Functional role of N-glycosylation from ADAM10 in processing, localization and activity of the enzyme

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A disintegrin and metalloprotease 10 (ADAM10) is a type I transmembrane glycoprotein with four potential N-glycosylation sites (N267, N278, N439 and N551), that cleaves several plasma membrane proteins. In this study, ADAM10 was found to contain high-mannose and complex-type glycans. Individual N-glycosylation site mutants S269A, T280A, S441A, T553A were constructed, and results indicated that all sites were occupied. T280A was found to accumulate in the endoplasmic reticulum as the non-processed precursor of the enzyme. Furthermore, it exhibited only residual levels of metalloprotease activity in vivo towards the L1 cell adhesion molecule, as well as in vitro, using a ProTNF-alpha peptide as substrate. S441A showed increased ADAM10 susceptibility to proteolysis. Mutation of N267, N439 and N551 did not completely abolish enzyme activity, however, reduced levels were found. ADAM10 is sorted into secretory vesicles, the exosomes. Here, a fraction of ADAM10 from exosomes was found to contain more processed N-linked glycans than the cellular enzyme. In conclusion, N-glycosylation is crucial for ADAM10 processing and resistance to proteolysis, and results suggest that it is required for full-enzyme activity.

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1. Introduction

ADAM (a disintegrin and metalloprotease) 10 is a type I transmembrane glycoprotein that belongs to the ADAMs protein family. It is characterized by a conserved domain structure, consisting of an N-terminal signal sequence that directs the protein to the secretory pathway followed by a prodomain, a metalloprotease and a disintegrin domain, a cysteine-rich region, a transmembrane domain and an SH3-enriched cytoplasmic tail [1]. In order to be catalytically active ADAM10 prodomain has to be cleaved by proprotein convertases furin and/or PC7 in the Golgi compartments [2–4].

ADAM10 metalloprotease domain is known to mediate the proteolytic cleavage of transmembrane proteins in their extracellular domain as a soluble form (sheddng). ADAM10 can cleave a variety of proteins with importance in development, cell signalling and disease, such as the cell adhesion molecule L1 [5], pro-tumor necrosis factor-alpha [6], type IV collagen [7], amyloid precursor protein (APP) [8], ephrin-A2 [9], epidermal growth factor receptor [10], notch [11,12], pro-heparin-binding epidermal growth factor [13], fractalkine [14], CD44 [15], N-cadherin [16], betacellulin [17], low-affinity immunoglobulin E receptor CD23 [18], among others. Moreover, ADAM10 is overexpressed in tumors, such as, uterine and ovarian carcinomas [19], human haematological malignancies [20], neuroblastomas [21], prostate cancer [22], gastric carcinoma cells [23], colon cancers [24] and oral squamous cell carcinoma [25] suggesting a role in tumor progression and dissemination.

ADAMs disintegrin and cysteine-rich domains were described as being directly involved in cell–cell adhesion processes through interactions with integrins and other receptors [26].

ADAM10 can be found in various cellular compartments. It has been shown to colocalize with Golgi markers and was found on the cell surface [8,27]. More recently, it was also detected in secreted vesicles identified as exosomes [27]. Exosomes are small membrane vesicles (30–100 nm diameter) secreted by various cell types as a consequence of fusion of multivesicular late endosomes/lysosomes with the plasma membrane. Exosomes secretion has first been reported in a variety of cells, including cancer cells, neurons, B lymphocytes, T lymphocytes, dendritic cells, mast cells and platelets [28]. Exosomes secreted from tumor cells could promote cellular invasion and migration during metastasis [29].

ADAM10 like other ADAMs is N-glycosylated. It has four potential N-glycosylation sites (N-X-S/T, X-Pro), three located in the metalloprotease domain (N267, N278 and N439) and one in the disintegrin domain (N551). Glycosylation is one of the most important post-translational modifications in newly synthesized proteins which may influence the physicochemical and biological properties of...
glycoproteins. N-glycosylation has been related with protein folding and quality control of glycoproteins in the endoplasmic reticulum with consequences for processing and trafficking (reviewed in [30,31]), targeting in the secretory pathway, namely, to the lysosome (via the mannose-6-phosphate receptor) [32], dynamics of plasma membrane proteins [33–35], cell–cell interactions, e.g., during metastases formation [36] or inflammation [36,37], among others.

