Association to HeLa Cells and Surface Behavior of Exogenous Gangliosides Studied with a Fluorescent Derivative of GM1†

M. Masserini,* A. Giuliani, P. Palestini, D. Acquotti, M. Pitto, V. Chigorno, and G. Tettamanti

Study Center for the Functional Biochemistry of Brain Lipids, Department of Medical Chemistry and Biochemistry, The Medical School, University of Milan, 20133 Milan, Italy

Received April 24, 1989; Revised Manuscript Received August 1, 1989

ABSTRACT: Cultured HeLa cells were incubated with pyrene-GM1/^{3}H-radiolabeled GM1 ganglioside (1:4 M/M) mixtures for various times. The process of association of pyrene-GM1 with cells was qualitatively and quantitatively the same as that of ^{3}H-GM1. The pyrene-GM1 and ^{3}H-GM1 proportions in the various forms of association with cells were similar to that of the starting ganglioside mixture. After 2-h incubation, the association of ganglioside with cells was well established whereas almost no metabolic processing had occurred. During a 24-h incubation, pyrene- and ^{3}H-GM1 underwent similar metabolic processing and gave rise to catabolic (GM2 and GM3) and anabolic (GD1a) derivatives. Fluorescence spectroscopy experiments carried out with the excimer formation technique on subcellular fractions containing plasma membranes showed that exogenous ganglioside was, in part, associated with the cells in a micellar form removable by trypsin treatment, and in part inserted in a seemingly molecular dispersion. Addition of Ca^{2+} salts caused aggregation of the ganglioside, as indicated by the increase of the excimer:monomer fluorescence ratio. The phenomenon was Ca^{2+} concentration dependent (maximum at 10 mM), and subsequent addition of EDTA had no effect. The saccharide portion of exogenously incorporated pyrene-GM1 was available to interact with external ligands, as shown by its ability to bind cholera toxin whose addition reduced the collision rate among the ganglioside lipid moieties.

A widely used approach for studying the functional implications of gangliosides is their addition to "in vitro" cellular systems, followed by determination of the induced biological effects. These are assumed to derive from the modifications of membrane properties triggered by ganglioside insertion (Moss et al., 1976; Radsak et al., 1982; Sonderfeld et al., 1985; Facci et al., 1984; Fishman, 1986; Chigorno et al., 1985). Studies of ganglioside association with cells (Radsak et al., 1982; Schwarzmann et al., 1983; Facci et al., 1984; Chigorno et al., 1985) showed that (a) the bulk of exogenous gangliosides binds loosely to the surface and can be removed by treatment with fetal calf serum or albumin solutions, (b) a smaller portion of gangliosides interacts with proteins protruding from the membrane surface and is released by trypsin treatment, and (c) an even smaller but well-defined portion of associated gangliosides is inserted into the membrane lipid layer. The latter information was obtained by making use of synthetic molecules resembling the ganglioside structure ("gangliosidoides") (Kanda et al., 1982; Schwarzmann et al., 1983) and, recently, with fluorescent or paramagnetic derivatives of gangliosides (Masserini & Freire, 1987; Ollmann et al., 1987; Acquotti et al., 1986). A point of caution is the legitimacy of using ganglioside derivatives in place of natural molecules and raises the interesting problem of the behavior of exogenously incorporated gangliosides on the host membrane.

We faced these problems studying the association with cells, metabolic processing, and behavior on the cell membrane of the fluorescent derivative of GM1[1] ganglioside, pyrene-GM1. This probe (Acquotti et al., 1986; Masserini & Freire, 1987; Ollman et al., 1987), by sounding the hydrophobic domain of the membrane, is particularly suitable for studying the aggregation of the labeled molecules since the spectral features of the monomers are quite different from those of the excited dimers (excimers) (Galla & Hartmann, 1980). High values of the excimer:monomer fluorescence intensity ratios (e:m) indicate high probability of pyrene–pyrene collisions. Cultured HeLa cells were used because of their ability to functionally incorporate exogenous GM1 ganglioside (Fishman & Atikkan, 1980).

