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Proteoglycan profiling of human, rat and mouse insulin-secreting cells

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Abstract

Proteoglycans (PGs) are proteins with glycosaminoglycan (GAG) chains, such as chondroitin sulfate (CS) or heparan sulfate (HS), attached to serine residues. We have earlier shown that prohormones can carry CS, constituting a novel class of PGs. The mapping of GAG modifications of proteins in endocrine cells may thus assist us in delineating possible roles of PGs in endocrine cellular physiology. With this aim, we applied a glycoproteomic approach to identify PGs, their GAG chains and their attachment sites in insulin-secreting cells. Glycopeptides carrying GAG chains were enriched from human pancreatic islets, rat (INS-1 832/13) and mouse (MIN6, NIT-1) insulinoma cell lines by exchange chromatography, depolymerized with GAG lyases, and analyzed by nanoflow liquid chromatography tandem mass spectrometry. We identified CS modifications of chromogranin-A (CgA), islet amyloid polypeptide, secretogranin-1 and secretogranin-2, immunoglobulin superfamily member 10, and protein AMBP. Additionally, we identified two HS-modified prohormones (CgA and secretogranin-1), which was surprising, as prohormones are not typically regarded as HSPGs. For CgA, the glycosylation site carried either CS or HS, making it a so-called hybrid site. Additional HS sites were found on syndecan-1, syndecan-4, nerurexin-2, protein NDNF and testican-1. These results demonstrate that several prohormones, and other constituents of the insulin-secreting cells are PGs. Cell-targeted mapping of the GAG glycoproteome forms an important basis for better understanding of endocrine cellular physiology, and the novel CS and HS sites presented here provide important knowledge for future studies.

Key words: chromogranin-A, glycosaminoglycan, insulinoma, islet cells, proteoglycan

Introduction

Proteoglycans (PGs) are complex macromolecules present in almost all tissues and are composed of a core protein to which one or more highly acidic glycosaminoglycan (GAG) side chains are covalently bound to specific serine residues in a semi-conserved consensus sequence (Lindahl et al. 2015). Repetitive serine glycine (Ser Gly) motifs, with a nearby cluster of acidic amino acids, in the PG core proteins favor the synthesis of heparan sulfate GAGs, while the presence of a single Ser Gly motif in close proximity to acidic residues seems to favor the chondroitin/dermatan sulfate assembly (Esko and Zhang 1996). GAGs are structurally divided into four major types; chondroitin/dermatan sulfate (CS/DS), heparin/heparan sulfate (HS), hyaluronic acid (HA) and keratan sulfate (KS) (Kjellen and Lindahl 1991). These GAGs are typically

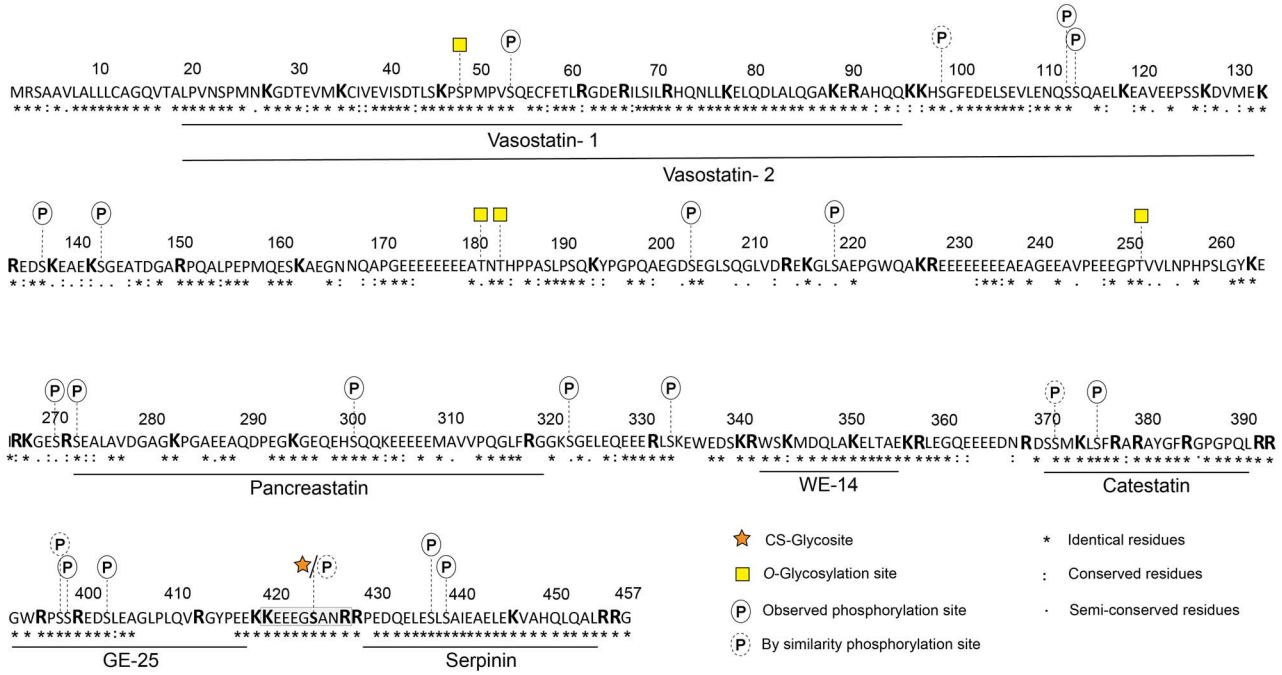

Fig. 1. Amino acid sequence, phosphorylation and *O*-glycosylation sites of human chromogranin-A pre-prohormone. Amino acids are shown as single letters, are numbered on top and indicated as bold at tryptic cleavage sites. Human, rat and mouse sequences were aligned using the multiple sequence alignment program Clustal Omega and the level of conservation among the three species is indicated below each residue. Sites of phosphorylation (P) and *O*-glycosylation (*O*-GalNAc; yellow squares and *O*-Xyl; orange star) of human CgA are marked above the corresponding amino acids. Established CgA-derived peptide hormones and fragments are annotated and their sequences underlined. The GAG site at Ser-424 (boxed sequence (Noborn et al. 2015) corresponds to Ser-433 of rat CgA and to Ser-430 of mouse CgA. Glycans are illustrated according to the Symbol Nomenclature for Glycans (Varki et al. 2015). This figure is available in black and white in print and in color at *Glycobiology* online.

composed of an ordered, repeating pattern of disaccharides such as 4GlcAβ1,3GalNAcβ1 and 4IdoAα1,3GalNAcβ1 for CS and DS, respectively; 4GlcAβ1,4GlcNAcα1 and 4IdoAα1,4GlcNAcα1 for heparin and HS; 4GlcAβ1,3GlcNAcβ1 for HA; and 3Galβ1,4GlcNAcβ1 for KS. An additional level of variation occurs by *N*-deacetylation and *N*-sulfation of GlcNAc residues in heparin/HS and by modifications with mainly sulfate groups but also with phosphate, sialic acid and fucose residues at various positions of the GAG chains (Sugahara et al. 2003; Prydz 2015). HA is unique among the GAG types in not being linked to a core protein and not being sulfated. PG heterogeneities are further associated with variations in core protein structures, types, lengths and numbers of GAG chains as well as the GAG attachment sites and the occupancy (i.e. level of GAG glycosylation) at the Ser residues. PGs not only serve to provide an extracellular matrix support in different tissues, but also are actively involved in a variety of physiological functions such as proliferation, differentiation, migration, adhesion and neural regeneration (Bishop et al. 2007; Malmstrom et al. 2012).

Mass spectrometry (MS) has been developed and applied as the preferred analytical tool for identification and quantitation of protein glycosylation in different biological samples (Thaysen-Andersen et al. 2016; Xiao et al. 2019). Our recent development of novel MS-based glycoproteomic approach for the global analysis of PGs has enabled us to identify PGs in complex biological fluids such as human urine, plasma and cerebrospinal fluid (CSF) (Noborn et al. 2015, 2016; Nilsson et al. 2017). By using this technique, we have been able to characterize CS and HS linkage regions and their modifications with sulfate, phosphate, sialic acid and fucose residues, the GAG attachment sites and the identities of the corresponding PG core proteins occurring in such fluids (Gomez Toledo et al. 2015; Noborn et al. 2016). Interestingly, we identified human prohormones as a novel class of PGs, including chromogranin-A (CgA), cholecystokinin, neuropeptide W, secretogranin-1 and secretogranin-3, all of which are stored and secreted from granules of endocrine cells (Noborn et al. 2015).

Prohormones are known to undergo various post-translational modifications (PTMs) such as phosphorylation and glycosylation and are processed by several proteolytic cleavages via the action of proprotein convertases to generate bioactive peptides (Seidah and Chretien 1999; Hook et al. 2008; Chen et al. 2018). Along with the primary amino acid sequence, PTMs have recently been shown to contribute to the functional processing of the natriuretic prohormones by blocking the action of such peptidases and also to affect the biological stability and activity of the released hormones (Fricker 2012; Hansen et al. 2019). However, the knowledge about the potential impact of GAG modifications on such processing, and if natively processed peptides may include any GAG modification are very limited, warranting further studies on this subject.

In this study, we applied our glycoproteomics methodology on cultured endocrine cells in addition to clinical samples to verify the previous findings using established cell model systems, which are known to produce and secrete a range of peptide hormones, including insulin and CgA. CgA is one of the main proteins of insulin secretory granules which costores and coreleases with insulin in normal human beta cells (Lukinius et al. 1992). It has been shown that CgA in endocrine cells stabilizes and prevents granule protein degradation from the Golgi apparatus, which leads to the promotion of secretory granule biogenesis (Kim and Loh 2006; D'Amico et al. 2014). The CgA-derived peptide, serpinin, has been shown to regulate this function (Koshimizu et al. 2011; Wollam et al. 2017).

Several O-phosphorylation and mucin type glycosylation modification sites have been detected along the CgA protein (Figure 1) (Gadroy et al. 1998; Beranova-Giorgianni et al. 2006). In contrast, information on the GAG glycosylation status of this protein or of any other of the proteins in secretory granules of insulin-secreting cells is very limited.

To fill up this information gap, we have characterized the GAG structures of insulin-secreting cells of rat, mouse and human origin. We used the methodology both with and without trypsin digestion in order to investigate native protease digestion sites distant and close to the GAG site of CgA. Also, with the use of the INS-1 832/13 cell line we estimated the GAG occupancy of CgA, which is likely to be important for understanding the influence of GAG chains on CgA prohormone processing. Moreover, the mapping of GAG modifications in endocrine cells was used to identify additional GAG sites (HS or CS) of prohormones, and would provide a theoretical framework for studying the role of PGs in endocrine cellular physiology.

Here, we used a glycoproteomics LC MS/MS approach, based on higher energy collision dissociation (HCD), to identify GAG type, linkage region structures and core proteins from three human beta islet samples and three rodent cell lines INS-1 832/13, MIN6 and NIT-1. In total, we identified four known CSPGs and two novel CSPGs (islet amyloid polypeptide; IAPP and immunoglobulin superfamily member 10; IgSF10), along with four known HSPGs and three novel HSPGs (CgA, secretogranin-1 and neurexin-2/neurexin-2-beta). The latter finding was surprising, as prohormones are not typically regarded as HSPGs. For the rodent CgA proteins, this HS site (Ser-433 in rat and Ser-430 in mouse) was found to be the same site as the CS site, making it a so-called hybrid site. The complex patterns of GAG modifications underline the importance of cell-targeted mapping of the GAG glycoproteome for better understanding of granule formation in endocrine cells and for the selection of cell models when studying diabetic pathogenesis.

Results

Characterization of CS modifications of CgA in human islet cells

In a previous study, we showed that tryptic glycopeptides of CgA in human urine and in CSF were modified with a CS linkage region at Ser-424 (Noborn et al. 2015). Now, we found similar glycopeptides of CgA in the human islet samples, also modified with a CS glycan at the same glycosite (Ser-424) (Figure 2). When searching for the characteristic MS^2 fragment ion at *m/z* 362.11, $[\Delta HexAHexNAc]^+$ typically found in GAGs depolymerized with chondroitinase ABC (ChABC), several glycopeptides appeared at different elution times in the extracted ion chromatogram (Figure 2A). The major peak had a precursor ion *m/z* at 1086.0616 (3+) and eluted at 20 min, corresponding to the peptide 419-KEEEGSANRRPEDQELES-436 of CgA modified with a hexameric CS glycan, including the canonical linkage region tetrasaccharide of GAGs, additionally carrying one sulfate and one phosphate group. The peptide sequence was identified by Mascot database searches for the typical b- and y-ions in the MS^2. Peptide ions were obtained from parent ion fragmentations at a higher normalized collision energy (NCE) of 40%, while the glycan sequence was obtained from fragmentation at a lower NCE of 30%, providing the diagnostic oxonium ion at *m/z* 362.11 (Figure 2C) (Noborn et al. 2015).

