from the indolizidine derivative CST. dNM was almost as potent as CST in its ability to inhibit the macroautophagic degradation of N-linked glycoproteins in $G_{\alpha i3}$-overexpressing cells (Figure 1A, inset) and in parental HT-29 cells (results not shown). In addition, the inhibitory effect of CST on glycoprotein degradation was not the consequence of a toxic side effect, because the inhibition of 3-MA-sensitive glycoprotein degradation was reversed after a wash-out period without CST in parental cells (Figure 1B) and in $G_{\alpha i3}$-overexpressing cells (results not shown).

## Lysosomal delivery of N-linked glycoproteins is impaired in CST-treated cells

The distribution of [$^3$H]mannose-labelled glycoproteins in parental HT-29 cells was analysed by subcellular fractionation on a Percoll gradient after a 10 min pulse and a 24 h chase period (Figure 2). These experiments were performed in the presence of 2 mM dMM to avoid the loss of radioactivity due to N-glycan trimming. Leupeptin (100 $\mu$g/ml) was also added to inhibit the lysosomal degradation of N-linked glycoproteins [18,20]. As shown previously, the radiolabelled material from both control and CST-treated cells seemed to be distributed mainly in two regions of the gradient corresponding to the position of the lysosomal marker ($\beta$-hexosaminidase) and ER/Golgi markers (glucose-6-phosphatase and $\beta$-1,4-galactosyltransferase) [18].