Materials and Methods

Reagents and Other Products. Commercial chemicals were of the purest quality available; solvents were distilled and water doubly distilled on a glass apparatus. Silica gel thin-layer plates (HPTLC, Kieselgel 60, 20 cm × 20 cm aluminum sheets) were from Merck (Darmstadt, FRG); 10-(1-pyrene)decanoic acid was from KSV Chemicals Oy (Helsinki, Finland); trypsin, *N*-acetylneuraminic acid (NeuAc), cholera toxin, and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO); all the solutions for HeLa cells culture and washing were from Flow Laboratories (Irwine, U.K.). Gangliosides GM1, GM2, GM3, and GD1a were purified from calf brain according to Tettamanti et al. (1973) and structurally characterized as described (Ghidoni et al., 1980). Gangliosides were tritium labeled at C3 of the long-chain base moiety and prepared as the erythro form according to Gazzotti

† This work was supported by Grant N.86.02190.27 from the Consiglio Nazionale delle Ricerche (CNR), Progetto Strategico Biotecnologie.

* Address correspondence to this author at the Dipartimento di Chimica e Biochimica Medica, Facoltà di Medicina e Chirurgia, Via Saldini 50, 20133 Milano, Italy.

[1] Abbreviations: NeuAc, *N*-acetylneuraminic acid; Cer, ceramide; GM1, II^{3}NeuAcGgOse$_4$Cer, Galβ1-3GalNAcβ1-4(NeuAcα2-3)-Galβ1-4Glcβ1-1′Cer; EMEM, Eagle's minimum essential medium with Earle's salts; FCS, fetal calf serum; HPTLC, high-performance thin-layer chromatography. Ganglioside nomenclature is in accordance with Svennerholm (1970) and the IUPAC–IUB Recommendations (1977, 1982).

0006-2960/90/0429-697$02.50/0

et al. (1984). The specific radioactivity ranged between 0.9 and 1.3 Ci/mmol, and the radioactivity purity was better than 99%. GM1, GM2, GM3, and GD1a gangliosides carrying a pyrenyldecanoyl residue in substitution of the fatty acyl moiety were synthesized and purified according to Acquotti et al. (1986). The concentration of pyrene-GM1 was estimated by sialic acid assay (see below) or by spectrophotometry (Levade & Gatt, 1987). The molar ratio of sialic acid to fluorescent fatty acid was virtually 1.

Cell Culture Conditions and Subcellular Fractionation of HeLa Cells. HeLa cells were propagated in Eagle's minimum essential medium with Earle's salts (EMEM) supplemented with 10% fetal calf serum (FCS), as described by Henneberry (1975), using 75 cm^2 plastic flasks. Subcultures were made on 35-mm-diameter culture dishes using the same medium. Cells were used for experiments at 80–90% confluency [110–150 μg of cell protein/dish, corresponding to about (5–6) $\times$ 10^5 cells]. Harvesting, rupture, homogenization, and subcellular fractionation of cells were carried out as described by Chigorno et al. (1986) for cultured fibroblasts. In particular, the "light" membrane subfraction was prepared starting from fraction P_2 obtained from the homogenate. Characterization of the obtained subcellular fractions was carried out by the enzyme marker technique following Chigorno et al. (1986). Cell viability was assessed by the Trypan Blue absorption method (Phillips, 1973). The morphology of cultured cells as well as the efficiency of the rupturing procedure was examined by using a phase-contrast microscope.

Treatment of HeLa Cells with Fluorescent and Tritium-Labeled GM1. A given amount of tritium-labeled GM1 (^{3}H-GM1) or of a pyrene-GM1/^{3}H-GM1 mixture (1:4 M/M) was dried from a chloroform/methanol (2:1 by volume) solution. The residue was dissolved in an appropriate volume of EMEM to obtain a final 5×10^{-5} M ganglioside concentration with a radioactivity of about 2×10^6 dpm/mL. After removal of the culture medium from each dish, followed by rapid washing with EMEM, 1 mL of the ganglioside solution was added (in the absence of FCS), and incubation was carried out at 37 °C for up to 24 h. At the end of incubation, the ganglioside solution was removed, and the cells were washed 3 times with 1.5 mL (each time) of 0.9% NaCl solution. Cells were then maintained at 37 °C for 30 min with 1 mL of 10% FCS–EMEM and, after being washed, treated with 1 mL of 0.9% NaCl solution containing 0.1% trypsin (Schwarzmann et al., 1983; Chigorno et al., 1986). The cell preparations obtained after FCS and after trypsin treatments contain the "serum stable" and "trypsin stable" fractions of associated ganglioside, respectively. Cells or cell subfractions collected as pellets after centrifugation (15000*g*, 20 min) were subjected to ganglioside extraction according to Tettamanti et al. (1973) using 1 mL of the extraction solvent/100 μg of cell protein. The amount of pyrene-ganglioside was estimated in the extract by spectrophotometry (Levade & Gatt, 1987). No signal of the excimer form (read at the emission wavelength at 480 nm) was present under these conditions. Blanks were extracts from cells not incubated with ganglioside, and quantification was made on the basis of a standard curve of pyrene-GM1 dissolved in the same solvent.

