Enzymatic Glycoprotein Synthesis: Preparation of Ribonuclease Glycoforms via Enzymatic Glycopeptide Condensation and Glycosylation

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Abstract: In order to study the effects carbohydrates have on glycoprotein structure and function, it is imperative to be able to synthesize the appropriate natural and non-natural glycoprotein variants in a single form. Because the available *in vivo* techniques provide only heterogeneous mixtures of different glycoforms, enzymatic *in vitro* methodologies have been pursued. Using the *N*-glycoprotein RNase B as a model system, the oligosaccharide was removed leaving only the *N*-acetylglucosamine as a "tag" to the site of glycosylation. Glycosyltransferases were then used to build a unique carbohydrate moiety. A new RNase glycoform containing the branched oligosaccharide, sialyl Lewis X or the Hg derivative, was synthesized enzymatically to demonstrate the feasibility of the method. In addition, the monoglycosylated protein was digested into several smaller pieces by subtilisin BPN′. These fragments were religated by subtilisin 8397 to the full length RNase by addition of glycerol; this method points to a new chemical-enzymatic process for the synthesis of glycoproteins using synthetic peptides and glycopeptides as substrates for enzymatic ligation followed by further enzymatic glycosylations.

The carbohydrate portions of glycoproteins are implicated as playing major roles in modulating protein stability and folding, protein targeting, cell adhesion, and many other intercellular recognition processes.[1,2] Glycoproteins, however, generally do not exist in a single form but as several different glycoforms (the peptide sequence is the same; only the attached carbohydrate varies), making the study of carbohydrate structure and function of glycoproteins difficult.

Currently, modern separation techniques, such as capillary electrophoresis, have made the isolation of certain individual glycoforms possible,[3] but the processes are very complicated and tedious and are limited to use with natural glycoproteins. Progress has been made toward the use of *in vivo* techniques such as glycosyl engineering[4,5] and cell-line mutations to prepare specific glycoproteins; however, these methods still produce only heterogeneous material.[6] *In vitro* enzymatic and chemical methods have been used in the preparation of certain glycoproteins in homogeneous forms. For example, homogeneous glucocerebrosidase used in the treatment of Gaucher's disease has been prepared via enzymatic removal of the heterogeneous sugar chains,[7] and neoglycoproteins have been prepared via chemical glycosylation at sites other than the normal N- and O-glycosylation sites.[8−10] We report here the enzymatic synthesis of glycoproteins using endoglycosidases, proteases, and glycosyltransferases. Shown in Scheme 1 are two strategies used in the synthesis of glycoproteins: one is to remove the heterogeneous carbohydrates of glycoproteins using endoglycosidases followed by addition of desired carbohydrates using glycosyltransferases; the second is to ligate synthetic peptides and glycopeptides using proteases followed by enzymatic glycosylations.

Ribonuclease B was chosen as the model system to investigate. It is a small protein (124 amino acids) and contains a single glycosylation site at asparagine 34. Several glycoforms exist in nature, but all are of the high mannose type (N-linked $Man_{5-9}GlcNAc_2$). The nonglycosylated form, RNase A, is also readily available and has been thoroughly characterized. In addition, Anfinsen showed in a classic paper that reduced, denatured RNase A can be refolded under mild oxidative conditions to the native structure, with all four native disulfide bonds reformed.[11]

We chose the tetrasaccharide sialyl Lewis X as the model oligosaccharide, since our laboratory has experience with the enzymatic synthesis of this molecule.[12] The anticipated product is shown in Figure 1, a computer-generated composite of the crystal structure[13] of ribonuclease A and the solution conformation of sialyl Lewis X.[12]

We envision that native RNase may be cut into fragments at certain exposed regions via enzymatic proteolysis and these fragments might be religated under thermodynamically controlled conditions with the same enzyme. Alternatively, synthetic peptide and glycopeptide esters may be ligated under kinetically controlled conditions catalyzed by a protease. The former strategy was recently used in the preparation of nonglycosylated triose phosphate isomerase,[14] and the latter strategy was used in the synthesis of RNase A.[15]