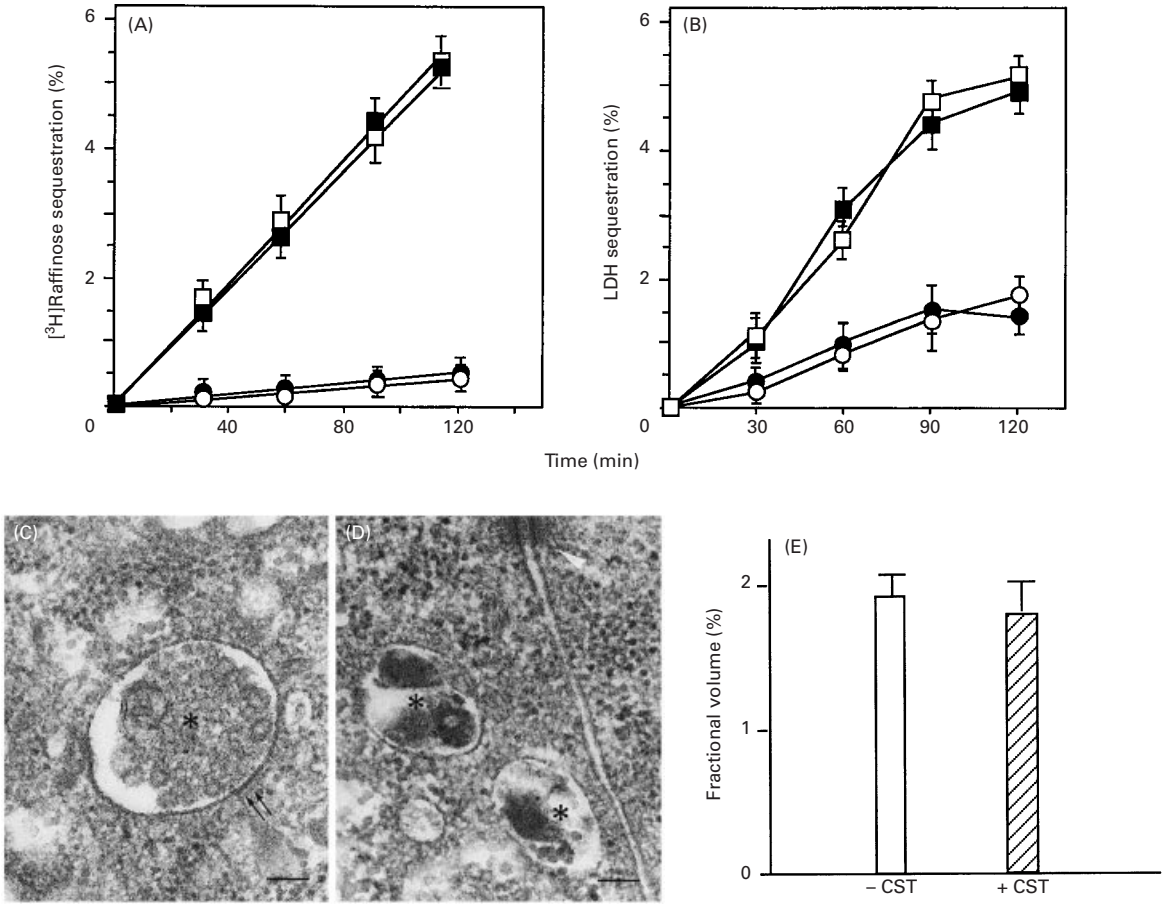


**Figure 4 Effect of CST on macroautophagy in HT-29 cells**

(**A**) HT-29 cells were electroloaded with 2 mCi of [$^3$H]raffinose, then incubated at 37 °C for various durations in the absence (□) or presence of 3-MA (○), CST (■) or CST plus 3-MA (●). After cell disruption and centrifugation, the radioactivity was measured in sedimentable material. (**B**) The activity of sequestered lactate dehydrogenase in sedimentable material was also measured in cells incubated in the absence (□) or presence of 2 mM CST (●), 100 $\mu$g/ml leupeptin (□) or CST plus leupeptin (■). Values are means ± S.E.M. for five separate experiments. Abbreviation: LDH, lactate dehydrogenase. (**C**, **D**) Representative electron micrographs of nascent (**C**) and degradative (**D**) autophagic vacuoles in HT-29 cells treated with CST. Note the double membrane delimiting the early autophagic vacuole (arrows) and more condensed intravacuolar material (asterisks) in the degradative vacuole (**D**) than in the nascent autophagic vacuole (**C**). Scale bars, 1 $\mu$m. (**E**) Columns represent the fractional volumes (as percentages) occupied by autophagic vacuoles in untreated (−CST) or CST-treated (+CST) HT-29 cells. Values are means ± S.E.M. for 10–14 morphological analyses.

However, their quantitative distributions differed. When the sum of the radioactivity recovered in the ER/Golgi region (fractions 12–17) and in the lysosomal regions (fractions 3–7) was set to 100 %, we calculated that 66 % and 82 % (a significant difference, $P < 0.001$) of the radiolabelled glycoproteins were present in the ER/Golgi region in untreated and CST-treated cells respectively. As CST and dNM inhibited the macroautophagic degradation of N-linked glycoproteins we reasoned that the lysosomal delivery of these molecular species should be impaired by glucosidase inhibitors.

### Inhibition of N-linked glycoprotein degradation is related to glucose trimming

To demonstrate that the CST-induced inhibition of the macroautophagic degradation of N-linked glycoproteins was due to the persistence of glucose residues on N-glycans, we first analysed the glucose content in N-glycans from CST-treated HT-29 cells; next we investigated the rate of autophagic N-linked glycoproteins degradation in a glucosidase-I-deficient cell line.

An analysis of the pattern of oligosaccharides released from [$^3$H]mannose-labelled glycoproteins by endo H showed an accumulation of glucosylated high-mannose oligosaccharides in CST-treated cells (Figure 3). In contrast, $Man_9GlcNAc_1$ and $Man_8GlcNAc_1$ were the main species observed in 3-MA-treated cells (Figure 3). This pattern was identical with that previously reported in HT-29 cells (results not shown) [20].

Among the different CHO mutants, the Lec23 cells deficient in glucosidase I activity have been shown to accumulate glycoproteins substituted with high-mannose glycans containing three glucose residues [23]. This feature is reminiscent to that observed in CHO cells treated with CST.

