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Requirement of *N*-glycosylation for the secretion of recombinant extracellular domain of human Fas in HeLa cells

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Abstract

Apoptosis has been shown to be associated with altered glycosylation patterns and biosynthesis of glycoproteins. A major cell surface receptor involved in the induction of apoptosis is Fas that is activated by binding Fas ligand but can also be activated by binding anti-Fas antibody. In order to determine whether the Fas receptor is glycosylated, the extracellular domain of human Fas (shFas) was expressed as a cleavable fusion protein (shFas-Fc) in HeLa cells. These cells were shown to express activities of glycosyltransferases involved in *N*- and *O*-glycan biosynthesis. The secreted shFas-Fc was shown to be a glycoprotein with heterogeneous glycan chains. MALDI mass spectrometry revealed a disperse molecular weight of shFas with an average of 23.4 kDa. Western blots of shFas-Fc secreted from tunicamycin treated transfected HeLa cells showed that only *N*-glycosylated glycoforms were secreted, while the unglycosylated shFas-Fc remained intracellular. The results suggest that both *N*-glycosylation sites of the extracellular domain of Fas are occupied with large *N*-glycans that play a role in the expression of the glycoprotein.

Keywords: Fas; Apoptosis; Glycoprotein; Glycosylation; HeLa cells; Glycosyltransferases

1. Introduction

Apoptosis mediated through the cell surface receptor Fas (Apo-1, CD95) is an important process in T-cell cytotoxicity (Hanabuchi et al., 1994), and has been implicated in disease (Debatin et al., 1994; Grodzicky & Elkon, 2000). Trimeric Fas ligand (FasL), a glycoprotein present on the surfaces of many leukocytes, binds Fas on cell surfaces. Fas molecules self-associate to form functional trimers which can induce intracellular cascades of apoptosis (Nagata & Goldstein, 1995; Nagata & Suda, 1995; Orlinick, Elkon, & Chao, 1997; Schneider et al., 1997; Schneider & Tschopp, 2000). Binding of either Fas ligand (FasL) or anti-Fas antibodies induces apoptosis.

There is ample evidence linking apoptosis and glycosylation (Brockhausen, Schutzbach, & Kuhns, 1998; Brockhausen et al., 2002; Hiraishi, Suzuki, Hakomori, & Adachi, 1993; Rapoport & Le Pendu, 1999). The

Abbreviations: Bn, benzyl; ConA, concanavalin A; Gal, galactose; GlcNAc, *N*-acetylglucosamine; HP, helix pomatia; MAA, Maackia amurensis agglutinin; MALDI, matrix-assisted laser desorption ionization; Man, mannose; MS, mass spectrometry; PHA, phaseolus vulgaris hemagglutinin; PNA, peanut agglutinin; SNA, Sambucus nigra agglutinin; WGA, wheat germ agglutinin

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induction of apoptosis appears to depend on cell surface glycosylation (Keppler et al., 1999). For example, plant or mammalian lectins that bind to cell surface carbohydrates have been shown to induce or control apoptosis (Gabius, 2001; Perillo, Marcus, & Baum, 1998; Rabinovich & Gruppi, 2005). Although the mechanisms are not yet known, it appears that *N*-glycans are important in this process, since the inhibition of *N*-glycosylation can induce apoptosis (Chang & Korolev, 1996; Martinez et al., 2000; Perez-Sala & Mollinedo, 1995; Yoshimi, Sekiguchi, Hara, & Nishimoto, 2000). There are many possibilities how *N*- and *O*-glycans can carry out their regulatory function at the cell surface. These include roles in the conformational properties of proteins, in oligomerization, expression and stability of glycoproteins, in the expression of specific glycoprotein epitopes, and in ligand binding and signaling. For example, *N*-glycans appear to regulate the expression level of FasL (Orlinick et al., 1997). Glycosylation is required for efficient secretion of soluble FasL from human embryonic kidney cells (Schneider et al., 1997). However, using the *Pichia pastoris* expression system for constructs of the extracellular domain of FasL, it was found that deletion of *N*-glycosylation sites had variable effects on the secretion of the recombinant protein. Thus in an N-terminal-tagged construct, deletion of one *N*-glycosylation site caused an increase in secretion, while no secreted protein was detected when all three sites were deleted (Muraki, 2006).

