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Synthesis of Proteins and Glycoproteins in Dengue Type 2 Virus-infected Vero and *Aedes albopictus* Cells

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SUMMARY

Fifteen proteins were detected in Vero cells infected by dengue type 2 (DEN-2) virus that were not observed in mock-infected cells, namely P98, p82, P67, GP60, gp54, GP46, p30, p28, gp22, GP20, p18, gp16, p15, p14 and gp13. With the exceptions of gp54 and gp13, polypeptides corresponding to those listed above were also observed in DEN-2 virus-infected *Aedes albopictus* C6/36 cells. Pulse-chase labelling experiments suggested a possible precursor-product relationship between p30 and p28, and between gp22 and GP20. Peptide mapping and immunoprecipitation experiments showed that the major glycoproteins GP60, GP46 and GP20 were unrelated. Immunoprecipitations of infected cells with antiserum prepared against the DEN-2 soluble complement-fixing (SCF) antigen demonstrated that this antigen is equivalent to the non-structural glycoprotein GP46. The envelope glycoprotein (E) from virus grown in C6/36 cells migrated faster through polyacrylamide gels containing SDS than E from virus grown in Vero cells. [³H]Mannose-labelled glycopeptides of GP60, GP46 and GP20 were separated by gel filtration and by electrophoresis in Tris-borate gels; in addition, the polypeptides synthesized in infected cells in the presence of tunicamycin were analysed. The results revealed heterogeneity among the glycan units of GP60 and GP46.

INTRODUCTION

The *Flavivirus* genus of the family *Togaviridae* comprises approximately 60 virus species which have been divided into seven subgroups or complexes (Porterfield, 1980; Matthews, 1982). The four serotypes of dengue virus constitute the dengue subgroup. Purified virions of dengue type 2 (DEN-2) virus contain a single envelope glycoprotein E and two smaller proteins C and M (Stollar, 1969). The flavivirus genomic RNA is a polycistronic single-stranded molecule of approximately 4.2×10^6 mol. wt., having a 5' cap and lacking a poly(A) tail at the 3' end (Boulton & Westaway, 1972; Wengler *et al.*, 1978; Cleaves & Dubin, 1979). The number and functions of non-structural proteins and glycoproteins synthesized in dengue virus-infected cells are not well characterized (Westaway, 1973; Stohlman *et al.*, 1975, 1976; Schlesinger, 1977, 1981); more information is available on the virus-specified polypeptides detected in cells infected by viruses of the West Nile-Murray Valley encephalitis subgroup of the *Flavivirus* genus (for review, see Westaway, 1980; Wright *et al.*, 1981). No subgenomic mRNA has been detected in cells infected by any flavivirus, and it has been proposed that internal initiation of protein synthesis occurs on the genomic mRNA (Westaway, 1977, 1980; Westaway *et al.*, 1984).

In this paper we describe the proteins and glycoproteins detected in mammalian (Vero) and mosquito (*Aedes albopictus* C6/36) cell lines infected by DEN-2 virus, and partially characterize the glycans of the three major virus-specified glycoproteins, i.e. GP60, GP46 and GP20, purified from infected Vero cells. We also demonstrate that GP46 is the soluble complement-fixing (SCF) antigen of DEN-2 virus-infected cells (McCloud *et al.*, 1971; Smith *et al.*, 1970; Cardiff *et al.*, 1970, 1971; Russell *et al.*, 1980).

METHODS

Cells and virus. Vero cells were grown at 37 °C in Medium 199 supplemented with 7.5% foetal calf serum (FCS, heat-inactivated at 56 °C for 30 min) and then maintained after infection at 37 °C in Eagle's MEM containing 0.1% bovine serum albumin (BSA). *Aedes albopictus* C6/36 cells (Igarashi, 1978) were grown at 28 °C in Eagle's basal medium supplemented with 10% FCS and non-essential amino acids (0.1 mM of each) and maintained after infection at 28 °C in MEM containing 0.1% BSA and non-essential amino acids (0.1 mM of each). The New Guinea 'C' strain of DEN-2 virus was obtained from Dr J. G. Aaskov of the Queensland Institute of Medical Research (Brisbane, Australia). Cells were infected with a 10% (w/v) suspension of DEN-2 virus-infected suckling mouse brain (25th passage).

Purification of radiolabelled virions. Radiolabelled virions were purified from cell culture fluids by polyethylene glycol precipitation and sedimentation through 5 to 25% (w/v) sucrose gradients (Westaway & Reedman, 1969). Confluent monolayers of Vero cells or C6/36 cells were infected with DEN-2 virus at a multiplicity of 0.1 p.f.u./cell. To grow [³H]methionine-labelled DEN-2 virus in Vero cells, the culture fluid was replaced 72 h after infection with MEM containing one-hundredth the normal concentration of methionine and 10 µCi/ml of radioactive precursor. The culture fluid was harvested at 96 h and 112 h after infection and the virions purified as above. To grow [³H]methionine-labelled DEN-2 virus in C6/36 cells, the culture fluid was replaced 96 h after infection (and later at 144 h) with maintenance medium containing [³H]methionine at 10 to 15 µCi/ml. The culture fluid was harvested at 144 h and 160 h after infection, the harvests pooled and the virions purified as above.

Preparation of radiolabelled cells. Vero cells and C6/36 cells were infected with DEN-2 virus at 1 p.f.u./cell. At 72 h after infection of Vero cells the maintenance medium was replaced with MEM containing actinomycin D (AMD) at a concentration of 3 µg/ml, and containing one-hundredth the normal concentration of methionine or lacking glucose for subsequent labelling with radioactive methionine or carbohydrate, respectively. Radiolabelling of cells commenced at 75 h after infection. For C6/36 cells the treatment with AMD commenced at 120 h after infection; the procedures and labelling media used were the same as for Vero cells, except that the media contained non-essential amino acids (0.1 mM of each). Where cells were to be labelled in the presence of tunicamycin (Eli Lilly & Co.) the inhibitor was added to the medium at the same time as AMD. In short pulse experiments the labelling medium contained 28 mM-HEPES and [³H]mannose at 125 µCi/ml, or [³⁵S]methionine at 20 µCi/ml. The concentrations of glucose and methionine in 'chase' media were 0.4% and 75 µg/ml, respectively. Cells for immunoprecipitation experiments were labelled with [³⁵S]methionine at 250 µCi/ml, with [³H]mannose at 500 µCi/ml or with [³H]glucosamine at 450 µCi/ml in 0.3 ml of medium in the 16 mm wells of a multidish Dispo-Tray (Linbro).

