Rotational Mobility of Sendai Virus Glycoproteins in Membranes of Fused Human Erythrocytes and in the Envelopes of Cell-Bound Virions†

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ABSTRACT: The rotational mobility of Sendai virus envelope glycoproteins (F, the fusion protein, and HN, the hemagglutinin/neuraminidase) was determined by using erythrosin (Er)-labeled monovalent Fab′ antibody fragments directed specifically against either F or HN. By use of time-resolved phosphorescence anisotropy, the rotational mobility of Er–Fab′–viral glycoprotein complexes was studied both in the envelopes of unfused virions bound to erythrocyte ghosts and in the target cell membrane after fusion had occurred. The rotational correlation times (ϕ) of Er–Fab′-labeled F and HN were rather similar in the envelopes of bound unfused virions, but highly different in membranes of fused cells. The different ϕ values indicate that F and HN diffuse separately in the target cell membrane and for the major part are not complexed together. The temperature dependence of the ϕ values of the Er–Fab′–viral glycoprotein complexes revealed a breakpoint at 22 °C for the F proteins both in bound virions and in the membranes of fused cells, and for the HN proteins in the envelopes of bound virions. In all these cases, the ϕ values increased between 4 and 22 °C, demonstrating a reduction in the rate of rotational diffusion. Further elevation of the temperature reversed the direction of the change in ϕ. This phenomenon may reflect a temperature-dependent microaggregation of F and HN saturating at ca. 22 °C and presumably related to the fusion mechanism since the breakpoint temperature correlates closely with the threshold temperature for virus–cell and cell–cell fusion. Both of the latter reactions proceed at negligible rates at about 20 °C. Such an interpretation is also in accordance with the temperature dependence of the lateral diffusion of F and HN in the target cell membrane after fusion. The latter measurements demonstrate that the fractions of laterally mobile F and HN in the target cell membrane increase with temperature up to 22 °C but level off at higher temperatures, a finding consistent with the notion that lateral mobility of the two envelope proteins in the target cell membrane is required for the induction of cell–cell fusion.

Enveloped animal viruses utilize membrane fusion to introduce their genome into the host cell cytoplasm during the initial stages of infection (White et al., 1983; Poste & Pasternak, 1978). Virally mediated fusion is the best-characterized system involving the fusion of biological membranes, and the fusogenic activity can be clearly assigned to specific viral envelope glycoproteins (Post & Pasternak, 1978; Volsky & Loyter, 1978; Hsu et al., 1979; White et al., 1983; Florkiewicz & Rose, 1984). These properties make virally mediated membrane fusion a highly suitable system for studies on the role of fusogenic proteins in the fusion mechanism.

Among the paramyxoviruses which fuse with the host-cell plasma membrane at neutral pH values, the fusogenic activities of Sendai virus have been investigated most extensively. The envelope of Sendai virions contains two glycoproteins: the fusion protein (F),[1] which mediates virus–cell and cell–cell fusion (Post & Pasternak, 1978; White et al., 1983), and the hemagglutinin/neuraminidase protein (HN), which mediates virus binding to membrane receptors and cell agglutination (Poste & Pasternak, 1978; Choppin & Scheid, 1980). Recent studies have suggested that the HN protein may also play an active role in the fusion process (Ozawa et al., 1979; Miura et al., 1982; Nakanishi et al., 1982; Gitman & Loyter, 1984; Citovsky et al., 1986; Henis et al., 1989).

The mechanism of biological membrane fusion processes including those mediated by virions is still obscure. Recent studies in our laboratory have suggested that lateral motion of Sendai virus envelope proteins in the target cell membrane plays an essential role in virally mediated fusion of human erythrocytes. Employing fluorescence photobleaching recovery (FPR), we have demonstrated that both F and HN become laterally mobile in the target cell membrane following fusion (Henis et al., 1985) and that a strict correlation exists between the concentration of *laterally mobile* viral glycoproteins in the cell membrane and the extent of cell–cell fusion (Henis & Gutman, 1987; Aroeti & Henis, 1988; Henis et al., 1989). Lateral mobility of both F and HN appears to be required for effective cell–cell fusion, since lateral immobilization of either glycoprotein was sufficient to inhibit this process (Henis et al., 1989).

These findings emphasize the need for information on the possible difference between the organization of the viral glycoproteins in the cell membrane (after virus–cell fusion) and in the viral envelope (prior to fusion), especially in view of the distinctions between cell–cell fusion and viral envelope–cell fusion with regard to the sensitivity to cross-linking and lateral immobilization of the viral glycoproteins (Henis et al., 1989).

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[1] Abbreviations: F, fusion protein; HN, hemagglutinin/neuraminidase protein; FPR, fluorescence photobleaching recovery; Er, erythrosin; TMR, tetramethylrhodamine; HAU, hemagglutinating unit(s); PMSF, phenylmethanesulfonyl fluoride; D, lateral diffusion coefficient; R_f, mobile fraction.

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However, FPR measurements cannot provide information on the dynamic properties of the viral glycoproteins in the viral envelope itself, since they measure lateral motion on a scale determined by the size of the laser beam spot on the membrane (several square micrometers, significantly larger than the diameter of a viral particle). Moreover, the dependence of the lateral diffusion of membrane proteins on molecular size is very weak (Saffman, 1976), making FPR measurements incapable of detecting mild changes in the oligomerization state of the viral envelope proteins. Neither limitation applies to rotational mobility measurements that are independent of the dimensions of the light source and are highly sensitive to the size of the rotating membrane protein (Jovin & Vaz, 1989).

