The Synthesis and Maturation of a Non-structural Extracellular Antigen from Tick-borne Encephalitis Virus and Its Relationship to the Intracellular NS1 Protein

By JOHN M. LEE, ALAN J. CROOKS AND JOHN R. STEPHENSON*

Division of Biologics, PHLS, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, U.K.

(Accepted 13 October 1988)

SUMMARY

The replication of flaviviruses results in the secretion of four virus-coded proteins into the extracellular environment. Three of these proteins, E, C and M (or pre-M), are found in purified virions. A fourth virus-specified extracellular protein which was not present in either the slowly sedimenting haemagglutinin particles or in virions is described. The relationship of this protein to the intracellular NS1 polypeptide was investigated along with its similarity to the soluble complement-fixing antigen (SCF) reported for mosquito-borne flaviviruses. The difference in the M, of NS1 and SCF is the result of additional glycosylation of SCF, mostly by the addition of fucose molecules. The synthesis of E and NS1 is sequential but their secretion is simultaneous, suggesting a role for NS1 in virion protein transport or virion release.

INTRODUCTION

Viruses of the tick-borne encephalitis (TBE) complex are responsible for the most important arthropod-borne virus diseases in Europe (Westaway, 1987). The structural proteins of TBE virus (TBEV) particles (Heinz & Kunz, 1977) conform to the general pattern described for most flaviviruses (see Russell et al., 1980 for review), having a major envelope protein E, a minor envelope protein or membrane protein M (or its precursor) and a core protein C. Previous work in our laboratory has demonstrated that not only is the major envelope protein immunogenic, but another, non-virion protein (the 51K polypeptide) which is antigenically unrelated to E, is also a major immunogen in several mammalian species (Hambleton et al., 1983; Stephenson et al., 1984). Similar virus-specific, non-virion antigens have been described for other flaviviruses (Shapiro et al., 1971). Brandt et al. (1970) and Cardiff et al. (1970) both described an extracellular antigen of dengue virus which fixed complement, but was not present in virus particles. More recently evidence has been presented that the soluble complement-fixing antigen (SCF) of dengue virus is related to an intracellular, virus-specific protein, gp46 (Smith & Wright, 1985). Furthermore, Schlesinger et al. (1983) have demonstrated that the virus-specific, non-structural glycoprotein of yellow fever virus (YFV), NS1, is immunogenic and monoclonal antibodies (MAbs) raised against it demonstrate complement-fixing activity. The same is true of the 51K polypeptide of TBEV (Phillpotts et al., 1986).

The functions of the non-structural glycoproteins of flaviviruses are unknown at present, but recent work with YFV and dengue virus have suggested they could play a role in the control of disease. Experiments with YFV (Gould et al., 1986) have shown that antibodies directed against NS1 can have a protective function. Moreover, Schlesinger et al. (1987) have demonstrated that immunization with purified NS1 from dengue virus-infected cells can partially protect mice from an intracerebral challenge with dengue virus. Schlesinger et al. (1986) have shown a similar effect for YFV NS1 in monkeys.
We report here that cells infected with TBEV secrete an extracellular non-virion antigen as well as virus particles and the slowly sedimenting haemagglutinin (SHA) described previously. The molecular composition of this antigen, its relationship to NS1 and its kinetics of secretion are discussed.

**METHODS**

**Cells and viruses.** Monolayers of porcine kidney cells (PS cells) were infected with the Neudorfl isolate of TBEV as described previously (Stephenson et al., 1984).

**Infectivity assays.** Plaque assays in monolayers of PS cells were performed in 6 x 24-well cluster plates as described by de Madrid & Porterfield (1974).

**Preparation of radiolabelled proteins from infected cells and cell supernatants.** At 20 h post-infection (p.i.) the cell culture medium was replaced with 5 ml of phosphate-buffered saline (PBS) per 25 cm² flask. Two h later this was replaced with 2 ml of fresh PBS containing 500 µCi/ml [35S]methionine (> 800 Ci/mmol), 1 mCi/ml [3H]labelled sugars (10 to 40 Ci/mmol) or 50 µCi/ml [14C]-labelled amino acids (25 mCi/milligramatom of carbon). At 42 h p.i. the supernatant was removed and clarified three times by centrifugation at 10000 g, 4 °C for 2 min. The cell monolayer was covered with radiimmune precipitation buffer (RIP buffer) (0.15 M-NaCl, 10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 0.01% sodium azide, 1% NP40, 500 units aprotinin/ml) and stored at −20 °C.

