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Definitive evidence that a single *N*-glycan among three glycans on inducible costimulator is required for proper protein trafficking and ligand binding ☆

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ABSTRACT

Glycosylation is a widespread post-translational modification found in glycoproteins. Glycans play key roles in protein folding, quality control in the endoplasmic reticulum (ER) and protein trafficking within cells. However, it remains unclear whether all positions of protein glycosylation are involved in glycan functions, or if specific positions have individual roles. Here we demonstrate the integral involvement of a specific *N*-glycan from amongst the three glycans present on inducible costimulator (ICOS), a T-cell costimulatory molecule, in proper protein folding and intracellular trafficking to the cell surface membrane. We found that glycosylation-defective mutant proteins lacking *N*-glycan at amino-acid position 89 (N89), but not proteins lacking either N23 or N110, were retained within the cell and were not detected on the cell surface membrane. Additional evidence suggested that N89 glycosylation was indirectly involved in ICOS ligand binding. These data suggest that amongst the three putative ICOS glycosylation sites, N89 is required for proper ICOS protein folding in the ER, intracellular trafficking and ligand binding activity. This study represents a substantial contribution to the current mechanistic understanding of the necessity and potential functions of a specific *N*-glycan among the multiple glycans of glycoproteins.

Introduction

Glycosylation is a widespread, post-translational modification found on glycoproteins and glycolipids [1,2]. Glycans are covalently attached to asparagine residues in the *N*-glycosylation consensus sequence (Asn-X-Ser/Thr, where X may be any amino acid except for Pro), serine or threonine residues of a polypeptide chain (*O*-glycosylation) or to a lipid backbone. Glycans are constructed to be attached in a mature form, with branched, bulky and highly hydrophilic units ordered in a sequential manner through the distinct substrate specificities of glycosyltransferases and glycosidase enzymes in the lumen of the endoplasmic reticulum (ER) and the Golgi apparatus. It has been well established that the glycans play essential roles in various biological processes, such as development, immune response and tissue formation [3–5]. The biochemical functions of *N*-glycan attached to a glycoprotein are also thought to include influencing protein solubility, stabilization of protein structure and receptor–ligand interactions [6,7].

Glycans are also important elements contributing to intracellular events in secretary pathways such as protein folding, quality control in the endoplasmic reticulum (ER) and protein trafficking through interactions between specific glycan structures and lectins [8–10]. The glycan provides an active mechanism for facilitating protein folding by recruiting the lectin-like chaperones calnexin (CNX) and calreticulin (CRT) within the ER [8,9]. On the contrary, misfolded proteins with immature *N*-glycan are recognized by the lectin ER-degradation enhancing α-mannosidase-like protein (EDEM) and then degraded by ER-associated degradation (ERAD)

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for ER-quality control [9]. Non-glycosylated proteins are associated with the molecular chaperone BiP (GRP78) to promote their folding [8]. The correctly folded glycoproteins are transported by the mannose-specific L-type lectins ERGIC-53 and VIP36 for in ER-to-Golgi trafficking [10]. These studies strongly suggest that specific glycan structures contribute to the proper execution of the various biological activities of the glycoprotein. It remains unclear whether all glycans on a glycoprotein contribute to protein function and proper trafficking to the cell surface membrane. Although molecular recognition mechanisms in ER-quality control have been established, there is currently no published evidence demonstrating the relationship between the qualitative protein changes caused by glycosylation and protein trafficking through ER–protein-quality control process.

Inducible costimulator (ICOS) is a costimulatory molecule and member of the CD28 family. This membrane-bound glycoprotein is inductively expressed on activated T-cells [11,12]. Previous studies have shown that ICOS plays important roles in the differentiation and regulation of effector and regulatory T-cells as well as in the pathogenesis of allergic disease, autoimmune disease and transplant rejection [13–16]. A FDPPPF amino-acid motif in the ICOS extracellular domain is essential for interaction with its ligand, B7h [12]. ICOS contains three putative *N*-glycosylation sites in the extracellular domain, and two of which have confirmed glycosylation [17]. However, the biological roles of ICOS *N*-glycosylation as related to both it biological activity and protein trafficking remain to be established.