Polyacrylamide gel electrophoresis of proteins. Labelled cells were washed twice with phosphate-buffered saline (PBS) and dissolved in 2% SDS. Proteins were separated in polyacrylamide gels using a discontinuous buffer system (Laemmli, 1970). Polypeptides were detected in analytical slab gels by fluorography (Bonner & Laskey, 1974). Concanavalin A (Con A)-extracted glycoproteins were separated in cylindrical gels, the gel sliced transversely, and the contents of each slice eluted into 0.1% SDS. The radioactivity in a one-tenth volume of the eluate of each slice was measured in a liquid scintillation counter.

Concanavalin A extraction of glycoproteins. Vero cells infected by DEN-2 virus and labelled with [³H]mannose were extracted with Con A-Sepharose (Pharmacia) following the method of Poliquin & Shore (1980). Infected cells were washed twice in PBS at 4 °C, suspended in Con A lysing buffer [10 mM-Tris-acetate pH 7.6, 0.5 mM-magnesium acetate, 1.0 mM-dithiothreitol (DTT), 1% Triton X-100, 0.5% sodium deoxycholate] and disrupted in a glass Dounce homogenizer. The homogenates were diluted with an equal volume of buffer medium A (10 mM-Tris-acetate pH 7.6, 0.5 mM-magnesium acetate, 1.0 mM-DTT, 1.0 M-NaCl) and centrifuged for 5 min at 10000 g. One ml aliquots of the supernatants were mixed with 150 µl of packed Con A-Sepharose (in buffer medium A) for 90 min at room temperature. The Con A-Sepharose was then given two 15 min washes in buffer medium A followed by two 15 min washes in 0.1% SDS. Glycoproteins were eluted from the Sepharose pellets by adding 100 µl of 5% SDS containing 8 M-urea and heating at 100 °C for 3 min. The last step was repeated and the eluates pooled prior to electrophoresis.

Peptide mapping by limited proteolysis. The method for limited proteolysis of polypeptides was based on that of Cleveland *et al.* (1977). Samples for analysis were prepared by Con A extraction and gel electrophoresis (see above). Purified glycoproteins in 0.1% SDS were dialysed against 0.05 M-Tris-HCl pH 6.8 containing 0.1% SDS and 0.1 mM-EDTA. Samples were digested at 37 °C with either alpha-chymotrypsin (Worthington Biochemicals) or *Staphylococcus aureus* V8 protease (Miles Laboratories) and the products analysed on 15% polyacrylamide gels (Wright *et al.*, 1981).

Antiser preparation. Approximately 3×10^7 infected cells were extracted with Con A-Sepharose and the eluted glycoproteins separated on cylindrical gels. Trace amounts of radioactively labelled samples were used to follow polypeptides during the preparative procedures. The three major virus-specified glycoproteins, GP60, GP46 and GP20, were eluted into 0.1% SDS, reduced in volume to 1 ml with Aquacide II-A (Calbiochem), and dialysed

exhaustively against PBS. The glycoprotein antigens were emulsified with an equal volume of Freund's complete adjuvant and then used to immunize rabbits. Each rabbit received a total of ten intradermal injections of 0.1 ml of antigen preparation (approximately 2 µg of viral protein), five at each of two sites on the animal's flanks. Six weeks after the primary immunization each rabbit received a booster immunization consisting of six 0.1 ml intradermal injections of the appropriate glycoprotein antigens in Freund's incomplete adjuvant. After a further 4 weeks the rabbits were bled out by cardiac puncture and the sera collected. Hyperimmune rabbit antiserum prepared against DEN-2 virus-infected suckling mouse brain (SMB) was provided by Dr E. G. Westaway (Westaway, 1966). Rabbit antiserum prepared against the DEN-2 SCF antigen by Dr J. M. Dalrymple was made available by Dr W. E. Brandt. The DEN-2 SCF antigen used to immunize rabbits was purified from a DEN-2 virus-infected suckling mouse brain suspension by protamine and ammonium sulphate precipitation, gel filtration and isoelectric focusing (J. M. Dalrymple, personal communication).

Radioimmunoprecipitation. Infected and mock-infected cells were dissolved in 0.5 ml of lysing buffer (0.15 M-NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.015 M-Tris-HCl pH 7.4) following the method of Wechsler *et al.* (1979), and 100 µl aliquots immunoprecipitated as previously described (Wright *et al.*, 1981). The anti-SMB and anti-SCF sera were normally diluted 1 in 10 with Tris-saline (0.15 M-NaCl, 0.015 M-Tris-HCl pH 7.4) for use in immunoprecipitations, whereas the antisera prepared against GP60, GP46 and GP20 were diluted 1 in 3.

Pronase digestion and glycopeptide analyses. [³H]Mannose-labelled GP60, GP46 and GP20 were extracted from infected Vero cells with Con A-Sepharose, separated in cylindrical gels and then eluted into 0.1% SDS as described above. BSA (200 µg) was added to the eluates of the purified glycoproteins and the volume of each reduced to 0.5 ml with Aquacide II-A. The preparations were dialysed exhaustively against 0.1 M-ammonium bicarbonate pH 8.0 and then digested at 60 °C with Pronase-CB (Calbiochem) as described by Weitzman *et al.* (1979). The Pronase-digested samples were then split into two, lyophilized, and the glycopeptides in each half analysed by a different technique. The first was molecular sieve chromatography using a 0.9 × 86 cm column of Bio-Gel P-6 (200 to 400 mesh) and an elution buffer of 0.1 M-ammonium acetate pH 8.0. The second was Tris-borate PAGE, as described by Weitzman *et al.* (1979), using cylindrical gels containing 10% acrylamide and 0.1 M-Tris-borate pH 8.3. Following electrophoresis, the gels were sliced transversely and the radioactivity in each slice was measured in a liquid scintillation counter.

Radioisotopes. Radioactive materials were obtained from Amersham (Australia). The compounds used and their respective specific activities were D-[6-³H]glucosamine (26.8 Ci/mmol), D-[2-³H]mannose (13.4 Ci/mmol), L-[Me-³H]methionine (85 Ci/mmol) and L-[³⁵S]methionine (approx. 1440 Ci/mmol). The reference ¹⁴C-methylated protein mixture included myosin, mol. wt. 200 000 (200K), phosphorylase b (92.5K), BSA (69K), ovalbumin (46K), carbonic anhydrase (30K) and lysozyme (14.3K).

