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BEHAVIOR OF VESICULAR STOMATITIS VIRUS GLYCOPROTEIN IN MOUSE LM CELLS WITH MODIFIED MEMBRANE-PHOSPHOLIPIDS

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Summary

LM cells in which the membrane phospholipids had been modified with choline analogues were infected with vesicular stomatitis virus. The choline analogues tested were choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine and ethanolamine. These modifications per se did not affect the syntheses of individual viral proteins. The viral glycoprotein was detected in the plasma membranes of all the modified cells by pronase digestion in pulse-chase experiments, but the amount of glycoprotein susceptible to proteolysis varied, decreasing in these modified cells in the following order: *N,N'*-dimethylethanolamine- > choline- > *N*-monomethylethanolamine- > ethanolamine-treated cells. After a 4-h chase, glycoprotein was mainly distributed in the plasma membranes of cells modified with *N,N'*-dimethylethanolamine, whereas it was found in both the microsomes and plasma membranes of cells modified with other analogues. Fairly large amounts of glycoprotein were also found in the soluble fraction of ethanolamine-treated cells, but not in that of choline- or *N,N'*-dimethylethanolamine-treated cells. More precise experiments on the behaviour of glycoprotein with a short period of chase strongly suggested that migration of glycoprotein from the microsomes to the plasma membranes was fastest in cells modified with *N,N'*-dimethylethanolamine and slowest in cells modified with ethanolamine. Membrane lipid modifications also resulted in release of different numbers of progeny virions from the cells, release of virions from the cells decreasing in the following order: *N,N'*-dimethylethanolamine- > choline- > *N*-monomethylethanolamine- > ethanolamine-treated cells. These results indicate that modification of membrane phospholipids influences not only the insertion of glycoprotein into the microsomes and its migration to the plasma membranes, but also the production of progeny virions.

Introduction

Vesicular stomatitis virus is an enveloped virus with an envelope consisting of proteins and lipids [1,2]. The viral membrane proteins (glycoprotein and matrix protein) are coded in genome RNA, whereas the membrane lipids are derived from host membranes [1—3]. The membranes of the host cells are involved in many processes of viral infection including viral maturation [1,2]. Knipe et al. suggested that maturation of vesicular stomatitis virus consists of a series of reactions in which the viral proteins interact with the host membrane systems [4]. Of the proteins of vesicular stomatitis virus, glycoprotein is especially interesting because of its relation to the host membranes: it is synthesized on membrane-bound polysomes, inserted into the microsomal membrane and glycosylated, and then it migrates to the cell surface, where its sugar moiety becomes oriented to the outside of the cell. Furthermore, during biosynthesis of glycoprotein, the amino terminal was found to contain a signal sequence which is a prerequisite for transfer of glycoprotein across the membranes, but which is cleaved proteolytically before chain completion [5,6].

We are interested in the effects of modification of membrane lipids on the behavior of the glycoprotein of enveloped virus, because studies on these effects could provide information on the process of maturation of virus in relation to the structure and function of biological membranes. For this purpose we chose a combination of vesicular stomatitis virus and LM cells, since LM cells, which require choline for growth and can grow in serum-free chemically defined medium, readily incorporate choline analogues, such as *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine and ethanolamine, into their membrane phospholipids producing artificially modified membranes [7,8].

This paper describes studies on the effects of modifications of LM cells with choline analogues on the behavior of viral glycoprotein within the host cells. Detection of differences in the amounts of glycoprotein on the cell surface led to the finding that migration of glycoprotein from the microsomes to the plasma membranes varied in the efficiency in infected cells in which the membrane phospholipids had been modified by different choline analogues.

Materials and Methods

Virus, cells and media. LM cells, a strain of mouse fibroblasts, were provided by Dr. F. Schroeder. Cells were grown in suspension in serum-free, chemically defined Higuchi medium [9].

