Identification of Photolabeled Peptides for the Acceptor Substrate Binding Domain of β1,4-Galactosyltransferase

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We successfully applied a carbene-generating *N*-acetylglucosamine derivative carrying a biotinyl group to the radioisotope-free identification of peptides within bovine UDP-galactose: *N*-acetylglucosamine β1,4-galactosyltransferase (GalT, EC 2.4.1.38) catalytic domain. Owing to the low yield of cross-linking, conventional photoaffinity labeling experiments usually encounter a thorny problem in attempting to isolate labeled components from very complex mixtures. A biotin tag introduced with our photoaffinity probe enabled us to separate the photolabeled protein from a large amount of coexisting unlabeled GalT. The introduction of biotin was also useful for the radioisotope-free detection of a labeled protein based on a highly sensitive chemiluminescent technique. We developed a novel poly(vinylidene difluoride) membrane for the identification of labeled peptides in a simple dot blot assay. Using this membrane, we successfully identified biotinyl peptides among a number of HPLC separated fragments derived from the protease digestion of photolabeled GalT proteins. The sequence analysis revealed that the biotin tag was incorporated within a tryptic GalT fragment of Y197-R208. Our approach yields, for the first time, information on the acceptor substrate binding-site fragment in this enzyme, that has been difficult to obtain using other approaches. These data are consistent with previous suggestions concerning the GalT acceptor site and clearly demonstrate the effectiveness of our approach for rapid identification of photolabeled peptides.

Key words photoaffinity labeling; diazirine; avidin–biotin system; substrate binding region; β1,4-galactosyltransferase

Understanding the molecular mechanism of carbohydrate–protein interactions is important in the fundamental pharmacological fields such as microbial infections, inflammation and cancer. Glycosyltransferases catalyze biosynthesis of oligosaccharides, and the molecular basis for substrate recognition of these enzymes is the most important topic in glycobiology.[1] Many glycosyltransferases are already cloned and sequenced. The enzyme UDP-galactose: *N*-acetylglucosamine β1,4-galactosyltransferase (GalT, EC 2.4.1.38) catalyzes the transfer of galactose units from UDP-galactose to terminal acceptor sugars, *N*-acetylglucosamine or glucose, forming a β1,4-galactosyl bond. Among the cloned glycosyltransferases, GalT has been one of the most widely studied.[2] The acceptor substrate binding sites of GalT remain obscure, however, due to the low homology of the catalytic domain with related glycosyltransferases. In addition, the crystallographic analysis of GalT has not been reported to date. The method of photoaffinity labeling becomes increasingly important because it enables us to chemically cross-link the specific ligands to the binding site of a target molecule. To this end, a carbene-yielding radioactive probe has already been examined for possible use in photoaffinity labeling of GalT, but no labeled site identification has been reported yet.[3] Owing to the low yield of cross-linking, conventional photoaffinity labeling often results in the isolation of very low amounts of labeled products from a large number of unlabeled proteins or peptides. We developed a series of biotinyl probes for the photoaffinity labeling of biofunctional proteins. The attachment of a biotinyl tag to the target molecule by photoaffinity labeling followed by the use of avidin–biotin facilitated the structural analyses of photolabeled components.[4] Thus, we first developed an asparagine-linked *N*-acetylglucosamine derivative **1** (BDGA) bearing a biotinyl phenyldiazirine for photoaffinity biotinylation of GalT.[5] Using this probe, we recently described a novel approach, photoaffinity biotinylation, for the efficient identification of photolabeled sites within GalT protein. We now report the detailed results of the identification of labeled GalT peptides based on use of the photoaffinity biotinylation method.[6]

