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Isoform-specific O-glycosylation by murine UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase-T3, *in vivo*

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Multiple isoforms of UDP-GalNAc:polypeptide N-acetylgalactosaminyl- transferase (ppGaNTase) have been cloned and expressed from a variety of organisms. In general, these isoforms display different patterns of tissue-specific expression, but exhibit overlapping substrate specificities, *in vitro*. A peptide substrate, derived from the sequence of the V3 loop of the HIV gp120 protein (HIV peptide), has previously been shown to be glycosylated *in vitro* exclusively by the ppGaNTase-T3 (Bennett *et al.*, 1996). To determine if this isoform-specificity is maintained *in vivo*, we have examined the glycosylation of this substrate when it is expressed as a reporter peptide (rHIV) in a cell background (COS7 cells) which lacks detectable levels of the ppGaNTase-T3. Glycosylation of rHIV was greatly increased by coexpression of a recombinant ppGaNTase-T3. Overexpression of ppGaNTase-T1 yielded only partial glycosylation of the reporter. We have also determined that the introduction of a proline residue at the +3 position flanking the potential glycosylation site eliminated ppGaNTase-T3 selectivity toward rHIV observed both *in vivo* and *in vitro*.

Key words: glycosyltransferases/mucins/O-glycosylation

Introduction

The addition of the first sugar in mucin-type O-glycosylation is directed by the family of UDP GalNAc:polypeptide N-acetylgalactosaminyl-transferases (EC 2.4.1.41) (ppGaNTases). To date, five isoforms of the mammalian ppGaNTase family have been cloned and functionally expressed (ppGaNTase-T5, Ten Hagen *et al.*, unpublished observations; ppGaNTase-T4, Hagen *et al.*, 1997; ppGaNTase-T3, Bennett *et al.*, 1996; White *et al.*, 1996; Zara *et al.*, 1996; ppGaNTase-T2, White *et al.*, 1996; ppGaNTase-T1, Hagen *et al.*, 1993; Homa *et al.*, 1993). In general, these isoforms differ in their tissue-specific expression but display overlapping substrate specificities as measured by *in vitro* assays.

The complexity of the ppGaNTase gene family suggests that isoform-specific substrates must exist and that their glycosylation is biologically significant. However, few examples of unique ppGaNTase specificity have been demonstrated thus far. These include glycosylation of the sequence VTHPGY in fibronectin (Matsuura *et al.*, 1989), which has subsequently been shown to be glycosylated *in vitro* by ppGaNTase-T3 (H. Clausen, personal communication); and the *in vitro* glycosylation of the peptide CIRIQRGPGRFVTIGKIGNMR, derived from the V3 loop of the HIV gp120 protein (Bennett *et al.*, 1996) by ppGaNTase-T3. However, our previous work has shown that peptide substrates which are poorly glycosylated *in vitro*, may be glycosylated with high efficiency *in vivo* (Nehrke *et al.*, 1996, 1997).

To ascertain whether ppGaNTase-T3 specific glycosylation could be detected *in vivo*, we have taken advantage of COS7 cells, which lack detectable levels of ppGaNTase-T3 transcript (Nehrke *et al.*, 1997). When a peptide derived from the V3 loop of the HIV gp 120 protein (HIV) is expressed as a reporter-peptide (rHIV) in COS7 cells, it is poorly glycosylated. However, when rHIV is coexpressed with recombinant mouse ppGaNTase-T3, the reporter peptide becomes more fully occupied with O-glycan. We also analyzed mutants of rHIV to define positions in the sequences flanking the glycosylation site which determine isoform-specificity of ppGaNTase-T3. Our results clearly demonstrate that, despite overlapping specificities, unique amino acid preferences define -T3 and -T1 ppGaNTase isoforms, both *in vitro* and *in vivo*.

Results

The effect of ppGaNTase-T3 expression on the in vivo *glycosylation of rHIV in COS7 cells*

Reporter rHIV, consisting of peptide RGPGRAFVTIGKIGNMR, followed by a 6x His tag, a heart muscle kinase site, and the FLAG antibody recognition octamer on the carboxyl terminus (Nehrke *et al.*, 1996) was expressed in COS7 cells. Approximately 70% of the rHIV reporter was expressed as a secreted peptide with an apparent molecular mass of 4.0 kDa; this corresponds to unglycosylated peptide since rHIV +0G (rHIV in which glycine was substituted for threonine) has the same apparent mass (Figure 1, compare lanes 1 and 3). Approximately 30% of the reporter migrated with an apparent molecular mass of 5.5 kDa. This size shift was the result of O-glycosylation, as determined by glycosidase digestion (data not shown). The smaller species seen in Figure 1 are likely to be proteolytic degradation products of the reporter which is thought to be more susceptible in its naked (unglycosylated) form (Nehrke *et al.*, 1996, 1997).