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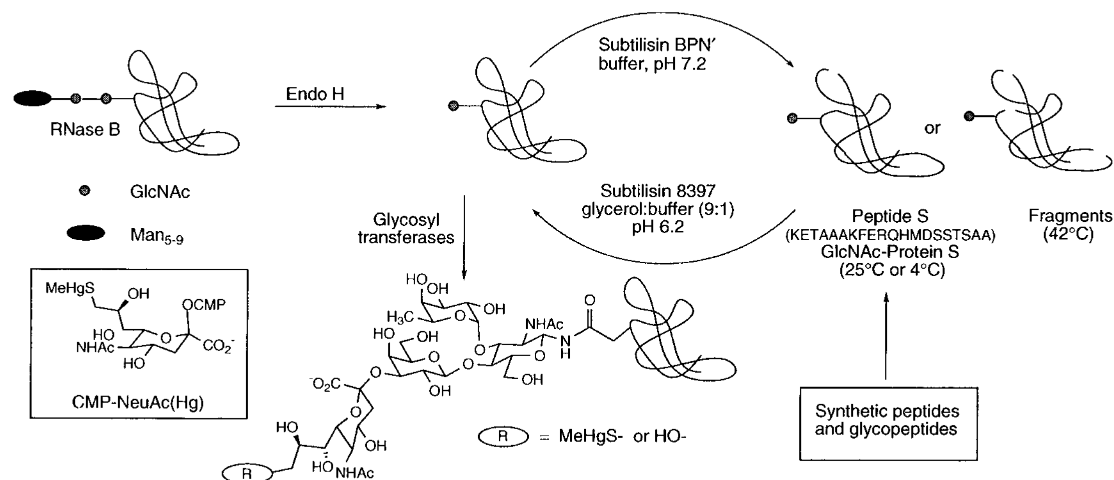
Scheme 1. Enzymatic Synthesis of Glycoproteins: Synthesis of a Ribonuclease B Glycoform Containing Sialyl Lewis X.



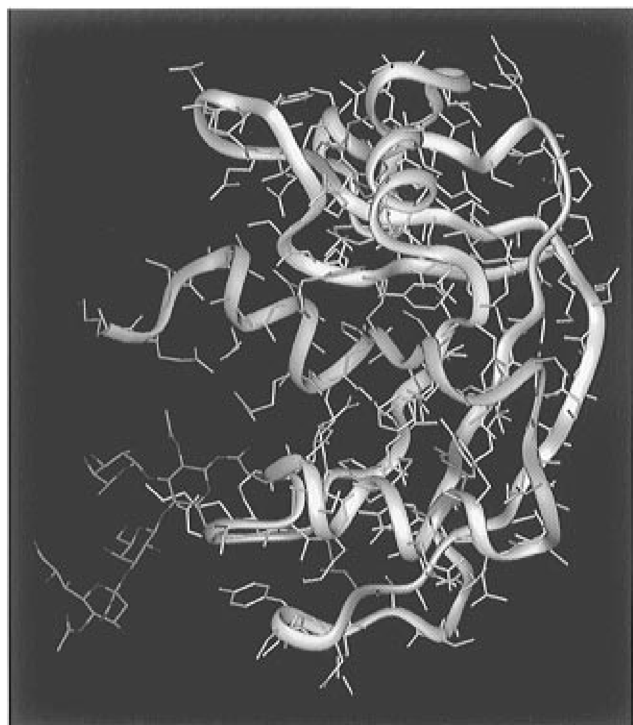


Figure 1. Hypothetical structure of SLe^x-RNase. The structure shown is a composite of the crystal structure of ribonuclease A[26] and the solution structure of sialyl Lewis X.[27] The pink regions are disulfide bonds and the yellow region is peptide S. Sialyl Lewis X is colored by atom (green is carbon, white is hydrogen, blue is nitrogen, and red is oxygen).