Results and Discussion

Isolation of Labeled GalT Protein from the Photolytic Mixture We have previously reported that a biotinyl group attached to the photoreactive asparagine-linked *N*-acetylglucosamine derivative was useful for the purification of photolabeled GalT protein produced in a low yield (4%).[5] The recovery yield of labeled GalT from an immobilized streptavidin, however, was only 61% because of very strong interactions within the streptavidin–biotin complex ($K_d = 10^{-15}$ M).[7] Instead of the strongly binding tetrameric streptavidin, we attempted to use an immobilized monomeric avidin, which has a weaker interaction with biotin ($K_d = 10^{-8}$ M),[8] for the im-

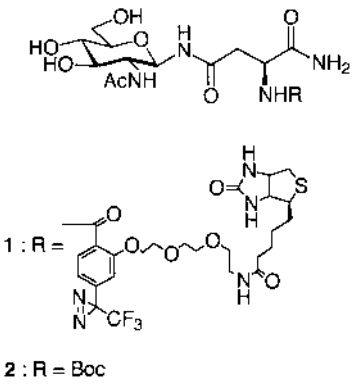

Fig. 1. Structures of BDGA **1** and Photolabel Inhibitor **2**

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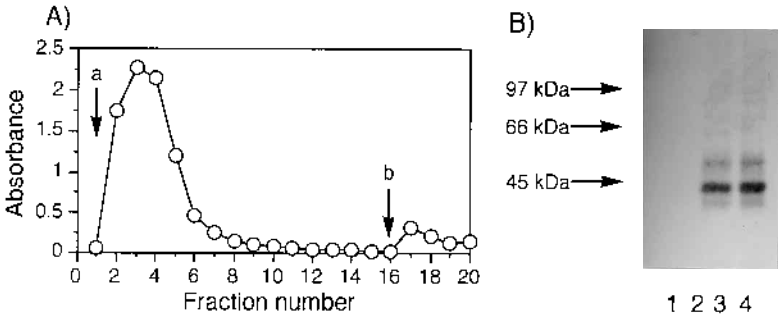

Fig. 2. Purification of Labeled Protein by Immobilized Monomeric Avidin

(A) Elution profile at 280 nm for immobilized monomeric avidin separation of the labeled mixture. Arrows a and b indicate treatment of the gel with NaP and 2 mM *d*-biotin in NaP, respectively. (B) Chemiluminescence detection of NaP fractions (lanes 1, 2) and 2 mM *d*-biotin fractions (lanes 3, 4). The unbound fraction was recycled for additional rounds of the photolabeling procedure (lanes 2, 4).

provement of the yield. The photolabeled protein mixture was loaded on an immobilized monomeric avidin column. The column was treated with a phosphate buffer (200 μl×15) and *d*-biotin in phosphate buffer (200 μl×5) successively. The adsorption at 280 nm of all fractions indicated that most of the protein was passed through the monomeric avidin column (Fig. 2A). Most of the GalT activity, which could have come from unlabeled protein, was recovered in these fractions (98±3%). Chemiluminescence revealed the biotinylated component, showing that the major portion (estimated above 80%) of labeled protein was recovered in *d*-biotin fractions (Fig. 2B, lane 3). These results indicated that monomeric avidin was more effective for purification of photoaffinity biotinylated components than for those of streptavidin. The unbound GalT was recycled for additional rounds of photoaffinity labeling, and the labeled protein was isolated by immobilized monomeric avidin in the same manner described above (Fig. 2B, lane 4). The monomeric avidin approach was useful for the purification as well as for the accumulation of photolabeled GalT proteins.

