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Membrane Fusion Activity of Tick-Borne Encephalitis Virus and Recombinant Subviral Particles in a Liposomal Model System

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We present a kinetic analysis of the membrane fusion activity of tick-borne encephalitis (TBE) virus and TBE-derived recombinant subviral particles (RSPs) in a liposomal model system. Fusion was monitored using a fluorescence assay involving pyrene-labeled phospholipids. Fusion was strictly dependent on low pH, with the optimum being at pH 5.3–5.5 and the threshold at pH 6.8. Fusion did not require a protein or carbohydrate receptor in the target liposomes. Preexposure to low pH of the virus alone resulted in inactivation of its fusion activity. At the optimum pH for fusion and 37°C, the rate and extent of fusion were very high, with more than 50% of the virus fusing within 2 s and the final extent of fusion being 70%. Lowering of the temperature did not result in a significant decrease in the rate and extent of fusion, suggesting that TBE virus fusion is a facile process with a low activation energy, possibly due to the flat orientation of the E glycoprotein on the viral surface facilitating the establishment of direct intermembrane contact. The fusion characteristics of TBE virus and RSPs were similar, indicating that RSPs provide a reliable and convenient model for further study of the membrane fusion properties of TBE virus.

INTRODUCTION

The entry of enveloped viruses into cells involves a membrane fusion step that occurs either at the cell plasma membrane, mediated by the interaction of viral envelope proteins and their receptors, or in the endosomal compartment after uptake of virions by receptor-mediated endocytosis (Hernandez *et al.,* 1996). In the latter case, it is generally the relatively low pH in the endosomal lumen that induces a change in the conformation or oligomeric state of the viral envelope proteins, initiating the fusion process (White, 1990; Gaudin *et al.,* 1995; Hernandez *et al.,* 1996).

The precise mechanism by which viral envelope proteins induce membrane fusion is still not well understood. Experimentally, model systems are needed in which kinetic data from fusion studies can be related to high-resolution protein structure information. For several enveloped viruses, the core structures of their membrane fusion proteins have been solved at the atomic level. These include not only the classic example of the hemagglutinin (HA) protein of influenza virus in both its neutral and acid-pH conformation but also the gp41 subunits of human and simian immunodeficiency viruses, the GP2 subunit of Ebola virus, and the TM subunit of Moloney murine leukemia virus (for reviews, see Skehel and Wiley, 1998; Weissenhorn *et al.,* 1999). Despite the different modes of cell entry of these viruses, it is interesting that their fusion proteins exhibit remarkable structural similarity (Skehel and Wiley, 1998; Weissenhorn *et al.,* 1999). The atomic structure of the envelope protein E of the flavivirus tick-borne encephalitis (TBE) virus has also been solved recently (Rey *et al.,* 1995). However, in contrast to the influenza virus HA, which is a homotrimeric spike, the TBE virus E protein in its native (neutral pH) conformation is an antiparallel homodimer that lies flat on the surface of the virion. Consistent with its role in mediating low-pH-induced fusion in endosomes, the E protein undergoes dramatic structural changes when exposed to an acidic pH (Rey *et al.,* 1995), and these changes are apparently involved in initial interactions with target membranes and possibly in driving the fusion process itself. Although the three-dimensional structure of the acidic form of the protein is still not known, it has been observed that the E dimers on the TBE virion surface undergo an irreversible quantitative rearrangement at the pH of fusion to form trimers, and this rearrangement appears to be involved in the fusion process (Allison *et al.,* 1995a; Stiasny *et al.,* 1996).

TBE virus is a medically important pathogen of the genus *Flavivirus*, which also includes yellow fever virus, Japanese encephalitis virus, and the dengue viruses. These viruses are composed of a nucleocapsid contain-

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ing the positive-strand RNA genome and the capsid protein C, surrounded by a lipid envelope containing the E protein and the small (~8 kDa) membrane protein M. As indicated, flaviviruses use the endocytic pathway to enter cells (Rice, 1996) and require a mildly acidic pH for the expression of membrane fusion activity. In the absence of target membranes, low-pH treatment of flaviviruses results in the loss of infectivity and fusogenicity due to irreversible structural changes in the E protein (Gollins and Porterfield, 1986b; Kimura and Ohyama, 1988; Guirakhoo *et al.,* 1992; Heinz *et al.,* 1994).

Flavivirus-infected cells release not only infectious whole virions but also noninfectious subviral particles containing the E and M proteins in a lipid membrane without the nucleocapsid (Russell *et al.,* 1980). Similar particles can also be produced using various eukaryotic expression systems (Mason *et al.,* 1991; Konishi *et al.,* 1992; Pincus *et al.,* 1992; Fonseca *et al.,* 1994; Pugachev *et al.,* 1995; Allison *et al.,* 1995b). Recombinant subviral particles (RSPs) from TBE virus have been shown to retain many of the structural and functional characteristics of the whole virion, including the ability to induce cell-cell fusion (Schalich *et al.,* 1996).