Fluorescence Spectroscopy Measurements. All fluorescence experiments were performed with a Jasco FP770 spectrofluorometer equipped with a thermostated cuvette holder. Excimer formation experiments were performed by measuring the fluorescence emission spectrum of pyrene-GM1 (excitation wavelength 343 nm). The emissions of monomer and excimer were taken at their maxima at 395 and 480 nm (Masserini & Freire, 1987), respectively.

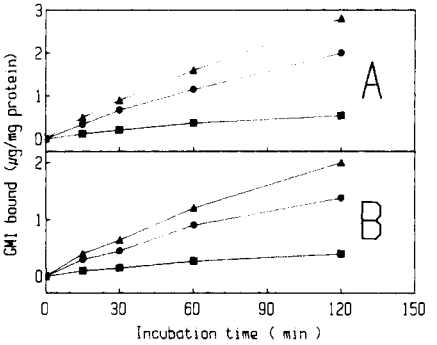

FIGURE 1: Time course of association to HeLa cells of ^{3}H-GM1 or the pyrene-GM1/^{3}H-GM1 mixture (1:4 M/M). Total ganglioside concentration 5×10^{-5} M. (Panel A) Serum-stable form of associated ganglioside; (panel B) trypsin-stable form of associated gangliosides. (▲) ^{3}H-GM1 incubated alone; (●) ^{3}H-GM1 incubated in mixture with pyrene-GM1; (■) pyrene-GM1 incubated in mixture with ^{3}H-GM1.

Treatment with Ca^{2+} or Cholera Toxin. In some experiments, the "light" membrane subfractions (see above) were washed and resuspended in 0.9% NaCl and thermostated at 37 °C, and Ca^{2+}, EDTA, or cholera toxin was added, Ca^{2+} was added from concentrated (1 M) $CaCl_2$ solutions and EDTA (disodium salt) from 0.3 M solutions. Cholera toxin was added in a stoichiometric amount (1:5 M/M; van Heyningen, 1983) to total GM1 (fluorescent or not) present in the cellular subfractions.

Metabolic Experiments. The occurrence of metabolic processing of radiolabeled and pyrene-GM1 was ascertained under the conditions used by Chigorno et al. (1985) for radioactive GM1 in fibroblasts. Cells incubated with ^{3}H-GM1 or pyrene-GM1/^{3}H-GM1 for 2, 8, and 24 h were subjected to ganglioside extraction, and the individual gangliosides were separated by TLC (see below).

Thin-Layer Chromatography. HPTLC plates were developed at room temperature with the solvent system chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11 by volume. ^{3}H-Ganglioside spots were made visible by autoradiography and quantified by radiochromatoscanning (Chigorno et al., 1986) using a Berthold TLC linear analyzer. Fluorescent bands were quantified by scanning with a CGA Fluorscan scanner (CGA, Florence, Italy).

Colorimetric Methods. Protein content was determined in solubilized cell pellets according to Peterson (1977), bovine serum albumin being used as the reference standard. Ganglioside/sialic acid was determined according to Svennerholm (1957).

Results

The viability of cells after exposure to pyrene-GM1/GM1 mixtures (1:4 molar ratio) up to 24 h was good and identical with that of the cells exposed to identical concentrations of the natural ganglioside.

