Glycoconj J (2012) 29:47–55
DOI 10.1007/s10719-011-9363-0

Asn^{54}-linked glycan is critical for functional folding of intercellular adhesion molecule-5

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Received: 6 October 2011 / Revised: 21 November 2011 / Accepted: 28 November 2011 / Published online: 21 December 2011

Abstract Intercellular adhesion molecule-5 (ICAM-5, telencephalin) is a dendritically polarized type I membrane glycoprotein, and promotes dendritic filopodia formation. Although we have determined the N-glycan structures of ICAM-5 in a previous report, their function is unknown. Here, we produced fifteen ICAM-5 gene constructs, in which each potential N-glycosylation site was mutated, to elucidate the function of the N-glycans of ICAM-5, and observed the effects of transfection of them on a neuronal cell line, Neuro-2a (N2a). Only the N54Q mutant, which is the mutant for the most N-terminal glycosylation site, failed to induce filopodia-like protrusions in N2a cells. Immunofluorescence staining and cell surface biotinylation revealed that N54Q ICAM-5 was confined to the ER and also could not be expressed on the cell surface. This is further supported by the biochemical evidence that almost all N-glycans of N54Q ICAM-5 were digested by Endo glycosidase H and peptide:N-glycanase, indicating that almost all of them retain high-mannose-type structures in ER. In additon, it also failed to form disulfide bonds or functional protein complexes. The stable transformants of N54Q ICAM-5 showed retarded cell growth, but it was interesting that there was no apparent ER stress, because the mutant was sequentially degraded via ER associated degradation pathway by comparing the susceptibilities of the responses to various inhibitors of this pathway in wild-type and N54Q ICAM-5 transfectants. Taken together, the Asn^{54}-linked glycan is necessary for normal trafficking and function of ICAM-5, but is unassociated with ER-associated degradation of it.

Keywords ICAM-5 · N-glycan · Site-directed mutagenesis · Protein trafficking

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Electronic supplementary material The online version of this article (doi:10.1007/s10719-011-9363-0) contains supplementary material, which is available to authorized users.

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Introduction

All five intercellular adhesion molecule (ICAM) family members have extracellular immunoglobulin-like domains and belong to the immunoglobulin superfamily. ICAMs 1–4 are expressed by the hematopoietic cell lineages: ICAM-1 and ICAM-2 on vascular endothelial cells and leukocytes [1–3]; ICAM-3 mainly on resting leukocytes [4–6]; ICAM-4 on red blood cells [7]. Only ICAM-5 is expressed on neural cells within the telencephalon [8, 9]. ICAM-5 is a single-pass transmembrane glycoprotein with an extracellular domain

consisting of nine immunoglobulin-like domains with 13–15 potential N-glycosylation sites; these sites are significantly conserved among mammals [10, 11]. ICAM-5 is specifically located in dendrites and slows dendritic spine maturation [12]. *In vitro*, ICAM-5 induces filopodia-like protrusions in N2a cells and hippocampal neurons. The protrusions result from the interaction between ezrin-radixin-moesin (ERM) proteins and ICAM-5′s carboxy-terminal region [13].

Some N-glycans are important for ICAMs' molecular functions. For example, removing ICAM-3′s N-glycans decreases its binding to lymphocyte function-associated antigen-1 (LFA-1), and dendritic cell-specific ICAM-3-grabbing nonintegrin recognizes the Lewis X-modified N-glycans on ICAM-3 [14]. ICAM-2′s high-mannose N-glycans are important for binding to LFA-1 [15]. The functions of ICAM-5′s N-glycans, however, are unknown. Previously, we determined the N-linked glycan structures in ICAM-5 from rat brain [16], and compared them with the N-glycans on human ICAM-1 expressed by mouse myeloma cells [17] and with those on ICAM-3 isolated from human T-cells [18, 19]. These comparisons showed ICAM-5′s high-mannose glycan content to be higher and its highly branched and sialylated glycan content lower than those of ICAM-1 and ICAM-3, suggesting that ICAM-5 N-glycans' functions might differ from those of ICAM-1 and ICAM-3.

Directed mutagenesis of a glycoprotein's potential N-glycosylation sites is an effective way to investigate the functions of its specific N-glycans. For example, ErbB3's Asn^{418}-linked glycan is crucial in preventing spontaneous heterodimerization and tumor promotion [20], and the human δ opioid receptor's Asn^{33}-linked glycan is important for its cell-surface stability [21]. In this study, we produced 15 site-directed mutations of ICAM-5 N-glycosylation sites, and observed the phenotypes of cells that expressed them. ICAM-5′s Asn^{54}-linked glycan was crucial for its transport from the endoplasmic reticulum (ER) to the Golgi apparatus, and for its ability to induce filopodia-like protrusions. At the protein level, the N54Q mutant failed to form disulfide bonds or at least some complexes, which probably account for its aberrant protein trafficking. Surprisingly, this mutant is degraded normally, via the ER-associated degradation (ERAD) pathway, without apparent ER stress (although its transfectants showed retarded cell growth), suggesting that the Asn^{54}-linked glycan is not directly involved in protein degradation.

