Glycophorin as a Receptor for Sendai Virus

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ABSTRACT: Glycophorin A was reconstituted into large unilamellar vesicles of egg phosphatidylcholine by detergent dialysis. The observed overall rate of Sendai virus fusion increased approximately 4-fold between 0 and 0.006 mol % glycophorin, roughly proportional to the glycophorin content. However, no further increase in rate was observed at 0.02 mol % glycophorin. Treatment of reassembled glycophorin−liposomes with neuraminidase resulted in a significant decrease in the percent of viral fusion, confirming that the presence of sialic acid residues on glycophorin is essential for its role as a receptor. The sialic acid-containing glycolipid, the ganglioside GD_{1a}, was also incorporated into phosphatidylcholine liposomes, either in addition to or in place of glycophorin A. Comparing, on the basis of sialic acid content, liposomes containing either glycophorin or GD_{1a}, comparable rates and extents of fusion were found. However, on a molar basis glycophorin is much more effective. It was found that the addition of GD_{1a} to glycophorin-containing liposomes only slightly increased the rate of fusion. This was largely due to an increase in the percent of virions capable of fusing.

The role of viral receptors in the process of viral fusion is not completely understood. We have been studying the fusion of Sendai virus with model liposomes. The use of these models allows us to limit the content and nature of receptor molecules present in the target membrane. Sialic acid moieties serve as a receptor for Sendai virus as a consequence of the presence of the HN protein in the viral envelop which has affinity for this species. It is thought that both sialic acid-containing glycolipids (Haywood, 1974a,b) as well as glycoproteins, such as the major erythrocyte glycoprotein glycophorin (Oku *et al.*, 1982; Gershoni *et al.*, 1985), can serve as viral receptor. Glycophorin is a 131 amino acid residue protein with 60% of its weight being carbohydrate, of which there are approximately 30 residues of sialic acid. Glycophorin dimerizes in a membrane as a consequence of specific interactions between protein subunits (Challou *et al.*, 1994; Smith & Bormann, 1995). The protein is surrounded by approximately 34 phospholipid molecules (Shan *et al.*, 1994). It had been suggested that receptor binding may play an active role in Sendai virus fusion (Citovsky *et al.*, 1986; Dallocchio *et al.*, 1995). Glycophorin-containing liposomes have also been shown to fuse with cells expressing the influenza HA protein in their plasma membrane (Ellens *et al.*, 1990). In the case of a sialic acid-containing glycolipid receptor for Sendai virus, we have shown that the effect of the receptor on the fusion rate constant is very dependent on the nature of the membrane in which the receptor is inserted but that the presence of a viral receptor always results in an increase in the aggregation rate constant (Epand *et al.*, 1995). There is also a report suggesting that the ganglioside GM_1 actually reduced the rate of fusion of Sendai virus with liposomes containing glycophorin (Umeda *et al.*, 1984). We have systematically studied the effect of glycophorin on the fusion parameters for Sendai virus and have compared it with GD_{1a} and with a combination of glycophorin with GD_{1a}. This comparison elucidates the effect of the extension of the receptor from the membrane on virus binding and fusion.

EXPERIMENTAL PROCEDURES

Materials

Chemicals. All phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). Gangliosides were purified according to Reed *et al.* (1987). All lipids showed one spot by TLC at a load of 50 μg. Octadecylrhodamine was purchased from Molecular Probes, Eugene, OR, and *N*-NBD-aminoundecanoyl-GD_{1a} (NBD-GD_{1a}) was purchased from I. Mikhalyov (Moscow, CIS). All other chemicals and solvents were of reagent grade. Neuraminidase (from *Clostridium perfringens*) was obtained from Sigma Chemical Co. (St. Louis, MO). The [9,10-^{3}H]dipalmitoylphosphatidylcholine was from NEN, Montréal, Quebec. Glycophorin was purified from human erythrocyte ghosts by the procedure of Segrest *et al.* (1979).

