Separation of Functional West Nile Virus Replication Complexes from Intracellular Membrane Fragments

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SUMMARY

Flaviviruses encode seven non-structural proteins for which functions have not yet been described. The identification of the viral and possible host proteins which may be involved in flavivirus replication has been impeded by the fact that the viral replication complexes are tightly associated with endoplasmic reticular membranes within infected cells and that in vitro polymerase activity is associated with large membrane fragments. To facilitate further study of flavivirus replication complexes, selected ultrapure detergents were analysed for their effect on West Nile virus (WNV) in vitro RNA-dependent RNA polymerase activity and for their ability to release functional replication complexes from partially purified intracellular BHK-21 membrane fragments. A few previous reports indicated that flavivirus in vitro polymerase activity was sensitive to detergent treatment. The present study indicates that WNV polymerase activity is variably inhibited depending on the concentration and identity of the detergent used. Of the five detergents (Tween 20, maltoside, octylglucoside, lubrol PX and sodium deoxycholate) tested, sodium deoxycholate was the most efficient at releasing functional viral replication complexes from intracellular membranes.

Flaviviruses are small, enveloped, positive-strand RNA viruses (Westaway et al., 1985). The RNA genome possesses a single open reading frame and serves as the only messenger RNA for the synthesis of viral proteins. The three structural proteins (C, M, E) are encoded in the 5' portion of the genome, while the seven non-structural proteins are encoded in the 3' portion (Rice et al., 1985, 1986; Speight et al., 1988; Westaway et al., 1985).

Little is known about the function of any of the flavivirus non-structural proteins. It has been postulated that the two largest non-structural proteins, NS5 (96K) and NS3 (67K) may be viral polymerase proteins, since they are hydrophilic and possess a net positive charge (Rice et al., 1985). Further, NS5 has been shown to share amino acid sequence homology with the putative polymerases of a number of plant and animal positive-strand RNA viruses (Rice et al., 1985, 1986). This homology is restricted to three short non-contiguous regions of the NS5 protein and may represent a conserved functional domain common to a particular class of RNA-dependent RNA polymerases. Although the function of the third largest non-structural protein, NS1, is unknown, it may not have a replicative function since it is glycosylated, present both intracellularly and on the plasma membrane of flavivirus-infected cells, and capable of fixing complement (Smith & Wright, 1985). The remaining four non-structural proteins vary in size and detectability depending on the particular flavivirus and host cell utilized (Heinz & Kunz, 1982; Rice et al., 1985; Speight et al., 1988). Flavivirus RNA synthesis has been localized to perinuclear endoplasmic reticular membranes of infected cells (Lubiniec & Henry, 1974; Ng et al., 1983) and the four small proteins (ns2a, ns2b, ns4a, ns4b) are membrane-associated and hydrophobic (Rice et al., 1985, 1986) and thus could be involved in maintaining the structural integrity and/or membrane association of the replication complexes.
As yet many aspects of flavivirus transcription remain to be elucidated. The components of flavivirus replication complexes have not yet been identified; nor is it known how flavivirus proteins interact with one another, the template RNA, and possibly cell proteins to form functional replication complexes. The identification of the functional components of the viral replication complexes has been impeded by the fact that the flavivirus \textit{in vitro} polymerase activity is tightly associated with intracellular membranes (Grun & Brinton, 1986, 1987). Studies have been further hampered because flavivirus replication complexes have a relatively low specific activity \textit{in vitro}.

Centrifugation of West Nile virus (WNV)-infected BHK-21 cell lysates through a 20\% glycerol cushion was used as an initial step in purification of the complexes (Grun & Brinton, 1987). This technique separated the majority of the largest WNV non-structural protein, NS5, as well as numerous host proteins from the cell fraction containing functional WNV-specific replication complexes without significant loss of \textit{in vitro} virus-specific RNA polymerase activity (Grun & Brinton, 1987). Further purification of functional replication complexes requires the separation of the complexes from intracellular membranes. Previous studies reported that detergent treatment inhibited flavivirus RNA-dependent RNA polymerase activity \textit{in vitro} (Cardiff \textit{et al.}, 1973; Chu & Westaway, 1987; Qureshi & Trent, 1972). The present study represents the first systematic evaluation of the relative efficiencies of five selected detergents to separate functional WNV replication complexes from partially purified intracellular membrane fragments. Of the five detergents tested, sodium deoxycholate (DOC) was the most efficient at releasing functional replication complexes.

Previous studies, in which the effect of detergents on flavivirus \textit{in vitro} polymerase activity was investigated, utilized two (Qureshi & Trent, 1972) or three (Cardiff \textit{et al.}, 1973) detergents. In both instances inhibition of polymerase activity was observed, and the authors postulated that inhibition might be due to the presence of contaminating nucleases. Chu & Westaway (1987) demonstrated the presence of RNase in NP40 by RNA product analysis. The addition of RNasin to their \textit{in vitro} polymerase reactions partially inhibited the RNase activity.

