Characterization of the Fusogenic Properties of Sendai Virus: Kinetics of Fusion with Erythrocyte Membranes

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ABSTRACT: A novel fluorescence assay [Hoekstra, D., De Boer, T., Klappe, K., & Wilschut, J. (1984) *Biochemistry 23*, 5675–5681] has been used to characterize the fusogenic properties of Sendai virus, using erythrocyte ghosts and liposomes as target membranes. This assay involves the incorporation of the "fusion-reporting" probe in the viral membrane, allowing continuous monitoring of the fusion process in a very sensitive manner. Fusion was inhibited upon pretreatment of Sendai virus with trypsin. Low concentrations of the reducing agent dithiothreitol (1 mM) almost completely abolished viral fusion activity, whereas virus binding was reduced by ca. 50%, indicating that the fusogenic properties of Sendai virus are strongly dependent on the integrity of intramolecular disulfide bonds in the fusion (F) protein. Pretreatment of erythrocyte ghosts with nonlabeled Sendai virus inhibited subsequent fusion of fluorophore-labeled virus irrespective of the removal of nonbound virus, thus suggesting that the initial binding of the virus to the target membrane is largely irreversible. As a function of pH, Sendai virus displayed optimal fusion activity around pH 7.5–8.0. Preincubation of the virus at suboptimal pH values resulted in an irreversible diminishment of its fusion capacity. Since virus binding was not affected by the pH, the results are consistent with a pH-induced irreversible conformational change in the molecular structure of the F protein, occurring under mild acidic and alkaline conditions. In contrast to virus binding, fusion appeared to be strongly dependent on temperature, increasing ca. 25-fold when the temperature was raised from 23 to 37 °C. A potential regulatory role of the viral binding protein HN in the fusion process, by modulating the ability of the F protein to interact with the target membrane bilayer in a temperature-dependent manner, is discussed.

Membrane fusion is a key event in the infectious pathway of enveloped viruses (Choppin & Scheid, 1980; Loyter & Volsky, 1982; White et al., 1983). Paramyxoviruses, such as Sendai virus and Newcastle disease virus, deliver their genomes into host cells for replication via a mechanism involving fusion between the viral envelope and the plasma membrane of the target cell. Although considerable progress has been made in elucidating the role of various viral proteins in this process (Choppin & Scheid, 1980; White et al., 1983; Hsu et al., 1981, 1982; Eidelman et al., 1984), the molecular mechanism underlying this event is still far from being clear. Paramyxoviruses contain two glycoproteins, HN and F, that comprise spikelike projections on the surface of the virus (Choppin & Scheid, 1980; Haywood, 1974). Fusion of these viruses involves an initial binding between the viral and target membranes. This process is mediated by the binding protein HN, which contains hemagglutination and neuraminidase activity. The fusion process itself is mediated by the F protein, which consists of two disulfide-linked subunits F_1 and F_2. The F protein is derived from an inactive precursor, F_0, and is formed upon proteolytic cleavage of F_0 by a host cell enzyme (Scheid & Choppin, 1979). As a result of this cleavage, a hydrophobic sequence of amino acids is exposed at the N-terminal region of the F_1 subunit. Beause of the presence and similarities of such hydrophobic segments in the fusion proteins of various families of viruses, a direct involvement of these segments in the fusion process per se has been suggested (Choppin & Scheid, 1980; White et al., 1983).

Insight into the mechanism of fusion between viruses and biological membranes is hampered not only by the complexity of such membranes but also by the lack of suitable assays which allow direct and continuous monitoring of the fusion process. Recently, we have described a novel fluorescent assay that provides the possibility to monitor fusion of biological membranes in a direct and continuous manner (Hoekstra et al., 1984). In the present work, we have utilized this assay to examine the kinetics of fusion between Sendai virus and erythrocyte membranes as well as liposomes. The fluorescent dye octadecyl Rhodamine B chloride was inserted into the viral membrane at a surface density that causes self-quenching of the fluorescent dye. Upon fusion between the labeled viral membranes and nonlabeled target membranes, relief of self-quenching will occur, resulting in a proportional increase in the fluorescence intensity. Hence, the assay allowed us to characterize kinetic and quantitative aspects of the fusion process between Sendai virus and erythrocyte membranes in a direct manner.

Experimental Procedures

Virus. Sendai virus [hemagglutinating virus of Japan (HVJ)],[1] Z strain, was grown for 72 h in the allantoic cavity of 10-day-old embryonated eggs, purified by differential centrifugation (Maeda et al., 1975), and stored at −70 °C in phosphate-buffered saline. The concentration of virus was determined by protein measurements.

Erythrocyte Ghosts. Human red blood cells (type A^+) were obtained from the Red Cross Blood Bank. Ghosts were prepared (Hoekstra et al., 1983, 1985) by hypotonic hemolysis in 5 mM sodium phosphate buffer, pH 8.0. Prior to being resealed at 37 °C for 45 min in the presence of 1 mM Mg^{2+}, albumin (5% w/v) was sequestered in the ghosts by a brief (15 min) incubation on ice. After being resealed nonentrapped

[1] Abbreviations: R_{18}, octadecyl Rhodamine B chloride; HVJ, hemagglutinating virus of Japan (Sendai virus); KNP, 120 mM KCl/30 mM NaCl buffered with 10 mM sodium phosphate, pH 7.4; LUV, large unilamellar vesicles; PS, phosphatidylserine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; Chol, cholesterol; EMA, eosinyl-5-maleimide; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane.

