THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 44, pp. 30004–30015, October 30, 2009


# Post-translational Modification of Thrombospondin Type-1 Repeats in ADAMTS-like 1/Punctin-1 by *C*-Mannosylation of Tryptophan*

Received for publication, June 26, 2009, and in revised form, August 3, 2009 Published, JBC Papers in Press, August 11, 2009, DOI 10.1074/jbc.M109.038059

**Lauren W. Wang[‡], Christina Leonhard-Melief[§], Robert S. Haltiwanger[§], and Suneel S. Apte[‡1]**

*From the [‡]Department of Biomedical Engineering, Cleveland Clinic, Cleveland, Ohio 44195 and the [§]Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, New York 11794*

**Protein *C*-mannosylation is the attachment of α-mannopyranose to tryptophan via a C-C linkage. This post-translational modification typically occurs within the sequence motif W*XX*W, which is frequently present in thrombospondin type-1 repeats (TSRs). TSRs are especially numerous in and a defining feature of the ADAMTS superfamily. We investigated the presence and functional significance of *C*-mannosylation of ADAMTS-like 1/punctin-1, which contains four TSRs (two with predicted *C*-mannosylation sites), using mass spectrometry, metabolic labeling, site-directed mutagenesis, and expression in *C*-mannosylation-defective Chinese hamster ovary cell variants. Analysis of tryptic fragments of recombinant human punctin-1 by mass spectrometry identified a peptide derived from TSR1 containing the $^{36}$WDAWGPWSECSRTC$^{49}$ sequence of interest modified with two mannose residues and a Glc-Fuc disaccharide (*O*-fucosylation). Tandem mass spectrometry (MS/MS) and MS/MS/MS analysis demonstrated the characteristic cross-ring cleavage of *C*-mannose and identified the modified residues as Trp$^{39}$ and Trp$^{42}$. *C*-Mannosylation of TSR1 of the related protease ADAMTS5 was also identified. Metabolic labeling of CHO-K1 cells or Lec35.1 cells demonstrated incorporation of D-[2,6-$^{3}$H]mannose in secreted punctin-1 from CHO-K1 cells but not Lec35.1 cells. Quantitation of punctin-1 secretion in Lec35.1 cells *versus* CHO-K1 cells suggested decreased secretion in Lec35.1 cells. Replacement of mannosylated Trp residues in TSR1 with either Ala or Phe affected punctin secretion efficiency. These data demonstrate that TSR1 from punctin-1 carries *C*-mannosylation in close proximity to *O*-linked fucose. Together, these modifications appear to provide a quality control mechanism for punctin-1 secretion.**

The ADAMTS (a disintegrin-like and metalloprotease domain with thrombospondin type-1 repeats) superfamily (1) consists of 19 secreted metalloproteases (ADAMTS proteases) and six ADAMTS-like proteins in humans. ADAMTS-like proteins closely resemble the ancillary domains of ADAMTS proteases and like them have a conserved modular organization containing one or more thrombospondin type-1 repeats (TSRs)[2] (2–5). TSRs are modules of ~50 amino acids having a characteristic six-cysteine signature. The prototypic ADAMTSL, ADAMTSL1, also referred to as punctin-1 because of its punctate distribution in the substratum of transfected cells, is a 525-residue glycoprotein containing four TSRs (4). A longer punctin-1 variant arising from alternative splicing, containing 13 TSRs and homologous to ADAMTSL3, is predicted by the human genome sequencing project (NM_001040272) but has not yet been physically cloned and expressed. The function of ADAMTSL1/punctin-1 is unknown. Recently, *ADAMTSL2* and *ADAMTSL4* mutations were identified in the genetic disorders geleophysic dysplasia (6) and recessive isolated ectopia lentis, respectively (2). In genome-wide analysis, the *ADAMTSL3* locus has been associated with variation in human height (7). Thus, in addition to known genetic disorders caused by ADAMTS mutations (8, 9), ADAMTSL family members are now also implicated in human disease. Among the ADAMTS proteases, ADAMTS5 and ADAMTS4 are strongly associated with cartilage destruction in arthritis (10–12).

Like most secreted proteins, the ADAMTS superfamily members undergo post-translational modification and are predicted to contain *N*-linked oligosaccharides. In addition, TSRs of ADAMTS superfamily members, by virtue of high sequence similarity to the corresponding motifs in thrombospondin 1 and properdin, are predicted to contain two uncommon types of glycosylation. Specifically, TSRs often contain the sequence motifs $W^{0}XXW^{+3}$ and $C^{1}X_{2-3}(S/T)C^{2}XXG$, which are consensus sites for protein *C*-mannosylation of the $W^{0}$ residue and *O*-fucosylation (of Ser/Thr) respectively, in close proximity to each other (13, 14). In recently published work, it was shown that ADAMTSL1 and ADAMTS13 are modified by *O*-fucosylation, the covalent attachment to Ser or Thr residues of fucose or a fucose-glucose disaccharide (15, 16). Punctin-1 contains consensus sequences for *O*-fucosylation in all four of its TSRs, but the presence of the glycans was previously only confirmed on TSR2, -3, and -4 (16). Addition of *O*-fucose is mediated by protein *O*-fucosyltransferase 2 (POFUT2), which is a distinct transferase from that which catalyzes addition of *O*-linked fucose to epidermal growth factor-like repeats (POFUT1) (17, 18). A β3-glucosyltransferase subsequently adds glucose to the

* This work was supported, in whole or in part, by National Institutes of Health Grants AR49930 and AR53890 (to S. S. A.) and CA12307101 (to R. S. H.).

[1] To whom correspondence should be addressed: Dept. of Biomedical Engineering, Cleveland Clinic, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-445-3278; Fax: 216-444-9198; E-mail: aptes@ccf.org.

[2] The abbreviations used are: TSR, thrombospondin repeat; TSR1, thrombospondin type-1 repeat; HPLC, high pressure liquid chromatography; Dol-P-Man, dolichyl-phosphate mannose; CHO, Chinese hamster ovary.

3′-OH of the fucose (19, 20). It was further demonstrated that *O*-fucosylation, which occurs after completion of TSR folding, was rate-limiting for secretion of punctin-1 and ADAMTS13 (15, 16). This role was inferred from the following two experimental observations. 1) Expression of wild-type punctin-1 and ADAMTS13 in Lec13 cells, which do not fucosylate proteins, led to their decreased secretion (15, 16). 2) Mutation of the modified Ser or Thr residues greatly reduced secretion of punctin-1 and ADAMTS13 (15, 16).

Protein *C*-mannosylation is the attachment of an α-mannopyranosyl residue to the indole C-2 of tryptophan via a C-C linkage (14, 21). Unlike *O*-fucosylation, it can utilize protein primary structure rather than tertiary structure as the determinant, *i.e.* mannose is added to unfolded polypeptides or unstructured synthetic peptides (22). *C*-Mannosylation uses dolichyl-phosphate mannose (Dol-P-Man) as the precursor and appears to be enzyme-catalyzed within the endoplasmic reticulum (23), but the responsible mannosyltransferase has not yet been identified. A variety of mammalian cell lines can perform this modification (24). Proteins known to be *C*-mannosylated include human RNase 2, interleukin 12, the mucins MUC5AC and MUC5B, and several proteins containing TSRs, such as thrombospondin-1, F-spondin, and components of complement (C6 and C7) and properdin (13, 21, 25–27).

Krieg *et al.* (22) proposed a model in which the *C*-mannosyltransferase bound directly to the $WXXW^{+3}$ motif, analogous to the Asn-*X*-(Thr/Ser) motif for *N*-glycosylation, and later analysis showed that both the Trp residues in the $W^{0}XXW^{+3}XXX$ motif and the sole Trp residue in a $(F/Y^{1})XXW^{+3}$ motif could be modified (13). Based on meta-analysis of the *C*-mannosylation literature, Julenius (28) used a neural network approach to develop a prediction algorithm for protein *C*-mannosylation, termed NetCGlyc. This analysis suggested that Cys was an acceptable substitute for Trp at the +3 position (*i.e.* permitting *C*-mannosylation of $W^{0}$ in a $W^{0}$SSC motif). Julenius (28) reported a clear preference for small and/or polar residues (Ser, Ala, Gly, and Thr) at the +1 position, whereas Phe and Leu were not allowed. The NetCGlyc algorithm provides a useful guide for prediction of *C*-mannosylation sites, especially in the ADAMTS superfamily, which has a large number of TSRs (Table 1). Nonetheless, this modification has not been experimentally identified nor functionally characterized in any ADAMTS superfamily member. In general, the functional significance of *C*-mannosylation is unclear, although a previous analysis of the MUC5AC and MUC5B Cys subdomains suggested it could have a role in regulation of protein secretion (27). Here we specifically inquired whether the short form of punctin-1, the prototypic ADAMTSL, is modified by *C*-mannosylation, analyzed the role of Trp residues in the punctin TSRs, and investigated its possible functional significance in punctin-1 biosynthesis. By demonstrating that TSR1 of ADAMTS5 is also *C*-mannosylated, we extended the analysis to identify this unusual modification in an ADAMTS protease.

## EXPERIMENTAL PROCEDURES

*Expression Plasmids, Site-directed Mutagenesis, and Cell Culture*—Mammalian expression plasmids for wild-type human punctin-1 or the *N*-glycosylation-defective punctin-1 mutant N251Q (punctin-NQ), each having a C-terminal tandem Myc and $His_6$ tag, were described previously (16). Site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA) was used to substitute Trp residues of interest within likely motifs ($Trp^{36}$, $Trp^{39}$, $Trp^{42}$, $Trp^{385}$, and $Trp^{445}$) with Ala or Phe. The primers used for mutagenesis are available on request. The CHO cell mutants Lec15.2 or Lec35.1, which lack Dol-P-Man synthase activity or the ability to utilize Dol-P-Man, respectively (23, 29), were kindly provided by Dr. Mark Lehrman, University of Texas Southwestern Medical Center. These mutants, and CHO cells, were routinely grown on tissue culture plastic in Ham's F-12 medium supplemented with 15 mM HEPES, pH 7.2, 2% fetal bovine serum, 8% calf serum, and antibiotics. Transient transfections of HEK293 cells, CHO-K1 cells (both from ATCC, Manassas, VA), Lec15.2, and Lec35.1 cells were done using FuGENE 6 (Roche Diagnostics) as per the manufacturer's directions.