Materials and methods

Reagents and antibodies

Brefeldin A (BFA), tunicamycin (TM), MG132, lactacystin, and deoxymannojirimycin (DMJ) were purchased from Invitrogen (Carlsbad, CA, USA), Wako (Tokyo, Japan), Peptide Institute (Osaka, Japan), Cayman Chemical Company (Ann Arbor, MI, USA), and Enzo Life Sciences (Plymouth Meeting, PA, USA), respectively. We produced ICAM-5-specific polyclonal antiserum in rabbits as previously described [16]. Mouse monoclonal anti-glucosidase II (GII) antibody was raised against the following sequence of mouse GII: EEEEEEEEEEEEAC. We used anti-calnexin (CNX) antibody (clone SPA-860F; Stressgen, Victoria, BC, Canada), anti-glucose-regulated protein 78 kDa (GRP78) antibody (clone N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Alexa Fluor 488 or 546-labeled secondary antibodies (Invitrogen), and HRP-conjugated secondary antibodies (DAKO, Glostru, Denmark).

Vector construction and *in vitro* mutagenesis

Rat ICAM-5 cDNA (NCBI accession no. XP_233737) was cloned by RT-PCR using rat whole brain total RNA (Clontech, Palo Alto, CA, USA) and inserted into pUSEamp(+) (Millipore, Billerica, MA, USA) with *Kpn* I and *Xba* I. To produce single mutants of ICAM-5 N-glycosylation sites, site-directed mutagenesis was performed by PCR. Primers used to replace asparagine with glutamine at the N-glycosylation sites are listed in Supplementary data 3. All mutations were verified by sequencing.

Cell culture and treatment

The mouse neuroblastoma cell line N2a was grown in Dulbecco's modified Eagle's medium /Ham's F12, supplemented with 10% (*v/v*) fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 0.1 mg/ml streptomycin sulfate. To maintain stable transfectants, the same medium was supplemented with 0.5 mg/ml G418 (Sigma-Aldrich, Gillingham, Dorset, UK). DNA contents were analyzed on a flow cytometer (FACS Caliber; Becton Dickinson, Franklin Lakes, NJ, USA); cells were fixed and permeabilized, treated with RNase, and stained with propidium iodide. DNA content in stable transfectants decreased slightly, because the transfectants were mono-cloned from heterotrophic cell groups (Fig. S1). Stable transfectants were treated with 10 μM MG132, 10 μM lactacystin, and 1 mM DMJ for 8 h.

Immunocytochemistry

N2a cells were seeded onto 6-well plates (Becton Dickinson) for transfection. To label ICAM-5 and GII, cells were fixed with 4% paraformaldehyde in PBS for 2 h, permeabilized with 0.1% Triton-X100 in PBS for 5 min, blocked with 3% bovine serum albumin for 1 h, incubated with primary antibody mixtures for 1 h, rinsed with PBS, incubated with fluorescence-

conjugated secondary antibodies diluted in PBS for 1 h, and washed with PBS, all at room temperature. Cells were mounted in Vectashield mounting medium with DAPI (Vector labs, Peterborough, UK), and observed under an Olympus IX70 fluorescence microscope (Olympus, Center Valley, PA, USA).

Biotinylating and cross-linking cell surface proteins

Cell-surface proteins were biotinylated and cross-linked as described previously [22]. Cells were triple-washed with PBS and then incubated in PBS containing 10 μg/ml Biotin-AC_5-OSu (Dojindo, Kumamoto, Japan) or 1 mM disuccinimidyl suberate (DSS) (Thermo Scientific, San Jose, CA, USA) for 30 min at 4°C. To quench excess biotin or DSS, Tris–HCl (pH 7.4) was added to a final concentration of 50 mM, and the cells were incubated for another 10 min at room temperature. The cells were then double-washed with PBS and collected. Total cell proteins were subjected to immunoblotting. Biotinylated proteins were detected by HRP-labeled streptavidin (SA) (GE Healthcare, Uppsala, Sweden).

Immunoprecipitation and co-immunoprecipitation

Approximately 8.5×10^5 cells transiently expressing ICAM-5 were solubilized with 500 μl TNE buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 10 mM EDTA) or TNC buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 1 mM $CaCl_2$) at 4°C overnight, and centrifuged at 20 000×*g* for 10 min; 2 μg anti-ICAM-5 antibody and 40 μl protein A-Agarose beads (GE Healthcare) were added to the supernatant, and the solution was rotated overnight at 4°C. The beads were triple-washed with wash buffer (20 mM Tris–HCl, pH 7.4; 0.15 M NaCl; 1 mM $CaCl_2$), 20 μl of 2 × SDS-PAGE sample buffer (0.125 M Tris–HCl, pH6.8; 10% 2-mercaptoethanol (2-ME); 4% SDS; 10% sucrose; 0.004% bromophenol blue) was added to release bound proteins, and the samples were spun at 20 000×*g* for 10 min at room temperature. The supernatants were subjected to immunoblotting.

Glycosidase digestion

Forty million cells transiently expressing ICAM-5 were solubilized with 20 μl enzymatic digestion buffer (50 mM sodium phosphate buffer, pH 7.5; 50 mM sodium citrate buffer, pH 5.5; 50 mM sodium acetate buffer, pH 5.0; and 50 mM sodium citrate buffer, pH 4.5) containing 0.5% SDS and 1% 2-ME and boiled for 3 min. An equal volume of enzymatic digestion buffer, a half-volume of 7.5% NP-40, 25 000 units/ml peptide:N-glycanase F (PNGase F, New England Biolabs, Ipswich, MA, USA), 25 000 units/ml Endo *N*-acetylhexosaminidase H (Endo H, New England Biolabs), and 2 500 units/ml sialidase (Nacalai Tesque, Kyoto, Japan) were added, and the mixture was incubated overnight at 37°C. The digested proteins were precipitated by adding three volumes of ice-cold ethanol. The proteins were dissolved in 1 × SDS-PAGE sample buffer and subjected to immunoblotting.

