Characterization of yellow fever virus proteins E and NS1 expressed in Vero and Spodoptera frugiperda cells

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The cDNA encoding the E and NS1 proteins of the yellow fever virus (YFV) was expressed in Spodoptera frugiperda cells via the recombinant baculovirus Ac-E. NS1 as a gp100 precursor which was cleaved to generate the recombinant proteins E and NS1 similar in size, folding and antigenicity to the authentic ones. Recombinant protein E exhibited immunodominant epitopes as judged by its reactivity with YFV-neutralizing MAbs. Using the Triton X-114 phase separation system, authentic and recombinant E proteins as well as the gp100 precursor exhibited hydrophobic properties similar to those of integral membrane proteins. Recombinant protein E was found neither in the extracellular medium nor on the cell surface, suggesting that it did not migrate within the secretory pathway of insect cells. Analysis of protein NS1 expressed in primate and insect cells revealed that the newly synthesized 48K NS1 glycoprotein was converted to a heat-labile gp72 homo-oligomeric form. This phenomenon did not require the presence of carbohydrate groups. Using the Triton X-114 phase separation system, the oligomeric form of NS1 was shown to be associated with cellular membranes although it appeared less hydrophobic than protein E and gp100. A small fraction of YFV NS1 oligomers were transported throughout the secretory pathway to be shed into the extracellular medium of primate cells. YFV NS1 oligomers migrated from the endoplasmic reticulum to the Golgi complex, whereas their N-oligosaccharides of the high-mannose type are processed to the complex-mannose type. Protein NS1 expressed by recombinant baculovirus-infected insect cells was not found in the extracellular medium but associated with the plasma membrane of the cells. Two recombinant NS1 forms were detected in insect cells: a major one with an apparent M, of 48K and a minor one of 47K in which N-linked glycans were probably processed to a trimannosyl core without further elongation. Thus, it appears that the transport strategy as well as the N-glycosylation of NS1 in insect cells infected with recombinant baculovirus were different from those of the NS1 in primate cells infected with YFV.

Introduction

Yellow fever virus (YFV) is the prototype of the family Flaviviridae, which includes some 70 viruses, many capable of producing severe illness such as dengue haemorrhagic fever, Japanese encephalitis (JE) and tick-borne encephalitis. YFV is an enveloped virus containing a positive-sense ssRNA genome of 10862 bases (Rice et al., 1985). The viral RNA is the only mRNA synthesized during the replicative cycle (Desprès et al., 1986). Complete nucleotide sequences of three YFV strains have been determined (Rice et al., 1985; Desprès et al., 1987; Hahn et al., 1987; Dupuy et al., 1989). Sequence analysis has revealed the presence of a single open reading frame of 10233 bases encoding a polyprotein of 3411 amino acid residues. The polyprotein precursor by viral and cellular proteases (Rice et al., 1985, 1986). Recently we confirmed the role of cellular signalases in the maturation of the structural proteins C (capsid), prM (precursor to membrane protein M) and E (envelope) and showed that the domains responsible for the translocation of prM and E inside the membranes of the endoplasmic reticulum (ER) were located in the hydrophobic regions preceding the N terminus of the respective mature proteins (Ruiz-Linardès et al., 1989; Desprès et al., 1988, 1990).

The envelope protein E present at the surface of the virion is the most important structural antigen; it is an integral membrane protein which binds to cellular receptors and induces neutralizing antibodies (Schlesinger et al., 1983; Buckley & Gould, 1985). Several neutralizing and non-neutralizing monoclonal antibodies (MAbs) to YFV E protein were shown to protect mice passively against a challenge with homologous virus (Brandriss et al., 1986; Gould et al., 1986).
The non-structural glycoprotein NS1 was identified originally as the soluble complement-fixing (SCF) antigen in the extracellular medium (Brandt et al., 1970; Russell et al., 1980) and later detected on the surface of flavivirus-infected cells (Stoholm et al., 1975; Cardiff & Lund, 1976; Gould et al., 1985). Its function is still unknown; it may play a role in virus assembly and maturation (Rice et al., 1986). Its role in protection might result from antibody-dependent, complement-mediated cytolysis of infected cells expressing NS1 on their membranes (Schlesinger et al., 1985, 1986, 1990; Gould et al., 1985; Mason, 1989). Whereas immunity to YFV infection has generally been related to the presence of neutralizing antibodies elicited against the virus envelope protein, immunization with the purified non-structural glycoprotein NS1 (Schlesinger et al., 1985, 1988) or bacterially-expressed NS1 polypeptides (Cane & Gould, 1988) has been shown to protect mice and primates against a challenge with YFV. Furthermore, several MAbs specific for YFV NS1 had a cytoplastic activity in the presence of complement and were capable of passively protecting animals from a challenge with homologous virus (Schlesinger et al., 1985, 1988; Gould et al., 1986). The data suggest strongly that to prevent infection, immune recognition of protein NS1 may provide an alternative to direct flavivirus neutralization.

