Fusion activity of flaviviruses: comparison of mature and immature (prM-containing) tick-borne encephalitis virions

Farshad Guirakhoo,† Franz X. Heinz,* Christian W. Mandl, Heidemarie Holzmann and Christian Kunz

Institute of Virology, University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria

The fusion activity of flaviviruses [tick-borne encephalitis (TBE) virus and Japanese encephalitis virus] was assessed by inducing fusion from without of C6/36 mosquito cells with purified virus preparations. Membrane fusion and polykaryocyte formation was observed only after incubating the viruses at acidic pH. Two groups of monoclonal antibodies reacting with distinct non-overlapping antigenic domains on the TBE virus protein E inhibited fusion from without. One of these domains contains the most highly conserved and putative fusion-active sequence of the flavivirus protein E. Of five TBE virus monoclonal antibody escape mutants, each defined by a single amino acid substitution in the envelope protein E, one revealed a reduced fusion activity and another one a lower pH threshold. TBE virus grown in the presence of ammonium chloride as well as Langat virus purified from the supernatant of infected chick embryo cells contained the precursor of protein M (prM) rather than M itself. These ‘immature’ virions did not cause fusion from without, suggesting that the proteolytic processing of prM may be necessary for the generation of fusion-competent virions.

Introduction

An essential early step in the interaction of enveloped viruses with their host cells is the fusion of the viral membrane with cellular membranes (for review see Marsh & Helenius, 1989). These fusion events are controlled by specific viral envelope proteins, which in many cases require proteolytic cleavage to become fusogenic. Fusion has been shown to take place either at the plasma membrane (paramyxoviruses, certain retroviruses including human immunodeficiency virus and herpesviruses) or in endosomes after uptake by receptor-mediated endocytosis (togaviruses, rhabdoviruses, orthomyxoviruses and coronaviruses). In the latter case the fusogenic potential of the fusion protein depends on a conformational change which is triggered by the acidic pH in endosomes.

There is considerable evidence that flaviviruses are taken up by receptor-mediated endocytosis (Gollins & Porterfield, 1984, 1985; Kimura et al., 1986; Ng & Lau, 1988), although direct fusion of Japanese encephalitis (JE) and dengue 2 viruses with the plasma membrane of mosquito cells has also been described (Hase et al., 1989a, b). Consistent with a penetration mechanism involving acidic vesicles, it was shown that weak bases can inhibit flavivirus replication in certain cells (Brandriss & Schlesinger, 1984; Gollins & Porterfield, 1984; Randolph & Stollar, 1990) and cause an accumulation of virus particles in endosomes (Collins & Porterfield, 1985). The pH-dependent fusion activity of certain flaviviruses was demonstrated with model membranes (Gollins & Porterfield, 1986), by fusion from without (FFWO) (Summers et al., 1989) and by fusion from within (Randolph & Stollar, 1990).

Fully processed mature flaviviruses contain only three structural proteins termed E (envelope), C (capsid) and M (membrane). As revealed by studies using trypsin digestion, monoclonal antibodies (MAbs) (Guirakhoo et al., 1989; Kimura & Ohyama, 1988) and antipeptide sera (Roehrig et al., 1990), the envelope protein E undergoes a conformational change at the acidic pH characteristic of endosomes. Immature virions found in infected cells contain an additional glycosylated protein (prM) which is the precursor of the M protein (reviewed by Rice et al., 1986). The proteolytic processing of prM to yield M apparently represents a late event in the maturation of flaviviruses and is required to obtain fully infectious virions (Shapiro et al., 1972; Wengler & Wengler, 1989). Cross-linking experiments indicate that the cleavage of prM may be associated with a rearrangement of the oligomeric structures on the surface of the virus (Wengler...
& Wengler, 1989). Recent data provide evidence that this proteolytic maturation event takes place in acidic post-Golgi vesicles and can be inhibited by acidotropic amines such as chloroquine, ammonium chloride or methylamine (Randolph & Stollar, 1990).

