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Site-specific characterization of envelope protein N-glycosylation on Sanofi Pasteur's tetravalent CYD dengue vaccine

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ABSTRACT

Recently, several virus studies have shown that protein glycosylation play a fundamental role in the virus–host cell interaction. Glycosylation characterization of the envelope proteins in both insect and mammalian cell-derived dengue virus (DENV) has established that two potential glycosylation residues, the asparagine 67 and 153 can potentially be glycosylated. Moreover, it appears that the glycosylation of these two residues can influence dramatically the virus production and the infection spreading in either mosquito or mammalian cells.

The Sanofi Pasteur tetravalent dengue vaccine (CYD) consists of four chimeric viruses produced in mammalian vero cells. As DENV, the CYDs are able to infect human monocyte-derived dendritic cells *in vitro via* C-type lectins cell-surface molecules. Despite the importance of this interaction, the specific glycosylation pattern of the DENV has not been clearly documented so far. In this paper, we investigated the structure of the N-linked glycans in the four CYD serotypes.

Using MALDI-TOF analysis, the N-linked glycans of CYDs were found to be a mix of high-mannose, hybrid and complex glycans. Site-specific N-glycosylation analysis of CYDs using nanoLC–ESI-MS/MS demonstrates that both asparagine residues 67 and 153 are glycosylated. Predominant glycoforms at asparagine 67 are high mannose-type structures while mainly complex- and hybrid-type structures are detected at asparagine 153. *In vitro* studies have shown that the immunological consequences of infection by the CYD dengue viruses 1–4 *versus* the wild type parents are comparable in human monocyte-derived dendritic cells. Our E-protein glycan characterizations of CYD are consistent with those observations from the wild type parents and thus support *in vitro* studies. In addition, these data provide new insights for the role of glycans in the dengue virus–host cell interactions.

1. Introduction

Dengue disease affects more than 250 million people each year in inter-tropical areas, causing approximately 25,000 deaths, mainly children [1]. Dengue viruses (DENV) are enveloped flaviviruses transmitted by *Aedes* mosquitoes [2]. During infection, DENV enters target cells *via* specific receptors. Both the identification and the exact role of these receptors remain not completely understood [3]. Recently, cell-surface glycosaminoglycans have been studied [4], TIM and TAM proteins were also identified as possible entry factors [5]. All these molecules might play a role for the DENV entry and could be used as receptors to enter different types of cells [6]. Additionally, several studies described the interaction of DENV with C-type lectins cell-surface like the DC-specific ICAM3-grabbing non-integrin DC-SIGN/CD209 [7]. So far, DC-SIGN is assumed to be the most important DENV co-receptor [8], and a target for antiviral therapy. DENV is able to infect many types of host cells, but some subtypes of human dendritic cells (DCs) are the primary targets of DENV. In this case, the infection is mediated partially by the binding of DENV to DC-SIGN.

The DENV particle is composed of three structural proteins: a capsid (C), a premembrane (prM) and the envelope (E) proteins [9]. These proteins are involved in both the assembly and the maturation of DENV [10]. The E-protein is associated with the prM in immature virions [11]. Through the cellular secretion pathway, the prM-protein is cleaved into M by cellular furin protease, followed with a E-protein conformational rearrangement. The E-protein is the major glycoprotein surface [12], responsible for virus attachment and fusion, and constitutes the primary protective antigen [13]. It is now acknowledged that N-glycosylation of

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viral proteins plays a central role in virus–host cells receptors and co-receptors interactions [14]. In this respect, characterization of the N-glycosylation state of viral glycoproteins becomes necessary for the understanding of the virus/receptors interaction.

The E-protein contains two potential N-linked glycosylation sites at asparagine residues Asn67 and Asn153. These glycosylation sites are strictly conserved between the different dengue serotypes. The most frequently glycosylation motif observed in the flavivirus E-protein occurs for Asn153 in domain I, which is located near the fusion peptide of the opposite E in the dimeric E conformation of the mature virion [15]. Glycosylation of Asn67 is specific from DENV and located in domain II. The different serotypes and host cell systems described in the literature are known to dramatically influence the presence of glycans and their glycosylation patterns. Furthermore, N-glycosylation characterization of DENV has been reported from lectin interaction studies, exoglycosidase treatment, mutation experiments, combined with cell infection. However, none of these studies investigate the complete glycosylation pattern of DENV.

In this context, some previous studies have demonstrated the presence of N-linked glycans on both sites for all four serotypes [16]. The analysis of the N-linked glycans composition on DENV strongly indicates that the virus has a heterogeneous population of both high-mannose and complex glycans in mammalian cells. On the other hand, E-protein is glycosylated at Asn67 and Asn153 for serotype 1 but only at Asn67 for serotype 2, when DENV is expressed from insect cells [17]. Glycan analysis of a soluble recombinant E-protein from DENV2 expressed in human embryonic kidney cells showed that it bears either complex or hybrid N-linked glycans with 40% sialylated and 25% fucosylated glycoforms [18].