Methods

Purification of Sendai Virus. The Cantell strain of Sendai virus was propagated by inoculation of the allantoic sac of 10-day-old embryonated chicken eggs. After 72 h of incubation at 33 °C, the allantoic fluid was harvested and

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clarified by centrifugation at 3000*g* for 30 min at 4 °C. The virus was pelleted at 60000*g* for 1.5 h at 4 °C. The pellet was dispersed in phosphate-buffered saline and centrifuged through a discontinuous gradient of 35%, 40%, 45%, and 60% sucrose at 60000*g* for 2.5 h at 4 °C. The virus was harvested from the band that formed at the 40−45% interface. The virus was pelleted at 60000*g* for 1.5 h and resuspended in HEPES-buffered saline, pH 7.4, to a viral protein concentration of 1 mg/mL.

Preparation of LUVs by Detergent Dialysis. Egg phosphatidylcholine (PC) was purchased from Avanti-Polar Lipids, Inc. A weighed amount of egg PC was dissolved in 2:1 (v/v) chloroform/methanol. Tracer amounts of [^{3}H]-DPPC were added to monitor lipid recovery. The phospholipid was dried down to a film under a gentle stream of nitrogen gas and then placed in a vacuum evaporator equipped with a liquid nitrogen trap for a minimum of 30 min to ensure complete removal of organic solvents. In a typical preparation, just under 7 mg of egg PC was used.

A stock glycophorin A solution was made by dissolving lyophilized glycophorin in HEPES/MES buffer (5 mM HEPES, 5 mM MES, 5 mM sodium citrate, 150 mM NaCl, and 1 mM EDTA, pH 7.4) containing 25 mM CHAPS, a readily dialyzable, zwitterionic detergent (Chu & Sharom, 1992). A stock GD_{1a} solution was made by dissolving NBD-GD_{1a} in HEPES/MES buffer containing 25 mM CHAPS. A volume of stock glycophorin A solution was added to the dried egg PC lipid sample. The lipid:protein ratio was varied by adding appropriate amounts of egg PC and glycophorin A. The total volume was adjusted to 1 mL with HEPES/MES buffer containing 25 mM CHAPS. When GD_{1a} was to be incorporated into the lipid vesicles, it was added to the lipid mixture in detergent, prior to dialysis. Six to eight small glass beads were placed in the test tube, and the mixture was vortexed gently. The sample was warmed in a water bath at 37 °C and vortexed occasionally until it was free of large particles. Before dialysis, a 10 μL sample of the lipid−protein mixture was taken so that the initial radioactivity from the phospholipid tracer could be determined by scintillation counting.

The lipid−protein mixture was transferred to a 0.5−3 mL dialysis cassette, with a 10 000 molecular weight cutoff membrane (Pierce Chemical Co., Rockford, IL). The sample was dialyzed against five changes of HEPES/MES buffer, pH 7.4, at 4 °C over a period of 44−48 h. When the cassette contained fluorescently labeled ganglioside, the entire beaker was wrapped in aluminum foil to protect the probe from photodecomposition.

Following dialysis, if any large precipitates were present, they were removed by centrifugation for 10 min at high speed on a desk-top clinical centrifuge. In order to remove glycophorin that was not incorporated into the LUVs, the dialyzed lipid−protein mixture was passed down a Sepharose 2B-300 column (1 × 10 cm; 7 mL bed volume, Sigma) which had been previously equilibrated with HEPES/MES buffer, pH 7.4. The LUVs were eluted with the same buffer, and the cloudy fractions were pooled together for a total volume of 2−3 mL. The same column was used with LUVs containing GD_{1a}. A 10 μL aliquot was taken to determine the final lipid recovery by scintillation counting. Reconstituted glycophorin LUV samples were stored in silanized test tubes at 4 °C. Samples containing fluorescently labeled ganglioside were wrapped in aluminum foil. Vesicles containing egg PC only were prepared by detergent dialysis as well. However, most of this sample precipitated after dialysis. Therefore, the egg PC suspension was dialyzed and then passed through two stacked 0.2 μm polycarbonate filters (Nuclepore, Pleasanton, CA) using an extrusion device (Lipex Biomembranes, Ottawa, ON).