The dual role of neutralizing and non-neutralizing mouse antibodies in flavivirus protection has been substantiated by injecting recombinant vaccinia virus or recombinant baculovirus-infected insect cells expressing protein(s) E and/or NS1 from dengue virus (DEN) (Zhao et al., 1987; Zhang et al., 1988). Protection was obtained when DEN protein E or NS1 was expressed alone, but no significant response to protein E was found in the mouse sera prior to virus challenge (Bray et al., 1989; Lai et al., 1989). The E protein of JE virus was also expressed via recombinant baculovirus (Matsuura et al., 1989) and vaccinia virus (Yasuda et al., 1990). Inoculated mice were protected against a challenge with infectious virus but in contrast to DEN, protein E induced a good level of neutralizing antibodies.

To elucidate further the mechanism of action of proteins E and NS1 in the induction of a protective response, abundant expression of these antigens is needed. We have chosen to express these gene products using the baculovirus expression system. In this study, the YFV cDNA encoding proteins E and NS1 with their signals for translocation into the ER and cleavage at the N and C termini, was expressed in infected Spodoptera frugiperda cells under the transcriptional regulation of the baculovirus polyhedrin gene promoter. As will be shown, insect cells infected with the recombinant baculovirus synthesized E- and NS1-related products similar in size, folding and antigenicity to those expressed in primate cells infected with YFV.

### Methods

**Cells and viruses.** S. frugiperda IPLB-SF21-AE clonal isolate 9 (designated Sf9) cells were propagated at 28°C as monolayers in Grace's medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Infection with wild-type Autographa californica nuclear polyhedrosis virus (AcNPV) or recombinant baculovirus was performed at a multiplicity of approximately 10 p.f.u./cell. Vero (African green monkey cell lines) cells were grown at 37°C in Eagle's MEM (Dulbecco) supplemented with 5% heat-inactivated FBS. Infections with the YFV 17D-204 Pasteur vaccine strain were performed at a multiplicity of approximately 10 p.f.u./cell (Desprès et al., 1987).

**Construction of plasmids.** This was performed using standard techniques (Maniatis et al., 1982). The sequence encoding YFV proteins E and NS1, which includes the signal peptide of E and the ATG initiating codon of simian virus 40 (SV40) protein VP1, was isolated from plasmid pSV-E NS1 as a 2.6 kbp DNA fragment (Desprès et al., 1990). Prior to insertion into the baculovirus vector, an in-frame stop codon was introduced at the 3' end of this cDNA by digesting plasmid pSV-E NS1 with XbaI (unique site in multilinker) and filling-in with the Klenow fragment. The blunt ends were then circularized with T4 DNA ligase, generating plasmid pSV-E NS1 (TAG) (Fig. 1a).

To facilitate its insertion into the transfer vector, the YFV cDNA was removed from pSV-E NS1 (TAG) in two fragments of 0.70 kbp and 1.90 kbp as described in Fig 1(a). The 0.70 kbp fragment, which contains the sequence coding for the N-terminal half of protein E including its signal peptide, was obtained by digestion with HindIII [nucleotide (nt) 1488 on the SV40 genome] and Apol (nt 1603 on the YFV genome), the HindIII site being filled in with Klenow enzyme. The 1.90 kbp fragment, which contains the sequence encoding the C-terminal half of protein E followed by the complete protein NS1, was obtained by digestion with BglII (3' end site of multilinker on recombinant SV40 DNA) and Apol, the BglII site being filled in with Klenow enzyme.

The two YFV cDNA fragments were ligated with the blunt-ended BamHI site of linearized plasmid pVL 941-poly (Fig 1a). Plasmid pVL 941-poly was modified from pVL 941 (Luckow & Summers, 1989) by inserting a multilinker at the 3' end of the BamHI site (P. Gonnet, unpublished results). A recombinant transfer vector containing the 2.6 kbp insert encoding YFV proteins E and NS1 was designated pAc-E NS1 (Fig. 1a). Plasmid pAc-E NS1 was cotransfected with the genome of the wild-type AcNPV into Sf9 cells to generate recombinant baculoviruses using standard calcium phosphate precipitation procedures (Summers & Smith, 1987). Occlusion-negative viruses were plaque-purified, and one of them, pAc-E NS1 (Ac-E NS1) was isolated from plasmid pSV-E NS1 (Fig. 1a).

To confirm that the entire YFV cDNA had been transferred into the baculovirus genome, the viral DNA was extracted from Ac-E NS1-infected Sf9 cells. To isolate the sequence encoding E and NS1, we took advantage of the presence of two EcoRV sites surrounding the YFV cDNA, one at position –96 in the polyhedrin gene (Rohrmann, 1986) and the other in the multilinker downstream from the insert. After migration of the digestion products in an agarose gel, the passenger DNA with the correct size was revealed using a specific cRNA probe.
were electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell). The signal peptide of protein E and the stop codon at the C terminus of protein NS1 was isolated from plasmid pSV-E NS1 (TAG) and introduced into transfer vector pVL 941-poly, generating plasmid pAc-E NS1.