RESULTS

Identification of DEN-2 virus-specified proteins and glycoproteins in Vero cells

The analyses of Vero cells infected with DEN-2 virus and labelled with [³H]mannose or [³⁵S]methionine are shown in Fig. 1(a). The major DEN-2 virus-specified proteins and glycoproteins are indicated for lanes 1 and 5, respectively. Three of the five glycoproteins shown in Fig. 1(a) (lane 5), namely those of 60K, 46K and 20K, were consistently detected in infected Vero cells labelled with [³H]mannose or with [³H]glucosamine. Preparations of these three glycoproteins labelled with [³H]mannose or [³⁵S]methionine were partially digested by *S. aureus* V8 protease and α-chymotrypsin in separate reactions, and analysed by gel electrophoresis (Fig. 2). A lack of relationship between the two large glycoproteins was clearly demonstrated (compare lane 4 with lane 5, and lane 7 with lane 8 in Fig. 2a and b). The uniqueness of the third glycoprotein (20K) with respect to the two large glycoproteins was best indicated by the proteolytic digests of the [³⁵S]methionine-labelled preparations (Fig. 2b). Thus, on the basis of their distinct peptide maps and their stability in pulse-chase experiments (see later in Fig. 5), the three glycoproteins were designated GP60, GP46 and GP20 in accordance with the proposed system of nomenclature for flavivirus-specified proteins (Westaway *et al.*, 1980). The DEN-2 virus-specified polypeptides P98 and P67 were given upper case prefixes as they represent the DEN-2 virus equivalents of two of the most distinct and well recognized flavivirus-specified non-structural proteins, namely NV5 and NV4 (Westaway *et al.*, 1980); all of the other DEN-2 virus-specified polypeptides were prefixed with a lower case 'p' or 'gp' as appropriate.

GP46, although clearly detected when labelled with [³H]mannose (Fig. 1a, lane 5), was difficult to resolve from co-migrating host proteins when labelled with [³⁵S]methionine (Fig. 1a,

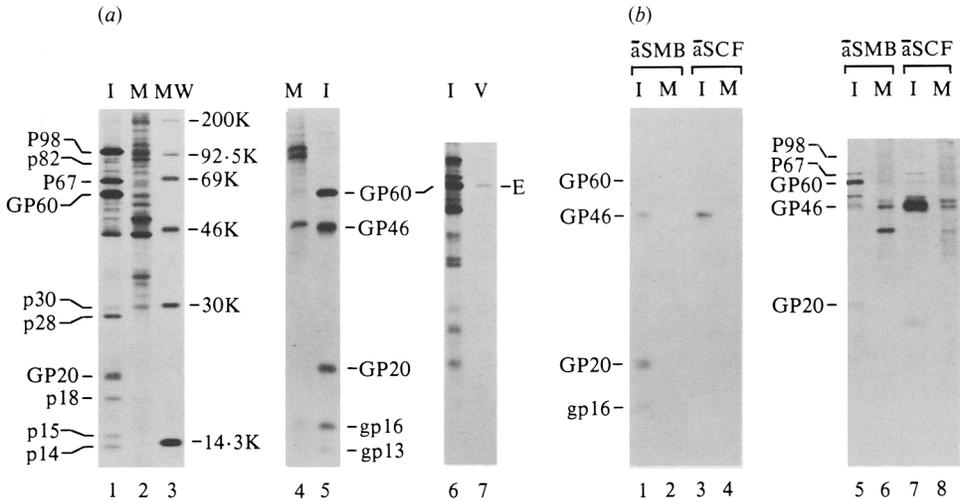


Fig. 1. Analyses in polyacrylamide gels of proteins and glycoproteins from DEN-2 virus-infected (I) and mock-infected (M) Vero cells. (a) Lanes 1 and 2, cells labelled for 30 min with [^{35}S]methionine and then chased with excess methionine for 150 min; lane 3, ^{14}C -labelled molecular weight markers (Amersham); lanes 4 and 5, cells labelled for 3 h with [^3H]mannose; lane 6, cells labelled for 3 h with [^{35}S]methionine; lane 7, [^3H]methionine-labelled DEN-2 virions (V). (b) Immunoprecipitates of DEN-2 virus-infected and mock-infected cells prepared using antiserum raised against DEN-2 virus-infected suckling mouse brain ($\bar{\text{a}}\text{SMB}$) or against the DEN-2 virus SCF antigen derived from infected suckling mouse brain ($\bar{\text{a}}\text{SCF}$). The cells used in lanes 1 to 4 were labelled for 3 h with [^3H]mannose and those in lanes 5 to 8 were labelled for 3 h with [^{35}S]methionine. Equal volumes of immunoprecipitated material were loaded in lanes 1 and 2, and in lanes 3 and 4. In lanes 5 and 6, and in lanes 7 and 8, equal amounts of radioactivity were loaded. The gels shown in lanes 1 to 5 of (a) and lanes 1 to 4 of (b) contained 12% acrylamide; the remainder contained 13% acrylamide.

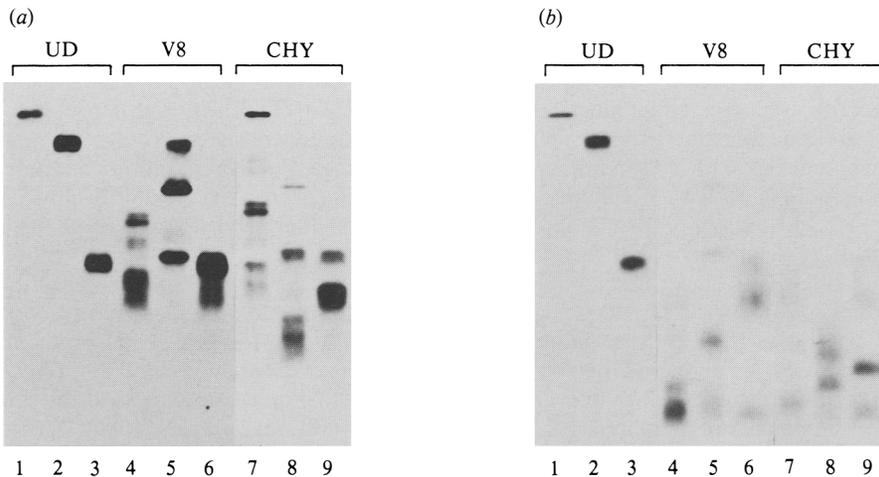


Fig. 2. Analyses in 15% polyacrylamide gels of limited proteolytic digests of (a) [^3H]mannose-labelled and (b) [^{35}S]methionine-labelled GP60 (lanes 1, 4 and 7), GP46 (lanes 2, 5 and 8) and GP20 (lanes 3, 6 and 9). Lanes 1 to 3, undigested samples (UD); lanes 4 to 6, samples digested with V8 protease; lanes 7 to 9, samples digested with α -chymotrypsin (CHY). The digestions were carried out at 37 $^\circ\text{C}$ for 15 min in (a) and 150 min in (b).

