Influence of N-linked oligosaccharide chains on the processing, cell surface expression and function of the measles virus fusion protein

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The fusion (F) glycoprotein of measles virus, a structural component of the virion envelope, contains four potential sites for attachment of N-linked oligosaccharides. Three are located in the F₂ subunit of the protein and one in the signal peptide. Four mutants were constructed by oligonucleotide-directed mutagenesis, in each case changing one N-linked glycosylation site from Asn-X-Ser/Thr to Ser-X-Ser/Thr. The wild-type and altered forms of the F protein were expressed in BHK-21 and HeLa T4 cells by use of the recombinant vaccinia virusencoding T7 polymerase system. Analysis of these proteins revealed that three (residues 29, 61 and 67) potential sites for addition of N-linked glycans in the F₂ subunit are actually utilized. The functional glycosylation sites were systematically removed in all possible combinations from the F protein to form a panel of mutants from which the role of carbohydrates, singly or in various combinations, could be evaluated. One single-site mutant protein lacking the glycosylation site of Asn-67 was processed, transported to the cell surface and could induce cell fusion. However, the other two single-site mutant proteins with deletions of glycosylation sites Asn-29 or Asn-61 exhibited a defect in processing, were not transported to cell surface and thus induced no cell fusion. The absence of any two of the three or of all three glycosylation sites resulted in protein retention in the endoplasmic reticulum. Therefore, it appears that glycosylation of sites Asn-29 and Asn-61 has important roles in maintaining the native structure of the F protein.

Measles virus (MV) is a member of the Morbillivirus genus within the Paramyxoviridae family of negative-strand enveloped RNA viruses. Two surface spike glycoproteins, the haemagglutinin (H) and the fusion (F) proteins, exist on the viral envelope (Norrby & Oxman, 1990). The H protein mediates attachment of virions to the receptor molecule CD46 (Dörrig et al., 1993; Naniche et al., 1993). The F protein functions in penetration of virus into the host cells by initiating fusion of the virion envelope with the cellular plasma membrane. The F glycoprotein is a type I integral membrane protein. The protein is synthesized as an inactive precursor (Fo) that is cleaved by a host cell proteolytic enzyme in the trans-Golgi (Morrison et al., 1985) to form a biologically active protein consisting of the disulphide-linked subunits F₁ and F₂ (Scheid & Choppin, 1974, 1977). Once the F protein has reached the plasma membrane, its hydrophobic F₁ amino-terminal (fusion) peptide mediates fusion of infected cells with adjacent cells.

The carbohydrate moieties attached to asparagine residues of the consensus sequence Asn–X–Ser/Thr (N-linked glycosylation) on polypeptides have been implicated in a variety of biological functions (Olden et al., 1982; Rademacher et al., 1988), such as protection of the protein from proteolytic degradation and establishment of native conformation through the accurate formation of disulphide bonds. The MV F protein contains four predicted sites for the addition of N-linked oligosaccharides at amino acid residues 6, 29, 61 and 67 (Richardson et al., 1986). However, it remains to be determined which sites are actually used. To address this question, we constructed mutants in which consensus sequences for N-linked glycosylation were altered. The mutations were made on plasmid peΔ5F1, which retains only the last 108 of the 573 nucleotides of the F gene 5' untranslated region (Spielhofer, 1990; Cattaneo & Rose, 1993). Four 25-mer oligonucleotides with the desired mismatch nucleotide in the middle of sequences complementary to F cDNA sequences encoding the four N-linked glycosylation consensus sites (Asn–X–Ser/Thr) were utilized to substitute codons for serine residues in place of those encoding asparagine residues. Oligonucleotide-directed site-specific mutagenesis was done as described previously (Hu et al., 1994a). The three sites used for N-linked glycosylation (see below) are desig-

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nated g1, g2 and g3, corresponding to Asn found at F protein residues 29, 61 and 67, respectively (Fig. 1a). The potential N-linked glycosylation site within the signal peptide is named gs. A total of eight N-linked glycosylation mutants were generated, each designated Fg(n), where n is a set of numbers defining the N-linked glycosylations that are used: e.g. a mutant lacking the g1 site is Fg23 and the nonglycosylated mutant is Fg0.