Macroautophagic degradation has been investigated in both CHO and Lec23 cells after pulse–chase experiments with [$^{14}$C]valine or [$^3$H]mannose as described in the Materials and methods section. The rate of macroautophagic degradation of [$^{14}$C]valine-labelled proteins was only slightly decreased in Lec23 cells in comparison with that observed in CHO cells (1.97 %/h compared with 1.52 %/h) (Table 1). In contrast, the rate of macroautophagic degradation of [$^3$H]mannose-labelled glycoproteins in Lec23 cells was less than one-third of that in CHO cells (Table 1). In the presence of CST a 70 % inhibition of the

macroautophagic degradation of [$^3$H]mannose-labelled glycoproteins was observed in CHO cells. In contrast, CST did not significantly affect the rate of macroautophagic degradation of [$^3$H]mannose-labelled glycoproteins in Lec23 cells. The decreased rate of degradation of [$^{14}$C]valine-labelled proteins measured in CST-treated CHO cells was in fact due to the incorporation of [$^{14}$C]valine in both N-linked glycoproteins and proteins.

The results reported above suggested that the inhibition of glucose trimming specifically impairs the autophagic degradation of N-linked glycoproteins without interfering with the autophagic degradation of non-glycosylated proteins. To investigate whether or not CST interfered with the overall macroautophagic pathway we next investigated the different parameters of the autophagic pathway. For this purpose we chose HT-29 cells because this cell line has a well-developed autophagic pathway, which facilitates the detection of potential modifications of autophagic parameters induced by CST.

### CST does not affect the macroautophagic capacity of HT-29 cells

Besides the rate of long-lived protein degradation, the macroautophagic pathway can be quantified by the analysis of (1) the rate of autophagic sequestration of cytosolic enzymes (lactate dehydrogenase) and of electroloaded [$^3$H]raffinose (a trisaccharide insensitive to mamalian lysosomal glycohydrolases) and (2) the fractional volume occupied by autophagic vacuoles in the cytoplasm.

Incubation of HT-29 cells with 2 mM CST did not change the rate of sequestration of electroloaded [$^3$H]raffinose measured in the presence or absence of 3-MA (Figure 4A). Similarly, CST did not affect the rate of lactate dehydrogenase sequestration measured in the presence or absence of leupeptin (Figure 4B). The low recovery of sequestered lactate dehydrogenase measured in the absence of leupeptin indicated that lysosomal degradation of cytosolic proteins is still active in CST-treated HT-29 cells. Consistent with these results was the observation of early and degradative autophagic vacuoles at the ultrastructural level in HT-29 cells treated with CST (Figures 4C and 4D). Moreover, calculation of the fractional volume occupied by autophagic vacuoles from electron micrographs showed that CST did not perturb the formation and maturation of autophagic vacuoles (Figure 4E).

From these results we concluded that CST did not alter the macroautophagic pathway but selectively decreased the sequestration of N-linked glycoproteins.

### Interaction of N-linked glycoproteins and BiP

To start to understand the mechanism by which CST inhibits the macroautophagic degradation of N-linked glycoproteins, we analysed their interaction with the ER chaperone BiP. Indeed, CST interferes with the quality control of newly synthesized N-linked glycoproteins in the ER [28]. Inhibition of the trimming of glucose residues impairs the interaction of monoglucosylated glycoproteins and calnexin and/or calreticulin and increases the interaction time with BiP. Thus the reduced sequestration of newly synthesized N-linked glycoproteins in autophagic vacuoles could be due to an extended duration of their interaction with the chaperone BiP. This hypothesis was explored in CHO cells, because none of the antibodies available satisfactorily immunoprecipitated BiP in the HT-29 cell model. Cells were pulse-labelled for 20 min with a [$^{35}$S]methionine/cysteine mix, then chased for 10 min. Samples were immunoprecipitated with the polyclonal anti-BiP antibody and immunoprecipitates were incubated with the mannose-specific lectin ConA. After a 10 min chase, the ConA-bound fraction was 2.6-fold higher in CST cells

