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Dissociation of NS5 from Cell Fractions Containing West Nile Virus-Specific Polymerase Activity

JANET B. GRUN AND MARGO A. BRINTON*
The Wistar Institute, Philadelphia, Pennsylvania 19104-4268

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West Nile virus replication complexes were partially purified from cytoplasmic extracts of virus-infected cells by centrifugation through a 20% glycerol cushion. Numerous cell proteins, as well as the largest nonstructural protein, NS5, were separated from the replication complexes without significant loss of in vitro West Nile virus polymerase activity.

Flaviviruses are small, enveloped, single-stranded RNA viruses of positive polarity. The genome RNA has a type 1 cap at the 5′ end and a stable stem-and-loop structure, rather than a poly(A) tract, at the 3′ terminus (3, 17, 24). This secondary structure is highly conserved among flaviviruses and may be important in RNA replication and encapsidation (3). The genome RNA possesses a single open reading frame and serves as the only mRNA for the synthesis of viral proteins. The three structural proteins (C, M, and E) are encoded at the 5′ end of the genome, whereas the 3′ portion of the genome encodes seven nonstructural proteins (17).

Little is currently known about the functions of flavivirus nonstructural proteins. The two largest nonstructural proteins, NS5, with a mass of 96 kilodaltons (kDa) and NS3 (67 kDa) are the most highly conserved proteins among flaviviruses (17, 21). Both NS5 and NS3 are hydrophilic and have a net positive charge (17), which may indicate a functional role in the replication of viral RNA. The NS5 protein has also been shown to share three short, noncontiguous stretches of amino acid sequence homology with the putative polymerases of a number of plant and animal plus-strand RNA viruses (17, 21). These sequences may represent functional domains of the polymerase protein. The glycosylated NS1 (48-kDa) protein is expressed on the plasma membrane of flavivirus-infected cells and is capable of fixing complement (19, 20). The remaining four nonstructural proteins, ns2a (21 kDa), ns2b (14 kDa), ns4a (26 kDa), and ns4b (12 kDa), vary in size and detectability depending on the particular flavivirus and host cell being studied (17). The lowercase designation indicates that these proteins have not been definitively mapped within the polyprotein precursor; proteolytic cleavage sites for their generation have so far only been predicted from the nucleic acid sequence. These four proteins are membrane associated and hydrophobic (17) and thus may be involved in maintaining the structural integrity or membrane association of the replication complexes.

To define the role of individual West Nile virus (WNV) nonstructural proteins in viral RNA replication, we are currently devising a protocol to purify functional replication complexes. Centrifugation of cell lysates through a 20% glycerol cushion was used as an initial step in purification; the majority of the largest WNV nonstructural protein, NS5, was separated from cell fractions containing WNV-specific replication complexes without significant loss of in vitro virus-specific RNA polymerase activity. As we previously demonstrated, the in vitro activity consists primarily of plus-strand RNA synthesis (9). These data suggest that the NS5 protein may not have a principal role in the synthesis of plus-strand RNA.

WNV E101 was propagated in BHK-21/W12 cells as previously described (9). Cells were infected at a multiplicity of infection of 1; at 16 h after infection, intracellular proteins were labeled with 15 μCi of [^{35}S]methionine (1,230 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml in the presence of 2 μg of actinomycin D per ml. The cells were harvested by being scraped into phosphate-buffered saline and were collected by centrifugation at 800 × *g*. Cells were allowed to swell for 10 min in ice-cold hypotonic buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride [Sigma Chemical Co., St. Louis, Mo.]) and were lysed by 20 strokes with a Dounce homogenizer. The disrupted cells were pelleted by centrifugation at 1,000 × *g* for 5 min, and the supernatant fraction (S1) containing cytoplasmic material and plasma membranes was reserved on ice. The nuclear pellet was suspended in 10 mM Tris hydrochloride (pH 8.0)–10 mM NaCl–100 kIU of aprotinin per ml–0.5 mM phenylmethylsulfonyl fluoride (TNAP). Additional endoplasmic reticular membranes were mechanically sheared from the intact nuclei by repeated passage through syringe needles as described elsewhere (9). Nuclei and large cell debris were pelleted at 1,000 × *g* for 10 min. The resulting supernatant fluid (S2) contained the fragmented outer nucleus-associated membranes.

