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Structural Proteins of Western Equine Encephalitis Virus: Amino Acid Compositions and N-Terminal Sequences

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The structural proteins of Western equine encephalitis virus, a member of the alphavirus group, have been characterized by the determination of their amino acid compositions and by N-terminal sequence analysis. More than 60 residues of the N-terminal sequences of each of the envelope glycoproteins have been determined. A comparison of these sequences with the previously determined sequences of two related alphaviruses, Sindbis virus and Semliki Forest virus, strongly supports the view that all three viruses have evolved from a common ancestor and provides information on the pattern of this evolution. The analysis of the capsid proteins of Western equine encephalitis virus shows that the nucleocapsid of this virus can accommodate a considerable degree of variability in its protein component and that at least some regions of alphavirus capsid proteins show more extensive differences between different viruses than do the envelope glycoproteins.

Western equine encephalitis virus (WEE) is one of about 20 viruses that have been assigned to the alphavirus genus of the Togavirus family (6). Most of the available information on the molecular details of the structure and replication of the alphaviruses has come from extensive work with two members, Sindbis virus (SIN) and Semliki Forest virus (SFV); the other alphaviruses have received considerably less attention. In contrast, the immunological cross-reactions of the viral antigens of the various alphaviruses have been extensively studied and have proven useful as a major criterion for the inclusion of a virus in the alphavirus group and for assessing the relationships among various members of the group (6). Thus, WEE and SIN have been found to be closely related antigenically and are included in the WEE subgroup of the alphaviruses.

The virion of WEE, like that of other alphaviruses, contains two envelope glycoproteins, each of about 50,000 molecular weight, and an internal capsid protein, C, of 30,000 molecular weight (22, 27). It seems certain that all three structural proteins are translated as a polyprotein precursor and processed by proteolytic cleavage and are present in the virion in equimolar ratios, although this has been examined experimentally only for SIN and SFV (25). However, WEE, unlike other alphaviruses, also contains a minor capsid protein, the X protein, 1,500 daltons smaller than the C protein. Comparisons of the peptides generated by proteolytic cleavage of these two proteins, and considerations based on the size of the subgenomic RNA coding for the viral structural protein, indicate that the X protein is a shortened form of the C protein (16).

Despite their similarities at the molecular level, alphaviruses are quite diverse in the severity of the diseases they cause in man and other vertebrates. WEE and others can cause a severe and sometimes fatal encephalitis in man and are significant (and economically important) pathogens in horses. In contrast, SIN and SFV cause only mild fever and rash or an asymptomatic infection in humans (26).

Recently, the complete sequences of the structural proteins of SIN and SFV have been deduced from the sequences of the subgenomic RNA coding for these proteins (9, 10, 24). We are interested in the corresponding sequences of WEE, to learn more about the common features, at the molecular level, of the members of the alphavirus group and to attempt to discern differences which may account for the wide range in the severity of the diseases they cause. WEE would seem to be a particularly appropriate choice for further study among the alphaviruses because a significant amount of genetic analysis has been performed on this virus. Temperature-sensitive mutants of WEE have been isolated and characterized by complementation analysis (11, 19), and WEE is the only alphavirus other

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than SIN for which complementation between mutants has been observed. Furthermore, interspecific complementation between mutants of WEE and SIN has recently been reported (E. G. Strauss, H. Tsukeda, and B. Simizu, manuscript in preparation). In this paper, we report the preliminary characterization of the structural proteins of WEE, including their amino acid compositions and extensive N-terminal sequence data from the two envelope glycoproteins.

MATERIALS AND METHODS

Virus growth and purification. The virus used in this study was the McMillan strain of WEE virus, originally obtained from the Virus Laboratory, the Rockefeller Foundation, New York, in 1957. The virus was passed in suckling mouse brains three times and then plaque purified several times by primary chick embryo fibroblast cultures in our laboratory. Primary chick embryo fibroblast cultures were infected with virus at a multiplicity of infection of 1 PFU per cell and incubated at 37°C. At 24 h after infection, the culture fluid was harvested and centrifuged at 3,000 rpm for 15 min. The supernatant was kept at −80°C and used as stock virus. Primary chick embryo fibroblast cultures were prepared as previously described (19) and used for virus propagation and plaque assays. Infected culture fluids were concentrated and purified by differential centrifugation and sucrose density centrifugation as described previously (12).