An earlier study (Lee et al., 1983) on the rotational diffusion of Sendai virus glycoproteins was limited to measurements on the viral envelope itself, and could not distinguish between the dynamic properties of F and HN due to the labeling method employed: the native virions were chemically modified with covalent triplet probes, resulting in labeling of both HN and F, as well as in some lipid labeling and up to 50% inactivation of the viral hemolytic activity. In the present study, we employed Er-labeled monovalent Fab′ fragments directed specifically against F or HN to investigate the rotational mobility of these proteins in virions attached to human erythrocytes (prior to fusion) and in the target cell membrane (after fusion). The temperature dependence of the rotational diffusion parameters was studied and compared with the lateral diffusion of F and HN in the target cell membrane. Our results demonstrate that F and HN reveal different rotational correlation times after fusion, that is, in the target cell membrane, suggesting that at least at this stage they are not resident in mutual complexes. The most likely interpretation for the temperature dependence of the rotational correlation times and the lateral diffusion coefficients of the viral glycoproteins is that a temperature-dependent aggregation of the viral glycoproteins occurs, saturating at about 22 °C. This phenomenon is observed for both F and HN in the envelopes of cell-bound, unfused virions, as well as for the F proteins embedded in the target cell membrane after fusion. It may be related to the viral fusogenic activity, as deduced from comparison to the thermal dependence of the fusogenic activities.

Materials and Methods

Reagents. Tetramethylrhodamine (TMR) 5,6-isothiocyanate and erythrosin (Er) 5,6-isothiocyanate were purchased from Molecular Probes (Junction City, OR). Glucose oxidase and catalase were from Sigma (St. Louis, MO).

Virus. Sendai virus (Z strain) was grown in the allantoic sac of 10–11-day-old chicken embryos, harvested 48 h after injection, and purified as previously described (Peretz et al., 1974; Hsu et al., 1982; Henis et al., 1985). The virus was resuspended in 160 mM NaCl/20 mM Tricine, pH 7.4 (solution A), and stored at −70 °C. Viral protein concentration was determined by a modified Lowry procedure (Markwell et al., 1978). Viral hemagglutinating activity was measured in hemagglutinating units (HAU) (Peretz et al., 1974) and was around 13 000 HAU/mg of viral protein. Treatment of Sendai virions with phenylmethanesulfonyl fluoride (PMSF) under conditions that inhibit their fusogenic activity but not virus binding and hemagglutination was performed as described earlier (Israel et al., 1983; Aroeti & Henis, 1986).

Interaction of Sendai Virions with Human Erythrocytes. Fresh human blood (type O, Rh positive) was obtained from a blood bank and stored with sodium citrate up to 7 days at 4 °C. Prior to use, erythrocytes were washed twice with solution A, diluted to 2% (v/v) (2×10^8 cell/mL) with the same buffer, and incubated (15 min, 4 °C) with 400 HAU/mL Sendai virions. The virions were earlier subjected to mild sonication and filtered through a 0.45-μm Acrodisc filter (Gelman, Ann Arbor, MI) to eliminate large viral aggregates (Henis et al., 1985). After being washed twice with cold solution A, the agglutinated cells were suspended in 1 mL of solution A and incubated 30 min at 37 °C to induce fusion. For measurement of the rotational mobility of viral glycoproteins in the envelopes of cell-bound, unfused virions, the 37 °C incubation was omitted, and measurements were performed on resealed erythrocyte ghosts formed by hypotonic lysis and resealing (Steck & Kant, 1974).

Antibodies and Fab′ Fragments against Viral Envelope Proteins. The antibodies employed in the present study were raised in rabbits by intracutaneous injections of vesicles containing either F or HN proteins (Henis et al., 1985). In order to eliminate IgG-mediated cross-linking of the antigens, monovalent Fab′ fragments were prepared from the IgG fraction (Henis et al., 1985; Henis & Gutman, 1987). The Fab′ were labeled with either TMR-isothiocyanate or Er-isothiocyanate to yield a dye:Fab′ molar ratio of 0.5:1, using standard labeling procedures (Brandtzaeg, 1973). The antibodies did not cross-react with erythrocyte membrane components, and no cross-reactivity between anti-F and anti-HN antibodies was detected (Henis et al., 1985; Katzir et al., 1989). In 70% sucrose and at 6 °C, the Er–Fab′ preparation yielded phosphorescence anisotropy decay characterized by a rotational correlation time of ca. 40 μs and an amplitude of 0.11–0.12 (see below for definitions of these parameters). We conclude that neither free dye nor aggregation was present.

Measurements of Rotational Diffusion by Time-Resolved Phosphorescence Anisotropy. Following incubation with the virions, the resulting ghosts (formed either by the virally mediated hemolysis or by hypotonic lysis and resealing) were labeled in suspension with anti-F or anti-HN Er–Fab′ fragments (100 μg/mL) in solution A containing 0.2% bovine serum albumin. In the case of unfused virions bound to ghosts, incubation with the Er–Fab′ fragments was carried out for 1 h at 4 °C (to eliminate virus–ghost fusion). In measurements on fused virions, labeling with Er–Fab′ (20 min, 22 °C) was performed after the completion of fusion. The samples were extensively washed from free untagged Er–Fab′ fragments by centrifugations (20000*g*, 10 min, 4 °C) and deoxygenated by resuspending the pellets in solution A containing 25 μg/mL glucose oxidase, 15 μg/mL catalase, and 50 mM glucose (Koloczek et al., 1987).

