Detection of Sendai virus fusion with human erythrocytes by fluorescence photobleaching recovery

Yoav I. Henis and Timothy M. Jenkins[+]

Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel Aviv 69 978, Israel

Received 23 November 1982

1. INTRODUCTION

Membrane fusion plays a role in a variety of biological processes [1]. Cell fusion with paramixoviruses, such as Sendai virus (SV), has received wide attention as a possible mechanism for virus entry into host cells, and as a means of introducing agents into cells [2,3] and into cell membranes [4,5]. These viruses also induce cell–cell fusion [6–8], employed to form heterokaryons in several biological studies [9,10].

In spite of the interest in virus–cell fusion, the fusion mechanism is still far from being clear, and the development of assays for the quantitative measurement of such events is still lacking. Procedures employed to detect fusion of unilamellar vesicles with each other [11,12] are not applicable to more complex systems. Resonance energy transfer [13] was recently used to monitor vesicle–cell fusion [14], but its use to investigate virus–cell fusion requires complicated labelling procedures.

Here we report the application of fluorescence photobleaching recovery (FPR) to study virus–cell fusion. Conditions whereby the lateral mobility of fluorescent-labelled components of SV can be studied on human erythrocytes were established. The labelled viral constituents were immobile when the virus was adsorbed to the cells, and became laterally mobile only after fusion had occurred. FPR measurements can therefore be used to monitor virus–cell fusion, and may be extended in the future to investigate the mechanism of this process.

[+] Exchange student from: The University of Bath, Bath Avon, England

2. EXPERIMENTAL

2.1. *Virus preparation*

Sendai virus (Z-strain) was grown in the allantoic sac of 10-day embryonated chicken eggs, harvested after 48 h, and pelleted twice (12000 × *g*, 2 h) in 160 mM NaCl, 20 mM tricine (Sigma, St Louis MO), pH 7.4 (SolNa) [15]. Purified virus was stored at −70°C.

2.2. *Fluorescent labelling*

SV (1.5 mg protein/ml) was reacted (12 h, 4°C) with 1 mg/ml fluorescein–isothiocyanate (FITC) (Molecular Probes, Junction City OR) in 150 mM NaCl, 10 mM $NaHCO_3$ (pH 9.5). The mixture was washed twice with the pH 9.5 buffer by centrifugation (12000 × *g*, 2 h) and twice with SolNa, and dialysed against SolNa. The distribution of the fluorescent label among viral components was estimated by sodium dodecyl sulfate–polyacrylamide (10% acrylamide) slab-gel electrophoresis [16], followed by fluorescence photography under laser illumination (488 nm, with a 510 nm barrier filter). Most of the fluorescence was found in the F (fusion factor), HN (hemagglutinating-neuraminidase), and M (matrix) proteins. F separates under the reducing conditions of the gel into two polypeptides, F_1 (M_r 51000) and F_2 (M_r 13000) [17]. F_1 and HN contain > 50% of the fluorescence (35% and 19%, respectively), while about 28% is associated with

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

the M-protein. Only minor labelling was detected in the nucleocapsid protein (NP) (<10%), and none in the P or A bands. Less than 10% of the label was found near the dye front, where free FITC, lipids and F_2 migrate. Thus, the lipid-associated fluorescence does not exceed a few percent. The fluorescent virus showed hemagglutination, fusogenic and hemolytic activities (measured as in [17,18]) similar to those of unlabelled virus.

2.3. *Interaction of Sendai virus with erythrocytes*

Fresh human blood (group O, Rh-positive) was stored up to 20 days at 4°C. Prior to use, erythrocytes were washed twice with SolNa and the buffy coat was removed. The erythrocyte pellet was diluted 50-fold with SolNa, and incubated with 400 hemagglutinating units/ml (30 μg/ml) fluorescent virus (10 min, 4°C), which formerly was sonicated 30 s in a bath sonicator to disrupt large aggregates. Unattached virions were removed by washing twice with SolNa. Half of the sample was kept on ice to avoid fusion, while the other half was incubated at 37°C (10–15 min) to induce fusion. Both samples were lysed by a 1:40 dilution in 5 mM phosphate (pH 8.0) at 4°C, and the pellets of the resulting ghosts were resuspended in SolNa to the original volume. The ghosts were attached to poly(L-lysine)coated coverslips, and were wet-mounted with SolNa for the FPR experiments. These experiments were performed on ghosts, in order to avoid cell heating and damage due to laser light absorption by haemoglobin.