*Metabolic Labeling, Autoradiography, and Immunoblotting*—CHO-K1 or Lec35.1 cells transiently transfected with punctin-NQ were cultured in serum-free, low glucose (0.5 mM) Dulbecco's modified Eagle's medium supplemented with 0.3 mM L-proline and 30 mCi/ml D-[2,6-$^{3}$H]mannose (23). After biosynthetic labeling for 18 h, the conditioned medium was collected, and punctin-NQ was affinity-purified using $Ni^{2+}$-agarose as described previously (4). 80% of the sample was used for reducing SDS-PAGE followed by gel autoradiography as described previously (16) The remaining 20% was used for reducing SDS-PAGE and Western blotting with anti-Myc antibody to ensure that punctin-1 was indeed affinity-isolated. For quantitative comparison of cellular protein content and secreted levels of punctin-NQ and Trp mutants, HEK293F or CHO cells were co-transfected with the appropriate punctin-1 plasmid and the plasmid HIgG-pRK5 for expression of the Fc portion of human IgG (16). Following Western blotting with anti-Myc polyclonal antibody (for punctin detection) and anti-IgG, the levels of punctin-1 and IgG were determined by densitometry. Punctin-1 levels normalized with respect to IgG were used for comparative analysis as described previously (16). Cellular levels of punctin-1 were similarly normalized to intracellular glyceraldehyde-3-phosphate dehydrogenase.

*Recombinant Protein and Mass Spectrometry*—Purification of His-tagged wild-type punctin-1 using $Ni^{2+}$-agarose was described previously (4). Recombinant ADAMTS5 produced in CHO cells and encompassing residues $Ser^{262}$–$Glu^{753}$ was a kind gift from Dr. Elisabeth Morris at Wyeth Pharmaceuticals. Purified proteins were subjected to in solution digest, adapted from the method of Stone and Williams (30). Briefly, ~1 μg of protein was precipitated with 4 volumes of acetone overnight at −20 °C. Air-dried proteins were suspended in 10 μl of 8 M urea, 10 mM tris(2-carboxyethyl)phosphine, and 0.4 M di-ammonium phosphate, pH 8.0, vortexed, and heated to 50 °C for 5 min. Samples were alkylated by adding 100 mM iodoacetamide, 50 mM Tris-HCl, pH 8.0, vortexed, and incubated in the dark for 30 min. The urea was diluted to 2 M, and samples were incubated overnight at 37 °C with 150 ng of trypsin. Digested samples were acidified (with 7 μl of 5% formic acid), and particulates were removed using a 0.22-μm spin filter.

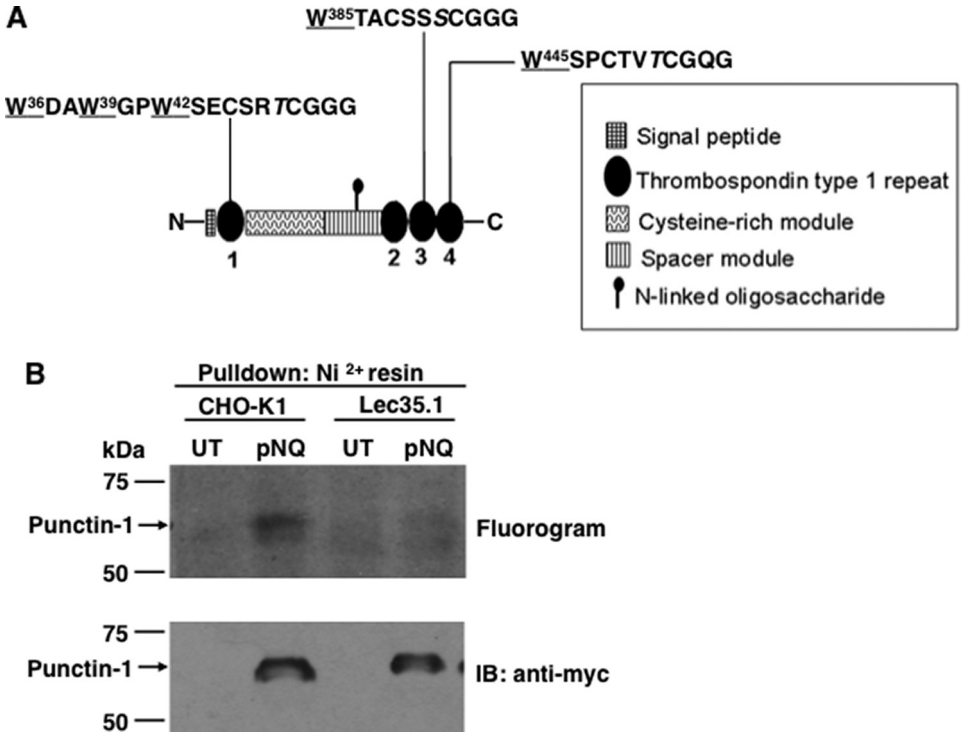



FIGURE 1. **Prediction of *C*-mannosylation and incorporation of radioactive mannose in punctin-1.** *A,* domain structure of punctin-1. The key to the various modules is shown in the *box at right*. The target residues within recognition motifs for *C*-mannosylation and *O*-fucosylation within each TSR are *underlined* and *italicized,* respectively. For these studies, the single *N*-glycosylation site was mutated (N251Q, this plasmid is named punctin-NQ or pNQ) to prevent incorporation of mannose into the *N*-linked oligosaccharide. *B,* CHO-K1 and Lec 35.1 cells were transfected with pNQ, metabolically labeled with [2,6-$^3$H]mannose, and punctin-NQ was affinity-isolated from the medium and analyzed as described under "Experimental Procedures." *UT* indicates untransfected cells (a negative control). The fluorogram, *top panel*, shows incorporation of [2,6-$^3$H]mannose in the punctin-NQ secreted by CHO-K1 cells. Lec35.1 cells failed to add mannose to punctin-1. The *bottom panel* is the corresponding Western blot using anti-Myc monoclonal antibody to illustrate that punctin-1 was efficiently pulled down from both samples. Note the reduced level of punctin in medium from Lec35.1 cells (compare with Fig. 3*A*). *IB*, immunoblot.

One- to 5-$\mu$l aliquots were analyzed by liquid chromatography/tandem mass spectrometry using an Agilent 6340 ion-trap mass spectrometer coupled to a nano-flow HPLC-CHIP system. Samples were separated using an Agilent (Zorbax 300SB) nano-CHIP C18 column at a flow rate of 450 nl/min with a 25-min linear gradient from 5 to 95% acetonitrile in 0.1% formic acid. Effluent from the CHIP was sprayed directly into the mass spectrometer. The drying gas (nitrogen) flow rate was 5 liters/min with a drying gas temperature of 325 °C. The capillary voltage was maintained at 1800 V. Full MS scans ($m/z$) 300-2200 were performed, and the three most abundant ions in each spectrum were selected for collision-induced dissociation (MS/MS).

*O-Fucosylation*—Peptides with *O*-fucose glycans were identified by neutral loss searches for the loss of glucose (hexose, 162 Da), fucose (deoxyhexose, 146 Da), or the sequential loss of glucose-fucose (308 Da) as described previously (16). Peptides identified as glycosylated by neutral loss were manually selected in subsequent runs for MS/MS/MS, in which the most abundant ion from MS/MS (usually the unglycosylated parent ion) is fragmented again, yielding high intensity b and y ions to match the predicted fragmentation of unglycosylated parent peptides. The mass of unglycosylated peptides was matched to predicted tryptic peptides which contain the consensus sequence $C^1X_{2-3}(S/T)C^2XXG$.

*C-Mannosylation*—Peptides that differed in mass by 162 Da (for each mannose) from predicted tryptic fragments that contained a W*XX*W (W*XX*W*XX*W) motif, with or without the consensus sequence for *O*-fucosylation, were subjected to MS/MS and/or MS/MS/MS. *C*-Mannosylation of Trp is highly stable, so modification was confirmed by both identification of b and y ions where Trp residues may have the additional 162 Da, but also by loss of 120 Da in MS/MS spectra, a characteristic cross-ring fragmentation product of aromatic *C*-glycosides, as well as loss of water molecules (31).

## RESULTS

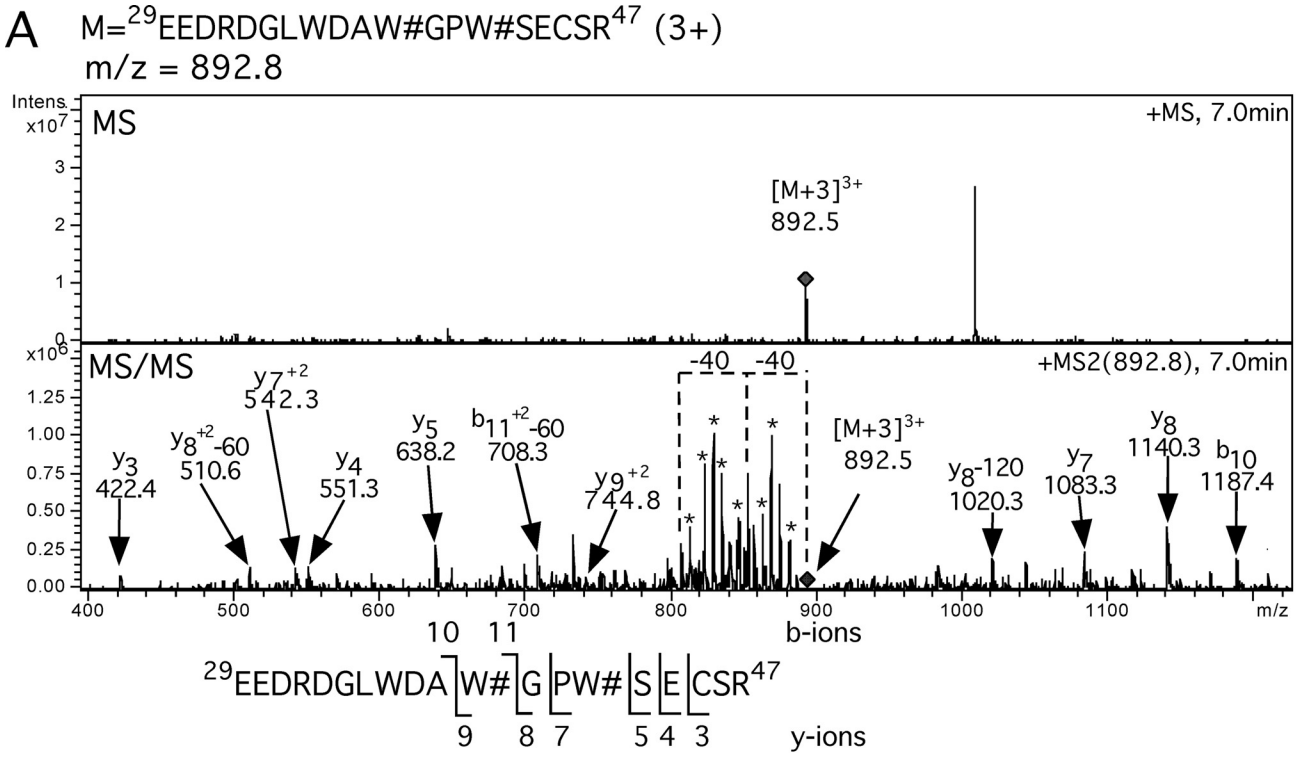
*Incorporation of Mannose during Biosynthesis of Punctin-1*—On examination of the sequences of the four TSRs present in the short form of punctin-1, we noticed that TSR1 contained the classic consensus sequence W*XX*W for *C*-mannosylation in tandem (Fig. 1*A*). TSR3 and TSR4 each contain a Trp residue with a Cys residue at the +3 position (Fig. 1*A*). TSR2 does not contain either the W*XX*W consensus sequence for *C*-mannosylation nor the W*XX*C variant motif present in TSR3 and TSR4. Analysis of human punctin-1 (GenBank$^{TM}$ accession number AF176313) at the NetCGLyc 1.0 server predicted that Trp$^{39}$ and Trp$^{42}$ in TSR1 and Trp$^{445}$ in TSR4 were likely to be modified (see Table 1). Surprisingly, in contrast to the predicted modification of $^{445}$WSPC, modification of the $^{385}$WTAC motif in TSR3 was not predicted. TSR1 contains tandem consensus *C*-mannosylation sequences, *i.e.* Trp$^{36}$-Asp-Trp$^{39}$-Gly-Pro-Trp$^{42}$-Ser-Glu-Cys, such that Trp$^{36}$, Trp$^{39}$, or Trp$^{42}$ could each serve as the target residue for *C*-mannosylation, and Trp$^{39}$ or Trp$^{42}$ could be the +3 residue for modification of Trp$^{36}$ and Trp$^{39}$, respectively.