The New Jersey serotype of vesicular stomatitis virus (kindly supplied by Dr. A. Oya, and originally donated by Dr. R.P. Hanson, University of Wisconsin) was adapted to LM cells and a single plaque was isolated for cloning. Stock virus was prepared with a low multiplicity of infection (0.1) in the presence of 5% calf serum (Flow Laboratories Inc., Rockville, MD, U.S.A.). After a 24-h incubation at 37° C, the medium with a titer of about 10^8 plaque-forming units/ml was harvested (1000 × *g*, 5 min) and stored at —70° C.

The phospholipids of LM cells were modified by incubating the cells with choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine or ethanolamine at a concentration of 40 μg/ml for three days as described previously

[8]. In all experiments, choline or one of its analogues was added to the medium and the incubation temperature was 37° C.

Plaque assay. Plaque assay was performed on monolayers of LM cells. Cells from a suspension culture were plated at approx. $5 \cdot 10^6$ cells per 60-mm petri dish and incubated for 1 or 2 days in 5 ml of Higuchi medium without methylcellulose and dextran sulfate. Diluted virus (0.2 ml/dish) was adsorbed to the resulting monolayers for 1 h at 37° C and then 5 ml of the first overlay medium (Earle's balanced salt solution [10] with 0.5% lactoalbumin hydrolysate and 0.1% yeast extract) containing 1% agar (Difco, Detroit, MI, U.S.A.) was added. Cultures were incubated for 48 h, and then 5 ml of the second overlay medium (Earle's balanced salt solution) containing 25 μg/ml of neutral red and 1% agar was added and plaques were counted 16 h later.

Preparation of membrane fractions. All procedures were carried out at 4° C. Cells were washed with phosphate-buffered saline [11] and suspended in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5). Swollen cells ($2.5 \cdot 10^7$ cells/ml) were disrupted by 30 strokes of a Dounce homogenizer with a tightly fitting pestle. Nuclei and undisrupted cells were removed by centrifugation ($1000 \times g$, 5 min), and the supernatant was recentrifuged at $8000 \times g$ for 15 min.

The upper layer of the pellet was carefully taken up and suspended in 8 ml of the same Tris-HCl buffer. Then it was layered above 2 ml of 36% sucrose in the buffer (w/w) and centrifuged at $90\,000 \times g$ for 2.5 h (SW41 rotor). An SW27 rotor was used for large scale preparation. The band on the sucrose layer was collected by pipette, centrifuged at $100\,000 \times g$ for 40 min and used as the preparation of plasma membranes. The pellet obtained by centrifugation on sucrose at $90\,000 \times g$ was used as mitochondria. Microsomes were precipitated from the $8000 \times g$ supernatant by centrifugation at $100\,000 \times g$ for 40 min, and the supernatant was used as the soluble fraction. These fractions were identified by assay of their marker enyme activities [8,12]. The results of a typical experiment are summarized in Table I. The specific activity of ouabain-sensitive ($Na^+ + K^+$)-ATPase, and the molar ratios of sterol to phospholipid (0.45), and of phosphatidylethanolamine to phosphatidylcholine (0.98) in our plasma membrane fraction were comparable with those in a preparation from LM cells obtained by a different method [8]. The marker enzymes were also concentrated in the corresponding subcellular fractions of the modified cells, indicating that this method is applicable to cells with modified membranes. This method has the advantage that it is much quicker than the previous one [8].

Enzyme assay. Published methods were used for assay of ouabain-sensitive ($Na^+ + K^+$)-ATPase (a marker of plasma membranes) [13] and glucose-6-phosphatase (a marker of microsomes) [14]. Oligomycin sensitive ATPase (a marker of mitochondria) was determined by the method of Futai et al. [15] in the presence of 0.06 nmol of oligomycin per 10 μg protein. 5'-Nucleotidase activity was measured in similar reaction mixture to that used for assay of ($Na^+ + K^+$)-ATPase, except that 10 mM AMP was added. Released phosphorus was determined by the method of Josse [16]. The method of Lowry et al. [17] was used for protein determination.