Identification of Labeled Peptides with Proteolysis of Labeled GalT The labeled and monomeric avidin purified GalT protein was blotted on poly(vinylidene difluoride) (PVDF) membrane, followed by sequential *in situ* digestion to effect better yield of peptide fragments.[9] The blotted membrane was treated with *Staphylococcus aureus* V8 protease and *N*-tosyl-phenylalanine chloromethyl ketone (TPCK)-trypsin successively. The peptides released in the digestion buffer were purified by HPLC. The fragments became less hydrophobic after the digestion and were not well retained on the PVDF membrane for chemiluminescent detection of biotinyl components. For improvement of the peptide retention properties, a commercial PVDF membrane was photochemically modified with nitrophenyldiazirine followed by the reduction of nitro group to lead an aminophenyl functional group. Peptide carboxy groups can be covalently connected to this aminophenyl group using water-soluble carbodiimide reagent.[6] Using the membrane, no chemiluminescence fraction was detected in V8 peptides (Fig. 3), but major chemiluminescence was detected in tryptic peptides (Fig. 4A). To confirm this result, the digest mixture was directly treated with immobilized streptavidin beads for removing biotinyl components from the mixture. Although the UV profiles of the supernatant were no different than they were before the treatment, no chemiluminescence could be detected in the peak position where the biotinyl component should be eluted (Fig. 4B). Furthermore, we separated a peak migrating in the same retention time from unlabeled GalT digestion, with no chemiluminescence. These findings indicate

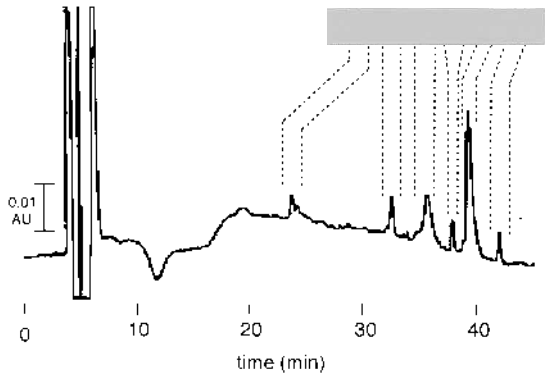

Fig. 3. HPLC Profile of Peptides from V8 Digest of Photolabeled GalT

The upper photograph is the chemiluminescence pattern of each fraction after immobilization on modified PVDF membrane.

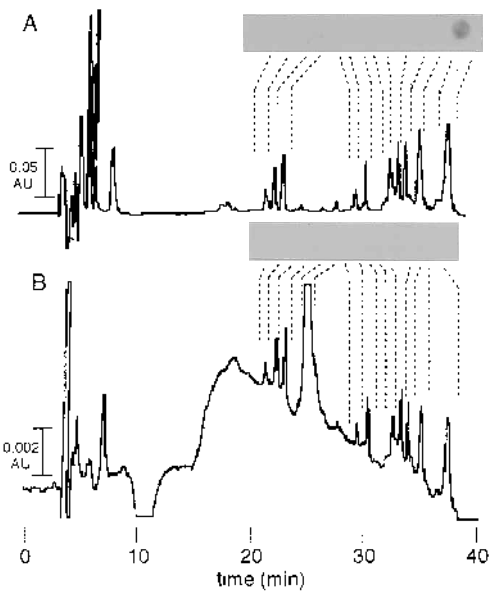

Fig. 4. HPLC Profile of (A) Peptides from Tryptic Digest of Photolabeled GalT and (B) Treated Tryptic Digests of Photolabeled GalT with Immobilized Streptavidin

The chemiluminescence patterns are similar to those in Fig. 3.

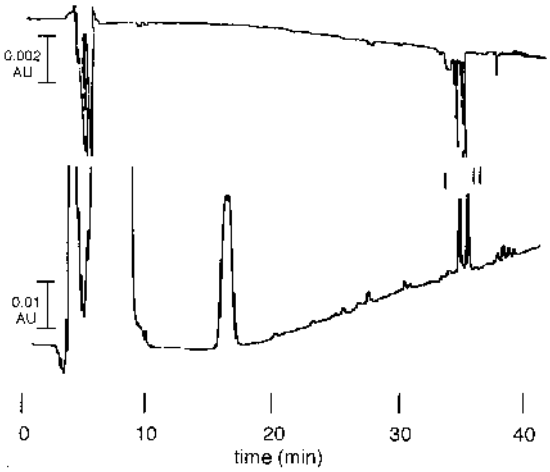

Fig. 5. HPLC Purification of Biotinyl Peptides from AP-I Digest of Photolabeled GalT with Immobilized Monomeric Avidin

The peptides are monitored at 215 nm (upward) and 280 nm (downward).