Protein labelling in SF9 and Vero cells. Before labelling, cells were washed twice and incubated in methionine-free medium for 60 min. SF9 cells were pulse-labelled for 180 min at 27 h post-infection (p.i.) with 100 μCi of [35S]methionine per ml. The YFV-infected Vero cells were pretreated at 8 h p.i. with 5 μg/ml of actinomycin D and labelled with 100 μCi of [35S]methionine per ml in the presence of actinomycin D. When labelled in the presence of tunicamycin (5 μg/ml), the cells were pretreated for 60 min with the drug. For pulse-chase experiments, infected cells were labelled for 30 min with 200 μCi of [35S]methionine per ml. After the labelling period, cells were washed and chased for various periods with an excess of unlabelled methionine (10 mM).

After labelling or chase, the proteins present in the supernatant were precipitated by the addition of nine volumes of 95% ethanol and immunoprecipitated using the protocol described by Ruiz-Linarés et al., 1985. The immune complexes were treated with Endo-β-N-acetylglucosaminidase H (Endo H) or a trpE-E fusion protein (S. Gerbaud, unpublished results). After several washings the membrane was incubated successively for 1 h at room temperature with a 1:50 dilution of rabbit immune serum directed against the NS1 protein (Schlesinger et al., 1985) or a 1:500 dilution of biotinylated horse-radish peroxidase complex (Amersham). After washing, bound antibodies were visualized by reacting the membrane with 0.06% of 4-chloro-1-naphthol (Sigma) and 0.015% of hydrogen peroxide.

Protein labelling in SF9 and Vero cells. Before labelling, cells were washed twice and incubated in methionine-free medium for 60 min. SF9 cells were pulse-labelled for 180 min at 27 h post-infection (p.i.) with 100 μCi of [35S]methionine per ml. The YFV-infected Vero cells were pretreated at 8 h p.i. with 5 μg/ml of actinomycin D and labelled with 100 μCi of [35S]methionine per ml in the presence of actinomycin D. When labelled in the presence of tunicamycin (5 μg/ml), the cells were pretreated for 60 min with the drug. For pulse-chase experiments, infected cells were labelled for 30 min with 200 μCi of [35S]methionine per ml. After the labelling period, cells were washed and chased for various periods with an excess of unlabelled methionine (10 mM).

After labelling or chase, the proteins present in the supernatant were precipitated by the addition of nine volumes of 95% ethanol and incubation at -20 °C for 18 h as described previously (Després et al., 1990). To analyse intracellular proteins, cells were washed twice with cold PBS and lysed with cold radioimmunoprecipitation assay (RIPA) buffer (50 mM-Tris-HCl pH 7.5, 150 mM-NaCl, 10 mM-EDTA, 0.1% SDS, 1% Triton X-100, 1% deoxycholate) containing 25 μg/ml aprotinin (Sigma). After 5 min incubation in ice, cellular extracts were clarified by centrifugation for 5 min in a minifuge. The proteins were immunoprecipitated using the protocol described by Ruiz-Linarés et al., 1989 and resolved in 12% SDS-polyacrylamide gels.

Western blot analysis. Proteins separated in SDS–polyacrylamide gels were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell). The membrane was saturated with washing buffer (20 mM-Tris-HCl pH 7.5, 500 mM-NaCl) containing 3% FBS and incubated overnight at 4 °C with a 1:50 dilution of rabbit immune serum directed against the NS1 protein (Schlesinger et al., 1985) or a trpE-E fusion protein (S. Gerbaud, unpublished results). After several washings the membrane was incubated successively for 1 h at room temperature with a 1:50 dilution of biotinylated anti-rabbit Ig donkey serum and with a 1:500 dilution of streptavidin-biotinylated horse-radish peroxidase complex (Amersham). After washing, bound antibodies were visualized by reacting the membrane with 0.06% of 4-chloro-1-naphthol (Sigma) and 0.015% of hydrogen peroxide.

Endo-β-N-acetylglucosaminidase H (Endo H) and F (Endo F) treatments. SF9 cells infected with the recombinant baculovirus or Vero cells infected with YFV were labelled as described above and cellular extracts immunoprecipitated using NS1-specific MAb 8G4 (Schlesinger et al., 1983). The immune complexes were treated with Endo H (20 milliunits (mU)/ml) or Endo F (1 mU/ml) (Boehringer Mannheim) according to the procedure described by Jarvis & Summers (1989).

Triton X-114 extraction. Cells were rinsed with PBS and suspended in a solution of 2% Triton X-114 (BDH) in 50 mM-Tris-HCl pH 7.5 containing 25 μg/ml of aprotinin and incubated for 10 min on ice. The aqueous and detergent phases were separated by warming and centrifuging at 37 °C in a minifuge at 3000 r.p.m. (Bordier, 1981; Mason, 1989). The aqueous phase was collected and after several washings with buffer E (10 mM-Tris-HCl pH 7.5, 150 mM-NaCl, 5 mM-EDTA), the detergent phase was obtained. Aqueous and

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Fig. 1. Construction of recombinant baculovirus Ac-E NS1. (a) The sequence encoding the YFV E and NS1 proteins including the start codon derived from SV40 protein VP1, the signal peptide of protein E and the stop codon at the C terminus of protein NS1 is reported. The ATG initiation codon and the TAG stop codon are overlined. The amino acid sequence at the N and C termini of the translated polypeptide is indicated with numbers below, referring to the N and C amino acids which are derived from the YFV polyprotein.