Semi-quantitative RT-PCR

Total RNA was extracted from N2a cells using Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX, USA) according to the manufacturer's protocol. Reverse transcription was performed using 2 μg total RNA, 2 μg oligo$(dT)_{12–18}$, 1 mM DTT, 0.5 mM dNTP, and 400 units/μl of M-MLV reverse transcriptase (Invitrogen) for each reaction. Specific PCR primers are listed as Supplementary data 3.

Results

The ICAM-5 Asn^{54}-linked N-glycan is important for ICAM-5 protein maturation

WT and mutant ICAM-5 molecular weights were determined by immunoblotting (Fig. 1). N54Q ICAM-5 was mainly detected at 110 kDa, whereas the other fourteen ICAM-5 mutants were detected primarily at 150 kDa; a smaller form detected at 110 kDa was identified as an immature form (see N54Q ICAM-5 is not transported from the ER to the Golgi apparatus.)

N54Q ICAM-5 is mainly confined to the ER and does not induce filopodia-like protrusions

Mock-transfected cells and those expressing WT ICAM-5 or its mutants were examined by immunocytochemistry (Fig. 2a-q). Filopodia-like protrusions were seen in only 7.6±3.4% cells expressing N54Q ICAM-5, but about 70–80% of N2a cells expressing WT ICAM-5 or mutants other than N54Q exhibited them (Fig. 2r). In addition, N54Q localized to the cells' extranuclear region, especially to the

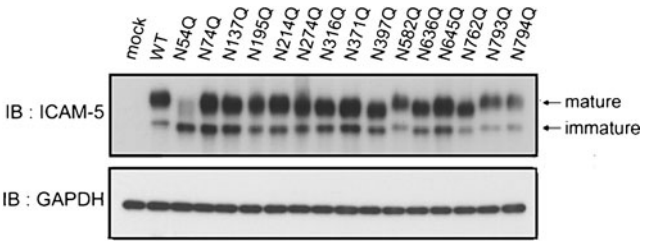

Fig. 1 ICAM-5 molecular weights. N2a cells were transfected with ICAM-5 plasmids or empty vector (mock). Total cell proteins were immunoblotted; GAPDH was the internal loading control