Vesicle Sizing by Quasi-Elastic Light Scattering. Prior to vesicle sizing, the entire sample of LUVs as well as a volume of HEPES/MES buffer, pH 7.4, used for dilution was passed through a 0.45 μm filter to remove dust particles. The LUVs were diluted to approximately 0.1 mg/mL.

The instrument used to measure the diameter of the LUVs was a Brookhaven Model B1 9000AT digital correlator. The vesicles were assumed to be spherical. Scattering at 514 nm was measured at an angle of 90° for 500 s. The data were analyzed by cumulant analysis and nonnegatively constrained least squares using software provided by the manufacturer.

Virus Fusion Assay. Sendai virus was labeled with octadecylrhodamine (R18) (Molecular Probes) according to the procedure of Hoekstra *et al.* (1984). Ten microliters of R18 (10 nmol) in ethanol was injected into 1 mL of a suspension of Sendai virus in HEPES/MES buffer. The mixture was allowed to incubate at room temperature for 1 h. Unincorporated R18 was then removed by passing the labeled virus through a Sephadex G-75 gel filtration column eluted with the HEPES/MES buffer, and collecting the virus in the void volume. The final viral protein concentration was determined using the BCA assay (Pierce Chemical Co., Rockford, IL). LUVs were diluted into 2 mL of HEPES/MES buffer, pH 7.4, maintained in silanized cuvettes in a thermostated cuvette holder at 37 °C with continual magnetic stirring. Five micrograms of R18-labeled Sendai virus was rapidly injected into the cuvette through a light-sealed septum. Fluorescence was recorded using an SLM AMINCO Bowman Series 2 luminescence spectrometer interfaced with a 386/20 IBM compatible computer. The instrument used a xenon arc light source with a 560 nm filter between the excitation slit and sample and a 590 nm cutoff filter between the sample and the photomultiplier tube to minimize any contribution of light scattering to the fluorescence signal. The excitation and emission monochromators were set at 565 and 600 nm, respectively. The fluorescence intensity immediately after addition of the labeled virus is taken as F_0. A 40 μL aliquot of 10% Triton X-100 was added in order to measure F_{100}. The percent of R18 dequenching was calculated at time t from:

$$\% \text{ R18 dequenching} = 100[(F_t - F_0)/(F_{100} - F_0)]$$

For kinetic experiments, the fluorescence was recorded over the first 10 min after initiation of fusion. For the final extents of fusion, the cuvettes are wrapped in foil, and the fluorescence was measured after 8 h of incubation at 37 °C in a shaking water bath. Both the kinetics and the final of viral fusion are presented as the averages of at least two determinations done on the same day, with the same batches of virus and liposomes. Duplicates were always within 10% of each other, and the kinetic runs were generally superimposable.

Analysis of Data. The analysis of the final extents of fusion of Sendai virus with liposomes was done as previously described (Nir *et al.* 1986a, 1990). In the analysis of the

kinetics of fusion, we have employed three parameters: *C*, the second-order rate constant of virus adhesion to cells or to liposomes; *f*, the first-order rate constant of the actual fusion of an adhered virus particle; *D*, the first-order dissociation rate constant.

Determination of Glycophorin. In order to determine the recovery of glycophorin in the reconstituted vesicles, a modification of the resorcinol−sialic acid assay published by Svennerholm (1957) was used. Aliquots of the LUV samples were pipetted into test tubes, and the amount of lipid present in the aliquot was calculated. Standards were made with weighed amounts of glycophorin A (25−100 μg) whose protein content had also been determined by the BCA assay. Aliquots of egg PC were added to the standard samples since it was found that the lipid alone gave a negative absorbancy reading compared with a solvent blank and it also lowered the readings for glycophorin. The same amount of lipid was added to the glycophorin standards as was present in the aliquot of LUVs used in this assay. Sample aliquots were diluted to a standard volume of 1 mL with distilled water. One milliliter of resorcinol reagent, prepared as described by Svennerholm (1957), was added to each test tube. The tubes were capped with marbles and placed in a boiling water bath for at least 20 min. After the samples were allowed to cool to room temperature, 2 mL of *N*-butylacetate/1-butanol (85:15, v/v, reagent grade) was added, and the samples were vortexed. The samples were then centrifuged at high speed for 10 min in a clinical centrifuge. The upper organic phase was transferred to glass cuvettes and read at 580 nm in a Perkin-Elmer Lambda 4B UV/VIS spectophotometer against distilled water. By comparing the absorbance reading of the unknown LUV sample to its corresponding standard glycophorin sample, the amount of glycophorin in the LUV aliquot was determined.

