Rice Golgi Proteome: Identification and Characterization of *N*-acetylglucosaminyltransferase- I - like Protein

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Summary

A shotgun proteomic analysis of the nucleoside diphosphatase (NDPase)-associated Golgi membranes isolated from rice suspension-cultured cells was performed by multi-dimensional liquid chromatography-tandem mass spectrometry. The Golgi membranes contained an *N*-acetylglucosaminyltransferase-I-like protein, in addition to reversibly glycosylated polypeptide, putative xylosyltransferase and vacuolar sorting receptor. *N*-acetylglucosaminyltransferase-I-like protein (GNTI-LP) synthesized by a cell-free transcription/translation system from the cDNA clone catalyzed the reaction forming (GlcNAcβ1-2) (Manα1-6) (Manα1-3) $(Man)_3$- $(GlcNAc)_2$- PA from (Manα1-6) (Manα1-3) $(Man)_3$- $(GlcNAc)_2$- PA and UDP-GlcNAc, indicating that the protein is an *N*-acetylglucosaminyltransferase-I. The optimal pH for the enzyme reaction was 8.0, but the enzyme activity was expressed at a broad pH range between 5 and 8. The optimal temperature was 30℃. The enzyme required Mn^{2+} and Mg^{2+}, and the activity was inhibited with EDTA. The *K*m value for UDP-GlcNAc was determined to be 0.58 μM. Furthermore, the *N*-terminal region including a transmembrane domain (M1 to Q120) was not essential for the enzyme activity. Western blot analyses with anti-GNTI-LP revealed that the Golgi membranes actually accumulate a protein that migrates as a 51 kDa band in SDS gels.

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Key words : Glycoprotein, Golgi, *N*-acetylglucosaminyltransferase-I, *Oryza sativa*, Proteome

The Golgi apparatus is a multifunctional organelle, responsible for the biosynthesis of complex cell-surface polysaccharides, the processing and modification of glycoproteins, and the sorting station of polysaccharides and proteins destined for different locations. The Golgi apparatus locates the center of main route of protein traffic, that is, numerous proteins come in the Golgi from the endoplasmic reticulum (ER), and come out to the plasma membrane and vacuole (Hawes and Satiat-Jeunemaitre, 1996). In addition to these, recent investigations have demonstrated that some glycoproteins are transported to the plastids from the ER-Golgi system through the secretory pathway (Asatsuma et al. 2005, Villarejo et al. 2005, Nanjo et al. 2006, Kitajima et al. 2009).

The proteome analysis is a useful tool for clarifying the dynamic function of Golgi apparatus. The Golgi apparatus is thought to be made up of approximately 1,000 proteins. So far, about 200 Golgi proteins have been identified from a variety of tissues from several animal species (Taylor et al. 2000). A proteomic analysis of proteins from rat hepatic Golgi fraction has been reported (Bell et al. 2001). A total of 81 proteins have been identified. They include Golgi-resident enzymes, Golgi lectin, anterograde cargo, KDEL receptors, p24 family members, SNAREs, RABs, and ARF-GEF. Dunkley et al. (2006) have performed a proteomic analysis of *Arabidopsis* Golgi by using the localization of organelle proteins by isotope tagging (LOPIT) technique. The 89 proteins assigned by LOPIT to the Golgi apparatus largely belonged to three main classes: predicted glycosyltransferases, EMP70 proteins, and putative methyltransferases. Our cell fractionation study of rice cis-Golgi membranes labeled with GFP-SYP31 (a cis-Golgi SNARE, Uemura et al. 2004) revealed that the cis-Golgi membranes contains membrane-bound α-mannosidase activities, and several membrane traffic-related proteins including a set of RAB (YPT1) family and ER resident proteins, but the Golgi-resident glycosyltransferases were hardly detectable (Asakura et al. 2006). Further exhaustive analysis of Golgi membrane proteins is required for clarifying the rice Golgi proteome in detail.

In the present study, we carried out a shotgun proteomic analysis of the NDPase-associated Golgi membranes isolated

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from rice suspension-cultured cells. We identified and characterized *N*-acetylglucosaminyltransferase-I-like protein in the NDPase-associated rice Golgi membranes.

MATERIALS AND METHODS

Materials

A full-length cDNA of rice *N*- acetylglucosaminyltransferase-I-like protein (GNTI-LP) (LamdaFLC-GNTI-LP; accession No. AK101526) was supplied from the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). Restriction enzymes and other enzymes were purchased from Toyobo (Tokyo, Japan) unless otherwise stated. Oligonucleotides were synthesized by Hokkaido System Science (Sapporo, Japan). The Ni-chelating column was from Sigma (St. Louis, MO). *Escherichia coli* Origami B and pETb vector were from Takara (Kyoto, Japan) and Novagen (Madison, WI), respectively.