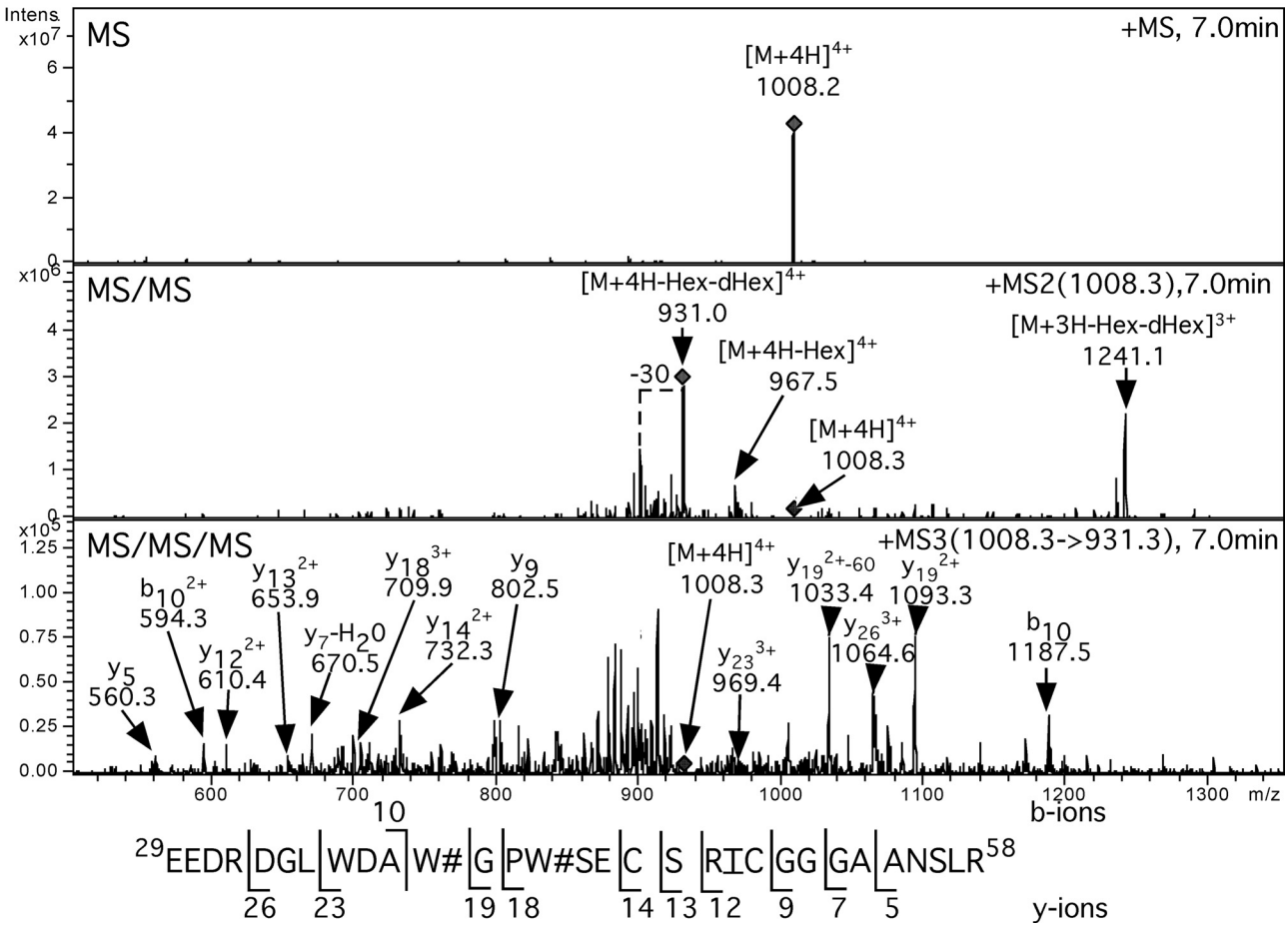
N251Q punctin-1 (punctin-NQ) was used for biosynthetic analysis because this mutation eliminates the possibility of incorporation of mannose into the single *N*-linked oligosaccharide present in punctin-1 (4) (Fig. 1*A*), and thus reports exclusively the incorporation of radiolabeled mannose at other sites. This *N*-glycan-deficient mutant is secreted at a lower level than wild-type punctin-1 but is nevertheless stable and is readily detected by Western blot in conditioned medium of transfected cells using anti-Myc monoclonal antibody (16). Affinity purification of punctin-NQ was done using the medium of metabolically labeled control CHO-K1 or mutant CHO cells that cannot utilize Dol-P-Man (Lec35.1) (29). This demonstrated incorporation of D-[2,6-$^3$H]mannose in punctin-NQ secreted

**TABLE 1**
**Predicted *C*-mannosylation sites[a] in the ADAMTS superfamily**

| | Predicted modified residues and location[b,c] |
|---|---|
| ADAMTS1 | W[546]GMW[549]GPW[552]GDCSRTC |
| ADAMTS2 | *W[448]SRCSQQEL*<br>W[564]GAW[567]SPFGSCSRTC<br>W[923]EPCSQTC<br>W[984]SQCSVTC |
| ADAMTS3 | *W[437]SRCSGQELKRYI*<br>W[974]SECSVTC |
| ADAMTS4 | *W[404]SPCSARFIT*<br>W[523]GPW[526]GPW[529]GDCSRTC |
| ADAMTS5 | *W[255]WRRRRRSIS*<br>*W[452]SKCTSATIT*<br>W[570]GSW[573]GSW[576]GQCSRSC<br>W[884]LACSRTC |
| ADAMTS6 | *W[444]SACSRDYITS*<br>WGPW[564]SLW[567]GECSRTC<br>WVTGDW[1027]GQCSAQC |
| ADAMTS7 | *W[428]SRCSRQYITR*<br>WSGW[544]SAW[547]SICSRSC<br>W[1045]SQCSVTCGEG<br>WVVGPW[1672]GQCSAPC |
| ADAMTS8 | WAPW[533]GPW[536]GECSRTC<br>WVLGDW[842]SECSSTC |
| ADAMTS9 | W[591]GSW[594]SPFGTCSRTC<br>WQAGPW[1116]VQCSVTC<br>WKALDW[1247]SSCSVTC<br>WRTGPW[1337]GACSSTC |
| ADAMTS10 | WGPW[583]TPW[586]GDCSRTC |
| ADAMTS12 | WGRW[548]SPW[551]SHCSRTC<br>WQYGHW[832]TECSVTC<br>WWAGEW[890]EACSATC<br>WIVGNW[1321]SECSTTC<br>*CGLGAYW[1334]KRVEC* |
| ADAMTS13 | W[356]SSW[359]GPRSPCSRSC<br>*W[511]DRCQVC* |
| ADAMTS14 | *W[97]PGRVG*<br>*W[438]SRCSKLEL*<br>W[555]SSW[558]TKFGSCSRSC<br>W[977]SQCSATC<br>WVAGEW[1042]GECSAQC |
| ADAMTS15 | *W[403]SACSAAIITDF*<br>WVAGSW[848]GPCSASC |
| ADAMTS16 | *W[471]SPCSRQYLHK*<br>W[589]SDW[592]SSW[595]SPCSRTC<br>WSVGNW[936]SACSRTC<br>W[995]AECSHTC |
| ADAMTS17 | WSPW[549]GAW[552]SMCSRTC<br>WVAGPW[870]SPCSATC |
| | WKTGDW[981]STCSSTC |
| ADAMTS18 | *W[473]SSCSRQYLKKF*<br>W[591]SAW[593]SKW[597]SECSRTC<br>*W[803]SIDWPGEFPF*<br>WSLGPW[999]SQCSKTC |
| ADAMTS19 | W[639]SLW[642]SPCSRTC<br>WMMTEW[985]TPCSRTC<br>WRMGDW[1096]SKCSITC |
| ADAMTS20 | *W[443]SNCSRKYVTE*<br>W[559]GPW[562]EPYSSCSRTC<br>WAASEW[1031]SECLVTC<br>WQVGPW[1086]GPCTTTC |
| ADAMTSL1 long form | WDAW[39]GPW[42]SECSRTC<br>WLAQEW[445]SPCTVTC<br>*W[531]SACTVTC*<br>WEIGKW[675]SPCSLTC<br>WAFSSW[1615]GQCNGPC |
| ADAMTSL2 | WETSSW[740]SECSRTC<br>WYTSPW[971]SECTKTC |
| ADAMTSL3 | WDAW[81]GDW[84]SDCSRTC<br>*W[438]SRCSGQELKR*<br>WIAMEW[486]SQCTVTC<br>W[572]SACSTTC<br>WHVGSW[711]GPCSATC<br>W[827]SKCSVSC<br>WKTGPW[975]SECSVTC<br>WEPGNW[1432]SHCSATC<br>WFTSVW[1491]SQCSVSC |
| ADAMTSL4 | WGPW[50]VQW[53]ASCSQPC<br>WEVGSPW[813]SQCSVRC |
| ADAMTSL5 | WTPW[41]VSWTRCSSSC |
| Papilin | WGPW[32]SQW[35]SPCSRTC<br>WSHGSW[286]SDCSAEC |

[a] The full-length human reference ADAMTS sequences from GenBank™ were analyzed at the NetCGly 1.0 server for prediction of *C*-mannosylation sites. For prediction of *O*-fucosylation sites in the same peptide, the consensus sequence $C^1X_{2-3}(S/T)C^2$ *XXG* was utilized.
[b] The sequence context in which the predicted modified Trp residue occurs is provided, and the residue with predicted modification is numbered. Ser/Thr residues predicted to be *O*-fucosylated based on the consensus sequence *CXX*(S/T)C are underlined.
[c] Sequences containing predicted *C*-mannosylation sites that are not within TSRs are shown in italics.