Protein purification. Purified virus preparations were treated with Nonidet P-40 to a final concentration of 1% to solubilize the envelope glycoproteins. Nucleocapsids were removed by centrifugation at 50,000 rpm in a Beckman type 50 Ti rotor for 60 min at 4°C, and the pelleted nucleocapsids were dissolved in 1% sodium dodecyl sulfate (SDS)–0.067 M Tris-chloride (pH 7)–0.5 mM EDTA. The solubilized envelope glycoproteins E1 and E2 were separated by chromatography on columns of glass wool (31). When necessary, a mixture of the capsid proteins X and C was prepared free of viral RNA by chromatography of SDS-disrupted nucleocapsids on hydroxylapatite in SDS (20) as described for the SIN capsid protein (2). Pure C protein and X protein were prepared by preparative polyacrylamide gel electrophoresis of nucleocapsids, using the buffer system of Laemmli (18), except that the gel buffer concentrations were reduced by a factor of two, with a separating gel consisting of a 12 to 20% exponential gradient of acrylamide (acrylamide-bisacrylamide, 37.5:1), and with 0.01% mercaptopropionic acid in the sample buffer and upper reservoir. Protein bands stained with Coomassie blue were excised from the slab gel, and the proteins were recovered by electroelution in SDS (15). Essentially the same system was used for analytical gel electrophoresis of capsid protein preparations.

Amino acid compositions. Amino acid compositions were determined on purified preparations of E1 and E2 and from nucleocapsid preparations (X plus C proteins) freed of viral RNA. Single 4-μg samples were hydrolyzed with 6 N HCl at 110°C in evacuated tubes for 24, 48, and 72 h, with the values for serine and threonine determined by extrapolation to zero time of hydrolysis, assuming first-order kinetics. Isoleucine and valine were determined primarily from the samples hydrolyzed for 72 h, but data from the 24- and 48-h hydrolyses were also considered. Cysteine was determined as cysteic acid after HCl hydrolysis for 24 h of single performic acid-oxidized samples. For E1 and E2, performic acid-oxidized samples gave values for methionine sulfoxide greater than for methionine in the unmodified proteins; the former are reported in Table 1. For the capsid proteins, the methionine value was greater and is the value reported. All hydrolysates were analyzed on a Durrum D-500 Mk. II amino acid analyzer, and tryptophan was not determined.

Protein sequencing. The mixture of the X and C proteins, as SDS-disrupted nucleocapsids, and E1 and E2 were subjected to automated Edman degradation on a noncommercial spinning cup sequencer (14). The purified X and C proteins were analyzed on the same instrument and also on a gas phase instrument with improved sensitivity (13). A modification of the reversed-phase high-pressure liquid chromatographic procedure previously described (17) was used to analyze the phenylthiohydantoin (PTH) amino acid derivatives released at each cycle, and the PTH derivatives were quantitated by comparison with a standard mixture. To permit the determination of cysteine, E2 was reduced with dithiothreitol before sequencing, and the cysteines were modified with iodoacetamide. Cysteine was not determined in the other proteins.