The rotational mobility of the viral glycoproteins was determined by time-resolved measurements of polarized delayed luminescence (Zidovetzki et al., 1981, 1986; Jovin & Vaz, 1989). The delayed luminescence spectrometer has been described (Matayoshi et al., 1983; Jovin & Vaz, 1989). Samples containing about 2×10^7 cells/mL and 5–30 nM erythrosin (as bound Er–Fab′) were excited at 515 nm by an excimer–dye laser combination, and emission was collected above 645 nm. Individual records were generated at 10 Hz. In all cases, 2000 records consisting of both parallel and perpendicular emission components were accomulated and averaged. Blank corrections were applied by subtracting records generated under identical conditions, but using virus-deficient erythrocyte ghosts incubated with Er–Fab′ fragments. The total phosphorescence emission, $S(t)$, was calculated as $S(t) = I_{\parallel} + 2I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ are the parallel and perpendicular polarized emission components, respectively. $I_{\perp}$ was corrected for the inequality of the gain of the photomultipliers and the finite aperture of the optics monitoring the

Table I: Best-Fit Parameters Derived from the Phosphorescence Intensity Decay Curves of Er–Fab′–Viral Glycoprotein Complexes[a]

viral glycoprotein	system	temp (°C)	PMSF treatment	τ_1 (μs)	τ_2 (μs)	β_1 (%)	β_2 (%)
F	bound virions	4	−	32 ± 0.5	268 ± 1.0	16 ± 0.4	84 ± 0.4
		4	+	31 ± 0.8	272 ± 2.0	13 ± 1.0	87 ± 1.0
		15	−	33 ± 0.5	247 ± 2.0	18 ± 0.5	82 ± 0.6
		22	−	35 ± 1.0	232 ± 1.0	19 ± 1.0	81 ± 1.0
		22	+	30 ± 0.4	233 ± 1.0	17 ± 1.0	83 ± 1.0
		37	+	29 ± 0.5	200 ± 2.0	22 ± 2.0	78 ± 1.0
HN	bound virions	4	−	29 ± 1.0	266 ± 2.0	15 ± 0.5	85 ± 0.5
		4	+	30 ± 0.7	256 ± 3.0	16 ± 0.4	84 ± 0.7
		15	−	31 ± 1.5	241 ± 2.0	18 ± 0.8	82 ± 0.8
		22	−	30 ± 0.7	225 ± 1.0	20 ± 0.5	80 ± 0.5
		22	+	30 ± 0.8	216 ± 2.0	22 ± 0.8	78 ± 0.8
		37	+	28 ± 0.5	186 ± 1.0	27 ± 1.0	73 ± 0.6
F	fused virions	4	−	30 ± 0.4	258 ± 1.0	17 ± 1.0	83 ± 1.0
		15	−	31 ± 0.5	240 ± 3.0	22 ± 1.0	78 ± 2.0
		22	−	30 ± 0.5	220 ± 2.0	23 ± 1.0	77 ± 1.0
		31	−	28 ± 0.2	200 ± 2.0	30 ± 2.0	70 ± 2.0
		37	−	27 ± 0.3	193 ± 2.0	29 ± 1.0	71 ± 1.0
HN	fused virions	4	−	30 ± 0.4	253 ± 2.0	20 ± 1.0	80 ± 1.0
		15	−	31 ± 0.3	253 ± 2.0	22 ± 0.5	78 ± 0.5
		22	−	29 ± 0.5	212 ± 2.0	27 ± 1.0	73 ± 1.0
		31	−	29 ± 0.5	196 ± 0.4	27 ± 1.0	73 ± 1.0
		37	−	27 ± 0.4	183 ± 1.0	32 ± 1.0	68 ± 1.0

[a] Binding and fusion of Sendai virions and human erythrocytes were as described under Materials and Methods. After being labeled with anti-F or anti-HN Er–Fab′ fragments, phosphorescence was measured at the indicated temperatures (see Materials and Methods). The decay of the phosphorescence intensity was fitted to the equation $S(t) = S_0\sum_i \beta_i \exp(-t/\tau_i)$ with $i = 1$), describing a biexponential decay process. τ_1 and τ_2 are the phosphorescence lifetimes, while β_1 and β_2 are the corresponding fractional amplitudes. The numbers in the table are mean ±SE of 8–12 measurements in each case.