In contrast to many other viruses (for review see Marsh & Helenius, 1989), information on the location of fusion-active sites in flavivirus proteins is limited. It is also not known whether proteolytic cleavage events in the course of maturation are necessary for achieving fusion competence. To study the fusion activity of tick-borne encephalitis (TBE) virus we have established an assay based on FFWO with C6/36 mosquito cells. The fusion characteristics of a TBE virus wild-type as well as several MAb escape mutants were determined and specific epitopes involved in fusion inhibition were identified. Experiments with prM-containing 'immature' virions grown in the presence of ammonium chloride provide evidence that the proteolytic cleavage of prM may be necessary for fusion activity.

Methods

Cells and media. BHK-21 and Vero cells were grown in MEM containing Earle's salts, sodium bicarbonate, 5% foetal calf serum (FCS), 2 mm-L-glutamine and 100 µg/ml neomycin. Aedes albopictus cells (clone C6/36) (obtained from Dr E. Gould, NERC Institute of Virology, Oxford, U.K.) were grown in MEM/Earle's, buffered with 20 mM-HEPES at pH 7.6, supplemented with 10% FCS, 10% tryptose phosphate broth, 2 mm-L-glutamine, and 100 µg/ml neomycin. BHK-21 and Vero cells were grown at 37 °C, C6/36 cells at 28 °C.

Viruses. TBE virus (European subtype, strain Neudoerfl), MAb escape mutants of strain Neudoerfl (termed VA3a, VA3b, VB1, VB4 and V2 according to the selecting MAb) (Holzmann et al., 1989), Langat and JE viruses were grown in primary chick embryo cells. PrM-containing TBE virus was produced by adding 20 mM-ammonium chloride to TBE virus-infected chick embryo cells at 24 h p.i. The virus in the cell supernatants was harvested at 42 h p.i., concentrated by ultracentrifugation, purified by two steps of sucrose density gradient centrifugation, and dialysed against TAN buffer (0.05 M-triethanolamine, 0.1 M-NaCl) as described previously (Heinz & Kunz, 1981). Since TAN buffer appeared to be toxic to the cells, the viruses were centrifuged down at 38000 g and resuspended in MEM/Earle's, buffered with 20 mM-HEPES at pH 7.6 containing 0.1% bovine serum albumin (BSA) to a final concentration of 400 µg/ml.

Antibodies. A panel of 19 MAbs defining three domains (A, B and C) and three isolated epitopes (i1, i2 and i3) on the protein E of TBE virus was used. The serological specificities and functional activities of these MAbs as well as the structural properties and topological relationships of the corresponding epitopes have been described (Guirakhoo et al., 1989). The locations of the epitopes in a structural model of protein E have been assigned (Mandl et al., 1989). A MAb directed to the human transferrin receptor (kindly provided by Dr O. Majdic, Institute of Immunology, University of Vienna, Austria) was used as a negative control.

All MAbs were purified from mouse ascites using either Protein A affinity chromatography (MAPS II kit, Bio-Rad) or ammonium sulphate precipitation as described previously (Guirakhoo et al., 1989).

Concentrations of specific antibody in these preparations were determined by densitometric evaluation of the paraprotein separated on an SPE-agarose gel (Beckman).

Fusion from without. The fusion assay described by Mann et al. (1983) was modified as follows: BHK-21, Vero and A. albopictus (C6/36) cell monolayers were grown in 96-well microtitreplates (Costar). The cells were precooled for 45 min at 4 °C and washed with serum-free medium. Then 0.03 ml purified virus at a concentration of 100 to 400 µg/ml was added to the cells and incubated at 4 °C for 1 h. After removal of the virus suspension, prewarmed fusion medium (40 °C) was added. The fusion medium consisted of MEM/Earle's without sodium bicarbonate, buffered with either 20 mM-HEPES at pH 6.8 and above or 20 mM-MES at pH 6.6 or below. The cells were incubated for 2 min with the fusion medium which was then replaced by the normal C6/36 cell growth medium (pH 7.6) described above. The cells were further incubated for 2 h at 40 °C, washed with PBS (pH 7.4), fixed with absolute methanol, stained with Giemsa, and examined for the appearance of multinucleated cells. The number of nuclei and the number of cells in five microscopic visual fields (magnification 100-fold) were counted either after staining or in the phase-contrast microscope, and the fusion index [1 – (number of cells/number of nuclei)] was calculated.

