O-Fucosylation of Thrombospondin Type 1 Repeats in ADAMTS-like-1/Punctin-1 Regulates Secretion

IMPLICATIONS FOR THE ADAMTS SUPERFAMILY

The ADAMTS superfamily contains several metalloproteases (ADAMTS proteases) as well as ADAMTS-like molecules that lack proteolytic activity. Their common feature is the presence of one or more thrombospondin type-1 repeats (TSRs) within a characteristic modular organization. ADAMTS like-1/punctin-1 has four TSRs. Previously, O-fucosylation on Ser or Thr mediated by the endoplasmic reticulum-localized enzyme protein-O-fucosyltransferase 2 (POFUT2) was described for TSRs of thrombospondin-1, properdin, and F-spondin within the sequence Cys-Xaa1-Xaa2-(Ser/Thr)-Cys-Xaa-Xaa-Gly (where the fucosylated residue is underlined). On mass spectrometric analysis of tryptic peptides from recombinant secreted human punctin-1, the appropriate peptides from TSR2, TSR3, and TSR4 were found to bear either a fucose monosaccharide (TSR3, TSR4) or a fucose-glucose disaccharide (TSR2, TSR3, TSR4). Although mass spectral analysis did not unambiguously identify the relevant peptide from TSR1, metabolic labeling of cells expressing TSR1 and the cysteine-rich module led to incorporation of [3H]fucose into this construct. Mutation of the putative modified Ser/Thr residues in TSR2, TSR3, and TSR4 led to significantly decreased levels of secreted punctin-1. Similarly, expression of punctin-1 in Lec-13 cells that are deficient of the putative modified Ser/Thr residues in TSR2, TSR3, and TSR4 led to decreased levels of secreted protein, which were restored upon incubation of the cell lysate with a bacterial fucosidase. In addition, mutational analysis of Thr in TSR2, TSR3, and TSR4 led to decreased levels of secreted punctin-1. Taken together, the data define a critical role for N-glycosylation and O-fucosylation in the biosynthesis of punctin-1. From a broad perspective, these data suggest that O-fucosylation may be a widespread post-translational modification in members of the ADAMTS superfamily with possible regulatory consequences.

In humans, the ADAMTS superfamily contains 19 ADAMTS proteases and at least five ADAMTS-like proteins. ADAMTS proteases consist of a metalloprotease zymogen domain attached to a C-terminal ancillary domain. The modular construction of the ancillary domain, which includes one or more thrombospondin-type-1 repeats (TSRs), is a hallmark of the ADAMTS superfamily. TSRs were initially discovered in the matricellular protein thrombospondin-1 and were subsequently identified in several other molecules. ADAMTS-like proteins closely resemble the ancillary domains of ADAMTS proteases in their modular content (including the presence of TSRs) and primary sequence but lack the metalloprotease domain, and thus, do not have protease activity. ADAMTS-like proteins are not alternatively spliced variants arising from ADAMTS genes, but they are the products of distinct genes. They are present in chordates as well as non-chordates, implying conserved functions, although these are presently unknown. Currently, it is considered that some ADAMTS-like proteins may be extracellular matrix components, although a potential regulatory role vis-à-vis ADAMTS proteases is also supported.

ADAMTS-like 1 (ADAMTSL1, also known as punctin-1), the focus of this investigation, is one of a pair of closely related molecules (the other being ADAMTSL3/punctin-2), which are secreted glycoproteins having an affinity for extracellular matrix. Although its function is unknown, we have been interested in it as a structural and biochemical model for the ancillary domain of the ADAMTS proteases. Here, we specifically investigated the post-translational modification of punctin-1 by O-fucosylation of its TSRs. Punctin-1 is particularly well suited for such analysis since it contains four TSRs, yet it is relatively small (60 kDa) when compared with ADAMTS proteases bearing the same number of TSRs, which facilitates its purification as a recombinant protein. Like other members of the superfamily, its TSRs range from 50 to 60 amino acids in length and contain 6 conserved cysteines. Punctin-1 has four consensus sites for O-fucosylation and one site for N-linked oligosaccharide attachment, but it lacks predicted sites for mucin-type O-linked oligosaccharide attachment.