emission. The multiexponential analysis of $S(t)$ yields the phosphorescence (triplet) lifetimes and their amplitudes. The emission anisotropy was calculated as $r(t) = (I_\parallel - I_\perp)/S(t)$. The decay of the emission anisotropy, $r(t)$, is a measure of the rate of the rotational relaxation of the phosphorescence probe bound to a macromolecule and can be represented in general as a sum of exponential terms:

$$r(t) = \sum_i \alpha_i \exp(-t/\phi_i) + r_\infty \quad (1)$$

where ϕ_i, the rotational correlation time, depends on the rotational diffusion coefficients (reflecting the segmental, wobbling, and global motions of the macromolecule and the attached probe). The coefficients α_i are the respective amplitudes. The use of eq 1 is justified in the absence of molecular heterogeneity reflected by distinct anisotropy functions associated with the different emission decay components (Jovin & Vaz, 1989). In such a case, $r(t)$ is independent of $S(t)$. The constant limiting anisotropy, r_∞, depends on the degree of orientational constraints on the movements. The initial anisotropy $r_{in.} = \sum\alpha_i + r_\infty$. A more detailed description of the formalism used is presented in former studies (Cherry, 1979; Zidovetzki et al., 1981; Jovin & Vaz, 1989). In the current experiments, the anisotropy decay curves were fit to the monoexponential expression $r(t) = \alpha \exp(-t/\phi) + r_\infty$.

Fluorescence Photobleaching Recovery (FPR). The lateral diffusion coefficients (D) and mobile fractions (R_f) of the viral envelope proteins in the cell membrane were measured by FPR (Koppel et al., 1976; Axelrod et al., 1976) using a previously described apparatus (Henis & Gutman, 1983). The bleaching conditions in FPR experiments were shown not to alter the lateral mobility measured (Wolf et al., 1980; Koppel & Sheetz, 1981). Following incubation with the virions under fusion-promoting conditions, the ghosts formed by the viral hemolytic activity were attached to glass coverslips precoated with poly(L-lysine) (Katzir et al., 1989). The cells were labeled with either anti-F or anti-HN TMR–Fab′ fragments (100 μg/mL, 20 min, 22 °C, in solution A containing 0.2% bovine serum albumin), washed twice, and wet-mounted in the same buffer on a temperature-controlled microscope stage for the FPR experiments.

The monitoring laser beam (529.5 nm, 1 μW, argon ion laser) was focused through the microscope to a Gaussian radius of 0.93 μm with a 100× oil-immersion objective. A brief pulse (5 mW, 20 ms) bleached over 50–70% of the fluorescence in the illuminated region. The time course of fluorescence recovery was followed by the attenuated monitoring beam. D and R_f were determined from the fluorescence recovery curves by nonlinear regression analysis (Petersen et al., 1986). Incomplete recovery was interpreted to represent fluorophores which are immobile on the experimental time scale ($D \leq 5 \times 10^{-12}$ cm^2/s).

Results

Phosphorescence Lifetimes and Emission Anisotropy of Er–Fab′ Bound to Viral Glycoproteins. Anti-F or anti-HN Er–Fab′ was employed to label the viral F or HN envelope proteins, either in the viral membrane (labeling Sendai virions bound to resealed erythrocyte ghosts) or in the ghost membrane (after fusion with human erythrocytes). In all cases, the decay of the total phosphorescence intensity, $S(t)$, was biexponential. Typical curves are shown in Figure 1, and the emission lifetimes and the corresponding fractional amplitudes derived from such curves for the various experimental conditions employed in the present study (adsorbed vs fused virions, F protein vs HN protein) are summarized in Table I. Both the phosphorescence lifetimes and their fractional amplitudes were only slightly dependent on temperature. The long lifetimes and the multiexponential character of the phosphorescence decays are characteristic of triplet probes conjugated with proteins (Garland & Moore, 1979; Cherry, 1979; Zidovetzki et al., 1981; Jovin & Vaz, 1989). Almost all of the phosphorescence signal was associated with the ghosts even after incubation for the duration of the measurements (Figure 1d), indicating that dissociated Er–Fab′ in solution do not affect significantly the anisotropy values measured. Moreover, the level of signal attributed to nonspecific labeling of erythrocyte membrane components by the Er–Fab′ frag-

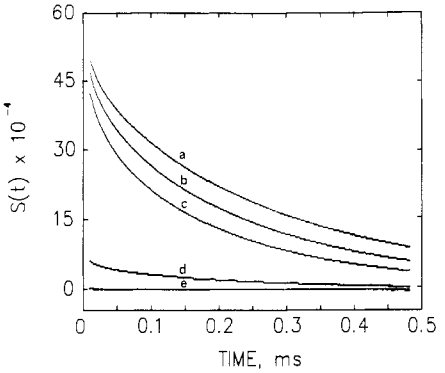

FIGURE 1: Decay of total phosphorescence intensity [$S(t)$] of membrane-bound anti-F Er–Fab′ after fusion of erythrocytes with Sendai virions. Human erythrocytes were fused with Sendai virions and labeled with anti-F Er–Fab′ as described under Materials and Methods. Phosphorescence emission levels were measured by utilizing the delayed luminescence spectrometer (see Materials and Methods). The measurements were performed at (a) 4 °C, (b) 22 °C, and (c) 37 °C. Curve d shows the intensity (at 22 °C) emitted from the supernatant after pelleting down the ghosts, while (e) is the emission of resealed ghosts (not exposed to virus) incubated with anti-F Er–Fab′. Similar results were obtained for the HN viral protein labeled with anti-HN Er–Fab′. Data analysis and the parameters obtained for each temperature are shown in Table I.