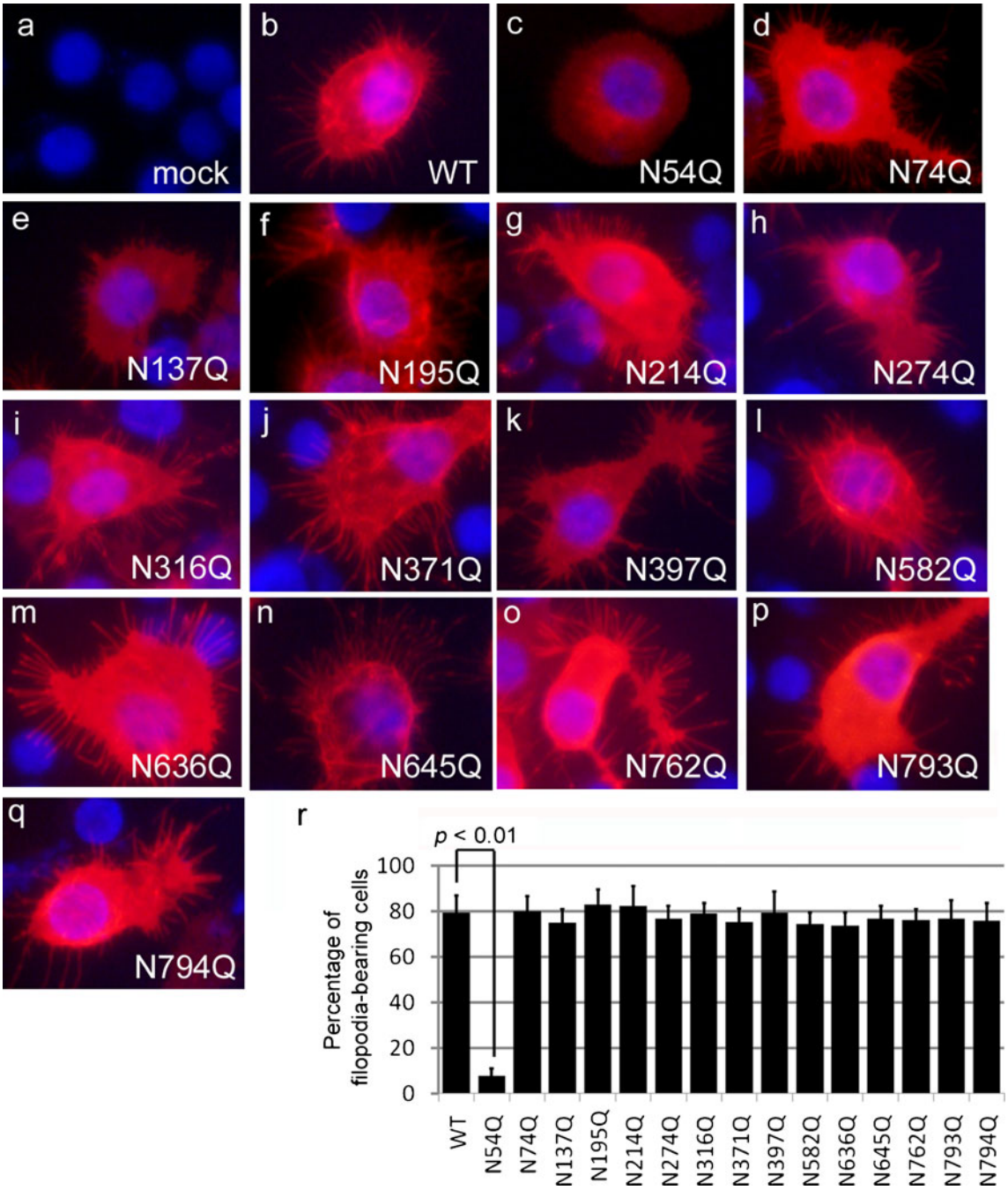

Fig. 2 Cellular phenotypes induced by ICAM-5 mutants. N2a cells transfected with empty vector (mock, **a**), WT ICAM-5 (**b**) or mutant ICAM-5 (**c**-**q**) plasmids and stained with anti-ICAM-5 antibody and DAPI. (**r**) Percentage of ICAM-5-positive filopodia-bearing cells calculated for each construct from 10 randomly selected visual fields. The p value was calculated using Student's two-tailed t test. Error bars indicate SD

peripheral nuclear zone, suggesting that it was confined to the ER. Co-immunostaining analysis revealed that, unlike WT ICAM-5, N54Q co-localized with an ER marker protein, GII (Fig. 3a and b). WT and N54Q ICAM-5 cell-surface localization analysis (Fig. 3c) showed much more biotinylation of mature WT ICAM-5 than of mature N54Q, and immature WT and N54Q ICAM-5 were not biotinylated (lanes 3, 4 upper panel; lanes 1, 2 lower panel). Thus, immature ICAM-5 was not expressed on cell surfaces, and only a minute amount of N54Q ICAM-5 was trafficked to the plasma membrane. To examine any residual function of N54Q ICAM-5, we attempted to detect protein interactions using a cross-linker. Protein complexes were specifically observed in WT but not N54Q ICAM-5-transfected cells (Fig. 3d, lanes 5