2 The abbreviations used are: TSR, type-1 repeats; POFUT1, protein-O-fucosyltransferase 1; POFUT2, protein-O-fucosyltransferase 2; CRD, cysteine-rich domain; CHO, Chinese hamster ovary; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MS/MS, tandem mass spectrometry; LC-MS/MS, laser chromatography-tandem mass spectrometry; CID, collision-induced dissociation; Hex, hexose; dHex, deoxyhexose; EGF, epidermal growth factor; GlcNAc-TI, N-acetylgalactosaminytransferase I.
O-Fucosylation, the covalent linkage of Fuc by O-linkage to Ser or Thr residues (9), has been extensively studied on the epidermal growth factor (EGF) repeats of the Notch signaling receptor, where it plays a critical role (10, 11). The enzyme responsible for O-fucosylation of EGF repeats in Notch is protein-O-fucosyltransferase 1 (POFUT1) (12). O-Fucose on EGF repeats can be extended by β1,3-N-acetylgalcosaminyltransferases of the Fringe family (13, 14). Mutations in either POFUT1 or Fringe enzymes demonstrated an essential role for O-fucosylation in Notch signaling (10, 11). O-Fucosylation was subsequently identified in the TSRs of thrombospondin-1, properdin, and F-spondin (15, 16). The addition of O-fucose to TSRs is mediated by a distinct enzyme: protein-O-fucosyltransferase 2 (POFUT2) (17, 18). In contrast to EGF repeats, O-fucose on TSRs cannot be modified by the Fringe family of enzymes (18). Instead, O-fucose on TSRs is extended by a novel β1,3-glucosyltransferase to generate the disaccharide glucose-β1,3-fucose (18–20). Elimination of O-fucose modification sites reduces secretion of soluble constructs of the Drosophila Notch extracellular domain (21, 22) or cell-surface expression of full-length mouse Notch1 (23). It is not yet known whether O-fucosylation of TSRs has a similar effect. Interestingly, both POFUT1 and POFUT2 are endoplasmic reticulum-localized enzymes that only modify properly folded EGF repeats or TSRs, respectively. Both enzymes have been proposed to function in protein folding and quality control (17, 18, 22, 24).

Analysis of fucosylated peptides from thrombospondin-1, F-spondin, and properdin suggested a consensus sequence within the TSRs of F-spondin and properdin. The addition of O-fucose on TSRs cannot be modified by the Fringe family of enzymes (17, 18). Instead, O-fucose on TSRs is extended by a novel β1,3-glucosyltransferase to generate the disaccharide glucose-β1,3-fucose (18–20). Elimination of O-fucose modification sites reduces secretion of soluble constructs of the Drosophila Notch extracellular domain (21, 22) or cell-surface expression of full-length mouse Notch1 (23). It is not yet known whether O-fucosylation of TSRs has a similar effect. Interestingly, both POFUT1 and POFUT2 are endoplasmic reticulum-localized enzymes that only modify properly folded EGF repeats or TSRs, respectively. Both enzymes have been proposed to function in protein folding and quality control (17, 18, 22, 24).

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cysteine-rich domain (CRD) was generated by PCR using the full-length punctin-1 construct as template, and the sequence was verified and cloned into pFLAG-CMV-5c (Sigma-Aldrich) for expression with a C-terminal FLAG tag. Wild-type, truncated, and mutant plasmids were expressed in the various cell lines described below.

Cell Culture and Transfections—HEK293F, COS-1, CHO, and Lec1 cells were obtained from ATCC (Manassas, VA) and were routinely maintained as described previously (4, 34). Lec1 cells are CHO derivatives that are deficient in the activity of the Golgi enzyme N-acetylglucosaminyltransferase I (GlcNAc-T1), which is required for the synthesis of hybrid and complex-type N-linked oligosaccharides (35, 36). Since the catalytic step mediated by this enzyme is a prerequisite for the addition of fucose to N-linked oligosaccharides, Lec1 cells incorporate fucose at sites of O-fucosylation but not N-glycosylation (37). Lec1 cells can, however, modify proteins with a simple oligomannose-type N-linked oligosaccharide (37). Lec13 cells (kindly provided by Dr. Pamela Stanley, Albert Einstein College of Medicine, New York, NY) are CHO derivatives that are deficient in the conversion of GDP-mannose to GDP-fucose because of a mutation in GDP-mannose 4,6-dehydratase (34).

For expression with a C-terminal FLAG tag. Wild-type, truncated, and mutant plasmids were expressed in the various cell lines described below.