In the cases in which the LUVs contained both glycophorin and GD_{1a}, and as an occasional verification of the Svennerholm assay, the glycophorin content was determined by Trp fluorescence by comparing the fluorescence emission from the glycophorin-containing LUVs with that from a standard solution of glycophorin whose protein concentration was determined by the BCA assay. Fluorescent readings were corrected for minor contributions from solvent and lipid.

Determination of GD_{1a} Incorporation. NBD-GD_{1a} was used as a probe to determine the recovery of ganglioside after dialysis and gel filtration. A standard curve was prepared by measuring the fluorescence of predetermined amounts of NBD-GD_{1a} dissolved in 2 mL of 1% Triton X-100. Fluorescence was recorded at 37 °C by the same instrument used for the virus fusion assay, with the excitation and emission monochromators set at 468 and 543 nm, respectively. A 420 nm cutoff filter was placed in front of the excitation slit, and a 460 nm filter was placed in between the sample and the photomultiplier tube. The standard curve was analyzed by least-squares linear regression and used to determine the amount of ganglioside present in the LUV aliquots. It was found that the recovery of ganglioside was proportional to the recovery of phospholipid. In separate experiments, we determined that NBD-GD_{1a} promoted less fusion than unlabeled GD_{1a}. We therefore used the unlabeled ganglioside for the viral fusion studies, assuming that the recovery of GD_{1a} was comparable to those of phospholipid and of NBD-GD_{1a} which were found to be almost quantitative.

Neuraminidase Treatment of the Glycophorin Liposomes. Following the procedure of Ellens *et al*. (1990), glycophorin liposomes (183:1 egg PC:glycophorin ratio) were incubated for 1 h at 37 °C in HEPES/MES buffer, pH 6.1, with 1.1 units of neuraminidase/mL, which corresponds to an estimated 4.5 units of neuraminidase/mg of glycophorin. After the incubation, the liposomes were passed down a Sephadex G-75 column (1.5 × 20 cm; 30 mL bed volume; Pharmacia), previously equilibrated with HEPES/MES buffer, pH 7.4, to remove the neuraminidase. The column was eluted with the same buffer, and the vesicles were recovered in the void volume. The pH of the recovered vesicle solution was found to be 7.33. LUVs were diluted into 2 mL of HEPES/MES buffer, pH 7.4, and used for kinetic experiments.

Fusion Product Experiments. In order to study the nature of the fusion product, Sendai virus was allowed to fuse with reconstituted glycophorin−LUVs at a 1:1 ratio (virus:vesicle ratio). The amount of virus required was calculated using a virion weight of 5×10^8 Da and an LUV radius of 110 nm. A lipid concentration of 25 μM and 28.5 μg of Sendai viral protein were used. The LUVs with glycophorin were allowed to fuse for 1 h with R18-labeled Sendai virus, at which time either (1) an additional 25 μM of fresh glycophorin−LUVs was injected, or (2) an additional 50 μg of R18-labeled Sendai virus was added, or (3) 50 μg of unlabeled Sendai virus was injected. Samples were incubated for an additional 700 s, and then 160 μL of 10% (v/v) Triton X-100 was added to the cuvette.