Since the release of functional flavivirus replication complexes from intracellular membranes is a prerequisite for their further purification, a systematic investigation of six detergents was undertaken. WNV, strain E101, was propagated in BHK-21/WI2 cells as previously described (Grun & Brinton, 1986). Sixteen h post-infection, the cells were harvested and then allowed to swell for 10 min in ice-cold hypotonic TNP buffer [10 mM-Tris–HCl pH 8.0, 10 mM-NaCl, 100 KIU of aprotinin per ml and 0.5 mM-PMSF (Sigma)]. The cells were disrupted with a Dounce homogenizer and then centrifuged at 1000 g for 5 min. The supernatant fraction (S1), containing cytoplasmic material and plasma membranes, was reserved on ice. The nuclear pellet was resuspended in TNP buffer and additional endoplasmic reticular membranes were mechanically sheared from the intact nuclei (Grun & Brinton, 1986). Nuclei and large cell debris were then pelleted at 1000 g for 10 min. The resulting supernatant fluid (S2) contained fragmented outer nucleus-associated membranes. Since we have previously shown that both the S1 and S2 fractions contain WNV-specific polymerase activity (Grun & Brinton, 1986), these two fractions were combined and layered onto a 2.0 ml cushion of 20\% glycerol in TNP buffer. The functional replication complexes and associated membrane fragments were then pelleted by centrifugation at 70000 g for 30 min in an SW50 rotor (Beckman Instruments). The pellet was resuspended in TNP buffer (100 \mu l/10^8 cell equivalents) and aliquots were incubated at 4 °C for 2 h with an equal volume of one of the following detergents: Tween 20 (Pierce Chemicals, Rockford, Ill., U.S.A.), dodecyl-\beta-D-maltoside (Calbiochem), n-octyl-\beta-D-glucoside (Calbiochem), lubrol PX (Pierce) or DOC (Sigma). Each detergent was obtained in the highest purity available. When pretested for activity, Tween 20, maltoside, octylglucoside, lubrol PX and DOC were found to be free of nuclease; NP40, obtained from several different suppliers, was always contaminated with nuclease as indicated by its degradation of single-stranded viral RNA (data not shown). Only the five RNase-free detergents were further evaluated.

Detergent-treated samples were assessed for virus-specific polymerase activity utilizing an \textit{in vitro} transcription assay; the conditions and components of this assay were previously optimized for WNV polymerase activity (Grun & Brinton, 1986). Briefly, 25 \mu l of each detergent-treated
Fig. 1. Effect of detergent on activity and release of WNV replication complexes from intracellular membrane fragments. Partially purified membrane fragments from WNV-infected BHK cells were treated for 2 h at 4 °C with Tween 20 (a, f, k), maltoside (b, g, l), octylglucoside (c, h, m), lubrol PX (d, i, n) or DOC (e, j, o). (a to e) At the end of the incubation period, the polymerase activity in each detergent-treated fraction was determined. The dashed lines represent polymerase activity in the absence of detergent. (f to j) Detergent-treated samples were centrifuged at 7000 g for 10 min. Each supernatant fraction was then assayed for WNV-specific polymerase activity. The dashed lines represent polymerase activity in the supernatant fraction of samples not treated with detergent. (k to o) Percentage protein solubilization was determined by the BCA protein assay. The dashed lines (k to o) indicate the amount of protein released into the soluble fraction from samples not treated with detergents. Data are representative of three experiments in which duplicate samples were assayed.

fraction was added to 25 μl of a reaction mixture composed of buffered salts, dithiothreitol, protease inhibitors and ribonucleoside triphosphates. RNasin was not included in the reaction mixtures, since cell extracts did not contain detectable levels of RNase. Following incubation at 30 °C for 60 min, reactions were terminated by the addition of SDS to a final concentration of 1-0%. The samples were phenol-extracted and the in vitro labelled RNA products were ethanol-precipitated at -70 °C and then electrophoresed on non-denaturing 0.8% agarose gels. Subsequently, the gels were dried and autoradiographed to locate the positions of the virus-specific RNA products on the gel. [32P]GMP incorporated into virus-specific RNAs (replicative form/replicative intermediate and genome RNA) was then quantified by counting radioactivity in excised regions of the dried gels containing the viral RNAs in the presence of liquid scintillation cocktail (Grun & Brinton, 1986).

