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Evidence that maturation of the N-linked glycans of the respiratory syncytial virus (RSV) glycoproteins is required for virus-mediated cell fusion: The effect of α-mannosidase inhibitors on RSV infectivity

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Abstract

Glycan heterogeneity of the respiratory syncytial virus (RSV) fusion (F) protein was demonstrated by proteomics. The effect of maturation of the virus glycoproteins-associated glycans on virus infectivity was therefore examined using the α-mannosidase inhibitors deoxymannojirimycin (DMJ) and swainsonine (SW). In the presence of SW the N-linked glycans on the F protein appeared in a partially mature form, whereas in the presence of DMJ no maturation of the glycans was observed. Neither inhibitor had a significant effect on G protein processing or on the formation of progeny virus. Although the level of infectious virus and syncytia formation was not significantly affected by SW-treatment, DMJ-treatment correlated with a one hundred-fold reduction in virus infectivity. Our data suggest that glycan maturation of the RSV glycoproteins, in particular those on the F protein, is an important step in virus maturation and is required for virus infectivity.

Keywords: Respiratory syncytial virus; Virus assembly; Fusion protein; Glycosylation; Swainsonine; Deoxymannojirimycin; Proteomics

Introduction

Human respiratory syncytial virus (hRSV) encodes three integral membrane proteins, namely the fusion (F), attachment (G) and small hydrophobic (SH) proteins. The G protein mediates attachment of the virus to the cell during virus entry, while the role played by the SH protein during virus replication remains to be established. During virus entry, the F protein plays a pivotal role by mediating fusion of the virus and host-cell membranes. Studies employing reverse genetics have shown that whereas the F protein is essential for replication in tissue culture, the G and SH proteins are dispensable (Bukreyev et al., 1997; Karron et al., 1997; Whitehead et al., 1999; Techaarpornkul et al., 2001). The G protein is extensively modified by O-linked glycosylation, but it is not clear to what extent it is modified by N-linked glycosylation. In addition, only minor amounts of the SH protein are N-linked glycosylated. In contrast, of the three virus glycoproteins, the F protein has relatively high levels of N-linked glycans, and evidence suggests a role for N-linked glycosylation of the F protein in mediating virus-mediated cell fusion (Zimmer et al., 2001b).

The F protein is synthesised initially as an inactive precursor (F0), which undergoes proteolytic cleavage by cellular factors in the trans-Golgi compartment, (Bolt et al., 2001; Gonzalez-Reyes et al., 2001; Sugrue et al., 2001; Zimmer et al., 2001a). This generates the mature and active form of the protein which consists of the disulphide-linked subunits, F1 and F2 (Scheid and Choppin, 1977; Gruber and Levine, 1985; Collins and Mottet, 1991; Anderson et al., 1992a, 1992b). During its

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transport through the secretory pathway, the F protein is glycosylated by several enzyme activities in the endoplasmic reticulum (ER) and Golgi complex (Lambert, 1988). Depending on the RSV isolate, the F protein amino acid sequence can contain up to 5 potential N-linked glycosylation sites. However, experimental evidence suggests that in the mature F protein, the F1 subunit is glycosylated at a single site, N500, while the F2 subunit is glycosylated at two different sites, namely N27 and N70 (Collins et al., 1984; Baybutt and Pringle, 1987; Lopez et al., 1988; Lerch et al., 1991; Zimmer et al., 2001b). Recent reports suggest that N500 plays a role in F protein-mediated fusion (Zimmer et al., 2001b) possibly by interacting with the heptad repeat regions. In contrast, the role played by N27 and N70 in the functionality of the F protein remains to be established.