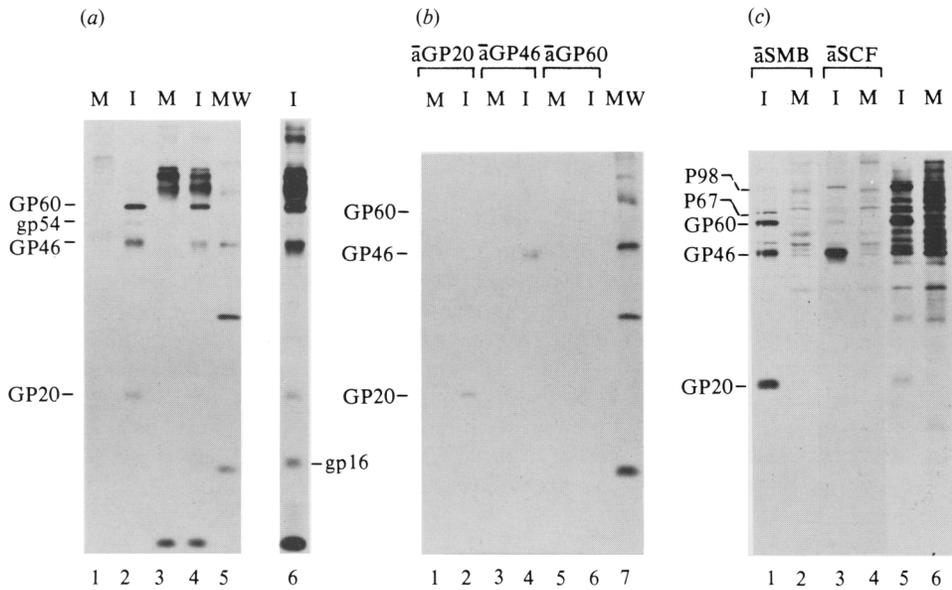


Fig. 3. Analyses in polyacrylamide gels of proteins and glycoproteins from DEN-2 virus-infected (I) and mock-infected (M) C6/36 cells and Vero cells. (a) Lanes 1 and 2, Vero cells labelled for 3 h with [3H]mannose; lanes 3 and 4, C6/36 cells labelled for 3 h with [3H]glucosamine; lane 5, ^{14}C -labelled molecular weight markers; lane 6, longer exposure of the material in lane 4. (b) Mock-infected and DEN-2 virus-infected C6/36 cells labelled for 3 h with [3H]glucosamine and immunoprecipitated with antisera raised against GP20, GP46 and GP60 purified from infected Vero cells. Equal volumes of immunoprecipitated material were loaded in each pair of lanes. Lane 7, ^{14}C -labelled molecular weight markers. (c) DEN-2 virus-infected and mock-infected C6/36 cells labelled for 3 h with [^{35}S]methionine and immunoprecipitated with antiserum raised against DEN-2 virus-infected suckling mouse brain ($\bar{a}SMB$) or against the DEN-2 SCF antigen ($\bar{a}SCF$). Lanes 5 and 6, the starting material from which the immunoprecipitates were prepared. The concentration of acrylamide in the gels was 12% in (a) and 13% in (b) and (c).

lanes 1 and 2). The small glycoproteins gp16 and gp13 were not detected in all gels and it remains to be established whether or not they represent unique, virus-specified polypeptides. In some experiments a minor glycoprotein with a mol. wt. of approximately 54K was also detected (see later in Fig. 3a, lane 2; Fig. 5a, lanes 3 to 7). Since GP60 was strongly and stably labelled, and co-migrated with the envelope glycoprotein (E) from purified DEN-2 virus labelled with [3H]methionine (Fig. 1a, lanes 6 and 7), or with [3H]mannose (data not shown), we assume it represents the intracellular counterpart of E. Confirmation of this assumption was prevented by our inability to obtain sufficient radioactively labelled E for peptide mapping.

The results of the immunoprecipitation experiments for infected Vero cells are shown in Fig. 1(b). [3H]Mannose-labelled GP60, GP46, GP20 and gp16 were all precipitated by anti-SMB serum; in most experiments, GP60 was more readily detected than in the example shown (Fig. 1b, lane 1). GP46 was the only [3H]mannose-labelled glycoprotein detected following immunoprecipitation by anti-SCF serum (Fig. 1b, lane 3). This finding demonstrates that GP46 (NV3 in the old nomenclature) represents the SCF antigen. The major virus-specified proteins and glycoproteins immunoprecipitated from [^{35}S]methionine-labelled cells by anti-SMB serum were P98, P67, GP60, GP46 and GP20 (Fig. 1b, lane 5). Although the largest protein P98 was consistently detected in immunoprecipitates prepared with the anti-SMB serum, it was always present in smaller proportions than the other immunoprecipitated polypeptides (see Fig. 1b, lane 5 and Fig. 3c, lane 1), possibly because of proteolytic degradation of P98 that occurred during the immunoprecipitation procedure. Analysis of immunoprecipitates of [^{35}S]methionine-labelled cells prepared with anti-SCF serum revealed that although GP46 was the major

virus-specified protein precipitated (approximately 80% of label present as determined by densitometry), very small amounts of several others were also present (Fig. 1*b*, lane 7). A possible explanation for this apparently non-specific precipitation is that the cell extracts used in the immunoprecipitations were not completely dissociated by the triple-detergent lysis buffer; proteins associated with small fragments of membranes containing GP46 were thus precipitated together with GP46 by the anti-SCF serum. It is also possible that radiolabelled proteins bound non-specifically to the Protein A, to the Sepharose CL-4B beads or to both (Roehrig *et al.*, 1982; Schlesinger *et al.*, 1983). In most immunoprecipitates of cells labelled with [³⁵S]methionine (e.g. Fig. 1*b*, lanes 5 to 8), two host-coded proteins that migrated close to GP46 were observed. The smaller of the two is probably actin (Hayes *et al.*, 1978). The recoveries of radioactivity in the immunoprecipitates of infected cells shown in lanes 5 and 7 of Fig. 1(*b*) were approximately sixfold greater than the recoveries obtained for the corresponding immunoprecipitates of mock-infected cells shown in lanes 6 and 8. Samples containing equal amounts of radioactivity were electrophoresed in lanes 5 and 6 and in lanes 7 and 8; thus, the intensities of the bands in lanes 6 and 8 representing polypeptides immunoprecipitated from mock-infected cells have been enhanced approximately sixfold relative to the intensities that would have been obtained if equal volumes of infected and mock-infected samples had been loaded.