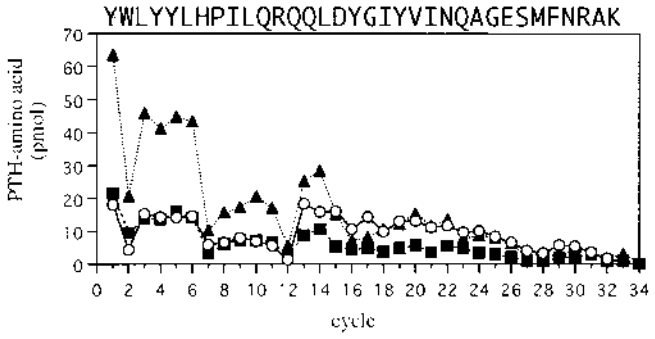

Fig. 6. Sequence Analysis of Photolabeled Peptides

▲: peptide I, ■: peptide II, ○: unlabeled Y197-K230.

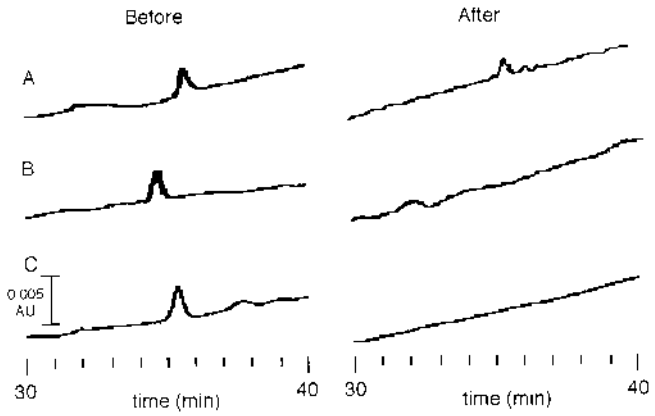

Fig. 7. HPLC Profiles of Photolabeled AP-I Peptides with Immobilized Streptavidin

(A) unlabeled peptide (Y197-K230), (B) labeled peptide I, (C) labeled peptide II.

that labeled peptides could coexist with some unlabeled peptides and the biotinyl fragment was absorbed by immobilized streptavidin selectively. Sequence analysis of the chemiluninescent positive fraction revealed the presence of three peptides: two major components predicted as tryptic fragments of GalT S96-R129 and A98-R129 from the first 20 residues sequence and the third fragment sequence of the first 6 residues, YWLYYL, corresponding to the predicted GalT fragment Y197-R208, which has a very low initial yield (1 pmol). We performed a sequence analysis of the peak after treatment with immobilized streptavidin to distinguish the photolabeled peptide, which revealed that only the Y197-R208 peptide disappeared specifically.

AP-I Digestion of Photolysis GalT Mixture and Purified Labeled Peptides To confirm the results obtained with the modified dot blot analysis, the photolabeled mixture was directly subjected to digestion. We used the protease *Achromobactor lyticus* endoproteinase Lys-C (AP-I), since it did not produce the S96-R129 fragment that interfered with the analysis of the tryptic fragments. Biotinylated fragments from the AP-I digest were isolated by immobilized monomeric avidin using the method described for the isolation of labeled GalT protein. This process revealed that the bound fraction consisted of two strong peaks showing UV absorption at 215 as well as 280 nm (Fig. 5, I, II) in HPLC. No corresponding peak was observed in the avidin treated digest mixture when GalT was photolabeled in the presence of inhibitor **2**.[6] Sequence analysis revealed that both peaks consisted of the same region Y197-K230 (Fig. 6), although photolabeled amino acid residues, which may be different between the two peptides, were not observed due to the chemical instability of the cross-link during the conditions of sequence analysis. The unlabeled Y197-K230 peptide was isolated from AP-I digestion of unlabeled GalT for control experiments and had no chemiluminescence. The two labeled peptides and corresponding unlabeled peptide were treated with immobilized streptavidin, and both labeled peptides could be bound to the immobilized streptavidin but the unlabeled peptide could not (Fig. 7). These observations clearly indicated that the Y197-K230 sequence was biotinylated, as expected from the tryptic fragments.