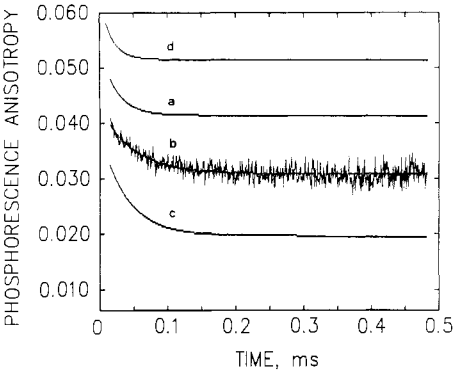

FIGURE 2: Representative anisotropy decay curves of Er–Fab′-tagged F protein in the membranes of fused human erythrocytes. Smooth lines represent computer fits to a monoexponential decay function (see Materials and Methods). Curve b shows both original data and the fitted curve; all others show only the fitted curves. Measurements were performed at 4 °C (a), 22 °C (b and d), or 37 °C (c). In curve d, cell-bound Er–Fab′ were cross-linked by goat anti-rabbit IgG (50 μg/mL, 20 min, 22 °C) after fusion. In this case, the best-fit parameters obtained were $r_\infty = 0.051$, $\alpha = 0.011$, and $\phi = 17$ μs. The parameters characterizing all the other curves are depicted in Figures 3 and 4.

ments was negligible, as indicated by the very low phosphoresence level emitted from resealed ghosts incubated with Er–Fab′ in the absence of virions (Figure 1e).

The phosphorescence emitted from the Er–Fab′ bound to either viral glycoprotein (F or HN) was polarized. Representative phosphorescence anisotropy decay curves obtained for anti-F Er–Fab′ bound to erythrocytes which were fused with Sendai virions are shown in Figure 2. The results of fitting such curves (for F or HN proteins after fusion in the erythrocyte membrane, or in the envelope of unfused virions attached to erythrocyte ghosts) to the monoexponential decay law are depicted in Figures 3 and 4. In all the cases examined and at all measurement temperatures, the emission anisotropy decayed from an initial value (r_{in}) to a limiting anisotropy (r_∞). The latter was finite (>0), reflecting either the influence of orientational constraints on the rotational motions of the Er–Fab′–viral protein complexes or the existence of a certain percentage of immobile viral glycoprotein species. Indeed, cross-linking of Er–Fab′ bound to viral glycoproteins embedded in the target cell membrane by bivalent goat IgG directed against rabbit IgG led to a dramatic increase in the absolute values of r_{in} and r_∞ (Figure 2d). The values of r_{in} (0.03–0.06) are relatively low compared to the value of 0.25 obtained for the phosphorescence anisotropy of Er immobilized in poly(methyl methacrylate) (Garland & Moore, 1979; Corin et al., 1987), suggesting that the Er–Fab′ conjugate bound to either F or HN can undergo local and/or segmental motion faster than the experimental time scale (<1 μs). Such low r_{in} values have been encountered for other cell membrane receptors (Zidovetzki et al., 1981, 1986). The positive amplitudes of the anisotropy decay (i.e., in the direction of decreasing anisotropy) observed under all labeling and measurement conditions employed suggest that the orientation of the Er probe relative to the rotation axis is relatively random. This may reflect the existence of several Fab′ binding sites on each viral glycoprotein type and/or the occurrence of several alternative sites on the Fab′ that can be coupled to erythrosin isothiocyanate.

Comparison of the Temperature Dependence of the Anisotropy Decay Parameters of Er–Fab′-Labeled Viral Glycoproteins. The temperature dependence of the rotational correlation times (ϕ) of F and HN labeled with Er–Fab′ (Figure 4) deserves special attention. This parameter shows completely different patterns for F and HN embedded in the target cell membrane (after fusion) (Figure 4B,D). Thus, whereas the ϕ values for Er–Fab′–F increased with the measurement temperature between 4 and 22 °C and decreased upon further elevation of the temperature, the rotational correlation times for Er–Fab′–HN exhibited a very weak dependence on temperature. In the case of bound, unfused virions, the ϕ values for the two Er–Fab′-labeled viral glycoproteins were rather similar up to 22 °C (Figure 4A,C). Such measurements could not be performed at higher temperatures, due to the induction of fusion above 22 °C. This similarity (especially at 22 °C, where the difference between the ϕ values for the fused F and HN proteins was highest) indicates that the differences between the ϕ values of fused F and HN in the target cell membrane indeed reflect differences in the rotational motion of the viral glycoproteins, and are not simply due to the use of different antibody preparations for labeling each viral glycoprotein species, nor due to changes in the photophysical decay kinetics.

In order to examine whether a breakpoint in the temperature dependence of the ϕ values of F (and possibly also of HN) occurs also in the viral membrane, it was required to generate conditions where fusion could not occur even at temperatures above 22 °C. To achieve this, we employed PMSF-treated Sendai virions; the PMSF treatment abolishes the viral fusogenic activities but does not interfere with virus binding and with the virus-mediated hemagglutination (Israel et al., 1983; Aroeti & Henis, 1986). For both F and HN labeled with the appropriate Er–Fab′ fragments, the ϕ values measured for PMSF-treated virions bound to ghosts increased between 4 and 22 °C and decreased between 22 and 37 °C, as in the case of F proteins after fusion with erythrocytes (Figure 4A,C). It should be noted that below 22 °C, where measurements could also be performed on untreated bound virions, the PMSF treatment induced an increase in the ϕ values measured for Er–Fab′-labeled F proteins (but not for the HN proteins). However, this elevation was rather similar at 4 and at 22 °C, and did not alter the pattern of the temperature dependence of the ϕ values at this temperature range.