Analysis of lipids. Lipids were extracted by the method of Folch et al. [18]. Phospholipid was ashed and analyzed as phosphorus by the method of Chen et al. [19]. Sterol was determined by the method of Zak [20]. Phospholipids

TABLE I

DISTRIBUTION OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS OF LM CELLS

LM cells were grown in suspension in the presence of choline (40 μg/ml). Enzyme activities are expressed as nmol of P_i released/min per mg protein. A sample of $1.8 \cdot 10^9$ cells was fractionated. Preparation of the fractions and enzyme assays are described in Materials and Methods. The yields of marker enzyme activities in each fraction as percentages of those in the postnuclear fraction were as follows: $(Na^+ + K^+)$-ATPase in plasma membranes, 55%; 5′-nucleotidase and glucose-6-phosphatase in microsomes, 19% and 33%, respectively, Mg^{2+}-ATPase in mitochondria, 110%.

Fraction	Protein (mg)	Enzyme activity (nmol P_i released/min per mg protein)			
		$(Na^+ + K^+)$-ATPase *	5′-Nucleotidase	Glucose-6-phosphatase	Mg^{2+}-ATPase **
Postnuclear fraction	263	6.7	8.3	2.7	8.2
Plasma membranes	10.2	82.6	5.2	4.9	7.4
Microsomes	21.5	3.1	16.3	9.7	1.5
Mitochondria	18.6	4.2	8.5	4.0	110.9
Soluble	185	0	6.3 ***	0	0

* Ouabain-sensitive activity was measured.
** Oligomycin-sensitive activity was measured.
*** 5′-Nucleotidase was also present in the soluble fraction obtained by our procedure.

were separated by two-dimensional thin-layer chromatography (Kieselgel 60, E. Merck, Darmstadt, F.R.G.) with $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) as solvent in the first dimension, and butanol/acetic acid/H_2O (6 : 2 : 2, v/v) in the second. Spots were detected with iodine vapor. The spots were scraped off and extracted with 1.5% $NH_4OH/CHCl_3/CH_3OH$ (1 : 6 : 5, v/v) and the phosphorus content of the extract was measured.

Analysis of radioactive viral proteins. In our experimental system (a combination of LM cells and New Jersey serotype of vesicular stomatitis virus) syntheses of viral macromolecules and release of mature virions continued for about 12 h after infection. Cells were infected with virus in culture medium with choline analogues. Infected cells were pulse-labeled with radioactive amino acid mixture at 5 or 6 h postinfection and chased for the indicated times as described in the text. Detailed experimental conditions are described in the legends of the corresponding tables and the figure. Subcellular fractions were obtained as described above. Protein in the soluble fraction was precipitated by adding 9 vols. of ice-cold acetone.

Cells or subcellular fractions were heat-denatured in the presence of sodium dodecylsulfate and β-mercaptoethanol, and analyzed by gel electrophoresis as described by Laemmli [21]. After electrophoresis, slices of gel were dissolved in H_2O_2 [22] and their radioactivities were measured in Triton-toluene scintillation fluid. Samples were also analyzed by slab gel electrophoresis. After electrophoresis, the gel was dried and subjected to autoradiography with X-ray film (Fuji Photo Film Co., Tokyo, Japan) and the radioactivities in the bands corresponding to each viral protein were determined by densitometric scanning.

Chemicals. [5-^{3}H]Uridine (30.2 Ci/mmol), ^{3}H-labeled amino acid mixture and ^{14}C-labeled amino acid mixture were obtained from New England Nuclear

Corp., Boston, MA, U.S.A. Ouabain, oligomycin, actinomycin D, pronase, ATP and AMP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucose-6-phosphate was from PL Biochemicals, Inc., Milwaukee, WI, U.S.A. Acrylamide, *N,N'*-methylene bisacrylamide and the choline analogues were from Eastman Kodak Co., Rochester, NY, U.S.A. Sodium dodecyl sulfate was from Nakarai Chemical Co., Kyoto, Japan. All other chemicals used were of analytical grade.