Although b- and y-ions encompassing the assumed glycosylation site were observed, these ions were completely deglycosylated at all

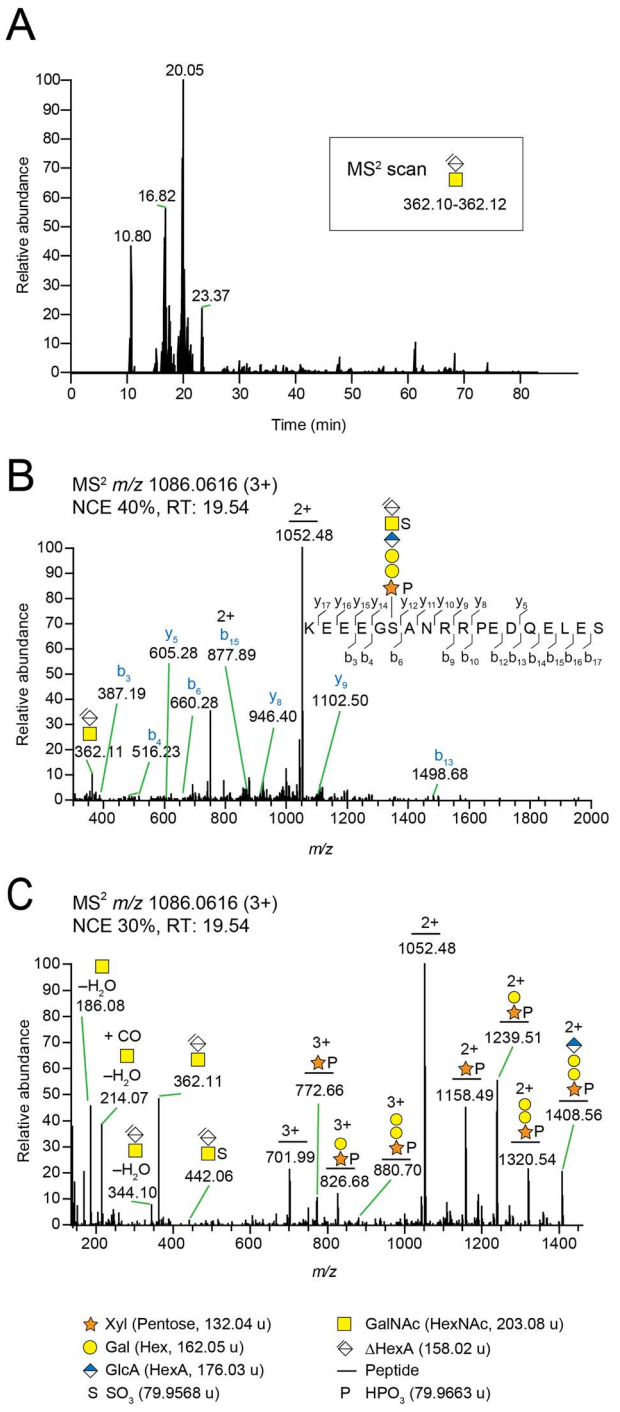

Fig. 2. MS^2 scan and spectra of GAG-glycopeptides of chromogranin-A from human islet cells. The spectral file was filtered for the MS^2 diagnostic ion at *m/z* 362.11, corresponding to the $[\Delta HexAHexNAc]^+$ ion (**A**). The MS^2 spectrum of the major glycopeptide is shown for NCE at 40%, to display the peptide sequence fragmentation (**B**) and at NCE 30% providing better glycan fragmentation (**C**). The positioning and distinction of sulfate and phosphate groups were made by manually evaluating the MS^2 spectra. The MS^2 spectrum of the precursor ion in (**C**) displays a mass shift of 79.958 u between the ions at *m/z* 362.110 and *m/z* 442.068, demonstrating the presence of a sulfate group on the GalNAc residue. A mass shift of 1153.1945 u between the precursor ion, *m/z* 1086.0616 (3+), and the peptide ions, *m/z* 1052.4841 (2+) and *m/z* 701.9922 (3+), demonstrates the presence of one sulfate group on the GalNAc residue and one phosphate group on the Xyl residue.

NCE settings. Therefore, the Ser attachment sites could not experimentally be proven when the peptide contained several Ser residues, but were assumed to be present on Ser Gly and Ser Ala based on their predominance in previously described GAG sites.

It should also be noted that the monoisotopic *m/z* values of precursor ions at four decimals are provided in the heading of the MS^2 spectra in this study. The *m/z* values of fragment ions are from the largest isotope peaks, not always from the monoisotopic ion. Thus, delta masses in the figures occasionally differ by ±1 u from calculated values. The nonmodified peptide ion at *m/z* 1052.48 was dominating in both spectra, but at NCE 30%, additional glycopeptide ions gave information on the glycan sequence and on the phosphate modification of xylose (Xyl) of the linkage region. Specifically, the [peptide+Xyl + phosphate] fragment ion, here at *m/z* 772.66 (3+) and *m/z* 1158.49 (2+), including an intact phosphate group on the Xyl residue were characteristic and can be efficiently used for pinpointing this modification in any linkage region of PG-derived glycopeptides. In contrast, sulfate groups are relatively labile, but the oxonium ion at *m/z* 442.06 gave support for its localization to ΔHexAGalNAc of the hexameric CS structure of this glycopeptide (Figure 2C); and based on the conclusive presence of genuine CS/DS structures, where sulfation of GalNAc is dominating, and the assumption of conserved biosynthetic pathways, the sulfate group was assigned to be positioned on the GalNAc residue. However, when additional sulfation was identified, based on the increase of the precursor ion mass and corresponding to the mass of a sulfate group, brackets were used to indicate that either of the Gal residues could be sulfated. Additional variants of CgA CS-glycopeptides were identified in the same way and are all displayed in Supplementary Table SI and summarized in Table I. Eight different glycopeptides of human CgA, covering the same glycosite were identified and the glycans were either unmodified, carrying one sulfate or one phosphate or both one sulfate and one phosphate group. MS support was found for sulfate groups attached either to the ΔHexAGalNAc or to one of the Gal residues. There are three Ser Gly motifs (Ser-98, Ser-142 and Ser-322) and three Ser Ala motifs (Ser-218, Ser-424 and Ser-438) within the human CgA protein sequence, and despite searching for any of the other corresponding CS-glycopeptides, we found only the Ser Ala site at Ser-424 modified with a CS hexasaccharide. CgA was the only protein in the human islets that was identified with a CS modification.

Table I. CS and HS modified glycopeptides identified in human islets, rat and mouse beta cells

Sample type	UniProt	Protein name	Peptide sequences[a]	Glycan modifications[b]
Human Islet	P10645	Chromogranin-A	^{419}K.KEEEGSANRRPEDQELES.L^{436}	+ 0, 1 or 2 Ⓢ/Ⓟ
	P31431	Syndecan-4	^{37}R.YFSGALPDDEDVVGPGQE.S^{54} ^{93}R.AGSGSQVPTEPK.K^{104}	
INS-1 832/13	P10354	Chromogranin-A	^{422}R.GDFEEKKEEEGSAN.R^{435}	+ 0, 1 or 2 Ⓢ/Ⓟ + 0 or 1 Ⓟ
	P12969	Islet Amyloid Polypeptide	^{24}A.TPVGSGTNPQVDKR.K^{37}	+ 0, 1 or 2 Ⓢ/Ⓟ
	Q63374	Neurexin-2 / Neurexin-2-beta	^{1485}R.DDSDCEEPVEASGF.A^{1498}	
	P35314	Secretogranin-1	^{230}R.EQSSQESGEETR.R^{241}	
	P26260	Syndecan-1	^{27}T.ANVPPEDQDGSGDDSDNF.S^{44} ^{45}F.SGSGTGALPDMTL.S^{57}	+ 0 or 1 Ⓟ
	P34901	Syndecan-4	^{44}F.SGALPDDEDAGGLEQDSDF.E^{62} ^{63}F.ELSGSGDLDDTEEPR.T^{77}	
MIN6	P26339	Chromogranin-A	^{419}R.SDFEEKKEEEGSAN.R^{432}	+ 1 or 2 Ⓢ/Ⓟ
	Q3V1M1	Immunoglobulin superfamily member 10	^{676}D.IDGSGLEEPKPSVLLK.Q^{691}	+ 1 Ⓢ/Ⓟ
	Q07456	Protein AMBP	^{205}R.AVLPQESEGSGTEPLITGTLKK.E^{226}	+ 1 or 2 Ⓢ/Ⓟ
	Q03517	Secretogranin-2	^{72}R.EESSPDYNPYQGVSVPLQLK.E^{91}	+ 2 Ⓢ/Ⓟ
NIT-1	P26339	Chromogranin-A	^{419}R.SDFEEKKEEEGSANR.R^{433}	+ 0, 1 or 2 Ⓢ/Ⓟ Ⓟ
	Q3V1M1	Immunoglobulin superfamily member 10	^{669}R.TIEHDRDIDGSGLEEPKPSVLL.K^{690}	+ 1 Ⓢ
	Q07456	Protein AMBP	^{205}R.AVLPQESEGSGTEPLITGTLKK.E^{226}	+ 1 or 2 Ⓢ/Ⓟ
	Q8C119	Protein NDNF	^{90}K.LSLQELHEGSSADGSGDPELLDQQK.Q^{114}	+ 0 or 1 Ⓟ
	P16014	Secretogranin-1	^{85}R.LLRDPADASGTR. W^{96}	+ 1 or 2 Ⓢ/Ⓟ
	Q62288	Testican-1	^{373}R.KQGTVSCEEEQETSGDFGSGGSVVLLDDLEDERELGPK.D^{410}	+ 0 or 1 Ⓟ

[a] CS and HS glycopeptides with the highest Mascot scores identified in this study are shown. Dots in the sequences correspond to cleavage sites and neighboring N- and C-terminal amino acids are specified to facilitate comparisons of peptide sequences. Bold and underlined serine residues represent identified attachment sites. Peptide sequences that are observed only upon combined trypsin and chymotrypsin digestions are shaded grey.

[b] Modifications with sulfate and/or phosphate and their numbers (0-2) are indicated by encircled letters S and P and is a summary of glycan variants shown in detail in Supplemental Table S1.

Finding an experimental model of CgA GAG modifications related to insulin secretion

Given that research on human pancreatic beta cells is hampered by the limited availability and limited long-term viability of these primary cells in vitro, there is a need for other more accessible experimental models to study functional effects of prohormone GAG modifications (Skelin et al. 2010). Therefore, we investigated if insulin-secreting cell lines also express GAG-modified CgA and possibly any additional GAG-modified prohormones. With this aim, we choose one rat cell line (INS-1 832/13) and two mouse cell lines (MIN6 and NIT-1), all of which secrete insulin upon stimulation and are commonly used in diabetic research. These cell lines are all established from insulinoma cells (Miyazaki et al. 1990; Hamaguchi et al. 1991; Hohmeier et al. 2000). The INS-1 832/13 clone originates from INS-1 cells and was selected among several clones transfected with a human insulin construct to produce stable and high levels of both human and rat insulins (Hohmeier et al. 2000). Our analyses of the different samples, i.e. cells and culture media from these three cell lines, revealed a series of CS-glycopeptides of the CgA protein (Table I). We identified the corresponding Ser glycosite in the rat and mouse samples as in human islet cells (Supplementary Figure S1) and glycopeptides of slightly different lengths carrying glycans with and without sulfate or phosphate modifications of the hexameric CS glycan (Supplementary Table SI). These findings demonstrated that all the three cell lines recapitulate the CS glycosylation modification of CgA found in human islet cells. Additionally, we identified a CS linkage hexasaccharide in rat INS-1 832/13 cells modified with an *N*-acetylneuraminic acid (Neu5Ac) residue (Supplementary Table SI, Supplementary Figure S2). Two distinct oxonium ions at *m/z* 292.10 $[Neu5Ac]^+$ and *m/z* 274.09 $[Neu5Ac–H_2O]^+$ at a NCEs 30% and 20% were observed, which are diagnostic for the presence of a Neu5Ac residue. Also, based on the presence of the fragment ion $[Peptide+Xyl + Gal+Neu5Ac + 2H]^{2+}$ at *m/z* 1155.48, the linkage site of this sialic acid was assigned to the inner Gal residue (Supplementary Figure S2B). These spectra show that a sialylated CS GAG modification of the rat CgA glycopeptide 422-GDFEEKKEEEGSANR-436 appears in the insulinoma cells, similar to what was earlier found as a modification of the CS linkage region of human bikunin in urine (Lu et al. 2010; Gomez Toledo et al. 2015).