COS7 cells do not express detectable amounts of ppGaNTase-T3 transcript (Nehrke *et al.*, 1997). We therefore modified the repertoire of COS7 cell ppGaNTases by transfection of a full-length ppGaNTase-T3 expression construct. Successful transfections were confirmed by the appearance of ppGaNTase-T3 activity in the COS7 cell extracts (see Table I). When the rHIV reporter was expressed in this modified COS7 background, the predominant product (approximately 85%) migrated with an apparent molecular mass of 5.5 kDa, consistent with the addition of O-glycan (Figure 1, lane 2). The increased overall recovery of signal in lane 2 compared to lane 1 is most likely due to a greater stability or efficiency of secretion of the reporter in its glycosy-

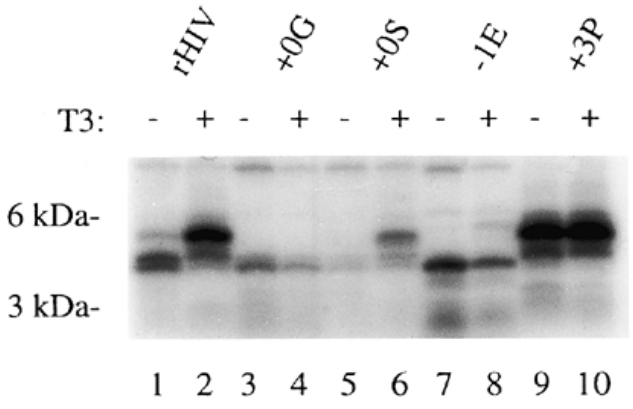

Fig. 1. Tricine SDS–PAGE analysis of the rHIV reporter protein secreted from COS7 cells and the effect of cotransfecting a recombinant full length ppGaNTase-T3 expression construct on O-glycosylation of the reporter. The sequence RGPGRAFVTIGKIGNMR from the V3 loop of the HIV gp120 protein was expressed as a fusion protein containing the insulin secretory signal, a 6x His nickel affinity tag, a heart muscle kinase site and the FLAG antibody recognition signal. The rHIV reporter was immunoprecipitated from transiently-transfected COS7 cell media and labeled with γ^{32}P-rATP using bovine heart muscle kinase. rHIV reporter protein (lanes 1 and 2) and derivatives +0G (lanes 3 and 4), +0S (lanes 5 and 6), -1E (lanes 7 and 8), or +3P (lanes 9 and 10) were analyzed from COS7 cells cotransfected with pSVL (lanes 1, 3, 5, 7, and 9) or pSVLmT3full (lanes 2, 4, 6, 8, and 10).

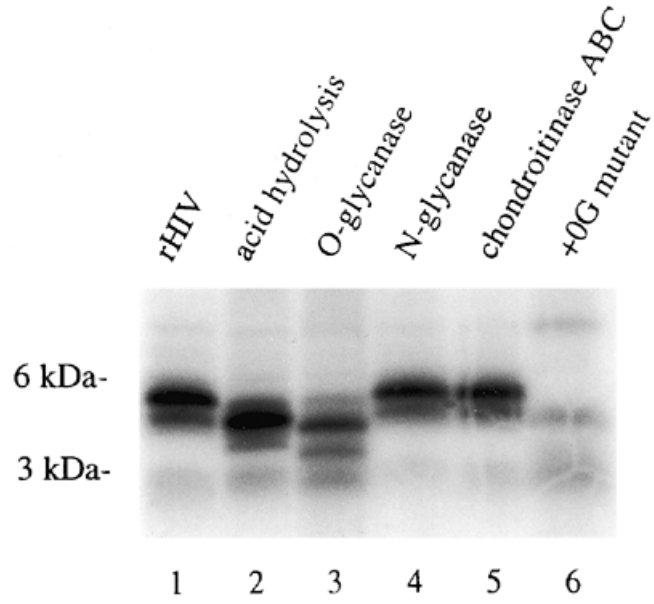

Fig. 2. Tricine SDS–PAGE analysis of endoglycosidase digestions of the rHIV reporter protein recovered after coexpression with ppGaNTase-T3. Immunoprecipitated, labeled material from the media of COS7 cells coexpressing the rHIV reporter and the ppGaNTase-T3 was acid hydrolyzed (lane 2) and then digested with the endoglycosidase O-glycanase (lane 3) or alternatively digested with N-glycanase (lane 4) or chondroitinase ABC (lane 5). The +0G mutant is included as a nonglycosylated control (lane 6).