RESULTS

The average diameters measured by QUELS for the various LUVs containing glycophorin were within 10% of 190 nm. The size of the reconstituted glycophorin vesicles did not vary as a function of protein concentration. However, the vesicles with only egg PC had a lower diameter of 143 nm. In addition, because the egg PC LUVs were aggregated after detergent dialysis and because the glycophorin-containing LUVs could not be prepared by extrusion, the fusion results with the pure egg PC vesicles are not directly comparable to the others. The diameters measured for the reassembled glycophorin LUVs were consistent with those reported by Mimms *et al.* (1981).

The overall rate of Sendai fusion with the glycophorin-containing liposomes increases markedly at lower lipid to glycophorin ratios (Figure 1). The increase is most noticeable at low mole fractions of glycophorin. Above a 1:171 glycophorin to lipid ratio, the increase in fusion rate is relatively modest with a 3-fold change in the glycophorin to lipid ratio. Sendai virus showed relatively little fusion in the absence of receptor. Apparently, glycophorin A plays a significant role in the fusion of Sendai virus.

The final extents of fusion did not depend appreciably on the amount of incorporated glycophorin (Table 1). Only the vesicles not containing receptor showed a drastic decrease in the final extent of fusion, giving a value of 46% compared to the average value of 65% for the reconstituted glycophorin−LUVs.

In order to obtain a more detailed evaluation of the role of glycophorin on the fusion of Sendai virus, we have compared the kinetics of fusion of pure PC liposomes with two batches of reconstituted glycophorin in egg PC at 50:1 and at 415:1 lipid to protein ratios (Table 2). The viral fusion

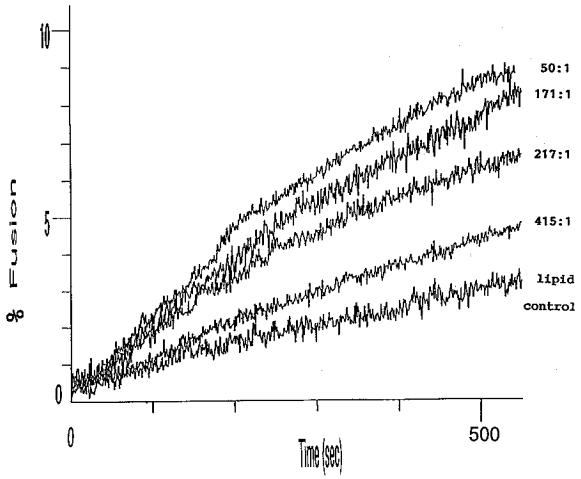

FIGURE 1: Fusion of Sendai virus with LUVs of egg PC containing the indicated lipid to glycophorin mole ratio. Assays were performed at 37 °C in HEPES/MES buffer, pH 7.4, using 5 μg of Sendai viral protein and 50 μM LUVs.

Table 1: Observed Final Extents of Fusion at 50 μM Lipid Concentration

egg PC:glycophorin ratio	final extent (%)
50:1	67
171:1	71
217:1	64
415:1	59
1:0 (control)	46

Table 2: Role of Glycophorin in the Fusion of Sendai Virus[a]

PC/ glycophorin molar ratio	vesicle diameter (nm)	% viruses capable of fusing	C ($M^{-1}s^{-1}$)	f (s^{-1})	D (s^{-1})
pure egg PC	145[b]	50	4×10^7	8×10^{-4}	0.005
415	196	50	1.8×10^8	9×10^{-4}	0.01
50	185	65	3.0×10^8	13×10^{-4}	0.015

[a] C is the second-order rate constant for the association of virus and liposome, f is the first-order rate constant of membrane fusion, and D is the first-order rate constant for the dissociation of the bound virus from the liposome. The estimated error in the viruses capable of fusing is 5%, for C and f it is 30%, and for D, 50%. [b] These vesicles required extrusion after detergent dialysis (see text).