Peptide characterization of CgA from human islets and rat INS-1 832/13 cells

To obtain an overview of the natively protease-cleaved peptide profiles of our samples, we omitted the use of trypsin in the workflow, mapped all peptides of CgA from human and rat insulin-secreting cells by nanoflow liquid chromatography–tandem mass spectrometry (nLC MS/MS) and compared the effects of various enrichment steps included in the sample preparation. The naturally occurring unmodified peptides (Figure 3A) of human islets generally had conserved N-termini, often at the start of the mature hormones (Figure 1), but with varying extensions towards the C-termini and usually not covering the whole sequences of the mature hormones. We observed the same pattern of CgA processing in rat INS-1 832/13 cells (Figure 3D) with intact serpinin as the only obvious exception. Some regions of the sequences were not represented as peptides (Figure 3A-F), and, in general, these regions correlated with a low level of conserved amino acids between the species (Figure 1). When including a strong anion exchange (SAX) chromatography step, the acidic peptides were enriched together with CS-modified glycopeptides (Figure 3B and E) and when adding trypsin, the longer peptides were, as expected, further digested and the CS-modified glycopeptides further enriched (Figure 3C and F). Additionally, six different phosphorylation sites of CgA were identified in the human cells (Ser-136, Ser-142, Ser-300, Ser-333, Ser-398 and Ser-402) and five in the rat cells (Ser-312, Ser-353, Ser-417, Ser-447 and a novel site at Ser-413) (Supplementary Figure S3). In human islets, five out of six and in rat INS-1 832/13 cells three out of five identified phosphorylation sites were located within Ser-X-Glu motifs. Lietz et al. (2018) have shown that in dense core secretory vesicles the Ser-X-Glu motif is the most common phosphorylation site motif, which correspond to the sites of phosphorylation by Fam20C, a predominant protein kinase of the secretory pathway. In addition to the CS-glycosylation, *O*-glycosylated peptides of CgA with preferentially sialylated core 1 type glycans (marked yellow in Figure 3) were found in both human and rat cells. From these results, we could conclude that the CgA prohormone and its peptide hormones were endogenously processed by several endo- and exo-proteases and that the natively cleaved CS-glycopeptides were naturally occurring glycopeptides of CgA from human and rat insulin-secreting cells. All the peptides and phosphopeptides (marked light blue in Figure 3) from CgA of human and rat INS-1 832/13 cells, identified and mapped in Figure 3, are listed in Supplementary File S3. *O*-glycosylated peptides are presented in Supplementary File S4.

Estimating the degree of CS modifications of CgA for INS-1 832/13 cells at different growth conditions

To obtain an estimation of the degree of CS modification of intact CgA and to investigate whether the concentration of glucose had any effect on the GAG production, the INS-1 832/13 cell line was grown under different concentrations of glucose (3–25 mM) for 6, 24 and 48 h, respectively (Figure 4). Extracted protein samples of cell media without fetal calf serum, were analyzed with western blots before and after ChABC plus heparinase II and III depolymerization. The analyses revealed a major band at 70 kDa, corresponding to the estimated weight of rat CgA, but additionally a broad smear of bands up to 95 kDa in the untreated samples, more clearly seen after 24 and 48 h of incubations (Figure 4A and B and Supplementary Figure S4). Upon enzymatic depolymerization with ChABC the smear of heavier bands was virtually gone supporting the GAG character of these glycoforms (Figure 4C and D and Supplementary Figure S4). In contrast, the major distinct band at 70 kDa and the more fast-moving bands 60 kDa remained intact upon depolymerization and were thus resistant to this procedure.

Estimating the relative levels of GAG modifications of CgA at different concentrations of glucose and after different intervals of growth by scanning the western blot did not reveal any obvious effects by altering the glucose concentrations (Figure 4). Thus, the mean values of occupancy for the four concentrations of glucose were 21.0 ± 11.9% at 3 mM (n = 4), 26.8 ±14.7% at 11 mM (n = 4), 27.3 ± 14.6% at 17 mM (n = 4) and 26.0 ± 12.7% at 25 mM (n = 4). However, when looking at the different time points, there was an increase in occupancy after extended growth. Thus, the mean values of occupancy for the three time intervals over all four concentrations of glucose were 13.7 ± 2.9% after 6 h (n = 8), 35.5 ± 3.1% after 24 h (n = 4) and 38.0 ± 5.6% after 48 h (n = 4). As a comparison, we also estimated the relative levels of GAG modifications based on the relative intensities of CS modified CgA glycopeptides versus those of the corresponding unmodified peptides in the nLC MS/MS analyses of the extracts of human islet cells, not subjected to trypsin digestion or SAX enrichment (compare Figure 3A).

It is known that glycopeptides do not ionize as well as their naked peptides and this may be even more so for the sulfated and carboxylated glycopeptides studied here and thus any level of occupancy would be an underestimation of the true level of occupancy. Any changes of relative levels would however be possible to follow and thus lacking relevant ionization efficiency data for individual pairs of peptides and glycopeptides of CgA, we calculated the occupancy under the assumption that the pairs possessed similar ionization efficiencies. These results indicated a level of GAG occupancy of (at least) 33.0% for the human CgA protein calculated from the unglycosylated versus the glycosylated peptide 413-GYPEEKKEEEGSANRRPEDQELESLS-438, which is in the same range as the results obtained from the western blot analyses of the rat INS-1 832/13 cells grown for 24 or 48 h. However, quantification by western blot is also equivocal when dealing with smears typical for glycoprotein heterogeneities.

Identifying Ser-433 of CgA in rat INS-1 832/13 cells and Ser-430 of mouse cells as a hybrid site carrying both CS and HS

To determine whether the CS-modified site of CgA or any other CSPGs in the three rodent cell lines, may alternatively be substituted with HS structures, we enriched GAG-glycopeptides from culture media by SAX chromatography and digested the collected fractions with both heparinases II and III and ChABC. Complete HS depolymerization using heparinases II and III is expected to generate tetrasaccharide linkage regions. Using heparinases II and III, we observed a distinct ion at m/z 173.04 in all MS^2 spectra of glycopeptides modified with a HS linkage region tetrasaccharide (Figure 5). This unique ion is interpreted to have a $[Hex–H_2O + CO]^+$ composition and is analogous to the ion at m/z 214.07, corresponding to a $[HexNAc–H_2O + CO]^+$ composition, present in the MS^2 spectra of CS hexasaccharide glycopeptides observed after ChABC treatment (see for instance Figure 2C) (Nilsson et al. 2017). Also, with the use of heparinases II and III only tetrasaccharide structures were identified. Thus, filtering the MS^2-data, from the combined CS and HS depolymerization experiment, for the diagnostic fragment ions at m/z 173.04 and 362.11 $[\Delta HexAHexNAc]^+$, we could identify two series of glycopeptides appearing at 11–12 min (exemplified for INS-1 832/13 cells in Figure 5A). The earlier eluting peak corre-

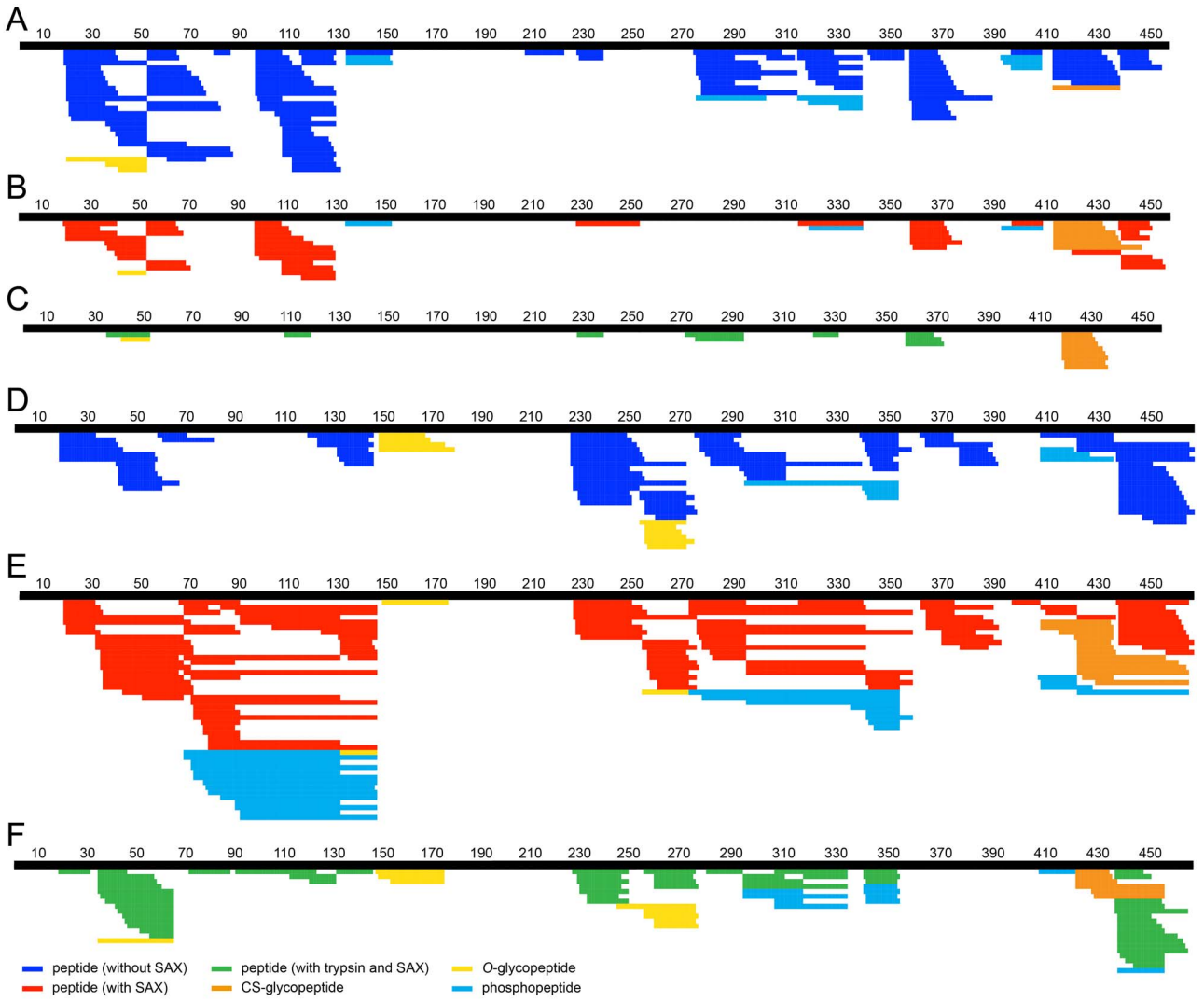

Fig. 3. Comparison of unmodified and modified peptides of chromogranin-A from different preparation protocols of human islets and rat INS-1 832/13 cells. Peptides were prepared from human islets (**A**-**C**) or rat INS-1 832/13 cells (**D**-**F**) either without (**A**, **B**, **D** and **E**) or with (**C**, **F**) digestion with trypsin and either not enriched (**A**, **D**) or enriched (**B**, **C**, **E** and **F**) on SAX-columns, and finally depolymerized with ChABC. The identified CgA peptides are aligned under the corresponding sequences and the type of peptide is illustrated with unique colors (unmodified; dark blue (without SAX), red (with SAX) and green (with trypsin and SAX), CS-glycopeptide; orange, *O*-glycopeptide; yellow and phosphopeptide; light blue). This figure is available in black and white in print and in color at *Glycobiology* online.

sponded mainly to the HS tetrasaccharide linkage region precursor ion at *m/z* 728.2854 (3+) (Figure 5B and C) and the later eluting one to a mixture of CS hexasaccharide linkage region precursor ions at *m/z* 933.3440 (3+) at 11.07 min and at *m/z* 881.3116 (3+) at 11.77 min (Figure 5B and D). The glycopeptide at 11.07 min had the sequence 422-GDFEEKKEEEGSANR-436, whereas the ones at 11.02 and 11.77 min had the amino acid sequence 422-GDFEEKKEEEGSAN-435 (Figure 5E and F). The relative distribution of the two glycoforms was estimated by comparing the relative abundances of the precursor ions; again lacking exact knowledge of the ionization efficiencies and under the admittedly rough assumption that glycopeptides with the same peptide sequence ionize similarly and irrespective of their glycan structures (Figure 5B). Thus, when comparing only the triply charged precursor ions (as in Figure 5B) there was 17% of HS and 83% of CS glycans of this peptide sequence. When comparing the sums of the triply charged precursor ions of both unmodified and modified glycans (including phosphorylated HS and monosulfated CS; Table I) the distribution was 18% of HS and 82% of CS, and finally when calculating all the doubly and triply charged precursor ions of the same two peptide sequences the distribution was 11% of HS and 89% of CS. These data clearly show that the C-terminal site of rat CgA is indeed a "hybrid PG site," allowing for alternative modifications with either HS or CS at the same amino acid position. Although we did not detect any phosphate group at this Ser-433 in the INS-1 832/13 cells, others have found this particular site phosphorylated but only in rat stomach and not in rat pancreas or in any other rat tissues (Lundby et al. 2012), which suggests a critical regulatory function for this particular Ser residue of CgA. In human islet cells, we did neither detect HS nor phosphate but only CS at this site of CgA.