Since we have previously shown that both the S1 and S2 fractions contain WNV-specific polymerase activity (9), these two fractions were combined. Replication complexes and associated membrane fragments contained in the combined S1 and S2 fractions were then concentrated by high-speed centrifugation (70,000 × *g* for 30 min) in an SW50 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The pellet was resuspended in TNAP (100 μl per 10^8 cell equivalents) prior to analysis (Fig. 1, lanes 1 and 2). In an alternate procedure, the combined S1 and S2 fractions were layered onto a 2.0-ml cushion of 20% glycerol in TNAP buffer and then subjected to high-speed centrifugation as described above. Four fractions were generated: (i) a TNAP supernatant fraction containing soluble proteins which were concentrated by ethanol precipitation at −70°C prior to protein analysis (Fig. 1, lane 3); (ii) a visible band at the TNAP–20% glycerol interface, from which, after dilution in TNAP, fragments of light membranes were further concentrated by centrifugation at 70,000 × *g* for 30 min and resuspension of the resulting pellet in TNAP (50 μl per 10^8 cell equivalents;

* Corresponding author.

TABLE 1. WNV-specific polymerase activity in cell fractions[a]

Cell fraction	Amt of protein		[32P]GMP incorporated		
	Per reaction (μg)	Total (10^3 μg)	Per reaction (cpm)	cpm/μg of protein	Total (10^3 cpm)
Total cytoplasmic extract	90.0	18	2,170	24.1	434
Generated by high-speed centrifugation					
A. Supernatant	28.9	13	150	5.2	7
B. Pellet	161.0	2.9	11,404	71.3	205
Generated by centrifugation on 20% glycerol cushion					
C. TNAP supernatant	40.4	9.1	110	2.7	24
D. TNAP-glycerol interface	62.9	3.3	240	3.8	12
E. Glycerol cushion	7.4	1.2	310	41.9	47
F. Pellet I	144.0	2.6	12,392	86.1	223
G. Pellet II[b]	116.0	2.1	11,433	98.6	206

[a] In vitro polymerase reactions were performed in triplicate.
[b] Sample was pelleted twice through 20% glycerol cushions.

Fig. 1, lanes 4 and 5); (iii) the 20% glycerol layer (not shown); and (iv) the pellet, which was either resuspended in TNAP and analyzed directly (not shown) or pelleted through a second discontinuous glycerol gradient and then analyzed (Fig. 1, lanes 6 and 7).

Electrophoresis of proteins on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels was performed as previously described (9), by the method of Laemmli (11). The gels were fixed in 7% acetic acid, stained with 0.25% Coomassie blue, enhanced with Enlightening (New England Nuclear Corp., Boston, Mass.), dried, and fluorographed.

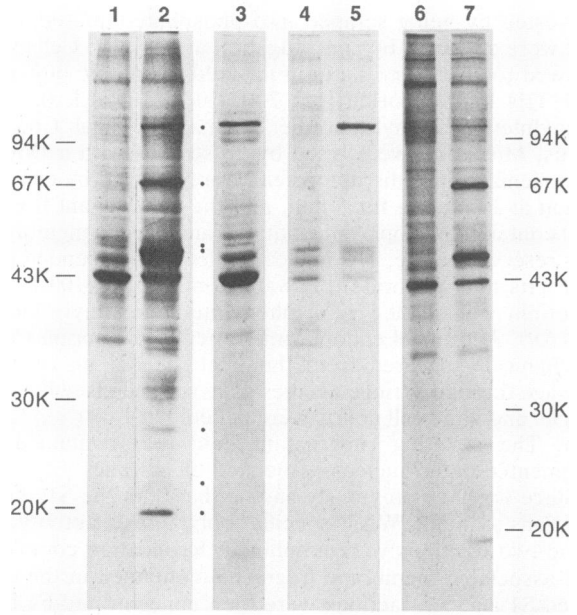

FIG. 1. Protein content of various cell fractions. WNV-infected (lanes 2, 3, 5, 7) and uninfected (lanes 1, 4, 6) BHK cells were fractionated as described in the text. Membrane-associated proteins were concentrated from cytoplasmic extracts by centrifugation at 70,000 × *g* for 30 min (lanes 1 and 2). Alternatively, cytoplasmic extracts were layered on 20% glycerol cushions and centrifuged. Proteins contained in three of the four resulting fractions are shown: TNAP supernatant fraction (lane 3); TNAP-glycerol interface (lanes 4 and 5); and the pellet fraction, twice centrifuged through glycerol cushions prior to analysis (lanes 6 and 7). Samples were electrophoresed on SDS–10% polyacrylamide gels, and the gels were fluorographed. Molecular mass markers are indicated on the left for lanes 1 and 2 and on the right for lanes 3 through 7 (94K is 94 kDa). The dots to the right of lane 2 indicate the following virus-specific proteins (from top to bottom); NS5 (96 kDa), NS3 (67 kDa), E (48 kDa), NS1 (47 kDa), ns4a (26 kDa), ns2a (21 kDa), and pre-M (19 kDa).