Fas is a type I membrane protein (37 kDa) with an extracellular domain containing three Cys-rich domains, a transmembrane domain and a cytoplasmic signaling death domain with caspase binding sites (Oehm et al., 1992). Fas has two *N*-glycosylation sites as well as a Thr-rich region that may possibly form *O*-glycosylation sites. The epitope of Fas (Fig. 1) representing the binding site of FasL depends on the presence of specific amino acids that are mainly found N-terminal to the first *N*-glycosylation site of the second domain, D2. However, amino acids in other domains also contribute to epitope recognition (Starling et al., 1997; Starling, Kiener, Aruffo, & Bajorath, 1998). Anti-Fas antibodies induce Fas clustering although the binding epitope is distinct from that of FasL (Fadeel, Lindberg, Achour, & Chiodi, 1998; Fadeel, Thorpe, & Chiodi, 1995). A linear epitope for these anti-Fas antibodies has been identified that directly precedes and appears to include the Asn residue of the second *N*-glycosylation site (Bajorath, 1999; Starling et al., 1997, 1998). However, internal disulfide bond formation is essential to maintain the anti-Fas antibody epitope (Fadeel et al., 1998). Modeling studies of these binding sites revealed that epitopes for FasL and anti-Fas antibodies are exposed at different protein surfaces, with the linear epitope of anti-Fas antibody being external, and the FasL binding epitope being on the internal side of the protein. It is thought that neither FasL nor anti-Fas antibodies, which have equivalent intracellular effects, induce specific conformational changes transmitted to the cytoplasmic moiety of Fas, but rather function in the recruitment of Fas at the cell surface to form functional trimers (Nagata & Goldstein, 1995). Because of the proximity of the *N*-glycosylation sites of Fas to its FasL binding epitopes, it is possible that *N*-glycans play a role in the biological activity of Fas. However, the glycosylation status of Fas has not yet been elucidated.

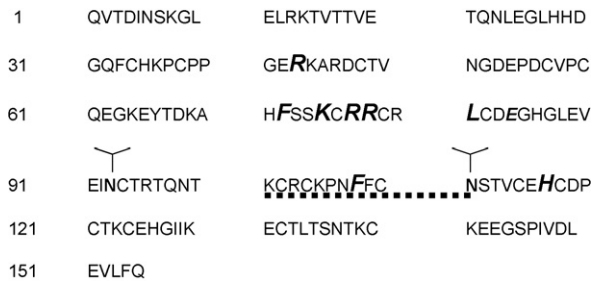

Fig. 1. Amino acid sequence of the soluble extracellular domain construct of human Fas (shFas). The amino acid sequence of the extracellular domain of shFas after cleavage from shFas-Fc shows two potential *N*-glycosylation sites at Asn93 and Asn111 (bold, the Y depicts an *N*-glycan), and a Thr-rich TVTTVET motif (Thr15 to Thr21) that resembles *O*-glycosylated mucin type sequences. The amino acids found to be important for Fas ligand binding are indicated in italic, bold, large font. The linear epitope for anti-Fas antibodies is underlined with a dotted line. Both anti-Fas antibody and Fas ligand bind near the two *N*-glycosylation sites (Fadeel et al., 1998; Starling et al., 1997, 1998).

Human cervical carcinoma HeLa cells have functional Fas-mediated apoptosis pathways (Yotsuyanagi et al., 1998). They also produce a variety of glycoproteins and glycosyltransferases (Axelsson et al., 2001; Berger, Thurnher, & Muller, 1987; Berger et al., 1993; Borsig, Kleene, Dinter, & Berger, 1996; Nilsson et al., 1993, 1994; Prescott, Lucocq, James, Lister, & Ponnambalam, 1997). In this study, HeLa cells were used to express the soluble extracellular domain of human Fas (shFas, Fig. 1) as a fusion protein with a cleavable human Fc (IgG1) tag at the C-terminus (shFas-Fc). We determined that shFas is a heterogeneous glycoprotein having both *N*-glycosylation sites occupied and that *N*-glycans play a role in the secretion of shFas-Fc. These studies contribute to our understanding of the role of glycosylation and the relationship between aberrant apoptosis and glycosylation in disease.