Although, we could identify the hybrid PG site at the Ser-430 of CgA of the two mouse cell lines, MIN6 and NIT-1, we did not

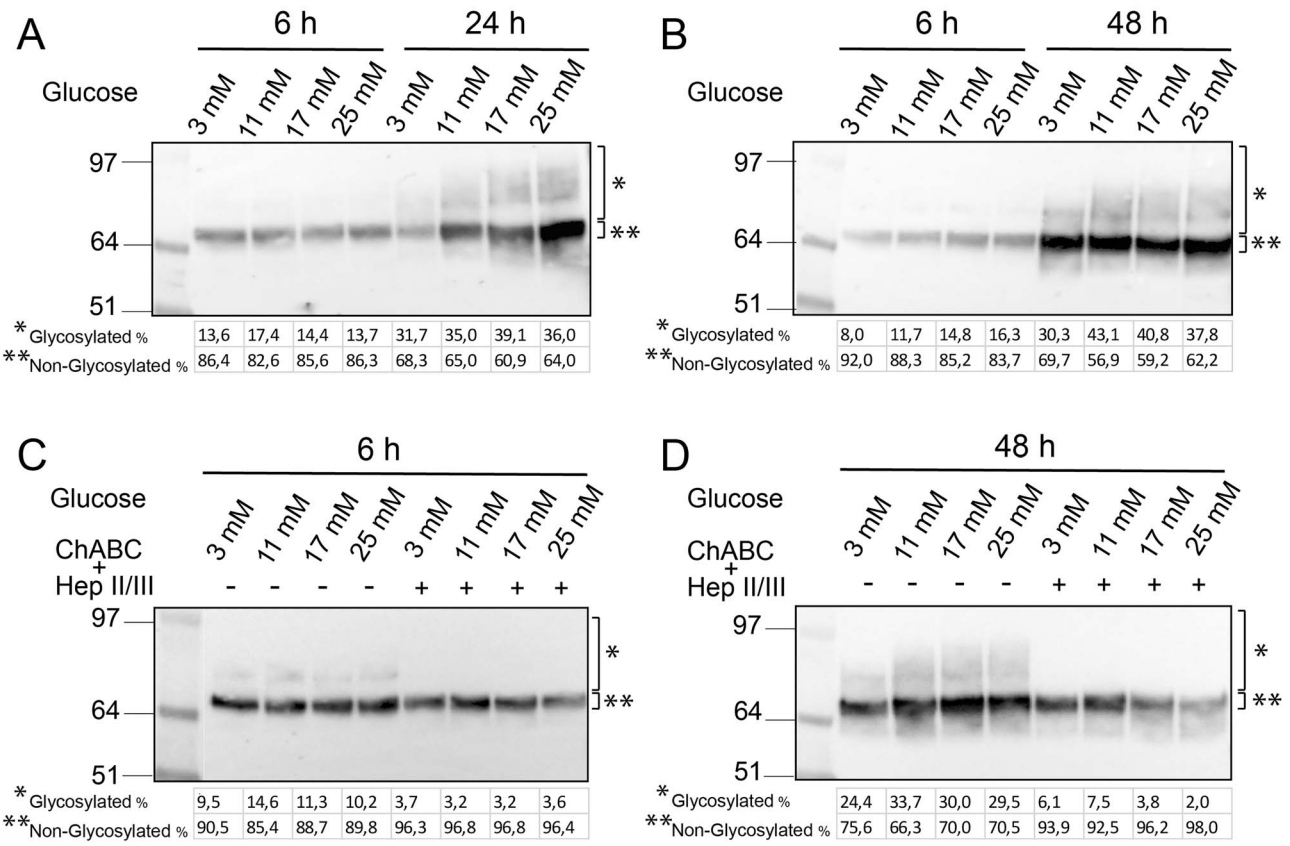

Fig. 4. Western blot analysis of chromogranin-A from INS-1 832/13 cell culture media after cultivation with different concentrations of glucose and different incubation times. Cells were incubated in 3, 11, 17 or 25 mM concentrations of glucose and the cell culture media collected after 6, 24 and 48 h (**A**, **B**). Western blot analysis was done with or without ChABC and heparinases II and III (Hep II/III) depolymerization on the fractions collected after 6 and 48 h (**C**, **D**). The numbers under the blots represent the relative distribution of glycosylated and nonglycosylated CgA isoforms (**A**-**D**).

estimate the relative distributions of CS and HS glycans in these cells.

Identification of additional PGs in human and rodent insulinoma cells

Several additional CS-glycopeptides carrying different modifications were identified in the media and cells of the three cell lines studied (Table I). We could thus identify a glycopeptide of IAPP (24-TPVGSGTNPQVDK-36) with a CS hexasaccharide carrying 0–2 modifications of sulfate and phosphate from rat INS-1 832/13 cells (Figure 6A and B). In addition, a monosulfated linkage region CS-glycopeptide from immunoglobulin superfamily member 10 (IgSF10) (676-IDGSGLEEPKPSVLLK-691) (Figure 6C and D) and a secretogranin-2 (SCG2) glycopeptide (72-EESSPDYNPYQGVSVPLQLK-91) modified with a disulfated CS glycan were identified from mouse MIN6 cells. Furthermore, a secretogranin-1 (SCG1) glycopeptide (85-LLRDPADASGTR-96), modified with a monosulfated CS hexasaccharide linkage region, was identified from mouse NIT-1 cells. In addition, a monosulfated hexasaccharide glycopeptide of IgSF10 (669-TIEHDEDIDGSGLEEPKPSVLL-690), and a hexasaccharide glycopeptide of protein AMBP, also known as bikunin, (205-AVLPQESEGSGTEPLITGTLKK-226) modified with one sulfate group and/or one phosphate group were identified from NIT-1 mouse cells (MS^2 spectra are shown with annotations in Supplementary Figure S5).

To enable identification of any additional HSPGs from human islets or from insulinoma cell lines, trypsin-digested, SAX-enriched and heparinases II and III-treated samples were analyzed with nLC MS/MS and the resulting spectra filtered for the presence of the HS-specific oxonium ion at *m/z* 173.04. The Mascot-assisted search analysis enabled the identification of HS-glycopeptides derived from several core proteins including chromogranin-A (CgA in rat and mouse, as already described), secretogranin-1 (SCG1 in rat and mouse), syndecan-1 (SDC1 in rat), syndecan-4 (SDC4 in human and rat), neurexin-2/neurexin-2-beta (Nrxn2 in rat), neuron-derived neurotrophic factor (protein NDNF, also known as epidermacan in mouse NIT-1 cells) and testican-1 (TICN1 in NIT-1 cells) (Table I and Supplementary Table SI). SDC1, SDC4, protein NDNF and TICN1 core proteins in INS-1 832/13 and NIT-1 cells were found to carry two HS GAG chains (Supplementary Figure S5C-K). This was also true for SCD4 found in human islet cells, one of which had the same attachment site (Ser-39) as the rat SCD4 (Ser-44) (Figure 7). Interestingly, two glycopeptides of SDC1 and SDC4 core proteins in INS-1 832/13 cells were found to carry two neighboring HS GAG chains attached to Ser Gly Ser Gly motifs in highly conserved sequences found in all three species (Supplementary Figure S8).

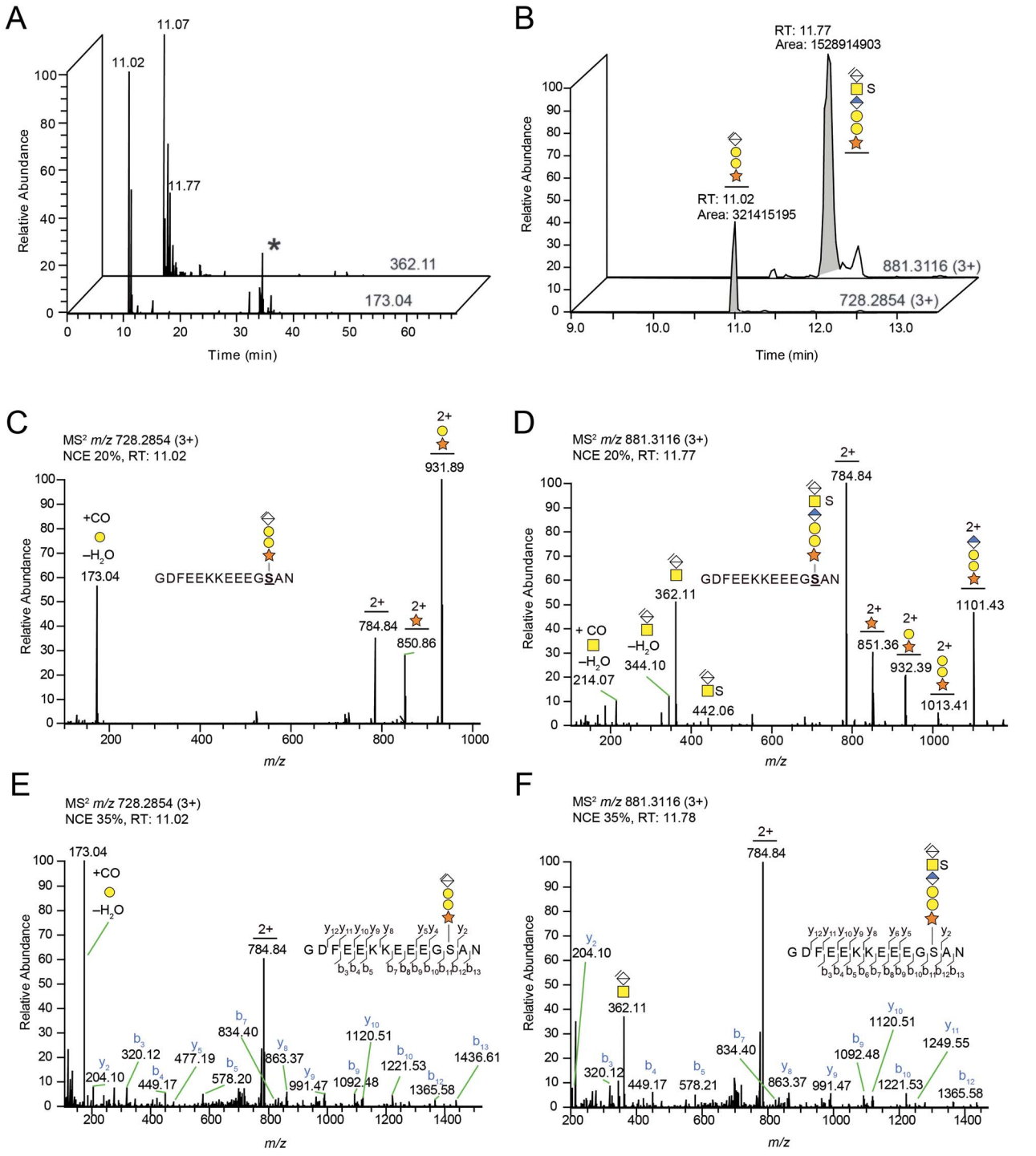

Fig. 5. Identification of chromogranin-A as a hybrid PG with one single glycosite in rat INS-1 832/13 cells. Extracted ion chromatograms (XIC) of ChABC- and heparinases II and III-depolymerized INS-1 832/13 cell culture media were obtained by filtering for the presence of the MS^2 diagnostic ions at *m/z* 362.11 and at *m/z* 173.04 (**A**). XIC of the ions of *m/z* 881.3116 (3+) and *m/z* 728.2854 (3+) utilized for comparing the areas under the peaks corresponding to the relative distribution of CS- and HS-glycopeptides of the same peptide sequence (**B**). MS^2 spectra of the HS and CS CgA glycopeptides, taken at 11.02 and 11.77 min at NCE 20% (**C**, **D**) and at NCE 35% (**E**, **F**). The asterisk in panel (**A**) indicates an unknown HS-glycopeptide eluting at 34 min. The major glycopeptide eluting at 11.07 min corresponds to a monosulfated CS-hexasaccharide linkage region attached to the peptide 422-GDFEEKKEEEGSANR-436.