In this study, we clarify the biological roles of one *N*-glycan on ICOS using a series of ICOS glycosylation-defective mutant proteins. We report that the *N*-glycan attached to the asparagine residue at the amino-acid position 89 (N89), a typical biantennary complex-type glycan, plays essential roles in the conformational stability of protein structure and the binding to the ligand. We also demonstrate that the *N*-glycan attached to N89 is essential for ICOS protein trafficking to the cell surface membrane. Our results indicate that attachment of a glycan moiety at ICOS N89 plays a pivotal role in executing the biological functions of this glycoprotein through stabilization of the protein structure.

Materials and methods

Preparation of N-glycosylation-defective or amino-acid deletion mutants. *N*-Glycosylation-defective mutations or amino-acid deletion in the ICOS binding motif (ΔPPP) were introduced into ICOS with an ICOS-IgFc fusion protein cDNA or a His-ICOS cDNA using site-directed mutagenesis. Each mutant was expressed separately in CHO-K1 or Jurkat cells and then utilized in subsequent experiments (see SI Materials and Methods).

Binding assay. Binding activity of glycosylation-defective ICOS-IgFc mutants to B7h-IgFc was measured by the solid-phase binding

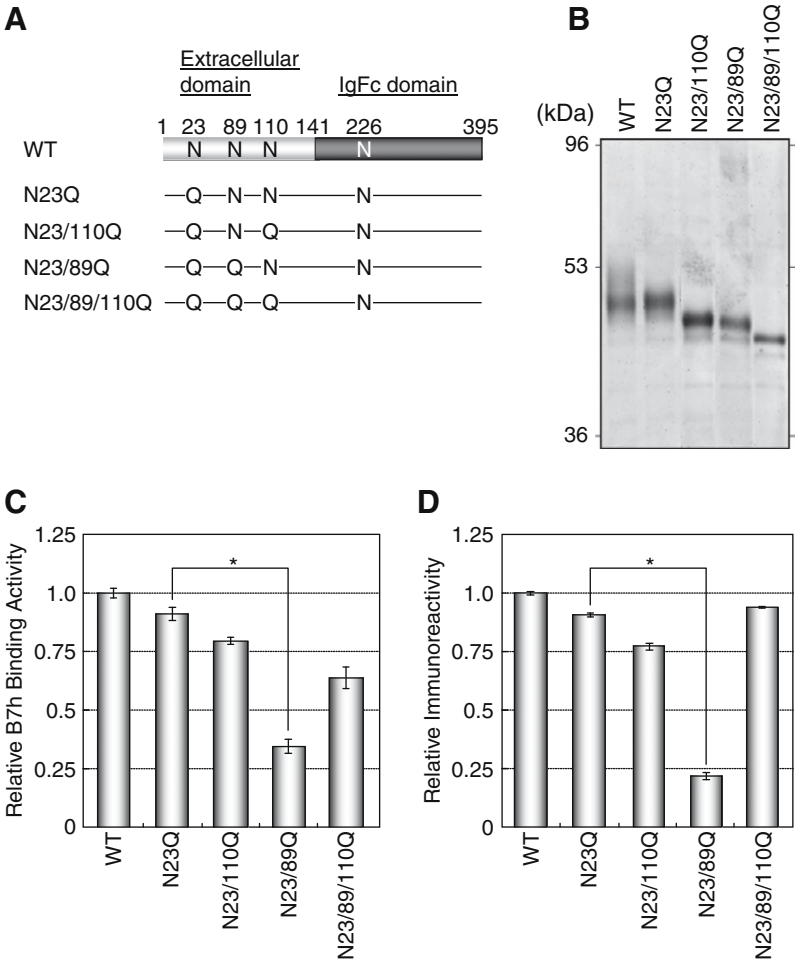

Fig. 1. *N*-Glycosylation at N89 is essential for both ICOS binding to B7h and the stability of the protein structure. (A) Schematic view of the glycosylation-defective ICOS-IgFc mutants N23Q, N23/89Q, N23/110Q and N23/89/110Q. The gray and black bars represent the ICOS extracellular domain and the human IgG_1 Fc region, respectively. Potential *N*-glycosylation sites (N) were mutated to glutamine (Q). (B) Analysis of glycosylation-defective ICOS-IgFc mutants by SDS–PAGE. (C) Binding activity of glycosylation-defective ICOS-IgFc to B7h-IgFc. $^*p < 0.005$. (D) Reactivity of an anti-ICOS antibody to ICOS-IgFc. $^*p < 0.005$.

assay. For the binding inhibition assay, asialo glycan, either disialo glycan or an anti-ICOS monoclonal antibody was added along with B7h-IgFc. Detailed methods are described in SI Materials and Methods.