**Radioimmune affinity chromatography (RIAC).** Radiolabelled antigens were prepared and RIAC analyses performed as described by Stephenson et al. (1987). Briefly, antigen and antibody were incubated overnight in RIP buffer at 4 °C and applied to a column of Protein A-Sepharose. After incubation at 4 °C for 1 h the columns were washed in RIP buffer and the antigen eluted in 8 M-urea, 10% SDS and 2% 2-mercaptoethanol.

**Purification of antigens for peptide mapping.** Affinity columns were prepared by reacting 2.5 mg of TBEV-specific MAbs in 5 ml PBS with 1 ml of preswollen cyanogen bromide-activated Sepharose. Antigen solutions were prepared as for RIAC and 1 ml was passed over a 1 ml affinity column at room temperature. The column was washed three times with 5 ml of RIP buffer (pH 8.0) containing 0.01% NP40 and the proteins were eluted with 1 ml of 0.05 M-acetic acid. The eluate was immediately neutralized by the addition of sodium hydroxide. The eluates were then concentrated to about 120 µl on Centricon 30 membranes (Amicon) and 50 µl was added to either 2.5 µl V8 protease (1 mg/ml) or 2.5 µl chymotrypsin (1 mg/ml). The samples were then incubated at 37 °C for 30 min and the digest was analysed by PAGE on 25% gels.

**PAGE.** Samples were run at 250 V for 2 h on 15% discontinuous SDS gels under denaturing conditions (Stephenson et al., 1977).

**Sucrose density gradient analysis.** Clarified infected cell supernatants were made 0.1% with respect to bovine serum albumin and 5% with respect to PEG 8000. After stirring for 1 h at 4 °C, the samples were pelleted at 10000 g, 4 °C for 10 min and each pellet was resuspended in 1 ml of phosphate buffer (0.185 M-Na2HPO4, 0.114 M-KH2PO4, 0.13 M-NaCl, pH 8.0). Samples were analysed by centrifugation in a Sorvall TT-4114 rotor at 80000 g, 4 °C for 4 hr on 10 to 30% linear sucrose gradients in phosphate buffer with a 50% sucrose cushion. All gradient fractions were stored at −20 °C before analysis. Sedimentation coefficients were estimated using ribosomal markers as standards.

**Preparation of MAbs.** MAbs T9 (anti-E), T12 (anti-NS1), T15 (anti-NS1) and T33/3 (anti-NS1) were prepared and characterized as described in Stephenson et al. (1984).

**Reagents.** [35S]Methionine, [3H]labelled sugars, [14C]-labelled algal hydrolysates, and [14C]methylated protein M markers were obtained from Amersham. Aprotinin was from Sigma and Protein A-linked Sepharose 4B and cyanogen bromide-activated Sepharose were from Pharmacia. *Staphylococcus aureus* V8 protease was from Miles Laboratories and a-chymotrypsin (from bovine pancreas) was from Serva.

**RESULTS**

**Virus-specific extracellular antigens**

When clarified supernatants from infected cells labelled with [35S]methionine were analysed by PAGE three polypeptides were detected which were not found in similar preparations from uninfected cell supernatants (Fig. 1). The polypeptide with an apparent Mr of 58K corresponds in mobility to a similar species (E) found in infected cells (Fig. 1, lane 4) and virions (Heinz & Kunz, 1977; Stephenson et al., 1987). The virion capsid protein (C) was seen migrating with an apparent Mr of 16K but the minor envelope protein M could not be detected. The third virus-specific polypeptide migrated as a heterogeneous species with an apparent Mr between 56K and 54K. This species does not correspond in Mr to any virus-specific species detected in virions or in infected cells.
Identity of extracellular antigens

The virus-specific extracellular antigens were identified by reacting infected cell supernatants with MAbs in an RIAC assay. Antibody T9 which displays neutralization and protective activities (Phillpotts et al., 1985, 1986) reacts only with the E glycoproteins of virus particles (Stephenson et al., 1987), the 58K intracellular polypeptide and the 56K extracellular polypeptide (Fig. 2a). Antibodies T12, T15 and T33/3 which react exclusively with the intracellular NS1 polypeptide (Stephenson et al., 1984) precipitate only the heterogeneous species migrating with an apparent $M_r$ between 56K and 54K (Fig. 2a). The relationship between these intracellular and extracellular polypeptides was confirmed as follows. Preparations of each protein were purified by affinity chromatography on columns containing either T9 (anti-E) or T12 (anti-NS1) MAbs as in Methods. The acid eluates were digested with either V8 protease or chymotrypsin and the digests were analysed by PAGE (Fig. 2b). Peptides of the E protein from both extracellular sources and intracellular sources appeared to be
identical. The peptides generated by NS1 and the heterogeneous extracellular antigen showed broad similarities, but some differences were observed. Thus both the intracellular and extracellular forms of E appear identical and NS1 is related both antigenically and in its peptide structure to the heterogeneous extracellular 56K to 54K protein and is therefore probably its intracellular precursor.