The plasmids containing F glycosylation mutant genes were introduced into HeLa T4 or BHK-21 cells by use of lipofectin (Gibco BRL). The genes were expressed by the recombinant vaccinia virus-encoding T7 polymerase system (Fuerst et al., 1986) and the proteins were analysed by immunoblotting. Transfected cells were harvested in lysis buffer (50 mM-Tris, pH 8.0, 62.5 mM-EDTA, 1% NP40, 0-4% deoxycholate) 20 h post-transfection. Different samples from equivalent numbers of cells were subjected to SDS–PAGE and the separated proteins were transferred semi-dry to Immobilon PVDF membranes (Millipore) which were blocked with 1% BSA/0.5% skim milk powder in TBST (10 mM-Tris, pH 8.0, 150 mM-NaCl, 0.05% Tween 20) for 1 h at room temperature and then incubated with an antipeptide antiserum overnight at 4 °C. This antiserum was raised against a peptide derived from the carboxy terminus of the F protein, NH2-(C)PDLTGTSKSYRSL-COOH (a cysteine was attached to the amino terminus of the peptide in order to couple the peptide to keyhole limpet haemocyanin for immunization of rabbits). After extensive washing with TBST, membranes were incubated with horseradish peroxidase conjugated swine anti-rabbit IgG (Dakopatts) for 1 h at room temperature. Proteins
Fig. 2. Flow cytometry analyses of wild-type and mutant F proteins. Transfected HeLa T4 cells were reacted with primary antibody and subsequently with FITC-conjugated goat anti-rabbit IgG for indirect staining of $2 \times 10^5$ cells. Cell surface expression of the F protein was measured on a flow cytometer. Background fluorescence shows staining of the cells transfected with an irrelevant plasmid after infection with vaccinia virus (VV). The designations of the wild-type and mutant F proteins are indicated within the histogram. The values represent the percentage of positively stained cells within the gated area of live cells.

were visualized using the enhanced chemiluminescence system (ECL, Amersham). The results derived from the study of the transfected HeLa T4 cell lysates are shown in Fig. 1(b). Since the blots were probed with an antiserum directed against the F protein carboxy terminus, both the F$_0$ precursor and the F$_1$ subunit, but not the F$_2$ subunit, could be detected. When the wild-type plasmid was used for transfection (Fgwt), two forms of F$_0$ precursor were clearly observed: a major product migrating with an estimated molecular mass of 53 kDa and a minor product with a molecular mass of 51 kDa [Fig. 1 b(ii)]. The same products were also observed in MV-infected cells (data not shown). Not unexpectedly, the Fgs mutant in which only the glycosylation site situated in the signal sequence had been altered produced the same profile of F$_0$ proteins as Fgwt.

Considering that an N-linked glycan residue has an average molecular mass of about 2 kDa, it is expected that mutations resulting in alteration of functional glycosylation sites generate a 2 kDa downshift of the F$_0$ products. As shown in Fig. 1 (b)(i), the F$_0$ proteins of mutants Fg12 and Fg13 had 2 kDa downshifts of both the 53 kDa and 51 kDa proteins compared with the wild-type F protein. These data suggest that site 3 (absent in mutant Fg12) and site 2 (absent in mutant Fg13) are both efficiently used. On the other hand, the F$_0$ proteins of mutant Fg23 also shifted down by 2 kDa, but not quantitatively. This suggests that site 1 is used in only part of the protein. The above conclusions were further confirmed by the analysis of the double- and triple-site mutants. Mutant Fg0, without potential glycosylation sites, produces one single 47 kDa protein. As expected, Fg1 has two F$_0$ bands of 47 and 49 kDa, whereas Fg2 and Fg3 have a single F$_0$ major product of 49 kDa.

To confirm that size differences monitored in our analysis are due to the addition of oligosaccharide chains to the F$_0$ precursor, we digested all the proteins from our panel of mutants with endoglycosidase H [endo H, Fig. 1b(ii)]. This enzyme cleaves high-mannose oligosaccharides that are added in the endoplasmic reticulum, but does not cleave oligosaccharides modified by two Golgi enzymes. Cell lysates were adjusted to 75 mM-sodium citrate pH 5-6, incubated with 3 mU endo H (Boehringer Mannheim) for 6 h at 37 °C and subjected to SDS-PAGE and Western blotting analyses. Since the F$_0$ precursor is found largely in the endoplasmic reticulum, endo H is expected to cleave F$_0$ oligosaccharides to completion. Only the size of the F$_0$ protein from the mutant without potential glycosylation sites (Fg0) remained unchanged after endo H digestion (47 kDa). In all glycosylated mutant proteins, however, endo H digestion was partial: about half of the material remained at 49 kDa when conditions resulting in complete digestion of other glycoproteins were used. The fraction of completely digested protein was only marginally increased by the use of concentrated enzyme or longer digestion times. Moreover endo F, an enzyme able to cleave complex oligosaccharides also, failed to eliminate the 49 kDa upper band (data not shown), suggesting an intrinsic resistance to glycosidase digestion.