Immunoreactivity. Reactivity of ICOS-IgFc or glycosylation-defective ICOS-IgFc mutant to an anti-ICOS antibody (BD Pharmingen) was examined by the solid-phase assay (see SI Materials and Methods).

Structural analysis of glycans attached onto ICOS. The structure of *N*-glycan from ICOS was analyzed using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) and matrix-assisted laser desorption/ionization time-off-light mass spectrometry (MALDI-TOF MS) according to previously reported methods [18,19]. Detailed methods are presented in SI Materials and Methods.

Flow cytometry. The glycosylation-defective mutants were labeled with an anti-His antibody (GE Healthcare) and a phycoerythrin-conjugated anti-mouse IgG antibody (Rockland). Labeled cells were analyzed by flow cytometry using a FACS Calibur™ (BD Bioscience, San Jose, CA, USA). The details of this analysis are presented in SI Materials and Methods.

Fluorescence microscopy. Cells expressing glycosylation-defective mutants were stained with an anti-His antibody (GE Healthcare) and a rhodamine conjugated anti-mouse IgG antibody (BD Bioscience). The cells were then fixed and analyzed using an Axiovert S100 fluorescent microscope (Carl Zeiss, Meditec, Jena, Germany). The details of this analysis are presented in SI Materials and Methods.

Results

Involvement of N-glycan on ICOS for the binding to its ligand, B7h

ICOS contains three putative asparagine *N*-glycosylation sites at amino-acid positions 23 (N23), 89 (N89) and 110 (N110) in the extracellular domain (Fig. 1A). Of these sites, two of them (N89 and N110) have been confirmed as being glycosylated [17]. Previous reports have shown that ICOS has proper binding affinity through self dimerization [11,15]. To examine the involvement of the ICOS *N*-glycans in ligand binding, we prepared a soluble form of ICOS (ICOS-IgFc), which consists of a homodimeric fusion protein between the ICOS extracellular domain and the Fc region of human IgG1 (Fig. 1A) [20]. Using this fusion protein, we first investigated whether *N*-glycosylation on ICOS plays an essential role in ICOS ligand binding activity. A series of ICOS mutants were created in which one or more of the three asparagine *N*-glycosylation sites were mutated to glutamine, and then wild type and mutant fusion proteins were individually expressed in CHO-K1 and purified for subsequent experiments (Fig. 1A). SDS–PAGE analysis under reducing conditions showed that the purified wild type ICOS-IgFc (WT) separated into a 49-kDa and a 53-kDa polypeptide chain, whereas the N23Q ICOS-IgFc mutant was detected as a single band of 49-kDa protein (Fig. 1B). These results indicate that the 53-kDa protein in the recombinant WT ICOS-IgFc could be glycosylated at N23 in addition to the previously reported glycosylation at N89 and N110 [17]. In the assay of binding between ICOS-IgFc mutant and B7h-IgFc, the N23Q ICOS-IgFc mutant showed the same binding activity as WT ICOS-IgFc (Fig. 1C). Therefore, we used N23Q ICOS-IgFc as a native form of ICOS to avoid artificial *N*-glycosylation at N23. The other fusion protein mutants were also detected as a band with a reduced molecular mass reflecting the number of *N*-glycosylation sites on the gel (Fig. 1B).

In binding assays using the ICOS-IgFc mutants and B7h-IgFc, N23/89Q ICOS-IgFc exhibited a significant reduction in B7h-IgFc binding affinity as compared with N23Q ICOS-IgFc, while the binding activity of N23/110Q ICOS-IgFc was similar to that of N23Q ICOS-IgFc. These reproducible differences were present despite conservation of the essential FDPPPF binding motif in all mutants (Fig. 1C). The binding activity of unglycosylated N23/89/110Q ICOS-IgFc was slightly reduced by approximately 30% as compared with that of WT and N23Q-IgFc.

