Association between the pH-dependent Conformational Change of West Nile Flavivirus E Protein and Virus-mediated Membrane Fusion

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SUMMARY

The major envelope protein (E) of West Nile virus mediates fusion between the membranes of the viral envelope and the target cell at optimum pH values of just below neutrality. The fusion is critical for the entry mechanism, allowing virus to escape from the acidic endosomal compartment. To define the role of the viral E protein in the fusion reaction, the conformational change in E and concomitant change of viral infectivity were studied quantitatively, using protease digestion of the E protein and assay of viral infectivity. The results showed that the conformational change occurred in a pH-dependent manner with an upper threshold of pH 7.0 and maximum conversion occurring at pH 6.4 and below. The conversion was rapid and reached a half-maximal value within 15 s after acidification. The exposure of free or cell-bound virions to acid pH resulted in the loss of infectivity in an almost identical pH-dependent manner. Based on these findings, it is suggested that there are two distinct viral modes of entry into macrophages, i.e. infectious endocytosis and non-infectious viral fusion with plasma membranes, with the pH of the extracellular medium determining which of these predominates. The implications of these observations for the role of the E protein in membrane fusion and the probable localization of fusion epitopes are discussed.

INTRODUCTION

West Nile virus (WNV), an animal virus of the genus flavivirus, family Flaviviridae, has a simple lipid–protein membrane enveloping an isometric nucleocapsid that contains the viral RNA (Westaway et al., 1985, Heinz et al., 1986). It contains four types of polypeptides: a core protein (C), a membrane-associated protein (M) and its precursor (preM), and a major membrane-associated protein (E) (Wengler et al., 1985). The E protein (M, 52263 as determined by Wengler et al., 1985) is composed of the segments R1, L1, R2, L2 and R3, followed by a membrane anchor region at the carboxy-terminal region of the molecule (Nowak & Wengler, 1987).

Biochemical and electron microscopic data have shown that, when WNV attaches to cells of the mouse macrophage-like cell line P388D1 at 0 °C at pH 7-6 and the mixture is warmed to 37 °C at pH 6-4 or below, viral and cell membranes fuse (Kimura et al., 1986, 1987), and this plays a central role in the entry of WNV into macrophages (Gollins & Porterfield, 1986b). However, the molecular mechanisms underlying fusion are obscure and there is a need for a suitable model to study this process.

The present experiments explored two aspects of the response of the E polypeptide (Wengler et al., 1985) to decreasing pH, namely the conformational change induced in the polypeptide and the nature of its subsequent interaction with host cell membranes. A series of experiments were conducted, at the molecular level, to determine the pH dependence of the conformational change of WNV E protein and to study any change in viral infectivity. The probable localization within the segments of E molecule of the epitopes which are responsible for the fusion reaction was also determined.

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METHODS

Cells. Vero and P388D1 cells were maintained as described previously (Kimura et al., 1986).

Virus. WNV (Egypt 101 strain) was prepared as described in Kimura et al. (1986), with the exception that Jcl:ICR mice, 4 days old (Clea Japan, Tokyo, Japan) were used following intracerebral inoculation. This preparation contained $3 \times 10^{10}$ p.f.u. per ml (assayed in P388D1 cells).

$[^{35}S]$Methionine-labelled virus. $[^{35}S]$Methionine-labelled WNV ($[^{35}S]$-WNV) was prepared and purified as described previously (Kimura et al., 1986). The stock of virus contained $5.2 \times 10^6$ c.p.m./ml and $4.7 \times 10^7$ p.f.u./ml (assayed in P388D1 cells).

Gel electrophoresis. Samples for gel electrophoresis were concentrated by precipitation in trichloroacetic acid (10% final concentration), washed twice in ice-cold acetone and analysed on modified Laemmli gels (Blobel & Dobberstein, 1979) with an acrylamide gradient from 7.5 to 15%. Fluorography was performed essentially by the method of Chamberlain (1979). The radioactivity of each $[^{35}S]$-labelled gel band was directly measured as described by Miwa et al. (1986) using an Argus 100 autoradiographic ultrasensitive system (Hamamatsu Photonics, Hamamatsu, Japan). The background was always less than 100 c.p.m., and, after this had been subtracted, the ratio between E and its major digestion product E1* was calculated.