Metabolic Labeling, Affinity Purification, and Fluorography—Metabolic labeling with [3H]fucose was done essentially as described by Nita-Lazar and Haltiwanger (40). HEK293F cells were cultured in 6-well plates containing 2 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum/well. They were transfected with full-length punctin-1 or TSR1-CRD plasmids when 60% confluent. 16 h following transfection, the medium was changed to 1 ml of serum-free Dulbecco’s modified Eagle’s medium containing 10 μCi/ml L-[6-3H]fucose (American Radiolabeled Chemicals, St. Louis, MO), and cells were incubated for a further 48 h and then washed three times with phosphate-buffered saline. 100 μl of cell culture lysate reagent (Promega Corp, Madison, WI) with Complete protease inhibitor mixture (Roche Diagnostics) was added to the wells. FLAG-tagged proteins were affinity-purified from the cell lysates using EZ-View Red anti-FLAG M2 affinity gel as per the manufacturer’s protocol (30 μl gel/100 μl lysate, Sigma-Aldrich). Untransfected metabolically labeled cells were processed similarly as a negative control. Bound proteins were eluted by boiling, electrophoresed by reducing SDS-PAGE, and detected either by Western blotting using an anti-FLAG polyclonal antibody (Sigma-Aldrich) or by fluorography. For fluorography, eluted proteins were electrophoresed by reducing SDS-PAGE. The gel was soaked in Enlightening™ rapid autoradiography enhancer (PerkinElmer Life Sciences) and exposed to film for 10 days.

Purification of Recombinant ADAMTSL1/Punctin-1—Of several stably transfected HEK-293F clones that were isolated, the clone with the highest production level was selected for protein purification. Cells were grown in serum-free medium (SFM II, Invitrogen) supplemented with 1-glutamine in triple-tier flasks (Nunc, Rochester, NY), and the medium was pooled for subsequent purification. Punctin-1 was purified from serum-free conditioned medium by affinity chromatography on nickel-Sepharose (ProBond resin, Invitrogen), utilizing the C-terminal His6 tag, essentially as described previously (4). Protein purity was assessed by silver staining under reducing and non-reducing conditions.

Analysis of Tryptic Peptides by Ion Trap Mass Spectrometry—Tryptic peptides were generated from 1 μg of reduced and alkylated punctin-1, and the resulting tryptic peptides were analyzed by LC-MS/MS using an Agilent XCT ion trap mass spectrometer as described (41). Briefly, the peptides were separated on a Zorbax 300SB-C8 column (3.5-μm beads, 150 × 0.3 mm, Agilent) with the following gradient: 0–5 min, 5% buffer B; 5–85 min, 5–35% buffer B; 85–105 min, 35–95% buffer B; 105–115 min, 95% buffer B (where buffer A = 0.1% formic acid, and buffer B = 95% acetonitrile in 0.1% formic acid). The effluent from the column was sprayed directly into the ion trap mass spectrometer under the conditions described in Ref. 41. The scanning range was 400–2200 m/z, and collision-induced dissociation (CID) fragmentation (MS/MS) was performed on the two most intense ions in each MS spectrum, with exclusion...
after two spectra. Peptides modified with O-fucose glycans were identified by searching the data set for CID spectra that exhibit the characteristic neutral loss of the fucose-glucose disaccharide (dHex-Hex). The loss of this species is recognized as a 308-Da loss from the peptide molecular ion, taking into account the charge state of the respective ions in the CID spectrum. For instance, with doubly charged peptide ions, this loss produces a doubly charged fragment ion with an m/z difference of 154. Similarly, with triply charged peptide ions, this loss produces a triply charged fragment ion with an m/z difference of 102.7. Sequential loss of the glucose (Hex) followed by the fucose (dHex) gives a characteristic fragmentation pattern, permitting facile identification of modified peptides, where the most abundant fragment ion in the CID spectrum is the unglycosylated peptide (see Fig. 2) (41). This feature allows one to deduce the molecular weight of the unmodified peptide from the CID spectrum and determine which peptide is modified based on the known amino acid sequence of the protein and the specificity of the trypsin protease. The masses of the unmodified peptides, determined in the CID spectra shown in Fig. 2 and in the supplemental Data (and summarized in Table 1), were matched to the masses of the predicted tryptic peptides from human punctin-1 that contain a predicted O-fucose modification site (Cys\(^{\text{Xaa}}\)-Xaa-Ser/Thr-Cys\(^{\text{Xaa}}\)-Xaa-Gly). Since each of these peptides has a unique mass (see Table 1), there were no ambiguities in assigning the spectra. The presence of b- and/or y-ions in the MS/MS data from fragmentation of the unglycosylated peptide confirmed the assignments. MS/MS/MS analysis of the unglycosylated peptides offered further confirmation of each peptide (data not shown). Due to the lability of the glycosidic linkage, assignment of the modified serine or threonine could not be reliably done. Thus, the assignment of the modified residue was based on the consensus sequence. Once glycopeptides were identified, additional searches were performed for unmodified peptides or for glycopeptides modified with fucose only.