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albumin was removed by repeated centrifugation. The concentration of ghosts (milligrams of protein per milliliter) was determined by measuring protein (prior to addition of albumin) or lipid phosphorus after lipid extraction (Folch et al., 1957), assuming 674 nmol of phospholipid/mg of protein (Cohen & Solomon, 1976). One milligram of ghost protein equals approximately 1.8×10^9 cells.

Incorporation of Octadecyl Rhodamine B Chloride into HVJ Membranes. An ethanolic solution of octadecyl Rhodamine B chloride (R_{18}; Molecular Probes Inc., Junction City, OR) was injected, under vortexing, into 1 mL of 120 mM KCl/30 mM NaCl/10 mM sodium phosphate (KNP buffer), pH 7.4, containing approximately 1 mg of HVJ protein. The final concentrations of the probe and ethanol were 10–20 μM and 1% (v/v), respectively. The mixture was incubated in the dark for 1 h at room temperature. R_{18}-labeled virus was purified from noninserted fluorophore by chromatography on Sephadex G-75. The amount of R_{18} incorporated into the virus membrane was quantitated by fluorescence measurements, after lysis of the virus in 1% (v/v) Triton X-100 and comparing the measured fluorescence to a standard curve prepared from known amounts of fluorophore solubilized in 1% Triton X-100 (Hoekstra et al., 1984). Phospholipid concentrations were determined by phosphorus determination on the extract of viral lipids or were calculated on the basis of protein content (275 nmol of phospholipid/mg of protein). In all experiments, the fluorophore constituted 2–3 mol % of the total viral phospholipids. Further details have been presented elsewhere (Hoekstra et al., 1984).

Fusion of R_{18}-Labeled HVJ with Ghosts. The principle of the assay relies upon self-quenching of the fluorophore R_{18}, occurring when the probe is incorporated into membranes at sufficiently high concentrations (1–9 mol % with respect to total lipid). Upon fusion of R_{18}-labeled membranes with membranes devoid of the probe, the surface density of R_{18} will decrease as a result of rapid mixing of the viral and target membrane components. At the probe concentration used, the decrease in self-quenching and, hence, the increase in fluorescence intensity are proportionally related to the extent of probe dilution (Hoekstra et al., 1984). To allow virus attachment, R_{18}-labeled HVJ was mixed with erythrocyte ghosts in a small volume of KNP buffer, pH 7.4. The mixture was incubated on ice for 10 min. Prewarmed buffer was then added so that the temperature was raised to 37 °C. The mixture was immediately transferred to a cuvette, and the relief of fluorescence self-quenching was continuously monitored in the fluorometer. Alternatively, R_{18}-labeled HVJ was suspended in KNP buffer, pH 7.4, at 37 °C. The cuvette was placed in the fluorometer, and the fusion reaction was initiated by rapid injection of ghosts into the medium. In all fusion experiments, the final incubation volume was 2 mL. Continuous monitoring of R_{18} fluorescence (excitation and emission wavelengths 560 and 590 nm, respectively) was carried out with a Perkin-Elmer MPF 43 spectrophotofluorometer. The sample chamber was equipped with a magnetic stirrer, and the temperature was controlled with a thermostated circulating water bath. Monitoring of fluorescence was done at 37 °C, unless indicated otherwise. The fluorescence scale was calibrated such that the residual fluorescence of R_{18}-labeled HVJ was taken as the zero level and the value obtained after addition of Triton X-100 (1% v/v), corrected for sample dilution, as 100% (infinite dilution). The extent of fluorescence self-quenching was calculated from the ratio of fluorescence measured before and after addition of Triton X-100 while initial fusion rates were determined from the slopes of the steepest part of the fluorescence recordings.

Binding of HVJ to Erythrocyte Ghosts. Fluorescently tagged HVJ was mixed with ghosts and incubated on ice, as described above. The mixture was transferred to Eppendorf cups, and bound virus and nonbound virus were separated by centrifugation in an Eppendorf table centrifuge (4 min, 15000*g* at 4 °C). The ghost pellet was washed twice with ice-cold KNP buffer, pH 7.4. R_{18} fluorescence in the supernatant (nonbound virus) and in the pellet was determined after addition of Triton X-100 (1% v/v). Alternatively, Sendai virus was labeled with eosinyl-5-maleimide (EMA; Molecular Probes, Junction City, OR; 0.1 mg/mg of viral protein), which reacts with sulfhydryl groups contained in the viral proteins. The labeling procedure was as described for R_{18}, and EMA-labeled virus showed hemagglutination and hemolytic activities identical with those of unlabeled virus. Binding experiments were performed as described above, and the fluorescence in the supernatant and in the pellet was determined (λ_{ex} = 520 nm; λ_{em} = 550 nm) after addition of Triton X-100 (1% v/v). To exclude the possibility of cosedimentation of aggregated virus particles, the virus preparations were centrifuged, under similar conditions as described above, prior to their incubation with the ghosts.