The largest nonstructural protein, NS5, was one of the most soluble WNV nonstructural proteins. Although some NS5 remained associated with the pelleted S1 and S2 membrane fragments (Fig. 1, lane 2) (9), most of the NS5 protein was found in the supernatant fraction. After centrifugation of the S1-S2 extracts through a 20% glycerol cushion, the majority of NS5 was found either in the TNAP supernatant fraction (Fig. 1, lane 3) or at the TNAP-glycerol interface (Fig. 1, lane 5). Additional NS5 was dissociated from the fraction F pellet by centrifugation of this sample through a second glycerol cushion (data not shown). Only a small quantity of NS5 remained associated with the pellet fraction G (Fig. 1, lane 7).

Each of the cytoplasmic or gradient fractions described above was assessed for total protein concentration by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.) and for WNV-specific RNA-dependent RNA polymerase activity by the in vitro polymerase assay described previously (9). Soluble proteins contained in dilute supernatant fractions were not concentrated by ethanol precipitation prior to assay as they were before gel electrophoresis (Fig. 1, lane 3) because such treatment destroys the WNV-specific polymerase activity (Grun and Brinton, unpublished observations). Polymerase reactions were incubated at 30°C for 60 min in a total volume of 50 μl (9). Reactions were stopped by the addition of protease and (10 min later) SDS to final concentrations of 40 μg per ml and 0.1%, respectively. The samples were phenol extracted, and the RNA products were ethanol precipitated at −70°C. Polymerase activity was measured by the amount of [32P]GMP incorporated into viral RNA products after electrophoresis on nondenaturing 0.8% agarose gels, as described previously (9). The values shown in Table 1 are expressed as amounts of [32P]GMP incorporated per reaction, per microgram of protein, and per fraction.

After high-speed centrifugation through a 20% glycerol cushion, the majority of the WNV polymerase activity was associated with the pellet fractions (Table 1, fractions B, F, and G). The glycerol cushion (fraction E) accounted for only about 10% of the total polymerase activity and contained 7.4 μg of protein. By analysis on SDS-polyacrylamide gels, the

proteins in fraction E appeared to be identical to those in fractions F and G (data not shown). Thus the activity detected in fraction E appears to be associated with membrane fragments which did not pellet during centrifugation. Only minimal polymerase activity was associated with the supernatant fractions (Table 1, fractions A and C) or the TNAP-glycerol interface (Table 1, fraction D). When S1-S2 fractions were subjected to discontinuous sucrose density gradient centrifugation by the methods of Caliguiri and Tamm (5), more than 60% of the WNV polymerase activity was associated with fractions containing endoplasmic reticular membrane fragments (Grun and Brinton, unpublished observations). These data are consistent with previous reports which determined that flavivirus RNA synthesis takes place on endoplasmic reticular membranes in the perinuclear region of infected cells (13, 15).

Although polymerase activity increased more than fivefold when membrane fragments were concentrated by centrifugation, only 47 to 51% of the total activity in the original S1-S2 extract could be accounted for. It is possible that some of the replication complexes were irreversibly disrupted by mechanical forces during centrifugation. Alternatively, an unknown host cell factor(s) may be loosely associated with the viral replication complexes and lost during the partial purification procedure. Cellular proteins have previously been reported to function in the transcription of other viruses; initiation and recognition of Qβ bacteriophage RNA templates are functions provided by host cell proteins (2, 12, 25), and a cellular terminal uridylyl transferase has been reported to be involved in poliovirus template initiation (1, 8, 14).

