Biochimica et Biophysica Acta, 733 (1983) 286–290
Elsevier

BBA Report

BBA 70116

MEMBRANE FUSION ACTIVITY OF RECONSTITUTED VESICLES OF INFLUENZA VIRUS HEMAGGLUTININ GLYCOPROTEINS

KAZUNORI KAWASAKI, SATOSHI B. SATO and SHUN-ICHI OHNISHI

Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606 (Japan)

(Received April 19th, 1983)

Key words: Influenza virus hemagglutinin; Membrane fusion; pH induction; Phospholipid vesicle

Reconstituted vesicles of hemagglutinin glycoproteins into egg yolk phosphatidylcholine/spin-labeled phosphatidylcholine/cholesterol (molar ratio 1.6:0.4:1) were prepared by dialysis. Preparations at appropriate protein-to-lipid ratios (1:44 and 1:105 mol/mol) contained vesicles with a diameter of 100–300 nm and a high density of spikes on the surface. These vesicles showed low pH-induced membrane fusion activity. At pH 5.2 and 37°C, fusion with erythrocyte membranes took place very rapidly within 1–2 min and reached a plateau at 63–66% fusion. The fusion was negligibly small at neutral pH and was induced to occur at pH values lower than 6.0. The reconstituted vesicles caused hemolysis and fusion of human erythrocyte cells in the same pH range as that of the fusion with erythrocyte membranes. The low pH-induced fusion activity of the reconstituted vesicles is essentially the same as that of the parent virus. These vesicles can be used to deliver some reagents or drugs into target cell cytoplasm via fusion at lysosomes.

A discovery of the low-pH-induced fusion of influenza virus envelope with cell membranes had led to a new infectious cell entry mechanism of this virus [1–4]. The virus particles are endocytosed into coated vesicles which are then fused with lysosomes. The viral genome RNAs are released into the target cell cytoplasm upon fusion of the viral envelope with the secondary lysosome [4–6] or endosomes because of the low intralysosomal pH. The molecule responsible for the low pH-induced fusion activity is hemagglutinin glycoprotein on the viral envelope. We have studied functions of isolated hemagglutinin proteins and reconstituted vesicles of hemagglutinin. The hemagglutinin proteins by themselves showed low-pH-induced hemolytic activity similar to that of the parent virus [7]. They did not cause fusion of erythrocyte cells, however. In the present paper, we describe results of our study on reconstituted vesicles of hemagglutinin proteins.

Hemagglutinin was solubilized from influenza virus A_0PR_8 using Triton X-100 and partially purified by sucrose density gradient centrifugation as described previously [7]. Neuraminidase was not removed by this procedure and contained at some reduced amount [7]. Triton X-100 was added to the isolated hemagglutinin at a ratio of 32 mg per 4 mg hemagglutinin in 1.5 ml of Pipes buffer (5 mM Pipes-NaOH/145 mM NaCl, pH adjusted to 7.5) and kept at room temperature for 30 min for solubilization. A lipid mixture consisting of egg yolk phosphatidylcholine/spin-labeled phosphatidylcholine/cholesterol at a molar ratio of 1.6 : 0.4 : 1 was used for reconstitution. Spin-labeled phosphatidylcholine was included for the assay of fusion with cell membranes [6,8]. The lipid mixture (7 mg) was suspended in 3.5 mg Pipes buffer and dispersed with a Vortex mixer. Triton X-100 (105 mg) was added to the mixture and kept for 30 min

Abbreviation: Pipes, 1,4-piperazinediethanesulfonic acid.

0005-2736/83/$03.00 © 1983 Elsevier Science Publishers B.V.

TABLE I

SOME CHARACTERISTICS OF RECONSTITUTED VESICLES

Protein/phospholipid [a]/cholesterol [b] (protein/phospholipid molar ratio)		Hemagglutination unit per mg protein	Normalized ESR peak height [c]	Fusion efficiency (%) [d]
Preparation mixture	Reconstituted vesicles			
(A) 1:11	1:31			
(B) 1:29	1:69	53000	0.26	66
(C) 1:70	1:120	87000	0.21	63
(D) 1:180	1:190	27000	0.18	40
(E) 1:450	1:410	91000	0.14	17

[a] A mixture of egg yolk phosphatidylcholine and spin-labeled phosphatidylcholine at a 4:1 molar ratio.

[b] Cholesterol was contained at 50 ml% of phospholipid in the preparation mixture. Cholesterol content in reconstituted vesicles was not determined.