Although membrane fusion activity under mildly acidic conditions has been demonstrated for several flaviviruses (Gollins and Porterfield, 1986a; Summers *et al.,* 1989; Randolph and Stollar, 1990; Guirakhoo *et al.,* 1991, 1993; Vorovitch *et al.,* 1991; Desprès *et al.,* 1993), detailed kinetic analyses of the fusion process have not been performed due to the lack of adequately sensitive techniques. However, using virus with a fluorescent probe incorporated in its membranes, it is possible to monitor fusion of the viral and liposomal membranes online, allowing fusion kinetics to be studied in detail. Pyrene-labeled lipids have been shown in several studies to be especially well suited for monitoring lipid mixing during viral fusion (Wahlberg *et al.,* 1992; Bron *et al.,* 1993; Stegmann *et al.,* 1993; Nieva *et al.,* 1994). Pyrene phospholipids can be incorporated into the viral membrane *in vivo* by biosynthetic labeling, ensuring that the fluorophore will remain stably integrated in the membrane (Stegmann *et al.,* 1993). Here, we present a detailed analysis of fusion of TBE virus and RSPs, biosynthetically labeled with pyrene phospholipids, in a liposomal model system. It is demonstrated that the fusion of TBE virus is a very fast and efficient process. It is triggered by a mildly acidic pH but does not require the presence of a protein or carbohydrate receptor or specific lipids, such as cholesterol (Chol) and sphingomyelin (SPM), in the target membrane.

RESULTS

Fluorescent labeling of TBE virus with pyrene

To investigate whether the membrane of TBE virus can be metabolically labeled *in vivo* with pyrene-conjugated lipid, TBE virus was grown in primary chicken embryo cells cultured beforehand in the presence of pyrene-hexadecanoic acid, as described in Materials and Methods, and purified by centrifugation on sucrose density gradients. The fluorescence spectrum of the purified pyrene-labeled virus (Fig. 1) clearly shows the characteristic fluorescence peaks for the pyrene monomer at 379 and 395 nm as well as an excimer peak at 480 nm. The excimer fluorescence arises from probe–probe interactions, and the appearance of a significant excimer intensity in the TBE virus preparation thus indicates that the label had been efficiently incorporated into the viral membrane phospholipids. At 37°C, the excimer/monomer ratio, defined as the ratio of the fluorescence intensities at 480 and 379 nm, respectively, was 0.38, similar to results achieved earlier with another enveloped virus, Semliki Forest virus (SFV) (Bron *et al.,* 1993). Disruption of the viral membrane with the detergent octa(ethylene glycol) *n*-dodecyl monoether ($C_{12}E_8$) resulted in the loss of the excimer peak and an increase in the intensity of the monomer peaks, due to essentially infinite dilution of the probe.

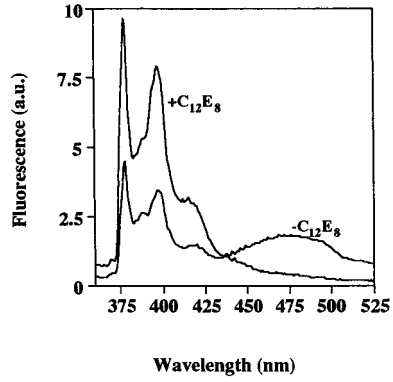

FIG. 1. Fluorescence emission spectrum of pyrene-labeled TBE virus. Pyrene-labeled virus was suspended in HNE buffer (pH 7.4) at a concentration of 1 μM phospholipd. The fluorescence emission spectrum at an excitation wavelength of 343 nm was recorded before and after the addition of $C_{12}E_8$ to a concentration of 10 mM.

Low-pH-dependent fusion of TBE virus with liposomes

Pyrene-labeled TBE virus was then studied for its ability to undergo fusion with artificial membranes (unilamellar liposomes with an average diameter of 0.2 μm, composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), SPM, and Chol, in a molar ratio of 1:1:1:1.5). Because the membrane surface area of a virus particle is about 10-fold smaller than that of an average liposome, fusion of a single virion with a liposome is expected to result in an approximate 10-fold dilution of pyrene-labeled phospholipids from the viral into the liposomal membrane and a corresponding 10-fold decrease of the pyrene excimer fluorescence intensity of the virion involved. As shown in Fig. 2 (curve a), extremely rapid

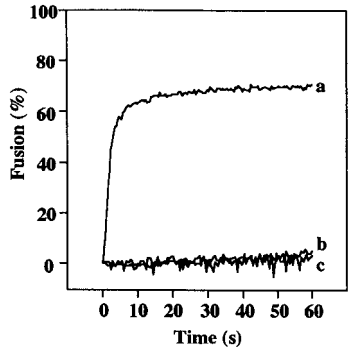

FIG. 2. Fusion of TBE virus with liposomes consisting of PC/PE/SPM/Chol (molar ratio 1:1:1:1.5). Pyrene-labeled virus (1.0 μM phospholipid) was mixed with liposomes (0.2 mM phospholipid) at 37°C. Subsequently, the medium was acidified to pH 5.3 by injection of small volume of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, 0.1 M acetic acid (pH 4.9) (curve a) or kept at pH 7.4 (curve b). Alternatively, virus in the absence of liposomes was acidified to pH 5.3 for 1 min at 37°C, after which liposomes were added while the pH was kept at 5.3 (curve c). All measurements were carried out in duplicate or triplicate with comparable results.