A
M=29EEDRDGLWDAW#GPW#SECSR47 (3+)
m/z = 892.8
MS
+MS, 7.0min
[M+3]3+
892.5
MS/MS
+MS2(892.8), 7.0min
-40
-40
y7+2
542.3
y8+2-60
510.6
y3
422.4
y4
551.3
y5
638.2
b11+2-60
708.3
y9+2
744.8
[M+3]3+
892.5
y8-120
1020.3
y7
1083.3
y8
1140.3
b10
1187.4
b-ions
29EEDRDGLWDAW#GPW#SECSR47
y-ions


B
M=29EEDRDGLWDAW#GPW#SECSRICGGGAANSLR58+dHex+Hex (4+)
m/z = 1008.3
MS
+MS, 7.0min
[M+4H]4+
1008.2
MS/MS
+MS2(1008.3),7.0min
[M+4H-Hex-dHex]4+
931.0
[M+4H-Hex]4+
967.5
[M+4H]4+
1008.3
[M+3H-Hex-dHex]3+
1241.1
-30
MS/MS/MS
+MS3(1008.3->931.3), 7.0min
b10 2+
594.3
y5
560.3
y12 2+
610.4
y13 2+
653.9
y7-H2O
670.5
y18 3+
709.9
y14 2+
732.3
y9
802.5
[M+4H]4+
1008.3
y23 3+
969.4
y19 2+-60
1033.4
y19 2+
1093.3
y26 3+
1064.6
b10
1187.5
b-ions
29EEDRDGLWDAW#GPW#SECSRICGGGAANSLR58
y-ions

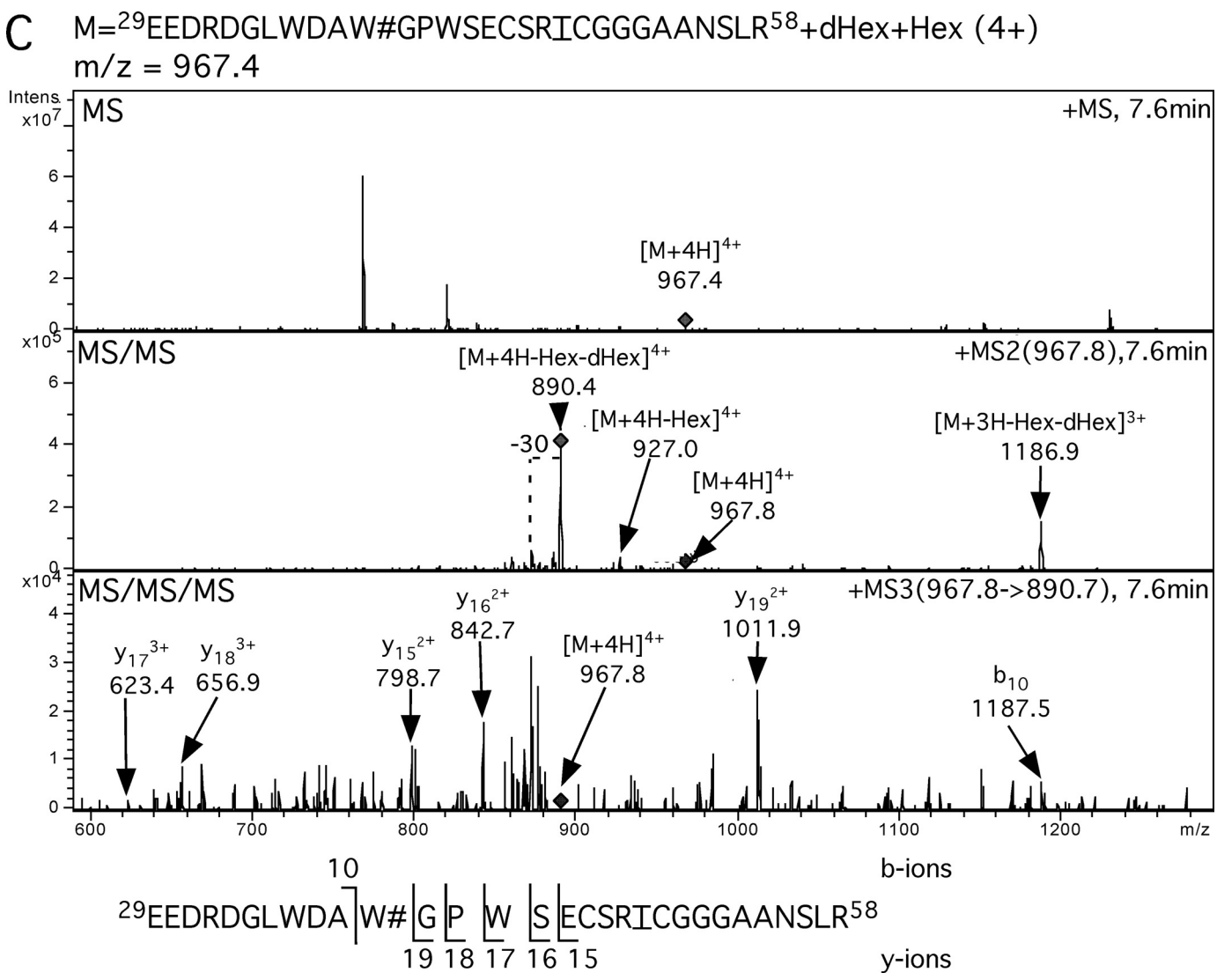



FIGURE 2—*continued*

from CHO-K1 cells but not from Lec35.1 cells, despite successful isolation of punctin-NQ from the medium of both cell types (Fig. 1*B*). These data strongly suggested that mannose is incorporated into punctin-1 at one or more sites independent of *N*-glycosylation.

*Identification of the Mannose Attachment Sites and Linkages on Punctin-1 and ADAMTS5 Using MS*—For analysis by mass spectrometry, punctin-1 was purified by nickel-Sepharose chromatography, digested with trypsin, and subjected to liquid chromatography/tandem mass spectrometry. Analysis of tryptic peptides from recombinant human punctin-1 by MS identified an ion with $m/z = 892.5$ (Fig. 2*A*, *MS*). This ion was consistent with the mass of the triply charged form of the peptide $^{29}$EEDRDGLWDAWGPWSECSR$^{47}$ derived from TSR1 and modified by two mannose residues. MS$^2$ fragmentation of this peptide showed characteristic cross-ring cleavage of *C*-man-

FIGURE 2. **TSR1 of punctin-1 is modified with *C*-mannose and a fucose-glucose disaccharide.** *A, top panel,* MS spectrum at 7 min of an HPLC run. The ion at *m/z* 892.5 corresponds to the triply charged form of the glycopeptide defined as *M*. *Bottom panel,* MS/MS spectrum of *m/z* 892.5 from the *top panel*. Cross-ring cleavage product from *C*-mannosylation is indicated by −40 (loss of 120 Da from triply charged ion). Two such losses are observed, consistent with the presence of two *C*-mannosylated Trp residues. Loss of water molecules (also characteristic of *C*-mannose fragmentation (31) is indicated with an *asterisk*. Fragment ions resulting from cleavage of peptide bonds (b and y ions) are indicated, confirming the identity of the peptide and the location of *C*-mannoses on both Trp[39] and Trp[42]. *C*-Mannosylated Trp is indicated by *W#*. *B, top panel,* MS spectrum at 7 min of an HPLC run. The major ion, *m/z* 1008.2, corresponds to the quadruply charged form of the glycopeptide defined as *M*. *Middle panel,* MS/MS spectrum of *m/z* 1008.2 from the *top panel*. Major ions representing the sequential loss of a hexose (*M* + *4H-Hex*) and deoxyhexose (*M* + *4H-Hex-dHex*) from the parent ion (*M* + *4H*) are indicated. Cross-ring cleavage product from *C*-mannosylation is indicated by −30 (loss of 120 Da from quadruply charged ion). *Bottom panel,* MS/MS/MS of the peptide without the hexose and deoxyhexose (*M* + *4H-Hex-dHex*) provides b and y ions verifying the identity of the peptide and presence of *C*-mannoses on Trp[39] and Trp[42]. *C*-Mannosylated Trp is indicated by *W#* and *O*-fucosylation site by *underlining*. *C,* TSR1 of punctin-1 can be modified with one *C*-mannose and an *O*-fucose disaccharide. *Top panel,* MS spectrum at 7.6 min of an HPLC run. The ion *m/z* 967.4 corresponds to the quadruply charged form of the glycopeptide defined as *M*. *Middle panel,* MS/MS spectrum of *m/z* 967.4 from the *top panel*. Major ions representing the sequential loss of a hexose (*M* + *4H-Hex*) and deoxyhexose (*M* + *4H-Hex-dHex*) from the parent ion (*M* + *4H*) are indicated. Cross-ring cleavage product from *C*-mannosylation is indicated by −30 (loss of 120 Da from quadruply charged ion). *Bottom panel,* MS/MS/MS of the peptide without the hexose and deoxyhexose (*M* + *4H-Hex-dHex*) provides b and y ions verifying the identity of the peptide and the presence of *C*-mannose on Trp[39]. *C*-Mannosylated Trp is indicated by *W#*, and *O*-fucosylation site by *underlining*.