[c] ESR central peak height divided by double-integrated area of the whole spectrum on a relative scale.

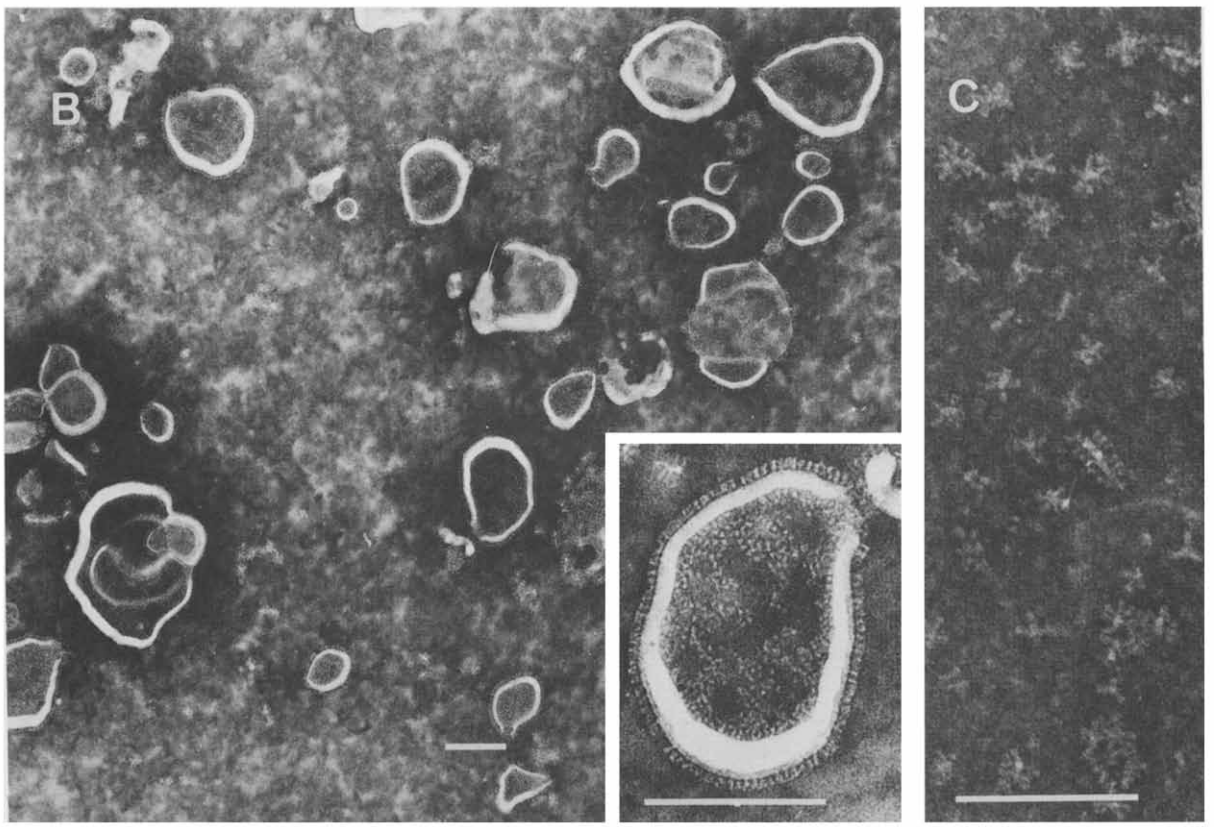
[d] Fraction of reconstituted vesicles fused with erythrocyte membrane after 5 min at 37°C at pH 5.2.

at room temperature for solubilization. The hemagglutinin and lipid solutions were mixed at various ratios to change the protein-to-lipid ratios as given in Table I and stood for 2 h at room temperature. The mixture was put into a Spectrapor membrane tubing 2 (25 mm, Spectrum Medical Industries) and dialyzed against Pipes buffer containing 2 mM each of $CaCl_2$ and $MgCl_2$ and 3 g of Bio-beads SM-2 (Bio-Rad Laboratories) at room temperature for 4 h and then at 4°C for about 80 h, in a manner similar to that of Volsky and Loyter [9]. After a low-speed centrifugation at $1\,800 \times g$ for 15 min, the supernatant was centrifuged at 4°C for 1 h at $130\,000 \times g$. The pellet was resuspended in Pipes buffer and passed through a column of Sephacryl S-500 (Pharmacia). Electron microscopic observation of the fractions showed that the reconstituted vesicles eluted at the void fractions (see Fig. 1A). The subsequent fractions contained unincorporated hemagglutinin rosettes (Fig. 1C). The void fractions were collected, pelleted dwn by centrifugation at $130\,000 \times g$ for 1 h, and resuspended in 145 mM NaCl (pH adjusted to 7.0 with dilute NH_4OH) for use in the following experiments.