fusion of the virus with the liposomes was observed when a virus–liposome mixture was acidified to pH 5.3 at 37°C. Under these conditions, the pyrene excimer fluorescence intensity decreased by more than 50% within the first 2 s, with an extent at 1 min of about 70%. This implies that within 1 min at least 70% of the virus particles had fused at least once with a target liposome. No fusion was observed when the experiment was carried out at pH 7.4 (curve b), consistent with earlier reports that TBE virus requires an acidic pH for fusion (Guirakhoo *et al.,* 1991; Allison *et al.,* 1995a). Although Fig. 2 presents fusion curves for one batch of pyrene-labeled virus, similar results were obtained with another batch of virus and other batches of liposomes (results not shown). All virus fusion data presented below, however, were generated with the batch of virus also used in the experiment presented in Fig. 2.

To investigate the pH dependence of fusion in more detail, fusion assays were performed with the same liposome preparation at different pH values, and the initial rate and final extent of fusion for each pH value were determined. As shown in Fig. 3, the pH dependence exhibited a threshold around pH 6.8. The rate of fusion was optimal at pH 5.3–5.5 (approximately 35–40% per s in this particular experiment), although it should be noted that very high rates were already achieved at pH 6.0–6.2. An optimal extent of fusion at 1 min of 60–70% was seen in a broad pH range of 5.0–6.2. The initial rates and the extents of fusion under optimal conditions varied somewhat between individual measurements and experiments, with the rates fluctuating between 30% and 40% per s and the extents at 1 min fluctuating between 60% and 70%.

Kinetics of fusion inactivation

It has been shown previously that exposure of TBE virus to an acidic pH in the absence of target membranes results in a rapid loss of viral infectivity (Gollins and Porterfield, 1986b; Kimura and Ohyama, 1988; Guirakhoo *et al.,* 1992; Heinz *et al.,* 1994). We now examined whether this loss of infectivity correlates with a loss of fusogenicity in the liposomal model system. Pyrene-labeled virus was preexposed to pH 5.3 for 1 min in the absence of target liposomes. Subsequent addition of liposomes demonstrated that this low-pH preincubation had almost completely inactivated the fusion capacity of the virus (Fig. 2, curve c).

To examine the kinetics of fusion inactivation in more detail, the virus was preincubated at pH 5.3 for different lengths of time in the absence of liposomes, and the remaining fusion activity was measured after the addition of liposomes. Figure 4 shows the rate and extent of fusion at each time point as a percentage of the control. Both the rate and extent of fusion fell below the half-maximum level within the first 10 s of incubation, but the initial rate of fusion decreased more sharply with time than did the extent of fusion.

The loss of fusion activity on low-pH exposure of the virus in the absence of target membranes is presumably due to irreversible rearrangements of the viral envelope glycoproteins and indicates that the fusion-active state of the viral E protein is of a very transient nature.

Temperature dependence of fusion

The effect of temperature on the rate of fusion with PC/PE/SPM/Chol liposomes at pH 5.3 was investigated at 37°, 20°, 15°, and 4°C, and the results are shown in Fig. 5. The rate of fusion decreased with decreasing temperature (Figs. 5A and 5B), but the overall effect of temperature was

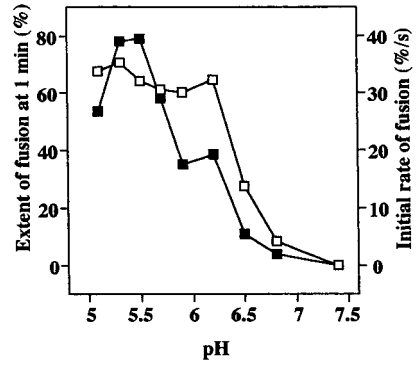

FIG. 3. Extent and rate of TBE virus fusion with liposomes as a function of the pH. Fusion of pyrene-labeled TBE virus with PC/PE/SPM/Chol liposomes was measured as in Fig. 2. The initial rates of fusion (filled symbols) were determined from the tangents to the steepest parts of the fusion curves. The extents of fusion (open symbols) were determined at 1 min after acidification of the virus-liposome mixtures. All measurements were carried out in duplicate, with the data shown representing average values.