Time course of digestion of WNV by TPCK-treated trypsin. Aliquots (30 µl) of WNV, containing $5.4 \times 10^6$ c.p.m. $[^{35}S]$-WNV and 73 ng viral protein, were dissolved in polyoxyethylene (10) octylphenyl ether (POE, 1% final concentration) (Wako, Osaka, Japan). The mixture was left on ice in a cold-room for 1 min. TPCK-treated trypsin (TPCK-trypsin, Sigma) was added to a final concentration of 10 µg/ml and the mixture incubated for various times in an ice-cold water-bath with constant rocking. The digestion was stopped by adding a 10-fold excess of soybean trypsin inhibitor (STI), and aprotinin to a final concentration of 2% (v/v) (all from Sigma), and incubated for 10 min at 0 °C to inactivate protease. After 10 min on ice, the samples were acid-precipitated and processed for gel electrophoresis as above.

Protease assays of the acid-induced conformational change. Aliquots (30 µl) of virus, as above, were adjusted to the required pH with 0.14 M-NaCl containing 50 mM-HEPES and 50 mM-MES (all from Nakarai Chemicals, Kyoto, Japan), incubated for 10 min at 37 °C with constant rocking, brought to pH 7.4 by the addition of 0.05 M-NaOH, and kept on ice until protease digestion. TPCK-trypsin (10 µg/ml final concentration) was then added with 1% POE present, as indicated. After incubation for 16 min at 0 °C, the digestion was stopped, as described above. The samples were acid-precipitated and processed for gel electrophoresis as described above.

In the kinetic analysis of the conversion of $[^{35}S]$-labelled E protein to the protease-resistant form, the samples were incubated for various times at 37 °C and digested as described above.

Assay for the effect of pH on infectivity of WNV prebound on P388D1 cells. WNV was bound to P388D1 cell monolayers in 24-well plates for 2 h at 0 °C, in 0.2 ml of binding medium, pH 7.4 (Kimura et al., 1986). Cells were then washed twice with binding medium at 0 °C, 2 ml of pre-warmed (37 °C) assay medium (Kimura et al., 1986) at various pH values was added to monolayers to allow synchronized infection, and plates were floated in a 37 °C water-bath for 90 s. This medium was then replaced with the relevant growth medium (37 °C, pH 7.4), and cells were incubated for a further 4 h at 37 °C. At the end of this period, cells were washed three times with binding medium, pH 7.4, and incubated for a further 3 days with growth medium containing 1.5% CM-cellulose. After staining with naphtalene black the number of viral plaques was counted.

Assay for the effect on binding of WNV particles, pretreated at various pH values, to P388D1 cells. The binding assay used was a modified version of the method described earlier (Kimura et al., 1986). $[^{35}S]$-WNV was pretreated for 10 min at 37 °C, either at pH 6.0 or pH 7.4, and was brought, where required, to pH 7.4. It was then incubated with P388D1 cells on ice in a cold-room. At various times after the start of adsorption, the cells were washed three times with binding medium, pH 7.4. The final cell pellet was resuspended in phosphate-buffered saline and added to scintillation vials containing 10% SDS (Gollins & Porterfield, 1984). The cell-bound viral radioactivity was measured by a Packard Tri-Carb liquid scintillation spectrometer. Three 10 min counts were taken and the average c.p.m. was calculated. The background was about 35 c.p.m. and was subtracted to give the bound radioactivity. All determinations of each value and virus-free backgrounds were carried out in duplicate and the mean values were used for calculations.

RESULTS

Time course of protease digestion of WNV proteins

When WNV was solubilized with POE and treated with TPCK-trypsin at 0 °C, there was partial proteolysis of all structural proteins. The fluorograph in Fig. 1 shows that the E polypeptide (M, 52000 by our estimates) was cleaved to give several distinct bands. These were shown to have an apparent Mr of 41500 (E1*) and 28000 (E3*). Other products of M, 33000 (E2*) and M, 15500 (E4*) are not clearly seen in Fig. 1 but are present in Fig. 2. As E was digested, E1* appeared and increased throughout the incubation period and E3* appeared after
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Fig. 1. Time course of digestion of WNV by TPCK-trypsin. 