(b) Structural map of the recombinant baculovirus Ac-E NS1. Nucleotide sequence at the junction between the polyhedrin gene, the 5' end of the region coding for protein E (including its signal peptide) and the 3' end of the region coding for NS1 is reported. The ATG start codon derived from SV40 protein VP1, the signal peptide of protein E and the stop codon at the C terminus of protein NS1 was synthesized from plasmid pGX4-E1 (Ruiz-Linarés et al., 1989) (not shown).
detergent phases were diluted with five volumes of buffer E, and the proteins were precipitated overnight with nine volumes of 95% ethanol at -20 °C and analysed in polyacrylamide gels.

**Immunofluorescence assay.** SF9 cells grown on coverslips were infected with AcNPV or recombinant virus for 24 h at 27 °C. The cells were fixed with formaldehyde and, when necessary, permeabilized with 0.1% Triton X-100 in PBS, as described previously (Després et al., 1988). The fixed cells, permeabilized or not, were incubated for 20 min at room temperature with a mouse immune serum directed against YFV (1:40 dilution) or with a 1:20 dilution of either the E-specific MAb 864 (Gould et al., 1985) or the NS1-specific MAB 8G4. Fluorescein isothiocyanate conjugated with anti-mouse immunoglobulin (Silenius) was used as the second antibody.

**Results**

**Construction of recombinant baculovirus Ac-E.NS1**

Recently, we described a SV40 recombinant called SV-E.NS1 containing a 2.6 kbp fragment of 17D-204 strain of YFV cDNA encoding proteins E and NS1 (Després et al., 1990). The N-terminal residue of the E protein [amino acid (aa) 286 on the YFV polyprotein] is preceded by its signal sequence derived from the preceding M protein (aa 271 to 285 on the YFV polyprotein precursor) and which is responsible for translocation into the ER (Després et al., 1990). The YFV cDNA encoding proteins E and NS1 was inserted into the baculovirus genome under the control of the polyhedrin promoter. This produced the recombinant baculovirus Ac-E.NS1 (Fig. 1b) in which the translation initiating codon was derived from the SV40 VP1 gene (Fig. 1b). According to data reported by Kozak (1986), Rohrmann (1986) and Luckow & Summers (1988), translation of the polyhedrin-linked mRNA encoding YFV proteins should initiate at the expected AUG start codon since it is in a favoured context. The 3′ end of the insert was chosen to represent the correct C terminus of the NS1 protein. Our choice was guided by the structure of DEN-2 protein NS1. Its C terminus has been sequenced and shown to be followed immediately by the N terminus of protein NS2A (Wright et al., 1989). In the case of YFV, only the N terminus of the NS2A protein has been sequenced and positioned on the polyprotein (Chambers et al., 1989). Therefore, based on sequence alignments, we assumed that the C terminus of YFV NS1 protein was located at residue 1130 in the polyprotein. A stop codon was introduced downstream from this sequence, generating the extraneous sequence GGSSS at the carboxy terminus of the NS1 protein (Fig. 1b).

**Detection of recombinant proteins E and NS1**

Recombinant Ac-E.NS1 contains YFV cDNA that encodes a polyprotein precursor to proteins E and NS1 with a theoretical Mr of 100K (865 amino acid residues). To confirm that these proteins were expressed and processed correctly in SF9 cells, Ac-E.NS1-infected insect cells were labelled with [35S]methionine for 3 h at 27 h p.i. This period was found to be optimal for labelling the recombinant proteins.

As shown in Fig. 2, when the viral proteins were immunoprecipitated with a mouse immune serum directed against YFV or with E- and NS1-specific MABs, recombinant proteins E and NS1 migrated with the same mobilities (apparent Mr, 54K and 48K respectively) as the authentic proteins from YFV-infected Vero cells. When the cells were treated with tunicamycin, an inhibitor of N-glycosylation of proteins, the authentic and recombinant NS1 comigrated with an apparent Mr of 43K (Fig. 2). We confirmed here that neither the recombinant nor the authentic E protein was glycylated as judged from the absence of any effect of the drug on their electrophoretic mobility (Schlesinger et al., 1983; Ruiz Linares et al., 1989; Després et al., 1990).