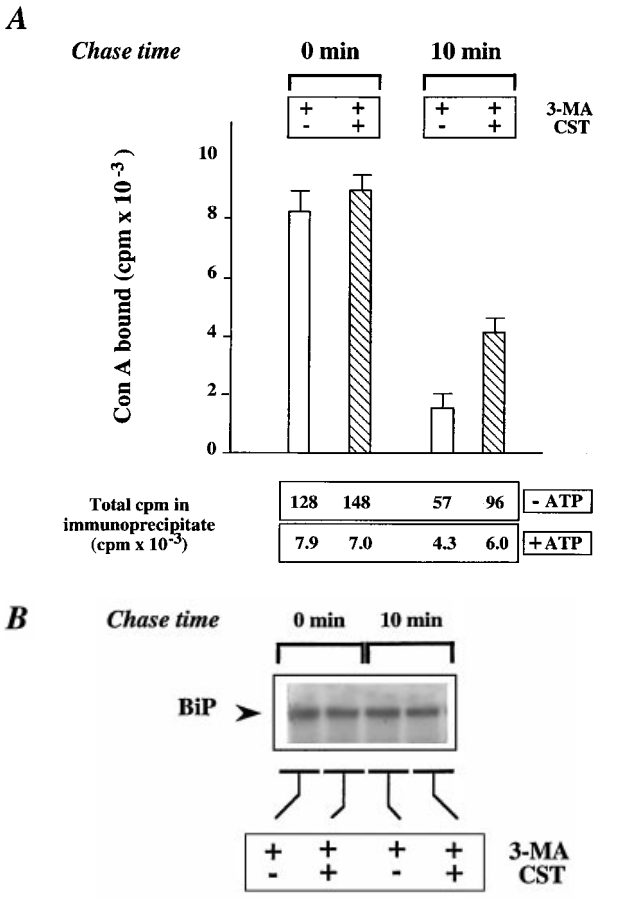


**Figure 5 Interaction of [$^{35}$S]methionine-labelled glycoproteins and BiP**

(**A**) The quantity of [$^{35}$S]methionine-labelled high-mannose glycoproteins interacting with BiP was determined on CHO cell homogenates immunoprecipitated with the polyclonal anti-BiP antibody. Immunoprecipitates were incubated with immobilized ConA. 3-MA-treated or 3-MA plus CST-treated cells were radiolabelled with 0.2 mCi/ml [$^{35}$S]methionine/cysteine mix for 20 min and thereafter chased for 10 min. Values are means ± S.E.M. for three separate experiments. (**B**) Illustration of a Western blot of immunoprecipitates performed with the monoclonal anti-BiP antibody in the absence of ATP.

than in untreated cells (Figure 5A). The smaller amount of radioactivity in the ConA-binding material in untreated cells was due neither to variations in the content of BiP recovered after immunoprecipitation, as demonstrated by Western blotting with the monoclonal anti-BiP antibody (Figure 5B), nor to the autophagic degradation of ER-associated material, because 3-MA was present during the experiment (Figure 5A).

The specificity of the assay was controlled by the ATP-dependence of the interaction between BiP and ConA-binding material. ATP has been shown to favour the dissociation of BiP from its substrates [30]. The depletion of ATP from the assay by treatment with apyrase favoured the interaction of ConA-retained material with BiP. In contrast, when apyrase was replaced by 10 mM ATP in the assay the recovery of ConA-retained material in the anti-BiP immunoprecipitate was markedly decreased (Figure 5A).

