Glycosylation mutants of dengue virus NS1 protein

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The non-structural glycoprotein NS1 of dengue virus type 2 contains sites for N-linked glycosylation at Asn-130 and Asn-207. NS1 synthesized in infected cells is glycosylated at both locations. We have now examined the dimerization and secretion of NS1 lacking one or both of these sites by transient expression of mutagenized cDNA inserted into a simian virus 40-based vector. Immunoblotting and radioimmunoprecipitation were used to detect NS1 associated with transfected cells and in the extracellular medium. Elimination of one or both glycosylation sites did not abolish dimerization and secretion of NS1. However, NS1 lacking Asn-207 showed reduced dimer stability and secretion. Treatment of secreted NS1 with endoglycosidase H demonstrated that complex glycans were attached at Asn-130 and high-mannose glycans at Asn-207.

Dengue virus type 2 (DEN-2) is a member of the Flaviviridae. The genome encodes two structural glycoproteins, prM and E, and a non-structural glycoprotein, NS1 (Smith & Wright, 1985). E is the envelope protein of the virion and prM is the precursor of the membrane-associated protein M. The function of NS1 has yet to be determined but a role in virus assembly and maturation is proposed (Rice et al., 1986; Mason, 1989). During its synthesis, NS1 is inserted into the endoplasmic reticulum (ER) where it forms dimers (Winkler et al., 1988). Although NS1 is found associated with cellular membranes, it lacks an identifiable anchoring domain. It has been suggested that an increase in hydrophobicity of NS1, promoted by dimerization, may facilitate membrane association (Winkler et al., 1989). Dimerization of NS1 precedes movement of the protein to the Golgi complex (Winkler et al., 1989) as observed with other viral glycoproteins (Doms et al., 1993). NS1 is subsequently transported to the cell surface and secreted into the extracellular medium (Stohlman et al., 1975; Cardiff & Lund, 1976; Westaway & Goodman, 1987).

As with most of the mosquito-borne flaviviruses, DEN-2 NS1 contains two glycosylation sites for N-linked glycans (Irie et al., 1989; Hahn et al., 1988), both of which are utilized (Smith & Wright, 1985). Endoglycosidases have been used to characterize the glycans attached at these sites (Winkler et al., 1988). Enzyme treatment of intracellular NS1 isolated from DEN-2-infected Vero cells reveals only high-mannose glycans (Winkler et al., 1988). However, in NS1 molecules secreted to the extracellular medium, one of the glycans is converted to a complex form (Winkler et al., 1988). These observations are consistent with results obtained by the endoglycosidase treatment of the NS1 of other flaviviruses (Mason, 1989; Jacobs et al., 1992; Flamand et al., 1992), although extracellular yellow fever (YF) virus NS1 may have two complex N-linked glycans (Post et al., 1990; Despres et al., 1991). The lack of complex glycans attached to intracellular NS1 suggests that the rate of transport of NS1 through the secretory pathway is dependent on the movement of NS1 from the ER to the Golgi system. Once in the Golgi system the glycan at one of the glycosylation sites in DEN-2 NS1 is further processed to a complex-type structure, immediately preceding release from the cell. However, which of the two glycans is modified, and whether it is the same glycan in each molecule of NS1, cannot be determined by the use of endoglycosidases alone. Answers to these questions require the use of site-specific mutagenesis as reported in this paper.

Experiments employing tunicamycin have shown that glycosylation of NS1 in DEN-2-infected BHK cells is not essential for secretion of the protein (Winkler et al., 1989). Similar experiments have been performed using YF virus-infected Vero cells (Despres et al., 1991) and transiently expressed NS1 of tick-borne encephalitis (TBE) virus (Jacobs et al., 1992). In both cases, lack of glycosylation did not block secretion. However tunicamycin prevented secretion of NS1 from Japanese encephalitis (JE) virus-infected Vero cells (Mason, 1989). This contrasting result for unglycosylated JE virus NS1 may reflect a true difference for this virus species, but also engenders caution in interpreting results obtained using cells treated with tunicamycin, which has biological activities in addition to the inhibition of N-linked glycosylation (Mahoney & Duskin, 1979).

The role of the carbohydrate chains of viral glycoproteins may vary for different proteins. Of particular
relevance to NS1 is their potential importance in the proper folding, oligomerization and transport of secretory and membrane proteins (for reviews see Olden et al., 1982; Doms et al., 1993). In the current study we used oligonucleotide-directed mutagenesis of the DEN-2 NS1 gene inserted into a simian virus 40-based expression vector, firstly to address the role of N-linked glycans in determining the dimerization and intracellular transport of NS1, and secondly to identify which of the two N-linked glycans in the molecule is processed to the complex form.