Other Procedures. Virus-induced hemolysis was determined by measuring hemoglobin in the supernatant after removal of intact cells by centrifugation. As a measure of the hemoglobin concentration, the absorbance at 540 nm was determined. Total release was accomplished by lysis of the cells in the presence of ammonia (Peretz et al., 1974). Trypsin treatment of HVJ was carried out as described by Shimizu & Ishida (1975). The virus was incubated with trypsin in 100 μL of 10 mM sodium phosphate buffer, pH 7.2, for 20 min at 37 °C, followed by addition of a 2-fold excess of soybean trypsin inhibitor. Treatment of HVJ with dithiothreitol (DTT) was done by preincubating the virus with various concentrations of the reducing agent in a final volume of 100 μL at 37 °C for 15 min. Subsequently, KNP buffer was added, and the mixture was transferred to a cuvette which was then placed in the fluorometer. The fusion reaction was initiated by addition of ghosts. Glycophorin was isolated as described by Taraschi et al. (1982).

Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation (Düzgünes et al., 1984). The vesicles were sized to an average diameter of 0.2 μm through Unipore polycarbonate filters (Bio-Rad). Vesicle compositions were as described in the legends. All phospholipids were obtained from Avanti Polar Lipids Inc. (Birmingham, AL); cholesterol and ganglioside GD_{1a} were from Sigma and Supelco, respectively.

RESULTS

Properties of R_{18}-Labeled Sendai Virus. As demonstrated elsewhere (Hoekstra et al., 1984), R_{18} becomes readily incorporated into the viral membrane when an ethanolic solution of R_{18} is injected into the virus-containing medium. With the conditions described under Experimental Procedures, the efficiency of incorporation, relative to the amount of probe added, is ca. 50–70%, while the degree of self-quenching varies between 60% and 80%. We have shown that the fluorophore is predominantly located in the *outer leaflet* of the viral bilayer (Hoekstra et al., 1984).

The presence of the dye does not affect the properties of the virus per se, as revealed by the following experiments. During a 15-min incubation on ice (25 μg of viral protein/100 μg of ghost protein), R_{18}-labeled HVJ bound to erythrocyte ghosts to the same extent as Sendai virus labeled with trace

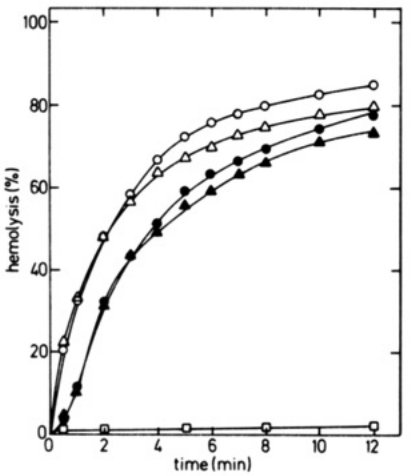

FIGURE 1: Effect of R_{18} on HVJ-induced hemolysis of human erythrocytes. R_{18}-labeled HVJ (13.2 μg/mL; ○, ●) or nonlabeled HVJ (13.2 μg/mL; △, ▲) was incubated with erythrocytes (10^8 cells/mL), and hemolysis was determined at 37 °C as a function of time, as described under Experimental Procedures. The virus was directly mixed with the erythrocytes at 37 °C (closed symbols) or was preincubated with the erythrocytes at 2 °C for 15 min prior to the monitoring of hemolysis at 37 °C (open symbols). In the latter experiment, nonbound virus was removed by centrifugation after the preincubation at 2 °C. (□) Hemolysis in the absence of HVJ.

amounts of the fluorophore EMA, which labels viral proteins. At this temperature, fusion does not take place (see below), and the extent of binding can thus readily be determined by measuring the total fluorescence after removal of nonbound virus by centrifugation. On the basis of fluorescence, 52.5% of R_{18}-labeled virus and 53.1% of EMA-labeled Sendai virus were bound to the erythrocyte membrane. Furthermore, as shown in Figure 1, the kinetics of hemolysis of erythrocytes as induced by either nonlabeled or R_{18}-labeled HVJ were very similar.

Assuming that hemolysis of erythrocytes results from fusion of the virus with the target membrane (Loyter & Volsky, 1982), the data in Figure 1 indicate that fusion took place at 37 °C irrespective of whether viruses and ghosts had been preincubated at 4 °C. Without a low-temperature incubation, a delay of ca. 20 s in the onset of hemolysis was observed. The subsequent rates of release were similar.

Interaction between R_{18}-Labeled HVJ and Erythrocyte Ghosts. When fluorophore-containing HVJ was incubated with erythrocyte ghosts on ice (up to 2 h), no relief of fluorescence self-quenching was seen (cf. Figure 6). Examination of virus-treated ghosts in the fluorescence microscope showed the presence of numerous fluorescent spots at the cell periphery, reflecting the adherence of the virus particles (Figure 2). This patchy fluorescence pattern was largely transformed to a uniform ring of fluorescence (Figure 2b,c) upon warming the sample to 37 °C, indicating the dispersal of viral components in the membrane following fusion. Since the ghosts contained albumin (Kulka & Loyter, 1979; Sekiguchi et al., 1981), large polyghosts were seen. A similar fluorescence distribution pattern was observed when virus and ghosts were directly mixed at 37 °C, except that large polyghosts did not form under these conditions (not shown).