Identification of DEN-2 virus-specified proteins and glycoproteins in C6/36 cells

A comparison of the DEN-2 virus-specified glycoproteins detected in C6/36 cells and Vero cells is shown in Fig. 3(*a*). The three major glycoproteins observed in infected Vero cells labelled with [³H]mannose, namely GP60, GP46 and GP20 (Fig. 3*a*, lane 2), were also seen in infected C6/36 cells labelled with [³H]glucosamine (Fig. 3*a*, lanes 4 and 6). [³H]Glucosamine was used to label glycoproteins in C6/36 cells because [³H]mannose was poorly incorporated by this cell line. Although glycoproteins corresponding to GP60, GP46 and GP20 were detected in the infected mosquito cells they clearly did not represent the major proportion of the incorporated [³H]glucosamine. Several prominent glycoproteins with mol. wt. higher than 60K were observed in both infected and mock-infected C6/36 cells (Fig. 3*a*, lanes 4 and 3). Of the three minor [³H]mannose-labelled glycoproteins frequently (though not always) observed in DEN-2 virus-infected Vero cells, namely gp54, gp16 and gp13, gp54 and gp13, were not readily detected in DEN-2 virus-infected C6/36 cells labelled with [³H]glucosamine (Fig. 3, lane 6). A glycoprotein corresponding to gp16 was resolved, migrating slightly faster than its counterpart in infected Vero cells.

To confirm the identity of the virus-specified glycoproteins in infected C6/36 cells, and to seek possible precursor-product relationships among the glycoproteins, antisera were raised against GP60, GP46 and GP20 purified from DEN-2 virus-infected Vero cells. Immunoprecipitates of DEN-2 virus-infected C6/36 cells labelled with [³H]glucosamine were then prepared using the anti-GP60, anti-GP46 and anti-GP20 sera. The analyses of the immunoprecipitates are shown in Fig. 3(*b*). For each antiserum a single glycoprotein was precipitated from infected but not from mock-infected C6/36 cells. These findings confirmed that the three [³H]glucosamine-labelled glycoproteins were the equivalents of those seen in infected Vero cells labelled with [³H]mannose and suggested that they were not derived from the larger glycoproteins detected in infected C6/36 cells (Fig. 3*a*, lane 4). These results also supported the finding that GP60, GP46 and GP20 were unrelated by peptide mapping (Fig. 2). The identification of the other major non-structural proteins in DEN-2 virus-infected C6/36 cells (namely P98 and P67) was achieved by immunoprecipitation of [³⁵S]methionine-labelled infected cells with anti-SMB serum (Fig. 3*c*, lane 1). GP46 was the major virus-specified protein present in immunoprecipitates of infected C6/36 cells prepared with anti-SCF serum (Fig. 3*c*, lane 3). The overall recoveries of radioactivity after immunoprecipitation of infected C6/36 cells labelled with [³⁵S]methionine were generally lower than those obtained with Vero cells but were approximately threefold greater than the recoveries obtained with the corresponding mock-infected cells. Thus, the bands in lanes representing immunoprecipitates of mock-infected cells in Fig. 3(*c*) have been intensified by loading equal amounts of radioactivity as in Fig. 1(*b*).

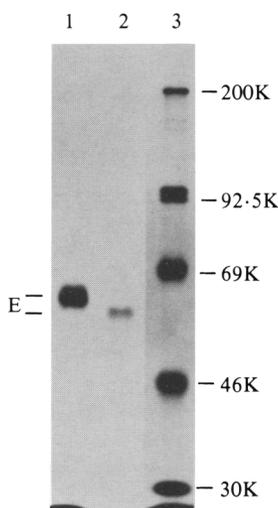


Fig. 4. Analysis in an 8% polyacrylamide gel of the envelope protein of [^3H]methionine-labelled DEN-2 virus grown in Vero cells (lane 1) and C6/36 cells (lane 2). ^{14}C -labelled molecular weight markers were also included (lane 3).

DEN-2 virions were labelled with [^3H]methionine during growth in Vero or C6/36 cells and the purified virions analysed by gel electrophoresis (Fig. 4). The E protein from virus grown in C6/36 cells (Fig. 4, lane 2) migrated slightly faster than the E protein from virus grown in Vero cells (Fig. 4, lane 1). The C and M proteins migrated at the gel front.

Pulse-labelling experiments

The experiments described in the previous sections were generally carried out using relatively long labelling periods (up to 3 h). Shorter labelling periods were also used with a view to detecting any possible precursor-product relationships among the proteins and glycoproteins specified in DEN-2 virus-infected Vero cells. Of particular interest in this regard was the intracellular form of the E protein since this appears to exist as a complex of interrelated proteins in cells infected with Kunjin virus (Wright *et al.*, 1981) or West Nile virus (Wright & Warr, 1985). DEN-2 virus-infected Vero cells were labelled with [^3H]mannose for 15 min and then incubated in medium containing excess glucose for varying periods up to 45 min (Fig. 5*a*). Twenty min after the addition of label, GP60, GP46 and GP20 were clearly present (Fig. 5*a*, lane 4) and there was an additional glycoprotein present with a mol. wt. of approximately 22000 (gp22). During the incubation in excess glucose, the amount of label in gp22 decreased as that in GP20 increased. Additional experiments with either infected Vero or C6/36 cells, using [^{35}S]methionine as label and pulse times as short as 5 min, also showed an apparent transfer of label from gp22 to GP20 during chase periods; furthermore, both gp22 and GP20 were immunoprecipitated with anti-GP20 serum (unpublished results). Thus, it is likely that gp22 is a precursor of GP20.