The antigenicity of the recombinant proteins was analysed using MABs directed against the E or NS1 protein. Neutralizing MABs 2C9 (Schlesinger et al., 1983) and 864 (Gould et al., 1985) recognize the envelope protein synthesized by the 17D-204 vaccine strain of YFV and react with the recombinant E protein expressed by an SV40–YFV hybrid virus (Després et al., 1990). As shown in Fig. 2, both MABs also reacted with the recombinant E protein synthesized in insect cells. Since these MABs recognize the native but not the denatured form of the E protein (Després et al., 1990), it can be concluded that the folding of recombinant E is similar to that of the authentic protein. The two MABs immunoprecipitated additional polypeptides which migrated faster than the E protein and may be similar to the degradation products already found in YFV-infected cells (Schlesinger et al., 1988; Cane & Gould, 1989). MAB 8G4 reacts specifically with the YFV NS1 protein (Schlesinger et al., 1983). It has been shown to play a role in complement-mediated lysis of infected cells and to protect mice passively from a lethal YFV challenge (Schlesinger et al., 1985, 1988). As shown in Fig. 2, MAB 8G4 immunoprecipitated the YFV protein NS1 produced in infected Vero cells as well as the unglycosylated form synthesized in the presence of tunicamycin. The recognition by MAB 8G4 was greatly altered when the YFV non-structural glycoprotein was treated with SDS and DTT, indicating that the epitope was conformation-dependent (data not shown). MAB 8G4 also reacted with the recombinant NS1 protein from Ac-E.NS1-infected SF9 cells (Fig. 2). In addition to the recombinant 48K protein, a minor polypeptide with an Mr of 47K was immunoprecipitated from insect cell lysates infected with Ac-E.NS1. As discussed below, it may represent a
Fig. 2. Expression of recombinant E and NS1 proteins. Sf9 cells infected with AcNPV (wt), Ac-E. NS1 (Ac) or mock-infected (m) were labelled for 3 h at 27 h p.i. with 100 μCi of [35S]methionine per ml. Similarly, Vero cells infected with the 17D-204 vaccine strain of YFV (17D) were labelled for 3 h at 27 h p.i. in the presence of actinomycin D (5 μg/ml). Tunicamycin (5 μg/ml) was present (+ Tun) or absent (−Tun) throughout the labelling period. The proteins were immunoprecipitated with a mouse immune serum directed against YFV, with the E-specific MAbs 2C9 and 864, or with the NS1-specific MAb 8G4 and analysed in a 12% SDS-polyacrylamide gel. The reactivity of E-specific MAbs was tested only on proteins from insect cell lysates infected with Ac-E. NS1. The polypeptide precursor to E and NS1 is indicated by a star (lane −Tun). Mr markers are indicated on the left.

recombinant NS1 form with a different oligosaccharide structure. In addition, we repeatedly observed that the recombinant E protein was immunoprecipitated nonspecifically by this MAb when the cells had been lysed with RIPA buffer.

Detailed analysis of Sf9 cells infected with recombinant baculovirus Ac-E. NS1 revealed the existence of two large polypeptides with MrS of approximately 100K (Fig. 2, star) which reacted with the mouse immune serum directed against YFV as well as with the E- and NS1-specific MAbs (Fig. 2). Fortuitously, the larger polypeptide with an Mr of 105K migrated with the same mobility as the YFV non-structural protein NS5 (Fig. 2, see lane 17D). When the recombinant proteins expressed by Ac-E. NS1 were synthesized in the presence of tunicamycin, only the faster migrating band with an Mr of 100K was detected (Fig. 2). These data as well as additional results presented below indicated that the 100K and 105K polypeptides were indeed the uncleaved precursors to recombinant E and NS1 in their unglycosylated and glycosylated forms respectively. The existence of these two forms suggests that glycosylation is limited in S. frugiperda cells.

Although the recombinant E and NS1 proteins and their precursor were detected easily by immune precipitation after [35S]methionine labelling, Coomassie blue staining of total proteins did not demonstrate the presence of specific YFV polypeptide bands (not shown). This indicated that the level of expression of the recombinant proteins was substantially lower than that of the polyhedrin produced by AcNPV and by recombinant baculoviruses expressing E or NS1 alone (P. Desprès et al., unpublished results). The reasons for the low level of YFV cDNA expression could be due to intrinsic characteristics of the YFV sequences or their products.

Together, these results indicate that recombinant E and NS1 proteins expressed by Ac-E. NS1 virus in S. frugiperda cells are similar in size, folding and antigenicity to those synthesized during infection with YFV in Vero cells.

Detection of oligomeric and membrane-associated forms of the NS1 protein

Oligomerization of NS1 in mosquito and mammalian cells infected by several flaviviruses has been described (Winkler et al., 1988, 1989; Mason, 1989; Schlesinger et al., 1990). The NS1 dimers formed during DEN infection are resistant to reduction and SDS treatments but sensitive to heat denaturation (Winkler et al., 1988). This raised the question of whether NS1 oligomerization also occurred in S. frugiperda cells infected with virus
Ac-E.NS1. Thus, it was necessary to establish the conditions for detection of NS1 oligomers in primate cells infected with YFV.

The proteins expressed by YFV in Vero cells were chase-labelled and analysed in SDS-polyacrylamide gels before and after heat denaturation. After a 30 min labelling period, the 48K NS1 protein was detected as a faint band in the denatured samples and became more prominent after a 60 min chase (data not shown). When the samples were not heat-denatured, the band corresponding to the 48K protein became weaker and weaker as the chase progressed. Concomitantly, a new viral band with an $M_r$ of 72K appeared, which became more and more intense during the chase. Fig. 3(a) shows the existence of the heat-labile 72K polypeptide after a 90 min chase. When tested by Western blotting, the 72K polypeptide reacted with a rabbit immune serum directed against YFV NS1 (Schlesinger et al., 1985) and, after heat denaturation, it dissociated to generate the 48K NS1 protein (Fig. 3b). This indicates that the 72K polypeptide represents an oligomeric form of the NS1 protein.