assays were done on the same day, using the same batch of R18-labeled virus, but varying the virus to liposome ratio, so that the kinetic constants could be calculated and so that the results are directly comparable within this pair. The final extent of fusion increased to 65% for the higher glycophorin content of the 50:1 lipid/protein liposomes compared with 50% for the other cases. The largest increase, however, was in the aggregation rate constant, C, which increased 4-fold for the LUVs with low glycophorin content and 7-fold for the liposomes with high glycophorin content, compared with the pure lipid control. While the comparison with the pure PC liposomes may not be quantitative, the large changes observed in C and the further increase with increasing glycophorin concentration make it clear that glycophorin acts as a Sendai receptor and promotes the binding of the virus. The fusion rate constant, f, increased only slightly for the 415:1 liposomes but showed a 2-fold increase for the 50:1 liposomes compared to egg PC alone.

The treatment of reconstituted glycophorin LUVs with the enzyme neuraminidase resulted in a severalfold decrease in the rate of fusion. This was as expected since neuraminidase removes glycophorin's sialic acid residues, which are thought to play an essential role in the fusion process (Figure 2).

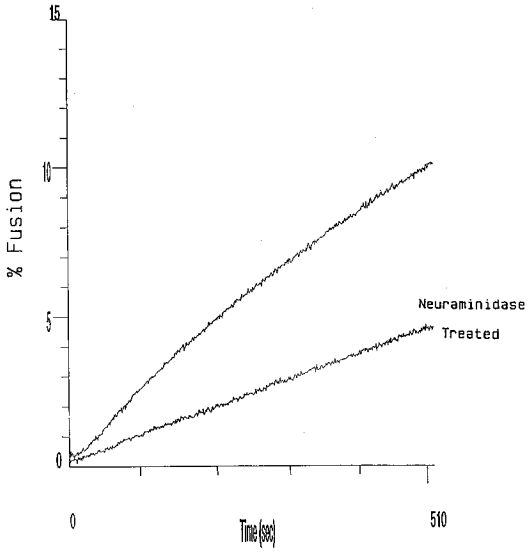

FIGURE 2: Fusion of Sendai virus (5 μg of viral protein) with 50 μM LUVs of egg PC/glycophorin (171:1 molar ratio). The neuraminidase-treated curve corresponds to LUVs that were treated for 1 h at 37 °C with neuraminidase prior to addition of the virus. The upper curve is the control in which the liposomes were treated identically except that neuraminidase was not included in the incubation mixture.

The fusion of Sendai virus with LUVs in a 1:1 ratio gave insight regarding the properties of the fusion products. The addition of Sendai virus to liposomes containing glycophorin (230:1 lipid:protein ratio) resulted in only a small decrease in the fluorescence intensity caused simply by dilution of the sample when the extra volume of the virus was added. This indicated that only one virion was able to fuse with a liposome; once a fusion product was formed, no other virion could fuse with it. In a separate experiment, when instead of unlabeled Sendai virus additional R18-labeled Sendai virus was added after 1 h to the 1:1 virus/liposome incubation mixture, the fluorescence intensity initially rose as a consequence of the addition of labeled virus. However, the fluorescence intensity level soon reached a maximal value and remained constant for the remainder of the incubation period. Addition of unlabeled virus to this mixture did not result in a significant increase in fluorescence. It can be concluded that not more than one Sendai virus can fuse with one liposome. These experiments also rule out dequenching of the probe due to exchange between membranes. The finding that a virion does not fuse with the fusion product of a virus with a liposome of a comparable size was established for influenza virus (Nir *et al.*, 1986b; Stegmann *et al.*, 1989), Sendai virus (Nir *et al.*, 1986a), and HIV-1 (Larsen *et al.*, 1993; Nir *et al.*, 1993). As formulated in Nir *et al.* (1990), this result could indicate a negative cooperativity, or repulsion, between glycoproteins in intact virus particles and those in the fusion products. Alternatively, it could also be just a saturation limit, as was favored for the case of Sendai virus (Nir *et al.*, 1993).