Of the six different PGs identified in the INS-1 832/13 cells, and the seven different PGs identified in mouse cell lines only CgA was of the hybrid type that could carry either a CS or a HS modification.

Discussion

In the present work, we applied a glycoproteomic approach to selectively purify GAG-substituted glycopeptides, either endogenously

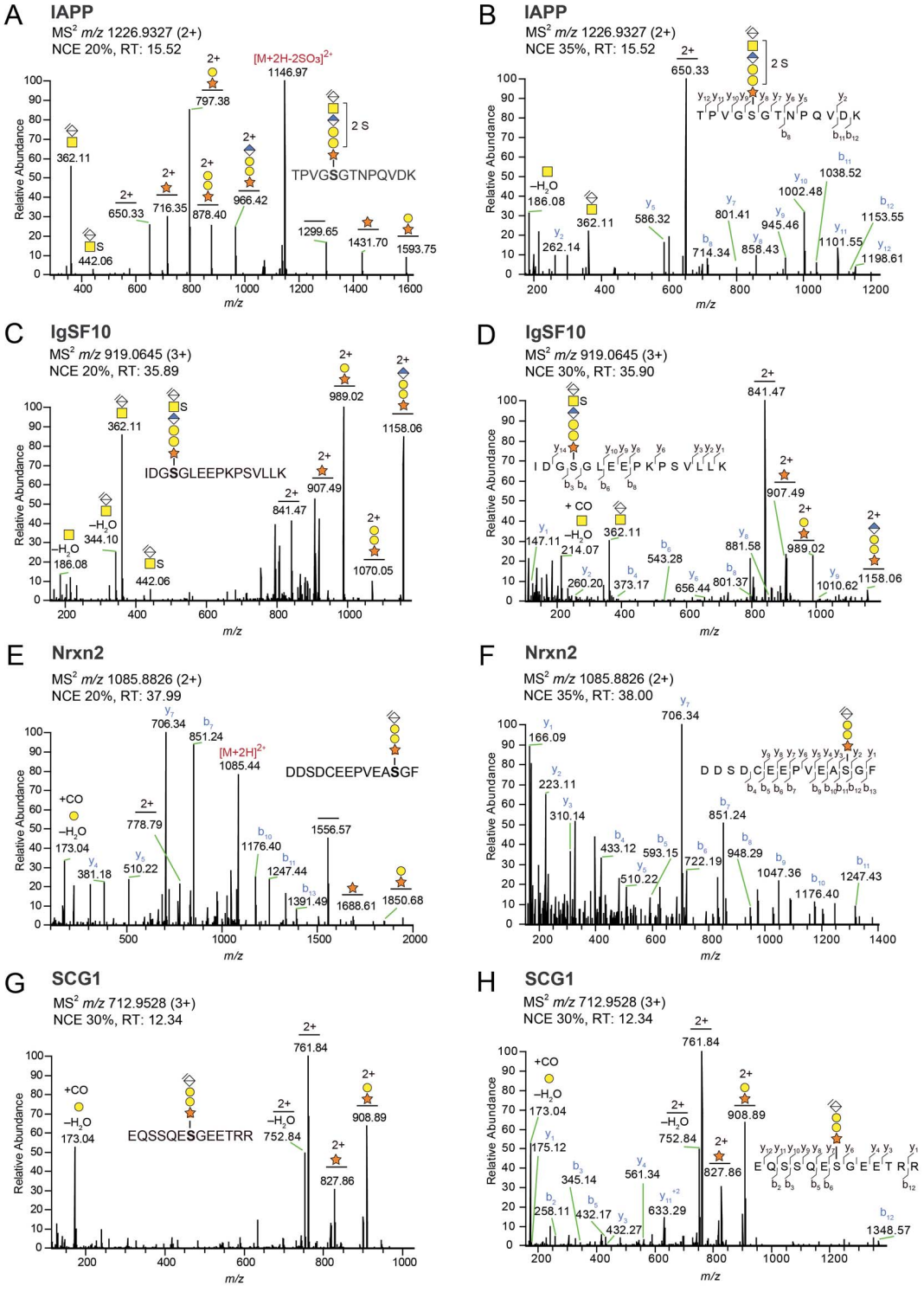

Fig. 6. MS^2 spectra showing the identification of novel CS- and HS-glycopeptides in rat INS-1 832/13, MIN6 and NIT-1 cells. The MS^2 spectra illustrate the identification of glycopeptides of islet amyloid polypeptide (**A**, **B**), neurexin-2/neurexin-2-beta (**E**, **F**) and secretogranin-1 (**G**, **H**) from rat INS-1 832/13 cells, and from a glycopeptide of immunoglobulin superfamily member 10 (**C**, **D**) from MIN6 and NIT-1 cells. The MS^2 spectra were acquired at NCE 20% for glycan fragmentation (left panels) and at NCE 35% for peptide fragmentation (right panels). The glycan and peptide fragmentation for secretogranin-1 are shown at NCE 30%, due to the lack of fragmentation at NCE 20% for this glycopeptides. Note the diagnostic ion at *m/z* 173.04 for HS-derived structures and the diagnostic ion at *m/z* 362.11 for CS-derived structures.

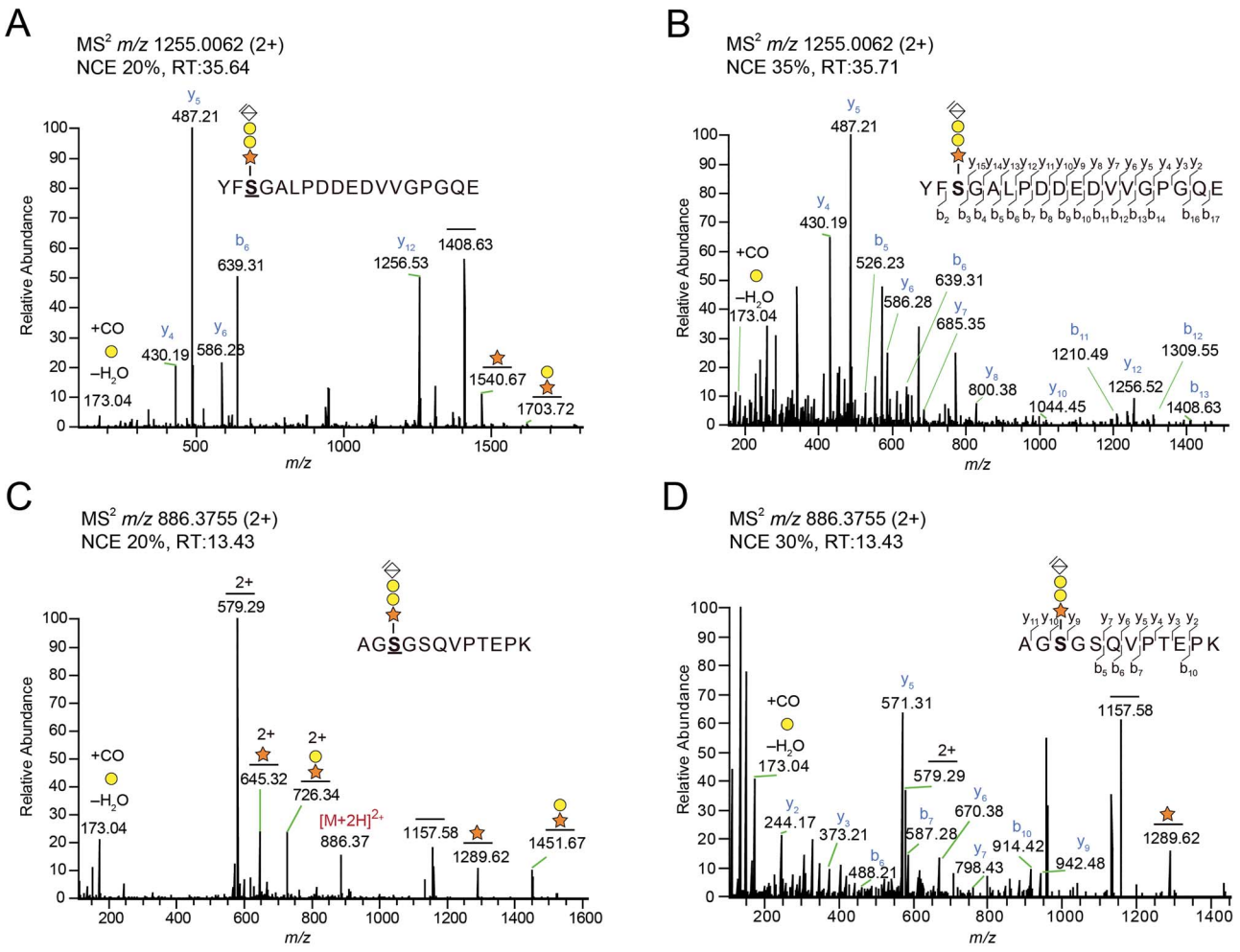

Fig. 7. Identification of two glycopeptides of human syndecan-4 modified with HS-tetrasaccharide linkage regions. MS^2 spectra of two different glycopeptides of SDC4 obtained from a human islet cell preparation, treated with both ChABC and heparinases II and III, and analyzed at NCE 20% for glycan characterization (**A**, **C**) and 30 or 35% (**B**, **D**) for peptide identification. The two precursor ions were extracted by filtering the MS^2 spectral file for the presence of the ion at *m/z* 173.04, diagnostic for HS structures. Only two major peaks appeared and the masses of the precursor ions, the glycan and peptide fragmentation patterns clearly identified HS tetrasaccharide linkage regions attached to these two glycopeptides.

formed during prohormone processing or after in vitro trypsin digestion, from human, rat and mouse insulin-secreting cell lines. The CS and HS chains were selectively depolymerized with ChABC and heparinases II and III, respectively. We performed the nLC MS/MS analysis with sequential fragmentation of precursor ions using initially a low NCE of 20% and then higher NCEs of 30–40%. At the higher NCEs the peptide parts were efficiently fragmented into fully deglycosylated b- and y-ions used for protein identification in Mascot database searches. The glycopeptide hits were then all manually verified by inspection of the MS^2 spectra especially at the NCE of 20%, where the presence of the intact peptide and the stepwise glycosidic fragmentations were used to verify the glycan and peptide identities. Also, phosphate modifications of the Xyl residue as well as modifications with sulfate groups and sialic acids were characterized manually, most often at lower NCE settings (Supplementary Figure S6).

From our results, it is obvious that many of the GAG-glycopeptides identified are "semitryptic." Our findings could thus be challenged as having a high false discovery rate (FDR). However, it is important to realize that we are here studying mainly prohormones which are subjected to endogenous proteolysis in the cells of interest. Additionally, we take advantage of the high resolution of the precursor ions obtained in MS1 and the fragmentation characteristics of the GAG-glycopeptides, verified by obligatory fragment ions of both glycans and of peptides in the manual analyses (Noborn et al. 2021), always following our database searches. Thus, employing this routine we reduce the risk of including false identities to a very low level and would rather like to underline the importance of using semitryptic searches in addition to tryptic searches when studying intracellularly processed glycopeptides.