Following the exit of the F protein from the ER, each attached glycan chain exists in a form that is characterised by a mannose core to which chains of mannose residues are attached. These mannose chains are removed subsequently in the Golgi complex by Golgi-resident α-mannosidases 1 and 2 and replaced with other terminal glycans, such as N-acetyl-glucosamine and fucose. This process changes the structure of the attached glycans from relatively simple structures to glycan chains that exhibit a high degree of complexity (Anderson et al., 1992a, 1992b; Collins and Mottet, 1991). N-linked glycosylation of the hRSV F protein does not appear to be required either for its proteolytic cleavage or its surface expression (Collins and Mottet, 1991). However, although there is no apparent requirement for glycosylation in F protein transport, evidence suggests that it may be required for post-assembly processes that involve the virus glycoproteins (e.g., membrane fusion, Zimmer et al., 2001b). The work described in this manuscript examines the effect of glycan maturation on virus infectivity. Our results suggest that maturation of the glycans on the virus glycoproteins is not required either for virus assembly or for the incorporation of these proteins into virus particles. However, glycan maturation is required for infectivity of progeny virus. The correlation between glycan maturation and at least one indicator of F protein functionality in tissue culture (i.e., virus infectivity) points to the F protein as the major site of action of DMJ. This suggests that the structure of the glycans that are attached to the F protein may be essential for determining the biological activity of this protein.

Results and discussion

The F protein exhibits heterogeneity in glycan maturation

The effect of glycosylation on F protein heterogeneity was examined by comparing the migration of the glycosylated and de-glycosylated forms of the F protein subunits by 2D SDS PAGE and Western blotting analysis. The F1 and F2 subunits were detected with MAb169 (Rixon et al., 2004) and PAb922, respectively.

RSV-infected cells were solubilised with denaturation buffer and incubated in the presence or absence of PNGase F as described in Materials and methods. This enzyme is able to remove N-linked glycans from proteins. The proteins in these lysates were separated by SDS PAGE, transferred by Western blotting on to PVDF membranes, and the membranes probed either with MAb169 or PAb922 (Fig. 1). Following treatment with PNGase F, the F1 subunit changed from a single protein species with a mass of 55 kDa to one of 50 kDa (Fig. 1A) while the F2 subunit changed from a single protein species with a mass of 20 kDa to one of 10 kDa (Fig. 1B). This showed that both subunits of the F protein were de-glycosylated efficiently

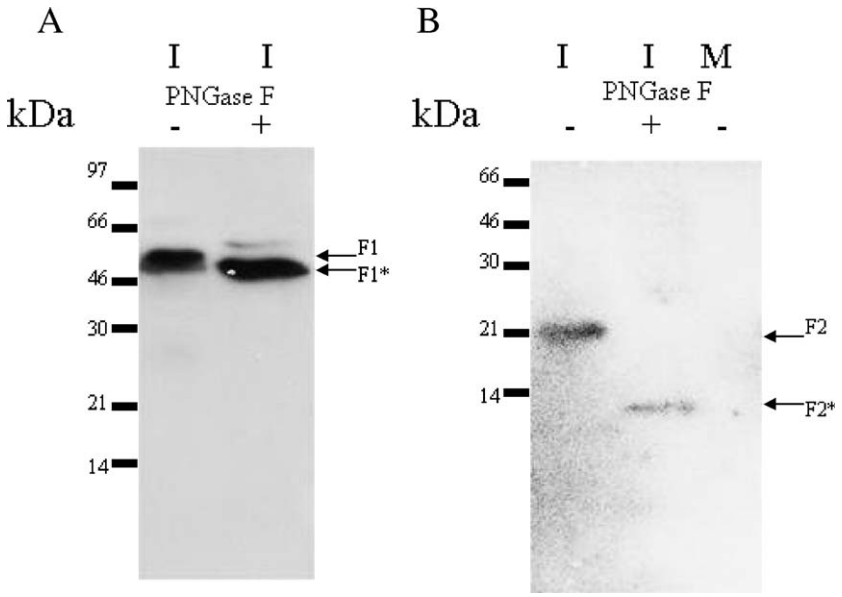

Fig. 1. Western blot analysis of RSV-infected Hep2 cells at 24 h PI. RSV-infected (I) cells were either untreated (−) or PNGase F-treated (+) and examined by Western blotting using (A) MAb169 or (B) PAb922. A comparison of mock- (M) and virus-infected cells shows the specificity of PAb922. The positions of the deglycosylated forms of the F protein subunits are highlighted (*), as are the positions of the molecular weight markers.