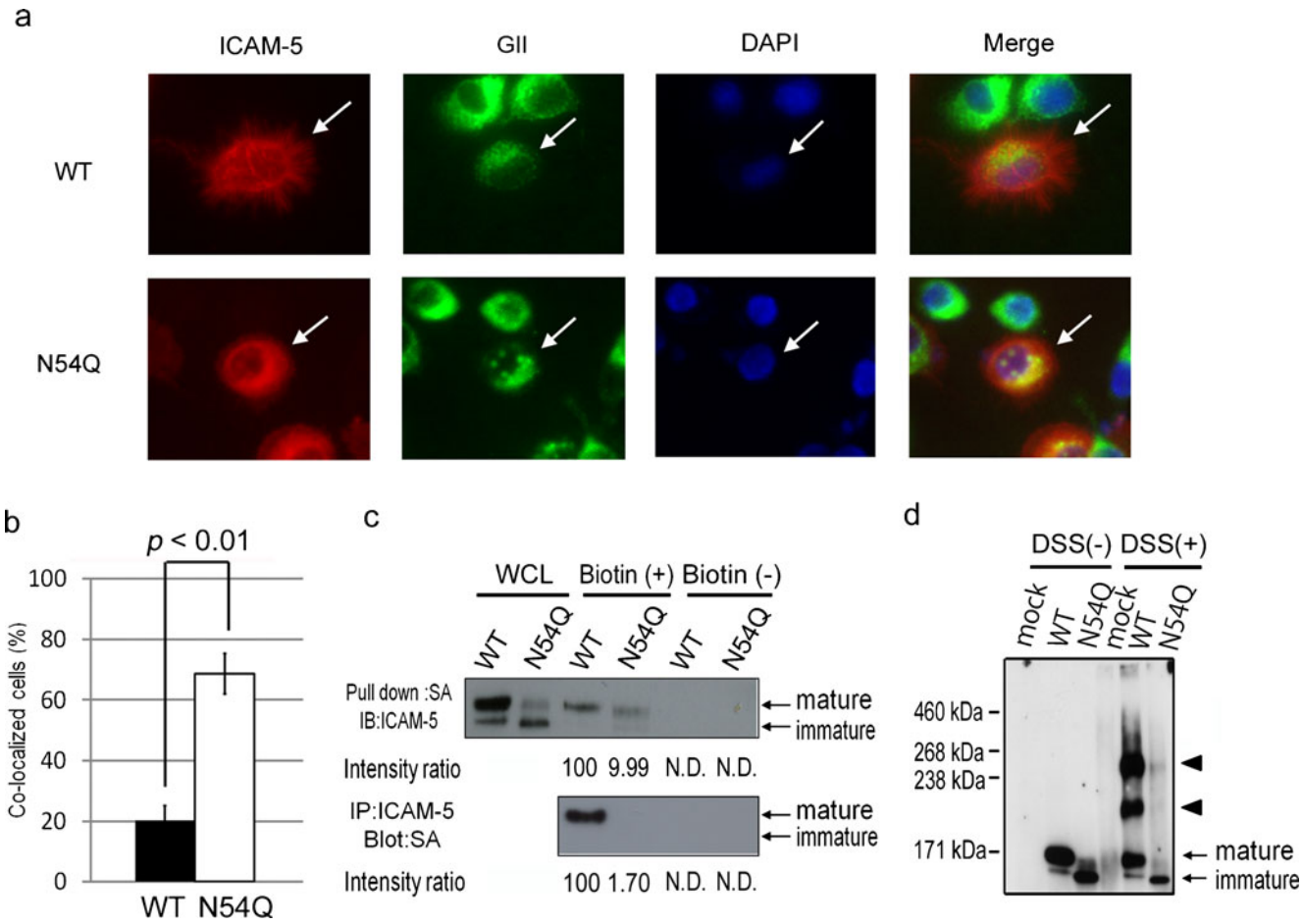

Fig. 3 WT and N54Q ICAM-5 localization. (**a**) N2a cells transiently expressing WT or N54Q ICAM-5 stained by DAPI and the indicated antibodies. White arrow indicates the same cell in each panel. (**b**) Percentage of cells showing co-localization of ICAM-5 with ER marker GII in ten arbitrarily selected visual fields. The p value was calculated using Student's two-tailed t test. Error bars indicate SD. (**c**) Cell-surface proteins labeled with Biotin-AC_5-OSu. Biotinylated ICAM-5 was pulled down or immunoprecipitated using streptavidin (SA) or anti-ICAM-5 antibody, blotted, and detected by anti-ICAM-5 antibody or SA. Band intensities were quantified by densitometry. N.D., not detectable. (**d**) Immunoblot of total proteins from cells treated with the cross-linker DSS, using anti-ICAM-5 antibody

and 6), showing that N54Q ICAM-5 did not form detectable cell-surface protein complexes.

N54Q ICAM-5 is not transported from the ER to the Golgi apparatus

Glycoproteins in the ER contain high-mannose-type N-glycans, whereas those residing in the Golgi apparatus contain complex and hybrid-type N-glycans. Therefore, we compared WT and N54Q ICAM-5 sensitivities to various glycosidases (Fig. 4a). First, we digested WT and N54Q ICAM-5 with PNGase F, which hydrolyzes nearly all glycoprotein N-glycan chain types. This treatment resulted in the N54Q and WT ICAM-5 bands showing the same molecular weight by SDS-PAGE (Fig. 4a), indicating that the molecular weight differences of the untreated glycoproteins were owing to their N-glycan structures. Since Endo H specifically digests high-mannose N-glycans, it digests the N-glycans of ER-resident glycoproteins. Like PNGase F, Endo H removed almost all the N54Q N-glycans, showing that they were almost all of the high-mannose type (Fig. 4a). Sialidase sensitivity marks glycoproteins with terminal sialic acids, which occur exclusively in complex and hybrid-type N-glycans. Sialidase digested WT ICAM-5, but not much of the N54Q ICAM-5 (Fig. 4a), showing that very little N54Q ICAM-5 had been modified by sialyltransferases, which are in the Golgi apparatus, and therefore most N54Q was in an immature form. To confirm this result, we used the reversible protein trafficking inhibitor BFA to examine glycosylation pattern switching in WT and N54Q ICAM-5 (Fig. 4b). The immature WT ICAM-5 that accumulated with BFA treatment matured when BFA was removed (Fig. 4c, lanes 1–5); however, the immature N54Q ICAM-5 could not be matured by removing BFA (Fig. 4c,