## DISCUSSION

It is now well established that N-glycans are important in the intracellular fate of glycoproteins including quality control in

the ER and lysosomal targeting [31,32]. The disposal of N-linked glycoproteins in the macroautophagic pathway is also dependent on the structure of their oligosaccharide chains. Pioneering works have shown that an accumulation of autophagic vacuoles occurs in the liver from rats treated with the mannosidase inhibitor swainsonine [33]. More recently, Tulsiani and Touster [34] demonstrated that swainsonine inhibits the fusion of autophagic vacuoles with the lysosomal compartment in liver from rats fed with this inhibitor. We also showed that swainsonine stabilizes the high-mannose glycoproteins trapped in the autophagic pathway in cultured HT-29 cells [35]. However, the mechanism by which swainsonine inhibits the maturation of autophagic vacuoles still remains poorly understood. Here we show that the inhibition of ER glucosidases impairs the degradation of N-linked glycoproteins along the macroautophagic pathway. The observation made in HT-29 cells was confirmed in two other cell lines, CHO cells and the glucosidase-I-deficient Lec23 cells. These results provide a line of evidence that the autophagic sequestration of ER-associated glycoproteins is not a bulk process but that selectivity exists, depending mainly on the structure of their carbohydrate moiety. Moreover, this selectivity seems to be related to the ER-associated quality control exerted on newly synthesized N-linked glycoproteins. This control is based on the recognition of polypeptides during their folding by chaperone molecules such as BiP [36], proteins involved in the disulphide bond formation (protein disulphide isomerase) [37] and the interaction of the ER lectins calnexin and calreticulin with monoglucosylated high-mannose oligosaccharides [31]. Newly synthesized N-linked glycoproteins have three glucose residues on their carbohydrate chains [38]. The inhibition of the removal of the two outermost residues has been shown to impair the interaction of glycoproteins with calnexin [39] and/or with its soluble counterpart calreticulin [40]. This has at least two consequences: the first to prolong the interaction of glycoproteins with BiP and delay their export from the ER [41,42], and the second to trigger the ER-associated degradation of some glycoproteins [43,44].

The marked effect of inhibition of the removal of the outermost glucose residues from N-glycans on the delivery of N-linked glycoproteins in the macroautophagic pathway is not a consequence of a non-specific impairment of the macroautophagic pathway. We fail to detect any significant change in the rate of autophagic sequestration of cytosolic markers, as well as in the rate of degradation of sequestered material in CST-treated cells. Accordingly, the fractional volume occupied by autophagic vacuoles is close to that observed in untreated HT-29 cells. These results suggest that glucosylated N-linked glycoproteins are retained in a region of the ER insensitive to autophagic sequestration.

Macroautophagy is considered to be a non-selective degradative mechanism of proteins including ER-associated proteins. This conclusion is based on the recovery of a large panel of resident ER proteins, including chaperones, in autophagic vacuoles [5,6,12,45]. ER chaperones are in large excess compared with newly synthesized (glyco)proteins and exist as a network in the lumen of the ER [46,47]. The results provided in the present study suggest that a selectivity exists in macroautophagic degradation depending on the N-glycan profile of ER-associated proteins.

Macroautophagy is rapidly modulated in response to changes occurring in the cell environment [3]. The impairment of sequestration of glycoproteins undergoing quality control in the ER would leave them the opportunity to be transported out of the ER when macroautophagy declines. This timing can also allow a rapid renewal of stress-damaged glycoproteins in a less energy-consuming manner in comparison with the non-selective degradation of the ER, which would require a synthesis of glycoproteins and ER-associated machinery *de novo*.

Phosphorylation of the ribosomal protein S6 inhibits autophagic sequestration [48]. It has been suggested that the phosphorylated S6 might stimulate ribosome binding to ER and decrease the ribosome-free region of ER engaged in the formation of autophagic vacuoles in higher eukaryotic cells [5]. Our results showing that N-linked glycoproteins that escape from autophagic sequestration have a prolonged interaction time with BiP give credence to the idea that ribosome binding to ER membrane and/or peptides emerging into the ER lumen are critical in the control of autophagic sequestration [5,48]. The chaperone BiP is known to have different functions in the ER. Besides its role in protein folding [36], BiP contributes to the bidirectional movement of polypeptides through the translocation apparatus [49] and maintains the permeability barrier of the rough ER membrane [50]. Further studies will be required to investigate the relationships between the different functions of BiP and autophagic sequestration.