**TABLE 2**

***C*-Mannosylated peptides from ADAMTSL1 and ADAMTS5**

# indicates mapped sites of *C*-mannosylation. Underlined S or T indicates sites of *O*-fucosylation. NA means not applicable.

| Protein | Glycopeptides identified | $[M + H]^+$ of parent | $[M + H]^+$ of product | Predicted $[M + H]^+$ | Parent-product |
|---|---|---|---|---|---|
| ADAMTSL1 | TSR1, $^{29}$EEDRDGLWDAW#GPW#SECSR$^{47}$ + Man + Man | 2676.4 | NA | 2767.59 | NA |
| | TSR1, $^{29}$EEDRDGLWDAW#GPW#SECSRTCGGGAANSLR$^{58}$ + Fuc-Glc + Man + Man | 4030.2 | 3721.0 | 3721.6 | 309.2 |
| | TSR1, $^{29}$EEDRDGLWDAW#GPWSECSRTCGGGAANSLR$^{58}$ + Fuc-Glc + Man | 3868.2 | 3558.6 | 3559.6 | 309.6 |
| ADAMTS5 | TSR1, $^{300}$YYSTSSHGNWGSW#GSW#GQCSRSCGGGVQFAYR$^{331}$ + Fuc-Glc + Man + Man | 4268.52 | 3958.2 | 3959.2 | 310.3 |

nose, resulting in sequential losses of 120 Da (40 Da for a triply charged peptide), supporting the presence of two *C*-mannose residues on this peptide (Fig. 2*A*, *MS*/*MS*). Multiple losses of water molecules (indicated by *) are also consistent with the presence of *C*-mannose (31). In addition, a series of y ions clearly reveals the presence of *C*-mannose on Trp$^{39}$ and Trp$^{42}$. In contrast, the b10 ion demonstrates Trp$^{36}$ is unmodified. These data support *C*-mannosylation of Trp$^{39}$ and Trp$^{42}$ but not Trp$^{36}$.

Another peptide containing these *C*-mannosylated Trp residues resulting from incomplete trypsin digestion was also detected (Fig. 2*B*, *MS*). This peptide ($m/z$ = 1008.2) also contains the predicted *O*-fucosylation site in TSR1. MS/MS fragmentation of this peptide showed sequential loss of glucose ($m/z$ 967.5) and fucose ($m/z$ 931.0) (Fig. 2*B*, *MS*$^2$), confirming the presence of the glucose-fucose disaccharide. A major ion characteristic of the cross-ring cleavage of a *C*-mannose on Trp was also observed (*−30, MS*/*MS,* Fig. 2*B*). MS$^3$ fragmentation of the $m/z$ 931.0 ion provided sufficient sequence information to confirm the *C*-mannose modifications on Trp$^{39}$ and Trp$^{42}$, as shown above (*MS*/*MS*/*MS,* Fig. 2*B*), although the fragmentation pattern is more complicated because of the larger size of the parent ion. The same peptide modified with a single mannose on Trp$^{39}$ and the glucose-fucose disaccharide was also identified (Table 2 and Fig. 2*C*), but no forms of this peptide lacking either mannose or *O*-fucose were found, suggesting that both modifications on this peptide occur at high levels of stoichiometry.

*Identification of C-Mannosylation and O-Fucosylation in ADAMTS5*—Analysis of the entire ADAMTS superfamily using the NetCGlyc 1.0 server predicted that each ADAMTS protease and each ADAMTS-like protein was likely to contain at least one modified Trp (Table 1). The majority of predicted sites were in TSR1 of the respective family members (Table 1). Accordingly, to extend the analysis of punctin-1 to additional members, we undertook MS analysis of recombinant ADAMTS5 as for punctin-1. We specifically asked whether like punctin-1, ADAMTS5 also contained *C*-mannosylation and *O*-fucosylation in TSR1. These experiments identified a peptide from TSR1 of ADAMTS5 modified with two *C*-mannoses and the glucose-fucose disaccharide (Table 2 and Fig. 3). Although the primary structure of ADAMTS5 predicts two TSRs (Table 1), recombinant ADAMTS5 frequently undergoes C-terminal processing that results in loss of TSR2, which was not present in the recombinant preparation used for this analysis.

*Effect of Lack of C-Mannosylation on Punctin-1 Secretion*—We investigated the consequence of inhibiting *C*-mannosylation of punctin-NQ using CHO-K1 variants, Lec15.2 and Lec35.1, and compared the levels of protein in the conditioned medium to that in parental CHO cells. Quantitation of secreted punctin-NQ was done in relation to the level of secreted co-transfected IgG. The punctin-NQ levels so normalized showed a statistically significant reduction of levels in medium from Lec35.1 cells but not in medium from Lec15.2 cells (Fig. 4*A*). Quantitative comparison also suggested accumulation of punctin-NQ intracellularly in Lec15.2 and Lec35.1 cells, but this was statistically significant only in Lec35.1 cells (Fig. 4*B*). Because we had previously shown that *O*-fucosylation led to decreased secretion, and because the *O*-fucosylation consensus sequences were intact in the expressed punctin-NQ, we asked whether supplementation with exogenous L-fucose (to ensure no deficiencies in GDP-fucose levels exist in these cells) would modify the secretion defect in Lec15.2 and Lec35.1 cells. However, the relative levels of punctin-NQ in the medium of CHO, Lec15.2, and Lec35.1 cells were unaffected by L-fucose supplementation (Fig. 4*C*).

*Site-directed Mutagenesis Suggests a Possible Dual Role for Trp Residues in Punctin-1*—In a previously reported analysis of *O*-fucosylation of punctin-1, mutagenesis of the modified Ser or Thr residues demonstrated that *O*-fucosylation was essential for secretion (16). A similar approach for analysis of *C*-mannosylation required consideration of the three-dimensional structure of TSR2 and TSR3 from thrombospondin-1, which had suggested that Trp residues at the N terminus of TSRs have a structural role through stacking with arginine residues present in the anti-parallel $\beta$-strand (32). The mutagenesis strategy used was therefore designed to minimize the potential structural consequences of mutating the Trp residues in the TSRs. In initial analysis, we replaced Trp$^{36}$, Trp$^{39}$, Trp$^{42}$, Trp$^{385}$, and Trp$^{445}$ in punctin-NQ individually with Ala. Each is the target Trp residue within a possible (Trp$^{36}$, Trp$^{385}$, and Trp$^{445}$) or experimentally determined (Trp$^{39}$ and Trp$^{42}$) *C*-mannosylation consensus sequence. In our previous analysis of punctin-1 *O*-fucosylation, we demonstrated that the peptides containing Trp$^{385}$ and Trp$^{445}$ are *O*-fucosylated (16), but we did not obtain any experimental evidence supporting *C*-mannosylation of these Trp residues.

Punctin-NQ with and without the specific Trp mutations was expressed in two cell lines, CHO and HEK293, to determine the consequence of the mutation for protein secretion and to elucidate the possible influence of cell- and species-specific effects. In HEK293F cells, W36A punctin-NQ showed a small but statistically significant reduction of secretion (Fig. 5, *A* and *B*), whereas W39A punctin-NQ and W42A punctin-NQ were not detectable in the medium, and therefore were not formally

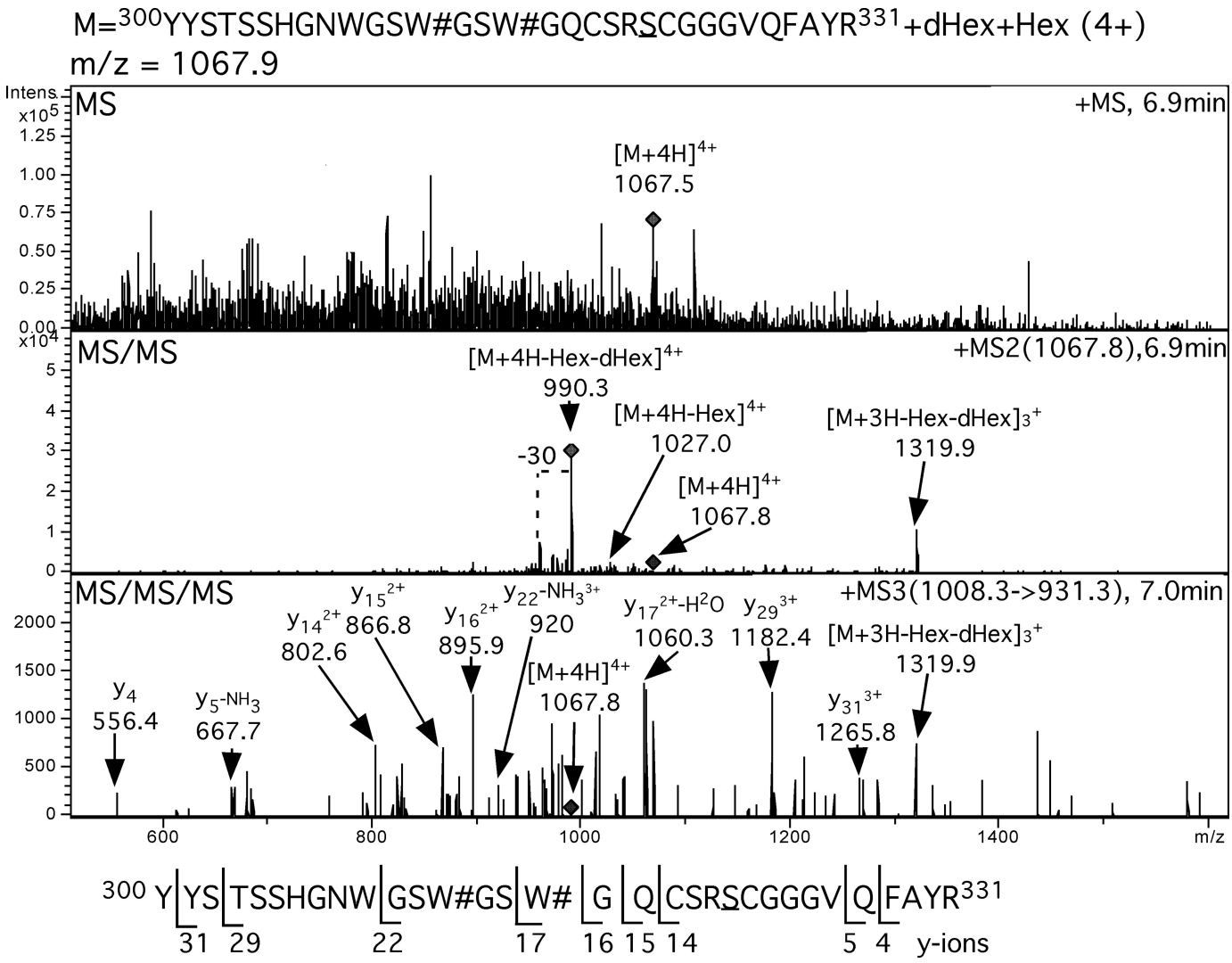



FIGURE 3. **TSR1 of ADAMTS5 is modified with two *C*-mannoses and an *O*-fucose disaccharide.** *Top panel,* MS spectrum at 6.9 min of an HPLC run. The ion *m/z* 1067.5 corresponds to the quadruply charged form of the glycopeptide defined as *M*. *Middle panel,* $MS^2$ spectrum of *m/z* 1067.5 from the *top panel*. Major ions representing the sequential loss of a hexose (*M* + *4H-Hex*) and deoxyhexose (*M* + *4H-Hex-dHex*) from the parent ion (*M* + *4H*) are indicated. Cross-ring cleavage product from *C*-mannosylation is indicated by −30 (loss of 120 Da from quadruply charged ion). *Bottom panel,* $MS^3$ of the peptide without the hexose and deoxyhexose (*M* + *4H-Hex-dHex*) provides b and y ions verifying the identity of the peptide and the presence of *C*-mannoses on $Trp^{312}$ and $Trp^{315}$. *C*-Mannosylated Trp is indicated by *W#* and *O*-fucosylation site by *underlining*.