Our initial approach has been to exploit the use of subtilisin BPN′ and glycosyltransferases in glycoprotein synthesis as we have been interested in the use of these enzymes in glycopeptide ligation. Several subtilisin variants have been engineered to meet specific needs.[16,17] We have also investigated the specificities of these enzymes using glycopeptides as substrates.[18−21]

Ribonuclease B, obtained from Sigma, contains approximately 10% ribonuclease A and a small amount of a brown impurity, and so was further purified using a cation exchange column with a borate−phosphate buffer system. Since borate is weakly chelated by diols, and since, when chelated, its pK_a drops from 9 to approximately 5, the presence of borate in the buffer differentiates glycoproteins from their nonglycosylated counterparts by adding negative charge. As a result, ribonuclease B elutes far in advance of ribonuclease A on a carboxymethylcellulose column equilibrated with 100 mM sodium phosphate/100 mM boric acid, pH 6.5 (see Figure 2).

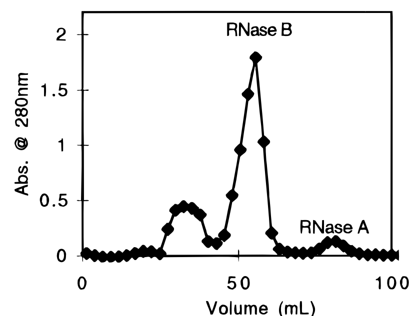


Figure 2. Purification of ribonuclease B using borate−phosphate cation exchange.

A variant of RNase possessing a single *N*-acetylglucosamine (GlcNAc) at asparagine 34 (Asn 34) was synthesized by treating RNase B (a mixture of glycoforms) with endoglycosidase H (Endo H) to produce the homogenous product GlcNAc-RNase. Endo H cleaved the high mannose core of the oligosaccharide specifically between the two most interior GlcNAc sugars.[22] This reaction was conducted under nondenaturing conditions and monitored by SDS−PAGE. Figure 3A shows a gel of the product of the reaction along with a control lane of RNase B.

A MES/HEPES buffer system was used to allow the glycosidase (optimal pH 5.5), the transferase (optimal pH 7.0), and the proteolysis (optimal at pH > 7) reactions to be carried out without a change of buffer by simply adjusting the pH. Dialyzing the reaction mixture against the MES/HEPES buffer system proved sufficient to remove the cleaved oligosaccharides.

The initial transferase reaction was mediated by β-1,4-galactosyltransferase (GalT). This enzyme is known to transfer galactose (Gal) from the sugar nucleotide, UDP-Gal, to the 4

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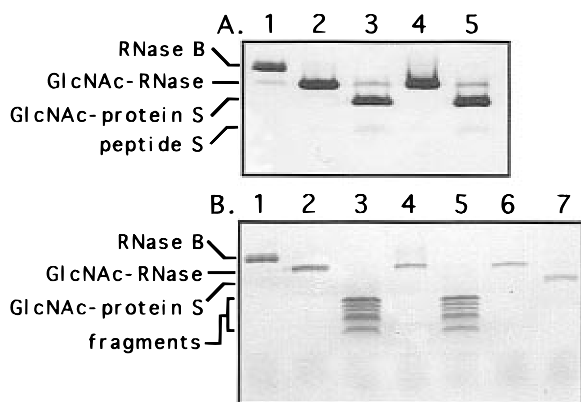

Figure 3. (A) Removal of carbohydrate, proteolysis at 4 °C, and subsequent religation of GlcNAc-RNase as visualized by a 20% tricine, denaturing gel: lane 1, RNase B; lane 2, GlcNAc-RNase; lane 3, proteolysis fragments GlcNAc-protein S and peptide S; lane 4, religation; lane 5, control in which PMSF was added prior to the organic solvent. (B) Removal of carbohydrate, proteolysis at 42 °C, and subsequent religation of GlcNAc-RNase as visualized by a 20% tricine, denaturing gel: lane 1, RNase B; lanes 2 and 4, GlcNAc-RNase; lane 3, fragments from proteolysis; lane 5, control in which PMSF was added prior to the DMSO; lane 6, control in which SDS and β-mercaptoethanol were not added; lane 7, GlcNAc-protein S as a reference.