In cancer, aberrant glycosylation, particularly of cell surface glycoconjugates, is a hallmark associated with the disease [36]. Altered glycosylation is the consequence of changes in glycosyltransferase expression and localization along the secretory pathway and also depends on the availability of sugar nucleotide donors in the Golgi lumen.

In the present work, the four potential N-glycosylation sites of baADAM10 were found to be occupied. By mutating each of those sites we observed that glycans from N278 were crucial for the intracellular processing of baADAM10, with the enzyme being accumulated as the precursor in the endoplasmic reticulum. Concerning N439, the N-glycans protected against proteolytic cleavage. N-Glycans from each mutant were required for full in vivo activity. On the other hand, a fraction of human endogenous ADAM10 from exosomes was found to contain more processed N-linked glycans than the cellular counterpart.

2. Materials and methods

2.1. DNA constructs

The plasmid pcDNA3-ADAM10wt [5] coding for wild-type bovine ADAM10 with the HA tag at the C-terminus was used as template for the construction of the N-glycosylation mutants: pBADAM10S269A, pBADAM10T280A, pBADAM10S441A and pBADAM10T553A. Site-directed mutagenesis was done using QuickChange cloning techniques (Stratagene, La Jolla, CA, USA), following the manufacturer’s instructions. The sense or antisense from the N-glycosylation site (N-X-T/S) were replaced by an alanine residue following a strategy previously used [31,38]. The PCR reactions were carried out with 50–115 ng pcDNA3-ADAM10wt as template, 2.5 mM dNTPs mix, 2.5 U/μl Phi Turbo DNA Polymerase and 10 μl of mutagenic primers. The primers used to obtain pBADAM10S269A, pBADAM10T280A, pBADAM10S441A and pBADAM10T553A were, respectively, as follows: 5′ GCA ATT CCT ATG TTC AAC CCC ACG ATG AG3′, 5′ CTA GTA AGA AAA TAT ACA AAT TCT GCT GGT CAG GAG CCG 3′, 5′ GAT CAA GCT GCA CAA GAA GCC ACA CTA CG3′, 5′ GAT CAA GCT GCA CAA GAA GCC ACA CTA CG3′. Standard molecular biology techniques were used for PCR product restriction and analysis. Mutations of the baADAM10 N-glycosylation sites were confirmed by automatic DNA sequencing analysis.

2.2. Cell culture and protein expression

Human ovarian cancer SKOV3 cells and human embryonic kidney HEK293 cells were grown at 37 °C, 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (Gibco), 1% penicillin/streptomycin solution (PS) (Gibco). SKOV3 and HEK293 were transfected with each of the plasmids indicated in 2.1 or pcDNA3-11 by the calcium phosphate technique using 5 μg of plasmid DNA and selection of stably transfected cells was performed using 2 and 1 μg/ml genetin sulphate (G-418) (Gibco), respectively. For transient expression, HEK293 cells were transfected with 2 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen), following manufacturer’s instructions.

2.3. Isolation of membrane vesicles

Confluent SKOV3 cells were cultivated for 24 h in serum-free medium. When indicated, cells were incubated with 10 μM TAPI-0 (Calbiochem) for 16 h. Supernatant was collected and centrifuged, at 4 °C, for 10 min, at 500 × g. For each picture, laser intensities and amplifications were measured in order to avoid pixel saturation. Each fluorophore used was excited independently and sequential detection was performed. Each picture consisted of a z-series of 20 images of 1024 × 1024 pixel resolution with a pinhole of 1.0 Airy unit. Colocalization analysis was performed using the open source Image J version 1.37 (http://rsb.info.nih.gov/ij/).

2.4. SDS-PAGE and Western blot analysis

Total proteins were precipitated with ethanol and solubilized in reducing SDS-PAGE sample buffer. Endogenous ADAM10 analysis was performed in non-reducing SDS-PAGE. For SDS-PAGE 7.5% acrylamide gels or precasted 4–12% acrylamide Bio–Tris gels (Invitrogen) were used. Western blot was performed using the following primary antibodies: mouse anti-ADAM10 monoclonal antibody MAB1427 (1:1000) (R&D), mouse anti-HA tag 6E2 monoclonal antibody (1:1000) (Cell Signalling), mouse anti-L1-11A (1:3), mouse anti-human CD9 (1:5000); mouse anti-annexin I monoclonal antibody (1:5000) (BD Biosciences), mouse anti-ubiquitin P4D1 monoclonal antibody (1:1000) (Santa Cruz Biotechnology). Secondary antibodies were: sheep anti-mouse IgG coupled to HRP (1:4000), donkey anti-rabbit IgG coupled to HRP (Amer sham) (1:3000). Antibody detection was accomplished with ECL Plus Western Blotting Detection Reagent (A mer sham).