Results

Viral protein synthesis and location of glycoprotein

Modification of the membrane phospholipids of LM cells with the choline analogues as reported previously [7,8] resulted in increase of the corresponding phospholipids up to 50% of the total phospholipids (Table II). LM cells with modified membrane phospholipids were infected with vesicular stomatitis virus and the effect of the modification on viral protein synthesis was examined. Pulse-labeling of LM cells with radioactive amino acids after viral infection showed that the syntheses of various viral proteins were similar in cells modified with different analogues (Table III). The transport activities of amino acids were also essentially the same in the various modified cells under the experimental conditions (0.03 pmol methionine incorporated/20 min per $5 \cdot 10^5$ cells). Thus the syntheses of viral proteins do not seem to be affected by modification of the membrane phospholipids of the host cells.

Though the synthesis of glycoprotein was almost the same in the various modified cells (Table III), the location of glycoprotein could be expected to be influenced by modification of membrane lipids since this protein is especially closely related to the host membranes during viral maturation. Thus we exam-

TABLE II

PHOSPHOLIPID COMPOSITIONS OF LM CELLS TREATED WITH CHOLINE ANALOGUES

Cells were grown in Higuchi medium supplemented with choline analogues for 3 days. Total lipids were extracted by the method of Folch et al. [18] and phospholipids were analyzed by two-dimensional thin-layer chromatography as described in Materials and Methods. —, not detected.

Analogue	Phospholipid composition (%)				
	Phosphatidyl-choline	Phosphatidyl-*N,N'*-dimethyl-ethanolamine	Phosphatidyl-*N*-monomethyl-ethanolamine	Phosphatidyl-ethanolamine	Other
Choline	51.7	—	—	36.0	12.3
N,N'-Dimethyl-ethanolamine	12.1	58.0	—	19.5	10.4
N-Monomethyl-ethanolamine	16.7	5.5 *	52.6	16.3	8.9
Ethanolamine	29.6	—	4.7 *	53.3	12.4

* On treatments with *N*-monomethylethanolamine and ethanolamine, small amounts of phosphatidyl-*N,N'*-dimethylethanolamine and phosphatidyl-*N*-monomethylethanolamine, respectively, were also detected. Formation of these phospholipids could be due to contamination of the *N*-monomethylethanolamine and ethanolamine preparations, or to very low activity, if it exists, of a methylation pathway in LM cells [7].

TABLE III

VIRAL PROTEIN SYNTHESIS IN CELLS WITH MODIFIED PHOSPHOLIPIDS

Cells ($5 \cdot 10^5$ cells/ml of the medium) were infected with virus (multiplicity of infection; 1.8) in 1 ml of culture medium and 6 h later transfered to 1 ml of phosphate-buffered saline. Cells were incubated for 30 min in the presence of 1 μCi ^{14}C-labeled amino acid mixture. Labeled proteins were analyzed by slab gel electrophoresis. Densitometric scans of the autoradiogram were made and the area under each peak corresponding to viral protein was calculated and expressed in arbitrary units. Glycoprotein, nucleocapsid protein, non-structural protein, matrix protein and transcriptase are generally designated as G protein, N protein, M protein and L protein, respectively [3]. The values for L protein were less than 60.

Analogue	Viral proteins (arbitrary units/$5 \cdot 10^5$ cells)				
	Glycoprotein	Nucleocapsid protein	Non-structural protein	Matrix protein	Total
Choline	256 ± 14	623 ± 20	205 ± 8	130 ± 10	1264
N,*N*'-Dimethyl-ethanolamine	276 ± 14	659 ± 9	213 ± 10	130 ± 3	1295
N-Monomethyl-ethanolamine	239 ± 7	616 ± 10	231 ± 9	147 ± 8	1260
Ethanolamine	227 ± 9	640 ± 8	219 ± 9	145 ± 7	1231

ined the amounts of radioactive glycoprotein on the cell surface after chase by digesting with pronase from the outside of the cells as reported previously [23], because the presence of glycoprotein on surface membranes is a prerequisite for the budding process. Typical profiles of radioactive viral proteins on