position of an *N*-acetylglucosamine moiety (GlcNAc) to form Galβ1,4GlcNAc.[23,24] A cofactor regeneration system was employed to limit the concentration of the byproduct UDP, a competitive inhibitor of the transferase reaction.[25] This system replenishes UDP-Gal through a multistep pathway which consumes UDP to produce UDP-Gal and drives the equilibrium toward the desired product. The reaction was monitored by following the transfer of the radiolabeled galactose to GlcNAc-RNase. It was found after 48 h at room temperature the reaction was complete; the product, which was further characterized by MALDI (matrix-assisted laser desorption ionization) mass analysis, was isolated in a 76% yield (calculated from radiolabel present). For comparison, without the recycling system, yields ranged between 40% and 50%. Three control reactions were carried out containing all the components of the above reaction with the following exceptions: (i) absence of GlcNAc-RNase and GalT, (ii) absence of GalT, (iii) substitution of RNase A for GlcNAc-RNase. All three showed no increase in radioactivity (over background) in the isolated RNase (Figure 4a). In addition, upon treatment of the presumed product, Galβ1,4GlcNAc-RNase, with β-galactosidase, the radiolabeled sugar was removed (Figure 4b). These results indicate the galactose was transferred enzymatically onto the nonreducing end of the GlcNAc moiety (which is N-linked to the RNase).

The oligosaccharide was further extended to a trisaccharide by use of two different glycosyltransferases (Figure 5). The enzyme α-1,3-fucosyltransferase V (FucT)[25] transfers the fucose group from GDP-fucose to the 3 position of GlcNAc to give the branched oligosaccharide Galβ1,4(Fucα1,3)GlcNAc-RNase, while α-2,3-sialyltransferase (SialT)[26] transfers the sialic acid moiety from CMP-sialic acid to the 2 position of the galactose moiety to produce NeuAcα2,3Galβ1,4GlcNAc-RNase. Carbon 14 labeled sugar nucleotides were used in these reactions as the donors. As can be seen in Figure 4b, the ^{14}C-labeled sugars

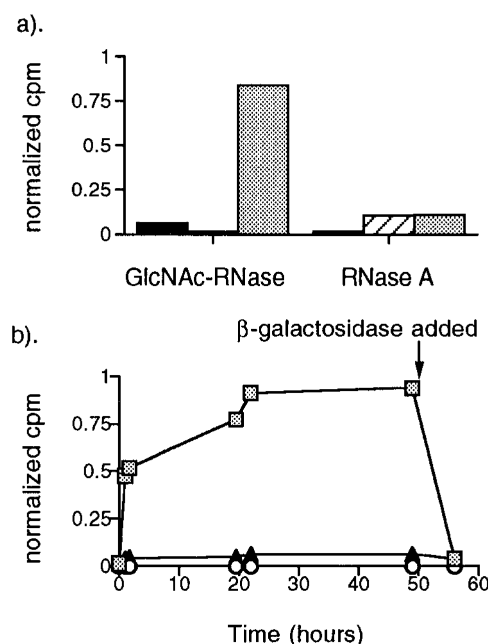

Figure 4. Galactosyltransferase-catalyzed reaction of GlcNAc-RNase with [^{3}H]UDP-Gal. Reaction progress was monitored by the transfer of radiolabeled galactose onto isolated RNase. (a) GalT reactions were run using both GlcNAc-RNase and RNase A (nonglycosylated control) as the substrate. The black bars represent reactions containing only sugar nucleotide. The striped bars represent reactions with sugar nucleotide and RNase but no transferase. The gray bars signify the isolated product of the full reaction. (b) Reaction course of GalT-catalyzed addition of galactose onto GlcNAc-RNase. Reaction conditions and controls are described above. The arrow indicates where β-galactosidase was added to catalyze the removal of the radiolabeled galactose.

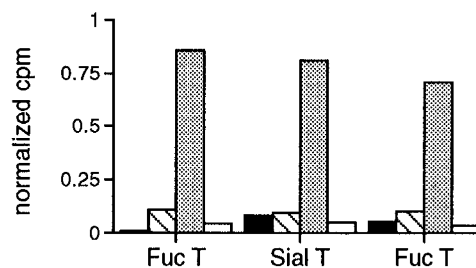

Figure 5. Isolated RNase products of three different transferase reactions as monitored by carbon 14 incorporation. All reactions have been normalized with respect to total radiolabel found in the crude reaction mixture. The first set of reactions is the FucT V-catalyzed synthesis of Galβ1,4(α1,3Fuc)GlcNAc-RNase. The second set is the SialT-catalyzed synthesis of NeuAcα2,3Galβ1,4GlcNAc-RNase, and the third is the FucT V-catalyzed synthesis of NeuAcα2,3Galβ1,4(α1,3Fuc)GlcNAc-RNase. In all of the above reactions the four bars correspond to (black) only labeled sugar nucleotide, (striped) labeled sugar nucleotide with GlcNAc-RNase but no transferase, (gray) transferase-catalyzed reaction, and (white) isolated RNase after treatment with appropriate glycosidase.