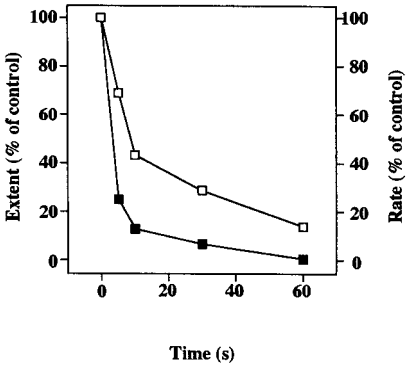

FIG. 4. Inactivation of membrane fusion activity by preincubation of TBE virus at low pH. Pyrene-labeled virus alone was preincubated at pH 5.3 for the periods of time indicated, after which PC/PE/SPM/Chol liposomes were added and the remaining fusion activity was determined, as in Fig. 2. Rates (filled symbols) and extents (open symbols) of fusion were determined as in Fig. 3 and are expressed as percentages of the control (fusion at pH 5.3 initiated in the presence of liposomes). All measurements were carried out in duplicate, with the data shown representing average values.

considerably less dramatic for TBE virus than has been shown previously for SFV (Bron *et al.,* 1993) or influenza virus (Stegmann *et al.,* 1990). For example, with TBE virus, the fusion curves at 37° and 20°C were almost indistinguishable (Fig. 5A), whereas at 15°C, the initial rate of fusion (17% per s) was reduced barely twofold relative to the rate of 30% per s at 37°C (Fig. 5B) without the appearance of any detectable lag phase preceding the onset of the actual fusion reaction (Fig. 5A). Only at the lowest temperature studied (4°C) could a brief (4.5 s) lag phase be observed between the time of acidification and the onset of fusion (Fig. 5A).

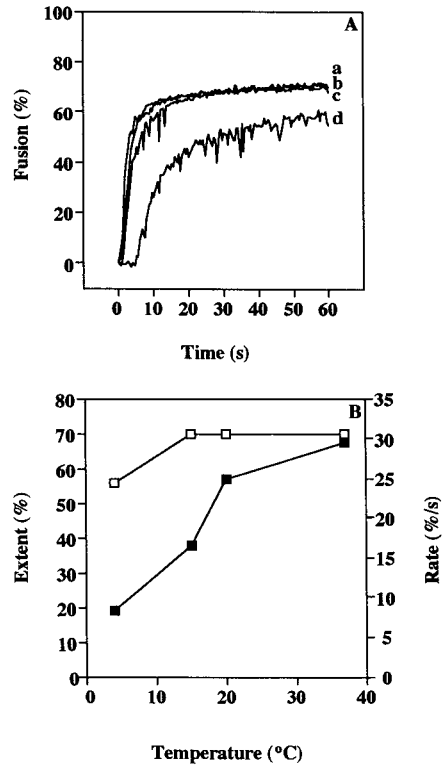

FIG. 5. Effect of temperature on fusion of TBE virus with liposomes. Fusion of pyrene-labeled virus with PC/PE/SPM/Chol liposomes was measured under conditions as in Fig. 2, but at the indicated temperatures. (A) Fusion curves at 37°C (curve a), 20°C (curve b), 15°C (curve c), and 4°C (curve d). (B) Rates (filled symbols) and extents (open symbols) of fusion, determined as in Fig. 3, as a function of the temperature. All measurements were carried out in duplicate, with the data shown representing average values.

Effect of membrane lipid composition

Some viruses have a specific requirement for particular lipids in the target membrane; for example, SFV and Sindbis virus (SIN) require Chol and sphingolipids (White and Helenius, 1980; Kielian and Helenius, 1984; Phalen and Kielian, 1991; Nieva *et al.,* 1994; Moesby *et al.,* 1995; Corver *et al.,* 1995; Smit *et al.,* 1999). To determine whether TBE virus also has a specific lipid requirement, we measured fusion of TBE virus with liposomes of several different compositions. As shown in Fig. 6, fusion of TBE virus with liposomes consisting of PC/PE/SPM/Chol (1:1:1:1.5) was fast and extensive (curve a). When PC/PE/Chol liposomes were used instead (curve b), the rate and extent of fusion remained essentially unchanged, indicating that SPM is not required for fusion. Omission of Chol from the liposomes, on the other hand, had a substantial influence on fusion. When PC/PE/SPM liposomes were used (curve c) the rate and extent of fusion were 9–10% per s and 49%, respectively, compared with approximately 32% per s and 70% observed with PC/PE/SPM/Chol liposomes. A further decrease was observed with liposomes consisting only of PC and PE (curve d). Thus the inclusion of Chol in the target membrane, although not absolutely essential, appears to facilitate fusion by TBE virus, whereas SPM can com-

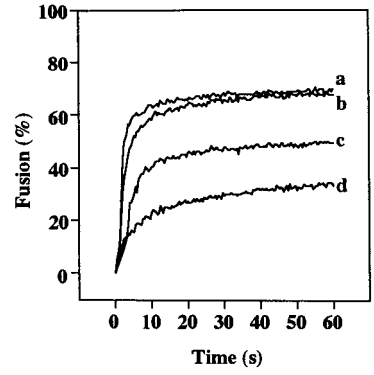

FIG. 6. Effect of the liposomal lipid composition on fusion of TBE virus with liposomes. Fusion of pyrene-labeled virus with liposomes of different lipid compositions was measured under conditions otherwise as in Fig. 2. Curve a, PC/PE/SPM/Chol (molar ratio, 1:1:1:1.5); curve b, PC/PE/Chol (molar ratio, 1:1:1); curve c, PC/PE/SPM (molar ratio, 1:1:1); and curve d, PC/PE (molar ratio, 1:1).