Of particular interest is the comparison of WNV-specific polymerase activity in the high-speed centrifugation pellet fraction (Table 1, fraction B; 11,404 cpm) with the activity in pellet fractions obtained after centrifugation through one or two glycerol cushions (Table 1, fractions F and G; 12,392 and 11,433 cpm, respectively). Although fractions F and G were progressively depleted of NS5, they contained essentially the same amount of WNV-specific polymerase activity as the NS5-rich, high-speed centrifugation pellet (cf. Fig. 1, lanes 2 and 7). It is possible that only a small amount of NS5 provides all of the observed polymerase activity. However, NS3, the second-largest nonstructural protein, could be responsible for the synthesis of plus-strand RNA from minus-strand templates since it remains associated with polymerase activity during purification.

It is interesting to note that at least three of the smaller nonstructural proteins (ns4a and ns2a [Fig. 1, lane 7] and ns2b [not shown]) also remained associated with viral polymerase activity. It is not known whether these small proteins have enzymatic functions or whether, by their hydrophobic nature, they act as membrane anchors for the replication complexes.

Fractions B, F, and G also contained a virus-specific band at 48 kDa. This band represents the WNV structural E protein and a small amount of the soluble nonstructural protein designated NS1. These two WNV proteins have previously been reported to comigrate on SDS-polyacrylamide gels (10). This phenomenon has so far been observed only for WNV, since the E and NS1 proteins of other flaviviruses differ significantly in their gel migrations. Both E and NS1 are glycosylated. The E protein is the virion envelope protein; NS1 has been identified as a complement-fixing protein located on the surface of flavivirus-infected cells (19, 20). For these reasons it is unlikely that either E or NS1 function in WNV-specific RNA replication.

Fractions rich in NS5 (Fig. 1, lanes 3 and 5; Table 1, fractions C and D) contained less than 5% of the polymerase activity. However, these fractions were also found to contain little template RNA (data not shown). Others have previously shown that purified poliovirus polymerase protein can elongate primed RNA templates (22, 23). When synthetic primer-template pairs such as poly(C)-oligo(G) or poly(A)-oligo(U) were added to in vitro reactions containing WNV NS5-rich fractions, no reactivity was detected. The addition of WNV genomic RNA also did not stimulate activity. Further, the addition of synthetic primer-template pairs and NS5-enriched fractions to reactions containing NS5-depleted pellet fractions also had no effect on activity. The inability to demonstrate NS5-directed elongation activity does not prove that NS5 is not a polymerase protein. Optimal assay conditions for exogenous template-dependent activity may be quite different from conditions which were optimal for in vitro plus-strand RNA synthesis from endogenous replication complexes.

We cannot currently rule out the possibility that small quantities of membrane-associated NS5 provide all the measured polymerase activity in pellet fractions B, F, and G. Since we have not yet been able to demonstrate a transcriptional activity for the soluble NS5, the presence within this protein of the postulated canonical RNA-dependent RNA polymerase sequence is the only evidence that indicates a polymerase function (17, 21). It is possible that the plus- and minus-strand viral RNAs are synthesized by two distinct replication complexes; NS5 may function solely in the synthesis of minus-strand RNA. Such minus-strand replication complexes may be extremely fragile and therefore may be disrupted by cell fractionation procedures. Alternatively, NS5 could function in the initiation and capping of nascent RNA strands but not in strand elongation.

The picornaviruses represent the only group of plus-strand RNA viruses for which a purified viral protein has been demonstrated to function in vitro as a polymerase (22, 23). The less-well-characterized alphaviruses, as well as several plus-strand RNA plant viruses, each possess a nonstructural protein with limited amino acid sequence homology to the picornavirus polymerases (21). On this basis, such proteins have been tentatively identified as polymerases. However, these plus-strand RNA viruses also encode two or more additional nonstructural proteins, which, despite their lack of sequence homology with the picornavirus polymerases, may also function as polymerase proteins. For example, the alphaviruses encode four nonstructural proteins (nsP1, nsP2, nsP3, and nsP4), three of which are associated with in vitro polymerase activity (4, 6, 16, 18). The major constituent of partially purified, active alphavirus replication complexes is the membrane-associated nsP1 protein. The nsP2 and nsP4 proteins are present only in low concentrations in the viral replication complexes (7). Specific functions have not yet been demonstrated for any of these proteins, but only the nsP4 protein of alphaviruses contains the conserved canonical polymerase sequences (17, 21).

Several classes of RNA-dependent RNA polymerases may exist, and flavivirus NS5, alphavirus nsP4, and picornavirus replicase may represent only one of these classes. Further studies are necessary to determine what roles additional types of polymerases may play in the replication strategies of various plus-strand RNA viruses.

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