were transferred to the protein only in the presence of the glycosyltransferases. Again the appropriate glycosidases removed the labeled sugars. It was found that 72% of the Galβ1,4GlcNAc-RNase was fucosylated upon treatment with FucT to give Galβ1,4(Fucα1,3)GlcNAc-RNase and 85% of the Galβ1,4GlcNAc-RNase was sialylated by SialT to give NeuAcα2,3Galβ1,4GlcNAc-RNase. For both of the above transferase reactions, only a nominal background level of ^{14}C was observed in either the absence of transferase or the absence of an oligosaccharide (i.e., control with RNase A, sugar nucleotide, and transferase).

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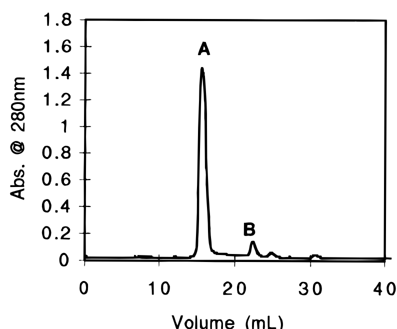

Figure 6. Sepharose 12 chromatography of RNase S. The initial peak (A), which elutes after approximately 17 mL, contains three components, the dominant of which has a mass ($M + H^+$) of 11 728, consistent with GlcNAc-RNase S-protein. The minor components have masses ($M + H^+$) of 13 885, consistent with GlcNAc-RNase, and 11 643, consistent with residues 22−124. The second peak (B) has a mass ($M + H^+$) of 2168, consistent with the peptide S, and a small amount of material with mass 2096, consistent with residues 1−19 (subtilisin is known[27] to cleave occasionally between residues 19 and 20, rather than between 20 and 21).

The synthesis of a larger, branched oligosaccharide was pursued to show the accessibility of the sugar chain and the feasibility of further manipulation (Figure 5). The product NeuAcα2,3Galβ1,4GlcNAc-RNase was fucosylated using the same fucosyltransferase and similar conditions as above to give NeuAcα2,3 Galβ1,4(Fucα1,3)GlcNAc-RNase, a protein-bound version of sialyl Lewis X in 74% yield. In a similar fashion, a Hg-labeled sialic acid was incorporated (Scheme 1).

GlcNAc-RNase was subjected to limited proteolysis by subtilisin BPN′ under two different sets of conditions to give varying results. Hydrolysis at either 4 or 25 °C, using 0.01 equiv of subtilisin BPN′, produced two fragments cleanly (Figure 3A). The major band was identified by N-terminal sequencing which revealed the terminal tetrapeptide of the fragment to be identical to residues 21−24 (four residues were analyzed to confirm the identity of the fragment) of RNase B. Protein S from ribonuclease has been known for some time and contains the residues 21−124.[27] (Subtilisin is also known to cleave occasionally between residues 19 and 20 to give a protein S species that has one additional alanine.[27]) Ligation of the two fragments by addition of 9 volumes of glycerol and subtilisin 8397[16,17] proceeded to completion as monitored by SDS−PAGE. In addition, a control was performed in which phenylmethanesulfonyl fluoride (PMSF) was added to the hydrolysis mixture before the DMSO. PMSF is a known inhibitor of the protease and prevented the religation from occurring.