Conclusion

The biosynthesis of oligosaccharides is dependent on a series of highly specific enzymes, glycosyltransferases, which recognize a specific sugar nucleotide and a glycoconjugate acceptor and transfer a glycosyl group in a defined anomeric configuration to a specific hydroxyl group in the acceptor substrate.[1,10] A major factor in determining the specific nature of the oligosaccharide components of glycoconjugates is the substrate specificity of the glycosyltransferases. On a few occasions, we observed that enzymes acting on similar substrates with the same mechanism, and classified in different families, displayed intriguing local similarities that could not be extended to the rest sequence.[11]

The enzyme GalT is one of the most extensively studied glycosyltransferases, and it was the first to be cloned and sequenced.[2] GalT utilizes UDP-galactose and GlcNAc as the donor and acceptor, respectively. The Galβ1,4GlcNAc α1,3-galactsyltransferase (α1,3-GlcT)[12,13] which utilizes UDP-galactose as the donor substrate exhibits low homology with GalT, but a hexapeptide (residues 349—354 of GalT) at the carboxyl-terminal part appears to be conserved as a possible region in donor binding.[12,14] cDNA cloning of *L. stagnalis* β1,4-*N*-acetylglucosaminyltransferase (β1,4-GlcNAcT), which shares the same acceptor substrate, revealed that the highest sequence identity (50%) is found between GalT in amino acids 181—326 of the bovine sequence,[15] and the GlcNAc acceptor binding region should be involved in this large sequence. A series of site-directed mu-

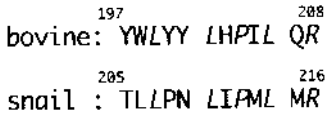

Fig. 8. Alignment of a Labeled Region of Bovine GalT from Photoaffinity Biotinylation with Similarly Located Regions of Snail β1,4-GlcNAcT

The italics indicate identical residues in the four sequences.

tagenesis in human GalT indicated that several aromatic amino acids (human Y284, Y309 and W310 corresponding to bovine Y286, Y311 and W312) are supposed to play a critical role in acceptor binding. One of these amino acids (Y309 in human) is also involved in UDP-galactose binding.[16] Furthermore, recent studies of site-directed mutagenesis indicated that the amino acids F305-N308 in human corresponding to bovine F307-N310 are important for GalT catalysis or are located close to the binding of UDP-galactose.[17]

The present status of most glycosyltransferases, however, suggests that the presence of low similarities for primary sequences between glycosyltransferases utilizing the same donor or acceptor. Almeida *et al.* reported in their studies of human GalT that specific functions of a newly homologous gene in a glycosyltransferase family are not easily predicted even though homologous glycosyltransferases recognize the same nucleotide of the donor substrate and form the same configuration of linkages.[18] Furthermore, alignment of a cloned 14 full-length β4GalT family cannot clearly predict the information of acceptor binding sites.[19] Therefore, it would be better preferred to reveal the substrates binding regions with other approaches.