2.4. *FPR*

Lateral diffusion coefficients (d) and mobile fractions (R_f) were measured by FPR [19,20] at 22°C, using an apparatus similar to the one in [21]. The monitoring laser beam (488 nm, 0.03 μW) from an argon ion laser was focused on the ghost membrane through a Zeiss Universal microscope to a spot of 0.85 μm radius, using a ×100 oil-immersion lens. 50–70% of the fluorescence in this region was irreversibly bleached by a 40 ms pulse of 0.3 mW intensity. The d and R_f values (representing an average over all fluorescent-labelled components) were derived from the rate of fluorescence recovery in the bleached spot [20]. Incomplete recovery was interpreted as indicating fluorophores immobile on the experimental time scale ($d \leq 5 \times 10^{-12}$ cm^2/s).

3. RESULTS AND DISCUSSION

The labelling of erythrocyte ghosts by fluorescent SV was dependent on the incubation conditions. Incubation at 4°C yielded a patchy fluorescence pattern, which became homogenous

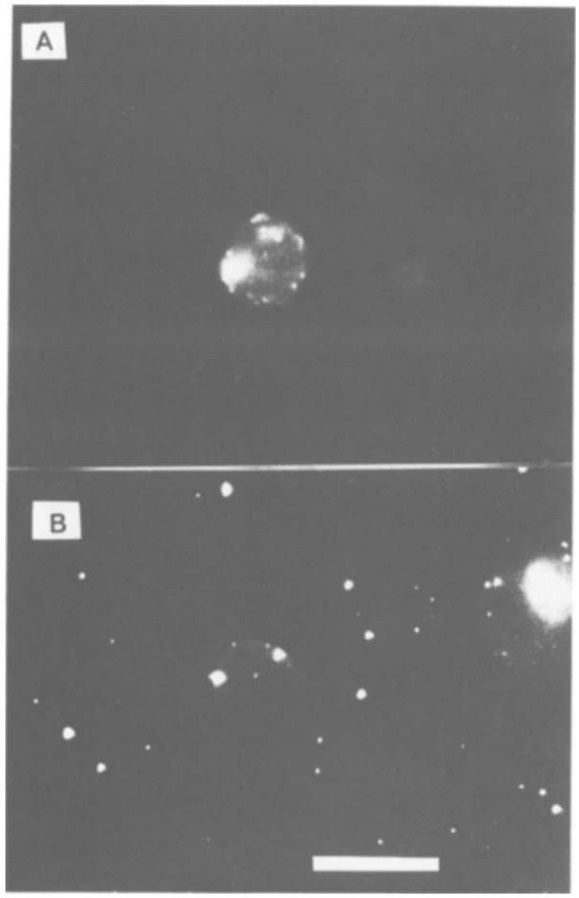

Fig.1. Fluorescence micrographs of human erythrocyte ghosts with fluorescent SV. SV was adsorbed to erythrocytes at 4°C (A), and fusion was induced by a further incubation at 37°C (B), as in section 2.3. The large fluorescent spots seen in B in addition to the homogenous labelling, are aggregates of fluorescent virus, which can be seen also on the coverslip next to the cell (excessive washing of the virus–cell complexes was avoided in order to show this point). These spots could easily be avoided in FPR expts due to the small size of the laser beam relative to the cell. Photographs were taken with 400 ASA film (Kodak Tri-X) under laser illumination (488 nm, 10 mW, beam expanded to 100 μm radius) using a ×100 oil-immersion lens (1.3 numerical aperture). Bar = 10 μm.

after warming the sample (10 min) to 37°C (fig.1). Similar observations were reported for human erythrocytes treated with SV and labelled with anti-virus antibodies [22]. Since incubation at 4°C yields only virus adsorption but not fusion [6], the diffuse fluorescence observed after incubation at 37°C (conditions under which fusion occurs) suggests the dispersal of viral components in the plane of the membrane following fusion, as was also indicated by gross morphological evidence [6,23].

FPR experiments on cells pre-incubated with virus at 4°C or at 4°C, and then at 37°C provide a direct comparison between the dynamic properties of adsorbed and fused SV, respectively (fig.2, table 1). Adsorbed fluorescent viral particles are laterally immobile ($d \leq 5 \times 10^{-12}$ cm^2/s). However, exposure of erythrocyte–SV conjugates to conditions where virus–cell fusion occurs (10 min at 37°C) is accompanied by the mobilization of fluorescent viral components. The mobile components comprise about 40% of the total fluorescence (table 1), and display a relatively high mobility ($d = 7.3 \times 10^{-10}$ cm^2/s). This value is considerably higher than that reported for native membrane proteins on human erythrocytes (2.2×10^{-11} cm^2/s) [24], but slower than that of the lipid probe *N*-4-nitrobenzo-2-oxa-1,3-diazolyl-phosphatidylethanolamine (obtained from Avanti, Birmingham AL) (3×10^{-9} cm^2/s at 22°C).