To investigate processing events that occurred over longer periods of time, infected Vero cells were labelled for 30 min with [^{35}S]methionine and then incubated in the presence of excess, unlabelled methionine for periods up to 150 min (Fig. 5*b*). The intensity of a band corresponding to p82 decreased during the chase (Fig. 5*b*, lanes 3 to 8). Svitkin *et al.* (1981) showed that in cells infected by tick-borne encephalitis (TBE) virus, a protein of 79K (NV41/2) was produced which was related to P69 (NV4) by peptide mapping. It is possible that p82 represented the DEN-2 virus counterpart of TBE virus p79, its disappearance being the result of conversion to P67. However, confirmation of this suggestion awaits peptide mapping of P67 and p82. Also evident in Fig. 5*b* was an apparent transfer of label from p30 to p28 (lanes 3 to 8), and from gp22 to GP20 (lanes 3 and 4) in agreement with the results presented in Fig. 5*a*.

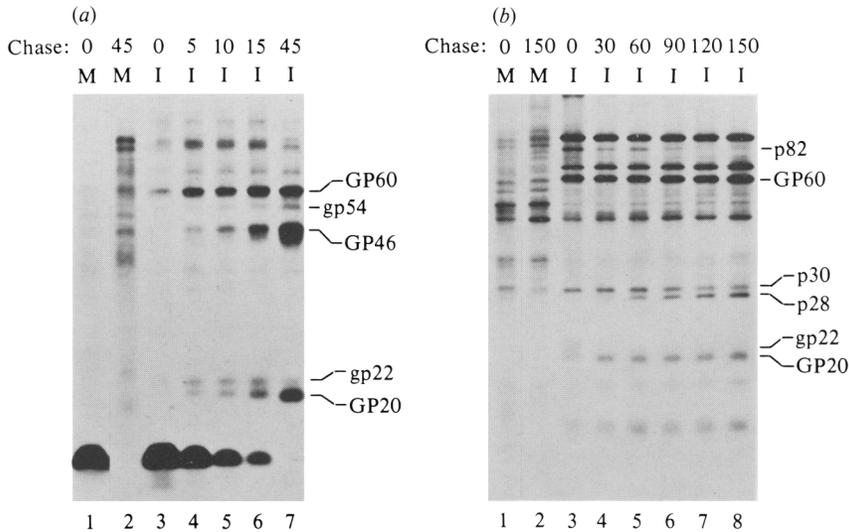


Fig. 5. Analysis in 12% polyacrylamide gels of proteins and glycoproteins from DEN-2 virus-infected (I) and mock-infected (M) Vero cells following pulse-labelling experiments. (a) Cells labelled for 15 min with [³H]mannose and then incubated in medium containing excess glucose for the times (min) shown. (b) Cells labelled for 30 min with [³⁵S]methionine and then incubated in medium containing excess methionine for the times (min) shown.

Tunicamycin treatment of infected and mock-infected cells

To obtain an estimate of (i) the total amount of *N*-linked carbohydrate and (ii) the number of *N*-linked glycan units for each of GP60, GP46 and GP20, DEN-2 virus-infected and mock-infected Vero cells were labelled with [³⁵S]methionine for 3 h in the presence of 0, 1 and 5 µg/ml of tunicamycin, an inhibitor of *N*-linked glycosylation. The reductions in incorporation of [³⁵S]methionine by the mock-infected Vero cells treated with 1 µg/ml and 5 µg/ml tunicamycin were 15% and 57% respectively; for the infected Vero cells the corresponding reductions in incorporation were 40% and 70%. Whereas tunicamycin had little effect on the polypeptides labelled in the mock-infected cells (Fig. 6*a*, lanes 1 to 3), marked changes took place in the polypeptide profiles of infected cells treated with tunicamycin (Fig. 6*a*, lanes 4 to 6). The addition of *N*-linked glycans to GP60, GP46 and GP20 was strongly inhibited by 5 µg/ml tunicamycin (Fig. 6*a*, lane 6). The molecular weights of the new polypeptides detected in treated cells were estimated and used to calculate the size of the total *N*-linked carbohydrate component of each glycoprotein. GP60 and GP46 each possessed about 4000 daltons of carbohydrate, whereas GP20 had approximately half this amount. The new forms of GP60, GP46 and GP20 lacking *N*-linked glycans were immunoprecipitated by the appropriate glycoprotein antiserum (data not shown). Although the form of GP20 synthesized in the presence of tunicamycin comigrated with p18, it was apparently not related to it since p18 was not immunoprecipitated by anti-GP20 serum (data not shown). At a tunicamycin concentration of 1 µg/ml, which was less than that required to inhibit glycosylation completely, an intermediate glycosylated form of GP60 was detected (Fig. 6*a*, lane 5). It was also likely that an intermediate glycosylated form of GP46 was produced, but was not readily detected because of the difficulties associated with the resolution of virus-specified proteins in this region of the gel. It appeared that no intermediate glycosylated forms of GP20 were synthesized at 1 µg/ml tunicamycin (Fig. 6*a*, lane 5). These observations suggested that GP60 had at least two *N*-linked glycans per molecule, that GP46 possibly had two, and that GP20 had one.

The effects of tunicamycin on the proteins synthesized in C6/36 cells (Fig. 6*b*) were similar to those observed in Vero cells (Fig. 6*a*). Not only was it difficult to resolve GP46 from co-migrating host proteins, but also its equivalents made in tunicamycin-treated cells (Fig. 6*b*,

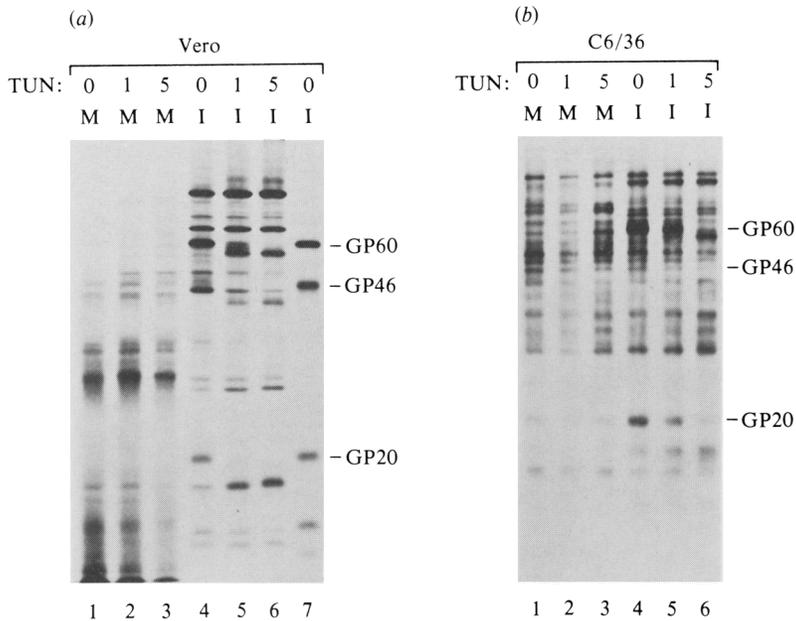


Fig. 6. Analyses in 12% polyacrylamide gels of DEN-2 virus-infected (I) and mock-infected (M) Vero cells and C6/36 cells treated with tunicamycin. (a) Vero cells and (b) C6/36 cells were labelled with [³⁵S]methionine for 3 h in the presence of tunicamycin (TUN) at the concentrations ($\mu\text{g/ml}$) indicated.

lanes 4 to 6). GP20 from infected C6/36 cells treated with tunicamycin appeared to migrate with, or slightly ahead of p18 (Fig. 6*b*, lane 6). Increasing the concentration of tunicamycin to 20 $\mu\text{g/ml}$ had no significant effect on the resolution of underglycosylated polypeptides in SDS gels.