The occurrence of fusion between R_{18}-labeled HVJ and ghosts, revealed as a relief of fluorescence self-quenching, can be directly monitored in the fluorometer. After HVJ and ghosts (25 μg of virus protein/43 μg of ghost protein) were preincubated at 4 °C, followed by addition of prewarmed buffer, or when the same amounts of virus and ghosts were directly mixed at 37 °C, a gradual increase in fluorescence

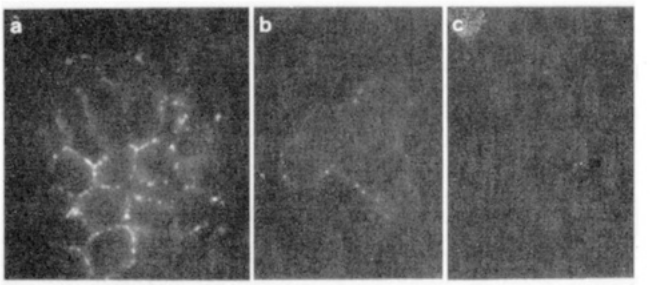

FIGURE 2: Fluorescence micrographs of erythrocyte ghosts after incubation with R_{18}-labeled HVJ. Albumin-loaded ghosts were incubated with R_{18}-labeled HVJ at 2 °C for 15 min. Nonbound virus was removed by centrifugation, and the cells were examined under a fluorescence microscope either immediately (a) or after a subsequent incubation at 37 °C for 30 min (b, c).

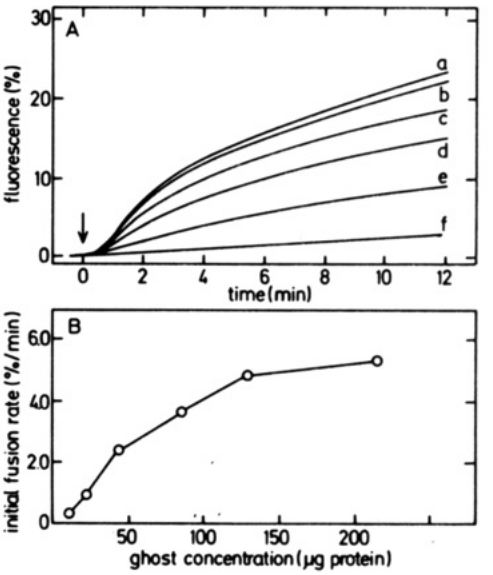

FIGURE 3: Interaction of R_{18}-labeled HVJ with erythrocyte ghosts as a function of ghost concentration. (A) R_{18}-labeled virus (26 μg of protein) was suspended in KNP buffer at 37 °C. At $t = 0$ (arrow), various amounts of erythrocyte ghosts (a–f) were injected into the medium, and fusion was monitored continuously as described under Experimental Procedures. Ghost concentrations were 216 (a), 130 (b), 86 (c), 43 (d), 22 (e), and 11 (f) μg of protein in a final volume of 2 mL. (B) Initial fusion rates were calculated from the slope of the steepest part of the fluorescence tracings shown in (A) and plotted as a function of the erythrocyte ghost concentration.

was seen initially (15–35 s), which was followed by a more rapid development of fluorescence (Figure 3), while after ca. 4–5 min, the rate of probe dilution decreased again. The apparent initial fusion rates, calculated from the slope of the steepest part of the curve, were 2.3 and 1.8 %/min, respectively, while the onset of the rapid development of fluorescence was slightly faster (ca. 15 s vs. ca. 35 s, respectively) when HVJ and ghosts had been preincubated at 4 °C. These differences presumably reflect the differences in binding kinetics at 4 and 37 °C, very similar to that observed above in the case of virus-induced hemolysis (Figure 1).

Effect of Ghost Concentration, Target Membrane, pH, and Temperature on HVJ Fusion Properties. Figure 3A shows the kinetics of fusion of HVJ with erythrocyte ghosts, when mixed directly at 37 °C, as a function of the ghost concentration. The initial fusion rate increased with increasing ghost concentration, up to ca. 120 μg, and leveled off at higher concentrations (Figure 3B). For convenience, the results are expressed as a percentage of the fluorescence that would be obtained upon infinite dilution of the fluorescent amphiphile, i.e., after addition of Triton X-100 (see Experimental Proce-

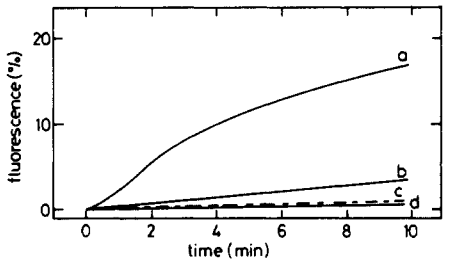

FIGURE 4: Fusion of HVJ with various target membranes. R_{18}-labeled HVJ (26 μg of protein) and (a) erythrocyte ghosts (86 μg of protein), (b) LUV consisting of DOPC/DOPE/Chol/PS/GD_{1a} (200 nmol of lipid, molar ratio 1:0.4:0.6:0.1:0.13), (c) DOPC LUV (200 nmol of lipid), or (d) nonlabeled HVJ (80 μg of protein) were mixed at 37 °C as described in the legend of Figure 3, and the kinetics of R_{18} dilution were monitored.

dures). However, when taking into account that (i) approximately 50% of the viral dose added becomes cell associated, (ii) during the early events only a fraction of the virus fuses with the ghost membrane, and (iii) HVJ contains 400 nmol of (total) lipid/mg of protein and ghosts 1000 nmol of (total) lipid/mg of protein, it can be calculated (Hoekstra et al., 1984) that, under the conditions in Figure 3, the early fusion events lead to infinite dilution of the R_{18} dye. Therefore, the percentage of R_{18} fluorescence development is directly proportional to the percentage of fusion (Hoekstra et al., 1984). For example, in the presence of 86 μg of ghost protein (curve c, Figure 3A), approximately 18% of the total virus population has fused after a time interval of 12 min. In a very similar fashion as described above for the initial fusion rate, the final extent of fusion, as determined after 20 h, also varied with the erythrocyte ghost concentration. The final extent of fusion was 22% at 50 μg of ghost protein, increased linearly to approximately 48% fusion at 100 μg, and leveled off to approximately 60% fusion at 150 μg of ghost protein. Using different techniques, others have reported similar results (Lyles & Landsberger, 1981; Maeda et al., 1981).