In addition to fusion, SV facilitates lipid exchange between the viral envelope and erythrocyte membranes [25]. The contribution of such a process to the mobilization of viral components cannot be significant in view of the low level of fluor-

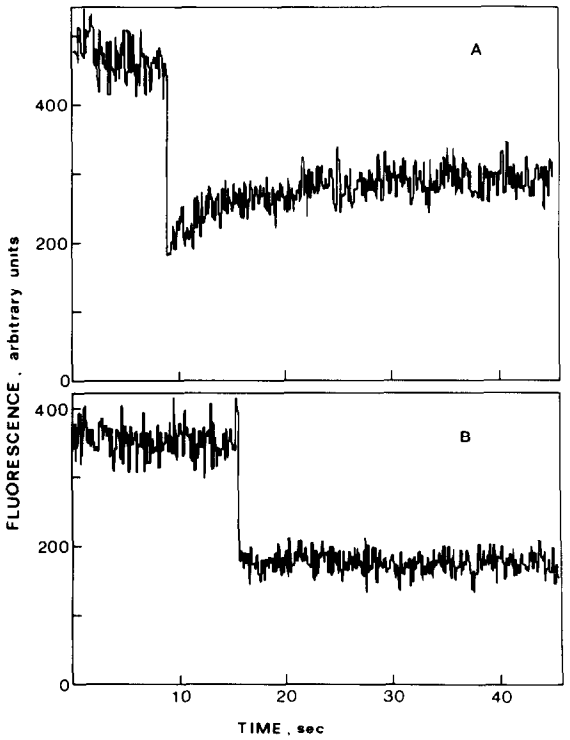

Fig.2. Representative FPR curves of fluorescent SV components on human erythrocyte ghosts. The measurements were performed on ghosts at 22°C, as in section 2. The expts were performed on ghosts prepared from erythrocytes which were incubated with SV at 4°C to achieve adsorption (B), and which were further incubated at 37°C to induce fusion (A). The lack of fluorescence recovery in B indicates immobility of the probe on the time scale of the experiment. The specific curve shown in A yielded $d = 7.0 \times 10^{-10}$ cm^2/s and a mobile fraction of 0.40.

Table 1

Mobility of fluorescent SV components on human erythrocyte ghosts

Treatment	Mobile fraction ± SE	Diffusion coefficient ± SE ($cm^2/s \times 10^{10}$)
4°C + 37°C	0.36 ± 0.04	7.3 ± 0.4
4°C	0.02 ± 0.01	–
Trypsinized virus, 4°C + 37°C	0.02 ± 0.01	–

FPR expt were performed at 22°C, as described in section 2. Results are averages on 10–20 cells. In the last two rows, the diffusion coefficient was too low to be measured ($\leq 5 \times 10^{-12}$ cm^2/s). Erythrocytes were either lysed and taken for the FPR experiment immediately after incubation with fluorescent SV at 4°C (designated '4°C'), or were pre-incubated 10 min at 37°C after the 4°C adsorption step, prior to the preparation of ghosts (designated '4°C + 37°C'). Trypsin treatment was performed by incubating 0.5 mg/ml SV with 60 μg/ml trypsin (twice recrystallized, Sigma, St Louis MO) in SolNa (60 min, 35°C). Soybeam trypsin inhibitor (Sigma) was added to a final concentration of 120 μg/ml, and the mixture was washed twice with SolNa

escence associated with the viral envelope lipids (section 2.2). In order to verify that other exchange processes do not contribute to the mobility observed following incubation at 37°C, we performed FPR experiments using fluorescent SV pre-treated with trypsin (table 1). This treatment cleaves the F-protein, rendering the virus capable of adsorption but not of fusion or hemolysis [26,27]. Trypsinized SV was laterally immobile even after incubation with erythrocytes at 37°C (table 1), lending further support to the notion that the mobilization of viral components is the result of fusion and not of exchange.

An important conclusion of the reported studies is that adsorbed virions are immobile (most likely due to their size), and that viral components become laterally mobile after dispersal in the membrane by fusion. The immobility of adsorbed virions ensures that their fluorescence will not interfere with the measurement of the mobility of fused viral components. Thus, FPR provides a method by which the fractions of fused and adsorbed virus on single cells can be evaluated. Quantitatively, the mobile fraction observed after fusion (table 1) yields a minimum value for the fraction of fused virus. This is due to the fact that a significant fraction of the fluorescence (about 40%) is associated with viral proteins which do not become part of the cellular plasma membrane after fusion, such as M or NP (see section 2.2). These proteins contribute to the fluorescence of adsorbed virus, but when fusion occurs they either remain immobile or are washed away from the cell following ghost preparation. Therefore, the fraction of fused virus may be higher than the R_f of 0.35 observed after fusion (table 1).