RESULTS

Amino acid composition. As the first step in the analysis of the structural proteins of WEE,

TABLE 1. Amino acid compositions of WEE structural proteins

Amino acid	Composition in the following protein(s)[a]		
	E1	E2	Capsid
Aspartic acid and asparagine	7.86 (34)	7.07 (30)	9.44 (25)
Threonine	8.14 (35)	9.22 (39)	5.00 (13)
Serine	8.85 (38)	6.96 (29)	3.94 (10)
Glutamic acid and glutamine	8.00 (35)	7.49 (31)	9.54 (25)
Proline	5.81 (25)	6.48 (27)	13.29 (35)
Glycine	6.37 (28)	6.66 (28)	8.84 (23)
Alanine	9.70 (42)	7.73 (32)	5.88 (15)
Cysteine	4.67 (20)	4.52 (19)	1.20 (3)
Valine	7.93 (34)	6.42 (27)	6.20 (16)
Methionine	1.06 (5)	1.07 (4)	2.96 (8)
Isoleucine	5.38 (23)	5.71 (24)	2.69 (7)
Leucine	6.66 (29)	6.78 (28)	5.97 (16)
Tyrosine	3.12 (14)	4.46 (19)	2.92 (8)
Phenylalanine	4.25 (18)	2.97 (12)	2.59 (7)
Histidine	3.68 (16)	4.64 (19)	1.30 (3)
Lysine	5.67 (25)	6.30 (26)	11.02 (29)
Arginine	3.12 (14)	5.77 (24)	7.22 (19)

[a] Reported as mole percents. Numbers in parentheses are the numbers of residues per molecule. These were calculated under the assumption that each protein had the same number of residues as the corresponding SIN protein (24). Values are rounded to the nearest integer.

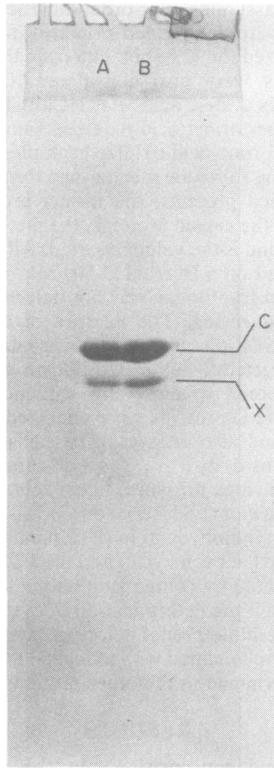

FIG. 1. SDS-polyacrylamide gel electrophoresis of WEE capsid proteins. After electrophoresis, gels were stained for protein with Coomassie blue. Lane A, SDS-disrupted nucleocapsids. Lane B, Capsid proteins prepared for amino acid analysis by hydroxylapatite chromatography.

their amino acid compositions were determined (Table 1). The precision of these values is estimated to be 5% or better. The E1 and E2 preparations used for the analysis were pure when examined by polyacrylamide gel electrophoresis, but the C protein preparation contained about 10% X protein (Fig. 1). This should not have significantly affected the results, since the X protein appears to be a shortened form of the C protein (16).

We note that the WEE capsid protein is relatively rich in proline and in the basic amino acids lysine and arginine, as is the case with SFV and SIN (9, 24). There are differences in the cysteine contents of the capsid proteins of the three alphaviruses, as WEE has three cysteine residues, SFV four, and SIN none. In addition, the amino acid compositions suggest that the envelope glycoproteins (but not the capsid proteins) of WEE are more similar to the SIN envelope proteins than to SFV (Table 2). Certainly this is a relatively insensitive measure of the differences between the proteins, since we can tabulate only the minimum number of amino acid replacements necessary to account for the differences in the compositions, rather than the actual number of differences in the sequences when they are compared position by position. Nonetheless, the results of Table 2 are quantitatively in agreement with the results of immunological studies and with the protein sequence data (see below).

N-termini of E1 and E2. We next determined the N-terminal sequences of WEE E1 and E2 by automated Edman degradation. For the sake of illustration, the results of the first 20 cycles of the analysis of E2 are shown in Fig. 2A, although the sequences were in fact determined by inspection of the high-pressure liquid chromatograms. The deduced sequences are shown in Fig. 3, which also shows the corresponding, previously determined sequences of SIN and SFV. As expected, there are no glycosylation sites or extensive hydrophobic regions in these portions of the WEE proteins. In the case of WEE E1, cysteine was not determined, but the failure to detect any PTH-amino acids at positions 49, 62, and 63, together with the overall homology among the three E1s, makes it probable that these residues are cysteines in WEE E1 also. The extent of the homology between WEE and SFV and especially SIN is striking (Table 3). Also, at least for SIN and SFV, the extent of the homology in the N-terminal region of E1 and E2 is similar to that observed in the entire proteins (Table 3).