We used pancreatic beta cells from human, rat and mouse in this work. Beta cells are located in pancreatic islets and are responsible for synthesizing, processing, storing and releasing insulin, which is the main hormone to maintain normal blood glucose levels. In this study, we structurally identified four known CSPGs (CgA, SCG1, SCG2 and protein AMBP) and two novel CSPGs (IAPP and IgSF10) together with four known HSPGs (SDC1, SDC4, protein NDNF and TICN1) and three novel HSPGs (CgA, SCG1 and Nrxn2). The identification of the three novel HSPGs was surprising as the numbers of core proteins known to carry HS are few, and until recently only 18 HSPGs have been described (Dreyfuss et al. 2009; Sarrazin et al. 2011; Xu and Esko 2014; Zhang et al. 2018). Several of the identified

PGs in this study are known as prohormones, including CgA, SCG1, SCG2 and IAPP, which is in line with our previous publication where prohormones were described as a new class of CSPGs. The finding that two prohormones (CgA and SCG1) carried HS was also unexpected, as prohormones are typically not regarded as HSPGs. These findings indicate a larger structural and functional complexity of PGs than previously perceived.

In the pancreatic beta islets, prohormones are costored, processed and then secreted into the circulation together with insulin. GAG modifications of these prohormones suggest a general function for negatively charged GAGs in the storage and/or processing of some cargo proteins within the secretory vesicles. Furthermore, the strict conservation of the glycosylation site in CgA in three different species, and the GAG glycosylation of CgA in various human tissues (beta cells, CSF and urine), indicates the importance of this GAG structure for protein function. Notably, genes encoding HSPG core proteins typically display a high degree of conservation throughout evolution (Noborn and Larson 2020). For instance, the recently identified HS site on the neurexin-1 gene is conserved from zebrafish to human (Zhang et al. 2018).

Among all identified PGs, CgA was the only one expressed in all three species studied. CgA serves as a prohormone, which upon proteolysis is cleaved into several biologically active peptides. The CgA-derived peptides such as vasostatin I, vasostatin II, pancreastatin, WE-14 and serpinin are involved in a variety of functions. Some of these peptides have similar functions while others exert contrary functions (Loh et al. 2012). Therefore, the regulation of the CgA proteolytic cleavage process is very important in order to maintain the balance of biological effects of these peptides (D'Amico et al. 2014). A study by Koshimizu et al. (2011) has shown that in endocrine (AtT-20) and neuroendocrine (PC12) cells, regulation of granule biogenesis is mediated by the C-terminus cleavage product of CgA, serpinin. In our study, we have shown that CgA is cleaved into several smaller peptides including serpinin that is released from a position in close proximity (+ 4 residues) to the GAG site that we have identified. Whether the GAG chain at this site could affect the cleavage of CgA and release of serpinin remains to be determined.

We have previously shown that CS-modified CgA glycopeptides are present in human urine and CSF (Noborn et al. 2015). Here, we confirm that in human pancreatic islets, CgA carries a GAG chain at the same glycosite (Ser-424). The CgA glycopeptides identified contain clusters of acidic amino acids in close proximity to a single Ser Ala motif, which is a variant of the consensus Ser Gly motif for GAG modifications of peptides. Additionally, in rat INS-1 832/13 cells, the combined use of ChABC and heparinases for the selective formation of hexasaccharide- and tetrasaccharide-substituted glycopeptides, respectively, enabled us to identify this attachment site (422-GDFEEKKEEEGSAN-435) in rat CgA as a "hybrid site." In addition, Ser-430 of CgA from the two mouse cell lines, MIN6 and NIT-1, was also identified as a hybrid site. The hybrid sites, which allow for the alternative presentation of either CS or HS at the same amino acid, is likely of biological importance since CS and HS often display opposite effects on cell function. For instance, it has been shown that CSPGs and HSPGs often exert opposite effects on neural extension (Coles et al. 2011; Katagiri et al. 2018). However, so far, we have detected this hybrid site in rat INS-1 832/13 and MIN6 and NIT-1 cells and not in human islets. Whether this reflects a significant functional difference for CgA among species is presently not known to us. One reason for finding only CS modifications and not HS modifications of CgA in human islets may be the limited amounts of the human samples that were available for our analysis and/or that the HS structures are considerably less abundant than the CS structures of CgA in human islets. For the rat cells, we found the ratio of CS to HS modifications to be around 85:15. Either way, we find the complexity of GAG modifications of CgA in INS-1 832/13 cells intriguing and worthy of further studies.

A major, but rarely addressed, issue in glycoproteomics is the occupancy at various sites of glycosylation. For CgA, we approached this in two ways. Using western blots of intact proteins of INS-1 832/13 cells grown at different concentrations of glucose, we found that the levels of GAG modifications of CgA were less dependent on glucose concentrations than on the actual growth period (Figure 4) and leveled off at ~35% occupancy after 24 h. In addition, the GAG occupancy in human islet cells was calculated from MS data comparing the relative intensities of the natively formed nonglycosylated vs glycosylated CgA peptide, 413-GYPEEKKEEEGSANRRPEDQELESLS-438, providing a similar value of about at least 33% occupancy. These two rough methods for estimating the relative levels of occupancy are however, both limited by their inherent weaknesses, as stated in the Result section.

The term part-time PG was coined for this phenomenon already in 1987 when Lars-Åke Fransson was studying thrombomodulin (Fransson 1987), a PG for which the antithrombotic effect has been shown to be strictly dependent on the presence of the GAG chain (Nadanaka et al. 1998; Izumikawa and Kitagawa 2015). More recently additional PGs, such as brevican, neuroglycan C and APP have also been considered as being part-time PGs (Yamada et al. 1994; Aono et al. 2004; Mihov et al. 2015).

We identified a novel CS GAG site at Ser-28 in the N-terminal domain of proIAPP in rat INS-1 832/13 cells (24-TPVGSGTNPQVDKR-37). The CS hexasaccharide was observed with and without Xyl phosphorylation and/or sulfation of the GalNAc residue. We have, however, not been able to identify a CS-glycopeptide of proIAPP either in the human beta cells or in the mouse cell lines (NIT-1 and MIN6). For the human and mouse proIAPP amino acid sequences, there are no typical CS- or HS-consensus motifs as found in the N-terminal domain of the rat sequence (Gly Ser Gly). This could explain why we detected the CS GAG site only in rat cells but not in human or mouse cells (Supplementary Figure S7).

We also identified a novel core protein, IgSF10 with a CS hexasaccharide in the mouse MIN6 and NIT-1 beta cells. This protein (2594 amino acids long) has several Gly Ser and Ser Gly motifs but just one Gly Ser Gly motif which is the one site decorated with a CS hexasaccharide (676-IDGSGLEEPKPSVLLK-691 in MIN6 cells and 669-TIEHDEDIDGSGLEEPKPSVLL-690 in NIT-1 cells). Alignment of human, rat and mouse IgSF10 shows that the Gly Ser Gly glycosite is a conserved site, but we did not observe the corresponding CS-glycopeptides in the human and rat samples. IgSF10 is known to be an *N*-glycosylated cell surface protein that has a role in gonadotropin-releasing hormone GnRH neuronal migration (Howard et al. 2016).

We additionally identified GAG-modified glycopeptides of the prohormone SCG1 from rat and mouse cell lines with either HS or CS GAG-modified sites. SCG1 is an acidic protein that belongs to the granin family. In addition to being a precursor of several bioactive peptides, SCG1 has a tendency to self-assemble into aggregates in the acidic milieu of secretory granules (Noborn et al. 2015). The study by Noborn et al. (2015) has shown that SCG1 is modified with CS chains at Ser-93 and Ser-239 in human CSF and urine samples. In this present study, we found SCG1 with an HS modification at Ser-236 (229-EQSSQESGEETR-241) in rat INS-1 832/13 cells (Figure 6G and H), which is at the same Ser residue found modified in the human CSF. In

addition, we identified a CS hexasaccharide of SCG1 in mouse NIT-1 cells at Ser-93 (85-LLRDPADASGTR-96) which corresponds to the same glycosite as already identified in human urine and CSF, again pointing to conserved GAG modification sites across species.

Another PG prohormone that we observed was secretogranin-2 (SCG2). Similarly, to CgA and SCG1, this prohormone belongs to the granin family. We found a CS modification in the 72-EESSPDYNPYQGVSVPLQLK-91 peptide from SCG2 in mouse MIN6 cells. Although the attachment site is ambiguous, this peptide harbors a novel serine glycosite, the importance of which remains to be determined.

In addition to the prohormones, we found SDC4 in human pancreatic islets and rat beta cells and SDC1 in rat beta cells, both modified with tetrasaccharide GAG linkage regions. It has earlier been shown that HS-modified SDC4 plays important role(s) in the glucose-induced insulin secretion of mouse pancreatic beta cells (Takahashi et al. 2018). We identified two HS-glycopeptides of SDC4 protein in human islet cells, one in the N-terminal domain of the SDC4 (37-YFSGALPDDEDVVGPGQE-54) and one containing a consensus glycosite at Ser-95 (93-AGSGSQVPTEPK-104) of SDC4. In rat INS-1 832/13 cells, we observed the HS GAG chains of SDC1 at Ser-44 (44-SGALPDDEDAGGLEQDSDF-62) and at Ser-65 and Ser-67 (63-ELSGSGDLDDTEEPR-77). It has been known for a long time that the Ser-39 of human SDC1 is a GAG attachment site (Esko and Zhang 1996) and recently, the 93-AGSGSQVPTEPK-104 sequence was identified as a CS-glycopeptide in human BeWo choriocarcinoma cells (Toledo et al. 2020). It has also been shown that SDC1 is uniquely localized inside the mouse beta cells, despite that HSPGs are typically expressed on cell surfaces or in intracellular matrices, or in basement membranes (Choong et al. 2015). HSPGs, including SDC1, are involved in beta cells maturation and are needed to maintain normal glucose-induced insulin secretion (Takahashi et al. 2009). Neurexin-2/neurexin-2-beta, which we identified with a HS GAG modification in INS-1 832/13 cells, is known to be expressed in INS-1 beta cells and to participate in the insulin secretory mechanism (Suckow et al. 2008). The importance of neurexin-2/neurexin-2-beta GAG modifications remains to be determined.

Another secretory protein identified in mouse NIT-1 cells was protein NDNF/epidermacan, from which we identified a glycopeptide carrying HS GAG modifications at both Ser-99 and Ser-104. NDNF is known to be expressed in brain and spinal cord of mouse and to be involved in neural functions (Kuang et al. 2010). In contrast to Ser-99, which is located in a Gly Ser motif, Ser-104 is located in a Gly Ser Gly motif, a site that is well conserved in several species. The functional significance of GAG modifications of protein NDNF remains to be clarified (Manabe et al. 2008).

In summary, we have identified a series of different CSPGs and HSPGs in human, rat and mouse insulin-secreting cells. In many cases, the GAG attachment sites are conserved in all three species and in some, such as in CgA from rat INS-1 832/13 cells, they constitute a hybrid site allowing for both CS and HS modifications. The roles of these GAG modifications for the packing, processing and secretion of peptide hormones, especially insulin in the secretory granules of islet beta cells, will be the object of future studies.

Materials and methods

Human islets

Human pancreatic islets from deceased donors were kindly provided by Prof. Olle Korsgren (Uppsala University, Sweden). Collection was performed under ethical permission of the Swedish Ethical Review Authority, Uppsala University, 2009/371 updated 20170712. The islet purity of the three preparations used were 80, 52 and 40%, respectively.

Cell culture

Rat INS-1 832/13 (Hohmeier et al. 2000) and mouse MIN6 (Ishihara et al. 1993) insulin-secreting cell lines were kindly provided by Prof. Lena Eliasson (Lund University, Sweden). INS-1 832/13 cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS), 10 mM HEPES, 100 IU/mL penicillin, 100 µg/mL streptomycin and 50 µM 2-mercaptoethanol. Mouse MIN6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 20 mM HEPES, and 50 µM 2-mercaptoethanol. Mouse NIT-1 insulinoma cells were kindly provided by Prof. Susanna Cardell (Gothenburg University, Sweden) (Hamaguchi et al. 1991). NIT-1 cells were cultured in Ham's F12K medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 10% heat-inactivated FBS. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2, and routinely verified as mycoplasma free. All cell culture reagents were purchased form Thermo Fischer Scientific (Waltham, MA, USA), except FBS, penicillin streptomycin, protease inhibitor cocktail and 2-mercaptoethanol, which were purchased from Sigma Aldrich (St. Louis, MO).