Fusion inhibition. MAbs were diluted in MEM/Earle's, 20 mM-HEPES, 0.1% BSA, pH 7.6 to yield final concentrations of 10, 50 and 100 µg/ml. In some of the assays 30 µl of each dilution was incubated with 30 µl of TBE virus (400 µg/ml) for 30 min at room temperature. The mixture was then added to precooled C6/36 cell monolayers and fusion assays were performed as described above. Alternatively, 30 µl of TBE virus (400 µg/ml) was allowed to adsorb to the cells at 4 °C for 1 h. After removal of the unbound virus, 30 µl of each MAb dilution was added and incubated for 30 min at room temperature. After removal of the MAb solution the cells were processed for fusion as described above.

Results

Establishment of the fusion assay

TBE and JE viruses were tested for the induction of FFWO with BHK, Vero and C6/36 mosquito cells at pH 7-6 and 5-5 as described in Methods. At pH 7-6 no fusion activity was detectable in any of the assays. The results obtained at pH 5-5 are presented in Fig. 1. Fusion with TBE and JE viruses was only observed with C6/36 cells, which formed multinucleated giant cells (Fig. 1 f, i). Incubation at 40 °C was required for optimal fusion activity. The efficiency of polykaryocyte formation was greater at 37 °C and did not occur at or below room temperature. Therefore for further experiments C6/36 cells and an incubation temperature of 40 °C were used.

Dependence of FFWO on pH

To determine the pH threshold of fusion activity, FFWO assays were performed at different pHs from 7-0 to 5-2 using predetermined optimal virus concentrations.
Fig. 1. Micrographs of Giemsa-stained BHK-21, Vero and C6/36 cells after FFWO by TBE and JE viruses. Fusion assays were performed at pH 5.5 as described in Methods. CTL, Control cells subjected to the assay procedure in the absence of virus.

Fig. 2. Dependence of FFWO on pH: (●) TBE virus, (△) JE virus. FFWO was performed at different pH values with constant virus concentrations (TBE virus, 300 μg/ml; JE, 100 μg/ml) as described in Methods.

(300 μg/ml for TBE virus; 100 μg/ml for JE). As seen in Fig. 2 the fusion activity of TBE and JE viruses started to become activated at pH 6.4, and 50% of maximal fusion activity was reached at pH 6.2 and 6.3, respectively. In some experiments with JE virus, peaks of fusion were observed at pH 6.0 and pH 5.2 with decreased fusion at intermediate pH values.

Inhibition of TBE virus-induced fusion with MAbs

In a structural model of the TBE virus protein E, 19 different MAb-defined epitopes have been assigned to distinct sequence elements and protein domains [Mandl et al. (1989); see also the Methods section]. To obtain some information on those structures which are possibly involved in fusion activity, we performed fusion inhibition assays with the panel of 19 MAbs. Six of them revealed fusion inhibition activity when added to the virus both before and after adsorption to the cells (see Methods). When mixed with the virus suspension before adsorption, however, only antibodies A3, A4 and i2 were capable of completely inhibiting fusion activity, whereas only partial inhibition was observed with the antibodies B1, B4 and B5 (Fig. 3).
Fusion inhibition by MAbs directed against the E protein of TBE virus. (▲) A3, (●) A4, (□) B1, (▲) B4, (▲) B5, (●) i2, (●) negative control (MAb against transferrin receptor).