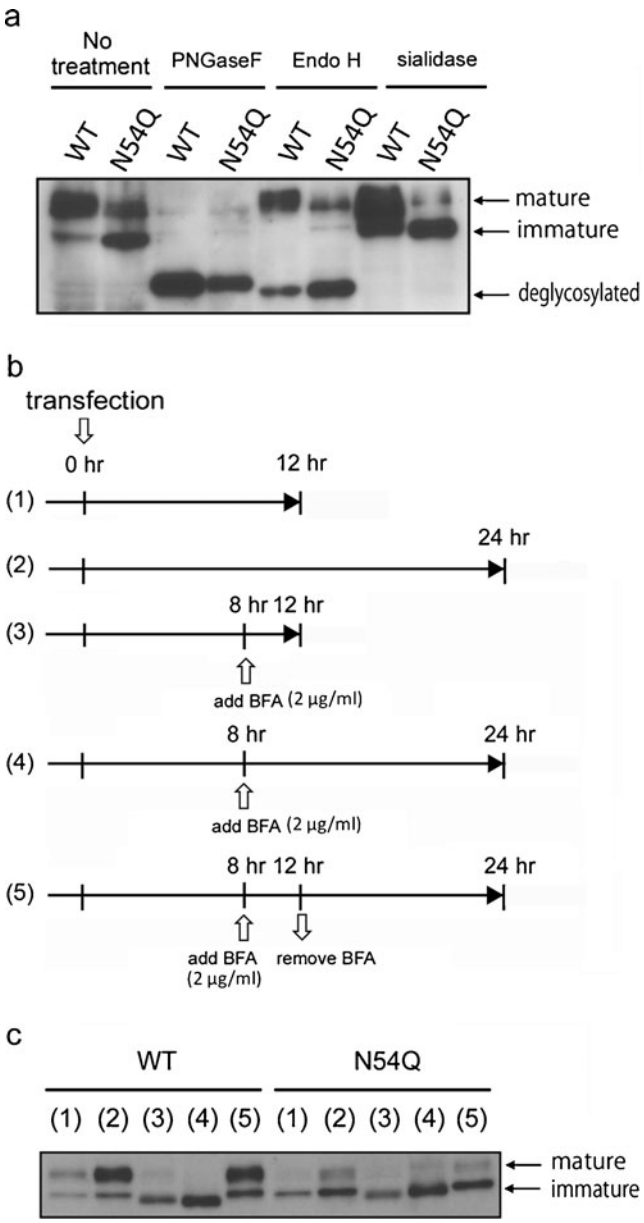

Fig. 4 N54Q ICAM-5 was not transported from the ER to the Golgi apparatus. (**a**) Total cell proteins were digested by glycosidases and immunoblotted with anti-ICAM-5 antibody. (**b**) Brefeldin A (BFA) treatment schedules after transfection. (**c**) Total cell lysates from transfected cells treated as indicated in the corresponding schematic in (**b**), immunoblotted with anti-ICAM-5 antibody

lanes 6–10). This shows that almost no N54Q ICAM-5 was transported from the ER to the Golgi apparatus.

N54Q ICAM-5 does not induce ER stress responses

The above results indicated that almost all N54Q ICAM-5 was confined to the ER. Generally, when abnormal proteins accumulate in the ER, ER stress inhibits cell growth. The calculated doubling times of the stable transfectants as compared to parental cells or mock transfectants showed obvious growth retardation (Fig. 5a and b); however, the levels of the ER stress indicators GRP78 and ATF6 were not elevated (Fig. 5c and d), indicating that this retardation was not induced by a typical ER stress response. We therefore speculated that the ER-confined N54Q ICAM-5 was degraded normally.

N54Q ICAM-5 is trapped in the calnexin cycle and is degraded normally via ERAD

The N-glycans of many glycoproteins are actively involved in protein folding, which occurs in association with CNX and calreticulin (CRT), chaperones that interact with newly synthesized glycoproteins in the ER and have an affinity for monoglucosylated high-mannose glycans ($Glc_1Man_9GlcNAc_2$) [23]. CNX recognizes the amino-terminal N-glycans of the membrane glycoprotein hemagglutinin, and CRT recognizes carboxy-terminal N-glycans [24]. We speculated that CNX strongly recognized and interacted with immature N54Q ICAM-5, and used a co-immunoprecipitation assay to test this hypothesis (Fig. 6a, lane 4 in right panel). Since N54Q ICAM-5 did not induce ER stress, it might be degraded normally via the ERAD pathway, *i.e.*, removed one mannose residue by α-mannosidase in ER, transported to the cytosol by EDEM, ubiquitinated, deglycosylated by cytosolic PNGase, and degraded by proteasomes [23]. Deglycosylated ICAM-5 (94 kDa) accumulated in stable N54Q ICAM-5-expressing transfectants treated with the proteasome inhibitor MG132 or lactacystin (Fig. 6b). Conversely, glycosylated ICAM-5 (110 kDa) accumulated in these transfectants when treated with the α-mannosidase inhibitor DMJ. Thus, although N54Q ICAM-5 was not trafficked from the ER to the Golgi apparatus, it was degraded normally via the ERAD pathway and did not accumulate abnormally.

N54Q ICAM-5 does not form disulfide bonds

Since N54Q ICAM-5 was trapped in the CNX cycle, we speculated that it was not correctly folded in the ER. Since abnormal disulfide bonds frequently cause misfolding, we separated WT and N54Q ICAM-5 by SDS-PAGE under reducing and non-reducing conditions, and found that WT ICAM-5 migrated more slowly in reducing than non-reducing conditions, but there was no difference in band mobility for N54Q ICAM-5 (Fig. 6c), indicating that N54Q ICAM-5 does not contain any disulfide bonds.

Discussion

To elucidate ICAM-5 N-glycans' functions, we constructed fifteen site-directed ICAM-5 mutants and observed the phenotypes of cells expressing them. We found that the mutant for the most N-terminal glycosylation site (N54Q) did not