To briefly characterize the ICOS-IgFc protein structures, we performed an immunoreactivity assay in which a neutralizing antibody against ICOS was incubated with the ICOS mutant proteins. Immunoreactivities to various ICOS mutants were relatively equivalent to the respective binding activities detected in the binding assay (Fig. 1D). Antibody immunoreactivity to N23/89Q ICOS-IgFc,

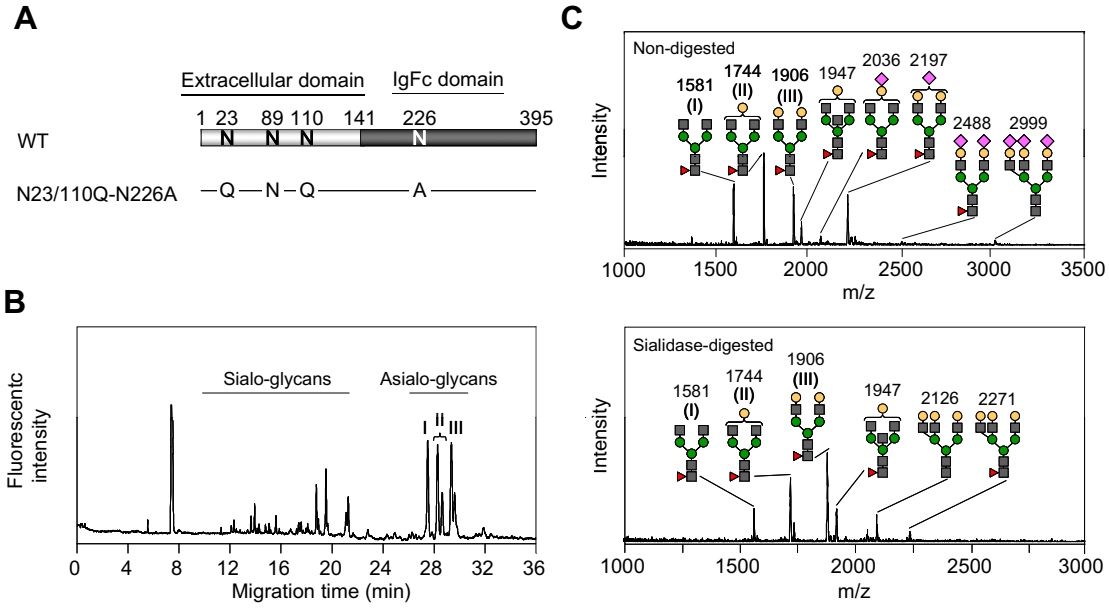

Fig. 2. Structural analysis of the glycan attached onto N89 on ICOS. (A) A schematic view of the *N*-glycosylation-defective ICOS-IgFc mutant N23/110Q-226A, which has an *N*-glycan only at N89 in the ICOS extracellular domain. The gray and black bars represent ICOS extracellular domain and the human IgG_1 Fc region, respectively. Potential *N*-glycosylation sites (N) were mutated to glutamine (Q) or alanine (A). (B) *N*-Glycan migration profiles of N23/110Q-N226A ICOS-IgFc by CE-LIF. (C) The mass spectra of 2-AA labeled *N*-glycans derived from N23/110Q-N226A ICOS-IgFc by MALDI-TOF MS. Arabic numerals represent observed masses. Roman numerals indicate the oligosaccharide structures (see Table S3). The abbreviations used for the structures are: ○, galactose; ■, *N*-acetylglucosamine; ●, mannose; ▲, fucose; ♦, *N*-acetylneuraminic acid.

but not N23Q or N23/110Q ICOS-IgFc, was significantly decreased (Fig. 1D) compared with to the immunoreactivity of WT ICOS-IgFc. In contrast, immunoreactivity to the unglycosylated mutant N23/89/110Q ICOS-IgFc was similar to that of N23Q ICOS-IgFc. These results indicated that the unglycosylated ICOS presented a protein structure that was similar to that of WT ICOS, but which exhibited a slight reduction in ligand binding. These observations raised the possibility that there may be direct involvement of *N*-glycans in the binding of ICOS to B7h in addition to the FDPPPF binding motif. Results also indicated that the absence of an *N*-glycan attached onto N89 caused a conformational change which reduced ligand binding. *N*-Glycosylation of ICOS at N89 could be essential for the binding activity of ICOS to B7h and/or stabilization of the protein structure.