Of particular interest are the first few residues of E1 and E2, since these N-termini are generated by proteolytic cleavage during the processing of the precursor polyprotein (25). The N-termini

TABLE 2. Differences in the amino acid compositions of alphavirus proteins[a]

Protein	Difference in no. of residues compared with:	
	SIN	SFV
E1		
WEE	36	65
SIN		57
E2		
WEE	32	60
SIN		54
Capsid		
WEE	56	62
SIN		62

[a] For each comparison, the difference in the number of residues present was calculated for each amino acid, excluding tryptophan, and these differences were summed without regard to sign. Calculations are based on data for WEE from Table 1 and on data for SIN and SFV as previously published (9, 10, 24).

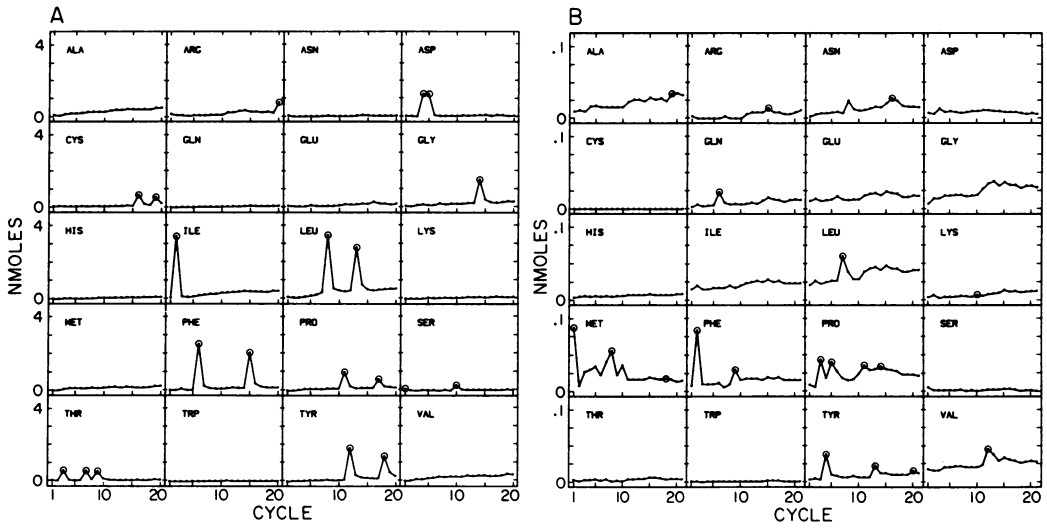

FIG. 2. Yields of amino acid PTH-derivatives from the spinning cup sequencer analysis of WEE proteins. Aliquots of each cycle were analyzed by high-pressure liquid chromatography, peaks were quantitated by comparison with a standard mixture of PTH-amino acids, and the yields were normalized to an injection of 100% of the sample. (A) The first 20 cycles of the analysis of 4.5 nmol of E2. (B) The analysis of 1 nmol of the capsid protein preparation containing both C and X proteins. Note the difference in the ordinate scales. Cysteine was not determined in (B).

of E1 and E2 are not identical in the three viruses, as there are several differences in the first four amino acids of each protein, although the differences are largely conservative.

N-termini of the C and X proteins. The results of Edman degradation of a C protein preparation containing about 10% X protein are shown in Fig. 2B, and the deduced sequence is shown in Fig. 3. Although the general background of PTH amino acid derivatives rose as expected, the yields of the specific PTH amino acids at each cycle were quite low, to the extent that the sequence was obtained from about 10% of the protein loaded on the sequencer, the rest of the protein being blocked. A tentative alignment of this sequence with the capsid protein of SIN is also shown in Fig. 3; this alignment requires the insertion of a large gap in the WEE sequence and a difference in the relative start points.

