



## Functional Analysis of the Human $\alpha$ 1,3/4-fucosyltransferases

*The role of potential N-glycosylation sites*

*By Lise Lotte Christensen, PhD, Medical Science*

This PhD thesis is based on studies carried out at the Molecular Diagnostic Laboratory, Department of Clinical Biochemistry, Aarhus University Hospital, Skejby Sygehus during the period October 1997 to December 2000. The thesis is based on three original papers, and on unpublished data.

The scientific group at the Molecular Diagnostic Laboratory has studied the cellular changes occurring during the development and progression of cancer for a long time. One of the major interests has been to investigate the altered glycosylation pattern of cancer cells. Therefore, it was highly relevant to study the enzymes involved in the alteration of glycosylation in cancer cells, e.g. the human  $\alpha$ 1,3/4-fucosyltransferases (hFucTs), as described in this thesis. The hFucTs are responsible for the final step in the synthesis of fucosylated glycoconjugates. The hFucTs are glycosylated themselves, and hence their enzyme activity could be influenced by the altered glycosylation pattern in cancerous tissue.

The aim of this PhD study was to analyse the glycosylation and functional role of the potential N-glycosylation sites of human  $\alpha$ 1,3/4-fucosyltransferase III (hFucTIII) (Asn154 and Asn185), human  $\alpha$ 1,3-fucosyltransferase V (hFucTV) (Asn60, Asn105, Asn167 and Asn198) and human  $\alpha$ 1,3-fucosyltransferase VI (hFucTVI) (Asn46, Asn91, Asn153 and Asn184). These analyses could shed light on the mechanisms of cellular localisation, turnover and folding of the hFucTs and glycosyltransferases in general. The main emphasis was on the role of N-glycosylation in hFucTIII. Furthermore, the thesis also describes an attempt to generate an hFucTIII specific monoclonal antibody in mice.

Initially, hFucTIII, -V and -VI were expressed in COS-7 cells in the presence of the N-glycosylation inhibitor tunicamycin. The presence of tunicamycin resulted in a decrease in the molecular mass of all three enzymes. In addition, the  $\alpha$ 1,3/4-fucosyltransferase activity was completely lost in tunicamycin-treated

cells, demonstrating that hFucTIII, -V and -VI require core glycosylation for the expression of their enzyme activity. The expression of the three enzymes in the presence of castanospermine, an inhibitor of N-glycan trimming, resulted in a slightly elevated molecular mass of all three enzymes. The  $\alpha$ 1,3/4-fucosyltransferase activity in castanospermine-treated cells was reduced to 40-55% of the activity of the native enzymes in untreated cells. Thus, although required for full enzyme activity, trimming of the glucose residues on the N-linked glycans is not essential for expression of hFucTIII, -V and -VI enzyme activity. To evaluate the potential N-glycosylation sites individually and in different combinations, 15 different N-glycosylation mutants of hFucTIII, -V and -VI were generated using site-directed mutagenesis. First of all, wt hFucTIII, -V and -VI and the mutants were all expressed in comparable amounts in transfected COS-7 cells. Secondly, it was demonstrated that the homologous potential N-glycosylation sites (hFucTIII: Asn154 and Asn185, hFucTV: Asn167 and Asn198 and hFucTVI: Asn153 and Asn184) are glycosylated in all three enzymes. The N-linked glycans at Asn154, Asn167 and Asn153 are essential for the production of catalytically active enzymes, whereas a lack of N-linked glycans at Asn185, Asn198 and Asn184 only resulted in a decrease in enzyme activity. Kinetic analysis of the hFucTIII N185Q mutant revealed that the affinity towards the oligosaccharide acceptor and the nucleotide sugar donor is not significantly affected by the elimination of Asn185. Hence, N-linked carbohydrates at this particular site are not involved in the interaction of the enzyme with either of its two substrates. The hFucTV and -VI enzymes each contain two potential N-glycosylation sites in addition to the homologous sites described above (hFucTV: Asn60 and Asn105 and hFucTVI: Asn46 and Asn91). Both sites were shown to be glycosylated in hFucTV, whereas only one of the sites (Asn91) is glycosylated in hFucTVI. Although their individual glycosylation does not contribute considerably to the expression of enzyme activity in any of the two enzymes, one of the sites in hFucTV must be glycosylated to obtain an enzyme activity comparable to that of wt hFucTV. Finally, analyses of the cellular localisation of hFucTIII wt and mutants fused to the yellow fluorescent protein (YFP) in living COS-7 cells were carried out. These analyses indicate that the hFucTIII N154Q-YFP and N154Q/N185Q-YFP fusion proteins are retained in the ER, whereas the hFucTIII N185Q-YFP protein is transported to the Golgi as the wt hFucTIII-YFP enzyme. These results are preliminary and must be confirmed using immunoelectron or immunofluorescence microscopy of COS-7 cells expressing either wt hFucTIII or the N-glycosylation mutants without

fusion to YFP. We speculate that the ER retained mutants are not correctly folded, and that the N-linked carbohydrates at Asn154 are more important for proper folding than the N-linked glycans at Asn185. Furthermore, pulse-chase analyses indicate that the lack of N-glycosylation has no apparent effect on the intracellular turnover of hFucTIII.

This PhD project also describes the generation of a monoclonal antibody directed against hFucTIII. The generation of monospecific antibodies against the hFucTs is a difficult task due to the high sequence similarity between the three enzymes. Unfortunately, the monoclonal antibody generated in this study also recognises hFucTV and to a lesser extent hFucTVI. Consequently, the antibody can only be used to detect hFucTIII in tissues/cells that exclusively express hFucTIII. In addition, the antibody did not show any reactivity in Western blotting; and nor did it show reactivity to any of the hFucTIII N-glycosylation mutants.

In conclusion, the results presented in the present thesis clearly demonstrate that N-linked glycans are indispensable for the expression of full enzyme activity of hFucTIII, -V and -VI. Furthermore, it was demonstrated that glycosylation of one of the homologous N-glycosylation sites was more important for expression of enzyme activity than of the other. Finally, it was shown that trimming of the glucose residues on the N-linked glycans was necessary for the expression of full enzyme activity of all three enzymes.