As demonstrated in Figure 4, the nature of the target membrane clearly determined the efficiency of HVJ fusion. No dilution of R_{18} was seen when R_{18}-labeled HVJ was incubated with an excess of DOPC phospholipid vesicles (curve c) or nonlabeled Sendai virus (curve d), while some fusion between viral and vesicle bilayers occurred (curve b) when the vesicles contained ganglioside GD_{1a} as a viral receptor (Haywood & Boyer, 1982). Both the initial fusion rate and the extent of fusion (determined after 10 min) increased considerably (approximately 8- and 6-fold, respectively) when ghost membranes (curve a), at a lipid concentration half of that of the phospholipid vesicles, constituted the target membrane for HVJ. No efforts were undertaken to determine whether the low fusion activity of HVJ with liposomes was caused by a diminished binding of the virus to the liposomal target membrane.

As indicated in Figure 5, fusion between HVJ and erythrocyte ghosts was dependent on pH, displaying a maximum around pH 7.4–8.0, with respect to both the initial fusion rate and the extent of fusion, as determined after 15 min. The same results were obtained when, after preincubation of the virus alone at the appropriate pH, the sample pH was readjusted to 7.4, thus excluding that an effect of pH on the erythrocyte membrane contributed to the observed pH dependence of fusion (not shown). Furthermore, the same effect of pH on HVJ fusion (at 37 °C) was seen when the virus had been preincubated at the appropriate pH at 4 °C, irrespective of a readjustment of the pH to 7.4, prior to monitoring fusion (not shown, cf. Figure 5).

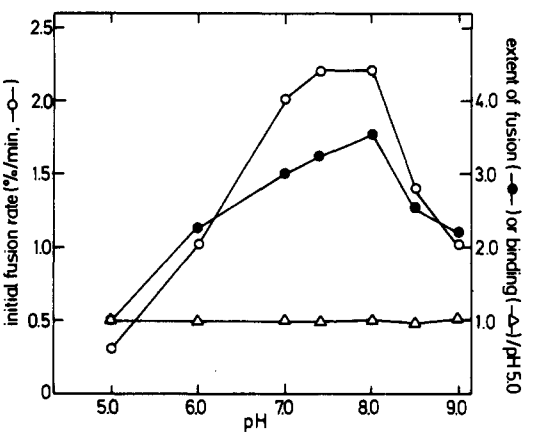

FIGURE 5: Effect of pH on binding and fusion properties of HVJ. R_{18}-labeled HVJ (25 μg of protein) was incubated in 110 mM KCl/20 mM NaCl/10 mM sodium acetate/10 mM sodium phosphate/10 mM Tris, adjusted to the appropriate pH, at 37 °C for 10 min. Subsequently, 45 μg of ghosts was injected into the medium (final volume 2 mL), and fusion was monitored. The initial fusion rates (○) were calculated and plotted as a function of pH. At each pH, the extent of fusion (●) was determined after *15 min* and was plotted relative to the extent of fusion obtained at pH 5.0. Binding of HVJ (△) as a function of pH was determined after incubating R_{18}-labeled HVJ ghosts in 300 μL of medium, adjusted to the desired pH, at 2 °C for 15 min. Nonbound virus was removed by centrifugation, and the cell pellet was washed twice. Cell-associated fluorescence was determined after addition of Triton X-100 (1% v/v) as described under Experimental Procedures, and the extent of binding at each pH was plotted relative to that obtained at pH 5.0.

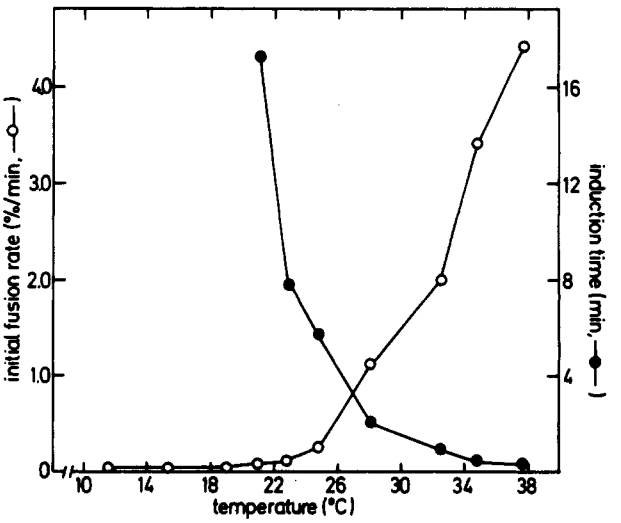

FIGURE 6: Temperature dependence of fusion of HVJ with erythrocyte ghosts. R_{18}-labeled HVJ (32 μg) was added to the KNP medium followed by addition of 100 μg of ghosts. The initial fusion rates (○) were calculated and plotted as a function of temperature. The induction time (●) of the fusion process was determined from the intercept of the tangents drawn to the fluorescence tracings and the *x* axis (time scale). The pH of the fusion medium (7.4) was corrected for temperature-dependent pH changes.