GM1 Association to Cultured HeLa Cells. When only ^{3}H-GM1 was used for incubation, the amount of serum-stable form of ganglioside associated after 2 h was 2.8 μg/mg of protein (Figure 1, panel A). Trypsin treatment (Figure 1, panel B) led to about 25% decrease of this amount. When identical conditions and a pyrene-GM1/^{3}H-GM1 (1:4 M/M) mixture were used, the total amount of GM1 associated after 2 h (sum of pyrene-GM1 and ^{3}H-GM1) did not change (Figure 1). Noteworthy, the radioactive and fluorescent ganglioside (serum-stable or trypsin-stable forms) proportions were always in the range 3:1 to 4:1 (M/M).

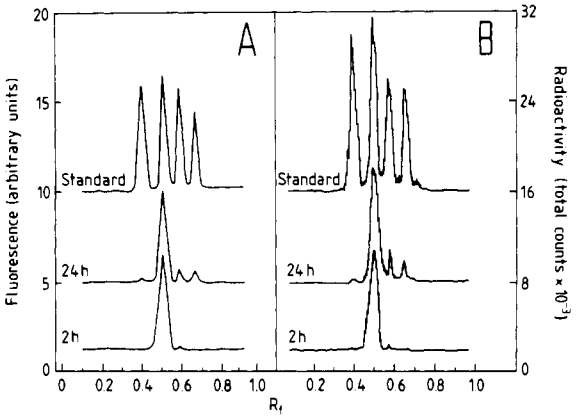

FIGURE 2: Fluorescence scanning (panel A) and radiochromatoscanning (panel B) of the HPTLC of gangliosides extracted from HeLa cells following incubation with a 5×10^{-5} M pyrene-GM1/^{3}H-GM1 mixture (1:4 M/M): 2-h incubation; 24-h incubation; standard pyrene- or ^{3}H-gangliosides. R_f of standard pyrene-gangliosides; GD1a, 0.4; GM1, 0.53; GM2, 0.59; GM3, 0.66. R_f of standard ^{3}H-gangliosides: GD1a, 0.39; GM1, 0.5; GM2, 0.57; GM3, 0.65.

Exogenous Ganglioside Metabolism. After 2-h incubation with the pyrene-GM1/^{3}H-GM1 mixture, the thin-layer chromatogram (Figure 2, panel A) showed the presence of a fluorescent band corresponding to pyrene-GM1 and of an extremely faint band (less than 1% of the total fluorescence) having the same R_f as pyrene-GM2. After prolongation of incubation to 24 h (Figure 2, panel A), the latter band accounted for 5% of the total, and two new fluorescent bands appeared, one having the same chromatographic behavior as pyrene-GM3 (about 5% of the total) and another having the same R_f as pyrene-GD1a (about 1% of the total fluorescence). After 8 h, the metabolism was intermediate between the situation at 2 and 24 h. The radiochromatoscanning of the same plate gave similar results (see Figure 2, panel B), indicating that the mode and apparent rate of metabolic processing of pyrene-GM1 were similar to those of the natural ganglioside.

Fluorescence Spectroscopy Experiments. The excimer formation technique was first used to determine the critical micellar concentration (cmc) of the mixture pyrene-GM1/^{3}H-GM1 (1:4 M/M), as described (Masserini & Freire, 1986; Ollmann et al., 1987). The analysis of the excimer:monomer fluorescence ratio (e:m) as a function of the total ganglioside concentration indicated that the monomer–micelle transition of this dispersion occurs at a concentration of 3×10^{-7} M (data not shown). The same technique was used to follow the association with cells of pyrene-GM1/^{3}H-GM1 mixtures. For this purpose, after incubation with ganglioside for 2 h, "light" membrane subfractions were prepared from treated cells. The activity of the marker enzyme (Na^+,K^+)-ATPase increased 3–4-fold with respect to the starting homogenate, suggesting a substantial enrichment in plasma membranes. The EMEM solution used for the incubation with gangliosides had an e:m value of 0.5; the "light" membrane subfraction had a value of 0.11 after FCS treatment (serum-stable form of associated ganglioside) and a value of 0.09 after trypsin treatment (trypsin-stable form of associated ganglioside). The trypsin solution withdrawn after cell treatment had an e:m value of 0.4.