2.5. Protein deglycosylation

For glycosidase hydrolysis cell lysates (approximately 1 × 106 cells) were incubated with endoglycosidase H (Endo H) (New England Biolabs) and peptide: N-glycosidase F (PNGase F) (New England Biolabs or Roche) according to supplier’s protocol. Briefly, cell lysates were denatured in glycoprotein denaturing buffer (0.5% SDS with or without 1% i-mercaptoethanol) at 100 °C for 10 min, and incubated with deglycosylation enzyme (PNGase F or Endo H) in the corresponding buffer. Hydrolysis was carried out at 37 °C for 3 h or overnight. The samples were further analyzed by SDS-PAGE and Western blot.

2.6. Immunoprecipitation

Exosomes were lysed with BOG buffer (20 mM Tris–HCl pH 8.0, 50 mM β- octylglucopyranoside (BOG), 1 mM phenylmethylsulphonylfluoride (PMSF), 10 mM sodium fluoride, 10 mM sodium orthovanadate, 1:500 protease inhibitors cocktail (Complete, Roche)) for 30 min at 4 °C with occasional vortexing. Proteins of lysates were incubated for 1 h at 4 °C with a rabbit anti-HA11 polyclonal antibody (Covance) previously coupled to protein A/G Plus agarose beads (Santa Cruz Biotechnologies). Immunoprecipitated proteins were washed 3 times with BOG buffer and were eluted at 100 °C for 5 min with sample buffer. Samples were analysed by SDS-PAGE and Western blot.

2.7. Confocal immunofluorescence microscopy

Cells were grown on glass coverslips until they reached 80% confluence, they were washed with PBS containing 0.5 mM MgCl2, fixed with 4% paraformaldehyde and permeabilized with methanol/acetone (1:1) solution for 10 min at –20 °C, then washed done with 1% BSA in PBS for 1 h and primary and secondary antibodies were incubated at room temperature for 2 and 1 h, respectively. Antibody solutions were prepared in blocking solution and washes were performed with PBS. Coverslips were mounted in Avarol and examined on a Leica Confocal (SP2+AOBS) microscope. Primary antibodies were: rabbit anti-HA11 (1:1000) (Covance), goat IgG anti-calnexin C-20 (1:500) (Santa Cruz), mouse IgG anti-ERClC3 (1:1000) (Alexis Biochemicals), mouse IgG anti-CM130 (1:200) (BD Biosciences), mouse IgG anti-EA1 (1:100) (BD Biosciences), mouse IgG anti-LAMP1 (1:100) (BD Biosciences). Secondary antibodies were: donkey anti-rabbit IgG AlexaFluor 594 (1:500); donkey anti-goat IgG AlexaFluor 488 (1:500) and donkey anti-mouse IgG AlexaFluor 488 (1:500) (Molecular Probes).

For each picture, laser intensities and amplifiers gains were adjusted in order to avoid pixel saturation. Each fluorophore used was excited independently and sequential detection was performed. Each picture consisted of a z-series of 20 images of 1024–1048 × 1024 pixel resolution with a pinhole of 1.0 Airy unit. Colocalization analysis was performed using the open source Image J version 1.37 (http://rsb.info.nih.gov/ij/).

2.8. Peptide cleavage assay

ADAM10-HA immuno-precipitates in 90 μl were incubated with 30 μl (final concentration 50 mM) fluorescent quenching peptide Abz-Leu-Ala-Gln-Ala-Val-Arg-FMK (Biosearch Technologies) for 20 h at room temperature for 2 and 1 h, respectively. Antibody solutions were prepared in blocking solution and washes were performed with PBS. Coverslips were mounted in Avarol and examined on a Leica Confocal (SP2+AOBS) microscope. Primary antibodies were: rabbit anti-HA11 (1:1000) (Covance), goat IgG anti-calnexin C-20 (1:500) (Santa Cruz), mouse IgG anti-ERClC3 (1:1000) (Alexis Biochemicals), mouse IgG anti-CM130 (1:200) (BD Biosciences), mouse IgG anti-EA1 (1:100) (BD Biosciences), mouse IgG anti-LAMP1 (1:100) (BD Biosciences). Secondary antibodies were: donkey anti-rabbit IgG AlexaFluor 594 (1:500); donkey anti-goat IgG AlexaFluor 488 (1:500) and donkey anti-mouse IgG AlexaFluor 488 (1:500) (Molecular Probes).

