THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 6, Issue of February 11, pp. 4035-4041, 1994
Printed in U.S.A.

Localization of *O*-Glycan Initiation, Sphingomyelin Synthesis, and Glucosylceramide Synthesis in Vero Cells with Respect to the Endoplasmic Reticulum-Golgi Intermediate Compartment*

(Received for publication, August 19, 1993, and in revised form, October 1, 1993)

Anja Schweizer‡, Henrik Clausen§, Gerrit van Meer¶, and Hans-Peter Hauri‖

From the Department of Pharmacology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland, the §School of Dentistry, Faculty of Health Sciences, DK-2200 Copenhagen, Denmark, and the ¶Department of Cell Biology, Medical School, University of Utrecht, 3584 CX Utrecht, The Netherlands

The identification of an endoplasmic reticulum-Golgi intermediate compartment (ERGIC), defined by the 53-kDa transmembrane marker protein ERGIC-53, has added to the complexity of the exocytic pathway of higher eukaryotic cells. Recently, a subcellular fractionation procedure was established for the isolation of the ERGIC from Vero cells (Schweizer, A., Matter, K., Ketcham, C. M., and Hauri, H.-P. (1991) *J. Cell Biol.* 113, 45–54) which provides a means to study more precisely the compartmentalization of the various enzymic functions along the early secretory pathway. Here, we have investigated if *O*-glycan initiation and sphingomyelin synthesis are associated with the ERGIC by analyzing both the responsible enzyme activities and their corresponding products. Moreover, the synthesis of glucosylceramide, the precursor of most glycosphingolipids, was also analyzed. In the purified ERGIC fraction UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (GalNAc transferase) was only minimally enriched, sphingomyelin synthase was not enriched, and UDP-glucose:ceramide-glucosyl transferase specific activity was lower than in the homogenate. On Percoll gradients all three enzymes cofractionated with Golgi markers rather than ERGIC-53. Accordingly, sphingomyelin concentrations were extremely low in the ERGIC fraction. Double immunofluorescence localization of core *N*-acetylgalactosamine, the product of GalNAc transferase, by monoclonal antibodies against GalNAc-Ser/Thr (Tn antigen) revealed only little apparent overlap with ERGIC-53. This was particularly evident in brefeldin A-treated cells which showed entirely different patterns of Tn antigens and ERGIC-53. The results suggest that in the secretory pathway of Vero cells *O*-glycan initiation and sphingomyelin as well as glucosylceramide synthesis mainly occur beyond the ERGIC in the Golgi apparatus.

The use of yeast genetics and the development of cell-free transport assays are providing exciting insights into the molecular machinery responsible for conveying proteins between the endoplasmic reticulum (ER)[1] and the Golgi apparatus (Balch, 1989, 1990; Pryer *et al.*, 1992; Mellman and Simons, 1992; Rothman and Orci, 1992). A pressing limitation in our understanding of the mechanisms underlying the ER to Golgi exocytic pathway concerns the difficulty of relating the various biochemical events to the structural organization of the endomembrane system (Hauri and Schweizer, 1992). This uncertainty is mainly a result of the lack of appropriate markers necessary to resolve all of the consecutive membrane compartments of the secretory pathway. In a first step toward resolving this issue we have identified a 53-kDa membrane marker protein (ERGIC-53) for an ER-Golgi intermediate compartment (ERGIC) in which protein transport from ER to Golgi is blocked at 15 °C (Schweizer *et al.*, 1988, 1990; Schindler *et al.*, 1993). Using ERGIC-53 as a marker protein, a procedure was developed for the isolation of the ERGIC from Vero cells (Schweizer *et al.*, 1991). The purified ERGIC fraction is only minimally contaminated with rough ER markers such as ribophorins, protein disulfide isomerase, and immunoglobulin-binding protein, and it is largely separable from the two *cis*-Golgi enzymes *N*-acetylglucosaminyl phosphotransferase and *N*-acetylglucosaminyl-1-phosphodiester-α-*N*-acetylglucosaminidase that generate the lysosomal targeting signal mannose 6-phosphate in a sequential manner. This membrane preparation allows, therefore, a more precise localization of the various functions associated with the ER to Golgi pathway.

In the present study we have focused our attention on *O*-glycan initiation as well as sphingomyelin and glucosylceramide synthesis. The key enzyme of *O*-glycan initiation is UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (GalNAc transferase). It catalyzes the transfer of *N*-acetylgalactosamine (GalNAc) to specific serine and threonine residues of the protein backbone (Elhammer and Kornfeld, 1986). The localization of *O*-glycan initiation mediated by GalNAc transferase remains controversial. Although a majority of investigators favor the *cis*-Golgi as the initiation site (Roth, 1983; Deschuyteneer *et al.*, 1988; Piller *et al.*, 1990 and references therein; Pascale *et al.*, 1992) other studies point to rough ER (Perez-Vilar *et al.*, 1991; Ellinger and Pavelka, 1992) or a post-ER pre-Golgi compartment, the budding compartment of mouse corona virus (Tooze *et al.*, 1988). These studies rely predominantly on biochemical evidences since the localization of GalNAc transferase by immunoelectron microscopy has not been achieved yet.

* This study was supported by the Kanton of Basel Stadt, the Swiss National Science Foundation, the Danish Medical Council, and the Lundbeck Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Hematology/Oncology, Washington University School of Medicine, St. Louis, MO 63110.

‖ To whom correspondence should be addressed: Dept. of Pharmacology, Biocenter of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel/Switzerland. Tel.: 41-61-267-2222 or 2229; Fax: 41-61-267-2208.

[1] The abbreviations used are: ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; GalNAc transferase, UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase; GlcCer, glucosylceramide; GlcCer synthase, UDP-glucose:ceramide glucosyl transferase; mAb, monoclonal antibody; Tn, GalNAc-α-Ser/Thr; sialosyl-Tn, NeuAc-α2–6GalNAc-Ser/Thr; TRITC, tetramethylrhodamine B isothiocyanate; FITC, fluorescein isothiocyanate.