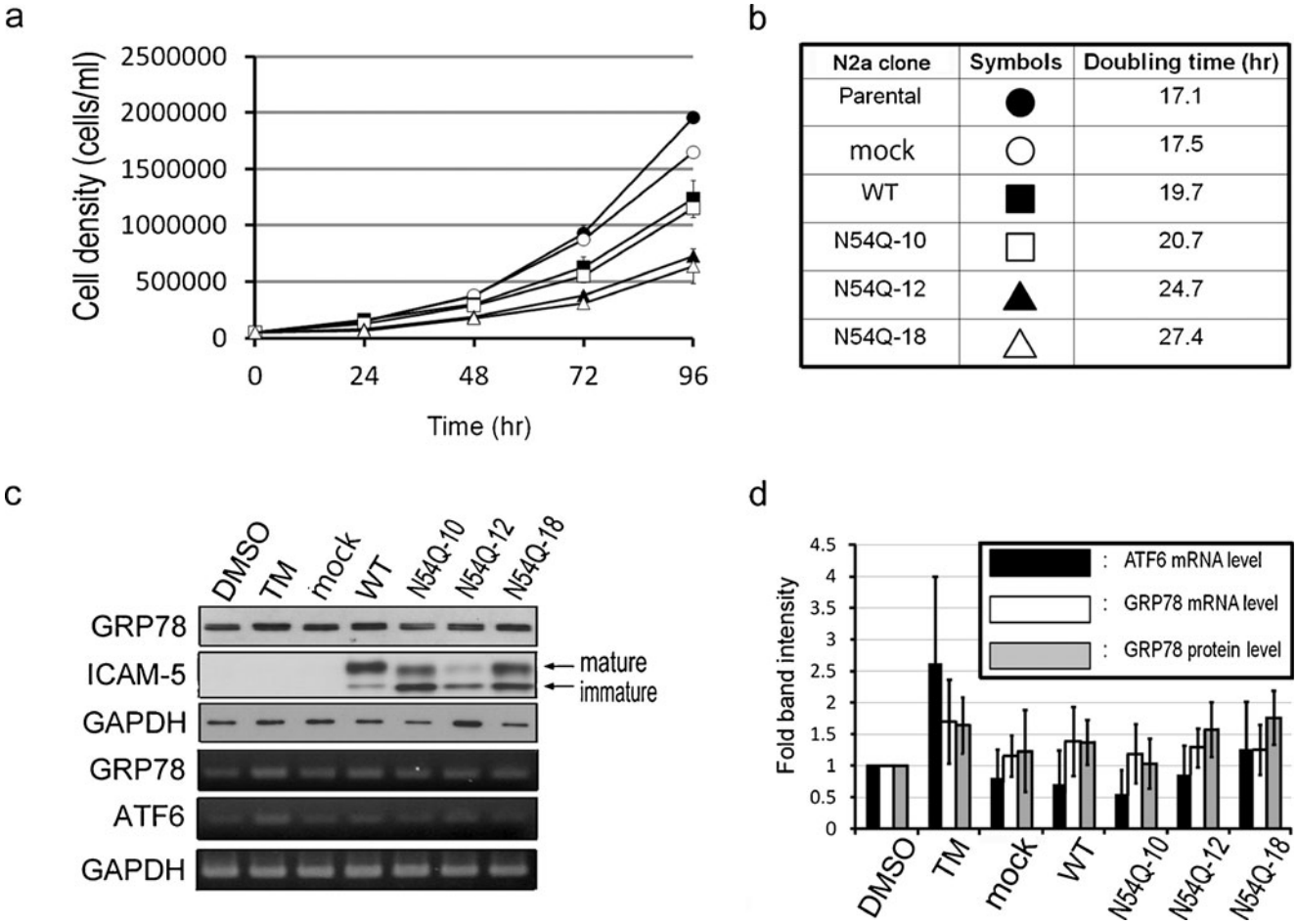

Fig. 5 N54Q ICAM-5 did not induce ER stress responses. (**a**, **b**) Growth curves and doubling times for parental N2a cells, N2a cells stably transfected with empty vector, or stable transfectants expressing WT ICAM-5 or N54Q. (**c**) ATF6, GRP78, and ICAM-5 levels detected by immunoblotting and semi-quantitative RT-PCR. GAPDH was an internal control. TM (3 μg/ml), which inhibits the formation of the dolichol-bound *N*-acetylglucosamine derivative, was the ER stress-inducer positive control. (**d**) Band intensities were quantified by densitometry. N54Q-10, N54Q-12, and N54Q-18 mean different stable clones

induce filopodia-like protrusions in N2a cells (Fig. 2), was rarely expressed at the cell surface, and failed to form certain functional complexes (260 kDa and 200 kDa) (Fig. 3c and d). Normally, ICAM-5 interacts with α-actinin and ERM on the cell surface to form dendritic filopodia [13, 25], suggesting that this interaction was disrupted in the N54Q mutant. Additionally, since the disulfide bonds could not be formed in N54Q ICAM-5, it is suggested that Asn^{54}-linked glycan is critical functional folding of ICAM-5 (Fig. 6c).

Pro-αIIb is also degraded through the CNX cycle via an amino-terminal N-glycan; deletion of a glycosylation site (N15Q) prevents it from forming a complex with the β3 subunit or undergoing proteasomal degradation [26]. ERp57, a major protein disulfide isomerase, interacts with CNX in mammalian cells [23], and N15Q Pro-αIIb does not bind to CNX, suggesting that ERp57 cannot access mutated Pro-αIIb. In contrast, N54Q ICAM-5 interacts with CNX (Fig. 5c), suggesting that N54Q ICAM-5 is modified by ERp57. This scenario points to two possible explanations for N54Q's failure to assemble disulfide bonds: 1) GII hydrolyzes glucose from the N54Q N-glycan ($Glc_1Man_9GlcNAc_2$), thereby dissociating ICAM-5 from CNX, too quickly to permit disulfide bond assembly; or 2) Asn^{54}-linked glycan is important for ERp57 access to ICAM-5.