For each picture, laser intensities and amplifiers gains were adjusted in order to avoid pixel saturation. Each fluorophore used was excited independently and sequential detection was performed. Each picture consisted of a z-series of 20 images of 1024–1048 × 1024 pixel resolution with a pinhole of 1.0 Airy unit. Colocalization analysis was performed using the open source Image J version 1.37 (http://rsb.info.nih.gov/ij/).
3. Results

3.1. The N-glycosylation sites of hADAM10 are occupied

ADAM10 contains four potential N-glycosylation sites in the mature form of the protein (Fig. 1). Three of these sites are located in the metalloprotease domain: N267, N278 and N439, and in the disintegrin domain (N551). Endogenous human ADAM10 from SKOV3 ovarian carcinoma cells appeared as a major band with an apparent molecular mass of approximately 68 kDa (Fig. 2A). By enzymatic digestion with endoglycosidase H (Endo H), that removes N-linked glycans of the high-mannose and hybrid-type, we observed that the mature form of hADAM10 underwent a downward shift of approximately 5 kDa. On the other hand, hydrolysis with peptide N-glycosidase F (PNGase F), that removes both high-mannose, hybrid-and complex-type N-linked glycans, reduced the molecular mass by 9 kDa to the expected value for the hADAM10 core protein without the prodomain and with no post-translational modifications (59 kDa) (Fig. 2A). These results indicated that ADAM10 contained high-mannose as well as complex-type N-glycans.

To investigate the functional role played by the N-linked glycans, each potential N-glycosylation site was mutated. We used HA-tagged bovine ADAM10, which shares high homology with human ADAM10 protein sequence (97%), since several attempts to mutate the N-glycosylation sites of hADAM10 were not successful. Serine or threonine residues of each bADAM10 N-glycosylation site (N-X-T/S) were replaced by alanine, creating four bADAM10 N-glycosylation mutants: S269A, T280A, S441A, T553A (Fig. 1).

SKOV3 ovarian cancer cells were stably transfected with the pcDNA3 vector coding for bADAM10wt or S269A, T280A, S441A and T553A mutants. Stable cell lines were established and recombinant protein expression was analysed by SDS-PAGE and Western blot, using a mouse anti-HA tag antibody (Fig. 2B). All mutants were detected at lower molecular masses than the wild-type form, which indicated that all N-glycosylation sites were occupied. Most striking was the observation that mutant T280A accumulated as the precursor form at approximately 100 kDa, the mature form being detected at very low levels, which indicated that N-linked glycans from N278 were required for bADAM10 processing. Mutant S441A underwent proteolysis with an abundant cleavage product at 52 kDa. Therefore, the N-linked glycans from N439 protected bADAM10 from proteolysis. The highest shift was observed for T553A, which suggested that this site contained complex-type glycans, whereas the others contained high-mannose type glycans.

To investigate the type of glycans present in each N-glycosylation site, Endo H and PNGase F digestion of bADAM10wt and mutants was performed (Fig. 2C). Forms containing only high-mannose glycans exhibited similar shifts in SDS-PAGE after deglycosylation with each of the enzymes, whereas forms containing high-mannose and complex glycans exhibited higher shifts after PNGase F than after Endo H deglycosylation. In view of this, it was concluded that the mature form of bADAM10wt contained high-mannose and complex-type glycans similarly to endogenous hADAM10. The precursor form of bADAM10wt contained high-mannose and complex-type glycans, which indicated that it was transported beyond the ER to the Golgi, where it is known to be processed. On the other hand, precursor T280A contained only high-mannose type glycans suggesting that it probably accumulated in the ER. Mature S441A and T553A only contained high-mannose N-glycans, which suggested that either N439 or N551 could contain complex-type N-glycans.
3.2. Intracellular localization of bADAM10 N-glycosylation mutants

In agreement with previous reports [11,16], the analysis of the localization of endogenous hADAM10 by confocal microscopy of SKOV3 ovarian carcinoma cells showed predominant localization of the protein at the perinuclear region and at the cell surface where it appeared as punctuated dots evenly distributed at the plasma membrane (results not shown).