lated form. A similar result was obtained with a rHIV +0S reporter in which serine was substituted for threonine (Figure 1, lanes 5 and 6). As expected, the rHIV +0G mutant was not affected by the expression of ppGaNTase-T3 (Figure 1, lane 4).

The 5.5 kDa species recovered upon coexpression of rHIV and ppGaNTase-T3 was treated by mild acid hydrolysis and then digested with O-glycanase. This resulted in the production of a species with a mobility identical to that of rHIV +0G (Figure 2, compare lanes 3 and 6). Incubation with several other glycosidases, including N-glycanase, did not result in a change in mobility (Figure 2, lanes 4 and 5). Taken together, these data indicate that the reporter peptide acquires a common O-glycan structure (consisting of NeuAc α2,3(6) Gal β1,3 GalNAc) to form the 5.5 kDa species.

Effect of flanking amino acids on the O-glycosylation of rHIV in COS7 cells

Previously, we have shown that the simultaneous presence of charged residues at positions -1 and +3 relative to single hydroxyamino acids inhibited acquisition of O-glycan (Nehrke *et al.*, 1996, 1997). When glutamic acid was substituted at position -1 for valine (rHIV -1E), both the endogenous COS7 cell-specific and ppGaNTase-T3 augmented glycosylation were reduced to negligible levels (Figure 1, compare lanes 1 and 2 with 7 and 8). Further, substitution of lysine by proline at position +3 (rHIV +3P) effectively abolished the ppGaNTase-T3 specificity of this substrate since the endogenous ppGaNTase activity of the native COS7 cells could fully glycosylate the rHIV +3P mutant reporter (Figure 1, lanes 9 and 10).

The rHIV series of reporter constructs was then cotransfected with a full-length ppGaNTase-T1 expression construct (Figure 3). The glycosylation of the rHIV reporter increased to 50%, suggesting that ppGaNTase-T1 was able to utilize the wild-type sequence, albeit with significantly less efficiency than ppGaNTase-T3 (Figure 3, lanes 1–3). As expected, the overexpression of ppGaNTase-T1 had no effect on the modification of the rHIV +0G, -1E, or +3P reporter substrates. However, neither the endogenous COS7 repertoire of ppGaNTases, nor over-expressed ppGaNTase-T1 in the COS7 cell background, was able to modify the rHIV +0S substrate. This suggests that a serine-containing substrate is not as readily recognized by ppGaNTase-T1.

In vitro *glycosylation of HIV peptide by recombinant ppGaNTase-T1 and -T3*

To compliment the *in vivo* analysis, we tested ppGaNTase-T1 and -T3 activity against synthetic peptides using either recombinant enzyme which was secreted in a truncated form (Hagen *et al.*, 1993, 1997) or extracts derived from COS7 cells cotransfected with the full-length glycosyltransferase expression-constructs and rHIV +0G, to mimic conditions under which the reporter peptides were analyzed *in vivo* (Zara *et al.*, 1996; Table I). The peptides tested were based on the HIV and HIV+3P sequences and on a rat submandibular gland apomucin repeat sequence (EA2) shown previously to be a good substrate for ppGaNTase-T1 (Hagen *et al.*, 1995). Recombinant ppGaNTase-T3 enzyme was able to glycosylate both the HIV and EA2 peptides, while recombinant ppGaNTase-T1 glycosylated only the EA2 peptide. We found, however, that by changing the lysine at the +3 position in the HIV peptide to a proline, substantial activity was now observed with the ppGaNTase-T1 enzyme *in vitro*, as was observed for the rHIV +3P substrate in the native COS7 cell background *in vivo* (Figure 1, lane 9). ppGaNTase-T3 also showed increased activity with the HIV +3P peptide. The pattern of glycosylation in transfected COS7 cell backgrounds mirrored the relative *in vitro* activity of the recombinant ppGaNTase-T1 and T3 proteins. This suggests that the truncated, secreted recombinant proteins retained the same specificity as the full length glycosyltransferases which were overexpressed *in vivo*.