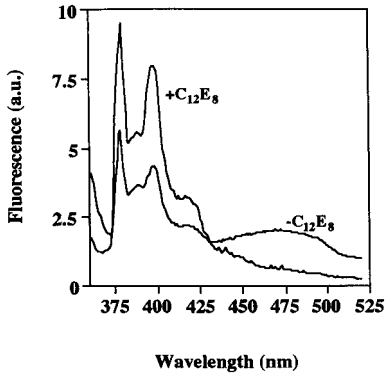

FIG. 7. Fluorescence emission spectrum of pyrene-labeled TBE RSPs. The spectrum was recorded under the conditions as in Fig. 1.

pensate somewhat for the lack of Chol when the latter is omitted.

Fusion characteristics of RSPs

In addition to the experiments with whole virions, we also investigated the fusion properties of TBE virus RSPs, which lack a nucleocapsid but retain many of the structural and functional characteristics of the virion envelope (Schalich *et al.,* 1996). The techniques used for metabolic labeling and purification of RSPs were similar to those used for whole virions, and purified pyrene-labeled RSPs yielded a fluorescence spectrum similar to that of the pyrene-labeled whole virus, with an excimer/monomer ratio of 0.2 (Fig. 7). Fusion of pyrene-labeled RSPs with PC/PE/SPM/Chol liposomes at pH 5.5, 37°C, is shown in Fig. 8A (curve a). The rate (30% per s) and extent (60%) of fusion were very similar to those obtained with whole virions, and like whole virions, RSPs required low pH for fusion (see pH 7.4 control, curve b). Furthermore, the pattern of target membrane lipid dependence for RSPs (Fig. 8B) was essentially identical to that shown in Fig. 6 for whole virions. RSPs, like whole virions, were irreversibly inactivated at low pH in the absence of target membranes (Fig. 9) and also showed a brief lag phase at low temperature (data not shown). The fusion characteristics of three individual pyrene-labeled RSP batches were very similar (comparative results not shown). Taken together, the data show that the fusion characteristics of pyrene-labeled RSPs in the liposome fusion system resemble those of whole virions.

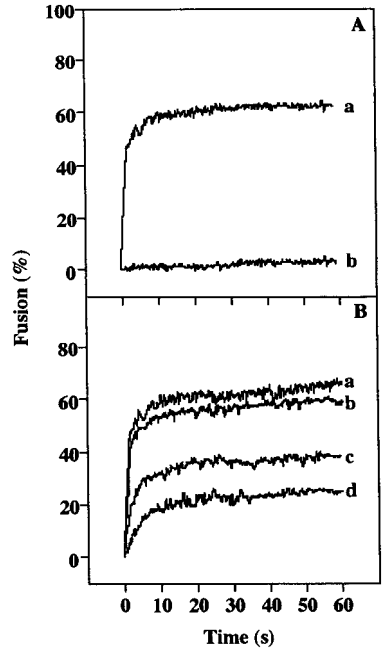

FIG. 8. Fusion of TBE RSPs with liposomes. Fusion of pyrene-labeled RSPs (0.5 μM phospholipid) was measured under the conditions essentially as in Fig. 2. (A) Fusion with PC/PE/SPM/Chol (molar ratio 1:1:1:1.5) liposomes. Curve a, pH 5.5; curve b, pH 7.4 control. (B) Fusion at pH 5.5 with liposomes of different lipid compositions. Curve a, PC/PE/SPM/Chol (molar ratio 1:1:1:1.5); curve b, PC/PE/Chol (molar ratio, 1:1:1); curve c, PC/PE/SPM (molar ratio, 1:1:1); and curve d, PC/PE (molar ratio, 1:1).

DISCUSSION

We present a detailed analysis of the membrane fusion activity of TBE virus and TBE-derived recombinant subviral particles in a liposomal model system. TBE virus enters its target cells through receptor-mediated endocytosis and subsequent low-pH-induced fusion of the viral envelope with the limiting membrane of the endosomal cell compartment (Heinz *et al.,* 1994). Accordingly, in the liposomal model system, fusion of pyrene-labeled TBE virus strictly required low pH. The fusion process exhibited a broad pH optimum between pH 5.0 and 6.2, whereas the pH threshold was around 6.8. A similar pH threshold for fusion (pH 6.7) has been reported for West Nile virus, another flavivirus (Gollins and Porterfield,

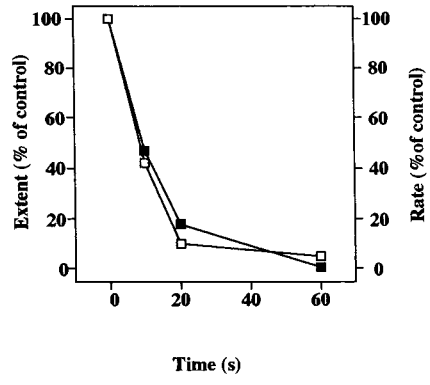

FIG. 9. Inactivation of membrane fusion activity by preincubation of TBE RSPs at low pH. Pyrene-labeled RSPs were preincubated at pH 5.5 in the absence of liposomes for the periods of time indicated. Subsequently, the residual fusion activity was measured as in Fig. 4. Rates (filled symbols) and extents (open symbols) of fusion are expressed as percentages of the control (fusion at pH 5.5 initiated in the presence of liposomes). All measurements were carried out in duplicate, with the data shown representing average values.