Since it has been reported that removal of N-glycosylation sites from proteins can affect protein transport and intracellular localization [43], bADAM10wt and mutants were colocalized with markers of the secretory pathway using confocal immunofluorescence microscopy. The markers used were: endoplasmic reticulum (ER)—calnexin, ER–Golgi intermediate compartment (ERGIC)—ERGIC53, Golgi—GM130, early endosomal antigen-1—EEA1, and late endosome/lysosome-associated membrane protein 1—LAMP1 (Fig. 3).

bADAM10wt colocalized with the EEA1 marker (Fig. 3D), which indicated that it was present in the endosomes. Low levels of protein were also found to colocalize with calnexin (Fig. 3A), and to a lower extent with GM130 (Fig. 3C) and ERGIC53 (Fig. 3B), probably corresponding to protein in traffic through the ER, Golgi and ERGIC. No colocalization with LAMP1 was observed (Fig. 3E). All mutants, with the exception of T280A, showed a similar staining profile (results not shown). T280A mutant showed higher colocalization with calnexin (Fig. 3F). These results showed that this mutant was retained in the ER and agreed with T280A precursor accumulation and its sensitivity to both Endo H and PNGase F.

Moreover, membrane protein biotinylation confirmed that most mature hADAM10 and bADAM10wt mature forms were localized at

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**Fig. 3.** Colocalization of bADAM10wt and T280A mutant, with markers of the secretory pathway by confocal immunofluorescence microscopy. SKOV3 cells stably transfected with bADAM10wt (red) were fixed in 1:1 methanol/acetone and double labelled with rabbit anti-HA tag antibody and one of the following markers (green): (A) goat anti-calnexin; (B) mouse anti-ERGIC53; (C) mouse anti-GM130; (D) mouse anti-EEA1 or (E) mouse anti-LAMP1, (F) T280A mutant (red) was double labelled with rabbit anti-HA tag antibody and goat anti-calnexin (green). Secondary antibodies were donkey anti-rabbit IgG AlexaFluor 594, donkey anti-mouse IgG AlexaFluor 488 and donkey anti-goat IgG AlexaFluor 488. Scale bars, 10 µm.
the plasma membrane. All N-glycosylation mutants showed comparable expression levels, at the cell surface, compared with bADAM10wt, with the exception of T280A that was almost undetectable (results not shown).

3.3. In vivo and in vitro activity of bADAM10wt and N-glycosylation mutants

The shedding activity of bADAM10wt and glycosylation mutants towards the L1 cell adhesion molecule was investigated. L1 is a 220 kDa type I membrane glycoprotein of the immunoglobulin family that is overexpressed in many human tumor cell lines, such as neuroblastomas, melanomas [44], colon [24] and ovarian carcinomas [19], as well as in neural, hematopoietic and certain epithelial cells. L1 cleavage by ADAM10 occurs in intracellular compartments, predominantly at the plasma membrane, and exosomes and results in two distinct fragments: a fragment of approximately 32 kDa that remains attached to the cell membrane and a soluble fragment of approximately 200 kDa that is released and can be detected in the supernatant.

HEK293 cells were selected for this study since they show low expression levels of endogenous hADAM10 and L1, and almost no L1 shedding (Fig. 4, Ctr). Therefore, HEK293 cells were stably transfected with pcDNA3-L1 (HEK-L1), and the selected stable cell lines were then transiently transfected with bADAM10wt and glycosylation mutants. Forty eight hours after transfection, L1 from the cell supernatants corresponding to a 24 h-production in serum depleted medium, was analysed by Western blot and a representative blot is shown (Fig. 4, upper panel). As controls L1 and bADAM10 from the corresponding cell extracts were also analysed by Western blot. Mature full-length L1 probably at the plasma membrane was detected (Fig. 4, middle panel). bADAM10wt and mutants were detected as mature form and precursor (Fig. 4, lower panel). Mutant T280A exhibited very low levels of mature form.

Transient overexpression of bADAM10wt in cells stably overexpressing L1 led to the detection of a higher level of shed L1 to the supernatant (Fig. 4). Mutation of N278 caused a decrease in the amount of shed L1 to almost undetectable levels. Transfection with the other mutants did not completely abolish the production of soluble L1, however, the levels appeared to decrease.