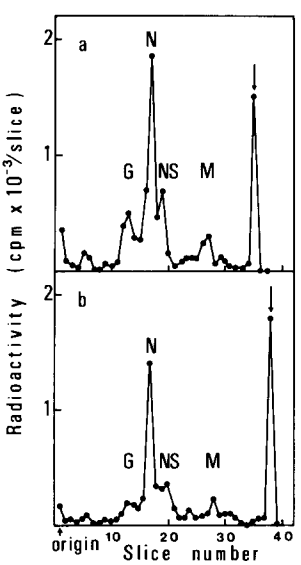

Fig. 1. Typical profiles of radioactive viral proteins of infected cells with or without pronase treatment. Choline-treated cells ($1.5 \cdot 10^6$ cells) were pulsed for 1 h in 0.5 ml of phosphate-buffered saline containing 5 μCi of ^{3}H-labeled amino acid mixture at 5 h postinfection (multiplicity of infection: 8) and then incubated with a further 0.5 ml of culture medium for additional 4 h. Pronase (300 μg) was added to the cell suspension and incubation was continued for 10 min. Cells were harvested and radioactive viral proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The arrows show the dye front of the gel. The symbols of G, N, NS, and M in the figure: see legend to Table III. (a) Without pronase, (b) with pronase.

TABLE IV

ESTIMATED AMOUNTS OF RADIOACTIVE GLYCOPROTEIN ON THE CELL SURFACE AFTER CHASE

The amounts of radioactive viral proteins were determined after sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described for Fig. 1. The molecular weights of the viral proteins calculated by comparison of their mobilities with that of *E. coli* ATPase on the gel were as follows: glycoprotein, 62 000 nucleocapsid protein, 51 000; matrix protein, 29 000. The molecular weight of glycoprotein in the infected cells was the same as that of periodic acid-Schiff-positive glycoprotein [24] containing the sugar moiety in virions, but it was lower than that reported previously [3].

Analogue	Pronase	Molar ratio of viral protein *		Glycoprotein susceptible to proteolysis ** (%)
		Glycoprotein	Matrix protein	
Choline	—	0.32 ± 0.027	0.36 ± 0.022	
	+	0.20 ± 0.016	0.35 ± 0.033	38
N,N'-Dimethyl-ethanolamine	—	0.47 ± 0.027	0.49 ± 0.034	
	+	0.23 ± 0.018	0.48 ± 0.032	52
N-Monomethyl-ethanolamine	—	0.40 ± 0.025	0,40 ± 0.030	
	+	0.32 ± 0.027	0.41 ± 0.029	20
Ethanolamine	—	0.35 ± 0.017	0.38 ± 0.037	
	+	0.29 ± 0.020	0.37 ± 0.035	18

* Expressed relative to nucleocapsid protein.

** Calculated as follows: 100 × [ratio(—pronase) — ratio(+pronase)]/ratio(—pronase).

sodium dodecyl sulfate-polyacrylamide gel with or without proteolysis are shown in Fig. 1. The molar ratios of glycoprotein and matrix protein to nucleocapsid protein in the presence and absence of pronase, calculated from the radioactivities, are summarized in Table IV. Glycoprotein was susceptible to proteolysis whereas matrix protein was not, indicating the presence of some glycoprotein on the cell surface of modified cells. Table IV also shows that the ratio of pronase-susceptible glycoprotein to total glycoprotein differed in the various modified cells, being largest in *N,N'*-dimethylethanolamine-treated cells and smallest in ethanolamine-treated cells. These results suggest that the subcellular localization of radioactive glycoprotein after chase may differ in cells modified with different analogues. For further studies of this phenomenon, we examined the subcellular distribution and the metabolic behavior of glycoprotein in infected cells. In subsequent experiments *N*-monomethylethanolamine-treated cells were however omitted from the series of modified cells since the amount of pronase-susceptible glycoprotein in *N*-monomethylethanolamine-treated cells was between the amounts in *N,N'*-dimethylethanolamine- and ethanolamine-treated cells.