Unlike the breakpoint observed in several cases in the temperature dependence of the ϕ values, r_∞ and the ratio r_∞/r_{in}

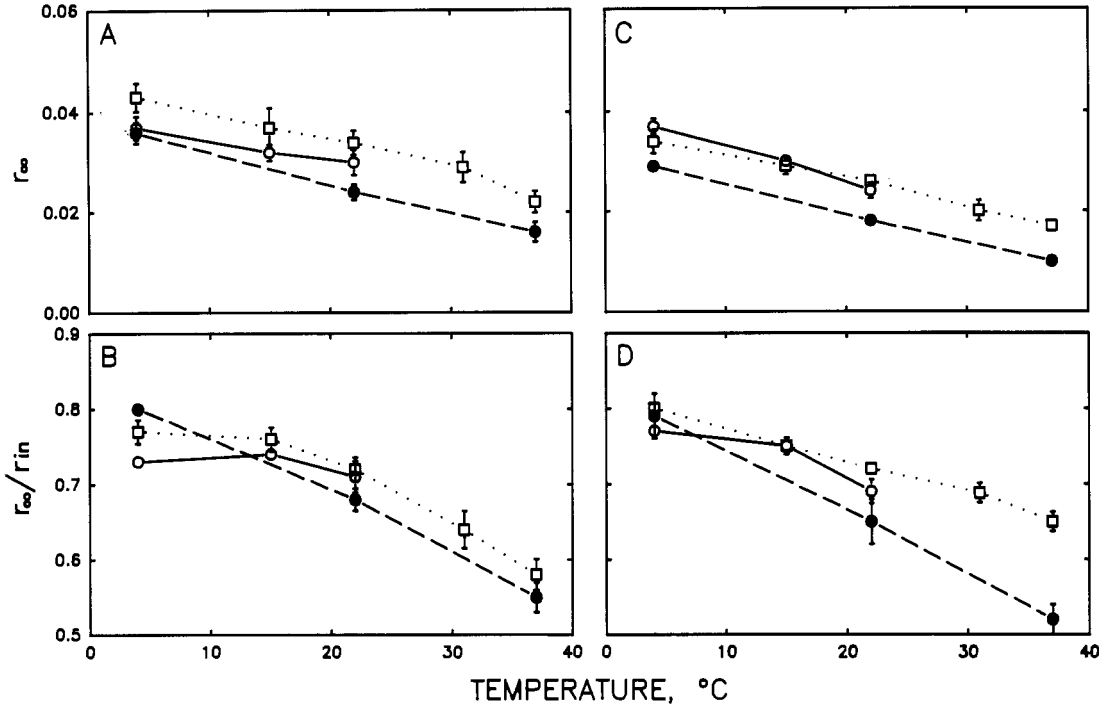

FIGURE 3: Temperature dependence of the anisotropy decay parameters of F (A and B) or HN (C and D) proteins tagged with Er–Fab′ in the viral envelope and in the membrane of erythrocytes fused with Sendai virus. For measurements of the proteins situated in the viral envelope, virions (native or PMSF-treated) were bound to ghosts in the cold and labeled with either anti-F or anti-HN Er–Fab′ fragments. For measurements on F or HN fused with the target cell membrane, the labeling was performed on ghosts formed following fusion (see Materials and Methods). The best-fit parameters were derived by fitting the anisotropy decay curves to a monoexponential expression. Results are the mean ± SE of 8–12 measurements in each case. Error bars are not shown where they are smaller than the symbol size. The α values did not change significantly with temperature; they were in the range of 0.009–0.010 for HN (either before or after fusion) and between 0.012 and 0.015 for F (fused or unfused). (O—O) Native virions bound to ghosts; (●---●) PMSF-treated virions bound to ghosts; (□···□) human erythrocytes fused with Sendai virions.