Since the in vivo activity is influenced by the catalytic activity of ADAM10 as well as its intracellular localization, we determined the proteolytic activity using a cell-free assay for mutants T280A and S269A that had the highest and the lowest alterations of in vivo activity, respectively. HEK293 cells were doubly transfected transiently with bADAM10wt or with S269A and T280A mutants solubilized in lysis buffer and subjected to immunoprecipitation with anti-HA antibody to isolate the proteins. After washing, the precipitated proteins were incubated with the fluorescent quenching peptide Abz-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Arg-Dap(dnp)-NH₂ containing the ADAM cleavage site of TNF-alpha. ADAM10wt and glycosylation mutant forms were efficiently purified from cell lysates of SKOV3 cells and were able to cleave the TNF-alpha peptide in a time dependent fashion. A reduced cleavage of the peptide to approximately 60% of the value obtained for bADAM10wt was found for S269A (Fig. 5). On the other hand, a reduction to 28% was observed for the T280A mutant, which could be due to an inactivating effect or to the lower level of mature form. Due to double transient transfection, high levels of precursor could be detected, which most likely did not contribute to cleavage since to this date no proteolytic activity has been described for the precursor form, and only minor amounts of mature ADAM10 were detected. Similar results were obtained when the proteins were expressed in SKOV3 cells (data not shown). In transient transfections ADAM17 also accumulates as the proform in the early secretory pathway, while little or no processed form can be detected (reviewed in [45]). These results corroborated the findings of the in vivo L1-cleavage assay.

3.4. Characterization of ADAM10 from exosomes

ADAM10 has been found in exosomes, where it promotes the shedding of several substrates, such as L1 and CD44 [27,29].

To investigate if glycosylation would play a role in glycoprotein sorting to exosomes, endogenous hADAM10 from cell extracts and exosomes, was analysed by SDS-PAGE and Western blot, and further digested with Endo H and PNGase F (Fig. 6A, B).

Exosomes were collected in the pellet after ultracentrifugation of cleared supernatants from SKOV3 cells followed by sucrose-density fractionation as previously described [29]. Since hADAM10 was only detected in fractions that are positive for the exosomal marker CD9 (Fig. 6A), the remaining studies were performed using exosomes obtained after ultracentrifugation without further fractionation.

Endogenous human ADAM10 from the exosomes appeared as a double band, the lighter one co-migrating with that from the cell extract (see Fig. 6A). The heavier band was resistant to digestion with Endo H but sensitive to PNGase F whereas the lighter band was sensitive to
both Endo H and PNGase F (Fig. 6B). The products of digestion with PNGase F also resulted in a double band. These results suggested that the heavier band contained complex-type glycans, whereas the lighter band contained both high-mannose and complex-type glycans similarly to hADAM10 from the cell extract. The presence of two bands after PNGase F digestion indicated an additional post-translational modification for the heavier band or proteolytic degradation.

On the other hand, a proteolytic product of hADAM10 was detected in exosomes but not in the cellular extract. Further experiments showed that such proteolysis was catalyzed by a metalloprotease since it was prevented when the cells were incubated with TAPI-0, a metalloprotease inhibitor [46] (Fig. 6C, upper panel). As control for the presence of exosomes the same blot was probed with anti-annexin I, an exosomal marker (Fig. 6C, lower panel).

Fig. 6. Western blot analysis of endogenous hADAM10 from SKOV3 cell extracts and exosomes. (A) Total protein from cell extracts (Ext; 1×10^6 cells/lane), exosomes (Exo; 1.5×10^7 cells), and exosomes fractionated by centrifugation in sucrose gradient, were analysed under non-reducing conditions using the mouse anti-ADAM10 antibody MAB1427. Exosomal marker CD9 was detected as a control using a different concentration gel. (B) Deglycosylation of hADAM10, from exosomes obtained after ultracentrifugation, with Endo H and PNGase F. (C) Exosomes were incubated for 16 h with the metalloprotease inhibitor TAPI-0. Annexin I, an exosomal marker was detected as an input control. Precursor (p) or mature (m) ADAM10 are indicated with closed arrowheads. Corresponding deglycosylated bands are shown with open arrowheads and * indicates ADAM10 cleavage product.