Sphingomyelin, an important lipid of the outer leaflet of the plasma membrane and the luminal leaflet of Golgi, endosomes, and lysosomes, is synthesized from ceramide by the transfer of phosphorylcholine directly from phosphatidylcholine, a reaction that is catalyzed by sphingomyelin synthase (Bernert and Ullman, 1981; Marggraf *et al.*, 1981; Voelker and Kennedy, 1982; Marggraf and Kanfer, 1984). Ceramide is synthesized at the cytosolic surface of the ER (Mandon *et al.*, 1992; Hirschberg *et al.*, 1993) and is transported to the Golgi apparatus by an as yet unknown mechanism (Collins and Warren, 1992; Moreau *et al.*, 1993). Recent subcellular fractionation experiments suggest that the *cis*/medial Golgi is the major site of sphingomyelin synthesis (Futerman *et al.*, 1990; Jeckel *et al.*, 1990, 1992), although some activity has been assigned to the plasma membrane (Futerman *et al.*, 1990; Kallen *et al.*, 1993; van Helvoort *et al.*, 1994). Newly synthesized sphingomyelin is subsequently transported to the plasma membrane by a vesicular route (see Koval and Pagano, 1991; van Meer and Burger, 1992; van Meer, 1993). The precise localization of sphingomyelin synthase at the ultrastructural level is unknown, and a contribution of the ERGIC to sphingomyelin synthesis may be suggested by the observation that sphingomyelin synthase did not redistribute to the ER in the presence of brefeldin A (van Meer and van 't Hof, 1993), a behavior that mimics that of ERGIC-53 but not that of *cis*-Golgi enzymes (Lippincott-Schwartz *et al.*, 1990; Schweizer *et al.*, 1993).

Ceramide also serves as a precursor for glucosylceramide (GlcCer) that in turn is the precursor of the complex glycosphingolipids (van Meer and Burger, 1992). The glucosyl transferase responsible for the synthesis of GlcCer, GlcCer synthase, has been localized to the Golgi apparatus by subcellular fractionation (Coste *et al.*, 1985; Futerman and Pagano, 1991; Jeckel *et al.*, 1992). Contrary to sphingomyelin synthase, GlcCer synthase activity was found not to be restricted to the *cis*-Golgi, and it has been postulated that GlcCer may be in part synthesized in an ER-Golgi intermediate compartment because significant GlcCer synthase activity was associated with a membrane fraction of density intermediate between ER and *cis*/medial Golgi apparatus (Futerman and Pagano, 1991). However, this membrane fraction was not characterized in terms of ERGIC markers.

Here we have studied the fractionation of membranes possessing GalNAc transferase, sphingomyelin synthase, or GlcCer synthase activities with respect to that of the ERGIC. Together with the assessment of the corresponding products this analysis suggests that the ERGIC of Vero cells does not appreciably contribute to *O*-glycan initiation, sphingomyelin synthesis, or GlcCer synthesis.

MATERIALS AND METHODS

Reagents—The following antibodies were used: mAb G1/93 or a rabbit polyclonal antibody against human ERGIC-53 (Schweizer *et al.*, 1988, 1991), a rabbit polyclonal antibody against human galactosyltransferase (kindly provided by Eric Berger, University of Zürich), mAbs IE3 (an IgG2A),[2] 5F4 (an IgM; Thurnher *et al.*, 1993), and TKH6 (an IgM)[3] against GalNAc-Ser/Thr (Tn antigen), mAbs TKH2 (an IgG1; Kjeldsen *et al.*, 1988) and 3F1 (an IgG1)[4] against NeuAc-α2–6GalNAc-Ser/Thr (sialosyl-Tn antigen), mAb 1C12 (an IgM),[4] recognizing both Tn and sialosyl-Tn. The anti-Tn mAbs were produced using asialo-ovine submaxillary mucin as immunogen essentially as described (Hirohashi *et al.*, 1985; Takahashi *et al.*, 1988) and found not to react with blood group A antigens.

Cell Culture—Vero cells were grown in Falcon Optilux dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 1% nonessential amino acids, 100 milliunits of penicillin, 100 μg/ml streptomycin, and 1 μg/ml fungizone under standard tissue culture conditions. For immunofluorescence experiments the Vero cells were grown in eight-well multichamber slides.

Immunofluorescence Microscopy—Single and double immunofluorescence experiments were performed as described in Schweizer *et al.* (1988). All of the primary mAbs were concentrated 10-fold from hybridoma supernatants by ammonium sulfate precipitation and used at appropriate dilutions. For double immunofluorescence localizations, the formaldehyde-fixed, saponin-permeabilized cells were sequentially incubated with an IgG mAb, goat anti-mouse IgG-TRITC, IgM mAb, goat anti-mouse IgM-FITC. In some experiments the sequence of incubations was rabbit polyclonal antibody, goat anti-rabbit-TRITC, IgM mAb, goat anti-mouse-FITC. The specimens were examined with a Reichert Polyvar microscope.

Subcellular Fractionation—The ERGIC fraction (F3 fraction) was isolated exactly as described (Schweizer *et al.*, 1991). In brief, Vero cells were homogenized in a ball-bearing cell cracker. A postnuclear supernatant was prepared and fractionated on a Percoll gradient (10.5% Percoll, 41 min at 36,900 × g_{av}) followed by equilibrium centrifugation on a discontinuous metrizamide gradient (18–20 h at 85,800 × g_{av}). The 18.5/27% interphase was pelleted in phosphate-buffered saline and recovered as F3 ERGIC fraction. ERGIC-53 was 41-fold enriched on average in the F3 fraction (Schweizer *et al.*, 1991). Fractions were analyzed immediately for marker proteins or frozen at −20 °C prior to analysis of GalNAc transferase, sphingomyelin synthase, and GlcCer synthase.

Enzyme Assays and Quantification of ERGIC-53—GalNAc transferase:Homogenate and fractions were diluted 1:1 in 1% Triton X-100, 20 mM Tris/HCl (pH 6.5), 100 mM NaCl, 10 mM $MnCl_2$, and sonicated for 30 s. In all assays 50 μl of this stock was used as enzyme source in a reaction volume of 100 μl. The final reaction mixture contained 50 mM Tris/HCl (pH 7.4), 50 mM NaCl, 5 mM $MnCl_2$, 250 μM UDP-[^{14}C]GalNAc (specific activity: 12,000 cpm/nmol or 29,281 cpm/nmol), 0.5% Triton X-100, and 10–50 μg of peptide (in most cases 20 μg of 16-amino acid peptide LSESTTQLPGGGPGCA, with the first 10 amino acids being the NH_2-terminal sequence of glycophorin A). The amino acid sequence and nomenclature of the peptide substrates used in the present study are as follows.

Glycophorin A: LSESTTQLPGGGPGCA.