Electron micrographs were taken with a JEOL Model 100B microscope. Negative staining was carried out using 3% phosphotungstate, pH adjusted to 7.4 with KOH. Hemagglutination assay was carried out by the pattern method of Salk [10]. Hemolytic activity was assayed spectrophotometrically at 520 nm as described previously [7].

Preparations B and C (see Table I) gave vesicles of the diameter ranging from 100 to 300 nm, 200 nm being most frequent, with a high density of spikes on the surface (see Fig. 1B). Vesicles prepared at lower protein-to-lipid ratios gave larger vesicles; for example, preparation E produced vesicles of 200–400 nm diameter. Only a few spikes were seen on most vesicles for such preparations. Unincorporated hemagglutinin rosettes were also present in all preparations though in small trace amounts (see Fig. 1B). Preparation A at a higher protein-to-lipid ratio gave few vesicles of smaller diameter and relatively abundant hemagglutinin rosettes (data not shown).

Membrane fusion activity of reconstituted vesicles was studied using erythrocyte ghosts as a target as described previously [6,8]. Reconstituted vesicles (0.5 ml, 30 μg phospholipid/ml) were mixed with 0.25 ml of human erythrocyte ghost in 145 mM NaCl (equivalent to 10% hematocrit) at 0°C and kept for 5 min for adsorption. Isotonic acetate buffer (0.25 ml, 20 mM sodium acetate and 145 mM NaCl) with various pH values was added to the mixture and centrifuged at 4°C for 7 min at $1800 \times g$. The pellet was taken into a capillary tube at 4°C and the ESR spectrum was measured at 37°C repeatedly. The ESR peak height will increase when the vesicles fuse with cell mem-



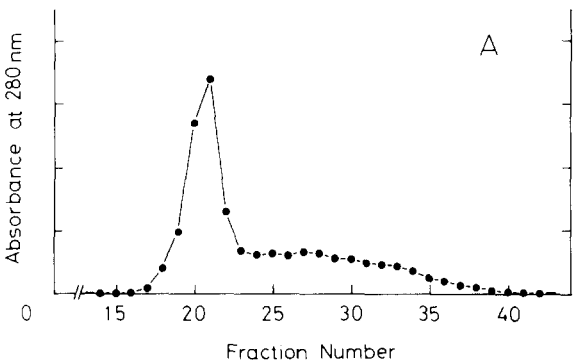


Fig. 1. Electron micrograph of reconstituted vesicles. A mixture of hemagglutinin and lipid in Triton-Pipes buffer at a molar ratio of 1 : 105 (preparation C in Table I) was dialyzed and the product was passed through Sephacryl S-500 column (25 cm length and 1.1 cm diameter) at a flow rate of 6 ml/h and 0.64 ml fractions were collected. (A) is the elution profile. (B) and (C) are electron micrgraphs of the void fractions and fractions 29–37, respectively. Negative staining was done by phosphotungstate at pH 7.4. Bar indicates 150 nm.

branes. Fig. 2 shows an example of the spectral change. The spectrum A for the reconstituted vesicles is broadened by the spin-spin interactions because of a high concentration of spin-labled phospholipid in the vesicle membranes (13 mol%). Spectrum B, after incubation of the vesicles with ghosts, has a higher peak height, since the spin-labeled phospholipids rapidly diffused into the ghost membranes after fusion and were diluted [8]. For quantitative analysis, we use normalized peak height defined as the central peak height divided by the double-integrated area of the whole ESR spectrum, which is equal to the relative peak height per unit number of spin labels. The normalized peak height for the reconstituted vesicles (designated as R) was 0.15–0.27, depending on the

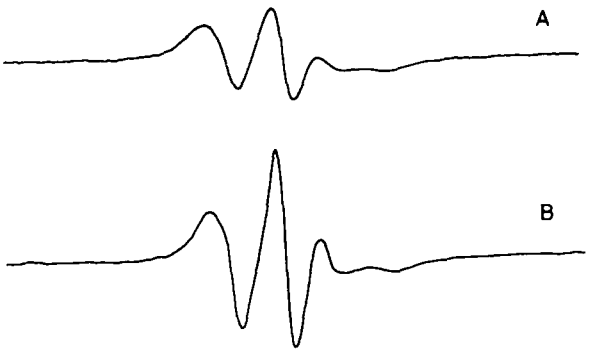


Fig. 2. ESR spectra of reconstituted vesicles (A) and those incubated with erythrocyte ghosts at pH 5.2 for 13 min at 37°C (B). Vesicles are from preparation C. The normalized peak height was 0.21 for (A) and 0.60 for (B).