We investigated the solubility of YFV E and NS1 proteins in the two-phase system from Triton X-114-extracted cell lysates, a method used to analyse the NS1 protein from DEN and JE virus-infected cells (Winkler et al., 1988, 1989; Mason, 1989). By direct analysis of the labelled cytoplasmic extract (data not shown) and Western blot analysis using a rabbit immune serum against the YFV E protein (Fig. 4a), we observed that the E protein was extracted completely into the detergent phase and none was found in the aqueous phase, as expected for an integral membrane protein. On the other hand, the heat-sensitive NS1 oligomers were present in both phases (Fig. 4a). Based on its solubility in both the Triton X-114 detergent and aqueous phases, the oligomeric form of NS1 must interact with the lipid bilayer but seems nevertheless to be less hydrophobic than a typical membrane protein. When N-glycosylation was blocked with tunicamycin, we observed the presence of a 62K polypeptide in the unboiled sample which was converted to the unglycosylated form of NS1 after heat denaturation (not shown). This result suggests that the 62K polypeptide is the unglycosylated form of the 72K polypeptide and that oligomerization of NS1 occurs without a requirement for N-linked oligosaccharide groups on the viral protein. No distinct band other than NS1 appeared after heating, suggesting that the 72K polypeptide is a homo-oligomer (not shown). On the basis of the $M_r$ of the denatured and undenatured forms (48K and 72K), we cannot rigorously establish that the high $M_r$ species represents a dimer, unless we assume that one or both forms have an unexpected electrophoretic mobility. However, this concept is supported by the fact that oligosaccharides contribute 10K to the oligomeric form. This would correspond to the mass of four oligosaccharides, since, as already reported, the NS1 monomer possesses two N-linked glycans which together contribute 5K (Després et al., 1990).

When Ac-E.NS1-infected Sf9 cells were labelled at 27 h.p.i. for 30 min and chased with unlabelled methionine, we were unable to detect the recombinant E and NS1 proteins from total baculovirus polypeptides because of the high cellular background level (not shown). Therefore, we tried to fractionate the proteins from Triton X-114-extracted cell lysates into Triton X-114 detergent...
YFV E and NS1 in primate and insect cells

(a) Western blotting on Triton X-114-extracted cell lysates. Vero cells infected with YFV (17D) or mock-infected (mock) were suspended in a solution of 50 mM-Tris-HCl pH 7.5 containing 2% Triton X-114. Detergent (Tx) and aqueous (Aq) phases were prepared as described in Methods. Proteins were analysed by Western blotting using rabbit immune sera directed against YFV E (anti-E) or NS1 (anti-NS1). (b) Western blotting on Triton X-114-extracted Sf9 cell lysates infected with AcNPV (Ac) or Ac-E. NS1 virus (Ac-E. NS1).

Fig. 4. Western blotting on Triton X-114-extracted cell lysates. (a) Vero cells infected with YFV (17D) or mock-infected (mock) were suspended in a solution of 50 mM-Tris-HCl pH 7.5 containing 2% Triton X-114. Detergent (Tx) and aqueous (Aq) phases were prepared as described in Methods. Proteins were analysed by Western blotting using rabbit immune sera directed against YFV E (anti-E) or NS1 (anti-NS1). (b) Western blotting on Triton X-114-extracted Sf9 cell lysates infected with AcNPV (Ac) or Ac-E. NS1 virus (Ac-E. NS1).

with tunicamycin, the level of synthesis of viral polypeptides was reduced so that the unglycosylated form of NS1 could not be visualized against the background of labelled proteins (not shown) unless the lysates were extracted with Triton X-114. In this case, the unglycosylated form of recombinant NS1 partitioned itself into both phases (Fig. 8).

The large recombinant 100K polypeptide was completely extracted into the Triton X-114 detergent phase and reacted with rabbit immune sera directed against E or NS1 proteins expressed by YFV (Fig. 4b). The result confirms that the polypeptide represents the uncleaved precursor to recombinant E and NS1 and indicates that it is associated strongly with intracellular membranes. Pulse–chase analysis showed that the cleavage to generate recombinant E and NS1 occurred after the precursor had been completely translocated into the lumen of the ER (not shown). One should also note that the processing of the precursor was unaffected by tunicamycin, since the E and the unglycosylated NS1 proteins were produced in the presence of the drug (Fig. 2).

Transport and secretion of authentic and recombinant NS1 proteins in infected cells

Since the NS1 proteins from several flaviviruses have been described as SCF antigens (Brandt et al., 1970) and later as glycoproteins secreted into the culture medium (Winkler et al., 1988, 1989; Mason, 1989), it was of interest to determine whether the NS1 protein synthesized in primate or S. frugiperda cells was also secreted into the extracellular medium.