We have also compared two representative sialic acid receptors, *i.e.*, glycophorin and GD_{1a}, present in the LUV either individually or in a combination of the two (Figure 3). The content of GD_{1a} in both the liposomes with only ganglioside as well as those with the combination of

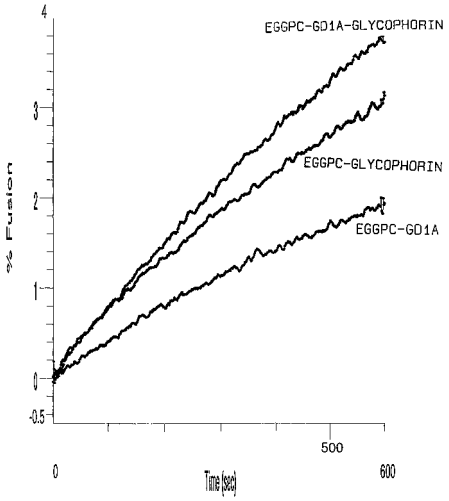

FIGURE 3: Fusion of Sendai virus (5 μg of viral protein) with 6 μM LUVs at 37 °C, pH 7.4. The LUVs were composed either of egg PC with 6 mol % GD_{1a}, egg PC with 0.25 mol % glycophorin (400:1, lipid:protein ratio), or egg PC with 6 mol % GD_{1a} plus 0.33 mol % glycophorin (300:1, lipid:protein ratio).

Table 3: Effect of Receptors on the Fusion of Sendai Virus to Liposomes of Egg PC[a]

receptor	content (mol %)	% viruses capable of fusing	C (M^{-1} s^{-1})	f (s^{-1})
GD_{1a}	6	40	1×10^8	7×10^{-4}
glycophorin	0.25	45	2.5×10^8	5.5×10^{-4}
GD_{1a} plus glycophorin	6			
	0.33	50	2.5×10^8	5.5×10^{-4}

[a] See Table 2 for estimate of errors. D is 0.01 s^{-1} for the last two cases and uncertain for the case of GD_{1a} alone. See Table 2 for definitions of C and f.

ganglioside and glycophorin was 6% with respect to the lipid composition. The phospholipid to glycophorin ratio was 400:1 in vesicles without ganglioside and 300:1 in vesicles with both receptors. The diameters of the vesicles were 268, 170, and 184 nm for the vesicles with GD_{1a}, glycophorin, or GD_{1a} plus glycophorin, respectively. The calculated kinetic fusion parameters are summarized in Table 3. These values are similar but not identical to those in Table 2. The error in the analysis within one experiment is indicated in a footnote in Table 2, but the deviations between experiments done with different lots of virus and different labelings with R18 are somewhat greater.

DISCUSSION

Glycophorin is a 31 kDa glycoprotein of which about 14 kDa corresponds to the protein portion. This protein is incorporated in the erythrocyte membrane with a cytoplasmic tail on the COOH end of the polypeptide chain having a molecular mass of 3.9 kDa, a transmembrane helical core of 3.3 kDa, with the remaining approximately 25 kDa on the extracellular surface of which 18 kDa is carbohydrate. Presumably in the reconstituted system this vectorial orientation is lost, but the virus would only interact with the fraction of glycophorin oriented with its sialic acid exposed to the external environment allowing the virus to bind. There is evidence that glycophorin can adopt one of two conformations, depending on the content of the protein in the membrane (Rüppel *et al.*, 1982). Below 0.8 mol % (125:1 lipid/protein), the protein spreads at the lipid−water interface to a surface area corresponding to a circle of 180 Å in diameter, while at higher concentrations (above 3.2 mol % or a lipid to protein ratio of 31) the protein protrudes further from the membrane surface and circumscribes a circle on the membrane surface of around 80 Å in diameter. At these mole fractions, the protein would cover the entire surface of the bilayer. Nevertheless, this apparently does not inhibit the fusion process.

