Maturation of the dengue-2 virus NS1 protein in insect cells: effects of downstream NS2A sequences on baculovirus-expressed gene constructs

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A series of recombinant baculoviruses was constructed in order to study the influence of downstream NS2A sequences on the processing of the dengue virus NS1 glycoprotein in insect cells. NS1 alone was expressed at a high level in its native dimeric form and processed efficiently through the Spodoptera frugiperda (Sf) cell secretory pathway. Recombinant NS1 was found associated with the plasma membrane and was also secreted into the extracellular medium. Although both intra- and extracellular NS1 were processed to an endo H-resistant form in Sf cells, Triton X-114 phase separation analysis further suggested that some modifications in addition to dimerization account for the hydrophobic properties of NS1, and that N-glycosylation was therefore not the only difference between the cell-associated and secreted forms. Cleavage at the NS1–NS2A junction of these recombinants demonstrated that as few as 26 amino acids from the N terminus of NS2A provide a sufficient, but not optimal, recognition sequence for a functional cleavage mediated by a protease present in Sf cells infected with recombinant Autographa californica nuclear polyhedrosis virus expressing NS1.

Dengue virus is a member of the family Flaviviridae which consists of a group of enveloped viruses with a single-stranded positive-sense RNA genome of about 11 kb. A single open reading frame encodes three structural and seven non-structural proteins which are derived by co-translational cleavage (Rice et al., 1986). The dengue virus non-structural protein NS1 is of particular interest because of several unexpected features (Winkler et al., 1988, 1989; Wright et al., 1989; Mason, 1989). Most notable for a non-structural protein is that it is both glycosylated (Smith & Wright, 1985) and a major viral immunogen which has been implicated in protection (Gould et al., 1986; Schlesinger et al., 1987; Cane & Gould, 1988; Henchel et al., 1988). The function of NS1 has yet to be determined but a role in viral assembly and maturation has been proposed (Rice et al., 1986; Mason, 1989) and recent studies with yellow fever (YF) virus suggest a role in viral replication (Muylaert et al., 1994). The protein is present at the surface of infected cells and is also secreted in mammalian cells with intraand extracellular NS1 differing in their N-glycosylation pattern (Gould et al., 1985; Winkler et al., 1988; Mason, 1989). NS1 is present in its native form as a dimer (Winkler et al., 1988), although a hexameric form has been described by Crooks et al. (1990). Newly-synthesized monomeric NS1 is a hydrophilic protein which becomes partially hydrophobic and membrane-associated during or shortly after dimerization (Winkler et al., 1989). The exact mechanism of NS1 association with membranes is not clearly understood, but the lack of any significant hydrophobic membrane-spanning domain suggests the involvement of some post-translational modification in anchoring NS1 to the membrane (Winkler et al., 1989).

A 24-amino-acid hydrophobic signal sequence at the C terminus of the E protein that precedes NS1 is necessary and sufficient to target NS1 to the endoplasmic reticulum (ER) (Falgout, 1989). The N terminus of NS1 is subsequently generated by cleavage at a signalase-like processing site that is highly conserved among the flaviviruses (Rice et al., 1986; Chambers et al., 1990). Studies on the cleavage between the C terminus of NS1 and the downstream NS2A, using recombinant vaccinia virus constructs, indicate that a novel mechanism which requires the last eight C-terminal residues of NS1 as well as the entire downstream NS2A in cis might be involved (Falgout et al., 1989; Hori & Lai, 1990). Furthermore, although the presence of a conserved V-X-A site at the NS1–NS2A junction suggest the possibility that cleavage is performed by a cell signalase, the site lacks the requisite upstream hydrophobic region (von Heinje,
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1983), indicating that a novel protease may be responsible (Speight et al., 1988; Chambers et al., 1989).

To study the maturation of the dengue virus NS1 protein in insect cells and in order to determine the effect of downstream NS2A gene sequences on processing, several NS1 gene constructs were expressed using recombinant baculoviruses. Synthesis of the NS1 glycoprotein, in each recombinant, was analysed in terms of N-glycosylation pattern, secretion and cleavage at the NS1–NS2A junction. Findings on the transport strategy of NS1 in insect cells as well as on the protease and sequence recognition involved in NS1–NS2A cleavage are presented.