The deletion of one of the three glycosylation sites used had different consequences for protein maturation: only the Fg12 mutant maintained partial cleavage, as monitored by the appearance of the F$_1$ protein, whereas the F$_0$ precursors of Fg13, Fg23 and of all double- and triple-site mutants were not efficiently cleaved (longer exposure of this gel revealed a cleavage level of approximately 1%).

The subcellular localization of the glycosylation mutant proteins was examined by indirect immunofluorescence. Analysis of the entire panel of mutants...
indicated that only mutant protein Fg12 displayed cell surface expression and the other mutant proteins, including mutants Fg13 and Fg23, did not reach the cell surface and exhibited exclusively endoplasmic reticulum-like staining (data not shown). The surface expression of the mutant proteins was also quantitatively determined by flow cytometry on a FACScan (Becton-Dickinson). The results are shown in Fig. 2 as percentage positively stained HeLa T4 cells. From transfection of the wild-type plasmid, 33% cells displayed positive staining. The mutant Fg12 exhibited a slightly lower level (28%) of mutant F protein at the cell surface compared with the wild type plasmid. The single-site mutants Fg13 (5%) and Fg23 (4%), the double-site mutant Fg1 (3%) and triple-site mutant Fg0 (2%) showed a level of fluorescence only marginally higher than the negative control (2%).

In the vaccinia virus T7 system and HeLa T4 cells, fusion by the MV F glycoprotein is completely dependent on the co-expression of the H protein (Cattaneo & Rose, 1993). To evaluate the possible contribution of N-linked oligosaccharide side chains on the H and F proteins to cell fusion, the F glycosylation mutants generated in this study and the previously produced H glycosylation mutants (Hu et al., 1994a) were co-expressed in HeLa T4 cells. All possible combinations between the F and H glycosylation mutants were used to cotransfect HeLa T4 cells and the capacity to cause cell fusion was determined under conditions described in Cattaneo & Rose (1993). Examples representing wild-type, single-, double- and triple-site F mutants cotransfected with H glycosylation mutant Hg12 are shown in Fig. 3. Again, only mutant Fg12 induced cell fusion (Fig. 3b). The other F mutants, e.g. Fg13 (Fig. 3c) and Fg23 (Fig. 3d), showed no capacity to cause cell fusion.

Deletion analysis indicated that three sites (asparagine 29, 61 and 67) were utilized for addition of N-linked oligosaccharide side-chains. It is not known if asparagine 6 was used because this site is located within the cleaved signal peptide. Our data indicate that the asparagine to
serine mutation did not have negative consequences on peptide cleavage. The three functional N-linked glycosylation sites defined for MV F protein are completely conserved in all morbilliviruses (Barrett et al., 1987; Kövamees et al., 1991; Richardson et al., 1986; Tsukiyama et al., 1988), suggesting that they have a functional significance. Inactivation of these glycosylation sites impaired the intracellular transport, as well as processing, of the F protein. The severity of the defect was dependent on both the position and the number of the deleted oligosaccharide side-chains. In the absence of glycosylation of Asn-67, the mutant protein (Fgl12) retained partial proteolytic cleavage, cell surface expression and fusion-inducing capacity. Hence it appeared that absence of this site had no significant effect on the overall F conformation.

When this paper was in preparation we learned that Alkhatib et al. (1994) performed functional studies on a small panel of MV F protein glycosylation mutants. Those authors substituted asparagine by the bulkier neutral amino acid glutamine rather than by the smaller neutral amino acid serine. They used human NCI-H460 and simian CV1 cells instead of human HeLa T4 cells and recombinant vaccinia viruses instead of the vaccinia virus–T7 RNA polymerase expression system. Importantly, in NCI-H460 cells fusion was not strictly dependent on H protein co-expression. In spite of these differences, both studies concluded that all three glycosylation sites in the F protein are used, that mutation of Asn-29 and Asn-61 had significant influence on the overall F conformation.

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References


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