We thank P. Stanley (Albert Einstein College of Medicine, New York, NY, U.S.A.) for the gift of the Lec23 cells; M. Laburthe (INSERM U410) for access to facilities during the course of this work; and S. E. H. Moore for critical reading of the manuscript. This work was supported by the INSERM and by grants from the Association pour la Recherche sur le Cancer (no. 9716) and the Association Vaincre les Maladies Lysosomales.

## REFERENCES

1 Dunn, Jr., W. A. (1994) Trends Cell Biol. **4**, 139–143
2 Seglen, P. O. and Bohley, P. (1992) Experientia **48**, 158–172
3 Mortimore, G. E. and Kadowaki, M. (1994) in Cellular Proteolytic Systems (Ciechanover, A. J. and Schwartz, A. L., eds.), pp. 65–87, Wiley-Liss, New York
4 Yamamoto, A., Masaki, R., Fukui, Y. and Tashiro, Y. (1990) J. Histochem. Cytochem. **38**, 1571–1581
5 Dunn, Jr., W. A. (1990) J. Cell Biol. **110**, 1923–1933
6 Ueno, T., Muno, D. and Kominami, E. (1991) J. Biol. Chem. **266**, 18995–18999
7 Seglen, P. O. (1987) in Lysosomes: Their Role in Protein Breakdown (Glauman, H. and Ballard, F. J., eds.), pp. 369–414, Academic Press, London
8 Dunn, Jr., W. A. (1990) J. Cell Biol. **110**, 1935–1945
9 Punnonen, E. L., Autio, S., Kaija, H. and Reunanen, H. (1993) Eur. J. Cell Biol. **61**, 54–66
10 Schworer, C. M., Shiffer, H. A. and Mortimore, G. E. (1981) J. Biol. Chem. **256**, 7652–7658
11 Kopitz, J., Kisen, G. Ø., Gordon, P. B., Bohley, P. and Seglen, P. O. (1990) J. Cell Biol. **111**, 941–953
12 Lenk, S. E., Bhat, D., Blakeney, W. and Dunn, Jr., W. A. (1992) Am. J. Physiol. **263**, E856–E862
13 Mortimore, G. E., Pösö, A. R., Kadowaki, M. and Wert, Jr., J. J. (1987) J. Biol. Chem. **262**, 16322–16327
14 Luiken, J. J. F. P., Vandenberg, M., Heikoop, J. C. and Meijer, A. J. (1992) FEBS Lett. **304**, 93–97
15 Partin, J. C., Schubert, W. K. and Partin, J. S. (1971) New Engl. J. Med. **285**, 1339–1343
16 Lardeux, B. R. and Mortimore, G. E. (1987) J. Biol. Chem. **262**, 14514–14519
17 Ghidoni, R., Houri, J. J., Giuliani, A., Ogier-Denis, E., Parolari, E., Botti, S., Bauvy, C. and Codogno, P. (1996) Eur. J. Biochem. **237**, 454–459
18 Houri, J. J., Ogier-Denis, E., De Stefanis, D., Bauvy, C., Baccino, F. M., Isidoro, I. and Codogno, P. (1995) Biochem. J. **309**, 521–527
19 Ogier-Denis, E., Couvineau, A., Maoret, J. J., Houri, J. J., Bauvy, C., De Stefanis, D., Isidoro, C., Laburthe, M. and Codogno, P. (1995) J. Biol. Chem. **270**, 13–16
20 Trugnan, G., Ogier-Denis, E., Sapin, C., Darmoul, D., Bauvy, C., Aubery, M. and Codogno, P. (1991) J. Biol. Chem. **266**, 20849–20855
21 Pan, Y. T., Hori, H., Saul, R., Sanford, B. A., Molyneux, R. J. and Elbein, A. D. (1983) Biochemistry **22**, 3975–3984
22 Saunier, B., Kilker, Jr., R. D., Tkacz, J. S., Quaroni, A. and Herscovics, A. (1982) J. Biol. Chem. **257**, 14155–14161
23 Ray, M. K., Yang, J., Sundaram, S. and Stanley, P. (1991) J. Biol. Chem. **266**, 22818–22825
24 Ogier-Denis, E., Houri, J. J., Bauvy, C. and Codogno, P. (1996) J. Biol. Chem. **271**, 28593–28600