A map of the sequences contained in the different NS1 constructs is shown in Fig. 1. All three constructs contain the NS1 coding sequence preceded by an N-terminal signal sequence of 28 amino acids, sufficient for the translocation of the NS1 protein into the ER (Falgout et al., 1989). The coding sequence starts with a methionine residue which provides a translation initiation site. The NS1 sequence of our isolate of dengue 2 virus (DEN-2V:PR159) has been reported previously (Leblois & Young, 1993). The constructs differed at the C terminus, containing either all of NS2A (Ac-NS1–NS2A), a 26-amino-acid C-terminal hydrophobic extension (Ac-NS1.H), consistent with the size of a membrane anchor domain, or no additional sequence (Ac-NS1). Expression of NS1 alone, following infection of Sf cells with the Ac-NS1 recombinant baculovirus was examined first, and then compared with the expression of the larger constructs.

Expression and secretion of recombinant NS1 were analysed by SDS–PAGE and immunoblotting (Fig. 2). Recombinant NS1 was detected at high levels in the culture fluid with the same apparent molecular mass as its intracellular counterpart which corresponded to the size of DEN-2V NS1 produced in the infected Aedes albopictus insect cell line C6/36 (Fig. 2a). When the samples were left unheated, NS1 appeared in its native dimeric form (Fig. 2a). Both cell-associated and secreted NS1 were purified from these cultures by immunoaffinity chromatography as described previously (Falconar & Young, 1990). Both intra- and extracellular recombinant NS1 were subjected to endo H and endo F treatment and compared with DEN-2V NS1 produced in C6/36 cells (Fig. 2b). As opposed to intracellular NS1 produced in DEN-2V infected cells, which was found in an endo H-sensitive form, both intra- and extracellular recombinant NS1 treated with endo H exhibited an intermediate molecular mass when compared to mock-treated and endo F-treated recombinant NS1. This indicated that one of the two high mannose linkages was trimmed in Sf cells to a trimannosyl core (Man3-Glc-NAc2), which is endo H-resistant (Tarentino & Maley, 1975) and probably represents the fully processed high-mannose glycan identified in baculovirus-infected Sf cells (Jarvis & Summers, 1989). Addition of complex carbohydrates does not appear to take place, owing to the absence of the requisite glycosyltransferase activities (Butters & Hughes, 1981).

In order to study the interaction of NS1 with cellular membranes, the hydrophobic properties of the intra- and extracellular forms of recombinant NS1 were examined using the TX-114 phase separation technique described.

Fig. 1. Map of the dengue-2 virus genome encoding regions of the E, NS1 and NS2A proteins used to prepare the three recombinant cDNA constructs, Ac-NS1, Ac-NS1.H and Ac-NS1.NS2A.
Fig. 2. Analysis of NS1 expression, glycosylation and secretion in Ac-NS1-infected Sf cells. (a) Sf cells were infected with recombinant baculovirus Ac-NS1 for 2 days while C6/36 cells were infected with dengue-2 virus (DEN-2V) for 4 days. Sf cell [Cells (Sf)] and C6/36 cell [Cells (C6/36)] extracts and cell culture supernatants [SN (Sf)], suspended in dissociation buffer, were either heated (+) or left unheated (−). These samples were then subjected to SDS–10% PAGE and subsequent immunoblot analysis using the DEN-2V NS1-specific MAb, 1H7.4. The positions of the NS1 monomer (NS1m) and NS1 dimer (NS1d) are shown. (b) Immunoaffinity purified cell-associated NS1 from Sf cells [Cells (Sf)] and C6/36 cells [Cells (C6/36)] and secreted NS1 from supernatants [SN(Sf)] were either mock-digested (−), digested with endo H (H) or with endo F (F). These samples were then subjected to SDS–10% PAGE and subsequent immunoblot analysis using the DEN-2V NS1-specific MAb, 1H7.4. The positions of cell-associated NS1 (►) and secreted NS1 (▶) are shown.

Fig. 3. Triton X-114 phase separation of dissociated or undissociated NS1 dimers. Both intracellular and extracellular purified recombinant NS1 were either heated to disrupt NS1 dimers (+) or left unheated (−). The samples were subsequently separated into aqueous (Aq) and detergent (Dt) phases using Triton X-114 as described by Bordier (1981). The positions of NS1 dimers (NS1d) and NS1 monomers (NS1m) are shown. (c) Intracellular purified recombinant NS1 was separated into aqueous (Aq) and detergent (Dt) phases. Both phases were subsequently re-extracted using Triton X-114. The position of the dimeric form of NS1 (NS1d) is shown. All samples were left unheated prior to analysis by SDS–10% PAGE and immunoblotting using the DEN-2V NS1-specific MAb, 1H7.4. The results showed that both NS1 species, in their native dimeric form, behaved very similarly and partitioned almost equally into both the aqueous and detergent phases (Fig. 3a, b). These results confirmed previous observations using dengue, Japanese encephalitis and yellow fever viruses, and recombinant constructs expressing NS1 proteins from these viruses, in mammalian (Winkler et al., 1989; Mason, 1989; Fan &
Mason, 1990; Després et al., 1991) or insect cells (Mason, 1989; Després et al., 1991; Flamand et al., 1992), indicating that NS1 is not a typical integral membrane protein. No difference was observed between the hydrophobic properties of NS1 in the three different baculovirus constructs, further suggesting that NS1 alone contains all the sequence requirements for membrane association (data not shown).