Analysis of the N-glycan structure attached onto N89 of ICOS

We next determined the *N*-glycan structure of ICOS at the position of N89, which is involved in the binding activity of ICOS. We prepared the N23/110Q-N226A ICOS-IgFc carrying *N*-glycan of N89 only, in which both N23 and N110 were substituted by glutamine and N226 in the IgFc region was replaced with alanine (Fig. 2A). The detailed structural analysis of glycans attached to WT ICOS-IgFc was performed using capillary electrophoresis, capillary affinity electrophoresis and MALDI-TOF MS (Fig. S1, Tables S2, S4 and see SI text in detail). On the capillary electrophoresis analysis of the *N*-glycans derived from N23/110Q-N226A ICOS-IgFc, the glycans were separated into major asialo glycans I, II, III and minor sialo glycans (Fig. 2B). After additional of MALDI-TOF MS analysis of sialidase-non-digested or sialidase-digested glycans, the results were compared with that of WT (Fig. 2C and Fig. S1). These results revealed that the major *N*-glycans attached onto N89 of ICOS were asialo glycans named I, II and III, which represented the same biantennary complex-type agalactosyl-, monogalactosyl- and digalactosyl-glycan structures, respectively, as were observed in the WT (Fig. 2C and Table S3). These results suggested that *N*-glycosylation of ICOS N89, and not the unusual glycan structure, would be essential for the ligand binding activity and protein structure of ICOS, since these were also typical glycan structures observed in the secretary glycoproteins produced in CHO-K1 cells [21,22].

ICOS N-glycans and B7h binding

We next examined the influence of the soluble *N*-glycans on the binding between ICOS and B7h. The asialo- or disialo-biantennary complex-type glycan (Fig. 3A), which possesses a *N*-acetyllactosamine or sialyl *N*-acetyllactosamine structure at the non-reducing end and is similar to the glycan at N89 (Fig. 2C), were assayed in the ICOS binding assay system described above. The neutralizing monoclonal antibody against ICOS, included as a control, significantly reduced the binding between ICOS and B7h in a dose-dependent manner. In contrast, both asialo- and disialo-biantennary complex-type glycans could not inhibit this binding, even at concentrations of 100-fold molar excess (Fig. 3B). These results suggested that *N*-glycans attached to N89 of ICOS contribute to the stabilization of the ICOS protein structure for the ICOS-B7h binding rather than a direct association between the *N*-glycans on ICOS and it ligand B7h.

ICOS N-glycans and protein trafficking

ICOS is an inducible costimulatory receptor localized on the cell surface of activated T-cells [11]. To clarify the involvement of *N*-glycosylation in the trafficking of ICOS to the cell surface membrane, we analyzed the subcellular localization of *N*-glycosylation-defective ICOS mutants. These fusion proteins carried a His-Tag and EGFP at