25 Ogier-Denis, E., Codogno, P., Chantret, I. and Trugnan, G. (1988) J. Biol. Chem. **263**, 6031–6037
26 Moore, S. E. H. and Spiro, R. G. (1994) J. Biol. Chem. **269**, 12715–12721
27 Weibel, E. R., Stäubli, W., Gnägi, H. R. and Hess, F. A. (1969) J. Cell Biol. **42**, 68–91
28 Hammond, C. and Helenius, A. (1994) Science **266**, 456–458
29 Seglen, P. O. and Gordon, P. B. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 1889–1892
30 Munro, S. and Pelham, H. R. B. (1986) Cell **46**, 291–300
31 Helenius, A., Trombetta, E. S., Hebert, D. N. and Simons, J. F. (1997) Trends Cell Biol. **7**, 193–200
32 Dahms, N. M., Lobel, P. and Kornfeld, S. (1989) J. Biol. Chem. **264**, 12115–12118
33 Novikoff, P. M., Touster, O., Novikoff, A. B. and Tulsiani, D. R. P. (1985) J. Cell Biol. **101**, 339–349
34 Tulsiani, D. R. P. and Touster, O. (1992) Arch. Biochem. Biophys. **296**, 556–561
35 Houri, J. J., Ogier-Denis, E., Bauvy, C., Aubery, M., Sapin, C., Trugnan, G. and Codogno, P. (1992) Eur. J. Biochem. **205**, 1169–1174
36 Haas, I. G. (1994) Experientia **50**, 1012–1020
37 Freedman, R. B. (1989) Cell **57**, 1069–1072
38 Kornfeld, R. and Kornfeld, S. (1985) Annu. Rev. Biochem. **54**, 631–663
39 Ware, F. E., Vassilakos, A., Peterson, P. A., Jackson, M. R., Lehrman, M. A. and Williams, D. B. (1995) J. Biol. Chem. **270**, 4697–4704
40 Spiro, R. G., Zhu, Q., Bhoyroo, V. and Soling, H. D. (1996) J. Biol. Chem. **271**, 11588–11594
41 Hammond, C. and Helenius, A. (1994) J. Cell Biol. **126**, 41–52
42 Muresan, Z. and Arvan, P. (1998) Mol. Endocrinol. **12**, 458–467
43 Kearse, K. P., Williams, D. B. and Singer, A. (1994) EMBO J. **13**, 3678–3686
44 Moore, S. E. H. and Spiro, R. G. (1993) J. Biol. Chem. **268**, 3809–3812
45 Marzella, L., Ahlberg, J. and Glaumann, H. (1982) J. Cell Biol. **93**, 144–154
46 Kuznetsov, G., Chen, L. B. and Nigam, S. K. (1997) J. Biol. Chem. **272**, 3057–3063
47 Tatu, U. and Helenius, A. (1997) J. Cell Biol. **136**, 555–565
48 Blommaart, E. F. C., Luiken, J. J. F. P., Blommaart, P. J. E., Vanwoerkom, G. M. and Meijer, A. J. (1995) J. Biol. Chem. **270**, 2320–2326
49 Nicchitta, C. V. and Blobel, G. (1993) Cell **73**, 989–998
50 Hamman, B. D., Hendershot, L. M. and Johnson, A. E. (1998) Cell **92**, 747–758

Received 11 August 1999/7 October 1999; accepted 5 November 1999