A better estimate of the fraction of fused virus may be obtained by following exclusively the lateral mobility of the viral envelope proteins F and HN. This can be achieved by performing FPR experiments on cells infected by SV and labelled by fluorescent, monovalent Fab[1] fragments of IgG directed against the viral envelope proteins. Such experiments are currently in progress in our laboratory.

FPR can supply a rapid and quantitative assay for virus–cell fusion. Such studies may be extended to investigate the involvement of alterations in the dynamics of viral and cell-surface proteins in the fusion process.

ACKNOWLEDGEMENTS

The authors thank Mrs N. Zakai and Professor A. Loyter (Dept. of Biological Chemistry, The Hebrew University of Jerusalem) for a gift of fluorescent SV and for helpful discussions.

REFERENCES

[1] Poste, G. and Pasternak, C.A. (1978) in: Cell Surface Reviews (Poste, G. and Nicolson, G.L. eds) vol. 5, pp. 306–369, Elsevier Biomedical, Amsterdam, New York.
[2] Furusawa, M., Nishimura, T., Yamaizumi, M. and Okada, Y. (1974) Nature 249, 449–450.
[3] Loyter, A., Zakai, N. and Kulka, R.C. (1975) J. Cell Biol. 66, 292–304.
[4] Volsky, D.J., Cabantchik, Z.I., Beigel, M. and Loyter, A. (1979) Proc. Natl. Acad. Sci. USA 76, 5440–5444.
[5] Prujansky-Jakobovits, A., Volsky, D.J., Loyter, A. and Sharon, N. (1980) Proc. Natl. Acad. Sci. USA 77, 7247–7251.
[6] Bächi, T., Aguet, M. and Howe, C.J. (1973) J. Virol. 11, 1004–1012.
[7] Miyake, Y., Kim, J. and Okada, Y. (1978) Exp. Cell Res. 116, 167–178.
[8] Seto, J.T., Becht, H. and Rott, R. (1974) Virology 61, 354–360.
[9] Edidin, M. and Wei, T. (1977) J. Cell Biol. 75, 475–482.
[10] Fowler, V. and Branton, D. (1977) Nature 268, 23–26.
[11] Miller, C. and Racker, E. (1976) J. Membr. Biol. 26, 319–333.
[12] Wilschut, J. and Papahadjopoulos, D. (1979) Nature 281, 690–692.
[13] Fung, B.K.-K. and Stryer, L. (1978) Biochemistry 17, 5241–5248.
[14] Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099.
[15] Hsu, M.-C., Scheid, A. and Choppin, P.W. (1979) Virology 95, 476–491.
[16] Weber, K. and Osborn, M. (1975) in: The Proteins (Neurath, H. and Hill, R.L. eds) vol. 1, 3rd edn. pp. 179–223, Academic Press, New York.
[17] Scheid, A. and Choppin, P.W. (1977) Virology 80, 54–66.
[18] Toister, A. and Loyter, A. (1973) J. Biol. Chem. 248, 422–432.
[19] Koppel, D.E., Axelrod, D., Schlessinger, J., Elson, E.L. and Webb, W.W. (1976) Biophys. J. 16, 1315–1329.

[20] Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E.L. and Webb, W.W. (1976) Biophys. J. 16, 1055–1069.
[21] Henis, Y.I. and Elson, E.L. (1981) Proc. Natl. Acad. Sci. USA 78, 1072–1076.
[22] Bächi, T., Eichenberger, G. and Hauri, H.P. (1978) Virology 85, 518–530.
[23] Shimizu, Y.K., Shimizu, K., Ishida, N. and Homma, M. (1976) Virology 71, 48–60.
[24] Schindler, M., Koppel, D.E. and Sheetz, M.P. (1980) Proc. Natl. Acad. Sci. USA 77, 1457–1461.
[25] Kuroda, K., Maeda, T. and Ohnishi, S.-I. (1980) Proc. Natl. Acad. Sci. USA 77, 804–807.
[26] Ozawa, M., Asano, A. and Okada, Y. (1979) Virology 99, 197–202.
[27] Maeda, T., Eldridge, C., Toyama, S., Ohnishi, S.-I., Elson, E.L. and Webb, W.W. (1979) Exp. Cell Res. 123, 333–343.