Furthermore, when both the aqueous and the detergent fractions were re-extracted using TX-114, each one partitioned again equally into the aqueous and detergent phases (Fig. 3 c), indicating that NS1 exhibits partial amphipathic properties rather than being comprised of subsets that are differentially associated with membranes (Mason, 1989; Flamand et al., 1992). When the samples were left unheated before SDS-PAGE and immunoblotting, a small fraction of intracellular NS1 was found in a monomeric form. This fraction was extracted in the aqueous phase only, which is in accordance with its essentially hydrophilic sequence (Fig. 3 a). As expected, no monomeric NS1 was detected in the extracellular medium as only dimeric NS1 is transported through the secretory pathway and secreted (Pryor & Wright, 1993).

While both forms of recombinant NS1 exhibited the same properties in their native dimer form, dissociation of mature dimers prior to TX-114 phase extraction did reveal differences in the detergent partitioning of intra- and extracellular NS1. Secreted NS1 dimer, once dissociated, behaved like newly synthesized NS1 and was essentially hydrophilic (Fig. 3 b). The increased reactivity of MAb 1H7.4 (Young & Falconar, 1990) with extracellular monomeric NS1 indicated an increased accessibility of this epitope and suggested that a reversible conformational change between the monomeric and dimeric forms of NS1 was responsible for the increased hydrophobicity of dimers (Fig. 3 b). Cell-associated NS1, on the other hand, retained its hydrophobic properties following dissociation, indicating that NS1 may undergo modifications in addition to dimerization which account for its hydrophobic properties (Fig. 3 a). Whether the structural differences observed between the intra- and extracellular forms, as seen in the increased reactivity of the MAb with extracellular monomeric NS1 and in epitope mapping studies (Young et al., 1993), were related to the differences in membrane association is unknown; however, the possibility of additional post-translational modifications has been suggested (Winkler et al., 1989).

To study cleavage at the NS1-NS2A junction in terms of the influence of downstream NS2A sequences on NS1 maturation, two additional constructs were expressed using recombinant baculoviruses (Fig. 1). The proteolytic processing and the secretion of the NS1 products expressed in Sf cells infected with these recombinant baculoviruses were studied in a pulse-chase labelling experiment (Fig. 4). The results showed that the NS1-NS2A precursor was cleaved efficiently to yield primarily NS1 and two additional minor NS1 products. No NS1 species corresponding to the theoretical size of an NS1-NS2A precursor (70 kDa) could be detected but the apparent molecular mass observed for the Kunjin and yellow fever virus NS2A was smaller than the predicted size for NS2A (Speight et al., 1988; Chambers et al., 1989). The larger NS1 product (NS1*) migrated with an apparent molecular mass of 63 kDa and the smaller (NS1**) was identified as a 48 kDa species. Two other minor NS1 products were also observed on immunoblots, with apparent molecular masses of 59 and 54 kDa (data not shown). Several bands below NS1, probably corresponding to NS1 breakdown products, were detected in the Ac-NS1-NS2A-infected Sf cells, whereas only faint bands were detectable with the Ac-NS1 and Ac-NS1.H constructs (Fig. 4). Surprisingly, a protein migrating to the same level as NS1 in the Ac-NS1-infected cell control was observed in the Ac-NS1.H-infected cells. This finding indicated that cleavage could occur at the NS1-NS2A junction, not only when the complete NS2A was present in cis but also with only the 26 N-terminal amino acid residues of NS2A (Fig. 4). Even after a 12 h chase, however, less than 50% of the NS1.H precursor had been cleaved. This differed from the results observed with the Ac-NS1.NS2A recombinant where the precursor protein NS1–NS2A was not...
detected. As few as 26 amino acids at the N terminus of NS2A were therefore sufficient to obtain cleavage at the NS1-NS2A junction. The presence of the full-length NS2A protein, however, provided an optimal recognition signal and/or increased the accessibility of this site to a specific protease present in the recombinant baculovirus-infected Sf cells.

