Peptide Mapping of Envelope-related Glycoproteins Specified by the Flaviviruses Kunjin and West Nile

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SUMMARY

Glycoproteins detected in Vero cells infected by the flaviviruses West Nile and Kunjin were examined by gel electrophoresis and peptide mapping. Two major glycoproteins, gp66 and gp54, were observed in West Nile virus-infected cells labelled for short time periods with \[^3H\]mannose. A third glycoprotein, gp58, was present in smaller amounts. Pulse-labelling experiments suggested that gp66 was a precursor of gp54. Peptide mapping of \[^3H\]leucine-labelled gp66, gp54 and the envelope glycoprotein E of West Nile virus demonstrated that gp66 and gp54 were related to E, and that the peptides of gp54 were a subset of those of gp66. Peptide mapping of the corresponding Kunjin virus-specified glycoproteins (gp66, gp59 and gp53) showed that the \[^3H\]leucine-labelled peptides of gp53 and gp59 were similar and were contained within gp66. Since we have shown previously that gp59 and gp53 are related to E of Kunjin virus, we conclude that cells infected by West Nile or Kunjin viruses contain a similar set of E-related glycoproteins.

Kunjin (KUN) and West Nile (WN) viruses belong to the same serological complex in the flavivirus genus within the family Togaviridae. Virions contain genomic RNA of positive polarity, two small polypeptides C and M, and one envelope protein E (for review, see Russell et al., 1980). Formerly, we showed by radioimmunoprecipitation using antiserum against E, and by limited proteolytic digestion, that KUN virus-infected Vero cells contain at least two glycoproteins related to E of KUN virus, namely gp53 and gp59. A third glycoprotein gp66 is an apparent precursor to gp53 in pulse-chase experiments, but is not immunoprecipitated by antiserum against E. Limited proteolytic digestion of \[^3H\]mannose-labelled gp53, gp59 and gp66 suggested that gp66 is probably related to the other two glycoproteins, but the results were not conclusive (Wright et al., 1981). Therefore, to confirm the relationship, we have now analysed the peptides produced by exhaustive proteolytic digestion of the \[^3H\]leucine-labelled glycoproteins.

In cells infected by WN virus, two glycoproteins similar in size to gp66 and gp53 specified by KUN virus were observed, but their relationships to E were not examined (Wright, 1982). In this paper, the two WN glycoproteins are compared with each other and with E of WN virus by peptide mapping. These experiments were undertaken to determine whether KUN virus is unique among flaviviruses in specifying more than one intracellular E-related glycoprotein, or whether the presence of such glycoproteins is a more general characteristic of flavivirus-infected cells.

The strains of KUN and WN viruses used in these studies, and the procedures followed to radiolabel virions and infect Vero cells have been described previously (Wright et al., 1981; Wright, 1982). Proteins were separated in polyacrylamide gels containing SDS using a discontinuous buffer system (Laemmli, 1970). To obtain suitable preparations of the intracellular viral glycoproteins for peptide mapping, infected cells were labelled with \[^3H\]leucine or \[^3H\]mannose for 30 min, the cells harvested in 2% SDS, and the lysed cells were dialysed against 0.05% SDS, 140 mM-NaCl in 100 mM-Tris-HCl pH 7.5 (SNT). The cell harvest was mixed with concanavalin A (Con A)-agarose (Miles Laboratories) in SNT for 1.5 h and...
bound glycoproteins were recovered using SDS and urea as described by Poliquin & Shore (1980). Four to 6% of the [3H]leucine-labelled samples, and approximately 55% of the [3H]mannose-labelled material bound specifically to the Con A–agarose and were subsequently eluted from it. The recovered material was electrophoresed through preparative 7% polyacrylamide gels. Labelled glycoproteins were eluted from the gels, 2 mg ovalbumin carrier was added, and the proteins were then reduced by dithiothreitol and alkylated by iodoacetamide. The alkylated glycoproteins were dissolved in 0.1 M-ammonium bicarbonate and digested with α-chymotrypsin (Worthington) for 26 h; 0.2 mg of enzyme was added at 0, 4 and 21 h (Wright et al., 1977, 1983).

Digested glycoproteins were analysed either on a column of Technicon type P chromobead ion-exchange resin held at 50°C, or by using a Waters HPLC system and a microbondapak C18 column at 25°C (Wright et al., 1983). Internal markers were monitored in both types of separations. For ion-exchange chromatography, each 2H-labelled preparation of digested glycoprotein was co-run with the same 35S-labelled tryptic digest of P71 of KUN virus; for HPLC, peptides of carrier ovalbumin were monitored at 210 nm. The complete internal marker profiles are not plotted in the figures, but the positions of major peaks of the markers are indicated, and demonstrate the reproducibility of the separation systems.