RESULTS

Characterization of Recombinant Human Punctin-1 Expressed in Mammalian Cells—Silver staining demonstrated that recombinant punctin-1 obtained from stably transfected HEK293F cells was substantially pure (Fig. 1B). Under non-reducing conditions, the mobility of punctin-1 was significantly altered, compatible with the presence of disulfide bonds (Fig. 1B, left-hand panel), since it is a multidomain protein in which the cysteine-rich module and each of the TSRs contain 10 and 6 cysteines, respectively. Treatment with peptide-N-glycanase F led to more rapid in-gel migration, which confirmed glycosylation at a single N-linked consensus oligosaccharide attachment site (Asn\(^{\text{Xaa}}\)-Ser\(^{\text{Xaa}}\)-Ser\(^{\text{Xaa}}\)) in the spacer module (Fig. 1A and B, right-hand panel). Expression of wild-type punctin-1 in HEK293F, COS-1, and CHO-K1 cells demonstrated that it was normally modified at Asn\(^{\text{251}}\)-Ser\(^{\text{252}}\)-Ser\(^{\text{253}}\) in the spacer module (Fig. 1A). When normalized to the levels of co-transfected IgG, the levels of N251Q-substituted punctin-1 in conditioned medium were invariably reduced in comparison with wild-type punctin-1 in these three cell types (ranging from to 22 to 55%) (Fig. 1C). Thus, mutagenesis of the Asn\(^{251}\)-Ser\(^{252}\)-Ser\(^{253}\) consensus sequence suggested that the core N-linked oligosaccharide was essential for optimal levels of secreted protein, but its absence did not abrogate protein secretion. Similarly, punctin-1 was secreted from Lec1 cells at lower levels (Fig. 1C), showing that its secretion was compromised by the inability to form complex, hybrid-type N-linked oligosaccharides (37). The size of punctin-1 secreted from Lec1 cells was intermediate in size to wild-type and N251Q-substituted punctin-1.

O-Linked Fucose Is Present in the Thrombospondin Type 1 Repeats of Punctin-1—Tryptic peptides from punctin-1 were analyzed by LC-MS/MS to determine whether the O-fucose consensus sequences within the TSRs were modified with O-fucose. Fragmentation of peptides modified with O-fucose glycans resulted in loss of the sugars, and the major product ion is the unglycosylated peptide (41). Thus, O-fucose modified peptides can be identified by searching MS/MS data for ions losing masses corresponding to the O-fucose glycan from multiply charged forms of peptide ions. Fig. 2A shows analysis of a tryptic peptide from TSR2 of human punctin-1 using this method. The top panel shows an MS spectrum of the effluent from the reversed-phase capillary high pressure liquid chromatography column at 65.7 min. The two major molecular ions correspond to triply ([M+3H]\(^{3+}\)) and quadruply ([M+4H]\(^{4+}\)) charged forms of a predicted tryptic peptide from TSR2 modified with the fucose-glucose disaccharide. The ion at m/z 1219.6 was selected for CID fragmentation, and the bottom panel shows the resulting MS/MS spectrum (Fig. 2A, bottom). The two major molecular ions correspond to sequential losses of the glucose ([M+3H-Hex]\(^{3+}\), m/z 1165.3) and the fucose ([M+3H-Hex-dHex]\(^{3+}\), m/z 1116.9) from the parent ion ([M+3H]\(^{3+}\)). The molecular ion at m/z 1116.9 matches the predicted mass of the triply charged form of a tryptic peptide containing an O-fucose consensus sequence from TSR2 of human punctin-1 (Table 1). Additional confirmation of the assignment comes from the identification of several b- and/or y-ions from the unglycosylated peptide in the MS/MS spectrum (Fig. 2A, bottom). In a separate experiment, the m/z 1116.9 was selected for MS\(^3\) fragmentation, which provided further confirmation of the assignment (not shown). Peptides modified with the fucose-glucose disaccharide from TSR3 (supplemental Fig. S1) and TSR4 (supplemental Fig. S3) were also identified using this approach. Additional searches revealed that the peptides from TSR3 (supplemental Fig. S2) and TSR4 (supplemental Fig. S4) also exist in the monosaccharide form. These data are summarized in Table 1. A molecular ion potentially matching a predicted peptide from TSR1 bearing the fucose-glucose disaccharide was also identified (not shown). Interestingly, this ion was 162 Da larger than predicted, suggesting the presence of an additional hexose. The peptide contains the consensus sequence for the addition of C-mannose (Trp-Xaa-Xaa-Trp) (16), providing a potential explanation for the additional 162 Da. Nonetheless, fragmentation data for this ion were ambiguous and did not conclusively support this assignment. Thus, further work must be done to determine whether the consensus sequence within TSR1 is modified (see below). The data for peptides from TSR2, TSR3, and TSR4 suggests that they are all modified with the O-fucose disaccharide. None of the peptides
were found unmodified, suggesting that the glycosylation machinery is very efficient, even upon forced overexpression of punctin-1.