quantitated (Fig. 5*A*). The levels of both W385A punctin-NQ and W445A punctin-NQ in conditioned medium were reduced (Fig. 5, *A* and *B*). Analysis of cellular levels of punctin-NQ and the Trp mutants demonstrated that there was an elevated cellular content of each mutant (Fig. 4*C*). In addition, lysates of cells expressing W36A, W39A, and W42A, and to a lesser extent W385A but not W445A or pNQ, showed the presence of molecular species of higher than the expected mass (Fig. 5*C*), suggesting the formation of aberrant complexes. The molecular mass observed for the putative complexes, 120 and 180 kDa, corresponds to the expected mass of punctin dimers and trimers, although we cannot exclude the possibility that these molecular species could represent punctin-1 complexed with another protein. Similar results were observed when the same mutants were transfected in CHO-K1 cells. Specifically, in CHO-K1 cells, there was a modest reduction of secretion of W36A and W42A, whereas there was statistically significant reduction of secretion of W39A and W385A with corresponding increases in cellular levels (data not shown). Overall, secretion of the mutants in CHO cells showed a similar trend as in HEK293F cells, but with a milder effect, indicating that effects of mutagenesis were dependent on the cell type used for the experiments. Because HEK293F cells showed a more severe effect on the punctin-NQ Trp substitutions, we used this cell line for further analysis.

We interpreted the results of this set of Ala for Trp substitutions as suggesting that mutation of $Trp^{39}$ and $Trp^{42}$ had severe consequences because these residues were themselves mannosylated, with $Trp^{42}$ also serving as the +3 residue for mannosylation at $Trp^{39}$, constituting in effect a double knock-out of *C*-mannosylation. However, an alternative interpretation was that structural consequences of W39A and W42A, *i.e.* lack of structural stability resulting from absence of the Trp aromatic side chain, could underlie the observed effect. Therefore, Ala substitution might be potentially unable to distinguish between

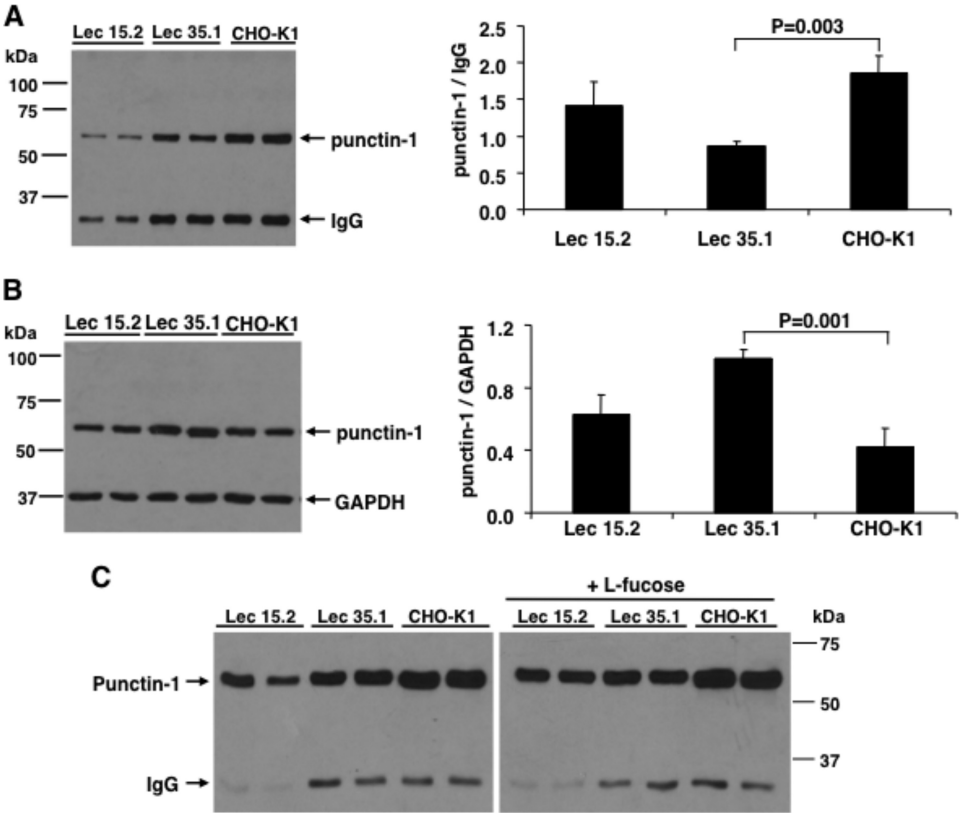



FIGURE 4. **Expression of N251Q-substituted punctin-1 (pNQ) in CHO-K1, Lec15.2 and Lec35.1 cells.** *A,* Western blot analysis (*left-hand panel*) and quantitation by densitometry (*right-hand panel*) show that the level of punctin-1 in the medium from Lec35.1 cells normalized to secreted IgG was significantly lower than that from CHO-K1 cells (*right-hand panel*, two-tailed Student *t* test, $n = 8$). *B,* amount of punctin-1 relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) detected in the Lec 35.1 cells by Western blotting (*left-hand panel*) was significantly higher than that in the CHO-K1 cells (*right-hand panel*, $n = 8$). The changes observed in Lec15.2 cells were not statistically significant ($n = 8$), although they followed the same trend as in Lec35.1 cells. Human IgG was used as a control for transfection and secretion efficiency and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a control for cell numbers. *C,* secretion efficiency of punctin-NQ in Lec15.2 cells, Lec35.1 cells, and CHO-K1 cells was compared in the absence (*left-hand panel*) or presence (*right-hand panel*) of supplemental L-fucose. The relative levels of punctin-NQ in the medium were unaffected by L-fucose supplementation.

the effects of *C*-mannosylation deficiency or the effects of structural perturbation.

It was previously shown that substitution of Ala for Trp at the +3 position in the W*XX*W consensus position of RNase 2 abolished *C*-mannosylation, whereas substitution of Phe for this Trp residue partially allowed the C-mannosylation of RNase 2 (up to 23%) (22). Phe has not, however, been reported to be a substrate for the putative mannosyltransferase. In view of this and because Phe substitution for Trp constitutes a structurally conservative substitution for Trp (both residues have an aromatic side chain), we undertook substitution with Phe. We substituted $Trp^{39}$ and $Trp^{42}$ with Phe because Ala substitution of these residues, which were shown to be modified by *C*-mannosylation, abolished punctin-1 secretion. We also substituted Phe for $Trp^{385}$, because Ala substitution had a more severe effect on this residue than $Trp^{445}$ (Fig. 5). Phe for Trp at position 385 (*i.e.* $^{385}$FTAC, which contains no Trp residues, and cannot be *C*-mannosylated) rescued the secretion defect observed in W385A punctin-NQ, whereas W39F punctin-NQ and W42F punctin-NQ were not detected in the medium (Fig. 6, *A* and *B*). However, we did not detect significant differences in the cellular levels (Fig. 6*C*). These results suggested that the role of Trp at position 385 was predominantly structural, whereas the mutations of $Trp^{39}$ and $Trp^{42}$ possibly reported the effects of both a structural perturbation as well as lack of *C*-mannosylation. Interestingly, the higher molecular mass species observed intracellularly with Ala substitutions (Fig. 5*C*) were not seen in any of the Phe substitutions (Fig. 6*C*), suggesting less of a structural perturbation by Phe.

## DISCUSSION

The results described here thus identify a new post-translational modification in two members of the ADAMTS superfamily, punctin-1 and ADAMTS5. Taken together, the data showing biosynthetic incorporation of tritiated mannose and detection of the characteristic cross-ring cleavage of the *C*-mannose, indicated *C*-mannosylation of $Trp^{39}$ and $Trp^{42}$, but not $Trp^{36}$ in punctin-1. Thus, the consensus sequence by itself does not ensure a 100% likelihood of *C*-mannosylation, which may be influenced by other sequence determinants or by the presence of adjacent *C*-mannosylation or other modifications, as previously proposed (13). The experimental observations for TSR1 matched the predictions of the NetCGlyc 1.0 server (Table 1), but we have not obtained evidence in support of the predicted modification of punctin-1 at $Trp^{445}$ in TSR4 (see Table 1 of Wang *et al.* (16)). Consensus sites for *C*-mannosylation are present in every member of the ADAMTS superfamily (Table 1). The majority of predicted sites in the ADAMTS-superfamily are in TSR1, consistent with the remarkable conservation of this TSR within the family, and its close resemblance to the TSRs of thrombospondins. This was supported by MS analysis of ADAMTS5, which also had *C*-mannosylation in TSR1.