Vero cells infected with YFV were pulse-labelled with [35S]methionine for 30 min and chased with unlabelled methionine. Authentic NS1 protein was present in the culture fluid after 2 h of chase, then a plateau was reached rapidly (Fig. 5). The secreted form of NS1 migrated as a diffuse band with an apparent $M_r$ slightly higher than its cellular counterpart. The extracellular form was detected as the 72K oligomer when analysed before heat denaturation (not shown). Although the proportion of NS1 in the extracellular and intracellular fractions was not quantified, analysis of the autoradiograph clearly indicated that a large amount remained intracellular (Fig. 5). From the pulse–chase analysis, it can be concluded that the secretion of NS1 is a rather slow process, since more than 5 h are required for half of the pulse-labelled NS1 protein to be released into the cell culture medium. Tunicamycin treatment did not abolish the secretion of NS1 which was still detected as an unglycosylated oligomeric form ($M_r$, 62K) in the culture medium of primate cells treated with the drug (data not shown).
In contrast to Vero cells infected with YFV, the protein NS1 expressed in Sf9 cells infected with Ac-E.NS1 was not secreted, because none of these recombinant polypeptides could be detected in the extracellular fraction after a 30 min pulse followed by a 3 h chase (not shown). The cells were not chased for longer periods because of possible cell lysis due to the baculovirus infection. Although recombinant NS1 oligomers were not secreted, non-permeabilized Ac-E.NS1-infected S. frugiperda cells (Fig. 6h) gave a clear positive reaction when tested with the NS1-specific MAb 8G4 by immunofluorescence at 24 h p.i., suggesting that they migrated from the ER to the Golgi complex and were transported to the plasma membrane with which they became associated. This phenomenon did not occur with recombinant E, which was not detected on the cell surface (Fig. 6e,f). This observation was confirmed by indirect immunofluorescence using the E-specific MAb 864 on unfixed insect cells infected with Ac-E. NS1 (not shown).

N-glycosylation of authentic and recombinant NS1 proteins

Endo H is known to remove N-linked high-mannose sugar residues but not the complex type of N-linked glycans (Tarentino & Maley, 1974), whereas Endo F cleaves high-mannose and complex oligosaccharides equally well (Elder & Alexander, 1982; Tarentino et al., 1985). Resistance to removal of carbohydrate side chains by Endo H is acquired after the conversion of GlcNAc2-Man3 to GlcNAc2-Man2-GlcNAc, an event that occurs in the medial region of the Golgi system (Kornfeld & Kornfeld, 1985). Thus, sensitivity or resistance to Endo H treatments of the N-linked oligosaccharide groups present on the NS1 protein expressed by YFV or by Ac-E. NS1 virus should give us an insight into its transport into the intracellular secretory pathway and an indication of the compartment where oligomerization occurs.

The intracellular and extracellular samples from the 2 h chase experiment (Fig. 5) were analysed for their reactivity with endoglycosidases. The extracellular NS1 protein of YFV appeared to be almost completely resistant to Endo H and showed the same heterogeneity as the untreated form but, as expected, was sensitive to Endo F (Fig. 7). This indicates that NS1 must traverse the secretory pathway where high-mannose oligosaccharides are processed to Endo H-resistant complex sugars. The heterogeneity of the released NS1 probably results from multiple modifications of the N-linked oligosaccharides. In contrast to the secreted form, the intracellular NS1 protein was fully sensitive to Endo H, indicating that it did not move to the Golgi complex but
stayed in the ER. Since the majority of intracellular NS1 was present as an oligomeric form, these results strongly suggested that folding and oligomerization occurred probably in the ER or in a compartment that precedes the trans-Golgi.

To characterize the nature of the N-linked oligosaccharides, the recombinant NS1 protein extracted from an Ac-E. NS1-infected Sf9 cell lysate by Triton X-114 detergent was immunoprecipitated with NS1-specific MAb 8G4. As shown in Fig. 8, similar results were obtained with the Triton X-114 detergent and aqueous phases. The recombinant NS1 migrated as two bands, a major one corresponding to a gp48 form and a minor one, gp47 (see first paragraph). When treated with Endo H, two bands were still observed but one migrated with the unglycosylated p43 form and the other with the gp47. These two bands were sensitive to Endo F which reduced their size to 43K. These results suggest that gp48 is the Endo H-sensitive form whereas the gp47 represents the Endo H-resistant form. The existence of gp47 suggested that, although the majority of N-glycosylation sites on the recombinant NS1 are of the high-mannose type, some of the N-linked oligosaccharides were trimmed without further elongation. If this is so, this protein had been transported from the ER to the Golgi system where N-linked glycans were possibly trimmed to Man₃GlcNAc₂ without further elongation. This phenomenon has been described to occur in insect cells (Hsieh & Robbins, 1984; Jarvis & Summers, 1989). In addition, such glycans are known to be poor substrates for Endo H (Tarentino & Maley, 1975). Thus it appears that glycosylation of recombinant NS1 expressed in insect cells was different from that of the authentic NS1 protein synthesized in primate cells.