Since the relevant peptide from TSR1 was not clearly identified in the mass spectral analysis, we used an alternative approach to ask whether TSR1 could be modified by O-fucosylation of Punctin-1.
were generated by sequential site-directed mutagenesis of single TSR mutants. We initially analyzed these mutants by expression in HEK293F cells and subsequently compared expression of the wild-type punctin and triple mutant in COS-1 cells and CHO-K1 cells as well. Substitutions of Ser/Thr within the consensus sequence in individual TSRs modestly affected the levels of punctin-1 in medium from transfected HEK293F cells (e.g. the effect of the mutation in TSR2 is shown in Fig. 3, A and B), but this was not statistically significant on analysis of three independent transfections. Tandem substitutions within TSR2 and TSR3 (T312A+S391A) led to an ~40% decrease in the levels of punctin-1 in the medium (Fig. 3, A and B). However, mutation of all three modified TSRs (T312A+S391A+T451A) led to a dramatic decrease in the levels of secreted punctin-1 in HEK293F cells (Fig. 3, A and B), COS-1 cells (Fig. 3, C and D), and CHO-K1 cells (data not shown). Western blotting of cell lysates from these experiments showed enhanced levels of intracellular protein in cells transfected with the triple mutant punctin-1 (Fig. 3, A–D). The inverse relationship between the levels of triple mutant punctin-1 in cell lysate and its levels in the medium strongly suggested that the lack of O-fucosylation led to diminished secretion of punctin-1 and that unmodified punctin-1 was retained intracellularly. Some decrease in the total amount of punctin-1 is also apparent, consistent with a possible effect of O-fucosylation on protein stability.

An alternative explanation for decreased secretion of triple mutant punctin-1 is that it may result from compromised folding and stability of the mutant polypeptide. Thus, as an alternative and complementary approach, we further evaluated the role of O-fucosylation by expression of wild-type punctin-1 and N251Q-substituted punctin-1 in Lec13 cells. Expression in Lec1 cells as shown above suggested that the addition of fucose to N-linked oligosaccharide was also essential for secretion since Lec1 cells are unable modify N-glycans with fucose (37). Lec13 cells are therefore expected to solely report the effects of lack of O-fucosylation in the TSRs upon expression of N251Q-substituted punctin-1. In six independent experiments in which we expressed punctin-1 and N251Q-substituted punctin-1 in fucose-starved Lec13 cells, it was either undetectable on Western analysis of 20 μl of unconcentrated conditioned medium. Metabolic radiolabeling of HEK293F cells expressing the TSR1-CRD construct, which contains neither the fucosylation sites detected by mass spectrometry (i.e. in TSR2, TSR3, and TSR4) nor the N-linked oligosaccharide attachment site, demonstrated incorporation of [3H]fucose into the protein encoded by this construct (Fig. 2B). TSR1-CRD was poorly secreted into the medium (data not shown). Thus, affinity purification of the metabolically labeled construct was done using the cell lysate. As a positive control for the experiment, full-length punctin-1 showed incorporation of the radiolabel, whereas no signal was seen upon analysis of untransfected cells (Fig. 2B). Since the only consensus motif for O-fucosylation in the TSR1-CRD construct is present in TSR1 (Fig. 1A), this is strongly suggestive of O-fucosylation modification within this TSR.