In principle, the pH-dependent effect on fusion could have been caused by differences in the extent of virus binding. However, as shown in Figure 5, the fraction of bound virus appeared to be the same, irrespective of the pH of the incubation medium. It is concluded, therefore, that the pH-dependent fusion properties of HVJ are exclusively due to an effect of the pH on the fusion reaction per se.

Two phenomena became apparent when monitoring fusion between HVJ and ghosts as a function of temperature (Figure 6). First, the initiation of the fusion reaction was increasingly delayed with decreasing temperature. At temperatures below 18 °C, no significant dilution of R_{18} was observed during a

Table I: Effect of Nonlabeled HVJ on Fusion of R_{18}-Labeled HVJ with Erythrocyte Ghosts[a]

nonlabeled HVJ (μg of protein)	initial fusion rate (%/min)	inhibition (%)
0	3.6	0
5	2.7	25
15	1.7	53
25	1.3	64
50	1.2	66

[a] Ghosts (70 μg of protein) were incubated with various amounts of nonlabeled HVJ at 2 °C for 15 min. Nonbound virus was removed by centrifugation, and the ghosts were washed twice. The final pellet was resuspended in KNP buffer, pH 7.4, at 37 °C. After 30 s, 25.8 μg of R_{18}-labeled HVJ was added, and fusion was monitored as described.

time interval of at least 30 min. Second, both the rate (Figure 6) and the extent of fusion (not shown) increased almost linearly in the range of 24–37 °C. Compared to the initial fusion rate at 24 °C, the rate at 37 °C was approximately 25-fold higher.

Inhibition of HVJ Fusion. Prior binding of nonlabeled HVJ to erythrocyte membranes (at 2 °C) inhibited the subsequent ability of R_{18}-labeled HVJ to fuse with the erythrocyte membrane (Table I). Preliminary experiments revealed that even an incubation of ghosts with 50 μg of nonlabeled virus on ice or at 37 °C for only 3 min sufficed to cause an inhibition in the initial fusion rate of ca. 55%. These results indicated that HVJ binds readily to the membrane, at both 4 °C and 37 °C, and that, moreover, the majority of the cell-associated viral particles are bound in an essentially irreversible manner. This conclusion can be inferred from the observation that the inhibition of fusion of R_{18}-labeled HVJ by nonlabeled HVJ was very similar, whether or not nonbound HVJ was removed prior to addition of R_{18}-labeled HVJ. Supporting evidence for a rather irreversible association of HVJ particles with ghosts was obtained from an experiment in which ghosts were preincubated on ice with R_{18}-labeled HVJ. Upon removal of nonbound virus and subsequent incubation of the virus–ghost complex at 37 °C for 15 min, ca. 70% of the labeled virus particles remained cell associated. Hence, the interaction between HVJ and erythrocyte ghosts does not seem to display the properties of a dynamic equilibrium between bound and nonbound virus particles.

Trypsin specifically abolishes the fusion activity of HVJ by cleavage of the F protein (Shimizu & Ishida, 1975). Results obtained with the R_{18} assay were in accordance with this observation (Figure 7). Depending on the trypsin concentration used, a decline in the initial fusion rate was seen, and fusion could be inhibited by more than 90%.

Pretreatment of HVJ with the reducing agent dithiothreitol (DTT) also resulted in an inhibition of the fusion reaction (Figure 7). At a concentration as low as 0.01 mM, a significant inhibition of fusion was already observed (10%), while at 1.0 mM DTT, R_{18} fluorescence development was barely detectable. Since DTT reduces intermolecular disulfide bonds, which preserve the molecular structure of both F and HN (Ozawa et al., 1979), it was of interest to determine the cause of the observed inhibition. For this purpose, we determined after a time interval of 10 min the extent of HVJ-induced hemolysis, and the extent of fusion and binding of HVJ per se, after treatment of the virus with a low (0.1 mM) and a high (1.0 mM) DTT concentration. Relative to nontreated virus, hemolysis was reduced by 15.7%, fusion by 16.6%, and binding by 6%, using 0.1 mM DTT-treated virus. The extent of inhibition of virus-induced hemolysis, fusion, and binding of virus treated with 1.0 mM DTT was 90%, 86.4%, and 57%, respectively. These results indicated that fusion and hemolysis

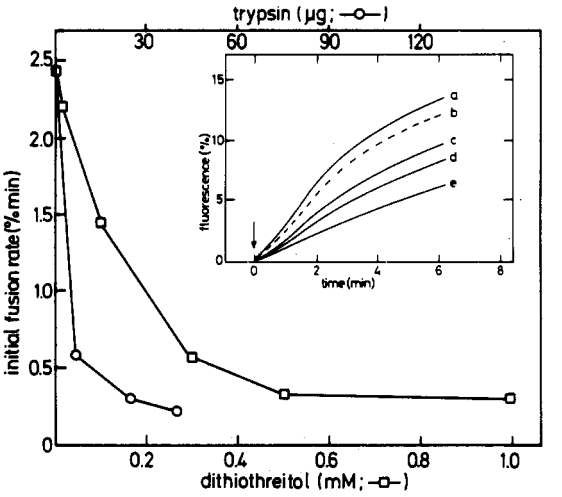

FIGURE 7: Inhibition of HVJ–ghost fusion upon pretreatment of the virus with trypsin and DTT; effect of glycophorin. R_{18}-labeled HVJ was pretreated with various concentrations of trypsin or DTT as described under Experimental Procedures. The virus (23.8 μg) was subsequently suspended in KNP medium at 37 °C, and fusion was initiated by adding 45 μg of ghosts. The initial fusion rates were determined and plotted as a function of the trypsin (○) or DTT (□) concentration. Insert: R_{18}-labeled HVJ (43 μg) was incubated with various amounts of glycophorin [(a) 0 μg; (c) 25 μg; (d) 50 μg; (e) 100 μg] at 2 °C in a total incubation volume of 400 μL. After 15 min, KNP buffer was added, and the temperature was adjusted to 37 °C. At $t = 0$ (arrow), 110 μg of ghosts was added to initiate the fusion process. Fluorescence tracing b was obtained when HVJ had been preincubated with 50 μg of glycophorin at 37 °C under otherwise identical conditions as described above.