1986a). This pH threshold is relatively high compared with that of fusion of alphaviruses, such as SFV or SIN. In a similar liposomal fusion model, we have observed a pH threshold of 6.2 for fusion of SFV (Bron *et al.,* 1993) and of 6.0 for fusion of SIN (Smit *et al.,* 1999). Also, the pH threshold for fusion of influenza virus, although it varies somewhat between virus strains, is generally lower than the threshold observed here for TBE virus (Stegmann *et al.,* 1986, 1987, 1990). The comparatively high pH threshold for fusion implies that flaviviruses may fuse from within early endosomes (Heinz *et al.,* 1994).

Fusion of TBE virus does not require the presence of a protein or carbohydrate receptor in the target membrane, as fusion proceeded efficiently with liposomes consisting of just nonglycosylated lipids. This indicates that receptor binding and fusion activity are separate functions of the TBE virus E protein and that receptor binding is not a molecular requirement for expression of membrane fusion activity by E. It has been demonstrated that other low-pH-dependent enveloped viruses, such as influenza virus (Stegmann *et al.,* 1986, 1987, 1989, 1995), SFV (Bron *et al.,* 1993; Nieva *et al.,* 1994; Moesby *et al.,* 1995; Wilschut *et al.,* 1995; Corver *et al.,* 1995, 1997), SIN (Smit *et al.,* 1999), and vesicular stomatitis virus (Moor *et al.,* 1999), do not need to bind to their receptors either to express membrane fusion activity. It, therefore, appears to be a common theme among low-pH-dependent viruses that receptor binding is not a mechanistic requirement for fusion and that a mildly acidic pH is the sole trigger of the fusion capacity of these viruses.

The present study clearly demonstrates that TBE virus fusion does not exhibit a specific requirement for Chol or sphingolipid (Fig. 5). For SFV and SIN, we and others have previously shown that both Chol and sphingolipids in the target membrane are absolutely required to establish fusion (White and Helenius, 1980; Phalen and Kielian, 1991; Klimjack *et al.,* 1994; Nieva *et al.,* 1994; Moesby *et al.,* 1995; Corver *et al.,* 1995; Smit *et al.,* 1999). Chol appears to be primarily involved in the low-pH-dependent binding of the virus to the liposomes, whereas the sphingolipid acts as a highly specific cofactor catalyzing the fusion event itself (Nieva *et al.,* 1994; Moesby *et al.,* 1995; Wilschut *et al.,* 1995; Corver *et al.,* 1995; Smit *et al.,* 1999). By analogy, it may be speculated that the decrease in TBE virus fusion seen with Chol-free liposomes is due to a lower extent of virus–liposome binding. On the other hand, omission of sphingolipid from the liposomes did not have a significant effect on the fusion process.

It has been demonstrated previously that RSPs derived from TBE virus retain many of the structural and functional properties of the whole virus, including fusion as assessed by cell-cell fusion assays (Schalich *et al.,* 1996). Here, we show that even the detailed characteristics of fusion of the virus and RSPs are very similar. Both are strictly pH dependent. Also, when exposed to low pH in the absence of target liposomes, RSPs exhibit a rapid inactivation of their fusion capacity with kinetics similar to those seen with the whole virus. Furthermore, virus and RSPs exhibit similar fusion behavior with respect to liposomes of different lipid compositions. Finally, the temperature dependence of fusion is the same for whole virus and RSPs. These similarities show that despite the fact that RSPs are smaller than virions and possibly have a slightly different surface lattice organization (Schalich *et al.,* 1996), the potential of the E dimer to undergo the conformational changes necessary of fusion remains unaffected. Therefore, RSPs provide a convenient model system for further study of the fusion properties of the TBE virus envelope glycoprotein E.

The most important outcome of this study relates to the fast kinetics of TBE virus fusion and the remarkable temperature dependence of the process. At 37°C, under the condition of our experiments, within 2–3 s on acidification, more than half of the virus particles have fused at least once, with the initial rate of fusion being close to 40% per s (Figs. 2 and 3). Furthermore, the rate and extent of fusion are only minimally affected by a lowering of the temperature from 37° to 15°C. Because our fusion assay is based on lipid mixing, we cannot formally rule the possibility that at lower temperatures, fusion is reduced with a concomitant increase of hemifusion. However, even if this were the case, the TBE virus fusion reaction differs markedly from SFV fusion or, in particular, influenza virus fusion in model systems, monitored with similar lipid mixing assays. For example, with SFV, typically the initial rate of fusion under comparable conditions is 25% per s at 37°C (Bron *et al.,* 1993; Nieva *et al.,* 1994; Moesby *et al.,* 1995), whereas at 20°C, this value is reduced to 1.5% per s, and at 10°C, it is reduced to 0.5% per s after lag phases between acidification and the onset of fusion of about 3 and 10 s, respectively (Bron *et al.,* 1993). Influenza virus has been shown to fuse even much more slowly with liposomes (Stegmann *et al.,* 1990). For example, in this latter study, an initial rate of fusion at 37°C of about 0.2% per s was observed, whereas at 0°C, this rate was reduced to 0.007% per s after a lag phase of about 8 min. A similar slow rate and long lag phase have been observed for influenza virus fusion with erythrocyte ghosts at 0°C (Stegmann *et al.,* 1990). In contrast, in the case of TBE virus fusion, on lowering of the temperature from 37° to 15°C, there was no detectable lag phase between acidification and the onset of fusion and the initial rate of fusion was decreased less than threefold. Only at temperatures as low as 4°C did we observe a brief lag phase and a further approximate twofold reduction in the rate of fusion. It is generally assumed (Stegmann *et al.,* 1990; Bron *et al.,* 1993; Hernandez *et al.,* 1996) that a lag between acidification and fusion represents the minimal time required for establishment of cooperation between a number of spikes, needed for initiation of fusion pore formation