O-Fucosylation of Punctin-1Facilitates Its Secretion—To determine whether O-fucosylation of punctin-1 was functionally significant, we substituted the predicted modified Ser/Thr residues in TSR2, TSR3, and TSR4 with Ala. Tandem substitutions of TSR2+TSR3 or TSR2+TSR3+TSR4 (triple mutant)
O-Fucosylation of Punctin-1

FIGURE 4. Reduced punctin secretion in Lec13 cells. A, punctin-1 or N251Q-substituted punctin-1 (NQ) was expressed in Lec13 cells cultured with (+) or without (−) 1 mM L-fucose, and Western blotting of conditioned medium was done using anti-Myc. This figure shows the highest expression (of six independent experiments) obtained in the absence of fucose. WT, wild type. B, bar graph showing the mean and standard deviation (six independent transfections) of the level of punctin-1 in conditioned medium. The constructs used and whether cells were grown in the presence or absence of L-fucose are indicated below the graph. The level of statistical significance is shown.

DISCUSSION

Previous work by Hofsteenge and colleagues (15, 16) had established a putative consensus sequence for O-fucosylation and identified the amino acid residue to which O-linkage occurred. A Ser or Thr residue present 3 residues downstream of the 1st Cys residue in the TSR. Hofsteenge and colleagues (15, 16) had suggested that the modification occurred within the trans-Golgi since it was the only secretory compartment known to contain the transporter for GDP-fucose. Subsequently, Luo et al. (17, 18) identified the TSR-specific O-fucosyltransferase, POFUT2, which was distinct from that previously implicated in fucosylation of EGF repeats, POFUT1. Surprisingly, like POFUT1, POFUT2 is localized in the endoplasmic reticulum (17, 19).

Here, we have extended these key observations to examine O-fucosylation in members of the largest potential group of substrates for POFUT2, and importantly, to determine the functional significance of this modification in punctin-1. We previously characterized recombinant punctin-1 from High-Five insect cells and showed that it was a glycoprotein with an affinity for extracellular matrix. Because insect cells glycosylate proteins differently from mammalian cells, we repurified punctin-1 from a stably transfected human cell line, HEK293F, that is commonly used for production of recombinant proteins. Mass spectral analysis demonstrated O-fucosylation of TSR2, TSR3, and TSR4. Interestingly, these TSRs did not have identically modified by O-fucosylation. Although mutation of a single O-fucosylation site in punctin-1 had modest effects, mutation of the sites in all three C-terminal TSRs dramatically diminished the levels of secreted protein, along with significant accumulation of punctin-1 intracellularly.

Further validation of an essential role for O-fucosylation was obtained using Lec13 cells. However, the use of these cells in our analysis first necessitated an evaluation of the role of N-linked glycosylation in punctin-1 since during protein biosynthesis, fucose is also commonly incorporated into complex or hybrid-type N-linked oligosaccharides, and more rarely, into mucin-type O-linked oligosaccharides (44). Most ADAMTS proteases and ADAMTS-like proteins (with the exception of ADAMTS4) contain at least one N-linked oligosaccharide. The N251Q substitution in punctin-1 prevents the co-translational attachment of the N-linked core oligosaccharide, which is probably essential for optimal folding and secretion of the protein. Subsequent to attachment of the core oligosaccharide in the endoplasmic reticulum, a further modification of the N-linked oligosaccharide by GlcNAc-TI in the Golgi apparatus is an essential prerequisite for the subsequent incorporation of fucose into complex or hybrid-type N-linked oligosaccharides (44). The level of punctin-1 in the medium of GlcNAc-TI-deficient Lec1 cells was decreased, further demonstrating that the N-linked oligosaccharide was essential for optimal secretion. Nevertheless, Lec13 cells, which are unable to incorporate fucose into any modification, would primarily report the effects of lack of incorporation of O-fucose into TSRs using N251Q-substituted punctin-1.