were affected to a similar degree and that, after a 1.0 mM DTT treatment, both fusion and hemolysis were almost completely inhibited, despite the fact that still some 40–50% of the viral fraction, relative to nontreated HVJ, became bound to the cell surface.

Glycophorin, known to function as a receptor site for HVJ in erythrocyte membranes (Bächi et al., 1977), inhibited HVJ–ghost fusion as shown in Figure 7 (insert). The efficiency of inhibition was dependent on the glycophorin concentration and increased with increasing protein concentration, culminating in approximately 70% inhibition at 100 μg of glycophorin. Interestingly, inhibition of fusion was more effective after a preincubation of the virus with glycophorin on ice. Only a limited degree of inhibition was seen when the protein was incubated with Sendai virus at 37 °C, followed by addition of the ghosts (curve b vs. curve d). These results indicated that, presumably, at higher temperature, the neuraminidase activity contained in the HN protein caused a rapid dissociation of the protein from the viral binding protein due to cleavage of the sialic acid residues.

Discussion

Fusion of viruses with artificial and biological membranes has been studied by using a variety of methods, including electron microscopy (Haywood, 1974; Oku et al., 1982), hemolysis of erythrocytes or leakage of vesicle contents (Maeda & Ohnishi, 1980; Kundrot et al., 1983), electron spin resonance techniques (Lyles & Landsberger, 1979; Maeda et al., 1981), and determination of the association of radiolabeled virus particles with the membranes (Haywood & Boyer, 1982; Wolf et al., 1980). Electron microscopy does not allow kinetic or quantitative assessments of the fusion process, while hemolysis or leakage need not necessarily accompany the fusion event: early harvested HVJ fuses with erythrocytes without significant hemolysis (Homma et al., 1976). Besides from being not an easily accessible technique, electron spin resonance procedures

produce results which are sometimes difficult to interpret, while proper quantitation is impossible in most cases (Umeda et al., 1983). The association of radiolabeled viruses with membranes may lead to ambiguous results as far as a distinction between binding and actual fusion is concerned while tedious procedures are required to remove nonfused viral particles. The assay applied in the present work (i) is simple and sensitive, (ii) does not require a separation of fused and nonfused particles, and (iii) provides the possibility to analyze the fusion kinetics between viruses and target membranes in an accurate manner. An additional advantage is that the fusion-reporting probe is located in the viral membrane: The fusion activity between HVJ and liposomes is rather low (Figure 4; Haywood & Boyer, 1982; Hsu et al., 1983), and a large excess of liposomes to viral particles is required (Haywood & Boyer, 1982; Hsu et al., 1983). With a fusion assay based on resonance energy transfer between two fluorophores which are inserted into the *liposomal* bilayer (Struck et al., 1981), the fluorescent signal, which should reveal the dilution of the probes upon fusion between the liposomal and viral membranes, was not distinctly different from the background fluorescence. A similar observation has been reported by Umeda et al. (1983) using spin-labeled liposomes.

One of the major problems in the quantitation of membrane fusion by lipid dilution is that intermembrane transfer of individual probe molecules may occur by a nonfusion mechanism involving either a transfer via a collision-mediated mechanism or a spontaneous transfer of free monomers through the aqueous phase (Wilschut & Hoekstra, 1984). However, previous experiments (Hoekstra et al., 1984) have indicated that R_{18} behaves as a nonexchangeable molecule after its insertion into various artificial and biological membranes, and the present observations corroborate and extend those described previously. Thus, the dependence of HVJ–ghost fusion on both the concentration and composition of the target membranes (Figures 3 and 4) argues against a mechanism involving spontaneous transfer [cf. Frank et al. (1983)], while the inhibition of fluorescence development upon trypsinization of Sendai virus (Figure 7) or upon incubation of HVJ with ghosts at temperatures below 18 °C (Figures 2 and 6) also opposes a mechanism involving contact-mediated transfer of individual R_{18} molecules. We conclude, therefore, that the dilution of R_{18} occurring upon HVJ–ghost interaction resulted from membrane fusion and subsequent randomization of viral and cellular components.