Sample preparation of CS/HS-glycopeptides

For preparation of human islet samples, ~0.5–1.0 x 10^5 cells with 40–80% islet purity was used for each experiment. For preparation of rat and mouse cell samples, ~4–5 x 10^7 cells were used for each experiment. Cells were cultured until confluence, the culture media were removed, and the cells were washed three times with PBS to remove the FBS. Fresh media without FBS were added to the cell cultures and the cells were incubated at 37°C for 16 h. The culture media then were collected and centrifuged at 200 x g for 5 min, the supernatants lyophilized and desalted with PD10 desalting column and stored at −20°C until use. Cells were detached using trypsin EDTA, washed with PBS and stored at −80°C until use. Cells were lysed by RIPA buffer containing protease inhibitor cocktail. All lysates were incubated on ice for 30 min and cleared by centrifugation at 14,000 x g for 15 min at 4°C. Enrichment of glycopeptides from cell lysate and/or cell culture media were performed as previously described (Noborn et al. 2015). Briefly, the samples (cell and/or cell culture media) were reduced (5 mM DTT) and alkylated (15 mM iodoacetamide). The protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA), and samples were subjected to trypsin digestion overnight (37°C) with sequencing grade modified trypsin (Cat: V5111, which is treated with TPCK (L-1-Tosylamide-2-phenylethyl chloromethyl ketone), Promega) to a protein ratio in the range of 1:20–1:100 (w/w). The trypsin-digested samples were then enriched for GAG-glycopeptides using SAX-chromatography (Vivapure, Q-mini H), as described previously (Gomez Toledo et al. 2015). The collected fractions from the SAX step were desalted and individually subjected to enzymatic depolymerization at 37°C for 16 h. For CS depolymerization, samples were incubated with 10 mU ChABC (C3667, Sigma-Aldrich) in digestion buffer (50 mM NH_4OAc, pH 8.0). Since this enzyme depolymerizes

both CS and CS/DS structures, irrespective of the presence or absence of IdoA residues in the GAG chains, we have in this paper and until we have provided data for the presence of IdoA in the chains, decided to refer to the PGs/GAGs as CSPGs/CS GAGs, respectively. For HS depolymerization, samples were incubated with 5 mU heparinase II (no EC number) and 5 mU heparinase III (EC 4.2.2.8) (both from *Pedobacter heparinus* overexpressed in *Escherichia coli*; kind gift from Prof. Jian Liu, University of North Carolina, USA) in HS digestion buffer (50 mM NH_4OAc, 4 mM $CaCl_2$, pH 7.2–7.4). The samples were again desalted using C18 spin columns (8 mg resin, Pierce) according to the manufacturer's protocol, dried and stored at −20°C until MS-analysis.

In one series of experiments, INS-1 832/13 cell media were treated simultaneously with trypsin and chymotrypsin (Sequencing Grade, Cat: V1061, Promega) overnight (37°C) and after enrichment for GAG-glycopeptides using the SAX-chromatography step, the GAGs were depolymerized as above with either ChABC, heparinases II and III or with the combination of these three enzymes in HS digestion buffer. The glycopeptides identified uniquely in this experiment are shaded in Table I and Supplementary Table SI.

LC MS/MS analysis

The samples were analyzed on an Orbitrap Fusion Tribrid MS coupled to an Easy-nLC 1200 system (Thermo Scientific, Waltham, MA, USA), as previously described (Noborn et al. 2015). Briefly, glycopeptides (3 µL injection volume) were trapped on an Acclaim PepMap C18 precolumn (20 × 0.1 mm I.D., 5 µm) and separated on an analytical column (350 × 0.075 mm I.D.) packed in-house with 3 µm Reprosil-Pur C18-AQ particles (Dr. Maisch GmbH, Ammerbuch, Germany). The following gradient was run at 300 nL/min: 10–50% B-solvent (80% acetonitrile, 0.2% formic acid in dH_2O) in A-solvent (0.2% formic acid in dH_2O) over 60 min, 50–100% B over 5 min, with a final hold at 100% B for 10 min. The column was coupled to a Flex nanospray ion source operating at 1.8 kV in positive ionization mode. MS scans were performed at 120,000 resolution (at *m/z* 200), an automatic gain control (AGC) target value of 10^6 with a mass range of *m/z* 600–2000. MS^2 analysis was performed in a data-dependent mode with the 3 s duty cycle time ("top-speed" setting) and the dynamic exclusion for 10 s, the most abundant doubly or multiply charged precursor ions in each MS scan were fragmented by HCD at NCE levels of 20, 30, 35 and 40%. MS^2 scans at each HCD energy level were acquired separately using the following parameters: one microscan performed at 30,000 resolution (at *m/z* 200), AGC 5.0 x 10^4 with a maximal injection time of 60 ms, a fixed first mass of *m/z* 100, an isolation window of 2.5 Da, maximum and minimum intensity threshold of 1.0 x 10^{20} and 1.0 x 10^4, respectively.

Data processing

The MS data were processed using Mascot Distiller and database searches for GAG-glycopeptides were performed as previously described (Gomez Toledo et al. 2015). Briefly, Mascot distiller (version 2.6.1.0, Matrix Science, London, UK) was used for the conversion of the HCD.raw spectra into singly protonated peak lists in Mascot. mgf format. Database searches were performed using an in-house Mascot server (version 2.5.1) with the enzyme specificity set to Trypsin, and then to Semitrypsin, allowing for up to two missed cleavages, in subsequent searches on rat, mouse and human sequences of the Swissprot database. The setting to Semitrypsin is important since CgA, and many other of the prohormones of these cells, are known to be endogenously processed into smaller peptides (Figure 3) often containing only one trypsin cleavage site. This natural processing of proteins thus contributes significantly to the high percentage of semitryptic peptides and glycopeptides identified in our study. The following constraints were included: peptide tolerance, 10 ppm; fragment tolerance 20 ppm; fixed carbamidomethyl modifications of Cys residues and variable Met oxidation. For glycopeptide identifications, variable modifications of Ser residues with the residual hexasaccharide structure [HexA(–H_2O)HexNAcHexAHexHexXyl-O-] without ($C_{37}H_{55}NO_{30}$, 993.2809 u), with one ($C_{37}H_{55}NO_{33}S$, 1073.2377 u), or with two ($C_{37}H_{55}NO_{36}S_2$, 1153.1945 u) sulfate groups attached. Additionally, searches were allowed to include variable modifications at Ser residues of a residual tetrasaccharide structure [HexA(–H_2O)HexHexXyl-O-] without ($C_{23}H_{34}O_{19}$, 614.169429 u) or with one phosphate group attached ($C_{23}H_{35}O_{22}P$, 694.135760 u). Moreover, neutral losses of the corresponding glycan masses were implemented in the modification settings. Sulfate and phosphate groups, with very similar masses of 79.9568 and 79.9663 u, respectively, were distinguished by manual evaluation of the MS^2 spectra. Additional modifications were allowed for phosphorylation at Ser, Thr and Tyr residues. We manually evaluated all the Mascot-generated GAG-glycopeptide hits according to previously established criteria (Gomez Toledo et al. 2015; Noborn et al. 2021). In summary, MS^2 spectra at low NCE setting (20%) had to include the presence of the fully deglycosylated peptide ion together with the peptide+Xyl ion and peptide+XylGal ion. Thus the loss in mass from the precursor to the peptide ion had to match the proposed glycan composition. Moreover, MS^2 spectra at higher NCE (30–40%) had to include the oxonium ion at *m/z* 362.10 $[HexA(–H_2O)GalNAc]^+$ for the hexasaccharide modifications, and the ion at *m/z* 173.04 $[Gal(–H_2O) + CO]^+$ for the tetrasaccharide modifications. Also, at least three b-ions and three y-ions had to be present in order to verify the peptide start and end residues. In order to identify *N*- and *O*-glycosylation sites, additional MS/MS database searches were done with the Byonic software (Protein metrics). Glycan modifications "57 *N*-glycans from plasma proteins" and "6 most common *O*-glycans" were allowed. A Byonic score cut off of 200 was used for glycopeptide hits and additional glycoforms sharing the same peptide sequence from the same LC MS/MS run were included even if the scores were much lower. Lists of peptide fragmentation ions were acquired with the MS tool at Protein Prospector (http://prospector.ucsf.edu/prospector/).

Glucose experiment

To investigate the effect of glucose concentration on the GAG occupancy of CgA, INS-1 832/13 cells were exposed to low and high glucose concentrations. The cells were initially cultured at 11.1 mM glucose concentration and allowed to grow to 50% confluence. Then, the cell culture media were removed, and cells were rinsed twice with PBS. The cells were incubated in media without glucose for 16 h, followed by incubation with media with increasing concentrations of glucose; 3, 11.1, 17 and 25 mM. After 6, 24 or 48 h, the media were collected and stored directly at −20°C until western blot analysis.

Western blotting

Sample protein concentrations were determined using the BCA protein assay kit. For CS and HS depolymerizations, samples were incubated with ChABC (10 mU) and heparinases II and III (10 mU of each), respectively, in 20 µL digestion buffer for 16 h at 37°C. The incubations were terminated by heat-inactivation of the samples

for 5 min. Approximately 20 µg of protein was used. Samples were combined with Laemmli sample buffer and subsequently heated for 10 min at 95°C. Samples were then separated on Bolt 8% Bis-Tris plus (Thermo Fischer Scientific) precast polyacrylamide gel and blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). The membranes were blocked using 5% nonfat milk in TBS containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Blots were incubated overnight at 4°C with primary rabbit polyclonal antibody against CgA (1:1000 dilution, ab15160, Abcam). The membranes were washed with TBS-T prior to incubating with the horseradish peroxidase–conjugated secondary goat anti-rabbit IgG antibody (1:5000 dilution, cat no. 7074 s, Cell signaling) at room temperature for 1 h. Blots were then washed in TBS-T and developed using the Supersignal West Dura Extended Duration Substrate detection system (Thermo Fischer Scientific). The relative distribution of CgA isoforms was determined from the band intensities using Image Lab software from Bio-Rad and normalized to the total amount of CgA in each lane.

Supplementary data

Supplementary data are available at *Glycobiology* online.

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Data availability

MS data have been deposited to the ProteomeXchange consortium via the PRIDE partner repository with the dataset identifier PXD024230.

Conflict of interest statement

The authors declare no conflict of interest.

Abbreviations

CS/DS: chondroitin/dermatan sulfate; CgA: chromogranin-A; GAG: glycosaminoglycan; GlcA: glucuronic acid; GalNAc: N-acetylgalactosamine; Gal: galactose; HCD: higher energy collision dissociation; HexA: hexuronic acid; IdoA: iduronic acid; NCE: normalized collision energy; Neu5Ac: N-acetylneuraminic acid; PG: proteoglycan; Xyl: xylose

References

Aono S, Tokita Y, Shuo T, Yamauchi S, Matsui F, Nakanishi K, Hirano K, Sano M, Oohira A. 2004. Glycosylation site for chondroitin sulfate on the neural part-time proteoglycan, neuroglycan C. *J Biol Chem*. 279:46536–46541.

Beranova-Giorgianni S, Zhao Y, Desiderio DM, Giorgianni F. 2006. Phosphoproteomic analysis of the human pituitary. *Pituitary*. 9:109–120.

Bishop JR, Schuksz M, Esko JD. 2007. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*. 446:1030–1037.

Chen YC, Taylor AJ, Verchere CB. 2018. Islet prohormone processing in health and disease. *Diabetes Obes Metab*. 20:64–76.

Choong FJ, Freeman C, Parish CR, Simeonovic CJ. 2015. Islet heparan sulfate but not heparan sulfate proteoglycan core protein is lost during islet isolation and undergoes recovery post-islet transplantation. *Am J Transplant*. 15:2851–2864.

Coles CH, Shen Y, Tenney AP, Siebold C, Sutton GC, Lu W, Gallagher JT, Jones EY, Flanagan JG, Aricescu AR. 2011. Proteoglycan-specific molecular switch for RPTPsigma clustering and neuronal extension. *Science*. 332:484–488.