Identification of *C*-mannosylation within the $^{42}$WSEC motif in TSR1 indicates that $Trp^{42}$ serves as both a recipient and +3 residue in the context of *C*-mannosylation. $Trp^{36}$ is followed by an Asp residue, which is rarely found at the +2 position of the *C*-mannosylation sequences (28) and may explain the lack of *C*-mannosylation of this residue. The lack of mannosylation of $Trp^{385}$ and $Trp^{445}$ despite the presence of a +3 Cys residue (28) is not currently explained. Notably, both of these residues are preceded by another Trp, which occurs five residues upstream and therefore does not constitute a W*XX*W motif. Indeed, although the W*XX*W motif is consistently seen in TSR1 of members of the ADAMTS superfamily, downstream TSRs frequently contain a W*XXX*W motif preceding the W*XX*C motif,

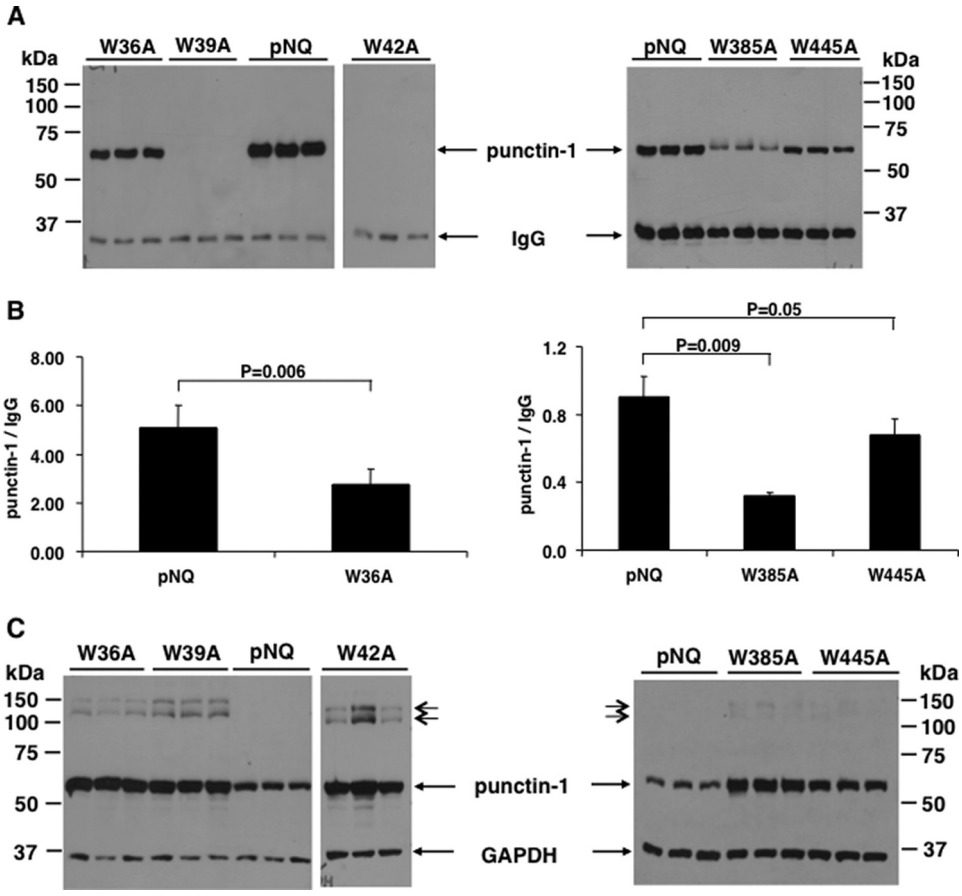



FIGURE 5. **Ala substitution of specific Trp residues in punctin-1 interferes with secretion.** *A* and *B,* Western blots of conditioned medium (*A*) from transfected HEK293F cells and quantitative analysis of the punctin levels in the medium normalized to secreted IgG (*B*). The levels of secreted protein obtained following transfection of the various Trp mutants were compared with the levels of punctin-1 NQ. W39A- and W42A-substituted punctin-1 are undetectable in medium. There is statistically significant reduction of W36A-, W385A-, and W445A-substituted punctin-NQ ($n = 5$). *C,* Western blot analysis of the corresponding cell lysate shows an increase in intracellular levels of all mutants relative to punctin-NQ. Note that Ala substitution of $Trp^{36}$, $Trp^{39}$, $Trp^{42}$, and $Trp^{385}$ led to formation of intracellular dimers and trimers (*small arrows*). *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

if present. The lack of conservation of W*XX*W in downstream TSRs is suggestive of low evolutionary pressure to retain *C*-mannosylation in the C-terminal TSRs.

Functional analysis of *C*-mannosylation is limited at the present time because the responsible *C*-mannosyltransferase has not yet been identified. Thus, specific tools such as inhibitory RNA, gene knock-out, and chemical inhibitors are not yet available, and the consequences of an organism-wide lack of *C*-mannosylation are not known. However, a potential role of *C*-mannosylation in regulation of punctin-1 secretion was investigated by expression in Lec15.2 and Lec35.1 cells. These cells are expected to have reduction of all modifications that depend on Dol-P-Man, such as *N*-glycosylation, glycosylphosphatidylinositol-anchor formation, and *O*-mannosylation (23, 29). By using the punctin-NQ mutant, possible interference with *N*-glycosylation was eliminated. Other types of mannosylation are not predicted to occur on Punctin-1. Punctin-1 does not have a C-terminal signal anchor sequence for glycosylphosphatidylinositol linkage (4), and *O*-mannosylation typically occurs in mucin-type domains (33) that punctin-1 lacks. Thus, it is likely that expression of the punctin-NQ variant reports exclusively the effect of *C*-mannosylation. The demonstration of tritiated mannose incorporation into punctin-NQ, taken together with the unequivocal demonstration of C-linkage of mannose to Trp residues within the appropriate consensus motifs, strongly suggests that the observed effect in the Lec variants are a consequence of the lack of *C*-mannosylation. Our previous work showed that interference with *O*-fucosylation also reduced secretion levels (16). The modifying enzyme, POFUT2, as well as the related enzyme POFUT1, which adds fucose to epidermal growth factor-like repeats, are believed to play roles in quality control during biosynthesis of their target proteins. Such a role is also feasible for the putative protein *C*-mannosyltransferase and can be tested once this enzyme is cloned.

*C*-Mannosylation is likely to be a co-translational process because it can occur on unfolded peptides and polypeptides (22), whereas *O*-fucosylation of TSRs requires prior protein folding (17). The *C*-mannosylation motif W*XX*W is consistently present in TSR1, the most N-terminal independently folding module in the ADAMTS-like proteins, indeed it is the most N-terminal glycosylation motif present in punctin-1 and other ADAMTS-like proteins. The fact that the peptide with *C*-mannose on $Trp^{39}$, but not $Trp^{42}$, is still *O*-fucosylated (Table 2) suggests that *C*-mannosylation of Trp*42* is not necessary for *O*-fucosylation. Further work will need to be done to determine whether complete loss of *C*-mannosylation affects addition of *O*-fucose. To rule out any defect in GDP-fucose biosynthesis in the Lec35.1 cells, we showed that supplementation of medium with L-fucose did not alter the secretion efficiency in these cells. We conclude that lack of *C*-mannosylation deficiency likely compromises protein folding, which in turn restricts secretion efficiency.

There are no naturally occurring or engineered mutations reported in the *C*-mannosylation consensus sequences in the ADAMTS superfamily, which motivated us to undertake substitution of Trp residues in punctin-NQ with Ala and Phe. In two instances of experimentally determined *C*-mannosylation in TSRs, $W^0$ is reportedly replaced with Phe or Tyr (22), with *C*-mannosylation occurring at $W^{+3}$. In addition, a detailed analysis of *C*-mannosylation of RNase 2 demonstrated that the Trp at position +3 could be replaced with Phe, with *C*-mannosylation occurring at $W^0$, albeit with a 3-fold decrease in modification (22, 28). In contrast, Ala is not acceptable at either

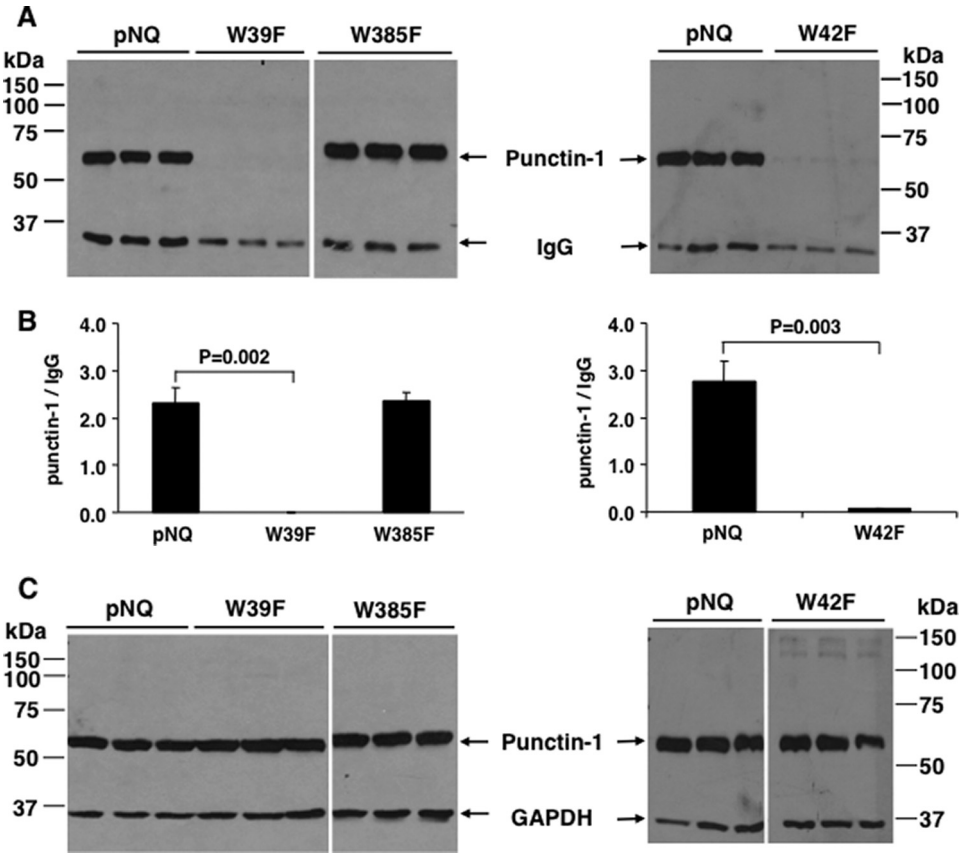



FIGURE 6. **Phe substitution of Trp$^{39}$ and Trp$^{42}$ but not Trp$^{385}$ interferes with secretion.** *A* and *B,* Western blots of conditioned medium (*A*) from transfected HEK293F cells and quantitative analysis of the punctin levels in the medium normalized to secreted IgG (*B*). The levels of secreted protein obtained following transfection of the various punctin-1 mutants ($n = 5$) were compared with the levels of punctin-1 NQ. Similar to W39A and W42A and W39F and W42F were either undetectable in conditioned medium (W39F) or barely detectable (W42F), whereas in contrast to W385A, the level of W385F in the medium was comparable with that of punctin-1 NQ. *C,* in the cell lysate there was no significant change in the level of any of the Phe substitutions of punctin-NQ, and none of the Phe substitutions showed the formation of intracellular complexes. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

$W^0$ or the +3 positions (13, 22). Consistent with these observations, we observed that Ala substitutions had a drastic effect on punctin-NQ secretion, but whether this was because of abolishing *C*-mannosylation, structural effects, or a combination of both was unclear. We observed that the effect of substituting an unconserved residue (Ala) for Trp was reduced or eliminated altogether at Trp$^{385}$ by the use of Phe, which we attributed a structural rescue, because Trp$^{385}$ is not modified. However, Phe substitution did not improve secretion of the Ala-substituted Trp$^{39}$ or Trp$^{42}$ punctin-NQ, suggesting that *C*-mannosylation of these residues may be critical for secretion of punctin. In this regard, it was intriguing that we identified a peptide from secreted punctin-1 lacking *C*-mannosylation at Trp$^{42}$ (Table 2), yet mutagenesis at this residue eliminated secretion. One possible explanation is that this mutation eliminated *C*-mannosylation at both Trp$^{39}$ and Trp$^{42}$. However, because mutagenesis of either residue clearly had a more dramatic effect than expression in Lec35.1 cells, the probability that Trp has a structural role at positions 39 and 42 is quite high.