In order for Sendai virus to fuse with the target liposomes, the phospholipids of the two membranes must come in contact. However, a consequence of the protrusion of the glycoprotein from the membrane surface is that the virus will initially be further from the surface of the target bilayer. The virus would also be further from the membrane surface than in the case of a liposome containing a glycolipid receptor. One would thus anticipate that membrane fusion would be made more difficult in the presence of glycophorin. For example, a membrane-tethered polymer, lipophosphoglycan, has been shown to greatly inhibit viral fusion (Miao *et al.*, 1995). The steric inhibition of fusion should also increase below a lipid to protein ratio of 125. We do not believe that this steric inhibition is an important factor. The rate of fusion increases with increased coverage of the membrane surface by glycophorin and with increased protrusion of this protein from the membrane surface. There is, however, a leveling off of the enhancement of fusion rate with increasing mole fraction of glycophorin, and steric factors may contribute to this phenomenon. The effectiveness of GD_{1a} as a receptor for Sendai virus also does not continue to increase at higher mole fractions, and in fact the rate decreases in certain cases (Epand *et al.*, 1995). We ascribed this effect to the receptor reducing negative curvature strain (Epand *et al.*, 1995). Glycophorin also shows a similar effect on lipid curvature (Taraschi *et al.*, 1982), and this may be another factor limiting the fusion rate at high mole fractions of receptor.

Comparing glycophorin and GD_{1a} as receptors for Sendai virus (Table 3) illustrates that the two very different receptors behave comparably, although under the conditions used glycophorin is a somewhat better receptor, especially if one considers that GD_{1a} is present at 6 mol % and glycophorin at only 0.25 mol %. Presumably, only half of the glycophorin is in the correct orientation in the membrane to promote fusion, and only half of the GD_{1a} is exposed on the outer monolayer. However, the amount of sialic acid present in the LUVs with each of the two receptors at these mole fractions is more comparable. There is 15 times as much sialic acid in a glycophorin molecule compared with GD_{1a}. This means that at the mole fractions used, GD_{1a} contributes 1.6 times the amount of sialic acid compared with glycophorin, yet the ganglioside is a poorer receptor. Thus, despite its greater steric bulk, glycophorin is at least as good and in fact a slightly more effective receptor than gangliosides. Although it is comparing two very different situations, the finding that glycoproteins are such effective receptors for Sendai virus may also explain why fusion rate constants measured with erythrocyte ghosts, in which there is a high concentration of glycoproteins, are 1 or 2 orders of magnitude greater than with liposomes containing GD_{1a}.

It is possible that glycophorin and ganglioside could act synergistically. Glycophorin has more sialic acid, and in addition, this sialic acid is more protruding into the aqueous

environment while the sialic acid of GD_{1a} is closer to the membrane interface. It is thus possible that the glycophorin could more readily bind to Sendai virus, while the GD_{1a} would be better situated to promote fusion. Hence, the virus could first bind to glycophorin and then be transferred to the ganglioside. There is some evidence from the literature showing increased vesicle lysis when a mixture of GD_{1a} and GD_{1b} was added to glycophorin-containing liposomes (Citovsky *et al.*, 1986). However, our results (Table 3) indicate that GD_{1a} only marginally increased the observed fusion rates and that this was largely a result of an increase in the percentage of virions capable of fusing. As was previously suggested (Nir *et al.*, 1990), the occurrence of unfused virions in the presence of an excess of liposomes is due to virions bound to liposomes via "inactive" sites on the viral membrane. Thus, the binding of Sendai virus to liposomes containing the receptors GD_{1a} or glycophorin reduces the chance of the virus binding irreversibly in a fusion-inactive state.

It has recently been suggested that the binding of viruses to receptors on the plasma membrane of cells at neutral pH can trigger a conformational change in a viral envelop protein that activates the viral fusion mechanism (Gilbert *et al.*, 1995). Thus, receptor binding would have the same triggering function for these viruses as acidification has for viruses, such as influenza, which fuse within endosomes. If such a mechanism also applied to Sendai virus, our results would suggest that the conformational change activating the viral protein was coupled to the association step rather than to the actual fusion step.

In summary, our results demonstrate that a large glycoprotein receptor can promote more viral fusion than a glycolipid receptor, despite its greater protrusion from the membrane surface.

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