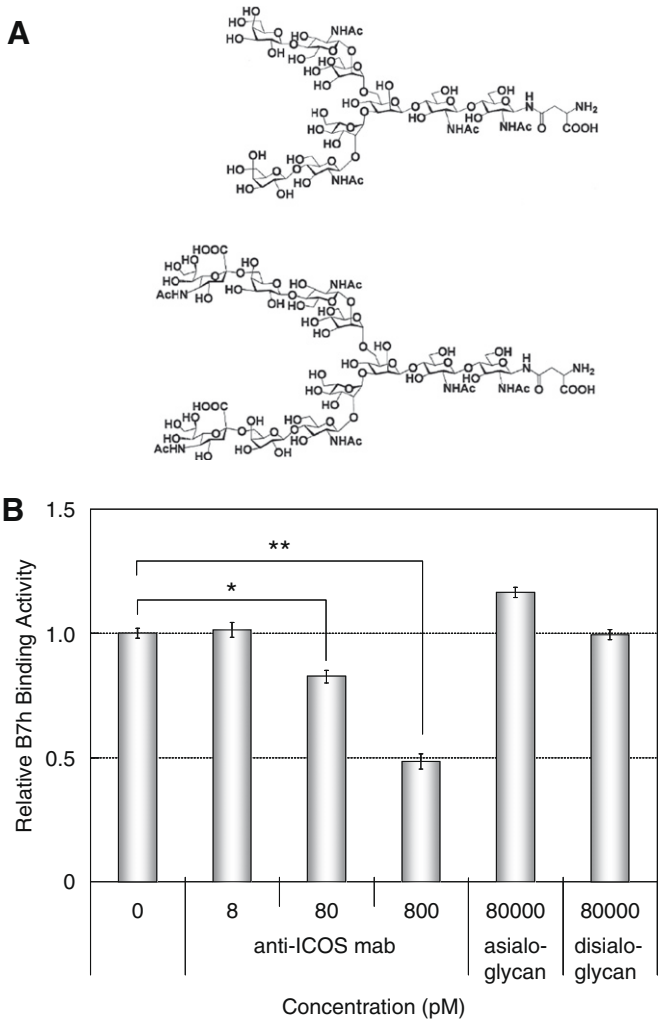

Fig. 3. Analysis of *N*-glycan contributions to binding between ICOS and B7h. (A) A schematic view of asialo (upper)- and disialo (lower)-biantennary complex-type glycans. (B) Inhibitory activity towards ICOS-IgFc binding to B7h-IgFc. $^{*}p < 0.001$, $^{**}p < 0.0001$.

the ICOS N- and C-terminals, respectively (Fig. 4A). Jurkat cells were transfected with individual expression vector encoding a mutant protein. Stably expressing cells were sorted according to EGFP expression level using a cell sorter in order to ensure that similar moles of mutant protein were expressed per cell in subsequent experiments (Fig. S2, left panel). The different stable transfectants were stained with an anti-His-Tag mAb and the cell surface localizations of *N*-glycosylation-defective ICOS mutants were analyzed cell surface fluorescence using flow cytometry and fluorescence microscopy (Fig. 4B, C and Fig. S2, right panel). Cell surface expression of both N23Q and N23/110Q ICOS mutants were similar to that of WT ICOS by flow cytometric analysis and microscopic observation; however, N23/89Q ICOS could not be detected on the cell surface using either method (Fig. 4B and C). In contrast to the cell surface expression of N23/89Q ICOS, the cell surface expression of N23/89/110Q ICOS, which was deleted all glycans, was partially observed at a relative 60% level of the expression detected for WT or N23Q ICOS (Fig. 4B). The cell surface expression of N23/89/110Q ICOS was also partially observed in microscopic observations (Fig. 4C). The deletion mutants WT ΔPPP ICOS and N23Q ΔPPP ICOS, which lacked three proline residues in the ligand binding motif FDPPPF and lost binding activity to B7h [23], were reduced in cell surface localization to a similar degree as was observed for N23/89/110Q (Fig. 4B and C). These results indicate that the protein sequence, which related to