Distribution and behavior of glycoprotein in modified cells

We first examined the subcellular distribution of radioactive glycoprotein in modified cells after chase for 4 h under the same experimental condition as shown in the legend of Table V and the results were as follows. In choline-treated cells, glycoprotein was mainly distributed in the microsomes and plasma membranes (plasma membranes, 8037; microsomes, 10 396; soluble fraction, 985; expressed as cpm/mg protein of each fraction). In ethanolamine-treated cells comparatively large amounts of glycoprotein were found in the soluble fraction as well as in the microsomes and plasma membranes (plasma

TABLE V

BEHAVIOR OF RADIOACTIVE GLYCOPROTEIN IN THE MICROSOMES AND THE PLASMA MEMBRANES AFTER CHASE

Modified cells ($5 \cdot 10^8$ cells in 50 ml of culture medium) were infected with virus (multiplicity of infection: 8). At 5 h postinfection, cells were suspended in 5 ml of amino acid-free medium and pulse-labeled with 50 μCi of ^{3}H-labeled amino acid mixture. After incubation for 1 h, the cell suspension was chased by addition of 9 vols. of fresh culture medium. Cells in 25 ml of medium were withdrawn for fractionation at the times indicated after chase and viral proteins were analyzed by sodium dodecy sulfate-polyacrylamide gel electrophoresis.

Analogue	Fraction	Radioactivity after chase (cpm/mg)		
		10 min	70 min	Δ *
Choline	Plasma membranes	2354 ± 167	3425 ± 154	1071 (100) **
	Microsomes	3180 ± 196	3481 ± 204	301
N,N'-Dimethyl-ethanolamine	Plasma membranes	2259 ± 84	4603 ± 185	2344 (218)
	Microsomes	4240 ± 376	2160 ± 170	−2080
Ethanolamine	Plasma membranes	1580 ± 115	2104 ± 131	524 (49)
	Microsomes	3393 ± 229	3703 ± 285	310

* Radioactivity of glycoprotein at 70 min minus at 10 min. For direct comparison of radioactive glycoprotein in the virus released during 60 min of chase, the total radioactivities in the virions (choline-treated cells, 792 cpm; *N,N'*-dimethylethanolamine-treated cells, 2593 cpm; ethanolamine-treated cells; 532 cpm) should be divided by twelve, because the radioactive glycoprotein should be one-fourth of the total radioactive proteins in the virions released and there should be 3 mg of plasma membranes in 2.5×10^8 cells judging from the recovery of marker enzyme (Table I).

** The values in parentheses show the amounts of migrated glycoprotein in percentage as compared with that of choline-treated cells.

membranes, 3442; microsomes, 5190; soluble fraction, 2433; expressed as cpm/mg protein of each fraction). *N,N'*-Dimethylethanolamine-treated cells contained less radioactivity in all subcellular fractions (plasma membranes, 3406; microsomes, 640; soluble fraction, 593; expressed as cpm/mg protein of each fraction) than cells modified with other analogues. As for radioactive glycoprotein in the microsomes, *N,N'*-dimethylethanolamine-treated cells contained about one-fifteenth of the radioactivity of choline-treated cells, while ethanolamine-treated cells contained about half that of choline-treated cells. Since viral protein synthesis is similar in these various modified cells, as shown in Table III, these results indicate that the radioactive glycoprotein was chased out from their microsomes more rapidly in *N,N'*-dimethylethanolamine-treated cells than in choline-treated cells. The low radioactivity of glycoprotein in the microsomes of ethanolamine-treated cells could not, however, be explained in the same way as that in *N,N'*-dimethylethanolamine-treated cells, since a relatively large amount of radioactive glycoprotein was found in the supernatant fraction. These results suggest that glycoprotein in the microsomes migrates to the plasma membranes more rapidly in *N,N'*-dimethylethanolamine-treated cells than in other modified cells.