Photoaffinity labeling[20] is frequently used to elucidate protein-ligand interaction without sequence similarities. A conventional radioactive diazirine photoprobe has been used for labeling of the acceptor binding site, but the labeled site was not identified.[3] One possible difficulty may be the relatively large-scale purification of labeled peptides using large quantities of radiolabel due to the low affinity to the enzyme. We have investigated the acceptor binding domain of GalT using photoaffinity biotinylation, which is radiochemical-free photoaffinity labeling. Photoaffinity biotinylation of bovine GalT using **1** provided the rapid identification of an acceptor binding region (Y197-R208) by means of the combined use of the dot blot analysis on aminophenyl modified PVDF membrane and the purification by monomeric avidin column. The region is included in the highest sequence identity between GalT and *L. stagnalis* β1,4-*N*-acetylglucosaminyltransferase. Sequence alignment analysis of bovine GalT and snail GlcNAcT is applied to this region, which is well identified between GalTs obtained from different source (Fig. 8). It revealed that three leucines (199, 202, 206), P204 and R208 in bovine were well conserved.

It has previously reported that the amount of photoincorporation **1** to GalT protein is influenced by the presence of donor analogue, UMP, and incubation temperature.[5,21] One of the possible explanations could be conformational changes which approach of N-(acceptor region including Y197-R208) and C-terminal (donor region including Y286, F307-N312) is caused by the presence of donor and acceptor in a same manner as bacteriophage β-glucosyltransferase with X-ray crystallographic analysis.[22]

These results clearly demonstrate the effectiveness of photoaffinity biotinylation for rapid identification of photolabeled peptides in substrate binding sites.[6] The systematic investigated methodology has been applied to glycobiologies[4a,b] and other fields.[4c,23]

Such an approach, combined with rapidly advancing molecular cloning and protein sequencing techniques, means that chemical identification of binding site sequences will be an attractive and challenging frontier for organic chemistry.

Experimental

Materials and Methods Bovine β1,4-galactosyltransferase was purchased from Sigma. Synthesis of photoaffinity label reagent **1** (Fig. 1) was reported previously.[5] Streptavidin-horseradish peroxidase conjugate and Hyper-film ECL were obtained from Amersham. Chemiluminescence detection reagents (Renaissance) were obtained from DuPont NEN. Immobilized streptavidin and monomeric avidin were Pierce UltraLink grade. The digest enzymes, *Staphylococcus aureus* V8, TPCK-trypsin and *Achromobactor lyticus* endoproteinase Lys-C (AP-I), were obtained from Boehringer, Worthington and Wako Chemical, respectively. PVDF membrane (Immobilon P) was obtained from Millipore, and the aminophenyl modification of PVDF membrane was described previously.[6] HPLC was performed with a JASCO system equipped with a Develosil C8 column (150 mm×4.6 mm i.d., Nomura Chemical) at a flow rate of 1 ml/min. The mobile phase consisted of eluent A, 5% CH_3CN/H_2O (v/v) containing 0.1% trifluoroacetic acid (TFA), and eluent B, 95% CH_3CN/H_2O (v/v) containing 0.08% TFA, which was eluted with a linear gradient programmed at 0—5 min, B, 5% and 5—75 min, B, 5—95%. The eluates were detected with a UV-VIS spectrophotometer (JASCO) at 215 and 280 nm. Sequencing of peptides was carried out on an Applied Biosystems 492 sequencer. All other chemicals were commercially available and used without further purification.

Purification Labeled GalT Protein with Immobilized Monomeric Avidin Photoaffinity labeling of bovine GalT (0.2 μmol) was carried out in the manner described previously.[5] After irradiation, the mixture was ultrafiltrated with microcon-10 at 4 °C to remove excess reagents, adjusted to 100 μl with 0.1 M sodium phosphate pH 7.8 (NaP) and subjected to an immobilized monomeric avidin column (200 μl gel). The column was treated with NaP (200 μl×10) and then 2 mM *d*-biotin in NaP (200 μl×6). Each fraction was concentrated with microcon-10 at 4 °C. Photoaffinity relabeling of unbound fractions was carried out in the same manner described above. The GalT activities were estimated spectrophotometrically using the method of Pierce *et al.*[24] The proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel) followed by Western blotting for chemiluminescence detection as described previously.[5]