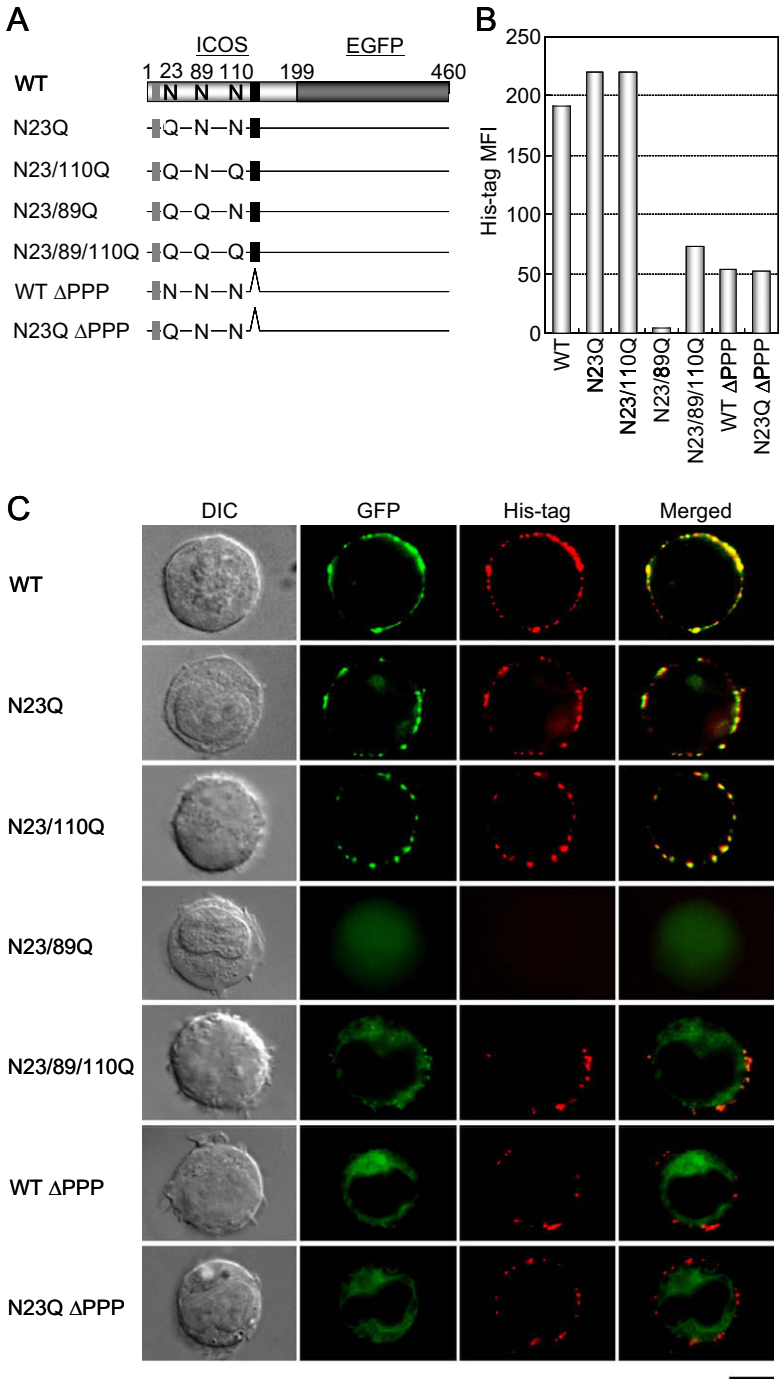

Fig. 4. ICOS cell membrane localization requires *N*-glycosylation at N89. (A) A schematic view of the ICOS-His–EGFP fusion protein. The white and gray bars represent ICOS and the EGFP tag, respectively. Histidine (His)-Tag (gray box) was inserted into the ICOS coding sequence just behind the signal sequence. Potential *N*-glycosylation sites (N) were mutated to glutamine (Q). Three proline residues in the FDPPPF motif (black box) were deleted. Each mutant was expressed separately in Jurkat cells. (B) Flow cytometric analysis of cells expressing ICOS. Cell surface expression was indicated by mean fluorescence intensity (MFI) (see SI Materials and Methods, Fig. S2). (C) Fluorescence microscopic analysis of cells expressing ICOS mutants. Cell surface expression of ICOS mutant was visualized by rhodamine (red signal) as described in SI Materials and Methods. Expression of EGFP (green signal) was observed as green fluorescence within the cells. Scale bar, 10 μm.

protein function and structure, is also involved in protein trafficking to the cell surface membrane. Furthermore, amongst of all of the mutant proteins, N23/89Q ICOS showed the most drastic reduction in cell surface expression. Thus *N*-glycosylation at N89 as well as the primary structure of the ICOS is essential for proper protein trafficking to the cell surface membrane. *N*-Glycosylation is also required not only to establish the primary protein structure, but also for proper glycoprotein function.

Discussion

In this study, we demonstrate the essential biological functions and physiological role of *N*-glycans attached to a T-cell costimulatory molecule, ICOS. Among the three putative ICOS *N*-glycosylation sites, the *N*-glycan attached at N89 is essential for proper protein trafficking to the cell surface membrane. *N*-Glycosylation at N89 also plays a pivotal role in B7h ligand binding through sta-

bilization of the protein structure. Our results successfully demonstrate that among multiple *N*-glycans on this glycoprotein, the specific N89 *N*-glycan regulates not only the biological functions but also the protein trafficking of ICOS.