For further confirmation of the behavior of glycoprotein, we carried out more detailed studies in which radioactive glycoprotein in the subcellular fractions was measured 10 min and 70 min after chase. Table V showed that in *N,N'*-dimethylethanolamine-treated cells the amount of radioactive glycoprotein decreased in the microsomes during the additional 60-min period of chase

and simultaneously increased in the plasma membranes. However, the radioactivity in both fractions increased in other modified cells. Negligible radioactive glycoprotein in virions was released from the host cells during the 60-min period of chase (see legend of Table V). Increase in radioactivity in the plasma membranes during the chase was greatest in *N,N'*-dimethylethanolamine-treated cells, followed in order by choline- and ethanolamine-treated cells (right column of Table V). Concerning the mobility of glycoprotein from the microsomes to the plasma membranes in the modified cells, the ratios of increase in radioactivity in the plasma membranes (right column of Table V) to the radioactivity in the microsomes 10 min after chase in the various modified cells were compared. The values in *N,N'*-dimethylethanolamine-, choline- and ethanolamine-treated cells were 0.55, 0.34 and 0.15, respectively. These results also strongly suggest that migration of glycoprotein from the microsomes to the plasma membranes is fastest in *N,N'*-dimethylethanolamine-treated cells and slowest in ethanolamine-treated cells.

Production of progeny virions

To determine whether the differences in migration of glycoprotein in the various modified cells reflect differences in production of progeny virions, we examined the plaque-forming activities in the culture media of these cells after infection. As shown in Table VI, the activity differed depending on the analogues used for modification, decreasing in the following order: *N,N'*-dimethylethanolamine- > choline- > *N*-monomethylethanolamine- > ethanolamine-treated cells. That is, the plaque-forming activity produced by *N,N'*-dimethylethanolamine-treated cells was the highest, and that produced by ethanolamine-treated cells was the lowest. These results were further confirmed by measurement of radioactive virions released from the modified cells (see legend of Table V). Preliminary experiment revealed there was no difference in infectivity of the virus with modified membrane-lipid-envelopes. These observations demonstrate that modification of membrane phospholipids of the host cells affects the efficiency of virion production in the cells and that this could be, at least in part, related to the different migration of glycoprotein within the cells.

TABLE VI

EFFECTS OF PHOSPHOLIPID MODIFICATION ON PROGENY FORMATION

Membrane phospholipids were modified as described in the legend to Table I. Cells ($1 \cdot 10^7$ cells in 2 ml of medium) were infected with virus (multiplicity of infection: 1.2). After adsorption for 1 h, cells were centrifuged at 60 × *g* for 5 min and suspended in 5 ml of fresh medium. Choline analogues were present throughout the experiment. Infected cells were kept in suspension for 26 h at 37° C and then removed by centrifugation (1000 × *g*, 5 min). Plaque-forming activity in the supernatant was assayed as described in Materials and Methods. The plaque-forming activity released from *N,N'*-dimethylethanolamine-treated cells was usually ten times released from ethanolamine-treated cells.

Analogue	Plaque forming units ($\times 10^8$/ml)
Choline	3.6
N,N'-Dimethylethanolamine	6.0
N-Monomethylethanolamine	2.4
Ethanolamine	0.9

Discussion

In this work the membrane phospholipids of LM cells were modified by treatment with choline or one of its analogues (Table II) and then the cells were infected with vesicular stomatitis virus. This system is useful for studies on biological membranes in situ, because the process of viral infection involves many membrane-associated reactions. From the standpoint of virus maturation, which is closely associated with host membranes, it seemed interesting to examine the behavior of glycoprotein in cells with modified membranes, since this protein is synthesized on membrane-bound polysomes, inserted into the microsomal membranes and glycosylated and then it migrates to the plasma membranes, where the assembly and budding of virus take place [4]. Modification of membrane phospholipids could affect all these steps.

Indeed, the results showed that the amounts of pronase-susceptible glycoprotein, which must be present in the surface membranes of the host cells, differed in cells modified with different analogues (Table IV). In choline-treated cells radioactive glycoprotein was distributed more evenly between the plasma membranes and microsomes whereas in *N,N'*-dimethylethanolamine-treated cells it was mainly distributed in the plasma membranes after 4 h chase, suggesting differences in the migration rates of glycoprotein from the microsomes in the various modified cells. Furthermore in ethanolamine-treated cells, large amounts of glycoprotein were found in the soluble fraction as well as in the microsomes and plasma membranes. Glycoprotein may accumulate in the soluble fraction by virtue of its inefficient insertion into the membranes, and this could also be one reason why ethanolamine-treated cells produced only low yields of virus.