In a subsequent experiment, Ca^{2+} influence on the ganglioside molecules associated to cells was investigated. As shown in Figure 3, both the serum-stable and trypsin-stable forms of associated ganglioside were sensitive to Ca^{2+}. In fact, the addition of the cation was accompanied by an increase of the e:m ratio, indicating the occurrence of an increase of pyrene–pyrene collisions. The effect of Ca^{2+} increased with cation

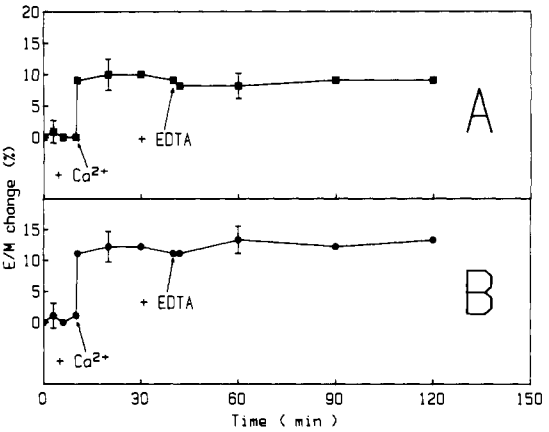

FIGURE 3: Effect of Ca^{2+} and EDTA addition on the e:m ratio of light membrane subfractions prepared from HeLa cells following 2-h incubation with a 5×10^{-5} M pyrene-GM1/^{3}H-GM1 mixture (1:4 M/M). (Panel A) Serum-stable form of associated gangliosides; (panel B) trypsin-stable form of associated gangliosides.

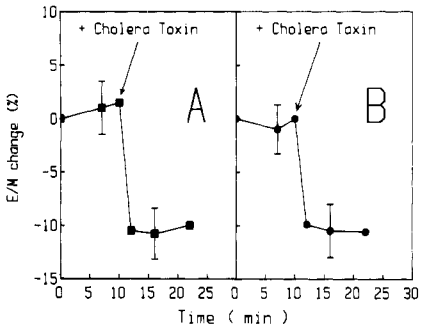

FIGURE 4: Effect of the addition of cholera toxin on the e:m ratio of light membrane subfractions prepared from HeLa cells following 2-h incubation with a 5×10^{-5} M pyrene-GM1/^{3}H-GM1 mixture (1:4 M/M). (Panel A) Serum-stable form of associated ganglioside; (panel B) trypsin-stable form of associated ganglioside.

concentration (maximum at 10 mM). The maximum increase in the e:m ratio, that was statistically significant ($p < 0.01$), was reached 1 min after the addition of the cation.

Subsequent addition of EDTA, in stoichiometric excess (20 mM) to the added Ca^{2+}, did not appreciably change, within 1 h, the e:m ratio value (Figure 3). The addition of EDTA or Ca^{2+}–EDTA mixtures to membrane preparations not treated with Ca^{2+} was used as the reference.

In further experiments, cholera toxin was added to the light membrane subfractions containing pyrene-GM1. As reported in Figure 4, a small but statistically significant decrease ($p < 0.01$) of the e:m ratio occurred for the light membrane subfractions carrying both the serum-stable and trypsin-stable forms of associated GM1.

Discussion

Upon incubation with HeLa cells, GM1 ganglioside definitely associates with them (Fishman & Atikkan, 1980). We show in this paper that also the fluorescent derivative of ganglioside GM1, pyrene-GM1, associates with the cells. The total amount of associated ganglioside is the same whether using a pyrene-GM1/^{3}H-GM1 mixture or using only the radiolabeled natural ganglioside. The fluorescent and radioactive GM1 proportion is the same as in the starting mixture at all cell association stages. These data suggest that the two gangliosides have similar behavior in the association process. If we remember that the critical micellar concentration (cmc) of the pyrene-GM1/^{3}H-GM1 (1:4 M/M) mixture is 10^{-7} M (present paper) and that of GM1 is 10^{-8}–10^{-9} M (Ulrich-Bott

& Wiegandt, 1984), we infer that most of the ganglioside is in micellar form at the concentration (5×10^{-5} M) used in our experiments.