Authentic samples of both the peptide S (residues 1−20) and GlcNAc-protein S (residues 21−124) were isolated by size-exclusion FPLC (Sepharose 12) and cation exchange chromatography, as discussed in the Experimental Section. Figure 6 shows the FPLC chromatogram of a low-temperature subtilisin digest of ribonuclease. The two fragments, which have no ribonuclease activity separately, show full activity when combined, as has been noted previously.[27] Addition of 0.01 equiv of subtilisin 8397 and 9 volumes of glycerol gave full-length religated GlcNAc-RNase which was characterized by MALDI and electrophoresis.

Further fragmentation was observed upon the enzymatic hydrolysis of GlcNAc-RNase by 0.005 equiv of subtilisin BPN′ at 42 °C in the presence of mildly reducing and denaturing conditions (0.45% SDS, 115 mM β-mercaptoethanol). For reference, standard denaturing conditions for SDS−PAGE are 2.5% SDS and 5% β-mercaptoethanol. As can be seen in Figure 3B, in the absence of SDS and β-mercaptoethanol no degradation is seen. However, in the presence of these reagents after 45 min the full-length protein is no longer present, and several new bands have appeared. Presumably the RNase is "relaxed" slightly under these conditions but not fully denatured. This allows the protease access to a larger number of bonds than under the low-temperature conditions above. Addition of 9 volumes of glycerol to the reaction mixture, however, did not produce religated full-length RNase.

The peptides from above were isolated as before and subjected to N-terminal sequencing to reveal that all contained the N-terminal sequence of the intact protein. It appears that, under these conditions, the bond between residues 20 and 21 is no longer the preferred cleavage site. Instead, amides near the C-terminal become preferable. We are currently identifying the exact cleavage sites.

In summary, we have demonstrated that homogeneous RNase glycoforms can be prepared enzymatically using two strategies described in this work. It is interesting to note that the three glycosyltransferases investigated in this study are not the enzymes involved in the biosynthesis of RNase B. They do, however, accept glycoproteins as good substrates. As the X-ray structure of RNase A[13] and the solution structure of sialyl Lewis X[12] are known, it would be interesting to know whether the structure of this new glycoform is the same as that predicted on the basis of combination of the two individual conformations (Figure 1). Work is in progress to investigate the kinetics of the glycosyltransferase reaction on macromolecular substrates, to prepare GlcNAc-protein S via subtilisin-catalyzed condensation of synthetic fragments under a kinetically controlled process, and to study the structures of synthetic glycoproteins.

Experimental Section

General Procedures. RNase A, RNase B, RNase protein S, and subtilisin BPN′ were obtained from Sigma. All transferases were obtained from Cytel Corp. Endo H was obtained from Oxford Glycosystems. SDS−PAGE gel and blots were run on Biorad Protean II system using a 20% tricine gel with 8% glycerol added.[28] A Beckman LS-3801 Scintillation counter was used to analyze isolated protein for both tritium and carbon 14 content. N-terminal sequencing was perform by the Core Facility at the Scripps Research Institute. Radiolabeled sugars and nucleotide-sugars were ordered from Amersham.

Purification of Ribonucleases A and B. Up to 300 mg of RNase B was loaded onto a 1.5 cm × 50 cm column of CM-52 (Whatman) (carboxymethyl)cellulose equilibrated with 100 mM sodium phosphate/100 mM boric acid, pH 6.5. The protein was eluted with the same buffer. Ribonuclease B elutes first, at approximately 50 mL, while RNase A elutes at after 75 mL.

Endoglycosidase Reaction To Give GlcNAc-RNase. RNase B (100 mg) was dissolved in 1 mL of 50 mM MES/HEPES at pH 5.5. To this solution, 1 μL of Endo H (500 U, New England Biolabs) was added. The reaction was kept at 37 °C for 3 h. The reaction was monitored by SDS−PAGE. After completion an aliquot (20 μL) was desalted by a Sephadex G-50 column equilibrated with 5 mM ammonium acetate (pH 7.5) for mass spectral analysis. MS (MALDI) expected 13 901, observed 13 871.