Muc2 (human intestinal mucin): PTTTPISTTTMVTPTPT.

Muc1 (human breast/pancreas mucin): APPAHGVTSAPDTRPAPGSTAPC.

Muc1a: PDTRPAPGSTAPPAC.

Muc1b: APPAHGVTSAPDTRPAPGC.

Human choriogonadotropin β-chain): PRFQDSSSSKAPPPSLPSPSRL.

The peptides were synthesized by Carlbiotech (Copenhagen, Denmark). The reaction product was isolated by chromatography on Dowex 1-X8 (Cl-form), and the radioactivity was counted in a liquid scintillation counter. An identical reaction mixture without peptide substrate was used to obtain a blank value that was subtracted from the values of the probes. In some cases the product was also analyzed by C-18 reverse phase fast pressure liquid chromatography on a Smart™ system (Pharmacia LKB Biotechnology Inc.) for control of incorporation of GalNAc into undegraded peptide. Generally no degradation was observed.

Sphingomyelin synthase and GlcCer synthase were measured as described previously (Jeckel *et al.*, 1992). All activities were measured in the linear range of the assay, and it was ensured that the concentrations of the liposomes containing the short chain ceramide and of UDP-glucose were saturating. Storage of gradient samples at −20 °C for up to 1 month did not affect the distribution of the enzyme activities over the gradient.

Galactosyltransferase was measured according to Stieger *et al.* (1988) and KCN-resistant NADH oxidoreductase according to Sottocasa *et al.* (1967). ERGIC-53 was quantified by densitometric scanning of immunoblots (Schweizer *et al.*, 1991).

Lipid Analysis—Lipids were extracted according to Bligh and Dyer (1959) and separated by two-dimensional thin layer chromatography (van Meer and van 't Hof, 1993). The lipids were visualized by iodine staining, and phosphate in the individual phospholipid spots was determined according to Rouser *et al.* (1970).

RESULTS

O-Glycan Initiation—To study if the ERGIC is the site of *O*-glycan initiation the distribution of GalNAc transferase was first studied by Percoll gradient centrifugation and compared with that of ERGIC-53 (Fig. 1). On a 10.5% Percoll gradient

[2] H. Clausen and S. Hakomori, unpublished data.

[3] T. Kjeldsen, S. Hakomori, and H. Clausen, unpublished data.

[4] T. Pallesen and H. Clausen, unpublished data.

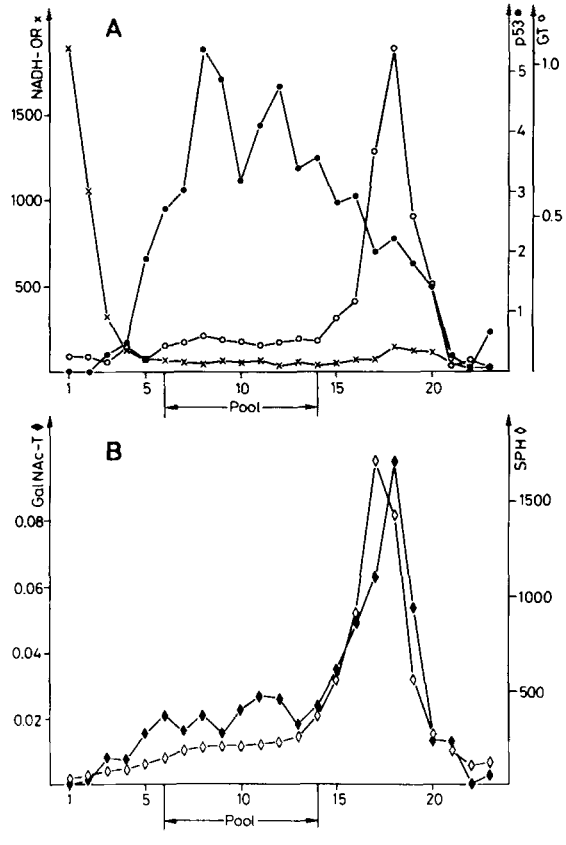

FIG. 1. **Distribution of GalNAc transferase, sphingomyelin synthase, and marker proteins on Percoll gradients.** Postnuclear supernatants from Vero cells were fractionated on 10.5% Percoll gradients. The gradients were divided into 23 fractions of 1.5 ml beginning at the bottom. *Panel A*, enzymatic activities of KCN-resistant NADH oxidoreductase (*NADH-OR*) and galactosyltransferase (*GT*) are given in milliunits/ml. ERGIC-53 (p53) was quantified by immunoblotting using a polyclonal antibody against ERGIC-53; values are given in arbitrary units/ml. *Panel B*, enzymatic profiles of GalNAc transferase (*GalNAc-T*), assayed with the glycophorin-derived peptide as a substrate given in milliunits/ml, and sphingomyelin synthase activity (*SPH*) given as pmol C_6-NBD-sphingomyelin/sample x 10 min. The two enzymes were assayed on two separate Percoll gradients that were run exactly as that shown in *panel A*. Fractions 6–14 were pooled (*Pool* in *panel A*) and refractionated on 5–30% metrizamide gradients to isolate the F3 ERGIC fraction (see Schweizer *et al.*, 1991 and Table I).

ERGIC-53 showed a broad peak in the center of the gradient which was flanked by a peak of KCN-resistant oxidoreductase at the bottom of the gradient and a peak of galactosyltransferase in the upper part of the gradient (Fig. 1*A*; Schweizer *et al.*, 1991). GalNAc transferase activity assayed on a parallel gradient with the glycophorin A-derived acceptor peptide as substrate (see "Materials and Methods") almost cofractionated with the *trans*-Golgi marker galactosyltransferase (Fig. 1*B*). The peak of GalNAc transferase coincided with that of galactosyltransferase, but there was always some trailing of the former enzyme into the broad peak of ERGIC-53. Hence the overall distribution of GalNAc- transferase was virtually identical to that reported previously for the *cis*-Golgi enzymes *N*-acetylglucosaminyl phosphotransferase and *N*-acetylglucosamine-1-phosphodiester-α-*N*-acetylglucosaminidase (Schweizer *et al.*, 1991). Five different acceptor substrates, including those derived from human glycophorin A, intestinal mucin, and breast/pancreas mucin, gave a similar distribution of GalNAc transferase on this Percoll gradient (not shown). The results suggest that GalNAc transferase codistributes with *cis*/medial Golgi markers rather than ERGIC-53.