WNV polymerase activity was inhibited to some extent by all five of the detergents tested (Fig. 1a to e). The detergent concentrations given in Fig. 1 represent the amount of detergent present during the 2 h detergent treatment step. The final detergent concentration present during the polymerase reaction was twofold less, since extracts were diluted by the addition of reaction components. WNV polymerase activity in control extracts, which were not treated with detergent, is indicated by a dashed line in Fig. 1(a to e). In general, polymerase activity was found to decrease with increasing concentrations of Tween 20, maltoside or octylglucoside (Fig. 1a to c). Polymerase activity in extracts treated with the lowest concentration (0-1%) of Tween
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20, maltoside or octylglucoside was inhibited by 41.1, 53.3 and 40.2% respectively, whereas in those extracts treated with 2% Tween 20, maltoside or octylglucoside, polymerase activity was inhibited by 84.1, 86.0 and 76.6% respectively. A different profile was seen with those extracts treated with lubrol PX. At the lowest detergent concentration tested (0.1%), the activity of the lubrol PX-treated extracts was inhibited to a greater degree (62.6%; Fig. 1d) than that of extracts treated with 0.1% Tween 20, maltoside or octylglucoside. The maximum inhibition of polymerase activity in lubrol PX-treated samples was 72.0% (1.0% lubrol PX). DOC was found to have the least inhibitory effect on WNV polymerase activity. In those extracts treated with 0.5% DOC, polymerase activity was decreased by only 18.7%. At other concentrations (0.1, 1.0 and 2.0%), DOC inhibited WNV in vitro transcription by 50.0, 25.2 and 34.0%, respectively.

To determine how much WNV polymerase activity had been released by detergent treatment, partially purified membrane fragments were incubated with detergent for 2 h as described above. Replication complexes were then separated from the remaining membrane fragments by centrifugation at 7000 g for 10 min. Preliminary results indicated that membrane fragments associated with polymerase activity in cell extracts not treated with detergent pelleted under these conditions. Although the sedimentation properties of the released complexes are as yet unknown, preliminary studies indicated that DOC-released activity had altered sedimentation properties since it remained in the top quarter of gradients containing 0.5% DOC and 20 to 70% sucrose after centrifugation for 2 h at 85000 g, while untreated complexes sedimented to the bottom of such gradients (data not shown).

Following centrifugation of the detergent-treated extracts at 7000 g for 10 min, each of the resulting supernatant fractions was evaluated for WNV-specific polymerase activity. As expected, supernatant fractions from control samples, not treated with detergents, contained very little polymerase activity (Fig. 1f to j, dashed line); less than 600 c.p.m. of [32P]GMP was incorporated into virus-specific RNA. The polymerase activity detected in Tween 20-solubilized fractions was not significantly greater than the control (200 to 800 c.p.m. [32P]GMP incorporated; Fig. 1f). The soluble polymerase activities of maltoside- and octylglucoside-treated supernatant fractions were similar to one another (Fig. 1g, h). The polymerase activity was approximately threefold greater than the control at maltoside or octylglucoside concentrations of 0.1, 0.5 and 1.0%. Those fractions solubilized with 0.1 to 2.0% lubrol PX contained five- to sixfold greater activity than untreated control samples (Fig. 1i). DOC-solubilized samples were found to have the highest WNV polymerase activity, showing a 10-fold increase over the control at 0.5% DOC (Fig. 1j).

As a means of further assessing the ability of each of the detergents to solubilize integral membrane proteins, the solubilized fractions were also analysed for total protein concentration by the bicinchoninic acid (BCA) protein assay (Pierce Chemicals). Approximately 25% of the protein in samples that were not treated with detergent remained soluble after centrifugation at 7000 g for 10 min (Fig. 1k to o, dashed line). This probably represents small protein aggregates, light membrane fragments or extrinsic membrane proteins which were eluted from the membranes during the 2 h incubation period under conditions of low ionic strength. When centrifugation conditions were changed from 7000 g for 10 min to 15000 g for 30 min, only an additional 5% of the total protein was pelleted (data not shown). Additionally, a number of detergent-treated and control samples were centrifuged at 15000 g for 30 min and then the supernatant fractions were analysed for polymerase activity. Under these conditions, enzyme activity profiles similar to those shown in Fig. 1 (f to j) were observed.