$^{35}$S-WNV was digested with 10 µg/ml trypsin in 1% POE at 0 °C. After 1, 2, 4, 8, 16 and 32 min (lanes 2 to 7, respectively), protease was inactivated with STI and aprotinin, and the digestion products were resolved by SDS-PAGE under reducing conditions. For the time 0 sample (lane 1), trypsin was pretreated with inhibitors. The proteolytic digestion products of E are termed $E1^*$ ($M_r$ 41500) and $E3^*$ ($M_r$ 28000).

Fig. 2. Trypsin sensitivity of viral proteins after treatment at pH 5.5, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0 and 7.4 (lanes 2 to 9 respectively). Aliquots of $^{35}$S-WNV in HEPES-MES-saline buffer were incubated for 10 min at 37 °C, brought to pH 7.4, and then digested with 10 µg/ml trypsin in POE for 16 min at 0 °C. The reaction was terminated by adding STI and aprotinin and the samples were processed for gel electrophoresis. The control lane (lane 1) shows virus reacted with pre-mixed trypsin and inhibitors. The proteolytic digestion products of E protein are termed as described in the legend for Fig. 1 with the addition of $E2^*$ ($M_r$ 33000) and $E4^*$ ($M_r$ 15500).
1 min of incubation and was resistant to further digestion. The preM glycoprotein and C had disappeared by 1 min of digestion. However, the time course of digestion of M was not clear since it overlapped with digestion products of other structural proteins with an identical molecular weight (Wengler et al., 1987; T. Kimura & A. Ohyama, unpublished data). After 1 min from the start of protease digestion at 0 °C, the ratio of E to E1* was 2.8; this decreased to 0.39 after 16 min and to 0.18 after 32 min. Using these results, an incubation period of 16 min was chosen for the experiments described below.

Trypsin sensitivity of viral proteins after treatment at various pH values

35S-WNV particles were treated with buffers of various pH values, brought to pH 7-4, digested with TPCK-trypsin and subjected to SDS–PAGE. When the digestion was performed at 0 °C in the presence of POE, it was found that acidic pretreatment had caused E to become trypsin-resistant in a pH-dependent manner. However, the same treatment did not affect the susceptibility of other viral proteins to trypsic digestion (Fig. 2). Direct quantification of both E and E1* bands after SDS–PAGE showed that the conversion into the trypsin-resistant form began at pH 7.0, reached a half-maximal value at a pH between 7.0 and 6.8, and was essentially complete at pH 6.4 (Fig. 3). There was a 4-6-fold difference in the ratio of E to E1* at pH values between 7.4 and 6.0. Using the results in Fig. 2 and 3, pH values 7.4 and 6.0 were chosen for the experiments described below.

In each case the conversion appeared to be irreversible, based on the fact that incubation at pH 7.4 at 37 °C for a further 10 min after the initial low pH treatment did not affect the fraction of trypsin-resistant E (data not shown).

Effect of pH on infectivity of WNV prebound to P388D1 cells

These experiments were performed to examine the contribution of acid-conversion to subsequent viral interaction with host cell membranes. WNV particles were allowed to bind to P388D1 cells in a cold-room on ice and were subsequently warmed to 37 °C at various pH values. The infectivity of prebound virus was then assayed by plaque titration at each pH value. Fig. 3 shows that prebound virus treated for 90 s started to lose infectivity at pH 7.0 and below, 50% being lost at a pH between 7.0 and 6.8. Less than 10% of the original infectivity remained at pH 6.6 and below. The pH dependence of the loss of infectivity was almost identical with that of the acid-induced change in trypsin sensitivity of the E molecule shown in Fig. 3.

WNV particles, initially at pH 8.0 and 0 °C, were brought to different pH values for 10 min at 37 °C, then the pH was brought back to 7.4. The infectivity of such viral suspensions was assayed in P388D1 cells. The pH-dependent loss of viral infectivity was also observed and its pH dependence was similar to that observed in the case of prebound virions (data not shown).

Effect on WNV binding to P388D1 cells of pretreatment at various pH values

WNV was pretreated either at pH 6.0 or 7.4 for 10 min at 37 °C and was brought to pH 7.4 at 0 °C. Virions were then allowed to bind to P388D1 cells on ice in a cold-room. When the time course of viral adsorption was assayed, it was demonstrated that low pH pretreatment had made WNV less efficient in binding to cells than pretreatment at pH 7.4 (Fig. 4). At 2 h, the cell-bound radioactivity was 525 c.p.m. at pH 6.0 and 1260 c.p.m. at pH 7.4.