Mutants of NS1 lacking either or both glycosylation sites were generated using the method of Kunkel et al. (1987); mutagenic oligonucleotides specified a change to Ala in the Asn residue at the consensus sequence for N-linked glycosylation, Asn-X-Thr/Ser. Ala has only a small side chain and causes minimal change in secondary structure (Cunningham & Wells, 1989). The mutated cDNAs were subcloned into the eukaryotic expression vector pSV.SPORT 1 (BRL, Life Technologies) and the NS1 mutant proteins resulting from the expression of these constructs were designated G1 (Asn-130 to Ala), G2 (Asn-207 to Ala) and G3 (Asn-130 and -207 to Ala) (Fig. 1). The wild-type protein containing the two original glycosylation sites was named 60 (Fig. 1) and the synthesis of this protein in COS cells has been described previously (Pryor & Wright, 1993). In addition to the NS1 gene, the expression vector contained nucleotides from the Kunjin virus 5' non-coding region, and nucleotides encoding the carboxy terminus of the DEN-2 E protein which acts as a signal sequence for the insertion of NS1 into the ER (Pryor & Wright, 1993). COS cells were transfected with recombinant plasmids and subsequently assayed for the presence of NS1 using immunoprecipitation or immunoblotting as described previously (Pryor & Wright, 1993). Digestion of NS1 protein with endoglycosidase H (endo H) was performed as recommended by the suppliers of the enzyme (New England Biolabs). When transfected cells were treated with tunicamycin, the drug was added to a final concentration of 5 μg/ml at 24 h post-transfection, and the cells were harvested 48 h later.

To investigate the effect of the absence of N-linked glycans on the dimerization of NS1, COS cells were transfected with the recombinant plasmids. Intracellular NS1 was detected by immunoblotting using rabbit polyclonal anti-NS1 antiserum (Smith & Wright, 1985). Protein samples were either heated for 2 min at 100 °C or not heated before analysis by SDS–PAGE (Fig. 2a). The absence of a glycosylation site in G1 and G2 caused an apparent decrease in size of both monomer and dimer of approximately 4000 Mr and 8000 Mr, respectively, compared with the wild-type protein 60. Correspondingly, elimination of both glycosylation sites in G3 resulted in a decrease in the size of the monomer of approximately 8000 Mr, and 16000 Mr in the dimer. The elimination of one or both N-linked glycans attached to the NS1 protein did not prevent the formation of dimers. However, for G2 and G3 in particular, this process was incomplete or, these dimers were unstable as monomers were detected in the unheated samples.

The stability of the dimers was investigated further by exposing protein samples to the following conditions before electrophoresis and immunoblotting: 22 °C for 2 h, 37 °C for 1 h, or 60 °C for 30 min. The intensity of the colour in the bands of the immunoblots (not shown) was measured in a densitometer and used to estimate dimer stability. The percentage of NS1 found in the dimeric form following each treatment is displayed in Fig. 2b. The average of three determinations is shown for each temperature. All mutants, and particularly G2, were more heat-sensitive than wild-type protein 60. For G2, only 10% of the protein was present as a dimer after 2 h at 22 °C. G3 was only slightly less stable than G1, but more stable than G2. Wild-type protein synthesized in the presence of tunicamycin and therefore unglycosylated, but with an unchanged polypeptide backbone, was also heat-sensitive. Thus removal of one or both N-linked glycans reduced the stability of the NS1 dimers, and in particular, removal of the second glycan alone (G2) caused the largest reduction in dimer stability.

To determine the importance of glycosylation on transport and secretion of NS1, protein in the extracellular medium of COS cells transfected by each mutant was radiolabelled and immunoprecipitated with rabbit anti-NS1 antiserum. The proteins were analysed using SDS–PAGE (Fig. 3). The presence of secreted NS1 dimers for all three mutants, differing in electrophoretic mobility according to glycosylation status, proved that glycosylation is not essential for secretion. As reported previously for other NS1 mutants, only the dimeric form of NS1, and not the monomer, was secreted from
Fig. 2. (a) Analysis by gel electrophoresis and immunoblotting of cell-associated NS1 dimers (d) and monomers (m) of proteins 60 (lanes 1 and 2), G1 (lanes 3 and 4), G2 (lanes 5 and 6) and G3 (lanes 7 and 8). Before electrophoresis, samples were treated with 2% SDS and 1% DTT. One half of each sample was heated at 100 °C for 2 min (lanes 1, 3, 5 and 7), the other half remained unheated (lanes 2, 4, 6 and 8). Polyclonal rabbit antiserum was used for immunoblotting. (b) The stability of NS1 dimers following treatment at 22 °C (2 h), 37 °C (1 h) and 60 °C (0–5 h). Note that these conditions are different from those used on the samples in (a). The proportion of each protein in dimeric form was calculated from the colour of the immunoblots obtained using rabbit polyclonal antiserum. 60 TUN, protein 60 synthesized in the presence of tunicamycin.