Glycoproteins (lane 1) and proteins (lane 2) greater than 30,000 M_r that were detected in WN virus-infected Vero cells are shown in Fig. 1. KUN virus-specified glycoproteins are analysed in lane 6. For consistency, previously assigned molecular weights and names have been given to polypeptides already described (Westaway et al., 1980; Wright et al., 1981). The approximate molecular weights of the polypeptides in the gel system used in Fig. 1 are apparent by reference to the markers in lane 7. Two major glycoproteins, gp66 and gp54, were consistently detected in cells infected by WN virus. [3H]Mannose-labelled gp54 co-migrated with the envelope glycoprotein E (Wright, 1982); sometimes it resolved into two closely migrating bands, as did gp53 of KUN in lane 6 of Fig. 1. Glycoprotein gp66 of WN and gp66 of KUN always co-migrated. A third glycoprotein, gp58 of WN, was usually detected, but only in minor proportions (see Fig. 2); it was observed in the gel of Fig. 1 after a longer exposure.

When WN virus-infected Vero cells were pulse-labelled with [3H]mannose for increasing periods of time, the proportion of label in gp66 decreased as the proportion in gp54 increased (Fig. 2). These results were similar to those reported for gp66 and gp53 of KUN (Wright et al., 1981), and were suggestive of a precursor–product relationship between the two WN glycoproteins. The proportion of gp58 relative to gp54 detected in WN virus-infected cells was less than the proportion of gp59 relative to gp53 observed in cells infected by KUN virus (Wright et al., 1981). No conclusions were possible concerning the involvement of WN gp58 either as a precursor of gp66 or gp54, or as an intermediate during processing of gp66.

For peptide mapping, KUN and WN virus-specified glycoproteins were labelled with [3H]leucine. We reasoned that if a polypeptide segment, possibly containing a hydrophobic core typical of a signal sequence, was removed during the processing of gp66 in WN and KUN virus-infected cells, then leucine, a common residue in polypeptides and particularly in signal peptides (Perlman & Halvorson, 1983), was probably present in the removed segment of gp66. Chymotrypsin rather than trypsin, which is a more specific protease, was used to digest exhaustively the glycoproteins for two reasons. Firstly, we found gp66 to be unusually resistant to proteolysis by trypsin under conditions which resulted in apparent complete digestion of other viral glycoproteins and proteins (Wright et al., 1983). Secondly, digestion of gp66 by chymotrypsin rather than trypsin was likely to produce more peptides characteristic of any short unique polypeptide segments contained in gp66. Preparations of glycoproteins for peptide mapping needed to be free of contaminating radiolabelled host proteins; it was also necessary to ensure that samples of gp66 were free of P71. Thus, the glycoproteins were purified using Con A and then electrophoresed as described above. Re-analysis of the preparations obtained after the two-step procedure demonstrated that they were electrophoretically pure.

Chymotryptic digests of gp66 and gp54 of WN were analysed by reverse-phase HPLC and compared with a digest of the envelope glycoprotein E of WN virus (Fig. 3), E having been prepared by elution from a gel following electrophoresis of purified [3H]leucine-labelled virions.
Fig. 1. Analyses in a 7% polyacrylamide gel of proteins and glycoproteins from virus-infected Vero cells labelled for 30 min with $^{3}H$mannose (lanes, 1, 4, 5, 6) or $^{3}H$leucine (lanes 2, 3) at 27 h after infection. Cells were infected with WN (lanes 1, 2, 5) or KUN (lane 6) viruses, or mock-infected (lanes 3, 4). Conditions for labelling and electrophoresis were as described previously (Wright et al., 1981), except that the sample buffer of this gel contained 1-6 M-urea. Lane 7 contains $^{14}C$-labelled molecular weight markers from Amersham. The lettering on the left refers to lanes 1 and 2; the lettering on the right refers to lanes 6 and 7. P98 and P71 are non-structural proteins, originally described as NV5 and NV4, respectively (Westaway et al., 1980).

Fig. 2. Analyses in a 7% polyacrylamide gel of $^{3}H$mannose-labelled glycoproteins from WN virus-infected (lanes 2, 4, 6) and mock-infected (lanes 1, 3, 5) cells. At 27 h after infection, cells were pulse-labelled for 10 min (lanes 1, 2), 20 min (lanes 3, 4), or 60 min (lanes 5, 6). Conditions for labelling have been described by Wright et al. (1981).