Fig. 4. Fusion activity of TBE virus MAb escape mutants. FFWO assays were performed at different pH values as described in Methods using a virus concentration of 400 µg/ml. (●) TBE virus, (▲) VA3a, (□) VA3b, (●) VB1, (●) VB4, (▲) Vi2.

Fusion phenotype of MAB-escape mutants
We have previously characterized MAb escape mutants of TBE virus (Holzmann et al., 1989), each being defined by a single amino acid substitution which maps to different structural domains of protein E (Mandl et al., 1989). To assess the possible consequences of these mutations on the fusion characteristics we have determined the pH optimum for FFWO of five of these variants (VA3a, VA3b, VB1, VB4 and Vi2) as compared to the wild-type strain Neudoerfl (for terminology see Methods). The results obtained are presented in Fig. 4. The mutants VA3a, VA3b, VB1 and VB4 had a pH optimum similar to that of the parent strain Neudoerfl, whereas VB4 and Vi2 required a significantly lower pH for maximal fusion activity. In addition, the fusion activity of VB4 appeared generally to be reduced.

Analysis of immature virions containing prM
Recently it has been shown that the proteolytic processing of prM to yield M probably takes place in acidic post-Golgi vesicles (Randolph et al., 1990) and can be blocked by the addition of acidotropic agents. To generate prM-containing virions we infected chick embryo cells and harvested the virus from the supernatant after addition of ammonium chloride late in the infection cycle (see...
Methods). The virions thus obtained lacked the M protein found in mature virions (Fig. 5a, lane 1) but contained an additional protein with an estimated $M$, of 28K to 30K (prM, lane 2). We had noticed previously that a similar protein pattern is also characteristic of Langat virus when grown in chick embryo cells in the absence of ammonium chloride (lane 3). These virus preparations were subjected to FFWO assays. As seen in Fig. 5b no fusion activity was observed with the prM-containing viruses under the conditions of the assay.

Discussion

By the use of C6/36 mosquito cells we have shown that purified preparations of TBE and JE viruses can cause FFWO, provided that the virions are exposed to acidic pH. This is consistent with the uptake of flaviviruses by receptor-mediated endocytosis followed by fusion of the viral membrane with the endosomal membrane (Gollins & Porterfield, 1984, 1985; Kimura et al., 1986; Ng & Lau, 1988). FFWO could not be demonstrated with Vero and BHK-21 cells. The reason for this restriction is unclear, but since mosquito cells are normally grown at 28 °C they may have a higher membrane fluidity at the assay temperature of 40 °C.

The dependence of the fusion activity on acidic pH suggests that conformational changes are required for its activation. Such changes have indeed been demonstrated in flavivirus E proteins by studies using trypsin digestion (Guirakhoo et al., 1989; Kimura & Ohyama, 1988), MAbs (Guirakhoo et al., 1989) and antipeptide sera (Roehrig et al., 1990). As revealed in a structural model of the TBE virus protein E (Mandl et al., 1989), these changes affect certain sites at or near antigenic and structural domain A. This domain also contains the most highly conserved sequence among all flavivirus E proteins, including a stretch of 12 strictly conserved amino acids (position 98 to 111). This moderately hydrophobic sequence contains the tetrapeptide G-L-F-G which is also found at the fusion-active amino terminus of the influenza virus HA2 (Wharton, 1987). A similar sequence is also found in the fusion proteins of paramyxovirus and retroviruses (Gallaher, 1987). One might therefore speculate that this sequence contributes to the fusion activity of flaviviruses after acid pH-induced conformational changes (Roehrig et al., 1989).