The observation that the e:m ratio of the incubation mixture is markedly higher than that of the serum-stable fraction is consistent with the hypothesis that the overall surface density of the ganglioside molecules associated with cells is markedly lower than in the micelles. In addition, the portion of gangliosides detached from the cells after trypsin treatment has a high e:m ratio, presumably given by GM1 linked to membrane proteins in a micellelike aggregation form, as already suggested (Schwarzmann et al., 1983; Chigorno et al., 1985). In contrast, the trypsin-stable form of association corresponds to ganglioside molecules dispersed on the membrane with a low probability of collision, in other words to a molecular dispersion of the gangliosides within the membrane. The metabolic fate of pyrene-GM1 reflects that of natural GM1, showing formation of GM2, GM3, and GD1a derivatives, in agreement with the general concepts of metabolism of exogenous gangliosides (Ghidoni et al., 1986; Sonderfeld et al., 1985). On the basis of the above, we presumed that, once inserted into the membrane, pyrene-GM1 behavior could be investigated as a way to inspect that of natural GM1. To this purpose, we employed a "light" membrane subfraction enriched in plasma membranes, prepared from cells incubated for 2 h with the pyrene-GM1/radioactive GM1 mixture. Under these conditions, the bulk of added ganglioside was at the membrane level, with no appreciable occurrence of metabolic processing. The addition of Ca^{2+} to the membranes causes an increase of the e:m ratio. According to Galla and Hartman (1980), the probability of molecular collisions between pyrene probes depends on their lateral diffusion coefficient as well as on their concentration. The first cause can be excluded to be responsible for the observed e:m increase, since Ca^{2+} is known to rigidify the bilayer (Borle & Selig, 1985; Akutsu & Selig, 1981). In addition, Ca^{2+} does not affect per se the rate of lateral diffusion of fluorescent GM1 (Goins et al., 1986; Ollmann et al., 1987). Therefore, the e:m increase is more likely due to an increase of the local probe concentration, reflecting a process of ganglioside aggregation. The fact that the addition of EDTA had no effect suggests that the aggregation of gangliosides may represent a condition of stability for the glycolipid in the membrane environment. Noteworthy, the effect induced by Ca^{2+} occurred at cation concentrations close to the physiological ones in the extracellular compartment, whereas in phospholipid vesicles carrying pyrene-gangliosides it was found (Ollmann et al., 1987) at much higher concentrations (over 100 mM). This marked difference can arise from any differences between artificial and natural membranes, like the presence in the latter ones of proteins or particular lipids. For instance, the interaction of Ca^{2+} with some proteins may induce changes in their conformation, increasing the binding affinity for gangliosides and leading to their aggregation. In turn, Ca^{2+} could induce clustering of acidic phospholipids (Galla & Sackmann, 1975; Ito et al., 1975) or cause aggregation of gangliosides by a phenomenon of passive exclusion in analogy with data obtained by using phospholipid vesicles (Masserini & Freire, 1986). It is worth noting that the effect following interaction with calcium is much stronger with pyrenyl acidic phospholipids, where 10-fold increases of the e:m ratio were found (Galla & Sackmann, 1975; Ito et al., 1975).

HeLa cells have an extremely low content of endogenous GM1 (Fishman, 1978; Markwell et al., 1984), and the insertion of exogenous ganglioside induces cellular responsiveness to cholera toxin (Fishman, 1978). Very little is known about the ganglioside dynamics on the membrane following interaction with the toxin (Goins et al., 1987). The data herein reported show that addition of the toxin to cells enriched with pyrene-GM1 leads to a decrease of the e:m ratio. These data show that pyrene-GM1, as well as the native ganglioside, is recognized and is available to interaction with bulk external ligands such as proteins. Moreover, it provides indirect evidence that the exogenous ganglioside has a definite degree of motion within the membrane.

In conclusion, the present investigation suggests that the behavior of pyrene-GM1 mimics that of native ganglioside with regard to association with HeLa cells, metabolic processing, and availability to interaction with external ligands. This makes sense for the use of the fluorescent ganglioside to inspect the behavior of natural ones in the membranes. Of course, this working hypothesis is inferred from a body of evidence, herein reported, but does not imply a general identity of behavior of the two gangliosides. From the data obtained, it appears that the lateral distribution, aggregative properties, and presumably the interactions of gangliosides with the membrane partners can be steadily influenced by the presence of Ca^{2+}. This intriguing issue deserves further attention and experimental exploration.