Fig. 3. Analysis of 35S-labelled immunoprecipitates of the extracellular fluid from cells secreting proteins 60 (lanes 3 and 4), G1 (lanes 5 and 6), G2 (lanes 7 and 8), G3 (lanes 9 and 10), or from mock-transfected cells (lanes 1 and 2). At 72 h after transfection, COS cells were radiolabelled with [35S]methionine for 6 h. The NS1 proteins were recovered from the culture medium by immunoprecipitation. Before electrophoresis, samples were treated with 2% SDS and 1% DTT. One half of each sample was heated to 100 °C for 2 min (lanes 1, 3, 5, 7 and 9), the other half remained unheated (lanes 2, 4, 6, 8 and 10). Positions of Mr markers are shown on the right.

transfected cells (Pryor & Wright, 1993). The testing of transfected cells by immunofluorescence also demonstrated the presence of each mutated NS1 on the cell surface (results not shown). Significantly, the amount of G2 and G3 immunoprecipitated from the medium was always at least 30% lower than the amount of 60 and G1. The most likely explanation is that since G2 and G3 dimers are unstable (Fig. 2a), and only dimers are secreted, the reduced secretion reflects reduced intracellular pools of the dimers. However it cannot be rigorously excluded that the lack of glycosylation at the second site inhibits secretion by a mechanism unrelated to dimerization.

To ascertain the nature of the glycans at the two glycosylation sites in NS1, mutated proteins were digested with endo H, which removes simple high-mannose, but not complex glycans. Digests of intracellular NS1 were examined by immunoblotting, revealing that the glycans were only of the high-mannose variety as previously described (Mason, 1989; Winkler et al., 1988; Jacobs et al., 1992; Post et al., 1990; Després et al., 1991) (data not shown). The extracellular proteins from transfected cells which had been radiolabelled were immunoprecipitated with rabbit polyclonal anti-NS1 antiserum and then digested with endo H (Fig. 4). Protein G2 was resistant to endo H (lane 6), protein G1 was sensitive (lane 4) and protein 60 was partially sensitive (lane 2). These results demonstrated that the glycan at Asn-130 of G2 was complex (endo H-resistant) whereas the glycan at Asn-207 of G1 was simple (endo H-sensitive). Dimers comprising monomers with one
complex glycan comigrated (lanes 2, 5 and 6) as broad bands (b) (see also lane 8 in Fig. 3), whereas dimers comprising unglycosylated monomers (lanes 4, 7 and 8) comigrated as sharp bands (d). Band c in lane 3 of Fig. 4 represents dimers comprising monomers with one simple glycan; these dimers migrated faster than those containing monomers with one complex glycan (band b). The lack of processing of the glycan at the second site to a complex form suggests that it is not accessible for modification during the passage of the dimer through the Golgi complex (Flamand et al., 1992).

We have shown that glycosylation of DEN-2 NS1 is not essential for dimerization and secretion of the protein. However, the absence of an N-linked glycan at the second glycosylation site (Asn-207) in G2 significantly reduced the stability and secretion of this NS1 dimer from COS cells. This finding provides a possible explanation for the lack of induction in mice of detectable antibody response against NS1 (R979,Igs0) encoding DEN-4 NS1 without the second glycan (similar to our G2 but with two amino acid changes) (Pletnev et al., 1993). In contrast, chimeric viruses encoding NS1 with two glycans or the second glycan induced measurable antibody. It is possible that cells infected with vNS1[R979,Igs0] secreted insufficient stable NS1 to effect a detectable antibody response in the mice.

Interestingly, it is the second glycosylation site which is conserved in the deduced amino acid sequence of both mosquito and tick-borne flaviviruses (Mandl et al., 1989; Chambers et al., 1990). In our experiments only high-mannose glycans were found at this site for DEN-2. It is not clear how these glycans contribute to the stability of the dimer. One possibility is that they form internal contacts within the dimer causing (i) increased stability, (ii) the glycans to be unavailable for modification in the Golgi complex (Flamand et al., 1992) and (iii) an increased hydrophobicity of the molecule (Winkler et al., 1989). Further studies on the importance of NS1 glycosylation in viral replication and the host immune response will be feasible when the above mutations are incorporated into infectious full-length RNA transcripts of the DEN-2 viral genome.

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References


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