We next determined to what extent GalNAc transferase is associated with the isolated ERGIC. The ERGIC of Vero cells can be purified 41-fold on average by Percoll followed by metrizamide gradient centrifugation as assessed by the relative enrichment of ERGIC-53. In this preparation rough ER markers such as ribophorins, protein disulfide isomerase, or immunoglobulin-binding protein and the *cis*-Golgi marker *N*-acetylglucosamine-1-phosphodiester-α-*N*-acetylglucosaminidase are maximally enriched 2–3-fold (Schweizer *et al.*, 1991). Five different acceptor substrates were used to measure GalNAc transferase activity in the homogenate and in the ERGIC fraction. Table I shows a 2.6–5.4-fold enrichment of GalNAc transferase activity in the isolated fraction, suggesting that the major localization of this enzyme in Vero cells is not the ERGIC.

The slight enrichment of GalNAc transferase activity in the ERGIC fraction could be caused either by cross-contamination of this fraction with elements of the *cis*-Golgi or by the presence of some GalNAc transferase activity in the ERGIC itself. In the latter case this activity may lead to GalNAc addition already in the ERGIC. To test for this possibility, we localized peptide-bound GalNAc residues, the products of GalNAc transferase, in Vero cells by means of mAbs against the Tn antigen in conjunction with immunofluorescence microscopy. The Tn antigen is defined as GalNAc residues directly linked *O*-glycosidically to serine or threonine residues of polypeptides (Dahr *et al.*, 1974; Hirohashi *et al.*, 1985).

Three Tn-specific mAbs and one mAb recognizing both Tn and sialosyl-Tn (*i.e.* NeuAc-α2–6GalNAc-Ser/Thr), named Tn/sialosyl-Tn, gave a specific reaction in Vero cells. Two mAbs recognizing sialosyl-Tn but not Tn gave no reaction, suggesting that sialosyl-Tn is not or only minimally present in Vero cells and that the signal produced by the mAb against Tn/sialosyl-Tn is exclusively the result of Tn antigens. Double immunofluorescence analysis with anti-Tn or anti-Tn/sialosyl-Tn and anti-ERGIC-53 showed different patterns for Tn and ERGIC-53 (Fig. 2). The Tn antigen was localized in a juxtanuclear site, whereas ERGIC-53, although also present at this site, was more spread into the cell periphery. The anti-Tn and the anti-Tn/sialosyl-Tn antibodies gave the same pattern with the exception that anti-Tn also produced some unidentified punctate labeling in the nucleus of some cells. The site of Tn labeling was identified as the Golgi apparatus by double immunofluorescence microscopy with antibodies against galactosyltransferase (Fig. 2, *e*, *f* and *g*, *h*).

To investigate if the partial overlap of ERGIC-53 and Tn was caused by the presence of some Tn in a subsite of the ERGIC, Vero cells were studied after exposure to brefeldin A. Brefeldin A is known to destroy the Golgi apparatus in most cells (Klausner *et al.*, 1992). In Vero cells brefeldin A leads to an almost uniform fine punctate staining of the cytoplasm of Golgi markers such as galactosyltransferase[5] or giantin (Linstedt and Hauri, 1993) without the appearance of a characteristic ER pattern. In contrast, ERGIC-53 redistributes into unique large dots in brefeldin-treated Vero cells (Schweizer *et al.*, 1993). Therefore, brefeldin A is useful to establish if a marker is associated with the Golgi apparatus or the ERGIC. Fig. 3 shows that Tn antigens in brefeldin-treated Vero cells redistribute like Golgi markers. Both anti-Tn and anti-Tn/sialosyl-Tn mAbs showed a fine punctate staining of the cytoplasm after brefeldin A treatment. There was no detectable Tn antigen associated with the ERGIC-53 dots, indicating that no detectable Tn is present in the ERGIC. We conclude that in Vero cells *O*-glycan initiation is an event that takes place beyond the ERGIC most likely in the *cis*-Golgi.

Sphingomyelin and Glucosylceramide Synthesis—Sphingomyelin synthase was found to cofractionate with galac-

[5] A. Schweizer and H.-P. Hauri, unpublished data.

TABLE I

Specific activities of GalNAC transferase, sphingomyelin synthase, and glucosylceramide synthase in the homogenate and in the ERGIC fraction (F3) of Vero cells

Enzyme activities are given milliunits/mg of protein for GalNAc-transferase and pmol/min/mg of protein for sphingomyelin synthase and glucosylceramide synthase. Substrates for GalNAc transferase are as specified under "Materials and Methods." The substrate for sphingomyelin synthase and glucosylceramide synthase was *N*-6(7-nitro-2,1,3-benzoxadiazol-4-yl)aminohexanyl ceramide (C_6-NBD-Cer). GphA, glycophorin A.

Enzyme and experiment no.	Substrate	Homogenate	ERGIC	Enrichment
GalNAc transferase				
1	GphA	0.012	0.036	3.0
2	GphA	0.017	0.064	3.8
	Muc2	0.026	0.129	4.9
3	Muc2	0.062	0.162	2.6
	Muc1	0.073	0.329	4.5
	Muc1a	0.073	0.396	5.4
	Muc1b	0.063	0.219	3.4
Sphingomyelin synthase				
1	C_6-NBD-Cer	12.1	15.4	1.3
2	C_6-NBD-Cer	12.2	14.2	1.2
Glucosylceramide synthase				
1	C_6-NBD-Cer	87.9	12.4	0.1
2	C_6-NBD-Cer	61.1	9.3	0.2

tosyltransferase on Percoll gradients (Fig. 1). Contrary to GalNAc transferase, however, there was little sphingomyelin synthase codistributing with ERGIC-53. Accordingly, sphingomyelin synthase was not significantly enriched in the isolated ERGIC fraction F3 (Table I). The distribution of GlcCer synthase on Percoll gradients was indistinguishable from that of sphingomyelin synthase or galactosyltransferase (not shown), and its specific activity was lower in the isolated ERGIC fraction F3 than in the corresponding homogenate (Table I).

An analysis of the lipid composition of the isolated ERGIC fraction F3 (Table II) showed a 20-fold lower sphingomyelin concentration for the ERGIC than for the homogenate, fully confirming the finding that sphingomyelin synthase is not enriched in the ERGIC fraction. The concentrations of other lipids were similar in ERGIC and homogenate with the exception of cardiolipin, which was not detectable in the ERGIC fraction. Since cardiolipin is a mitochondrial lipid, its absence from the ERGIC is not surprising. Overall these results indicate that the ERGIC does not contribute to sphingomyelin or GlcCer synthesis.