Registry No. GM2, 19600-01-2; GM3, 54827-14-4; GD1a, 12707-58-3; Ca, 7440-70-2.

References

Acquotti, D., Sonnino, S., Masserini, M., Casella, L., Fronza, G., & Tettamanti, G. (1986) *Chem. Phys. Lipids 40*, 71–86.

Akutsu, H., & Selig, J. (1981) *Biochemistry 20*, 7366–7373.

Borle, F., & Selig, J. (1985) *Chem. Phys. Lipids 36*, 263–283.

Chigorno, V., Pitto, M., Cardace, G., Acquotti, D., Kirschner, G., Sonnino, S., Ghidoni, G., & Tettamanti, G. (1985) *Glycoconjugate J. 2*, 279–291.

Chigorno, V., Cardace, G., Pitto, M., Sonnino, S., Ghidoni, R., & Tettamanti, G. (1986) *Anal. Biochem. 153*, 283–294.

Facci, L., Leon, A., Toffano, G., Sonnino, S., Ghidoni, R., & Tettamanti, G. (1984) *J. Neurochem. 42*, 299–305.

Fishman, P. H. (1986) *Chem. Phys. Lipids 42*, 137–151.

Fishman, P. H., & Atikkan, S. (1980) *J. Membr. Biol. 69*, 85–98.

Galla, H.-J., & Sackmann, E. (1975) *J. Am. Chem. Soc. 97*, 4114–4120.

Galla, H.-J., & Hartmann, W. (1980) *Chem. Phys. Lipids 27*, 199–219.

Gazzotti, G., Sonnino, S., Ghidoni, R., Orlando, P., & Tettamanti, G. (1984) *Glycoconjugate J. 1*, 111–121.

Ghidoni, R., Sonnino, S., Tettamanti, G., Baumann, N., Reuter, G., & Schauer, R. (1980) *J. Biol. Chem. 255*, 6990–6995.

Ghidoni, R., Trinchera, M., Venerando, B., Fiorilli, A., Sonnino, S., & Tettamanti, G. (1986) *Biochem. J. 237*, 147–155.

Goins, B., Masserini, M., Barisas, B. G., & Freire, E. (1986) *Biophys. J. 49*, 849–856.

Ito, T., Ohnishi, S., Ishinaga, M., & Kito, M. (1975) *Biochemistry 14*, 3064–3069.

IUPAC–IUB Recommendations for Lipid Nomenclature (1977) *Lipids 12*, 455–468.

IUPAC–IUB Recommendation for Lipid Nomenclature (1982) *J. Biol. Chem. 257*, 3347–3351.

Kanda, S., Inoue, K., Nojima, S., Utsumi, H., & Wiegandt, H. (1982) *J. Biochem. 91*, 2095–2098.

Levade, T., & Gatt, S. (1987) *Biochim. Biophys. Acta 918*, 250–259.

Masserini, M., & Freire, E. (1986) *Biochemistry 25*, 1043–1049.
Masserini, M., & Freire, E. (1987) *Biochemistry 26*, 237–242.
Moss, J., Fishman, P. H., Manganiello, V. C., Vaughan, N., & Brady, R. O. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1034–1037.
Ollmann, M., Schwarzmann, G., Sandhoff, K., & Galla, H.-J. (1987) *Biochemistry 26*, 5943–5952.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Phillips, H. J. (1973) in *Tissue culture methods and applications* (Kruse, P. F., & Patterson, M. K., Eds.) pp 221–228, Academic Press, New York.
Radsak, K., Schwarzmann, G., & Wiegandt, H. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 263–272.
Schwarzmann, G., Hoffmann-Bleihauer, P., Shubert, J., Sonderfeld, S., Conzelmann, E., Schwarzmann, G., Burg, J., Hinrichs, U., & Sandhoff, K. (1985) *Eur. J. Biochem. 149*, 247–255.
Svennerholm, L. (1957) *Biochim. Biophys. Acta 13*, 856–858.
Svennerholm, L. (1970) in *Handbook of Neurochemistry* (Lajta, A., Ed.) pp 425–452, Plenum, New York.
Tettamanti, G., Bonali, F., Marchesini, S., & Zambotti, V. (1973) *Biochim. Biophys. Acta 296*, 160–170.
Ulrich-Bott, B., & Wiegandt, H. (1984) *J. Lipid Res. 25*, 1233–1240.
Van Heyningen, S. (1983) in *Current Topics in Membrane and Transport* (Klinzeiler, A., & Mayhi, B., Eds.) pp 445–471, Academic Press, New York.