protein-to-lipid ratio used for the preparation (see Table I). The normalized peak height for ghosts labeled with a low concentration of spin-labeled phosphatidylcholine (G) was 0.82 and this value was used as that for spin labels diffused and diluted into ghost membranes on fusion. Therefore, the peak height should increase 3–5-times on complete fusion of the reconstituted vesicles with erythrocyte membranes.

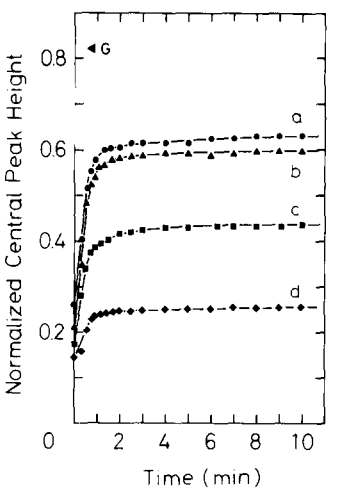


Fig. 3. Time-course of ESR peak-height increase on incubation of reconstituted vesicles with erythrocyte ghosts at pH 5.2 at 37°C. Vesicles were prepared at various protein-to-lipid ratios; (a) 1 : 44 (preparation B in Table I), (b) 1 : 105 (preparation C), (c) 1 : 270 (preparation D), and (d) 1 : 675 (preparation E). G indicates normalized peak height for ghosts labeled with a low concentration of spin-labeled phosphatidylcholine.

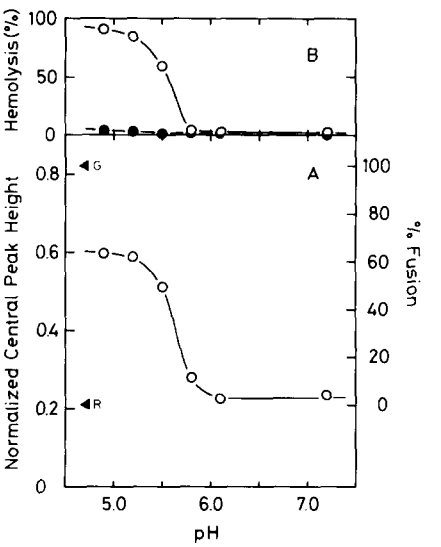


Fig. 4. pH dependence of fusion of reconstitured vesicles with erythrocyte membranes (A) and of hemolysis by reconstituted vesicles (B). The vesicles (preparation C) were incubated at various pH values with erythrocyte ghosts for 5 min at 37°C (A) or with human erythrocytes for 30 min at 37°C (B). The fusion efficiency was obtained from normalized ESR peak-height after 5 min incubation with reference to that for reconstituted vesicles (R) and for ghosts (G). The extent of hemolysis was determined spectrophotometrically. Filled circles (●) indicate autolysis in the absence of reconstituted vesicles.

It is interesting to note that the normalized peak height for the vesicles prepared at higher protein-to-lipid ratios was higher, since spin-labeled phospholipid are diluted more by the proteins in these vesicle membranes.

Fig. 3 shows the time-course of the peak-height increase when the reconstituted vesicles prepared at various protein-to-lipid ratios were incubated with ghosts at 37°C at pH 5.2. The peak height increased very rapidly within 1–2 min and reached a plateau. The plateau level was as high as 0.6 for preparations B and C. The fraction of the reconstituted vesicles that fused with erythrocyte membranes can be calculated by $(P-R)/(G-R)$ using normalized peak height for reconstituted vesicles (R), for ghosts (G), and for the reacting system at any time (P). The plateau level 0.63 for preparation B is equivalent to 66% fusion. This is very close to the fraction of influenza virus fused with erythrocyte ghosts at pH 5.2 (60%). The plateau level for reconstituted vesicles prepared at

lower protein to lipid ratios was lower (40% fusion for preparation D and 17% fusion for E (see Table I)), although the initial increase was similarly fast. The lower efficiency probably comes from smaller populations of active vesicles with a high density of spikes.