***In Situ* Digestions of Labeled GalT with V8 Protease and TPCK-Trypsin** The denaturation, *S*-pyridylethylation and *in situ* digestion were performed according to the method of Iwamatsu and Yoshida-Kubomura[9] with slight modification using 4-vinylpyridine instead of iodoacetic acid. The protein-blotted membrane was immersed in 10% acetonitrile–0.1 M NH_4HCO_3 pH 7.8 (300 μl) and incubated at 25 °C with V8 protease (20 pmol) for 17 h. The membrane was subjected to a second digestion of TPCK-trypsin (10 pmol) in 10 mM $CaCl_2$–0.1 M NH_4HCO_3 pH 7.8 (300 μl) at 25 °C for 17 h. Both digestion buffers were directly subjected to HPLC.

Chemiluminescence Detection of *in Situ* Digested Peptides A HPLC fraction of *in situ* digestion (10—25 μl/fraction) was blotted on the modified PVDF membrane,[6] dried up at 60 °C, blotted 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride in 5% CH_3CN/H_2O (20 mM, 10—25 μl) on the same place and reacted for over 2 h at room temperature. The membrane was subjected to chemiluminescence detection.

AP-I Digestion of the Photolyzed GalT Mixtures and Purified Labeled Peptides The preparative scale of photoaffinity labeling was carried out under the following conditions. The mixture of bovine GalT (0.75 μmol), 0.5 mM UMP, 2.0 mM $MnCl_2$, and 0.2 mM BDGA **1** in 50 mM *N*-ethylmorpholine pH 8.0 (3.8 ml) was incubated at 37 °C for 30 min under an argon atmosphere and cooled at 4 °C for 10 min in the dark, followed by irradiation with a 30-W black-light (Ultra-Violet Products Inc.) at 6 cm for 40 min on the ice. The labeled mixture was dialyzed against 0.1 M Tris–HCl pH 8.2 at 4 °C for 12 h, denatured and *S*-carboxymethylated with dithiothreitol (26.3 μl, 0.38 mmol) and guanidine hydrochloride (6.513 g, 68.2 mmol) at room temperature for 1.5 h and then with sodium iodoacetate (0.780 g, 3.75 mmol) at room temperature for 1 h, and subjected to a Sephadex G-50 column (3×20 cm, 0.1 M Tris–HCl pH 8.2) monitored with absorbance at

280 nm. The gel filtrated GalT (0.70 mmol), urea (0.12 g, 2.0 mmol) and AP-I protease (0.50 nmol) in a buffer of 0.1 M Tris–HCl pH 8.2 (19 ml) were incubated at 37 °C for 12 h and then subjected to a Sephadex G-10 column (4×18.5 cm, NaP). As a control experiment, the same treatment was carried out in the presence of label inhibitor **2** (100 mM). The eluants from the Sephadex G-10 column were subjected to an immobilized monomeric avidin column (0.2 ml gel) at 4 °C. The column was treated with NaP (0.5 ml×5), followed by treatment with *d*-biotin in NaP (2 mM, 0.2 ml×7). The fraction from the *d*-biotin treatment was directly analyzed with HPLC. We performed chemiluminescence detection of all HPLC fractions using the aminophenyl-modified PVDF membrane. The two chemiluminescence-positive fractions were analyzed for peptide sequence and identified as the Y190-K230 fragment of bovine GalT. Unlabeled Y197-K230 peptide was afforded from the Sephadex G-10 column from the control experiment in the presence of competitor **2**. Two labeled and unlabeled peptides were neutralized by sodium hydrogen phosphate (1 M, 0.1 volume of HPLC fraction), followed by suspension in immobilized streptavidin at room temperature for 2 h. After centrifugation, the supernatant was analyzed by HPLC.

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