DISCUSSION

O-Glycan Initiation—Two lines of evidence presented in this paper indicate that the ERGIC is not appreciably involved in *O*-glycan initiation in Vero cells. First, GalNAc transferase was only 2.6–5.4-fold enriched in a 41-fold enriched ERGIC fraction, and second, Tn-specific antibodies used to visualize GalNAc residues *O*-glycosidically linked to serine or threonine residues did not stain the ERGIC as visualized by immunofluorescence microscopy. Although we cannot exclude a minor contribution of the ERGIC to *O*-glycan initiation we consider it more likely that the slight enrichment of GalNAc transferase activity in the ERGIC fraction is caused by contamination with elements of the *cis*-Golgi. The use of the bona fide *cis*-Golgi marker enzyme *N*-acetylglucosamine-1-phosphodiester-α-*N*-acetylglucosaminidase in a previous study has suggested that such a cross-contamination may indeed result in an up to 3-fold enrichment of *cis*-Golgi elements in the ERGIC fraction (Schweizer *et al.*, 1991). The localization of GalNAc residues by Tn-specific antibodies confirms the lack of GalNAc transferase activity. After treatment with brefeldin A, Tn antigens were distributed like Golgi markers and did not enrich in elements of the ERGIC.

Recent evidence suggests that *O*-glycan initiation is mediated by at least two different GalNAc transferases since threonine-specific enzymes have been purified which were devoid of transferase activity toward serine-containing peptides (O'Connell *et al.*, 1992; Wang *et al.*, 1992). However, Homa *et al.* (1993) purified a bovine colostrum GalNAc transferase exhibiting both threonine and serine transferase activities, albeit the serine activity was considerably lower. In the present study we have utilized several different peptide substrates including both threonine- and serine-containing peptides to exclude that a subset of the transferase activity was left undetected. Therefore, the potential presence of more than one GalNAc transferase enzyme cannot explain our failure to find evidence of *O*-glycan initiation associated with the ERGIC of Vero cells. Moreover, anti-Tn antibodies do not select between threonine and serine linkages.

A majority of studies supports the notion that *O*-glycan initiation is an early Golgi event in most cells. Perhaps the best evidence is that *O*-glycosylation of newly synthesized proteins is prevented by cooling cells to 15 °C (Piller *et al.*, 1990; Pascale *et al.*, 1992). At that temperature protein transport from ER to Golgi is blocked in the ERGIC (Schweizer *et al.*, 1990; Lotti *et al.*, 1992). A few studies, however, favor a pre-Golgi site of *O*-glycan initiation (Pathak *et al.*, 1988; Tooze *et al.*, 1988; Perez-Vilar *et al.*, 1991; Ellinger and Pavelka, 1992). Although these studies may reflect cell type-specific variations of *O*-glycosylation other interpretations may explain the findings. Pathak *et al.* (1988) reported that a class 2 mutant of low density lipoprotein receptor which failed to reach the Golgi apparatus carries serine/threonine-linked core GalNAc residues. The receptor accumulated in the rough ER and in transitional elements of the rough ER. However, it cannot be excluded that the transport block of the receptor changed the normal compartmentalization of *O*-glycosylation or that the mutated receptor cycles between ER and Golgi similar to unassembled MHC class I molecules (Hsu *et al.*, 1991). Tooze *et al.* (1988) reported the addition of GalNAc residues to the E1 glycoprotein of budding mouse hepatitis virus A59. Since the budding of this virus preferentially occurs in smooth membrane elements interposed in between rough ER and stacked Golgi the authors concluded that *O*-glycosylation starts at a pre-Golgi site. Again it is unknown if such an artificial situation of virus infection had an impact on the compartmentalization of GalNAc addition or if there was a back-flow of Golgi-glycosylated E1 from the Golgi apparatus to the budding compartment. Perez-Vilar *et al.* (1991) reported the presence of GalNAc residues in dilated subregions of the rough ER of chondrocytes