As shown in Fig. 1 (k), of the five detergents tested Tween 20 was the least effective in dissociating membrane proteins. Over a concentration range of 0.1 to 2.0% Tween 20, only 20 to 40% of the total protein was detected in the supernatant fractions. In contrast, 70 to 80% of the total protein was present in supernatant fractions after treatment with 0.1 to 2.0% DOC (Fig. 1 o). Maltoside at concentrations of 0.1 to 2.0% solubilized 55 to 65% of the protein (Fig. 1f). Similar results were observed with extracts treated with 0.1 to 2.0% lubrol PX (Fig. 1n; 57 to 70% of the protein was solubilized). The protein solubility profile seen with octylglucoside showed the most dependence on detergent concentration. Seventy-five% of the protein was solubilized at concentrations of 1.0 to 2.0% octylglucoside, while at a concentration of 0.1%
detergent, only 20% of the protein was solubilized (Fig. 1m). It is likely that the inefficiency of octylglucoside, at low concentrations, to solubilize proteins is due to the high critical micelle concentration of this detergent (20 to 25 mM; Neugebauer, 1987). At concentrations above the critical micelle concentration (1 to 2%), octylglucoside was the most effective non-ionic detergent tested.

The amount of total protein released from membrane fragments by a particular detergent did not always correlate with the amount of WNV polymerase activity detected in the supernatant. For example, fractions solubilized with 0.1% DOC and fractions solubilized with 1.0 to 2.0% octylglucoside contained similar amounts of total protein (Fig. 1o and m, respectively); however, polymerase activity was much greater in the DOC-solubilized fraction (Fig. 1j and h). It is likely that the observed lack of correlation between total protein released and in vitro polymerase activity is a reflection not only of the fact that the amount of viral protein present in these extracts is small in comparison to the amount of cell protein present, but also that the detergents differ in the degree to which they inhibit polymerase activity (Fig. 1a to e). Proteins contained in the various detergent-treated supernatant fractions were further analysed by SDS–PAGE according to the methods of Laemmli (1970). Viral proteins were labelled intracellularly by incorporation of [35S]methionine (Amersham; 1230 Ci/mmol) in the

Fig. 2. Analysis of the protein content of detergent-treated extracts. Proteins contained in S1/S2 fractions from uninfected (lane 1) and WNV-infected (lane 2) BHK-21 cells were compared to proteins solubilized with 0.5% Tween 20 (lane 3), maltoside (lane 4), octylglucoside (lane 5), lubrol PX (lane 6) or DOC (lane 7). Following detergent treatment and centrifugation at 7000 g for 10 min, equivalent volumes of supernatant samples were loaded onto 10% SDS–polyacrylamide gels. This figure is a composite of three gels with the Mr markers for each gel indicated to the left, and the virus-specific bands indicated by the dots to the right of lane 2.
presence of 2 μg of actinomycin D per ml of methionine-deficient medium as described previously (Grun & Brinton, 1986). Radiolabelled proteins in untreated S1/S2 extracts from uninfected and WNV-infected BHK cells were compared with proteins contained in supernatant samples from extracts treated with 0.5% detergent (Fig. 2). The majority of NS5 (96K) had previously been shown to be separated from membrane fragments by the initial centrifugation through a 20% glycerol cushion (Grun & Brinton, 1987). Thus very little NS5 was detected in the detergent-treated samples (Fig. 2, lanes 3 to 7). The most prominent viral bands observed in the detergent-treated supernatants were NS3 (67K) and E/NS1 (47K to 48K). As shown previously (Grun & Brinton, 1987), the E and NS1 proteins of WNV do not differ significantly in their migration on 10% SDS–polyacrylamide gels. These viral proteins (NS3, E/NS1) were detected in all of the detergent-solubilized fractions. Occasionally, a viral band was detected at 56K. This band may represent alternative forms of E or NS1. The smaller viral proteins observed had calculated MrS of 26K, 21K (a doublet, shown here as a smear), 20K and 14K. On the basis of the most recent Mr predictions for the proteins of the closely related flavivirus, Kunjin (Speight et al., 1988), these proteins were designated ns4b, pre-M, ns2a and C respectively. The 14K protein was identified as the capsid protein by reference to the electrophoretic migration of virion structural proteins (data not shown). Proteins corresponding to ns2b and ns4a were not identified in these gels. Although each of the detergent-solubilized fractions was found to contain ns4b, ns2a, pre-M and C, some differences in the viral protein band intensities were noted. The 21K doublet was more prominent in the maltoside- and DOC-solubilized fractions (Fig. 2, lanes 4 and 7) as compared to the other detergent-solubilized fractions. The 26K viral band was less prominent in the octylglucoside-solubilized fraction (Fig. 2, lane 5). The significance of these apparent differences in the solubility of various viral proteins is as yet unknown. Soluble polymerase activity in samples treated with 0.5% maltoside or octylglucoside were similar (Fig. 1 g, h).

Further purification of flavivirus replication complexes is contingent on finding a means of releasing functional complexes from intracellular membrane fragments. The present study demonstrates that at least two detergents, DOC and octylglucoside, provide reasonable yields of released viral protein. However, the function of the complexes was best preserved by DOC treatment.

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REFERENCES


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