Table I. Activities of murine ppGaNTase isoforms T1 and T3 with EA2, HIV, and HIV +3P peptide substrates *in vitro* at 37°C

Isoform	Source	Peptide	Activity (c.p.m./h)
Mock	Media	EA2	132 (±35)
Truncated T1	Media	EA2	11262 (±160)
Truncated T3	Media	EA2	4299 (±154)
Mock	Lysate	EA2	213 (±21)
Full-length T1	Lysate	EA2	2808 (±240)
Full-length T3	Lysate	EA2	1670 (±321)
Mock	Media	HIV	115 (±23)
Truncated T1	Media	HIV	322 (±12)
Truncated T3	Media	HIV	6547 (±28)
Mock	Lysate	HIV	147 (±17)
Full-length T1	Lysate	HIV	196 (±13)
Full-length T3	Lysate	HIV	1579 (±89)
Mock	Media	HIV +3P	125 (±12)
Truncated T1	Media	HIV +3P	1140 (±82)
Truncated T3	Media	HIV +3P	9074 (±149)
Mock	Lysate	HIV +3P	160 (±19)
Full-length T1	Lysate	HIV +3P	314 (±49)
Full-length T3	Lysate	HIV +3P	1891 (±333)
Mock	Media	None	103 (±10)
Truncated T1	Media	None	301 (±23)
Truncated T3	Media	None	211 (±17)
Mock	Lysate	None	145 (±14)
Full-length T1	Lysate	None	171 (±29)
Full-length T3	Lysate	None	196 (±13)

Values shown represent the mean of duplicate assay points from two separate transfections, with the standard errors presented in parentheses. The activity of ppGaNTase isoforms -T1 and -T3 were determined using as an enzyme source either 1 µl of COS7 cell media from cells that had been transfected solely with the truncated, secreted ppGaNTase expression constructs pF3m-T1 or -T3 (at 1 µg per 35 mM well) or 2 µl of COS7 cell lysate from cells that had been transfected at a 1:20 ratio with the full-length, intracellular ppGaNTase expression constructs pSVLmT1full or -mT3full (at 0.05 µg per 35 mM well) and pKN83 +0G, the nonglycosylated rHIV reporter control (at 0.95 µg per 35 mM well). Mock controls were determined using cells that had been transfected with pSVL (at 1 µg per 35 mM well).

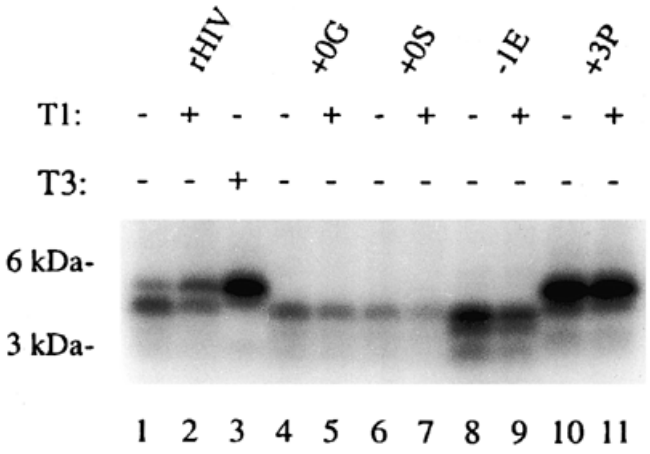

Fig. 3. Tricine SDS–PAGE analysis of reporter proteins secreted from COS7 cells cotransfected with a recombinant full length ppGaNTase-T1 expression construct on O-glycosylation of the reporter. rHIV reporter protein (lanes 1–3) and derivatives +0G (lanes 4 and 5), +0S (lanes 6 and 7), -1E (lanes 8 and 9), or +3P (lanes 10 and 11) were analyzed following immunopurification and labeling from the media of COS7 cells cotransfected with pSVL (lanes 1, 4, 6, 8, and 10), pSVLmT1full (lanes 2, 5, 7, 9, and 11), or pSVLmT3full (lane 3).