Characterization of acid-induced change in trypsin sensitivity of E protein

The kinetics of conversion of E to the trypsin-insensitive form were determined at pH 6.0. Fig. 5 shows that the ratio of E to E1* was 0.79 at 15 s, 0.88 at 1 min and 1.1 at 10 min and remained at approximately the same level for 1 h (data not shown). The ratio of E to E1* at pH 7.4 was about 0.35 at each of the indicated time points. Thus 80% of the total conversion occurred within the first minute. The rate of acid-induced conversion of E protein was similar to that previously observed for the kinetics of fusion between WNV and P388D1 cells (Kimura et al., 1986).

Further information on the change in trypsin sensitivity of the E polypeptide was obtained by analysis in a 7.5% gel. After incubation at pH 6.0 and digestion at pH 7.4, E1* consisted of two distinct bands (preE1* and E1*). However, the aliquot removed at 16 min demonstrated that no
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Fig. 3. Quantification of acid-induced change in trypsin sensitivity of E protein and concomitant loss of viral infectivity. Quantification of the E and E1* proteins in Fig. 2 is described in Methods and the ratio of E to E1* is plotted (•). Bars indicate the standard deviation from the mean. The effect of pH on infectivity of cell surface-bound WNV was assayed as follows. WNV particles prebound to P388D1 cells at 0 °C at pH 7-4 were warmed at 37 °C at the indicated pH for 90 s and then incubated for 3 days at 37 °C at pH 7-4. At the end of this period, the numbers of viral plaques were counted. Plaques per well formed by virus pretreated at each pH (O) are expressed as a percentage of plaques formed by virus pretreated at pH 7-4 (100%). The inoculum was serially diluted so that 50 to 100 plaques were counted.

Fig. 4. Effect of acidic pretreatment on binding of WNV at 0 °C. A solution containing 35S-WNV was incubated at 37 °C for 10 min either at pH 6-0 (O) or pH 7-4 (●), brought to pH 7-4 and then immediately incubated with P388D1 cells at 0 °C at pH 7-4. At various times after the start of incubation, 3 x 10^6 cells were removed from the mixture, washed three times and the cell-bound viral radioactivity was determined as described in Methods. The initial radioactivity added per 3 x 10^6 cells was 2 x 10^4 c.p.m.

Fig. 5. Kinetics of the conversion of E to the trypsin-resistant form. A solution containing 35S-WNV was exposed to pH 6-0 at 37 °C and at various time points aliquots were removed, brought back to pH 7-4 and digested with trypsin. The samples were analysed by SDS-PAGE. The ratio of E to E1* at the indicated time points was determined as described in Methods. The control experiment at pH 7-4 showed that the ratio was always about 0-35 at all the indicated time points. Bars indicate the standard deviation from the mean.

preE1* was present when E was pretreated at pH 7-4, as shown in Fig. 6. The apparent M, values were 43300 (preE1*) and 41500 (E1*), the latter being consistent with that shown in Fig. 2.

In Fig. 7 proteolytic cleavage was carried out in the presence of dithiothreitol (DTT, 10 mM final concentration), which reduces intramolecular disulphide bonds in the R1 and R2, but not R3 regions (Nowak & Wengler, 1987). The DTT treatment made E more trypsin-susceptible at pH 7-4. The ratio of E to E1* was 0-34 in the absence of DTT and 0-21 in its presence. However, the addition of 10 mM-DTT made the acid-induced conformational change in E more resistant to TPCK-trypsin cleavage. The ratio of E to E1* at pH 6-0 was 1-14 in the absence of DTT but 2-34 in its presence.

**DISCUSSION**

The results presented in this paper indicate that the E protein of WNV undergoes conformational change at pH 7-0 or below. Irreversible alterations were detected by biochemical means. The changes in E made it trypsin-resistant in a pH-dependent manner, with a threshold of pH 7-0 and maximum conversion occurring at pH 6-4 and below (Fig. 2 and 3). The alteration was rapid, with 50% of total conversion occurring within 15 s of acidification (Fig. 5). It is thus suggested that acidic pH pretreatment alters the exposure of sites for TPCK-trypsin cleavage in
E and that two critical cleavage sites, needed for generating preE1* and E1* (Fig. 6), are masked by an acid-induced conformational change in the E molecule (see below).