(Wright, 1982). Insufficient gp58 was obtained for peptide mapping. $^{3}H$Leucine-labelled peptides common to WN gp66, gp54 and E were numbers 1, 3, 4, 5, 10, 11, 13, 16, 18, 19 and 22, indicating that gp66 and gp54 are related to E. Furthermore, all peptides of gp54 were present in the digest of gp66; the peptides found in gp66 and not in gp54 were numbers 2, 6, 12, 15 and 17. Peptides 2, 12 and 17 were also absent from the digest of E, and were therefore unique to gp66. E contained peptides not found in either gp66 or gp54 (numbers 8, 9, 14, 20, 21, 23 and 24), but possible reasons for these unique peptides of E (e.g. glycosylation, phosphorylation, acylation) were not investigated. However, it may be relevant that the 17D strain of yellow fever virus contains at least two forms of the envelope protein, probably differing in glycan content (Schlesinger et al., 1983); similar heterogeneity for E of WN virus may explain the relatively large number of peptides unique to this protein.

$^{3}H$Leucine-labelled peptides of each KUN glycoprotein and an aliquot of marker peptides labelled with $^{35}S$methionine (Wright et al., 1983) were separated by ion-exchange chromatography (Fig. 4). The peptides of gp59 and gp53 had identical elution profiles, and were contained within the digest of gp66. Glycoprotein gp66 is therefore related to E of KUN, as it is known that gp53 and gp59 are E-related (Wright et al., 1981). Peptides unique to gp66 were numbers 2, 4, 6 and possibly 11, 13 and 15. The unique peptides were not the result of differences in glycosylation between gp66 and the other two glycoproteins, because $^{3}H$mannose-labelled...
Fig. 3. Analyses by HPLC of chymotryptic peptides of $[^3]$H-leucine-labelled glycoproteins of WN. (a) gp66, (b) gp54, (c) envelope protein E. Peptide samples were loaded on a microbondapak C18 column, and then eluted with a linear gradient of 0 to 43% acetonitrile in 0.05% trifluoroacetic acid at 25°C. The positions of unlabelled ovalbumin marker peptides monitored at 210 nm are indicated by vertical bars at the top of each graph, and confirm the reproducibility of the separations. The fractions containing the marker peptides are numbered in (a). The $[^3]$H-leucine-labelled peptides are numbered in the order of their elution.

Fig. 4. Analyses by ion-exchange chromatography of chymotryptic peptides of glycoproteins of KUN. (a) $[^3]$H-leucine-labelled gp66, (b) $[^3]$H-leucine-labelled gp59, (c) $[^3]$H-leucine-labelled gp53, (d) $[^3]$H-mannose-labelled gp66. Peptide samples were loaded on a column of Technicon type P chromobeads, and then eluted with a linear gradient of 0.2 M-pyridine-acetate (pH 3.1) to 2 M-pyridine-acetate (pH 5.0) at 50°C. The positions of $[^35]$S-methionine-labelled marker peptides are indicated by vertical bars at the top of each graph. Fraction numbers for the markers are shown in (a). $[^3]$H-leucine-labelled peptides are numbered in the order of their elution. The first peak in each profile represents unretained material.

gp53 and gp66, treated and analysed in the same manner as their $[^3]$H-leucine-labelled counterparts, both gave the same elution profile containing two major glycopeptides g1 and g2 (Fig. 4d). Furthermore, g1 and g2 did not elute in the position of any of the unique peptides of gp66.

The above results and our earlier report (Wright et al., 1981) together show that gp66 and gp54 of WN, and gp66, gp59 and gp53 of KUN are related to their respective E proteins. For both these flaviviruses, gp66 possesses extra peptides not found in the smaller glycoproteins. This conclusion was strengthened by the use of two different separation systems to resolve the
peptides. Using the above methods we failed to detect unique peptides of gp59 that were not contained in gp53 of KUN; it remains possible that a difference in glycosylation, or the presence of a small leucine-deficient peptide, or both of these alternatives account for the apparent difference in molecular weight between the two glycoproteins.

It is not known whether the extra peptides of gp66 are located at the C or N terminal end of gp66. If they are located at the N terminus, then they may be part of a polypeptide segment that functions as a signal or leader type sequence since the three E-related KUN glycoproteins are associated with membranes (Westaway \& Ng, 1980) and possess glycans similar in size and composition to N-linked glycans (Wright et al., 1980, 1981). Signal peptides are not necessarily degraded during glycoprotein synthesis, but may be contained in stable polypeptides as is the case for E3 and the 6K peptide of Semliki Forest virus (Garoff et al., 1980). There are at least four flavivirus-specified proteins less than about 15000 daltons in size (Westaway et al., 1980), and one or more of these may be a product of cleavage of gp66. Thus, we are currently re-examining the small polypeptides for possible relationships to gp66, and following the effects of inhibitors of glycosylation and proteolysis on the processing of gp66 and virus maturation.

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REFERENCES


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