(Ellens *et al.,* 1990; Danieli *et al.,* 1996). Therefore, the present observation that TBE virus fusion, in contrast to influenza virus or SFV fusion, exhibits a short lag phase only at low temperatures, while furthermore the rate of the fusion process is not drastically reduced, suggests that the TBE spike rearrangements required for fusion pore formation represent a very facile process with a low activation energy compared with that of other enveloped viruses. This low activation barrier could imply that the structural rearrangements in the E protein may also be induced at neutral pH and elevated temperatures. Yet, under physiological conditions, low pH would appear to be essential for triggering the fusion process.

It is likely that the above differences between the fusion kinetics of TBE virus and influenza virus are related to differences in the three-dimensional structures of their respective fusion proteins. While HA is a homotrimer with a perpendicular orientation with respect to the viral surface (Wilson *et al.,* 1981), the TBE virus E protein is a head-to-tail homodimer, lying flat on the viral membrane (Rey *et al.,* 1995). On exposure to low pH, both proteins undergo a series of conformational rearrangements, whereby the TBE E converts to a trimeric configuration (Allison *et al.,* 1995a). It is not clear whether all of these conformational changes are necessary for fusion, but an essential element appears to be the exposure of a fusion peptide sequence. In the HA trimer, the fusion peptides are located at the N-termini of the HA2 subunits, which in the neutral pH conformation of the protein are buried in the stem of the HA trimer close to the viral membrane (Wilson *et al.,* 1981). There is good evidence to indicate that the N-terminal HA2 fusion peptides interact with the target membrane in preparation for fusion (Harter *et al.,* 1989). Based on studies of synthetic peptides corresponding to a loop region in HA2 (Carr and Kim, 1993) and on the three-dimensional structure of a fragment of HA2 at low pH (Bullough *et al.,* 1994), a model has been proposed that involves acid-induced formation of an extended α-helical bundle and displacement of the HA2 fusion peptides from their buried position to the tip of the HA trimer (Hughson, 1997; Skehel and Wiley, 1998; Weissenhorn *et al.,* 1999). This model provides a plausible mechanism by which the fusion peptides may reach the target membrane. However, it is clear that extensive additional conformational changes in the HA molecule are required to juxtapose the fusion peptides and the transmembrane anchor segments of HA2 at one end of the trimer such that direct molecular contact between the interacting membranes is established (Hughson, 1997; Skehel and Wiley, 1998; Weissenhorn *et al.,* 1999).

In the TBE virus E protein, an internal fusion peptide has been proposed to be located in a loop region at the interface between the two E monomers (Rey *et al.,* 1995). Exposure of this sequence would require only dissociation of the homodimer rather than a major change in conformation. Indeed, a soluble dimeric ectodomain of E has recently been found to dissociate at low pH (Stiasny *et al.,* 1996) and to bind to liposomes as a monomer (unpublished observations), indicating that conversion to a trimer is not required for target membrane binding. It remains to be seen, however, whether the dissociation of the homodimer suffices to trigger the fusion event or whether further conformational changes (e.g., trimerization) are required to bring the membranes together. If major conformational changes are not required, the fast rate of TBE virus fusion and the low activation energy of this process, as discussed earlier, may be a consequence of the flat orientation of the E protein, which, by lying parallel to the interacting membranes, would facilitate the establishment of direct molecular contact between them. The E trimer ultimately formed under low-pH conditions may thus represent a final fusion-inactive structure. Indeed, the relatively slow kinetics of fusion inactivation (Fig. 4), compared with the fast kinetics of fusion (Figs. 2 and 3), would be entirely consistent with the notion that inactivation involves additional conformational changes in the E protein, although other explanations for the slow inactivation kinetics cannot be ruled out at this point. Thus at low pH, the TBE virus E protein would appear to be undergoing a series of conformational rearrangements, with fusion perhaps being mediated by an only minimal initial conformational change.