It has been shown recently that WNV can fuse efficiently with target membranes with an optimum pH of 6.4 and below (Kimura et al., 1986, 1987; Gollins & Porterfield, 1986a). The pH-dependent conformational change reported here occurs over the same pH range as that previously observed for efficient fusion of virus with the cell membrane (Kimura et al., 1986, 1987) and subsequent loss of infectivity (Gollins & Porterfield, 1986b; Fig. 3), and thus probably reflects the alteration in the protein that makes it active in fusion in vitro. Hence, it seems likely that when virus particles encounter pH values below neutrality, either in the pre-lysosomal endosomal compartments or on the plasma membranes, a conformational transition takes place in the E molecule that enables the virus particles to fuse with target membranes. However, if this acid-conversion takes place in the absence of cells, virus inactivation results in a pH-dependent manner similar to that which occurs in the presence of cells, and this is at least partially due to the appearance of virions that are inefficient at binding to P388D1 cells (Fig. 4).

Gollins & Porterfield (1986b) have suggested that the fusion reaction results in the uncoating of virus particles, although the location of this uncoating process (pre-lysosomal endosome or plasma membrane) determines whether the uncoated RNA will be infectious. In agreement with this, the results in Fig. 3 suggest that there are two distinct modes of entry of WNV, i.e. infectious adsorptive endocytosis (Marsh & Helenius, 1980) and non-infectious viral fusion to plasma membranes, with the pH of the extracellular medium determining which one predominates. In previous studies (Gollins & Porterfield, 1985; Kimura et al., 1986), adsorptive endocytosis was the only mode of entry which was observed at pH values of 7.4 and 8.0. However, as shown in Fig. 3, at pH 7.0 and below the proportion of endocytosis decreases and viral fusion is employed as the main mode of entry. This agrees with results obtained previously (Kimura et al., 1986).

Trypsin catalyses the hydrolysis of the peptide bond between the carboxyl group of arginine or lysine and the amino group of the adjacent amino acid (Smyth, 1967). In the light of this, and given the structure and amino acid sequence of the WNV E polypeptide (Nowak & Wengler, 1987), the Mr of preE1* (M, 43300) and E1* (M, 41500) indicated that the probable cleavage sites of E were either positions 84 lysine and 99 arginine in the R1 region or positions 402 lysine and 384 arginine in R3, or both.

To determine the sites in E cleaved by TPCK-trypsin, the electrophoretic mobility of E1*, after incubation of virus at pH 6.0 or 7.4, was analysed by comparing reduced and non-reduced samples. The fluorograph (not shown) showed that E1* did not comigrate with undigested E and that there was no substantial decrease in the electrophoretic mobility of non-reduced E1*. If
TPCK-trypsin cleaves the E molecule at positions 84 and 99 in R1, the disulphide linkages which connect cysteine residues at positions 74 and 105, and positions 92 and 116, would cause the amino-terminal fragment of E to remain linked with E1*, thereby decreasing the electrophoretic mobility of E1*. Thus, the result obtained suggests that the tryptic cleavage sites of the E molecule could be positions 402 (lysine) and 384 (arginine) in the R3 region. Similarly, Wengler et al. (1987) have shown that the E molecule is cleaved by trypsin to a fragment (p42) at the segment located in the R3 region, although digestion was carried out with 100 μg/ml of protease at pH 8.0 and 37°C.

The E glycoprotein of tick-borne encephalitis virus contains three distinct antigenic domains termed A, B and C which seem to be almost equivalent to R1, R2 and R3 in WNV (Heinz, 1986). Heinz et al. (1983) and F. Guirakhoo et al. (personal communication) have shown that domain A is highly conformation-dependent and that low pH abolishes antigenic reactivity. Conversely, domain C consists of epitopes which show no difference in antigenic reactivity after pretreatment at various pH values. Similar pretreatment of WNV at various pH values which resulted in the conformational change in E did not affect the association and dissociation constants of a neutralizing monoclonal antibody (F6/16A) directed against the viral E protein (Kimura et al., 1986).

The relevance of pH-dependent irreversible conformational change to the mechanism of virus-mediated membrane fusion is not known (Skehel et al., 1982). It may be that there is a substantial increase in the helical content of the protein resulting in the subsequent exposure of a hydrophobic face (Subbarao et al., 1987) which is assumed to play an important role in the initiation of the fusion reaction (White et al., 1983).

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