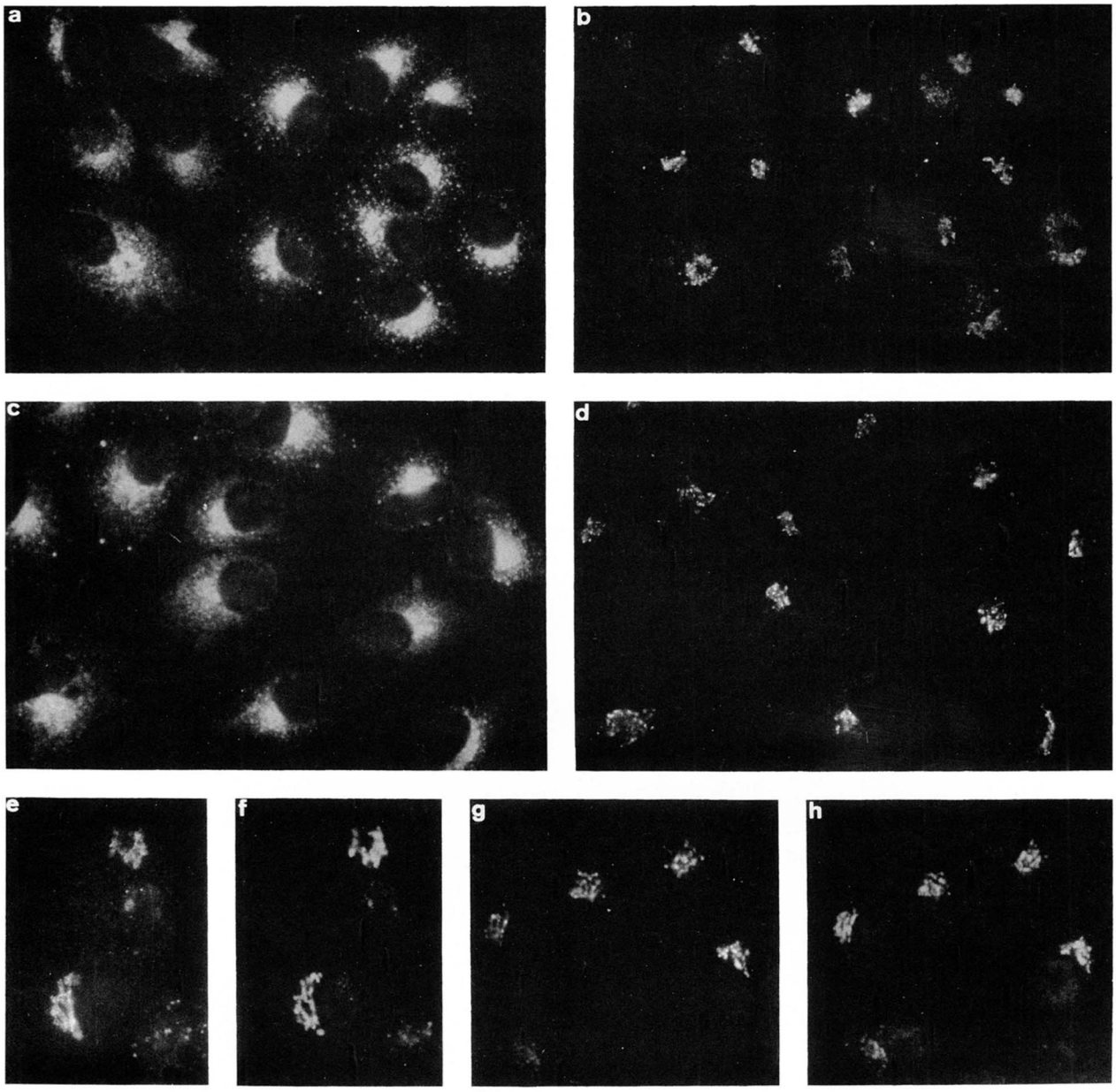

FIG. 2. **Immunofluorescence localization of Tn antigen, ERGIC-53, and galactosyltransferase in Vero cells.** *Panels a* and *b*, *c* and *d*, *e* and *f*, *g* and *h* are pairs of double immunofluorescence micrographs from experiments with mAb G1/93 against ERGIC-53 (*panels a* and *c*), mAb 5F4 against Tn (*panels b* and *e*), mAb 1C12 against Tn/sialosyl-Tn (*panels d* and *g*), and polyclonal anti-galactosyltransferase (*panels f* and *h*). The secondary antibody was rhodamine labeled in *panels a*, *c*, *f*, and *h* and fluorescein labeled in *panels b*, *d*, *e*, and *g*.

in primary culture as probed with GalNAc-specific lectins and Tn antibodies. The authors ascribed their findings to a differentiation process occurring during prolonged culture, but they may as well reflect early signs of deterioration of the primary culture. Ellinger and Pavelka (1992) found GalNAc-specific lectin reactivity in dilated rough ER subregions of colonic goblet cells *in vivo*. Unexplainably, in the ER the lectin binding was less competable with GalNAc monosaccharides than in the Golgi, raising some doubts about the specificity of the lectin reactions in this system.

Sphingomyelin and GlcCer Synthesis—Our results concerning sphingomyelin synthase are in line with the notion that sphingomyelin synthesis is a *cis*/medial Golgi event (Futerman *et al.*, 1990; Jeckel *et al.*, 1990, 1992). Sphingomyelin synthase was not enriched in the ERGIC fraction F3, and the sphingomyelin content of this fraction was extremely low (*cf.* Zambrano *et al.*, 1975; Koval and Pagano, 1991; Sodeik *et al.*, 1993). The low sphingomyelin concentration provides evidence that substantial transport of sphingomyelin from the organelle where it is synthesized to the ERGIC does not occur. If the two compartments are connected by retrograde membrane transport, this pathway must efficiently exclude sphingomyelin. Sphingomyelin synthesis in *cis*-Golgi would locate the lipid sorting event in the *cis*-Golgi (van Meer, 1993). It remains to be explained why, if sphingomyelin synthase is located in the *cis*-Golgi, it was found not to redistribute to the ER in the presence of brefeldin A (van Meer and van 't Hof, 1993). GlcCer synthase has been found to synthesize GlcCer on the cytosolic surface of Golgi membranes (Coste *et al.*, 1986; Futerman and Pagano, 1991; Trinchera *et al.*, 1991; Jeckel *et al.*, 1992) and can be solubilized by detergents (Durieux *et al.*, 1990). In a study on the subcellular fractionation of rat liver Futerman and Pagano (1991) concluded that GlcCer synthase is present in *cis*-Golgi but also in a different compartment that, judging from its density, may be a pre-Golgi compartment. In another study on rat liver Golgi it was shown that sphingomyelin synthase of rat