Similar to the situation described for the influenza virus haemagglutinin (Wharton, 1987), MAbs directed to different and non-overlapping sites in the protein E of TBE virus were capable of fusion inhibition. MAbs A3, A4 and i2 react with epitopes shown to undergo acid pH-induced conformational changes (Guirakhoo et al., 1989) and probably interfere directly with processes necessary for membrane fusion. MAbs B1, B4 and B5 recognize a different non-overlapping structural element [see structural model of protein E in Mandl et al. (1989)] and cause only partial inhibition of fusion, suggesting that they act by a different mechanism. One of the MAbs escape mutants (Vi2; Holzmann et al., 1989; Mandl et al., 1989), which is characterized by a Lys→Glu substitution at position 171 in the E protein, exhibited a significantly lower pH threshold for the induction of its fusion activity. In the case of the influenza haemagglutinin, mutations leading to a change of the pH optimum for fusion map either to the N terminus of HA2 or to the HA–trimer interphase (Wiley & Skehel, 1987). All the exchanges in the latter case result in charge changes which influence the stability of the trimer. Considering that charged residues are unlikely to be directly involved in membrane fusion, the Lys→Glu substitution in Vi2 may lead to a strengthening of the oligomeric E protein structure and to a lower pH requirement for the structural rearrangements necessary for membrane fusion. Further investigations including X-ray structure analysis are necessary to identify the structural basis of the flavivirus fusion activity.

The assembly of flaviviruses involves the intracellular generation of 'immature' virions which contain prM rather than M (Shapiro et al., 1972; Rice et al., 1986). The proteolytic cleavage of prM as a last step in virus maturation seems to be necessary to yield fully infectious virus (Wengler & Wengler, 1989). Recent data obtained with dengue, JE and Powassan viruses provide evidence that the processing of prM takes place in acidic vesicles of the post-Golgi network, as it can be inhibited by acidotropic amines (Randolph et al., 1990). We used ammonium chloride to generate and isolate prM-containing TBE virus from the supernatant of infected chick embryo cells. These particles did not cause FFWO under the assay conditions used. The acquisition of fusion activity may therefore also depend on the proteolytic cleavage of the prM protein. The latter occurs after a pair of basic amino acids (Rice et al., 1986) and probably is performed by a trypsin-like protease with the physiological role of processing prohormones during exocytosis (Orci et al., 1987). By recognizing the consensus sequence Arg/Lys-X-Arg/Lys the same enzyme appears to be responsible for the cleavage and activation of the fusogenic potential of a number of viral envelope glycoproteins (Strauss et al., 1987). The presence of prM in Langat virus grown in chick embryo cells, as shown in the present work, as well as the demonstration of prM in purified preparations of other flaviviruses (Henchal et al., 1985; Kimura-Kuroda & Yasui, 1983; Wengler et al., 1987) suggest that cleavage at the consensus sequence may depend on both host and viral factors.
In the case of myxo-, paramyxovirus and retroviruses, a highly conserved hydrophobic sequence is liberated at or near the amino terminus of the membrane-associated part of the cleaved proteins (Bosch et al., 1989; Gallaher, 1987; Morrison, 1988; Wharton, 1987). Depending on the virus, this structural element is thought to trigger fusion activity either at physiological pH or after acid pH-induced conformational changes of the protein (Marsh & Helenius, 1989). Flaviviruses expose only about 40 amino acids of the M protein outside the viral membrane after cleavage of prM. Sequence comparisons with the known fusion-active sequences cited above did not reveal a significant degree of homology with the TBE virus M protein. Since there is also no significant conservation of these sequences among flaviviruses it is likely that the E protein rather than the M protein is responsible for fusion activity.

The situation we encounter with flaviviruses is reminiscent of alphaviruses, where the proteolytic processing of the E2 precursor protein p62 has been suggested to be necessary for another protein (E1) to become fusion-active (Lobigs & Garoff, 1990). The presence of p62 seems to prevent the envelope glycoprotein complex from fusing cellular membranes during its transport through the acidic compartments of the endocytic pathway (Lobigs & Garoff, 1990). Further detailed studies will be necessary to elucidate the functional role of the flavivirus prM protein during transport and virus maturation.

References


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