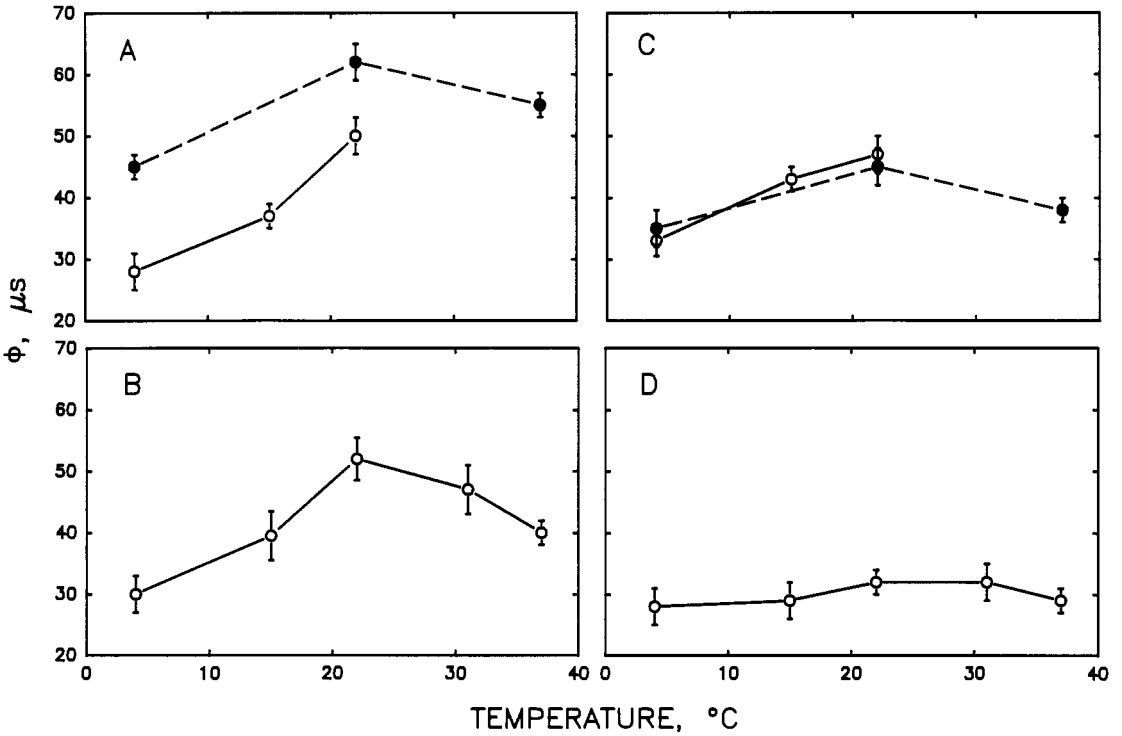

FIGURE 4: Temperature dependence of the rotational correlation times of Sendai virus glycoproteins labeled by Er–Fab′. Sendai virions (native or PMSF-treated) were bound to resealed erythrocyte ghosts in the cold (A and C) or fused (native virions only) with erythrocytes at 37 °C (B and D). The viral glycoproteins were then labeled with either anti-F (A and B) or anti-HN (C and D) Er–Fab′ fragments. The ϕ values (rotational correlation times) were derived as in Figure 3. Results are mean ± SE of 8–12 measurements in each case. (O—O) Native virions; (●---●) PMSF-treated virions.

were always higher at lower temperatures (Figure 3), as expected for a more restricted rotational motion of the Er–Fab′–viral glycoprotein complexes at lower temperatures. r_∞/r_{in} is a measure of the orientational constraints of the probe at equilibrium (Kinosita et al., 1984); it can be regarded as the normalized residual anisotropy, increasing along with the

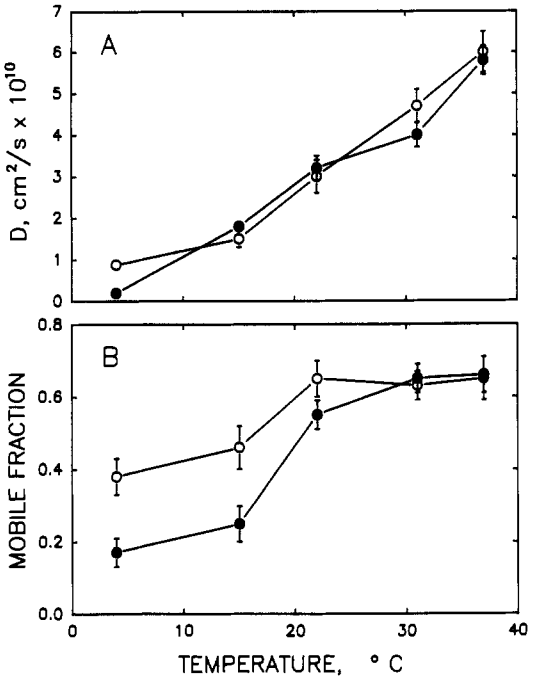

FIGURE 5: Temperature dependence of the lateral mobility of F and HN in the membranes of human erythrocytes fused with Sendai virions. Human erythrocytes were fused with Sendai virions at 37 °C as described under Materials and Methods. Anti-F and anti-HN TMR–Fab′ fragments were employed to label the F (○) and HN (●) proteins, respectively. The FPR measurements were conducted in solution A at 4, 15, 22, 31, or 37 °C. The results are the mean ± SE of 20–30 measurements in each case.

fraction of immobile glycoproteins. Finally, it should be noted that all the rotational mobility parameters were fully reversible upon temperature shifts, without regard as to whether the approach to the measurement temperature was from a lower or a higher temperature.

Temperature Dependence of the Lateral Diffusion of F and HN in Erythrocyte Membranes. The constraints imposed on the lateral diffusion of membrane proteins are very different from those governing rotational diffusion (Saffman & Delbruk, 1975; Saffman, 1976). It was therefore of interest to compare the temperature dependence of the rotational diffusion parameters with those characterizing the lateral diffusion of F and HN in the target cell membrane (after fusion). The results (Figure 5) demonstrate that unlike the breakpoint observed at 22 °C for ϕ of the F proteins after fusion with human erythrocytes, the lateral diffusion coefficients (D) of both F and HN always increased with the measurement temperature. Interestingly, the mobile fractions (R_f) of the two viral glycoproteins in the erythrocyte membrane also increased with the measurement temperature up to 22 °C. Thus, this temperature is also a breakpoint for these parameters, in as much as the R_f values do not increase upon further elevation of the measurement temperature (Figure 5B). As in the rotational mobility measurements, the temperature dependence of the R_f and D values of both viral glycoproteins demonstrated full reversibility, and identical values of these parameters were obtained following fast cooling of the sample from 37 to 4 °C or reheating.