Galactosyltransferase Reaction To Produce Galβ1,4GlcNAc-RNase. To 325 μL of GlcNAc-RNase (0.01 mmol in 50 mM MES/HEPES, pH 7.2) were added 5 μL of $MnCl_2$ (20 mM) and 5 μL of KCl (10 mM). To this solution were added pyruvate kinase (5 U), UDP glucose pyrophosphorylase (UDPGP, 1 U), inorganic pyrophosphorylase (PPase, 10 U), galactose-1-phosphate−uridyl transferase (G-1-P UT, 1 U), galactosyltransferase (GalT, 2 U), glucose-1-phosphate

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(glu-1-P, sodium salt, 3 μmol), uridyl diphosphate (UDP, sodium salt, 3 μmol), phosphoenolpyruvate (PEP, trisodium salt, 0.01 mmol), and dithiothreitol (DTT, 2 μmol). Reaction was readjusted to pH 7.2. Tritium-labeled galactose-1-phosphate (gal-1-P, dipotassium salt, 0.01 mmol) was then added, and the reaction was shaken at room temperature for 48 h. Radiolabeled and nonradiolabeled experiments were run in parallel. Protein product was isolated with a QAE Sephadex column equilibrated with 50 mM MES/HEPES, pH 7.2. Typically 30 000 cpm were loaded. Care was taken to make sure ^{3}H-UDP-Gal as well as ^{3}H-Gal did not coelute with the protein product. Nonradiolabeled product was sent for mass analysis. Yield: 76% calculated on the basis of radiolabel present in isolated product. MS (MALDI) expected 14 065; observed 14 034.

β-Galactosidase Removal of Galactose from Galβ1,4GlcNAc-RNase. A similar procedure was used for all glycosidases. To 20 μL of the above GalT reaction was added 0.5 μL of 1 M $MgCl_2$ (in 50 mM MES/HEPES, pH 7.0) and 2 μL of a stock of β-galactosidase (2 mg/mL in 50 mM MES/HEPES, pH 7.0). The mixture was shaken at 37 °C for 2 h. Protein product was isolated on a QAE Sephadex column as described above. MS (MALDI) expected 13 901; observed 13 873.

Fucosyltransferase V Reaction To Produce Galβ1,4(α1,3Fuc)-GlcNAc-RNase. To 30 μL of galactosyltransferase reaction mixture were added 1 μL of fucosyltransferase (obtained from Cytel and provided in 50% glycerol at 2.16 U/mL), 0.4 μL of 1.0 M $MnCl_2$ (50 mM MES/HEPES, pH 7.0), and 8.6 μL of 0.25 mM ^{14}C-labeled guanosine diphosphate fucose (GDP-Fuc, also in MES/HEPES buffer). The mixture was shaken at room temperature for 18 h. Protein product was isolated with a QAE Sephadex column as described above. Both tritium-labeled and non-radioactive Galβ1,4GlcNAc-RNase were carried through. Yield: 72%. Radiolabeled and nonradiolabeled experiments were run in parallel. MS (MALDI) expected 14 211; observed 14 180.

Sialyltransferase Reaction to Produce NeuAcα2,3Galβ1,4GlcNAc-RNase. To 40 μL of galactosyltransferase reaction mixture were added 1 μL of sialyltransferase (SialT; obtained from Cytel and provided in 50% glycerol at 1.23 U/mL), 2.5 μL of 100 mM $MnCl_2$ (50 mM MES/HEPES, pH 7.0), 1 μL of alkaline phosphatase (Sigma, 225 U/mL), and 7.5 μL of 1 mM ^{14}C-labeled cytidine monophosphate sialic acid (CMP-Sial, also in MES/HEPES buffer). The mixture was shaken at room temperature for 18 h. Protein product was isolated with a QAE Sephadex column as described above. Both tritium-labeled and nonradioactive Galβ1,4GlcNAc-RNase were carried through. Radiolabeled (^{14}C) and nonradiolabeled experiments were run in parallel. Yield: 85%. MS (MALDI) expected 14 356; observed 14 326. The expected Hg-labeled product was obtained using CMP-NeuAc(Hg)[29] as substrate.