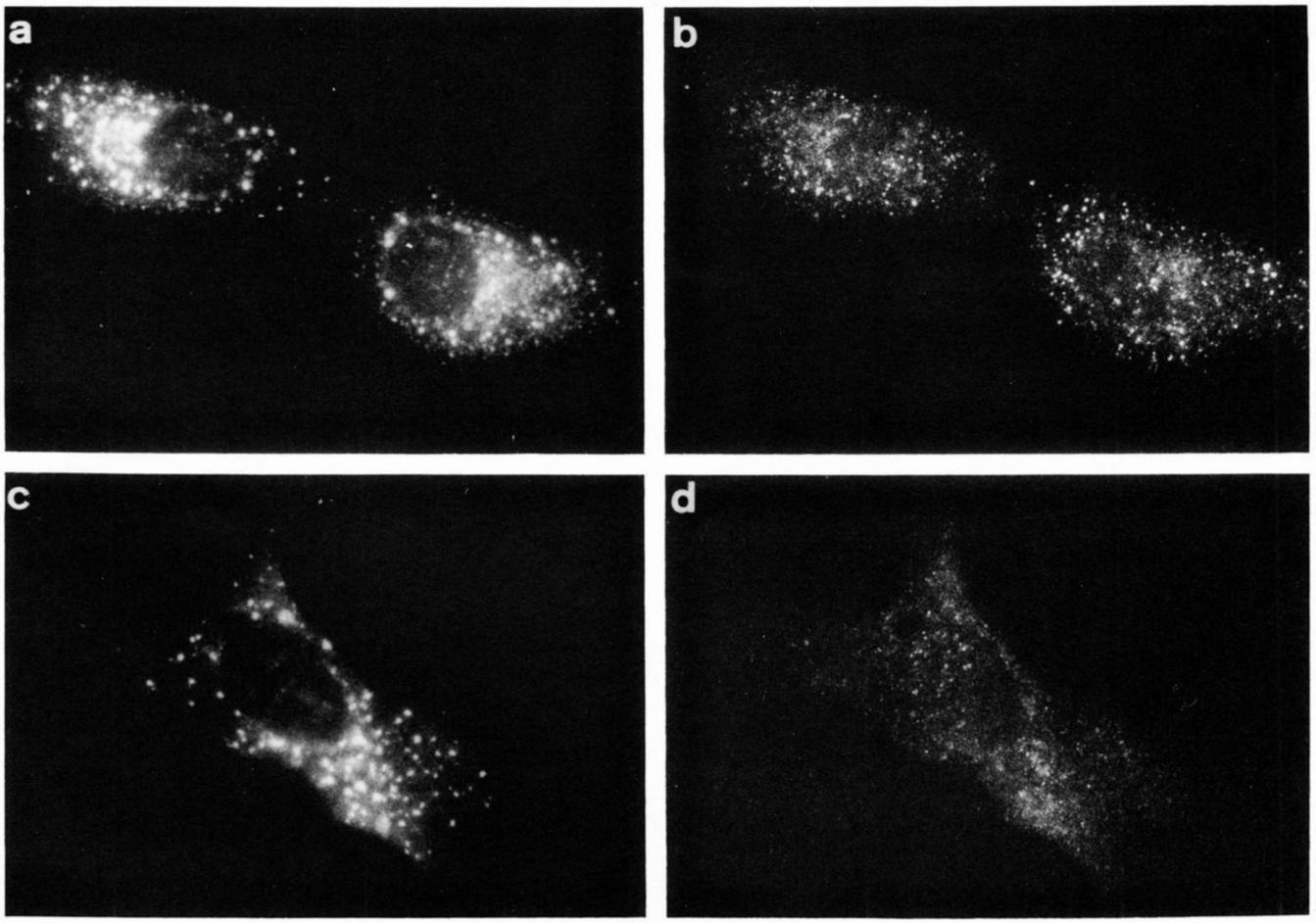

FIG. 3. **Redistribution of ERGIC-53 and Tn antigens in brefeldin A-treated Vero cells.** Vero cells were treated for 60 min with 10 μg/ml brefeldin A and then subjected to the double immunofluorescence microscopy procedure. Localization of ERGIC-53 by mAb G1/93 followed by goat anti-mouse-TRITC is shown in *panels a* and *c*; localization of Tn antigen by mAb 5F4 is shown in *panel b* and mAb 1C12 in *panel d* followed by goat anti-mouse-FITC. Note that there is no overlap of ERGIC-53 and Tn antigen.

TABLE II
Phospholipid composition of the ERGIC fraction F3 isolated from Vero cells

Numbers represent the mean of two independent experiments and are followed by the difference of the individual values from the mean. Values are given as percentage of total phosphate present in iodine detectable spots. ND, no iodine spot detected on TLC plate. The origin and front spots together contributed less than 1% to the total phosphate content.

Phospholipid	Homogenate	F3 fraction
Sphingomyelin	8.1 ± 0.3	0.4 ± 0.1
Phosphatidylcholine	44.8 ± 1.8	58.8 ± 2.2
Phosphatidylinositol	7.3 ± 0.1	8.6 ± 0.5
Phosphatidylserine	5.6 ± 0.6	3.6 ± 0.2
Phosphatidylethanolamine	29.5 ± 0.4	26.0 ± 1.3
Cardiolipin	4.1 ± 0.7	ND

liver Golgi membranes codistributes with the *cis*-Golgi marker *N*-acetylglucosaminyl phosphotransferase on sucrose gradients, whereas GlcCer synthase activity was more broadly distributed on the gradient with a second peak colocalizing with the *trans*-Golgi marker galactosyltransferase (Jeckel *et al.*, 1992). We have observed a similar distribution of the two enzymes on sucrose gradients of Vero cells (not shown). Our present evidence shows a very low activity of GlcCer synthase in the ERGIC fraction F3, even lower than that of galactosyltransferase, which is somewhat surprising for an enzyme associated, at least in part, with elements of the *cis*-Golgi. This leaves open the question of the nature of the second compartment containing GlcCer synthase activity and its implications for GlcCer transport and processing.

In conclusion, this study indicates that the ERGIC is neither the site of *O*-glycan initiation nor that of sphingomyelin or GlcCer synthesis. It is presently not possible to test whether our findings established for Vero cells can be generalized, because our attempts to isolate the ERGIC from other cell lines were unsuccessful.

Acknowledgments—We thank Henry Laning, Marion Thielemans, and Wouter van 't Hof for contributions to the lipid experiments; Eric Berger for the generous gift of antibodies to galactosyltransferase; and the Sandoz Company, Basel, for providing brefeldin A.

REFERENCES

Balch, W. E. (1989) *J. Biol. Chem.* **264,** 16965–16968
Balch, W. E. (1990) *Curr. Opin. Cell Biol.* **2,** 634–641
Bernert, J. T., and Ullman, M. D. (1981) *Biochim. Biophys. Acta* **666,** 99–109
Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
Collins, R. N., and Warren, G. (1992) *J. Biol. Chem.* **267,** 24906–24911
Coste, H., Martel, M.-B., Azzar, G., and Got, R. (1985) *Biochim. Biophys. Acta* **814,** 1–7
Coste, H., Martel, M. B., and Got, R. (1986) *Biochim. Biophys. Acta* **858,** 6–12
Dahr, W., Uhlenbruck, G., and Bird, G. W. G. (1974) *Vox Sang.* **27,** 29–42
Deschuyteneer, M., Eckhardt, A. E., Roth, J., and Hill, R. L. (1988) *J. Biol. Chem.* **263,** 2452–2459
Durieux, I., Martel, M. B., and Got, R. (1990) *Biochim. Biophys. Acta* **1024,** 263–266
Elhammer, A., and Kornfeld, S. (1986) *J. Biol. Chem.* **261,** 5249–5255
Ellinger, A., and Pavelka, M. (1992) *J. Histochem. Cytochem.* **40,** 919–930
Futerman, A. H., and Pagano, R. E. (1991) *Biochem. J.* **280,** 295–302
Futerman, A. H., Stieger, B., Hubbard, A. L., and Pagano, R. (1990) *J. Biol. Chem.* **265,** 8650–8657
Hauri, H.-P., and Schweizer, A. (1992) *Curr. Opin. Cell Biol.* **4,** 600–608
Hirohashi, S., Clausen, H., Yamada, T., Shimosato, Y., and Hakomori, S.-I. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7039–7043