Lec13 cells showed a dramatic decrease in secreted wild-type punctin-1 as well as N251Q-substituted punctin-1. Taken together with the mutagenesis data, we conclude that the lack of fucosylation in each TSR has an additive effect on restricting secretion of punctin-1. The inability to detect unfucosylated peptides from TSR2, TSR3, and TSR4 in the mass spectral analysis argues that these TSRs are constitutively modified. One consideration raised by these observations is whether there might exist one or more cellular chaperone proteins that are specifically responsive to the levels of TSR O-fucosylation and that are charged with facilitating secretion of only correctly
folded, and thus, fully fucosylated proteins. Relevant to this are the observations that POFTU2 only adds fucose to properly folded TSRs (18) and is present in the endoplasmic reticulum (17, 19), a compartment known to be involved in protein folding and quality control. Interestingly, OFUT1 (POFTU1 ortholog in Drosophila melanogaster) has been reported to have chaperone activity (22), suggesting that O-fucosylation of both EGF repeats and TSRs may play an important role in protein folding and/or quality control. It will also be important to investigate the role of O-fucosylation during stresses that induce the unfolded protein response since O-fucosylation may be affected under these conditions.

From the previous studies of Hofsteenge and colleagues (16), it is evident that two TSRs containing the target Ser or Thr residue but with a positively charged residue at the Xaa2 position (i.e., immediately prior to the predicted modification site) were not O-fucosylated. Interestingly, TSR1 from every member of the ADAMTS superfamily has the constant sequence Cys1-Ser-Arg-(Ser/Thr)-Cys2, where Arg precedes the pre-residue but with a positively charged residue at the Xaa2 position.

REFERENCES

1. Apte, S. S. (2004) Int. J. Biochem. Cell Biol. 36, 981–985
2. Porter, S., Clark, I. M., Kevorkian, L., and Edwards, D. R. (2005) Biochem. J. 386, 15–27
3. Hofsteenge, J., Huwiler, K. G., Macek, B., Hess, D., Lawler, J., Mosher, D. F., and Peter-Katalinic, J. (2001) J. Biol. Chem. 276, 40338–40345
4. Hirohata, S., Wang, L. W., Miyagi, M., Yan, L., Seldin, M. F., Keene, D. R., Crabb, J. W., and Apte, S. S. (2002) J. Clin. Investig. 109, 1664–1666
5. Dong, B., Su, J., Leung, J., and Apte, S. S. (2003) J. Biol. Chem. 278, 15–27
6. Wang, Y., Shao, L., Shi, S., Harris, R. I., Spellman, M. W., Stanley, P., and Haltiwanger, R. S. (2001) J. Biol. Chem. 276, 40338–40345
7. Kramerov, A. A., Kusche-Gullberg, M., Kramer, J. M., Ackley, B. D., Sibata, Y., Ellis, D. M., Ross, H., Wisswal, B. H., Murphy, K., Hillman, M. C., Jr., Hollis, G. F., and Arner, E. C. (1999) Science 284, 1664–1666
8. Kozma, K., Keusch, J. J., Hegemann, B., Luther, K. B., Klein, D., Hess, D., Haltiwanger, R. S., and Hofsteenge, J. (2006) J. Biol. Chem. 281, 36742–36751
9. Haltiwanger, R. S. (1999) J. Biol. Chem. 274, 30158–30165
10. Kozma, K., Keusch, J. J., Hegemann, B., Luther, K. B., Klein, D., Hess, D., Haltiwanger, R. S., and Hofsteenge, J. (2006) J. Biol. Chem. 281, 36742–36751
11. Litwin, J. M., and Apte, S. S. (2003) J. Biol. Chem. 278, 15–27
12. Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S., and Vogt, T. F. (2000) Nature 406, 369–375
13. Bruckner, K., Perez, L., Clausen, H., and Cohen, S. (2000) Nature 406, 411–415
14. Kozma, K., Keusch, J. J., Hegemann, B., Luther, K. B., Klein, D., Hess, D., Haltiwanger, R. S., and Hofsteenge, J. (2006) Mol. Cell. Proteomics 5, 11–18
15. Hofsteenge, J., Huwiler, K. G., Macek, B., Hess, D., Lawler, J., Mosher, D. F., and Peter-Katalinic, J. (2001) J. Biol. Chem. 276, 6485–6498
16. Gonzalez de Peredo, A., Klein, D., Macek, B., Hess, D., Peter-Katalinic, J., and Hofsteenge, J. (2002) Mol. Cell. Proteomics 1, 11–18