Fig. 7. Western blot analysis of bADAM10wt and N-glycosylation mutants from stably transfected SKOV3 cells. (A) Total protein from cell extracts (Ext; 1×10^6 cells/lane) and exosomes (Exo; 1.5×10^7 cells) were analysed using a mouse anti-HA tag antibody. Precursor (p) or mature (m) bADAM10 are indicated with closed arrowheads and * indicates bADAM10 cleavage product. (B) Protein ubiquitination of cell extract (100 μg total protein), corresponding exosomes (70 μg total protein) and immunoprecipitated exosomal bADAM10wt. Analysis was performed using mouse anti-Ubiquitin P4D1 (Ub) and mouse anti-HA tag antibodies (HA). Mature (m) bADAM10 is indicated with closed arrowhead and * indicates ubiquitinated proteins that interact with bADAM10.
For comparison, recombinant bADAM10wt and mutants over-expressed in SKOV3 cells were analysed (Fig. 7A). Precursor T280A was not found in the exosomes which is in agreement with the previous results indicating that it is retained in the ER. Mature forms of bADAM10wt, S269A, S441A and T553A were found in the exosomes, however, they were not detected as a double band, contrary to endogenous hADAM10, probably due to the low levels of bADAM10 expression. In addition, all they migrated at a slightly higher molecular mass than their cellular counterparts. The increased mass estimation could be due to post-translational modifications, such as N-glycosylation or ubiquitination. Immunoprecipitation of bADAM10wt followed by Western blot analysis using an anti-ubiquitin antibody allowed the identification of three bands (Fig. 7B). The heavier band comigrated with the mature form of bADAM10wt, therefore, suggesting that it was ubiquitinated. The other two bands possibly corresponded to other ubiquitinated proteins that may interact with bADAM10wt. As controls total cellular and exosomal proteins were also analysed (Fig. 7B). Exosomal proteins were heavily ubiquitinated in comparison with cellular proteins. High proteolytic activity from the exosomes hindered PNGase F digestion analysis.

Proteolytic cleavage also occurred for exosomal bADAM10wt and mutants similarly to endogenous hADAM10. This cleavage was particularly evident for mutant S441A. These results further support the importance of the N-linked glycans from N439 to protect the protein against proteolysis. Deglycosylation of exosomal bADAM10 remained inconclusive due to high level of protein degradation.

Detection of CD9, an exosomal marker, revealed no differences in the amount of secreted vesicles produced in all the four N-glycosylation mutants when compared with the bADAM10wt (results not shown).

3.5. Modelling of the 3D structure of bADAM10 metalloprotease domain

A model of bADAM10 metalloprotease domain based on its homology with ADAM17 (39% identity) was constructed to investigate the structural basis for the decreased in vitro activity of S269A, the absence of T280A processing and the susceptibility of S441A to proteolytic degradation, as well as significantly decreased in vivo activities of T280A and S441A (Fig. 8). Both N267 and N278 glycosylation sites are located in exposed zones before and after beta-strands, respectively, in loop regions as expected. The N267 residue has one glycosylation counterpart in ADAM17 (N264) in a spatially related region (not sequence related). The N278 has no counterpart on ADAM17. Surprisingly, the N439 glycosylation site is located inside an alpha-helix. In a related spatial zone in ADAM17, there is potential glycosylation site N452, which is, however, located at the beginning of the helix and not inside it as in the case of ADAM10.

4. Discussion

Glycosylation is an important post-translational modification that plays an important role in a number of physiological and biochemical properties of a glycoprotein including stability, folding, intracellular trafficking or activity [30]. However, N-linked glycosylation does not occur at every potential site and the role played by glycosylation in different proteins is highly variable and depends on the individual protein.

ADAM10 is a glycoprotein with four potential N-glycosylation sites: N267, N278, N439 and N551 but the functional role of ADAM10 N-glycosylation is unknown. To investigate if N-glycosylation has any role on ADAM10 processing, stability, activity and intracellular localization, the four potential sites were mutated and characterized individually.