**Sedimentation of virus-coded extracellular antigens**

Antigen preparations from infected cell supernatants were concentrated and analysed on sucrose gradients as in Methods. To facilitate the resolution from cellular proteins, gradient fractions were purified by RIAC and analysed by PAGE (Fig. 3a). The E protein sediments as two distinct species, one at 200S and the other at 90S, more clearly delineated in Fig. 3(b). The E protein migrating at 200S is coincident with viral infectivity (Fig. 3b) and therefore represents virus particles. The 90S species does not contain infectious virus and is the SHA described before for TBEV and several other flaviviruses. The 56K to 54K heterogeneous protein migrates very slowly at about 5S and is found neither in SHA nor infectious virus particles.

**Kinetics of synthesis and excretion of extracellular viral antigens**

The time courses of the synthesis and secretion of both the E and the 56K to 54K proteins were followed by labelling cells for various times, purifying the intracellular and extracellular products by RIAC and analysing them by PAGE (Fig. 4). The analysis of the kinetics of E
production demonstrates that a delay of at least 45 min occurs between the synthesis and the secretion of this protein (Fig. 4a). The analysis for the 56K to 54K protein revealed a similar delay of at least 45 min (Fig. 4b). Thus these polypeptides appear to be secreted simultaneously even though their synthesis is sequential.
Maturation of the NS1 protein to produce SCF

The migration of the extracellular form of NS1 (the 54K to 56K protein) on SDS-PAGE suggests that it has a higher $M_r$ than NS1 itself. This difference in migration is too small to be caused by oligomerization and the PAGE conditions would not detect a conformational change. As the extracellular form migrates heterogeneously on PAGE, a possible explanation is that NS1 is further glycosylated immediately before or during its release from the cell. To examine this possibility infected cells were labelled with either $[^3H]$galactose, $[^3H]$N-acetylglucosamine, $[^3H]$fucose or $[^3H]$mannose and extracellular antigens were prepared as in Methods. The antigen mixtures were purified by RIAC using either MAb T9 (anti-E) or MAb T12 (anti-NS1) and analysed by PAGE (Fig. 5). Both the E and the 54K to 56K extracellular antigens were labelled with all four sugars but the latter appeared to be labelled more intensely. In order to quantify the relative incorporation of $[^3H]$labelled sugars into intracellular and extracellular antigens, infected cell cultures were labelled with one of the $[^3H]$labelled sugars or with a $^{14}C$-labelled algal protein hydrolysate, purified by RIAC, and analysed by PAGE as before. The X-ray films were scanned and the intensity of each band was measured. The ratio of intensities of bands from $^{14}C$- and $[^3H]$labelled samples was calculated and the ratios from intracellular and extracellular proteins was compared (Table 1). As this method does not take into account intracellular sugar pools or the relative efficiency of their transport mechanisms, an absolute assessment of the amount of sugar in each protein cannot be obtained. However, it assesses relative amounts of sugar in proteins labelled under similar conditions. The $M_r$ of E appeared to differ little between the intracellular and extracellular forms and the ratios for N-acetylglucosamine, mannose and fucose remained similar. The exception was galactose and this may represent a limited secondary processing of the sugar chains in the Golgi apparatus. When the ratios for the intracellular and extracellular forms of NS1 were compared, again those for the core sugars mannose and N-acetylglucosamine remained constant. However, the ratios for the ‘Golgi sugars’, galactose and fucose, were dramatically raised in the SCF.

Fig. 4. PAGE analysis of proteins purified by RIAC from infected cells and cell supernatants, labelled with $[^35]S$methionine. (a) Proteins purified with MAb T9 (anti-E) and (b) proteins purified with MAb T12 (anti-NS1). Lanes 1 to 6, intracellular proteins; lanes 7 to 12, extracellular proteins. Fractions in lanes 1 and 6 were labelled for 30 min, lanes 2 and 8 for 45 min, lanes 3 and 9 for 60 min, lanes 4 and 10 for 75 min, lanes 5 and 11 for 90 min and lanes 6 and 12 for 105 min.
**TBE virus non-structural antigen**

1. **Fig. 5.** Glycosylation of extracellular antigens. Cells were infected with TBEV, labelled with tritiated sugars and extracellular antigens were purified by RIAC. Fluorographs of PAGE analyses were treated with PPO (Stephenson et al., 1977) and exposed for 4 days. Lanes 1, 3, 5 and 7, proteins purified with MAb T9 (E-specific); lanes 2, 4, 6 and 8, proteins purified with MAb T12 (NS1-specific). Samples in lanes 1 and 2 are labelled with N-[3H]acetylglucosamine, lanes 3 and 4 with [3H]mannose, lanes 5 and 6 with [3H]fucose and lanes 7 and 8 with [3H]galactose.