To determine which of the two proteins in the

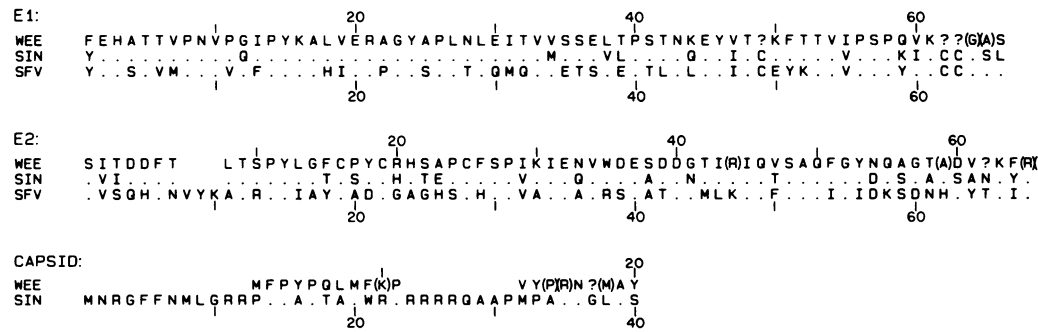

FIG. 3. The N-terminal sequences of the WEE structural proteins. Sequences are compared with the corresponding SFV (9, 10) and SIN sequences, with dots in the latter two sequences indicating positions of identity with the WEE sequences. The SIN E2 sequence shown differs at position 5 from the published sequence of our SIN HR strain (24) in the presence of D (characteristic of our SIN wild-type strain; J. R. Bell, unpublished data) rather than G. Gaps inserted in the sequences for alignment are indicated by blanks. For the WEE sequences, cysteine was determined only for E2. Parentheses indicate some uncertainty in the identification of residues, and question marks indicate that no residue could be identified. (The single letter amino acid code is: A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, Y = Tyr.)

TABLE 3. Homology between the N-terminal regions of alphavirus envelope glycoproteins

Protein	Homology (%)	
	WEE	SIN
E1		
SIN	81	
SFV	57	55 (53)
E2		
SIN	72	
SFV	40	44 (44)

[a] The values shown are the percentages of the positions in the aligned sequences of Fig. 2 which contain identical residues. Numbers in parentheses are the results of similar calculations applied to the sequences of the complete proteins as previously reported (10, 24), excluding the small, highly variable membrane-spanning roots (23).

capsid protein mixture contributed the sequence shown in Fig. 3, both the X and C proteins were purified by SDS-polyacrylamide gel electrophoresis and analyzed individually by Edman degradation. The purified C protein was found to contain the sequence of Fig. 3 at a somewhat lower level than in the mixture, probably due to the loss of free N-termini during polyacrylamide gel electrophoresis. No sequence could be detected from the purified X protein, but so little material was available for analysis that we were not confident of our ability to detect a sequence present at the 10% level. However, it was clear that the X protein was blocked to at least the same extent as the C protein.

DISCUSSION

The capsid of WEE is unusual in two respects when compared with other alphaviruses. Although we found most of the C protein of WEE to be blocked, we were able to detect and determine the N-terminal sequence of a small unblocked fraction of this protein. In contrast, the capsid proteins of SIN and SFV are blocked to the extent that no sequence can be detected (1, 4). We do not have any information on the structure of the N-terminus of the blocked fraction of the WEE C protein, but the simplest and most likely explanation is that it is an N-acetylated form of the unblocked fraction, since the N-terminal modification of the SIN capsid protein is known to be N^{α}-acetylation (3). Alternative explanations which could provide for different N-terminal amino acid residues, such as proteolytic cleavage of the blocked C protein to produce an unblocked C protein with a new N-terminus, would of course result in a difference in size in the two forms of the C protein, and we would have detected a minor C protein five residues or more shorter than the major component (see Fig. 1).