Our results showed that all four bADAM10 N-glycosylation sites were occupied with high-mannose or complex-type glycans. Moreover, the mutation of a single N-glycosylation consensus sequence at N278 profoundly affected the efficient processing and trafficking of bADAM10. It is well known that the interaction of glycoproteins with the lectin chaperones from the ER, calnexin or calreticulin, is required for their correct folding, reviewed in [30,31]. The time that a certain glycoprotein remains in the calnexin/calreticulin folding cycle in the ER depends on the protein, and it can vary from minutes to hours. If glycoproteins fail to attain the correct fold, they can either be accumulated in the ER as aggregates, be translocated to the cytosolic face of the ER and be degraded by the proteasome, or they can be transported to the Golgi apparatus and divert to the lysosome where they are degraded [30,48]. Our results suggest that the N-linked glycans from N278 are important for the folding of the protein probably mediating the interaction with calnexin. In the absence of this folding step ADAM10 would fail to attain its final conformation and would be retained in the ER. As a consequence the protein would remain as an inactive precursor protein not only because of impaired folding but also because further proteolytic processing to the mature form, which takes place in the Golgi, would not occur due to compartmentalization constraints. In agreement, T280A mutant showed reduced levels of activity both in vivo and in vitro. Since only the ADAM10 mature form is active [3,4] the residual activity observed for this mutant is the result of the small amount of detected mature form. The 3D-model of ADAM10 metalloprotease domain, built using comparative modelling techniques based on the 3D-structure of ADAM17, showed that the N278 site is located in an exposed zone, and, therefore, has a good possibility of being occupied, which agrees with the experimental results. In addition, results obtained by other authors supported that ADAM10 N278 site is occupied in human platelets [49]. Therefore, the N-linked glycans probably mediate interaction with calnexin.

Mutation of the N439 glycosylation site resulted in increased susceptibility of bADAM10 to proteases. The 3D model of ADAM10 showed that N439 was found in an alpha-helix, which is a rare event but has a probability of occurring of 10.5% [50] that is a non-negligible value. Therefore, mutation of this site probably induced a conformation alteration that exposed a particular region susceptible to protease degradation. Furthermore, since N439, as well as N267, are not located near the active site of ADAM10 from the 3D model, it is probable that mutating them did not have a direct effect on substrate binding, and therefore would not abolish enzyme activity, but could cause alterations in protein conformation, explaining the decreased activities. Changes in conformation caused by mutation of glycosylation sites with consequences for activity have been described for other...
proteins, for example, the Kv3.1 voltage-gated K⁺ channel [34] or the GABA-transporter [33].

N551 is located at the end of the disintegrin domain, juxtaposed to the Cys-rich domain. These domains have been described as mediators of protein–protein interactions and regulators of ADAM protease specificity [26]. Furthermore, Janes et al. [51], showed that the cystein-rich domain is the major responsible for substrate recognition and binding of the EphA3/ephrin complex. Even if the analysis of the crystallographic structure of the bADAM10 disintegrin and cystein-rich domains (PDB: 2a07) showed that N551 is located away from the identified substrate recognition site, it is possible that mutating this site caused conformational changes of these domains leading to decreased substrate recognition specificities. This could explain the decreased in vivo activity of TS53A.

Signals responsible for the sorting of proteins into vesicles to be released as exosomes, within multivesicular bodies are still being elucidated. Mono-ubiquitination of plasma membrane proteins acts as a signal for endocytosis and targeting to the endosomes. Furthermore, the sequestration of transmembrane proteins in exosomes could require (mono-)ubiquitination and the ESCRT I, II and III protein complexes [reviewed in 52,53]. Accordingly, in the present work, a comparatively higher amount of ubiquitinated proteins was found in the exosomes, particularly, recombinant exosomal bADAM10 appeared to be ubiquitinated. Concerning endogenous exosomal hADAM10, a fraction of the protein was detected at a higher molecular mass, which could be explained by ubiquitination or another post-translational modification. In addition, this fraction exclusively presented complex-type N-glysans differently from total cellular hADAM10, which contained a mixture of complex- and high-mannose-type oligosaccharides. Several possibilities can be admitted to explain this finding: i) more complex-type glycoforms of hADAM10 are preferentially enriched during sorting and trafficking from the plasma membrane to the multivesicular bodies; ii) preferential enrichment of complex-type glycoforms occurs during glycoprotein sorting into the exosomes; iii) increased proteolysis of less processed glycoforms occurs in the exosomes, thus revealing more processed glycoforms. Further experiments are required to clarify this matter, however, the low amounts of protein makes it a difficult task.

An ADAM10 cleavage product was specifically found in exosomes and it was identified as being the result of metalloprotease cleavage. It is possible that the ADAM10 and the unidentified metalloprotease are specifically sorted into the exosomes and only get in contact there. In conclusion, the work described here provides evidence for the functional role of N-glycans from each of the N-glycosylation sites from ADAM10. They are required for the processing and activation of the enzyme, to protect the protein from proteolytic degradation, and, finally, they are required for full enzyme activity.

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