2. **Table 1.** Semi-quantitative analysis of the incorporation of radiolabelled sugars into TBEV proteins

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* The ratios were calculated as follows. Cells were labelled overnight with 3H-labelled sugars or a 14C-labelled protein hydrolysate as described in Methods. Equal fractions of internal and external antigen preparations were purified by RIAC (see Methods) and analysed by PAGE. Comparable samples were run on the same gel and all X-ray films were exposed for 5 days. The relevant bands were scanned on a Joyce-Loebl densitometer and an integral was obtained. Each value is the ratio of such integrals.

**DISCUSSION**

Experiments reported here demonstrate that apart from the E, C and M polypeptides, PS cells infected with TBEV secrete another virus-specific polypeptide with an apparent Mr between 56K and 54K. Sucrose gradient analysis of radiolabelled extracellular antigens of TBEV demonstrated that the second extracellular glycoprotein was found neither in infectious virions.
nor in the SHA particles, and its sedimentation coefficient of 5S on sucrose gradients suggests it might exist as a dimer when secreted from the cell. Recent studies on dengue virus (Winkler et al., 1988) have shown that the intracellular form of NS1 can exist as a dimer.

As some MAbs that bind the 56K to 54K protein also fix complement (Phillpotts et al., 1986), this protein is glycosylated, secreted from the cell, but not found in either virions or the SHA, it is almost certainly equivalent to the SCF antigen described for dengue virus (Brandt et al., 1970), Japanese encephalitis virus (Rai & Ghosh, 1976) and West Nile virus (Lavrova, 1977). Results reported here have also indicated that the SCF of TBEV shares common peptides with the intracellular NS1 protein and both proteins contain similar antigenic sites. It is apparent therefore that the intracellular polypeptide, NS1, is a precursor of the extracellular SCF antigen. The possibility of intracellular NS1 itself being formed from a precursor involving NS2a sequences has been raised by the experiments of Mason et al. (1987). However such precursors were only detected when cloned genes from Japanese encephalitis virus were expressed in Escherichia coli and may only occur in artificial systems. No such precursors have yet been identified in vivo. Elucidating the steps in the maturation of NS1 to SCF does not appear to be straightforward. Originally Rice et al. (1985) suggested from cDNA sequence studies that the NS1 protein from YFV was attached to cell membranes by a hydrophobic C terminus. Recent results of Speight et al. (1988) have questioned this assumption by identifying cleavage sites between NS1 and NS2A such that NS1 no longer contains sequences coding for a hydrophobic C terminus. The resolution of these apparent discrepancies must await C-terminal analysis of purified proteins and further experiments on the subcellular location of NS1. Experiments reported here clearly demonstrate that extensive glycosylation of NS1 occurs, possibly as it passes through the Golgi apparatus, before its release from the cell. Such a process must be rapid as no intracellular forms of NS1 with the same glycosylation patterns as the external form, SCF, are found. Thus, unlike the E protein, where processing of the sugar chains in the Golgi appears limited, extensive processing of the sugar chains of NS1 appears to occur in the Golgi.

Finally, experiments to elucidate the kinetics of synthesis and secretion of these major viral immunogens reveal that at least 45 min elapse between the synthesis of E and its secretion into the medium. These experiments also indicated a similar lapse of time between the synthesis of NS1 and the appearance in the medium of its cleavage product, SCF. This time-lag probably reflects the time taken for the protein to travel through the Golgi apparatus to intracellular vesicles and the time taken for these vesicles to reach the cell surface. It is of interest that although NS1 is synthesized after E (Stephenson et al., 1987) the appearance of these two proteins in the extracellular medium is simultaneous.

At present the roles of the intracellular NS1 protein and its extracellular product the SCF antigen are unknown. As neither NS1 nor SCF can be found in virus particles it is difficult to see how either could be important for virion attachment. However, NS1 is secreted simultaneously with the virion protein E and it is possible therefore that NS1 could have a role in the transport of virus proteins or in the release of virus particles.

We wish to thank Mrs Denise Wright for preparing the manuscript.

REFERENCES
TBE virus non-structural antigen


(Received 13 May 1988)