MATERIALS AND METHODS

Lipids

PC from egg yolk, PE prepared by transphosphatidylation of egg PC, and SPM from bovine brain were obtained from Avanti Polar Lipids (Alabaster, AL). Chol was from Sigma Chemical Co. (St. Louis, MO). 1-Pyrenehexadecanoic acid was from Molecular Probes (Eugene, OR).

Virus and RSPs

The TBE virus prototype strain Neudoerfl (Mandl *et al.,* 1988) was grown in primary chicken embryo cells and purified as described previously (Heinz and Kunz, 1981). RSPs were generated by transfection of COS-1 cells with the recombinant plasmid SV-PEwt (Allison *et al.,* 1994), which contains the TBE virus prM and E genes under the control of the SV40 early promoter and has been shown previously to lead to secretion of RSPs when expressed in COS-1 cells (Allison *et al.,* 1995b). Virus and RSPs were purified on sucrose gradients as described previously (Schalich *et al.,* 1996).

Pyrene labeling of virus was carried out essentially as described previously for SFV (Wahlberg *et al.,* 1992; Bron *et al.,* 1993). Briefly, primary chicken embryo cells were grown for 16 h before infection in the presence of 15 μg/ml pyrene-hexadecanoic acid. After infection, the

cells were maintained until harvest in fresh serum-free medium, also containing the fluorescent label. For pyrene-labeled RSPs, COS-1 cells were labeled for 68 h with 10 μg/ml pyrene-hexadecanoic acid. After transfection, cells were grown for 17 h in fresh medium containing the fluorescent label and then for an additional 24 h in serum-free medium without label.

Liposomes

Liposomes (large unilamellar vesicles) were prepared by a freeze-thaw/extrusion procedure, as described previously (Bron *et al.,* 1993; Nieva *et al.,* 1994; Moesby *et al.,* 1995; Corver *et al.,* 1995, 1997). Briefly, lipid mixtures, dried from chloroform, were hydrated in HNE buffer (150 mM NaCl, 50 mM HEPES, and 0.1 mM EDTA, pH 7.4). Subsequently, the suspensions were subjected to five cycles of freeze-thawing (Mayer *et al.,* 1985) and extruded 21 times (Hope *et al.,* 1985) through a Unipore polycarbonate filter (pore size, 0.2 μm; Nuclepore, Pleasanton, CA) in a Liposofast extruder (Avestin, Ottawa, Canada). Liposomes consisted of a mixture of PC, PE, SPM, and Chol in a molar ratio of 1:1:1:1.5, unless indicated otherwise. Liposome concentrations were determined by phosphate analysis (Böttcher *et al.,* 1961).

Fusion assay

Fusion of TBE or RSPs with liposomes was monitored continuously in an AB2 fluorometer (SLM/Aminco, Urbana, IL) as a decrease of the pyrene excimer fluorescence at 480 nm with excitation at 343 nm (Wahlberg *et al.,* 1992; Bron *et al.,* 1993; Stegmann *et al.,* 1993; Nieva *et al.,* 1994; Moesby *et al.,* 1995; Corver *et al.,* 1995, 1997). Briefly, virus or RSPs (final concentration, 1 or 0.5 μM phospholipid, respectively) and liposomes (final concentration, 0.2 mM phospholipid) were mixed in the cuvette of the fluorometer in a final volume of 0.7 ml of HNE buffer (pH 7.4). The liposomes had on average a 10-fold larger membrane surface area per particle than the virus and a 40-fold larger surface area than the RSPs and were present at an approximate 20-fold excess over the virus and a 10-fold excess over the RSPs in terms of particle numbers. With SFV (Bron *et al.,* 1993) and SIN (Smit *et al.,* 1999), such an excess of target liposomes has been found to maximize the opportunity for an acid-activated virion to productively interact with a target liposome and thus to result in optimal fusion. The contents of the cuvette were stirred and kept at a temperature of 37°C, unless indicated otherwise. Fusion was initiated by injection of the volume of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, 0.1 M acetic acid (pH 4.9) required to achieve the desired pH. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence level and 100% fusion corresponded to the fluorescence after the addition of the detergent octa(ethylene glycol) *n*-dodecyl monoether $C_{12}E_8$ (Fluka, Buchs, Switzerland) to a final concentration of 10 mM, resulting in an infinite dilution of the fluorophore. Initial rates of fusion were determined from the tangents to the steepest parts of the fusion curves. There was a certain degree of variability among individual rate measurements carried out under the same conditions within one experiment. We estimate the uncertainty in the fusion rates to be ±5% of the corresponding fusion rate values; at very high rates of fusion (>10% per s), the uncertainty is estimated to be ±10%. The extent of fusion was determined at 1 min after acidification of the virus/RSP-liposome mixture. The uncertainty in the fusion extents is estimated to be ±% of the actual values.

Fusion inactivation assay

Virus or RSPs (1 or 0.5 μM phospholipid) were incubated at pH 5.3–5.5 and 37°C in the fluorometer cuvette for the different periods of time as indicated. Subsequently, liposomes (final concentration of 0.2 mM phospholipid) were added directly to the cuvette from a concentrated suspension (10 mM), and the remaining fusion capacity was measured for 1 min in a total volume of 0.7 ml.

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