Discussion

The envelope proteins of Sendai virus, F and HN, exist as oligomers in the viral envelope (Markwell & Fox, 1980; Sechoy et al., 1987). However, it is not clear whether F and HN interact in hetero complexes, although there are indications that not only F but also HN is required for the viral fusogenic activity (Ozawa et al., 1979; Miura et al., 1982; Gitman & Loyter, 1984; Katzir et al., 1989; Henis et al., 1989). Moreover, it is not clear whether or not the viral glycoproteins undergo changes in their organization in the membrane upon fusion with the target cell membrane. These questions have been explored in the present study by measuring the rotational mobility of F and HN in the viral envelope and in the target cell membrane (after fusion).

The temperature dependence of the rotational correlation times (ϕ) of the HN protein is clearly different in the envelope of cell-bound virions and in the fused cell membrane (compare panels C and D of Figure 4). On the other hand, no significant changes in the ϕ values of the Er–Fab′-tagged F protein are observed upon fusion (Figure 4A,B). These results suggest changes in the organization of the HN proteins upon fusion with the target cell membrane. The reduction in the ϕ values of HN upon fusion could indicate partial dissociation, or a shift in the equilibrium between the dimer and tetramer forms (Markwell & Fox, 1980) of the HN protein following fusion. Alternatively, the observed reduction in ϕ may also reflect a transfer from the more constricted phospholipid environment of the viral envelope (Abidi & Yeagle, 1984) to that of the erythrocyte plasma membrane, although the lack of change in the ϕ values of the F protein upon fusion argues against this possibility.

Although the temperature dependencies of φ of Er–Fab′-tagged F and HN are rather similar in the membrane of ghost-bound, unfused virions (Figure 4A,C), they are highly different in the erythrocyte membrane after fusion (Figure 4B,D). This demonstrates that F and HN diffuse separately in the target cell membrane, and for the major part are not complexed together following fusion. A similar conclusion was drawn from FPR experiments involving antibody-mediated cross-linking, which demonstrated that immobilization of either F or HN in the membranes of fused human erythrocytes did not affect the lateral mobility of the other viral glycoprotein type (Katzir et al., 1989). Our data do not rule out the possible existence of heterologous F–HN complexes in the viral envelope itself, in view of the rather similar ϕ values for F and HN in bound, unfused virions, at all measurement temperatures (Figure 4A,C).

The rotational correlation times (Figure 4) reveal a breakpoint at 22 °C for the F proteins before and after fusion and for the HN proteins in the envelope of ghost-bound virions. This phenomenon is present but significantly less pronounced in the case of HN proteins in the fused erythrocyte membrane (Figure 4D). Interestingly, the ϕ values increase between 4 and 22 °C, indicative of a reduction in the rate of rotational diffusion of the Er–Fab′–viral glycoprotein complex. This result is contrary to the expected effect of temperature on membrane fluidity. That is, the higher fluidity at elevated temperatures should facilitate the rotational mobility of membrane constituents, a trend observed only upon further elevation of the temperature between 22 and 37°C, reflecting faster rotational diffusion (reduced ϕ values). A possible interpretation for the 22 °C breakpoint in ϕ is a temperature-dependent microaggregation of F and HN that increases with temperature up to 22 °C and saturates at this temperature range; in such a case, membrane fluidity changes will become dominant only at higher temperatures, leading to a reduction of ϕ. It should be noted that temperature-sensitive microaggregation was proposed earlier to explain the elevation of the ϕ values with temperature for the rotation of Er-labeled epidermal growth factor bound to its receptor (Zidovetzki et al., 1981, 1986). In addition, the effect of protein aggregation

on rotation was shown to exceed that of lipid fluidity for rhodopsin in reconstituted membranes above the phase transition temperature (Kusumi et al., 1980; Kusumi & Hyde, 1982). The absence of an analogous breakpoint in the temperature dependence of the lateral diffusion coefficients of either F or HN in the target cell membrane (Figure 5) is in accordance with the proposed microaggregation of the viral glycoproteins, since unlike rotational diffusion, the lateral diffusion rate of membrane proteins is insensitive to the size of the diffusing protein (Saffman & Delbruck, 1975; Saffman, 1976).

The 22 °C breakpoint in ϕ discussed above is closely correlated with the temperature dependence of the viral fusogenic activities. The rates of both virus–cell and cell–cell fusion are reduced to negligible values at about 20 °C (Hoekstra et al., 1985; Aroeti and Henis, unpublished results). The possible aggregation phenomena suggested by the rotational mobility measurements may, therefore, play an important role both in virus–cell fusion and in virally-mediated cell fusion. In this context, it is interesting to note that a breakpoint at 22 °C is also detected in the fraction of laterally mobile F or HN proteins in the target cell membrane after fusion (Figure 5). This phenomenon is consistent with the notion that lateral mobility of both F and HN in the target cell membrane is essential for inducing cell–cell fusion of human erythrocytes, most likely due to the need to form higher aggregates of viral glycoproteins which are active in the fusion reaction (Henis et al., 1989; Morris et al., 1989).

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