Location of the Carbohydrates Present in the HK-ATPase Vesicles Isolated from Hog Gastric Mucosa†

Kathleen Hall,*,‡ Gonzalo Perez,‡ Debra Anderson,‡ Cecilia Gutierrez,‡ Keith Munson,‡ Steve J. Hersey,§ Jack H. Kaplan,∥ and George Sachs‡

CURE, Veterans Administration Medical Center, Los Angeles, California 90073, Department of Physiology, Emory University, Atlanta, Georgia 30322, and Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Received July 13, 1989; Revised Manuscript Received September 5, 1989

ABSTRACT: The glycosylation of H^+K^+-ATPase vesicles isolated from hog gastric mucosa was investigated by various methods. Following protein separation on sodium dodecyl sulfate reducing gels and transfer to poly(vinyl difluoride) membranes, binding of concanavalin A was confined to the 94-kDa band which corresponds to the catalytic subunit. In contrast, wheat germ agglutinin binding occurred in a region below the 94-kDa subunit, corresponding to the 60–85-kDa region, and also to protein just above the catalytic subunit. Treatment with glycopeptidase F removed most of the concanavalin A staining and also the wheat germ agglutinin staining found below the 94-kDa region, but spared the higher molecular weight wheat germ agglutinin reactive material. During the deglycosylation experiments a protein of 35-kDa was produced. Sequencing analysis of V8 protease generated peptide fragments of the 35-kDa protein show at least 30% homology with the Na^+K^+-ATPase β-subunits. Labeling of the carbohydrates by galactosyltransferase and [^{3}H]uridine diphosphate–galactose showed that the sites of labeling were extracellular and were confined to the wheat germ agglutinin staining regions. Two molecular weight regions, below the 94-kDa region, of 60 and 85 kDa were identified. Electron microscopy using postembedding staining techniques showed that both concanavalin A and wheat germ agglutinin staining occurred on the extracellular face of the gastric vesicles. It is concluded that there are three classes of glycosylated proteins in hog gastric vesicles: (1) the 94-kDa protein containing a core (simple) oligosaccharide, with no or few *N*-acetylglucosamine residues available for either wheat germ agglutinin binding or galactose transfer; (2) the 60–85-kDa region containing two protein bands that have complex N-linked oligosaccharides; and (3) a protein of higher molecular weight than the catalytic unit which contains O-linked complex oligosaccharides. All of these glycoproteins appear to be located on the extracellular face of the gastric vesicles.

The gastric HK-ATPase of mammalian parietal cells is isolated as a vesicular preparation that retains the ability to transport protons in exchange for K^+ upon the addition of ATP (Sachs et al., 1974). These vesicles are largely (94%) inside out (cytosol on the outside) and impermeable to ions such as K^+ and Cl^- (Saccomani et al., 1977). By use of these vesicles, studies on the structure of the HK-ATPase in relation to its orientation in the lipid bilayer can be carried out. The hydrolysis of ATP is accomplished by a protein that has an apparent molecular weight of 94 000 on reducing acrylamide gels. This protein has been shown by various techniques to be the catalytic unit of the HK-ATPase. For example, the 94-kDa protein when isolated on sodium dodecyl sulfate (SDS) gels is labeled by [γ-^{32}P]ATP (Helmich et al., 1985), and also [^{3}H]omeprazole, an inhibitor of the proton pump, covalently binds the 94-kDa protein specifically (Wallmark et al., 1984). The cDNA sequence of the HK-ATPase catalytic unit has been obtained from rat and hog and has about 60% homology

† This work supported by grants from the Smith Kline Beckman Fellowship Award, the SMI 001, Veterans Administration—Wadsworth, Los Angeles, CA, and NIH (GN 39500).
‡ Veterans Administration Medical Center.
§ Emory University.
∥ University of Pennsylvania School of Medicine.

0006-2960/90/0429-701$02.50/0