As described above, the presence of N-glycans from the E-protein could directly be related to its ability to interact with receptors. DC-SIGN receptor interacts with mannose and fucose-based oligosaccharides, particularly the Lewis blood group antigen [7]. In dengue virus, DC-SIGN preferentially binds to the terminal mannose residues, and this terminal mannosylation of E-protein is essential for DCs infection [19,20]. Several mutation experiments have shown that N-linked glycosylation at Asn67 is required for virus growth in mammalian cells and important for infectivity [21,22]. Recently, another group revealed that these results were influenced by the strain origin and the nature of the mutation [23]. Furthermore, the structure of an intact lipid-envelope virus DENV2 with the carbohydrate recognition domain of DC-SIGN was obtained [24]. Their cryoelectron microscopy reconstruction gave strong evidence for DC-SIGN binding to N-linked glycans at Asn67 of the two neighboring E-proteins. All these results are consistent and support the key role of the E-protein and glycosylation sites, mediating interaction with DC-SIGN molecules.

The Sanofi Pasteur tetravalent dengue vaccine (CYD dengue vaccine) is composed of four chimeric viruses [25] based on the backbone of the live attenuated yellow fever 17D vaccine, and expressing the major structural antigens prM and E of each dengue serotype. The CYD dengue vaccine candidate has been the subject of an extensive preclinical, clinical and industrial development [26,27]. Large Phase III efficacy studies in Asia and Latin America have recently demonstrated its ability to protect against disease [28,29]. As DENV, the CYD dengue viruses are able to infect human DCs *in vitro*, involving the cell-surface DC-SIGN molecules [30]. The CYD dengue viruses can replicate further in human primary cells such as monocyte-derived dendritic cells, and viremia can be detected *in vivo* after immunization, albeit at low levels [26]. Nevertheless, the first cycle of infection will impact subsequent ones *in vivo* (depending on its initial level and on the identity of target cells), and then the nature of the glycosylation obtained in vero cells has to be considered.

As mentioned above, glycosylation is a critical protein attribute for the ability of CYD dengue viruses to interact with DC-SIGN, which consequently needs further characterization. The glycosylation state of the E-protein from each considered serotype is described in this paper. Since N-glycosylation is dependent on cells subtype and various culture parameters [31], in-depth characterization of N-linked glycans from the E-protein of CYD produced in vero cells is particularly essential. We then considered the glycosylation state of the E-protein from each serotype.

In this work, N-linked oligosaccharides of the E-protein after in-gel deglycosylation have been characterized using mass spectrometry. N-glycosylation profiling of the E-protein was performed using MALDI-TOF, demonstrating that the CYD envelope had a heterogeneous pattern. Furthermore, a detailed study of both Asn67 and Asn153 sites was carried out from glycopeptides. The results show that high mannose glycans are predominant at Asn67 while mainly complex glycans are detected at Asn153.

2. Materials and methods

2.1. Purification of CYD virus particles

ChimeriVax™-DEN1-4 were produced on serum-free vero cells on microcarriers in bioreactor at Sanofi Pasteur (Marcy L'Etoile, France). The viruses were then precipitated on 7% polyethylene glycol-8000, and after 2 h of incubation at 4 °C, the pellets were collected by centrifugation at 16,000 × *g* at 4 °C for 1 h and suspended in 50 mM Tris pH 7.5, 75 mM NaCl, 0.1 mM EDTA. The concentrated virus suspension was loaded onto a 30–50% linear sucrose gradient in the same buffer and centrifuged for 18 h at 160,000 × *g* at 4 °C in a Beckman SW32 rotor. Sixty fractions of 500 μl were collected and the presence of virus was detected in each fraction by qRT-PCR targeted yellow fever 17D-NS5 gene [32]. Virions present in the fractions with the highest titers were pooled and diafiltrated twice on Amicon 100 kDa to eliminate sucrose and replace by buffer 50 mM Tris pH 7.5, 75 mM NaCl, 0.1 mM EDTA. Purified CYD viruses were stored at −80 °C.

2.2. Protein identification and Isolation of E protein from CYD virus particules

The proteins from purified CYD virus particules were separated using SDS-PAGE after protein reduction and alkylation. Gels were stained with GelCode® blue stain reagent. Analytical gel was done with 5 μg protein. All detected bands were excised and identifications were performed. Tryptic digestion was performed using DigestPro MS robot (Intavis AG Bioanalytical Instruments, Germany). Excised gel bands were washed alternatively with 50 mM ammonium bicarbonate, pH 8 and acetonitrile. Proteins were then reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide and then enzymatically digested with trypsin at 0.01 mg/ml diluted in 25 mM ammonium bicarbonate (Promega, USA). Samples were incubated at 37 °C during 4 h. Digestion was stopped by the addition of trifluoroacetic acid (TFA) at a final concentration 0.1% (v/v) and the resulting peptides were collected. The samples were then deposited onto a matrix layer of saturated α-cyano-4-hydroxycinnamic acid solution in acetone and TFA 0.1% (v/v), as described [33]. Analyses were performed on an Ultraflextreme MALDI-TOF mass spectrometer in reflectron positive mode (Bruker Daltonics, Billerica, MA). External calibration was done in each case using peptide calibrants (Bruker Daltonics, reference 222570). Mass spectra were acquired using sets of instrument parameters over 800–4000 *m*/*z*. Monoisotopic peptide masses determined by MALDI-TOF were submitted to the MASCOT