The second unusual feature of the WEE capsid is the presence of the X protein, a shortened form of the C protein (16). We were unable to determine the structure of the N-terminus of the X protein, except to note that it is blocked at least to the same extent as the C protein. This suggests that the N-termini of these two capsid proteins are the same, and that the difference lies at the C-terminal end, but evidence to support or refute this suggestion will require structural studies of the blocked N-termini of the X and C proteins.

The existence of these two types of variation in the WEE capsid is surprising in view of our current understanding of the alphavirus capsid structure. Host cell proteins are totally excluded from the virion during the budding of the SIN capsid through the host cell membrane (28), and the protein-protein interactions during budding are specific enough to result in an equimolar ratio of structural proteins in the alphavirus virion (25). In addition, neither vesicular stomatitis virus nor avian RNA tumor virus envelope glycoproteins can be incorporated into SIN virions, although SIN envelope proteins can be incorporated into the virions of either of these two unrelated viruses (32). These three observations imply very precise interactions between the capsid and the membrane glycoproteins of alphaviruses, and hence a high degree of order in the capsid structure. Nonetheless, the WEE capsid can accommodate a certain degree of variability in its protein components.

In this regard, it is interesting to note the cysteine contents of the capsid proteins of WEE and SIN. Cysteine residues are highly conserved among evolutionarily related eucaryotic proteins (8), among the HA proteins of different type A influenza viruses (29), among the envelope glycoproteins of SIN and SFV, except in hydrophobic membrane-spanning segments (10, 24), and among the N-termini of the envelope glycoproteins of WEE, SIN, and SFV (see below). Since this is in large part due to the unique role of cysteine in the formation of disulfide bridges which are part of the three-dimensional structure of proteins, the presence of three cysteine residues in the WEE capsid protein, and the lack of cysteine in the closely related SIN capsid, suggest that at least in some regions of the molecules the three-dimensional structures are quite different. However, there must also be regions where the structures are quite similar, since the sequences of the middle and C-terminal regions of the capsid proteins of SIN and SFV are highly homologous (5) and since alphavirus capsids carry broadly cross-reactive, group-specific antigenic determinants (7).

In contrast with the capsid protein, we were able to obtain extensive N-terminal sequence data from the WEE glycoproteins E1 and E2, and this allowed us to make detailed comparisons with the corresponding proteins of SIN and SFV. In particular, cysteine residues in the N-termini of the three alphavirus E2s are invariant, and our data indicate that they probably are conserved in the N-terminal region of the E1 proteins as well. The degree of homology among the three E1 proteins and among the three E2 proteins, including the conservation of cysteine residues, is impressive and lends strong support to the commonly held belief that the alphaviruses have descended from a common ancestor. The pattern of amino acid substitutions in the envelope proteins of the three viruses provides strong evidence that during evolution, WEE and SIN arose from a common ancestor, whereas SFV evolved from the ancestor of all three alphaviruses through a different lineage. In addition, we conclude from the data of Table 3 that WEE and SIN are equidistant, in an evolutionary sense, from SFV. The pattern of sequence homology is in agreement both with the results of immunological studies (6) and with a comparison of the amino acid compositions of the viral proteins, which suggests that WEE and SIN are much more closely related to each other than either is to SFV. However, the sequence comparison extends these findings to include a precise quantitative description of the relationships of these proteins in regions which are not subject to the mechanisms of selection operating on antigenic determinants.

Attempts to assess the relationship of various alphaviruses at the sequence level by nucleic acid hybridization (30) have been relatively uninformative and have reported extensive differences among most members of this group. It is clear from other work that this is due to the degeneracy of the genetic code and the fact that the third bases of the codons of the alphavirus RNAs show very little homology, except in a few regions of the molecule where the precise nucleotide sequence is obviously important (21). Thus, additional protein sequence information, either obtained directly or deduced from RNA sequences, will be most useful in evaluating the relationships of members of this virus group. Such an approach has yielded a great deal of information on the patterns and mechanisms of variation in the influenza viruses (29). We believe that it will continue to prove useful in describing the patterns and mechanisms of evolution in the alphavirus group.

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