Glycopeptide analyses

Although tunicamycin treatment of infected cells allowed estimates of the size of the *N*-linked carbohydrate component, and of the number of glycan units for each virus-specified glycoprotein, it did not provide any information about the number of structurally different glycan units that were attached to the polypeptide backbones of the glycoproteins. To investigate this area further, the glycopeptides derived from [³H]mannose-labelled GP60, GP46 and GP20 were analysed. The glycoproteins were exhaustively digested with Pronase and the resulting glycopeptides analysed by gel filtration (Fig. 7*a, b, c*) or by electrophoresis in Tris-borate gels (Fig. 7*d, e, f*). In each analysis by gel filtration, the void volume (V_0) eluted at fraction 22, and a [¹⁴C]mannose-labelled marker glycopeptide derived from Semliki Forest virus-specified p62 eluted at fraction 37. The molecular weight calibration of the column was carried out using stachyose and glycopeptides of ovalbumin and thyroglobulin (Wright *et al.*, 1980). GP60 had two major glycopeptide size classes with mol. wt. of approximately 2800 and 2300, and a minor glycopeptide size class with a mol. wt. of approximately 1500 (Fig. 7*a*). The glycopeptides of mol. wt. 2300 and 1500 were also present in the Pronase digests of GP46 (Fig. 7*b*) and GP20 (Fig. 7*c*). All of the glycopeptides were shown to be sensitive to digestion with endo- β -*N*-acetylglucosaminidase (endo H) (data not shown) and therefore possess mannose-rich glycans. Consistent with this conclusion was the observation that none of the glycopeptides was sensitive to mild acid hydrolysis and therefore they are presumably not sialylated.

Since glycopeptides of similar size but possessing glycans of different composition or structure may elute in the same position during gel filtration, the Pronase-digested glycoproteins were also analysed by Tris-borate gel electrophoresis. In this technique the separation of glycopeptides is based on their charge, which in turn is determined by the number of bound borate ions. The

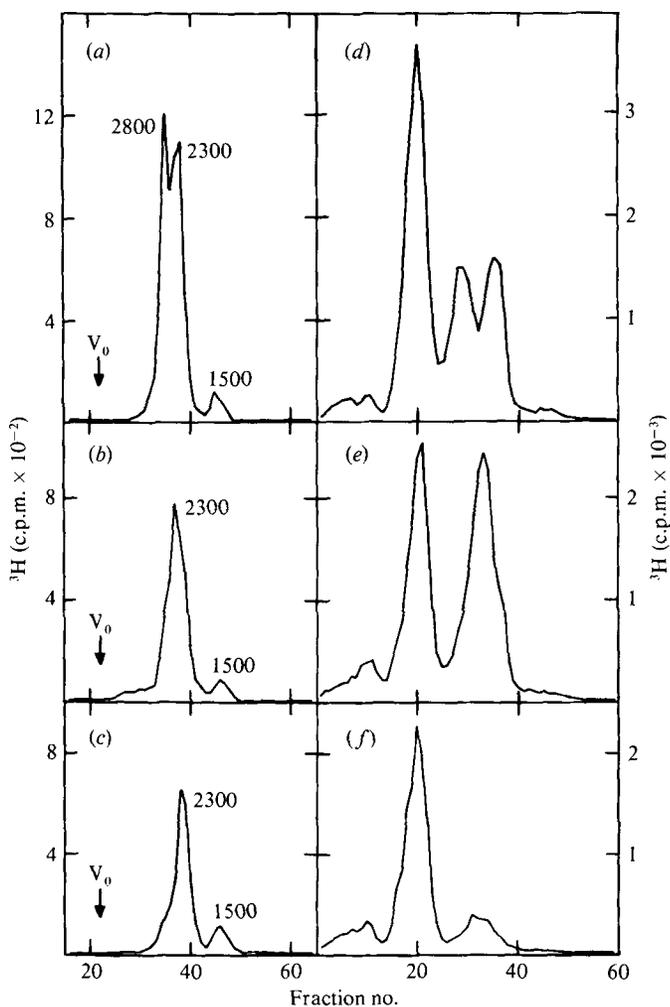


Fig. 7. Analyses of [^3H]mannose-labelled glycopeptides from Pronase-digested GP60, GP46 and GP20 either by gel filtration using Bio-Gel P-6 (*a, b, c*) or by Tris-borate gel electrophoresis (*d, e, f*). The three glycoproteins were prepared from DEN-2 virus-infected Vero cells that had been labelled for 3 h with [^3H]mannose. For the gel filtration profiles the void volume (V_0) is indicated. Electrophoresis was from left to right for the Tris-borate gel profiles.

composition, sequence and linkages of the glycans rather than the residual amino acid residues in glycopeptides influence the binding of the borate ions (Weitzman *et al.*, 1979). Analyses of the glycopeptides of GP60, GP46 and GP20 by this technique (Fig. 7*d, e, f*) revealed an additional degree of heterogeneity among them since more classes of glycopeptides for each glycoprotein were resolved by electrophoresis than by gel filtration. Comparison of Fig. 7(*a*) with Fig. 7(*d*) suggested that at least one of the major size classes of glycopeptides of GP60 (Fig. 7*a*) contained glycans of different structure or composition. Similarly, comparison of Fig. 7(*b*) with Fig. 7(*e*) suggested that the major size class of glycopeptides of GP46 (Fig. 7*b*) contained at least two different glycans.