**Discussion**

To elucidate the role of proteins E and NS1 in induction of protective immunity against yellow fever, we have sought to express these proteins via a recombinant baculovirus. *S. frugiperda* cells infected with recombinant baculovirus Ac-E. NS1 expressed a 100K glycoprotein which was cleaved to generate recombinant E and NS1. These recombinant proteins exhibited antigenic
properties and folding similar to the authentic proteins as judged from their reactivity with MAbs recognizing conformation-dependent epitopes. This raised the question of whether these expression products also exhibit immunological properties in vivo. Preliminary experiments indicate that mice immunized with *S. frugiperda* cell lysates infected with recombinant baculovirus Ac-E. NS1 were completely protected against fatal YFV encephalitis.

The recombinant baculovirus genome contains the YFV cDNA which encodes the polyprotein precursor to the E and NS1 with their signal sequences for translocation and insertion into the membranes of ER. Although the E-related products migrated very similarly in infected Vero and *S. frugiperda* cells, we do not know whether the signal peptide of E was cleaved off in insect cells, as seems to occur in primate cells (Desprès et al., 1990). Sequencing of the C terminus of the flavivirus envelope proteins revealed the presence of two long hydrophobic domains separated by an arginine residue (Rice et al., 1986). The first one would act as an internal stop-transfer signal which must serve to anchor the C terminus of E in the membranes and the second one as a signal peptide for translocation of NS1 (Wright et al., 1989; Nowak et al., 1989). The cleavage of this internal signal-like sequence by signal peptidases which occurs on the carboxy-side of the E protein may be the limiting step in the processing of the gp100 precursor. Mature recombinant protein E did not migrate throughout the secretory pathway of insect cells.

For several membrane proteins, oligomerization seems to be required for efficient transport from the ER through the Golgi system (Kreis & Lodish, 1986; Copeland et al., 1988). The existence of NS1 oligomers has been reported for different members of the flavivirus family (Winkler et al., 1988, 1989; Mason, 1989; Schlesinger et al., 1990). The present data support a similar conclusion: the NS1 protein expressed by YFV in primate cells or by a recombinant baculovirus in *S. frugiperda* cells is converted to a heat-labile oligomeric form (gp72), an event which occurs in the ER. Oligomerization appears to occur also in tunicamycin-treated cells, indicating that carbohydrates are not required for this process. As defined by Triton X-114 phase partitioning, the NS1 oligomer is membrane-associated although it appears less hydrophobic than a typical membrane protein such as the E protein. Winkler et al. (1989) have suggested that the process of

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**Fig. 8. Endoglycosidase treatments on recombinant NS1 protein.** Labelled proteins from Triton X-114-extracted Sf9 cell lysates infected with AcNPV (wt), Ac-E. NS1 virus (Ac-E. NS1) or mock-infected (m) were immunoprecipitated with the NS1-specific MAb 8G4. The immune complexes were treated with the buffer alone (O), with Endo H (H) or with Endo F (F). The unglycosylated NS1 protein from the detergent and aqueous phases of tunicamycin-treated cells was run as a control (Tun).
oligomerization in itself leads to the increase in hydrophobicity of NS1 which could become indirectly membrane-associated after non-covalent association with a membrane-anchored protein.

In addition to the oligomers, other NS1-related polypeptides have been described. JE virus-infected cells (Mason et al., 1987; Mason, 1989) contained the NS1 protein which contains extra amino acids from the NS2A region at its carboxy terminus. Chambers et al. (1989) reported that during YFV infection, there exists a high Mr, NS1 polypeptide which contains regions of NS2A. Such a polypeptide was not observed in YFV-infected Vero cells under our experimental conditions.

During YFV infection, a small percentage of the NS1 oligomers was secreted at a slow rate into the extracellular medium. From their resistance to Endo H treatments, it can be concluded that the secreted oligomers possess N-linked glycans of the complex-mannose type, whereas the intracellular ones contain a large majority of the high-mannose type. This probably reflects the fact that a high proportion of NS1 was retained in the ER or in a compartment preceding the trans-Golgi, whereas some NS1 oligomers escaped to enter the secretory pathway; their passage to the trans-Golgi must represent the rate-limiting step. Secretion of the NS1 oligomers was not abolished in the presence of tunicamycin suggesting that the unglycosylated form could be transported.

The NS1 molecules expressed by recombinant baculovirus also formed oligomers and most of them possessed N-linked glycans of the high-mannose type, except for a fraction which was possibly trimmed to a trimannosyl core without further elongation. Although the recombinant NS1 protein was detected at the cell surface, it was not secreted into the culture medium of infected S. frugiperda cells. This suggests that the recombinant NS1 oligomer migrates within the cellular secretory pathway, becoming associated with the plasma membrane without being released from the cell. This result differs from that of YFV-infected primate cells. The absence of secretion of NS1 from infected insect cells has also been reported for JE virus (Mason, 1989). It appears that some insect cell lines, such as S. frugiperda cells, are unable to produce the complex type of N-linked glycans required for the release of flavivirus NS1 proteins. Further experiments must be designed to determine whether the transport strategy of NS1 in primate and insect cells may be related to the difference in N-glycosylation.

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