Discussion

It has been unclear why so many forms of ppGaNTases have evolved. *In vitro* assays have suggested that subtle differences exist among functionally expressed isoforms, although there appears to be considerable overlap (Bennett *et al.*, 1996;White *et al.*, 1996; Zara *et al.*, 1996; Hagen *et al.* 1997). In this study, we present experimental evidence for substrate specificity of a ppGaNTase *in vivo*. We have shown that glycosylation of the rHIV substrate in COS7 cells (and particularly rHIV +0S, the threonine to serine mutant) is significantly increased by the coexpression of ppGaNTase-T3.

Our data also suggests that ppGaNTase-T3 is less affected by the presence of a charged residue at position +3 in the HIV substrate than other isoforms. With a lysine at this position, none of the ppGaNTase isoforms native to the COS7 cell background can substantially glycosylate this substrate; conversely, when proline is present at position +3, the site is occupied to a level of >85%. However, when ppGaNTase-T3 is overexpressed in COS7 cells, the glycosylation apparatus is unaffected by the presence of the charged residue at +3 in the rHIV reporter and the site is fully glycosylated.

The observation that overexpression of ppGaNTase-T1 leads to partial glycosylation of the rHIV reporter suggests that either ppGaNTase activities and/or UDP-GalNAc concentrations in COS7 cells are limiting. Supporting this possibility is that when COS7 cells are maintained at 23°C, which slows down transport and secretion, the glycosylation of reporter peptides that were shown to be poor substrates is enhanced (Nehrke *et al.*, 1996). An alternate possibility that we have not yet addressed is that overexpression of ppGaNTase-T1 induces the expression of ppGaNTase-T3 and/or some endogenous ppGaNTase. However, overexpression of ppGaNTase-T1 does not lead to measurable enhancement of rHIV +0S glycosylation, indicating that threonine is more effectively glycosylated in this peptide context than serine.

Collectively, our findings point toward specific components of the flanking amino acids serving as signals that selectively abrogate recognition by specific isoforms of ppGaNTase. Thus far we have characterized one such signal that appears to be wide-spread in its effect, namely charged amino acids at both the -1 and +3 positions relative to solitary (nonclustered) hydroxy-amino acids. However, other such signals may exist. We feel that it is possible that O-glycosylation may be a constitutive process unless it is inhibited by either one of the signals embedded within the primary sequence or by inaccessibility due to overall tertiary structure of the protein (Elliot *et al.*, 1994). Each isoform may react more or less severely to each of these signals, as is shown here by the comparison of ppGaNTase-T1 and T3. A complete analysis of the effects of peptide sequence on glycosylation will require work done both *in vitro*, where small changes are readily

apparent in terms of initial velocities of the addition of O-glycans, and *in vivo*, in a setting where competition between various isoforms, the rate of transport through the Golgi apparatus, and interactions with attenuating factors could potentially influence the final extent of glycosylation.

Materials and methods

Plasmid constructs

The rHIV expression construct pKN83 was created in several steps. First, complementary oligonucleotides encoding unique MluI and BglII sites were inserted into the pKN4 vector (Nehrke *et al.*, 1996) at the HpaI and AvrII sites to generate pKN66 (a reporter not used in this study). By replacing the sequence between the MluI and BglII sites with complementary oligonucleotides coding for each of the HIV gp120 protein wild-type and mutant sequences in frame with the upstream insulin secretory signal and the downstream 6x His, heart muscle kinase and FLAG octamer sequences, we created pKN83 and its +0G, +OS, -1E, and +3P mutant reporter expression vectors. The oligonucleotides used to create pKN66 were 5′ AACGCGTTAGCCGGCGCAGCAC-CCGGAGATCTC 3′ and its complement, containing an AvrII 5′ overhang. To create pKN83, the oligonucleotide 5′ cgcgTTGCGAGGGCCAGGTCGAGCCTTCGTA**ACC**ATCG GTAAAATCGGTAACATGAGA 3′ and its complement (capital letters only) containing a 5′ BglII overhang were inserted into the MluI and BglII sites of pKN66. The hydroxyamino acid codon is shown in bold. For the mutant derivatives of pKN83, the appropriate codons in the oligonucleotides were changed as follows: +0G (T to G mutation) ACC to GGA; +0S (T to S mutation) ACC to AGC; -1E (V to E at position -1) GTA to GAA; +3P (K to P mutation at position +3 relative to the hydroxyamino acid) AAA to CCC.