Fucosyltransferase V Reaction to Produce NeuAcα2,3Galβ1,4-(α1,3Fuc) GlcNAc-RNase. To 10 μL of the above reaction mixture were added 2 μL of fucosyltransferase (obtained from Cytel and provided in 50% glycerol at 2.16 U/mL), 0.3 μL of 1.0 M $MnCl_2$ (50 mM MES/HEPES, pH 7.0), 10 μL of 50 mM MES/HEPES buffer (pH 7.0), and 8.7 μL of 0.25 mM ^{14}C-labeled GDP-Fuc in MES/HEPES buffer. The mixture was shaken at room temperature for 24 h. Protein product was isolated with a QAE Sephadex column as described above. Both labeled and nonradioactive NeuAcα2,3Galβ1,4GlcNAc-RNase were carried through. Radiolabeled (^{14}C) and nonradiolabeled experiments were run in parallel. Yield: 74%. MS (MALDI) expected 14 502, observed 14 469. The Hg-SLe^x glycoprotein was prepared similarly.

(29) Prepared from 9-[(thiomethyl)mercuryl]sialic acid and CTP catalyzed by CMP-sialic acid synthetase.

Proteolytic Fragmentation Using Subtilisin BPN′ at 25 or 4 °C. A stock solution of 0.1 mg/mL of subtilisin BPN′ (Sigma) in 50 mM MES/HEPES, pH 7.2, was made. A small aliquot (9 μL) of a 3.3 mg/mL solution of GlcNAc-RNase was adjusted to pH 7.2, and the reaction was started by adding 1 μL of subtilisin stock. Reaction was allowed to proceed for 4 h at room temperature. An aliquot of the reaction (5 μL) was stopped by addition of 1 μL of a stock of PMSF (phenymethanesulfonyl fluoride, 5 mg/mL in CH_3CN, Aldrich). The remaining solution was used directly in the religation experiments described below. The 4 °C digestion was carried out in the same fashion, except for the temperature and the buffer, which was 100 mM HEPES, pH 8.0.

Purification of Peptide and Protein S. The proteolytic GlcNAc-RNase preparation described above was acidified to pH 3 by addition of 6 N HCl, and the fragments were separated by FPLC (Pharmacia) using an HR 10/30 Sepharose 12 column equilibrated with 200 mM acetic acid/150 mM sodium chloride (pH is approximately 2.75). The S-protein was not separated well from RNase, and so it was further purified via cation-exchange chromatography on a 1.5 cm × 50 cm CM-52 (Whatman) (carboxymethyl)cellulose column equilibrated with 200 mM sodium phosphate, pH 6.5. Peak identities were verified by mass spectrometry (MALDI).

Proteolytic Fragmentation Using Subtilisin BPN′ at 42 °C. A stock of 0.05 mg/mL of subtilisin BPN′ was made by dilution of the above stock. A 1 μL sample of this stock was added to 9 μL of the GlcNAc-RNase (3.3 mg/mL, 50 mM MES/HEPES, pH 7.2) and 1 μL of denaturing buffer (4% SDS, 10% β-mercaptoethanol). The reaction was heated to 42 °C for 45 min in a water bath. An aliquot of the reaction (5 μL) was terminated by addition of 1 μL of a stock of PMSF (phenymethanesulfonyl fluoride, 5 mg/mL in CH_3CN, Aldrich). The remaining solution was used directly in the re-ligation experiments described below.

Ligation of GlcNAc-protein S and Peptide S to give GlcNAc-RNase. Formation of RNase S complex was performed as described in the literature.[25,27b] After addition of 9 volumes of glycerol followed by the addition of 0.01 equiv of subtilisin 8397, the reaction was allowed to sit at room temperature for 3 days. After religation, 1 μL of PMSF stock (5 mg/mL in CH_3CN) was added to prevent hydrolysis upon addition of loading buffer.

Separation and Analysis of Fragments. Samples were denatured under standard conditions. SDS−PAGE was run as described in the General Procedures. The resulting gel was soaked in 10 mM CAPS (pH 11) for 5 min and transferred onto poly(vinylidene difluoride) (PVDF) membrane by tank electroblotting using the above CAPS buffer and Protean II blotting apparatus (Biorad). Membrane was stained for 1 min using standard Coomassie stain (0.1% Brilliant Blue R, 1% acetic acid, 40% methanol) and destained for 30 min with a solution of 50% methanol. Bands of interest were excised from the gel and sequenced by standard methods using a Procise Sequencer (Applied Biosystems, Inc.).

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