Since potential amino-terminal N-glycosylation sites are conserved in ICAMs (closed arrowhead in Fig. S2a), we speculated that the most amino-terminal N-glycan is generally important for their intracellular trafficking. However, we found that WT and N47Q ICAM-1 expressed in N2a cells reached the cell surface and were biotinylated (Fig. S2b), showing that this ICAM-1 N-glycan is unrelated to its trafficking. Similar results were obtained in the murine endothelial cell line MSS31 (data not shown). It is previously reported that Trp51 of human ICAM-1 interacts with N-terminal glycan, and these conserved glycan and Trp residue are critical for the proper conformation of the integrin binding domain of

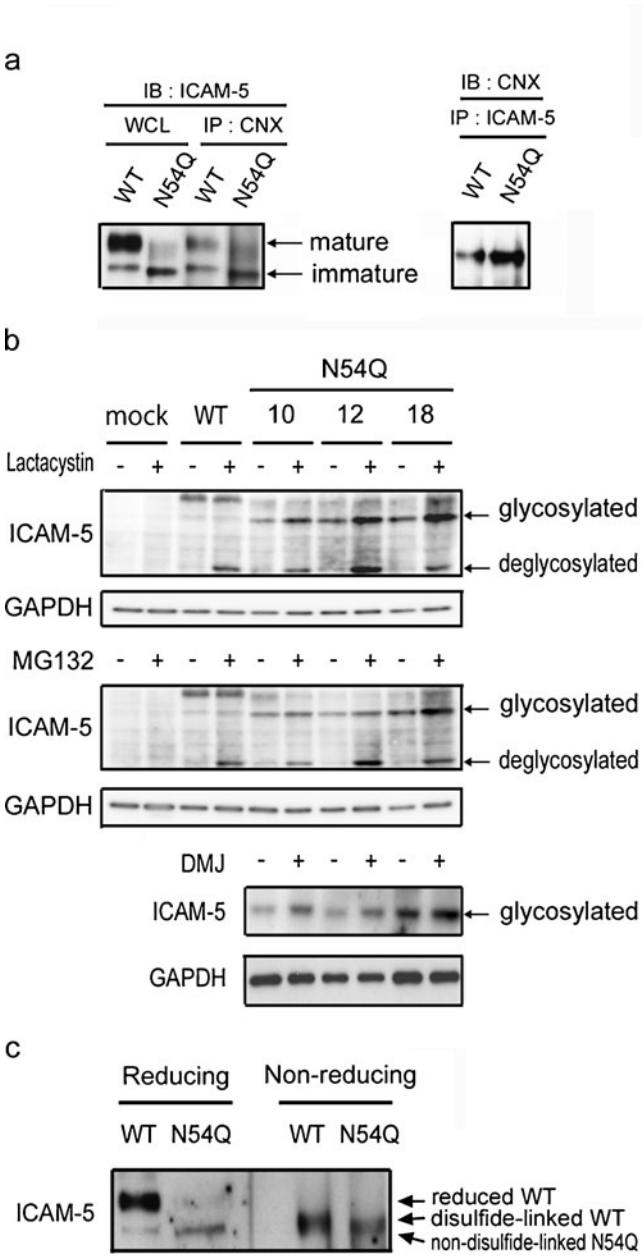

Fig. 6 Misfolded N54Q ICAM-5 was properly degraded through the ERAD pathway. (**a**) Co-immunoprecipitation analysis using anti-ICAM-5 or anti-CNX antibody followed by immunoblotting with anti-CNX or anti-ICAM-5 antibody, respectively. WCL, whole cell lysate. (**b**) Comparison of the responses to various inhibitors of ERAD pathway in parental N2a cells and transfectants. Total cell proteins from stable transfectants treated with various reagents were immunoblotted with anti-ICAM-5 antibody. GAPDH was an internal control. N54Q-10, N54Q-12, and N54Q-18 mean different stable clones. (**c**) Total cell proteins were subjected to immunoblotting with anti-ICAM-5 antibody under reducing (2-ME(+)) or non-reducing (2-ME(−)) conditions

ICAMs (glycan-W motif) [15]. Since ICAM-5 Trp^{82} is also conserved (open arrowhead in Fig. S2a), we speculated that it is important in the glycan-W motif. We examined W82F ICAM-5′s maturation and the cellular phenotype by immunocytochemistry and immunoblotting, and found that the construct matured and induced filopodia-like protrusions; thus, the glycan-W motif is not essential for protein trafficking either (data not shown).

Studies have reported decreased cell-surface levels of specific N-glycan-deleted G-protein coupled receptors, such as rhodopsin [27] and the β2-adrenergic [28], AT2 [29], calcium [30], VIP [31], LH-RH [32], and PAF receptors [33]. However, little has been reported regarding the contribution of N-glycans bound to cell adhesion molecules. E-cadherin mutated at the Asn^{633} N-glycosylation site accumulates in the ER and is degraded by ERAD [34]. However, Asn^{633} is not an amino-terminal N-glycosylation site, and protein trafficking mechanisms via amino-terminal N-glycans are not conserved among membrane glycoproteins. In addition, it is interesting that N-terminal five glycans of integrin α5 subunit are important for the cell surface expression, while Asn^{269} N-glycan, which is not the N-terminal one, is the most important for its expression on the cell surface [35, 36]. It is probable that membrane glycoproteins are trafficked via a specific N-glycan. Our results provide new insight into the intracellular trafficking mechanisms of these membrane glycoproteins.

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