D'Amico MA, Ghinassi B, Izzicupo P, Manzoli L, Di Baldassarre A. 2014. Biological function and clinical relevance of chromogranin A and derived peptides. *Endocr Connect*. 3:R45–R54.

Dreyfuss JL, Regatieri CV, Jarrouge TR, Cavalheiro RP, Sampaio LO, Nader HB. 2009. Heparan sulfate proteoglycans: Structure, protein interactions and cell signaling. *An Acad Bras Cienc*. 81:409–429.

Esko JD, Zhang L. 1996. Influence of core protein sequence on glycosaminoglycan assembly. *Curr Opin Struct Biol*. 6:663–670.

Fransson LA. 1987. Structure and function of cell-associated proteoglycans. *Trends Biochem Sci*. 12:406–411.

Fricker LD. 2012. *Neuropeptides and Other Bioactive Peptides: From Discovery to Function*. San Rafael (California): Morgan & Claypool Life Sciences.

Gadroy P, Stridsberg M, Capon C, Michalski JC, Strub JM, Van Dorsselaer A, Aunis D, Metz-Boutigue MH. 1998. Phosphorylation and O-glycosylation sites of human chromogranin A (CGA79-439) from urine of patients with carcinoid tumors. *J Biol Chem*. 273:34087–34097.

Gomez Toledo A, Nilsson J, Noborn F, Sihlbom C, Larson G. 2015. Positive mode LC-MS/MS analysis of chondroitin sulfate modified glycopeptides derived from light and heavy chains of the human inter-alpha-trypsin inhibitor complex. *Mol Cell Proteomics*. 14:3118–3131.

Hamaguchi K, Gaskins HR, Leiter EH. 1991. NIT-1, a pancreatic beta-cell line established from a transgenic NOD/Lt mouse. *Diabetes*. 40:842–849.

Hansen LH, Madsen TD, Goth CK, Clausen H, Chen Y, Dzhoyashvili N, Iyer SR, Sangaralingham SJ, Burnett JC Jr, Rehfeld JF, et al. 2019. Discovery of O-glycans on atrial natriuretic peptide (ANP) that affect both its proteolytic degradation and potency at its cognate receptor. *J Biol Chem*. 294:12567–12578.

Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. 2000. Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes*. 49:424–430.

Hook V, Funkelstein L, Lu D, Bark S, Wegrzyn J, Hwang SR. 2008. Proteases for processing proneuropeptides into peptide neurotransmitters and hormones. *Annu Rev Pharmacol Toxicol*. 48:393–423.

Howard SR, Guasti L, Ruiz-Babot G, Mancini A, David A, Storr HL, Metherell LA, Sternberg MJ, Cabrera CP, Warren HR, et al. 2016. IGSF10 mutations dysregulate gonadotropin-releasing hormone neuronal migration resulting in delayed puberty. *EMBO Mol Med*. 8:626–642.

Ishihara H, Asano T, Tsukuda K, Katagiri H, Inukai K, Anai M, Kikuchi M, Yazaki Y, Miyazaki JI, Oka Y. 1993. Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia*. 36:1139–1145.

Izumikawa T, Kitagawa H. 2015. Amino acid sequence surrounding the chondroitin sulfate attachment site of thrombomodulin regulates chondroitin polymerization. *Biochem Biophys Res Commun*. 460:233–237.

Katagiri Y, Morgan AA, Yu P, Bangayan NJ, Junka R, Geller HM. 2018. Identification of novel binding sites for heparin in receptor protein-tyrosine phosphatase (RPTPsigma): Implications for proteoglycan signaling. *J Biol Chem*. 293:11639–11647.

Kim T, Loh YP. 2006. Protease nexin-1 promotes secretory granule biogenesis by preventing granule protein degradation. *Mol Biol Cell*. 17:789–798.

Kjellen L, Lindahl U. 1991. Proteoglycans: Structures and interactions. *Annu Rev Biochem*. 60:443–475.

Koshimizu H, Cawley NX, Kim T, Yergey AL, Loh YP. 2011. Serpinin: A novel chromogranin A-derived, secreted peptide up-regulates protease nexin-1 expression and granule biogenesis in endocrine cells. *Mol Endocrinol*. 25:732–744.

Kuang XL, Zhao XM, Xu HF, Shi YY, Deng JB, Sun GT. 2010. Spatio-temporal expression of a novel neuron-derived neurotrophic factor (NDNF) in mouse brains during development. *BMC Neurosci*. 11:137.

Lietz CB, Toneff T, Mosier C, Podvin S, O'Donoghue AJ, Hook V. 2018. Phosphopeptidomics reveals differential phosphorylation states and novel SxE phosphosite motifs of neuropeptides in dense core secretory vesicles. *J Am Soc Mass Spectrom*. 29:935–947.

Lindahl U, Couchman J, Kimata K, Esko JD. 2015. Proteoglycans and sulfated glycosaminoglycans. In: rd VA, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Darvill AG, Kinoshita T, Packer NH, et al., editors. *Essentials of Glycobiology*: Cold Spring Harbor (NY). p. 207–221.

Loh YP, Cheng Y, Mahata SK, Corti A, Tota B. 2012. Chromogranin A and derived peptides in health and disease. *J Mol Neurosci*. 48:347–356.

Lu H, McDowell LM, Studelska DR, Zhang L. 2010. Glycosaminoglycans in human and bovine serum: Detection of twenty-four heparan sulfate and chondroitin sulfate motifs including a novel sialic acid-modified chondroitin sulfate linkage hexasaccharide. *Glycobiol Insights*. 2010:13–28.

Lukinius A, Wilander E, Eriksson B, Oberg K. 1992. A chromogranin peptide is co-stored with insulin in the human pancreatic islet B-cell granules. *Histochem J*. 24:679–684.

Lundby A, Secher A, Lage K, Nordsborg NB, Dmytriyev A, Lundby C, Olsen JV. 2012. Quantitative maps of protein phosphorylation sites across 14 different rat organs and tissues. *Nat Commun*. 3:876.

Malmstrom A, Bartolini B, Thelin MA, Pacheco B, Maccarana M. 2012. Iduronic acid in chondroitin/dermatan sulfate: Biosynthesis and biological function. *J Histochem Cytochem*. 60:916–925.

Manabe RI, Tsutsui K, Yamada T, Kimura M, Nakano I, Shimono C, Sanzen N, Furutani Y, Fukuda T, Oguri Y, et al. 2008. Transcriptome-based systematic identification of extracellular matrix proteins. *Proc Natl Acad Sci USA*. 105:12849–12854.

Mihov D, Raja E, Spiess M. 2015. Chondroitin sulfate accelerates trans-golgi-to-surface transport of proteoglycan amyloid precursor protein. *Traffic*. 16:853–870.

Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K. 1990. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: Special reference to expression of glucose transporter isoforms. *Endocrinology*. 127:126–132.

Nadanaka S, Kitagawa H, Sugahara K. 1998. Demonstration of the immature glycosaminoglycan tetrasaccharide sequence GlcAbeta1-3Galbeta1-3Galbeta1-4Xyl on recombinant soluble human alpha-thrombomodulin. An oligosaccharide structure on a "part-time" proteoglycan. *J Biol Chem*. 273:33728–33734.

Nilsson J, Noborn F, Gomez Toledo A, Nasir W, Sihlbom C, Larson G. 2017. Characterization of glycan structures of chondroitin sulfate-glycopeptides facilitated by sodium ion-pairing and positive mode LC-MS/MS. *J Am Soc Mass Spectrom*. 28:229–241.

Noborn F, Gomez Toledo A, Green A, Nasir W, Sihlbom C, Nilsson J, Larson G. 2016. Site-specific identification of heparan and chondroitin sulfate glycosaminoglycans in hybrid proteoglycans. *Sci Rep*. 6:34537.

Noborn F, Gomez Toledo A, Sihlbom C, Lengqvist J, Fries E, Kjellen L, Nilsson J, Larson G. 2015. Identification of chondroitin sulfate linkage region glycopeptides reveals prohormones as a novel class of proteoglycans. *Mol Cell Proteomics*. 14:41–49.

Noborn F, Larson G. 2020. Characterization of *C. elegans* chondroitin proteoglycans and their large functional and structural heterogeneity; evolutionary aspects on structural differences between humans and the nematode. *Adv Exp Med Biol*. 21:155–170.

Noborn F, Nikpour M, Persson A, Silhlbom C, Nilsson J, Larson G. 2021. A glycoproteomic approach to identify novel proteoglycans. *Methods Mol Biol*. (In press).

Prydz K. 2015. Determinants of glycosaminoglycan (GAG) structure. *Biomolecules*. 5:2003–2022.

Sarrazin S, Lamanna WC, Esko JD. 2011. Heparan sulfate proteoglycans. *Cold Spring Harb Perspect Biol*. 3:a004952.

Seidah NG, Chretien M. 1999. Proprotein and prohormone convertases: A family of subtilases generating diverse bioactive polypeptides. *Brain Res*. 848:45–62.

Skelin M, Rupnik M, Cencic A. 2010. Pancreatic beta cell lines and their applications in diabetes mellitus research. *ALTEX*. 27:105–113.

Suckow AT, Comoletti D, Waldrop MA, Mosedale M, Egodage S, Taylor P, Chessler SD. 2008. Expression of neurexin, neuroligin, and their cytoplasmic binding partners in the pancreatic beta-cells and the involvement of neuroligin in insulin secretion. *Endocrinology*. 149:6006–6017.

Sugahara K, Mikami T, Uyama T, Mizuguchi S, Nomura K, Kitagawa H. 2003. Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. *Curr Opin Struct Biol*. 13:612–620.

Takahashi I, Noguchi N, Nata K, Yamada S, Kaneiwa T, Mizumoto S, Ikeda T, Sugihara K, Asano M, Yoshikawa T, et al. 2009. Important role of heparan sulfate in postnatal islet growth and insulin secretion. *Biochem Biophys Res Commun*. 383:113–118.

Takahashi I, Yamada S, Nata K. 2018. Effects of heparan sulfate proteoglycan syndecan-4 on the insulin secretory response in a mouse pancreatic beta-cell line, MIN6. *Mol Cell Endocrinol*. 470:142–150.

Thaysen-Andersen M, Packer NH, Schulz BL. 2016. Maturing glycoproteomics technologies provide unique structural insights into the N-glycoproteome and its regulation in health and disease. *Mol Cell Proteomics*. 15:1773–1790.

Toledo AG, Pihl J, Spliid CB, Persson A, Nilsson J, Pereira MA, Gustavsson T, Choudhary S, Oo HZ, Black PC, et al. 2020. An affinity-chromatography and glycoproteomics workflow to profile the chondroitin sulfate proteoglycans that interact with malarial VAR2CSA in the placenta and in cancer. *Glycobiology*. 30:989–1002.

Varki A, Cummings RD, Aebi M, Packer NH, Seeberger PH, Esko JD, Stanley P, Hart G, Darvill A, Kinoshita T, et al. 2015. Symbol nomenclature for graphical representations of glycans. *Glycobiology*. 25:1323–1324.

Wollam J, Mahata S, Riopel M, Hernandez-Carretero A, Biswas A, Bandyopadhyay GK, Chi NW, Eiden LE, Mahapatra NR, Corti A, et al. 2017. Chromogranin A regulates vesicle storage and mitochondrial dynamics to influence insulin secretion. *Cell Tissue Res*. 368:487–501.

Xiao H, Sun F, Suttapitugsakul S, Wu R. 2019. Global and site-specific analysis of protein glycosylation in complex biological systems with mass spectrometry. *Mass Spectrom Rev*. 38:356–379.

Xu D, Esko JD. 2014. Demystifying heparan sulfate-protein interactions. *Annu Rev Biochem*. 83:129–157.

Yamada H, Watanabe K, Shimonaka M, Yamaguchi Y. 1994. Molecular-cloning of brevican, a novel brain proteoglycan of the aggrecan versican family. *J Biol Chem*. 269:10119–10126.

Zhang P, Lu H, Peixoto RT, Pines MK, Ge Y, Oku S, Siddiqui TJ, Xie Y, Wu W, Archer-Hartmann S, et al. 2018. Heparan sulfate organizes neuronal synapses through neurexin partnerships. *Cell*. 174:1450–1464 e1423.