The absence of cleavage reported with recombinant vaccinia viruses expressing NS1 with either 32, 107 (Falgout et al., 1989) or 61 (Parrish et al., 1991) N-terminal amino acids of NS2A may indicate differences between the two expression systems in either sequence recognition requirements or in the subcellular compartmentalization of the protease involved. Alternatively, presentation of the recognition sequence may be absolutely critical and even minor conformational variants may not act as suitable substrates. It is worth noting that both of the vaccinia constructs encoding truncated NS2A sequences reported by Falgout et al. (1989) incorporated additional C-terminal amino acids derived from vector sequences. The short NS2A extension encoded in the Ac-NS1.H recombinant included a hydrophobic domain from the N terminus of NS2A. This sequence may function as a membrane spanning domain which terminates at a potential trypsin-like (Arg/Lys-rich) cleavage site that is conserved amongst the flaviviruses (Putnak et al., 1988). Since a product of similar size (NS1**) was observed in both Ac-NS1.H- and Ac-NS1.H-infected Sf cells, this species may act as a viable intermediate substrate for the protease responsible for NS1-NS2A cleavage during natural dengue virus infections.

It has been suggested that NS2A may be a cis-acting protease that cleaves itself from NS1 (Falgout et al., 1989), but the fact that cleavage at the NS1-NS2A junction occurred with only a 26-amino-acid C-terminal extension did not support this hypothesis. The nature of the protease involved is still unknown and although delayed cleavage by a cell signalase has been proposed (Speight et al., 1988; Chambers et al., 1989), the absence of a prerequisite upstream hydrophobic region and conservation of the V-X-A cleavage site suggested that it is more likely that a novel protease may be responsible (Speight et al., 1988).

In addition, the NS1 produced with the NS1.H construct was processed efficiently through the secretory pathway of the Sf cells and found associated with the cell surface as well as secreted into the secretory pathway (data not shown; Fig. 4). Despite the presence of the additional species noted above, NS1 was the only product secreted by Ac-NS1.H- or Ac-NS1.H-infected cells. Comparison of relative amounts of extracellular NS1 found in the three different NS1 constructs showed that NS1 secretion was more efficient in Ac-NS1-infected cells. Quantification of the amount of labelled NS1 protein present in both intra- and extracellular fractions demonstrated that approximately 40% of the immunoprecipitable NS1 produced at 24 h post-infection was subsequently secreted in the next 16 h (Fig. 4). Cleavage at the NS1–NS2A junction appeared to limit the rate of NS1 secretion from infected Sf cells since similar ratios of cell-associated and extracellular NS1 were observed using both the Ac-NS1 and the Ac-NS1.H constructs. In contrast, despite the efficient cleavage at the NS1–NS2A junction observed using the Ac-NS1.H recombinant, only a low proportion of NS1 was identified in the supernatant. This latter result suggested that NS2A or the NS1 products may interfere with NS1 secretion. Similar observations were reported for Japanese encephalitis virus NS1 transiently expressed using vaccinia T7 constructs (Fan & Mason, 1990).

Alternative cleavage sites within the NS2A region, prior to the cleavage generating the C terminus of NS1, have previously been proposed to explain the generation of these species (Mason, 1989; Chambers et al., 1990). It has been suggested that delay in processing of this NS1 precursor may represent a requirement for conformational changes resulting from glycosylation, activation of the protease or entry of the precursor into a compartment containing the protease responsible (Chambers et al., 1990). It is possible that NS2A may play a role in transport or folding of NS1 during this processing event. To date, however, no such NS1 precursors have been reported for dengue virus, suggesting that if they do play a role in processing they must be relatively unstable or short-lived products. Whether the additional NS1–NS2A polypeptides observed following Ac-NS1.H infection have any precursor–product relationship with NS1 has not been studied in detail; however, their presence in infected cells after a 16 h chase period did not support this hypothesis. In the case of Japanese encephalitis virus, a 59 kDa species referred to as NS1' was detected in infected cells but did not seem to be a precursor of mature NS1 (Mason, 1989).

This work has demonstrated for the first time that cleavage at the NS1–NS2A junction can occur in Sf cells infected with an NS1 recombinant baculovirus construct possessing as few as 26 amino acids from the N terminus of NS2A. The nature of the protease involved and the minimal NS2A sequence requirement for its activity still need to be determined.

The study of NS1 processing in Sf cells has indicated that modifications in addition to dimerization are responsible for the hydrophobic properties of NS1 and that differences other than N-glycosylation exist between cell-associated and secreted forms. However, despite numerous studies detailing various features of the mature
forms of NS1 present in infected cells (Winkler et al., 1988, 1989; Wright et al., 1989; Mason, 1989; Fan & Mason, 1990; Falgout, 1989, Chambers et al., 1990), the post-translational processing and maturation of the NS1 protein are still not well understood. Addressing the issues of NS1 secretion and association with membranes in both mammalian and insect cells may help define the functional role of this non-structural protein in dengue virus infection; these studies are currently underway.

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