Punctin-1 was chosen for the present analysis in preference to ADAMTS5 for several reasons. Its smaller size relative to other ADAMTS superfamily proteins, such as ADAMTS5, makes it especially amenable to sensitive quantitation of secretion, because it is secreted efficiently from cells and it is relatively protease insensitive. Indeed, the ADAMTS5 preparation used here had lost the TSR2 by proteolysis. Punctin-1 contains only one *N*-linked oligosaccharide, which we mutated in punctin-1 NQ, and unlike many ADAMTS proteins only four TSRs, thus facilitating analysis by mutagenesis and mass spectrometry. ADAMTS5 contains several *N*-linked oligosaccharide attachment sites (1, 34). The functional implications of *C*-mannosylation in regard to secretion could not be explored in ADAMTS5, because its *N*-glycosylation is essential for secretion.[3]

The identification of the glucose-fucose disaccharide on TSR1 of punctin-1 (and ADAMTS5) also resolves a question regarding whether the presence of a positively charged amino acid (Lys, Arg) at the −1 position of the *O*-fucose glycosylation site ($CX^{-2}X^{-1}$(S/T)C*XX*G) inhibits *O*-fucosylation. No *O*-fucose had previously been detected on TSRs with a positive charge in this position (16). In our previous analysis of punctin-1 (16), we were unable to confirm the presence of *O*-fucose on TSR1. Here we have confirmed the presence of the Glc-Fuc disaccharide on peptides from TSR1 of both Punctin-1 and ADAMTS5 (Table 2), both of which have an Arg residue in the −1 position. No unmodified forms of these peptides were detected, suggesting that the presence of the Arg has no effect on the efficiency of *O*-fucosylation. The finding is significant, because with the exception of ADAMTSL4 and ADAMTSL5 (which have Pro and Ser at this position, respectively), all members of the ADAMTS superfamily have a positively charged residue at this position in TSR1.

In future studies it will be important to extend the present analysis to other members of the ADAMTS superfamily and determine the functional role of *C*-mannosylation through analysis of the consequences for proteolytic activity and intermolecular interactions. Such studies will be facilitated by the fundamental observations made here using punctin-1 as a prototype for the superfamily, but they are conditional upon identification of the mannosyltransferase, so that such effects can be studied by specifically suppressing its activity rather than by mutagenesis.

[3] D. R. McCulloch and S. S. Apte, unpublished data.

*Acknowledgments—We thank Dr. Mark Lehrman for the kind gift of Lec15.2 and Lec35.1 cells and Wyeth Pharmaceuticals for kindly providing ADAMTS5.*

## REFERENCES


1. Hurskainen, T. L., Hirohata, S., Seldin, M. F., and Apte, S. S. (1999) *J. Biol. Chem.* **274,** 25555–25563
2. Ahram, D., Sato, T. S., Kohilan, A., Tayeh, M., Chen, S., Leal, S., Al-Salem, M., and El-Shanti, H. (2009) *Am. J. Hum. Genet.* **84,** 274–278
3. Hall, N. G., Klenotic, P., Anand-Apte, B., and Apte, S. S. (2003) *Matrix Biol.* **22,** 501–510
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. Biol. Chem.* **277,** 12182–12189
5. Koo, B. H., Goff, C. L., Jungers, K. A., Vasanji, A., O'Flaherty, J., Weyman, C. M., and Apte, S. S. (2007) *Matrix Biol.* **26,** 431–441
6. Le Goff, C., Morice-Picard, F., Dagoneau, N., Wang, L. W., Perrot, C., Crow, Y. J., Bauer, F., Flori, E., Prost-Squarcioni, C., Krakow, D., Ge, G., Greenspan, D. S., Bonnet, D., Le Merrer, M., Munnich, A., Apte, S. S., and Cormier-Daire, V. (2008) *Nat. Genet.* **40,** 1119–1123
7. Weedon, M. N., Lango, H., Lindgren, C. M., Wallace, C., Evans, D. M., Mangino, M., Freathy, R. M., Perry, J. R., Stevens, S., Hall, A. S., Samani, N. J., Shields, B., Prokopenko, I., Farrall, M., Dominiczak, A., Johnson, T., Bergmann, S., Beckmann, J. S., Vollenweider, P., Waterworth, D. M., Mooser, V., Palmer, C. N., Morris, A. D., Ouwehand, W. H., Zhao, J. H., Li, S., Loos, R. J., Barroso, I., Deloukas, P., Sandhu, M. S., Wheeler, E., Soranzo, N., Inouye, M., Wareham, N. J., Caulfield, M., Munroe, P. B., Hattersley, A. T., McCarthy, M. I., and Frayling, T. M. (2008) *Nat. Genet.* **40,** 575–583
8. Apte, S. S. (2004) *Int. J. Biochem. Cell Biol.* **36,** 981–985
9. Porter, S., Clark, I. M., Kevorkian, L., and Edwards, D. R. (2005) *Biochem. J.* **386,** 15–27
10. Arner, E. C. (2002) *Curr. Opin. Pharmacol.* **2,** 322–329
11. Glasson, S. S., Askew, R., Sheppard, B., Carito, B., Blanchet, T., Ma, H. L., Flannery, C. R., Peluso, D., Kanki, K., Yang, Z., Majumdar, M. K., and Morris, E. A. (2005) *Nature* **434,** 644–648
12. Stanton, H., Rogerson, F. M., East, C. J., Golub, S. B., Lawlor, K. E., Meeker, C. T., Little, C. B., Last, K., Farmer, P. J., Campbell, I. K., Fourie, A. M., and Fosang, A. J. (2005) *Nature* **434,** 648–652
13. Gonzalez de Peredo, A., Klein, D., Macek, B., Hess, D., Peter-Katalinic, J., and Hofsteenge, J. (2002) *Mol. Cell. Proteomics* **1,** 11–18
14. 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
15. Ricketts, L. M., Dlugosz, M., Luther, K. B., Haltiwanger, R. S., and Majerus, E. M. (2007) *J. Biol. Chem.* **282,** 17014–17023
16. Wang, L. W., Dlugosz, M., Somerville, R. P., Raed, M., Haltiwanger, R. S., and Apte, S. S. (2007) *J. Biol. Chem.* **282,** 17024–17031
17. Luo, Y., Nita-Lazar, A., and Haltiwanger, R. S. (2006) *J. Biol. Chem.* **281,** 9385–9392
18. Luo, Y., Koles, K., Vorndam, W., Haltiwanger, R. S., and Panin, V. M. (2006) *J. Biol. Chem.* **281,** 9393–9399
19. 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
20. Sato, T., Sato, M., Kiyohara, K., Sogabe, M., Shikanai, T., Kikuchi, N., Togayachi, A., Ishida, H., Ito, H., Kameyama, A., Gotoh, M., and Narimatsu, H. (2006) *Glycobiology* **16,** 1194–1206
21. Hofsteenge, J., Blommers, M., Hess, D., Furmanek, A., and Miroshnichenko, O. (1999) *J. Biol. Chem.* **274,** 32786–32794
22. Krieg, J., Hartmann, S., Vicentini, A., Gläsner, W., Hess, D., and Hofsteenge, J. (1998) *Mol. Biol. Cell* **9,** 301–309
23. Doucey, M. A., Hess, D., Cacan, R., and Hofsteenge, J. (1998) *Mol. Biol. Cell* **9,** 291–300
24. Krieg, J., Gläsner, W., Vicentini, A., Doucey, M. A., Löffler, A., Hess, D., and Hofsteenge, J. (1997) *J. Biol. Chem.* **272,** 26687–26692
25. Doucey, M. A., Hess, D., Blommers, M. J., and Hofsteenge, J. (1999) *Glycobiology* **9,** 435–441
26. Furmanek, A., Hess, D., Rogniaux, H., and Hofsteenge, J. (2003) *Biochemistry* **42,** 8452–8458
27. Perez-Vilar, J., Randell, S. H., and Boucher, R. C. (2004) *Glycobiology* **14,** 325–337
28. Julenius, K. (2007) *Glycobiology* **17,** 868–876
29. Anand, M., Rush, J. S., Ray, S., Doucey, M. A., Weik, J., Ware, F. E., Hofsteenge, J., Waechter, C. J., and Lehrman, M. A. (2001) *Mol. Biol. Cell* **12,** 487–501
30. Stone, K. L., and Williams, K. R. (1993) in *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P., ed) pp. 43–69, Academic Press, San Diego, CA
31. Hofsteenge, J., Müller, D. R., de Beer, T., Löffler, A., Richter, W. J., and Vliegenthart, J. F. (1994) *Biochemistry* **33,** 13524–13530
32. Tan, K., Duquette, M., Liu, J. H., Dong, Y., Zhang, R., Joachimiak, A., Lawler, J., and Wang, J. H. (2002) *J. Cell Biol.* **159,** 373–382
33. Haltiwanger, R. S., and Lowe, J. B. (2004) *Annu. Rev. Biochem.* **73,** 491–537
34. Abbaszade, I., Liu, R. Q., Yang, F., Rosenfeld, S. A., Ross, O. H., Link, J. R., Ellis, D. M., Tortorella, M. D., Pratta, M. A., Hollis, J. M., Wynn, R., Duke, J. L., George, H. J., Hillman, M. C., Jr., Murphy, K., Wiswall, B. H., Copeland, R. A., Decicco, C. P., Bruckner, R., Nagase, H., Itoh, Y., Newton, R. C., Magolda, R. L., Trzaskos, J. M., and Burn, T. C. (1999) *J. Biol. Chem.* **274,** 23443–23450