The full-length murine ppGaNTase T1 expression vector pSVL-mT1full was created by inserting a SmaI–ApaI fragment from a full length cDNA clone of mT1 in pBSII SK+ into the pSVL derivative pIMKF3 (Hagen *et al.*, 1997) at the MscI and ApaI sites. pSVL-mT1full contains the β-globin 5′ UTR and the entire T1 coding region. The full-length murine ppGaNTase T3 expression vector pSVL-mT3full was created by inserting the entire coding region of the murine T3 isoform into the vector pSVL, as above. All of the expression constructs were sequenced to confirm reading frame and insertions. The secretable ppGaNTase expression constructs pF3mT1 and pF3mT3 have been described previously (Zara *et al.*, 1996; Hagen *et al.*, 1997).

Cell culture, transfections, and harvesting

Routine maintenance and transfection of COS7 cells was performed as detailed in Nehrke *et al.*, 1996. Briefly, LipofectA-MINE (Life Technologies) and 1 μg of cesium chloride–purified DNA (reporter construct and either pSVL or the pSVL-based ppGaNTase expression vectors at a 20:1 ratio) was used to transfect 35 mM plates of COS7 cells at >70% confluency. Media was harvested at 72 h posttransfection and clarified by spinning 5 min at 12,000 × *g*. Cells were harvested by adding 0.5 ml of extraction buffer (20 mM MES, pH6.5, 50 mM NaCl, 1% Triton X-100, 5% glycerol, and 1 mM DTT), incubating at 4°C for 15 min, then scraping cells from the plate and clarifying as above. Media and lysates were stored at -70°C until use.

Immunoprecipitation, radiolabeling, and gel analysis of reporter peptides

0.5 ml aliquots of the COS7 media were immunoprecipitated by rocking in the presence of 40 μl of anti-FLAG agarose (IBI) overnight at 4°C. The precipitate was washed twice with PBS-0.5% Tween 20, then incubated in 30 μl of 1× heart muscle kinase buffer (20 mM HEPES, pH 7.0, 75 mM NaCl, 15 mM $MgCl_2$) containing 5 U of heart muscle kinase (Sigma) and 5 μCi of γ^{32}P-ATP (Dupont-NEN) for 1 h at 37°C, washed twice more as above, and resuspended in 15 μl of 2× loading dye (8% SDS, 24% glycerol, 100 mM Tris, pH 7.0, 4% β-mercaptoethanol, and 0.02% Coomassie G-250). 2.5 μl of the loading dye was run on a Tricine SDS–polyacrylamide gel containing stacking (3%C/4%T), separating (3%C, 10%T), and resolving (6%C/16.5%T) phases, where %T refers to the total acrylamide concentration and %C refers to the concentration of the bis-acrylamide related to the total (Schagger and von Jagow, 1987). The gels were fixed, dried, and analyzed using a Molecular Dynamics PhosphorImager.

ppGaNTase enzyme assays

The enzyme activity was measured using the following assay conditions: a volume of 25 μl containing a final concentration of 500 μM peptide, 50 μM UDP-[^{14}C]-GalNAc (25,000 c.p.m.), 10 mM $MnCl_2$, 40 mM cacodylate pH 6.5, 40 mM 2-mercaptoethanol, and 0.1% Triton X-100. Peptide substrates (sequences in parentheses) used in these assays are: EA2 (PTTDSTTPAPTTK), HIV (RGPGRAFVTIGKIGNMR), and HIV +3P (RGPGRAFV-TIGPIGNMR). All enzyme assay points were performed in duplicate and these were repeated with media or lysate from duplicate, independent transfections. Glycosylated [^{14}C]-labeled peptides were separated from unincorporated UDP-[^{14}C]-GalNAc by anion exchange chromatography on formate form AG1-X8 resin spin columns (Bio-Rad).

Glycosidase digestions

Acid hydrolysis was done prior to immunoprecipitation by bringing the transfected COS7 media to 0.1 N H_2SO_4 for 1 h at 80°C, then neutralizing with 0.1 N NaOH and spinning for 5 min at 12,000 × *g*. Immunoprecipitated and labeled reporter protein was digested in 15 μl according to the recommendations of the manufacturer. O-Glycanase and N-glycanase were from Oxford Glycosystems, and chondroitinase ABC was from Seikagaku Inc.. The digested materials were brought to 2× loading dye concentrations and analyzed by Tricine SDS–PAGE.

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