The fusion of reconstituted vesicles was activated only in acidic media. Fig. 4A shows the pH dependence; fusion was induced at pH values lower than 6.0 and very extensive at pH 5.2. At neutral pH the fusion was negligibly small. This pH dependence is essentially the same as that of envelope fusion of the parent virus [1]. The reconstituted vesicles showed hemolytic activity in acidic media as shown in Fig. 4B. The pH dependence for hemolysis is quite similar to that for the fusion with erythrocyte membrane. The reconstituted vesicles also caused fusion of erythrocyte cells in essentially the same pH range as that for the membrane fusion (data not shown).

The results of our study clearly show that the reconstituted vesicles of hemagglutinin with contaminating amount of neuraminidase but without any other viral proteins have the low pH-induced fusion activity which is essentially the same as that of the parent virus. Since we can prepare the reconstituted vesicles containing some active reagents or drugs and also the vesicles containing some cell-specific markers such as antibody together with hemagglutinin in the membranes, these reconstituted vesicles can be used to deliver foreign reagents into some specific cell cytoplasm via fusion at lysosomes or endosomes.

The trigger for the fusion activity was ascribed to structural change in hemagglutinin induced in low pH media which would cause exposure of the N-terminal hydrophobic segment of hemagglutinin to be able to interact with the target membrane lipid core [4,7,11]. Hemagglutinin proteins by themselves can cause hemolysis in acidic media probably as a result of direct interaction of the hemagglutinin N-terminal segment with erythrocyte membrane. However, these proteins cannot cause fusion of erythrocyte cells without lipid membranes into which they were hold [7]. Therefore fusion of hemagglutinin-containing membranes (either viral or reconstituted) with target cell membranes appears to be prerequisite for further fusion of cells.

Huang et al. [12] were the first to prepare reconstituted vesicles of influenza viral glycoproteins using octylglucoside as detergent. They showed fusion of liposomes containing the cleaved hemagglutinin with cell membranes (chick embryo fibroblasts) at neutral pH, by electron-microscopic examination of thin-sectioned specimen and also by microinjection of fluorescent probe molecules preloaded into liposomes [13,14]. Their observation of fusion at neutral pH is in marked contrast to our present results. We do not know the reason for the discrepancy. However, we could point out that, in the electron-microscopic study, they did not examine the viral glycoproteins diffused into the target cell membrane using ferritin-labeled antibody. In the microinjection study, they observed diffusion of the fluorescent probe molecules into cell cytoplasm. However, such diffusion could also occur when the vesicles were endocytosed and fused in the lysosomes. Very recently, White et al. [15] have observed low-pH-induced fusion of Simian CV-1 cells infected with a recombinant SV40 virus and expressing the influenza HA gene on the cell surface. This may be in line with our present results.

References

1 Maeda, T. and Ohnishi, S. (1980) FEBS Lett. 122, 283–287
2 Huang, R.T.C., Rott, R. and Klenk, H.-D. (1981) Virology 110, 243–247
3 Lenard, J. and Miller, D.K. (1981) Virology 110, 479–482
4 Maeda, T., Kawasaki, K. and Ohnishi, S. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4133–4137
5 Matlin, K.S., Reggio, H., Helenius, A. and Simons, K. (1981) J. Cell Biol. 84, 404–420
6 Yoshimura, A., Kuroda, K., Kawasaki, K., Yamashina, S., Maeda, T. and Ohnishi, S. (1982) J. Viol. 43, 284–293
7 Sato, S.B., Kawasaki, K. and Ohnishi, S. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 3153–3157
8 Ohnishi, S. and Maeda, K. (1982) in Structure, Dynamics, and Biogenesis of Biomembranes (Sato, R. and Ohnsihi, S., eds.), pp. 33–55, Japan Scientific Societies Press, Tokyo, and Plenum Press, New York
9 Volsky, D.J. and Loyter, S. (1978) FEBS Lett. 92, 190–194
10 Salk, J.E. (1944) J. Immunol. 49, 87–98
11 Skehel, J.J., Bayley, P.M., Brown, E.B., Martin, S.R., Waterfield, M.D. White, J.M., Wilson, I.A. and Wiley, D.C. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 968–972
12 Huang, R.T.C., Wahn, K., Klenk, H.-D. and Rott, R. (1979) Virology 97, 212–217
13 Huang, R.T.C., Wahn, K., Klenk, H.-D. and Rott, R. (1980) Virology 104, 294–302
14 Huang, R.T.C., Rott, R., Wahn, K., Klenk, H.-D. and Kohama, T. (1980) Virology 107, 313–319
15 White, J., Helenius, A. and Gething, M.-J. (1982) Nature 300, 658–659