Among CD28 family members, ICOS shares with CD28 and CTLA-4 characteristic consensus residues encoding an immunoglobulin-like domain, a ligand binding domain and several *N*-glycosylation sites. These similar features suggest that the respective protein three-dimensional structures may also be similar [24]. CD28 and CTLA-4 *N*-glycosylations play essential roles in regulating protein activity [25,26]. A previous study demonstrated that mutation of all CD28 *N*-glycosylation sites led to a significant increase in both its ligand binding and costimulatory activities [25]. All of the *N*-glycosylation sites partially contributed to CTLA-4 dimerization, but not to co-localization with the T-cell receptor in the immunological synapse, or its inhibition of T-cell activation [26]. In this study, we demonstrated that among the three putative glycosylation sites in ICOS, the specific *N*-glycan attached to N89 was essential for both ligand binding and membrane trafficking. Based on the immunoreactivity of the neutralizing monoclonal antibody against ICOS, *N*-glycans on ICOS are also associated with the stability of the protein structure. These observations suggest that the *N*-glycans of costimulatory molecules have pivotal roles in ligand binding through stabilization of the protein structure.

Based on the crystal structure of a CTLA-4 and B7.1 complex, it has been suggested that CTLA-4 homodimers form a lattice-like array and bivalently bind B7.1, which is postulated to provide the stabilized receptor–ligand complex necessary for its inhibitory activity against T-cell activation [27]. Regarding ICOS, a three-dimensional model of the extracellular immunoglobulin-like domain was built based on the NMR structure of CTLA-4 which is highly similar to the crystal structure [23]. This model demonstrated that the extracellular domain of ICOS consists of two main surfaces: the A′GFCC′ face, which contains the ligand binding FDPPPF motif, and the BED face located on the opposite side, which is similar to other CD28 family members. In additional, a previous mutagenesis study showed that residue substitution in the A′GFCC′ face improved the protein's ligand binding activity [23]. These observations indicate that the structure of the A′GFCC′ face is crucial to the biological activity of ICOS. In the three-dimensional model of ICOS, the N89 *N*-glycosylation site resides on the BED face, and the N110 *N*-glycosylation site resides on the A′GFCC′ face. For this reason, we predicted that the N110 *N*-glycosylation site and not N89 would be essential for B7h binding activity. However, in this study, we demonstrated that *N*-glycosylation at N89 in the BED face was essential for ligand binding activity as well as for intracellular protein trafficking. In addition, the *N*-glycosylation-defective mutant N110Q, which lacks the *N*-glycan on the A′GFCC′ face, retained both its ligand binding activity as well as proper protein trafficking to the cell surface membrane. These results suggest that *N*-glycosylation at N89 in the BED face, and not N110 in the A′GFCC′ face, likely stabilizes the A′GFCC′ face from the opposite side. Of interest is the unglycosylated mutant N23/89/110Q ICOS, which showed ligand binding activity similar to that of the unglycosylated mutant CD28 [24], since the A′GFCC′ face in that mutant protein probably retained a similar structure to that of WT ICOS. From these results, the position of the *N*-glycosylation site in the glycoprotein is closely connected with both protein expression and function.

Protein quality control and trafficking through the secretory processes in the ER and Golgi complex are essential for proper protein expression and functions. The ER molecular chaperones BiP, CNX and CRT facilitate protein folding and play important roles in protein synthesis, quality control and proper maturation of proteins [28,29]. Glycosylated proteins are associated with CNX and CRT via *N*-glycans, while unglycosylated proteins are recognized by BiP. A previous study reported that BiP binds to early folding intermediates, while CNX and CRT bind after a short lag time to more mature, folded molecules [30]. In this study, the specific *N*-glycan at position N89, but not those at position N23 or N110, facilitates proper ICOS folding via a lection-like chaperone pathway such as CNX/CRT. In contrast, the unglycosylated N23/89/110Q ICOS mutant may have been folded roughly by another ER pathway such as BiP. The present results also suggest that N89 glycosylation, but not N23 and N110 glycosylation, and/or the protein structure which develops following N89 glycosylation, was essential for proper ER-quality control and for ICOS membrane trafficking.

This study demonstrates that specific *N*-glycans can play important roles in both protein trafficking and in protein activities. The precise relationship between a specific *N*-glycan amongst the sometimes multiple glycans of a particular glycoprotein and interactions with ER-quality control molecules through lectin-like chaperones or trafficking regulatory mechanisms involving movement from the ER to the cell surface membrane via the Golgi complex remain to be established. Therefore, further investigation is necessary for clarification of ER glycoprotein folding mechanisms involving recognition of a specific *N*-glycan by a molecular chaperone such as BiP, CNX or CRT. These studies will contribute to the current understanding of the roles of *N*-glycans in the functional regulation of glycoproteins.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.098.

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