Recently, several reports have been published indicating that Sendai virus exhibits pH-dependent hemolysis and cell–cell fusion activity (Hsu et al., 1982; Haywood & Boyer, 1982; Maeda & Ohnishi, 1980). Maeda & Ohnishi (1980) have shown that HVJ-induced hemolysis starts at pH values as low as 5.0 and displays a rather broad optimum between pH 6.0 and 9.0. On the other hand, Hsu et al. (1982) found the optimum for hemolysis and cell fusion to be centered around pH 9.0. Finally, Haywood & Boyer (1982), when investigating HVJ–liposome interaction, reported optimal HVJ fusion activity around pH 8.0. In the present study, *direct* information was obtained on the pH-dependent fusion activity of Sendai virus. The results, presented in Figure 5, indicate that the pH affected virus–ghost interaction exclusively at the level of the fusion reaction, presumably involving a pH-induced structural change in the F protein, since (i) the extent of binding was unaltered between pH 5 and 9 while (ii) separate incubations of the virus at various pH values excluded the possibility of pH-induced changes in the viral receptor activity at the erythrocyte membrane. The latter observation is consistent with results, previously reported by Hsu et al. (1982). Furthermore, the same effect of pH on the fusion activity was seen when the virus was incubated on ice or at 37 °C. These observations are consistent with a pH-induced *irreversible* conformational change in the F protein (Hsu et al., 1982) occurring when the virus is incubated at mild acidic and alkaline pH values, which moreover appears to be temperature independent. Finally, preliminary results on the fusogenic properties of reconstituted virus envelopes (M. C. Harmsen and D. Hoekstra unpublished results), which only contain HN and F, displayed a similar pH-dependent fusion activity. These results further corroborate the above suggestion that pH-induced changes in the functional properties of the F protein caused a diminished fusion activity rather than pH-induced changes of other viral components which could have indirectly affected the properties of the F protein.

Intramolecular disulfide bonds preserve the molecular structure of both F and HN (Ozawa et al., 1979). The method used in the present work allowed us to readily discriminate between an effect of the reducing DTT agent on the binding activity (HN protein), with potential consequences for the fusogenic ability of the virus, and/or on the fusion activity per se. It has been shown (Chejanovsky et al., 1984) that at relatively high DTT concentrations (50 mM), adsorbed virus particles can be dissociated from cell membranes. As shown here, only 1 mM DTT suffices to almost completely abolish the fusion activity, whereas some 40–50% of the DTT-treated viral fraction is still capable of binding to the cell surface. These results indicate that the F protein is much more sensitive toward reduction of intramolecular disulfide bonds than the HN protein, suggesting the absolute necessity of an intact disulfide link between F_1 and F_2 for proper functioning.

Attachment of Sendai virus particles to cell membrane receptors is a temperature-independent process (Loyter & Volsky, 1982). Indeed, preliminary results revealed that the fraction of HVJ, labeled with R_{18} or EMA, that became associated with the erythrocyte membrane after an incubation period of 15 min at various temperatures was relatively constant, varying between 40% and 55% (D. Hoekstra and K. Klappe, unpublished experiments). By contrast, *fusion* of HVJ with erythrocyte membranes is strongly dependent on temperature as shown in Figure 6. Most likely, the resistance of HVJ toward fusion at low temperature cannot be accounted for by a low-temperature-induced conformational change of the F protein causing inhibition of fusion. This can be inferred from the observation (Figure 5) that, irrespective of the incubation temperature, mild acidic and alkaline pH induced an irreversible conformational change in the F protein. As a result, a diminished fusion activity (at 37 °C) of HVJ was seen which, quantitatively, was the same regardless of whether (i) HVJ had been preincubated at 4 or 37° at various pH values or (ii) the pH had been readjusted to 7.4 prior to raising the temperature and monitoring fusion. This result would not have been expected if, after an incubation at the appropriate pH at 4 °C, a temperature-induced conformational change would be required to "activate" the F protein. If such a conformational change would be involved, one would expect that after exposure of the virus to low ($\leq$6.0) or high ($\geq$8.5) pH at *low* temperature, the subsequent conformational change, when shifting the temperature to 37 °C, would be either severely impaired or abolished due to the pH-induced structural alterations in the viral protein. As a result, a diminished or abolished fusion activity should then be seen at 37 °C, relative to the fusion activity determined after pretreating the virus directly at 37 °C at various pH values. Since the rate of fusion,

as a function of pH, was only dependent on the pH during the preincubation, i.e., irrespective of the preincubation temperature, the results argue strongly against the involvement of a temperature-dependent conformational change to activate the F protein and, hence, the viral fusion activity. Yet, despite a potentially "fusion-active" F protein at low temperature, merging of bilayers does not occur under those conditions (Figure 6), which would imply that some other factor prevents the F protein from interacting with the target membrane (Choppin & Scheid, 1980; White et al., 1983) at low temperature. This points to a possible regulatory role of the HN protein in the overall process eventually leading to fusion, which may involve its ability to refrain the viral membrane from coming into sufficiently close proximity of the target membrane, thus preventing a direct contact between the F protein and the membrane. Whatever the actual mechanism(s) by which HN may exert such an effect, it is likely that the dynamic behavior of HN could thus pose a temperature-dependent barrier for fusion. In this respect, it is noteworthy that a pronounced increase in the mobility of the viral glycoproteins occurs with increasing temperature (Lee et al., 1983) in a range corresponding to the dramatic increase in the fusion rate indicated in Figure 6. It has been suggested that at low temperature HN molecules self-aggregate into immobile complexes (Lee et al., 1983), being bound as such to the target membrane receptors. Because these HN complexes are located at the focal points of membrane contact and membrane fusion, they may well form a physical and/or sterical barrier for F proteins to come into direct contact with the target membrane. Since it is conceivable that the interaction between F proteins and target membrane is required to initiate fusion, it could thus be suggested that HN proteins regulate the fusion process indirectly in a temperature-dependent manner. These and other aspects of virus–erythrocyte membrane interaction are currently under investigation.

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