Migration of glycoprotein from the microsomes to the plasma membranes was examined further in more detailed experiments. The results showed that in *N,N'*-dimethylethanolamine-treated cells radioactive glycoprotein increased in the plasma membranes with simultaneous decrease in radioactivity in the microsomes and that 70 min after chase the plasma membranes contained more radioactive glycoprotein than the microsomes (Table V). In choline- and ethanolamine-treated cells, radioactive glycoprotein in the microsomes still increased 70 min after chase, possibly due to residual synthesis of radioactive glycoprotein. The different behaviors of radioactive glycoprotein observed in the microsomes of these modified cells could be explained by differences in the migration rates of glycoprotein from the microsomes in these cells. The mobility of glycoprotein from the microsomes to the plasma membranes can be expressed as the ratio of increase in radioactive glycoprotein in the plasma membranes during a 60-min period of chase to the radioactivity in the microsomes after a 10-min chase. The ratios obtained indicate that migration of glycoprotein from the microsomes to the plasma membranes is most rapid in *N,N'*-dimethylethanolamine-treated cells, followed in order by choline- and ethanolamine-treated cells. All these findings suggest that the presence of phosphatidyl-*N,N'*-dimethylethanolamine in the membranes favors the migration of glycoprotein of vesicular stomatitis virus from the microsomes to the plasma membranes, and that this could be related to the higher production of progeny virions in *N,N'*-dimethylethanolamine-treated cells.

Studies on the physico-chemical properties of cell membranes from the cells modified with choline analogues such as *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine and ethanolamine showed that the transition temperature was not affected by these modifications, suggesting that the cells have some sorts of compensation mechanism [8,25,26]. As for *N,N'*-dimethylethanolamine-treated cells, the fatty acid composition of phospholipids was altered with respect to chain length and degree of unsaturation [27] besides the altered contents of desmosterol [8] and as a consequence fluidity of membrane lipid was kept almost the same as compared with that of choline- or *N*-monomethylethanolamine-treated cells [8,25,26]. Phosphatidylethanolamine generally produces a more rigid membrane than phosphatidylcholine from the standpoint of membrane fluidity, because it has no methyl group on the amino nitrogen. However, large amounts of lysophosphatidylcholine are found in ethanolamine-treated cells with simultaneous alteration of fatty acid and sterol as well and these could increase membrane fluidity [8]. Despite the fact that there seems to be some compensation mechanism for regulating membrane fluidity, the mobility of glycoprotein was high in cells modified with *N,N'*-dimethylethanolamine, and low in cells modified with ethanolamine (Table V). The basal activity of adenylate cyclase, the membrane enzyme, is higher in ethanolamine-treated cells than in choline-treated cells [28]. Thus modification of the phospholipids influences not only the membrane enzyme activity but also migration of the membrane glycoprotein within the cells. These results suggested that membrane fluidity of the cells was not altered as a whole by supplementation with choline analogues but microviscosity surrounding the specific functional proteins must be affected by these treatments. Based on the idea of annular lipids presented recently [29], it is possible that membrane proteins may interact more strongly with certain specific lipids in the lipid bilayer. Both the stimulatory effect of phosphatidyl-*N,N'*-dimethylethanolamine and the inhibitory effect of phosphatidylethanolamine on the migration of glycoprotein from the microsomes to the plasma membranes are consistent with the idea of the existence of such annular lipids.

Two properties of the base moiety of phosphatidyl-*N,N'*-dimethylethanolamine may explain why this phospholipid favors the migration of glycoprotein: one is the number of methyl groups and the other is the absence of a positive charge on the amino nitrogen. An increased number of methyl groups increased the fluidity of membrane lipids. A moderate contribution to fluidity and the absence of a positive charge on phosphatidyl-*N,N'*-dimethylethanolamine could favor virus maturation.

As for the matured virions released from the cells modified with choline analogues the characteristic phospholipid compositions were found in their envelopes. However there seems to be no difference in the infectivity of these virions. All these results will be reported elsewhere.

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