Hirschberg, K., Rodger, J., and Futerman, A. H. (1993) *Biochem. J.* **290,** 751–757
Homa, F. L., Hollander, T., Lehman, D. J., Thomsen, D. R., and Elhammer, A. P. (1993) *J. Biol. Chem.* **268,** 12609–12616
Hsu, V. W., Yuan, L. C., Nuchtern, J. G., Lippincott-Schwartz, J., Hammerling, G. J., and Klausner, R. D. (1991) *Nature* **352,** 441–444
Jeckel, D., Karrenbauer, A., Birk., R., Schmidt, R., and Wieland, F. (1990) *FEBS Lett.* **261,** 155–157
Jeckel, D., Karrenbauer, A., Burger, K. N. J., van Meer, G., and Wieland, F. (1992) *J. Cell Biol.* **117,** 259–267
Kallen, J.-F., Quinn, P., and Allan, D. (1993) *Biochem. J.* **289,** 307–312
Kjeldsen, T., Clausen, H., Hirohashi, S., Ogawa, T., Iijima, H., and Hakomori, S. (1988) *Cancer Res.* **48,** 2214–2220
Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) *J. Cell Biol.* **116,** 1071–1080
Koval, M., and Pagano, R. E. (1991) *Biochim. Biophys. Acta* **1082,** 113–125
Linstedt, A., and Hauri, H.-P. (1993) *Mol. Biol. Cell* **4,** 679–693
Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E., Hauri, H.-P., Yuan, L. C., and Klausner, R. D. (1990) *Cell* **60,** 821–836
Lotti, L. V., Torrisi, M. R., Pascale, M. C., and Bonatti, S. (1992) *J. Cell Biol.* **118,** 43–50
Mandon, E. C., Ehses, I., Rother, J., van Echten, and Sandhoff, K. (1992) *J. Biol. Chem.* **267,** 11144–11148
Marggraf, W. D., and Kanfer, J. N. (1984) *Biochim. Biophys. Acta* **793,** 346–353
Marggraf, W. D., Anderer, F. A., and Kanfer, J. N. (1981) *Biochim. Biophys. Acta* **664,** 61–73
Mellman, I., and Simons, K. (1992) *Cell* **68,** 829–840
Moreau, P., Cassagne, C., Keenan, T. W., and Morré, D. J. (1993) *Biochim. Biophys. Acta* **1146,** 9–16
O'Connell, B. C., Hagen, F. K., and Tabak, L. A. (1992) *J. Biol. Chem.* **267,** 25010–25018
Pascale, M. C., Erra, M. C., Malagolini, N., Serafini-Cessi, F., Leone, A., and Bonatti, S. (1992) *J. Biol. Chem.* **267,** 25196–25202
Pathak, R. K., Merkle, R. K., Cummings, R. D., Goldstein, J. L., Brown, M. S., and Anderson, R. G. W. (1988) *J. Cell Biol.* **106,** 1831–1841
Perez-Vilar, J., Hidalgo, J., and Velasco, A. (1991) *J. Biol. Chem.* **266,** 23967–23976
Piller, V., Piller, F., and Fukuda, M. (1990) *J. Biol. Chem.* **265,** 9264–9271
Pryer, N. K., Wuestenhube, L. J., and Schekman, R. (1992) *Annu. Rev. Biochem.* **61,** 471–516
Roth, J. (1983) *J. Cell Biol.* **98,** 399–406
Rothman, J. E., and Orci, L. (1992) *Nature* **355,** 409–415
Rouser, G., Fleischer, S., and Yamamoto, A. (1970) *Lipids* **5,** 494–496
Schindler, R., Itin, C., Zerial, M., Lottspeich, F., and Hauri, H.-P. (1993) *Eur. J. Cell Biol.* **61,** 1–9
Schweizer, A., Fransen, J. A. M., Baechi, T., Ginsel, L., and Hauri, H.-P. (1988) *J. Cell Biol.* **107,** 1643–1653
Schweizer, A., Fransen, J. A. M., Matter, K., Kreis, T. E., Ginsel, L., and Hauri, H.-P. (1990) *Eur. J. Cell Biol.* **53,** 185–196
Schweizer, A., Matter, K., Ketcham, C. M., and Hauri, H.-P. (1991) *J. Cell Biol.* **113,** 45–54
Schweizer, A., Ericson, M., Bächi, T., Griffiths, G., and Hauri, H.-P. (1993) *J. Cell Sci.* **104,** 671–683
Sodeik, B., Doms, R. W., Ericsson, M., Hiller, G., Machamer, C. E., van 't Hof, W., van Meer, G., Moss, B., and Griffiths, G. (1993) *J. Cell Biol.* **121,** 521–541
Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967) *J. Cell Biol.* **32,** 415–438
Stieger, B., Matter, K., Baur, B., Bucher, K., Hoechli, M., and Hauri, H.-P. (1988) *J. Cell Biol.* **106,** 1853–1861
Takahashi, H. K., Metoki, R., and Hakomori, S. (1988) *Cancer Res.* **48,** 4361–4367
Thurnher, M., Clausen, H., Sharon, N., and Berger, E. G. (1993) *Immunol. Lett.*, in press
Tooze, S. A., Tooze, J., and Warren, G. (1988) J. Cell Biol. **106,** 1475–1488
Trinchera, M., Fabbri, M., and Ghidoni, R. (1991) *J. Biol. Chem.* **266,** 20907–20912
van Helvoort, A., van 't Hof, W., Ritsema, T., Sandra, A., and van Meer, G. (1994) *J. Biol. Chem.*, in press
van Meer, G. (1993) *Curr. Opin. Cell Biol.* **5,** 661–673
van Meer, G., and Burger, K. N. J. (1992) *Trends Cell Biol.* **2,** 332–337
van Meer, G., and van 't Hof, W. (1993) *J. Cell Sci.* **104,** 833–842
Voelker, D. R., and Kennedy, E. P. (1982) *Biochemistry* **21,** 2753–2759
Wang, Y., Abernethy, J. L., Eckhardt, A. E., and Hill, R. L. (1992) *J. Biol. Chem.* **267,** 12709–12716
Zambrano, F., Fleischer, S., and Fleischer, B. (1975) *Biochim. Biophys. Acta* **380,** 357–369