DISCUSSION

Fifteen proteins and glycoproteins were detected in DEN-2 virus-infected Vero cells that were not present in mock-infected cells, namely P98, p82, P67, GP60, gp54, GP46, p30, p28, gp22, GP20, p18, gp16, p15, p14 and gp13 (Fig. 1*a, 5a* and *5b*). With the exceptions of gp13, gp16 and

possibly gp54, proteins corresponding to the DEN-2 virus-specified polypeptides listed above have previously been observed in cells infected with DEN-2 virus or with other flaviviruses (Shapiro *et al.*, 1971; Trent & Qureshi, 1971; Westaway, 1973, 1975; Stohman *et al.*, 1975; Westaway *et al.*, 1977; Westaway & Shew, 1977; Schlesinger, 1981; Schlesinger *et al.*, 1983). Of the virus-specified polypeptides detected in infected Vero cells, only gp54 and gp13 were not resolved in the analyses of DEN-2 virus-infected C6/36 cells (Fig. 3*a, b, c*) possibly because of the difficulty in labelling viral glycoproteins in C6/36 cells with radioactive carbohydrates. The major virus-specified polypeptides (P98, P67, GP60, GP46 and GP20) were immunoprecipitated from extracts of infected cells by antiserum prepared against DEN-2 virus-infected suckling mouse brain (Fig. 1*b, 3c*). The glycoprotein GP46 was the major virus-specified polypeptide immunoprecipitated by antiserum raised against the SCF antigen of DEN-2, providing the strongest evidence thus far that it is the SCF antigen (Russell *et al.*, 1980).

The [³H]methionine-labelled E protein from DEN-2 virus grown in C6/36 cells migrated faster through polyacrylamide gels than E from virus grown in Vero cells (Fig. 4). Luukkonen *et al.* (1977) demonstrated that the envelope glycoproteins of Semliki Forest virus grown in mosquito cells migrated ahead of those from the same virus grown in hamster cells; the increased electrophoretic mobility of the glycoproteins was a result, at least in part, of the absence of sialic acid on the glycan units. Sindbis virus was also shown to lack sialic acid when grown in *A. albopictus* cells (Stollar *et al.*, 1976). The lack of sialic acid on virus-specified glycoproteins grown in cultured mosquito cells has been attributed to the absence of the enzyme sialyl transferase (Stollar *et al.*, 1976). However, the difference in migration between the E proteins of DEN-2 virus grown in Vero and C6/36 cells was not simply the consequence of a difference in sialic acid content for two reasons. Firstly, both E of virus grown in Vero cells and GP60 (the intracellular counterpart of E) detected in infected Vero cells co-migrated with GP60 synthesized in infected C6/36 cells. Secondly, the migration in Tris-borate gels of the glycopeptides derived from GP60 of infected Vero cells remained unchanged following acid hydrolysis under conditions that remove sialic acid from complex *N*-linked glycan units. Thus, at a stage late in the maturation of DEN-2 virions in C6/36 cells, E protein was modified by proteolysis, by processing of its glycans, or by both of these events to yield a glycoprotein of reduced molecular weight. More details of the maturation process are currently being sought.

Pulse-chase experiments with [³H]mannose and [³⁵S]methionine failed to detect any likely precursors of either GP46 or GP60. The lack of precursors of GP60 contrasted with the findings for cells infected with Kunjin or West Nile viruses where pulse-chase experiments and peptide mapping demonstrated precursor-product relationships among several E-related glycoproteins larger than E (Wright *et al.*, 1981; Wright & Warr, 1985). However, processing of gp22 to GP20 was detected during short pulse-chase experiments (Fig. 5*a*); both polypeptides were immunoprecipitated by anti-GP20 serum. As discussed in the preceding paragraph for the two forms of E protein, it is not known whether GP20 is derived from gp22 by proteolysis, by modification of glycan units, or by a combination of both.

The total coding potential of DEN-2 RNA is approximately 430 000 daltons of polypeptide, assuming overlapping genes are absent. This is adequate to account for the 15 polypeptides listed above, if the following assumptions and allowances are made: (i) related polypeptide backbones are shared by p82 and P67 (Svitkin *et al.*, 1981), by gp22 and GP20 (Fig. 5*a, b*), and by p30 and p28 (Fig. 5*b*) and (ii) the contribution of carbohydrate to the mol. wt. of the glycoproteins is approximately 4000 and 2000 for the large (GP60, gp54, GP46) and small (gp22, gp16, gp13) glycoproteins, respectively. More accurate estimates of the total unique polypeptide represented by the 15 proteins will be possible when the polypeptides with the prefixes 'p' and 'gp' have been further characterized by peptide mapping.

Analyses of the [³H]mannose-labelled glycopeptides of GP60, GP46 and GP20 by gel filtration showed that there were three size classes of glycopeptides with mol. wt. of approximately 2800, 2300 and 1500 (Fig. 7*a, b, c*). Glycopeptides of these sizes have also been reported for Kunjin virus-specified glycoproteins (Wright *et al.*, 1980). All three size classes were represented in the analyses of GP60, whereas the largest glycopeptides (mol. wt. 2800) were absent from the analyses of GP46 and GP20. The smallest glycopeptides (mol. wt. 1500) were

minor components, representing a small proportion of the total [^3H]mannose incorporated. Analyses of the glycopeptides by the technique of Tris-borate electrophoresis (Fig. 7*d, e, f*), which separated them on the basis of charge rather than size, revealed structural heterogeneity within the size classes. The results of the experiments with tunicamycin suggested (i) that the total contribution of *N*-linked carbohydrate to the molecular weights of the glycoproteins was approximately 4000 for each of GP60 and GP46, and 2000 for GP20, and (ii) that GP60 had at least two glycan units, GP46 probably had two and GP20 had only one. When considered with the number of different glycopeptides resolved for each glycoprotein in Fig. 7(*d, e, f*) (namely four for GP60, three for GP46 and three for GP20), these results indicated that the glycoproteins were not homogeneous with regard to the structure and the composition of their glycan units.

At present, little is known about proteins and glycoproteins specified by viruses of the other three dengue serotypes. Most of the work on flavivirus-infected cells that has been published to date has focused on cells infected by viruses of the West Nile-Murray Valley encephalitis subgroup (Westaway, 1980). Apparent unique features of DEN-2 virus-infected cells identified in this paper are the presence of glycoproteins smaller than GP20, the lack of several intracellular virus-specified glycoproteins related to E, the prominence of SCF antigen (GP46 or NV3) in [^3H]mannose-labelled cells, and the processing of p30 to p28. Further characterization of the virus-coded polypeptides and genome structure of all four dengue serotypes is needed to identify both the common and unique features in the replication of dengue viruses.

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