Rice seeds (*Oryza sativa* L. cv. Nipponbare) were supplied from the Niigata Agricultural Research Institute (Niigata, Japan).

Preparation of NDPase-associated Golgi membranes

Preparation procedure of Golgi membranes from rice suspension-cultured cells is summarized in Fig. 1. Rice cells (100 g) cultured for 6 days were gently homogenized with a half volume (50 ml) of 25 mM HEPES-KOH (pH 7.0), 1 mM EDTA, and 0.5 M mannitol in a mortar with pestle. The homogenate was passed through two layers of gauze and centrifuged at 1,000 x *g* for 20 min and 10,000 x *g* for 30 min, sequentially. The supernatant was layered on a 15% (w/w) sucrose layer and 50% sucrose cushion containing 25 mM HEPES- KOH (pH 7.0), and further centrifuged at 100,000 x *g* using a Beckman Type 55.2 Ti rotor for 3 h. The membrane fraction trapped on the 50% sucrose cushion was used as the microsome preparation.

The microsome preparation (approximately 330 mg proteins) was diluted with 25 mM HEPES-KOH (pH 7.0) to 26% sucrose, and adjusted with 1 M $MgCl_2$ to 5 mM $MgCl_2$. The microsomal membranes were layered on a 16 ml of discontinuous density gradient consisting of an equal volume of 30, 34, 38, and 42% sucrose solution containing 25 mM HEPES-KOH (pH 7.0) and 5 mM $MgCl_2$. The gradients were centrifuged at 100,000 x *g* using Type 55.2 Ti rotor for 3 h at 4℃. The NDPase-associated Golgi membranes were sedimented to 30% sucrose layer. The Golgi membrane fraction collected was adjusted to 30% sucrose again, layered to the phase between 28 and 34% sucrose of the second discontinuous sucrose gradient, and centrifuged at 100,000 x *g* for 3 h at 4℃.

Assays

Activities of Triton X-100-stimulated NDPase were determined by measuring phosphate liberated from IDP for 10 min at 37℃ in a reaction mixture consisting of 80 mM Tris-maleate-NaOH (pH 7.0), 4 mM $MgCl_2$, 1 mM sodium

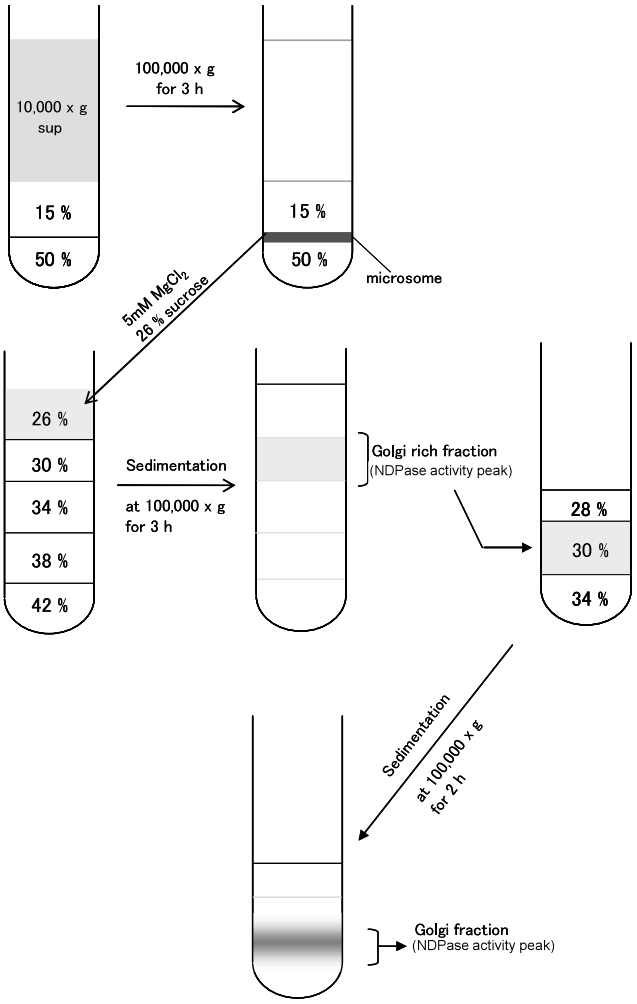

Fig. 1 Schematic illustration of fractionation procedure of rice microsomal membranes by discontinuous sucrose density gradient centrifugation. Microsome membrane preparation was layered on discontinuous sucrose gradient consisting of 30, 34, 38 and 42% sucrose. The NDPase Golgi membranes fractionated in the 30% sucrose layer by centrifugation. The membrane fraction was subjected again to the second discontinuous sucrose gradient consisting of 28, 30 and 34% sucrose. Further details of fractionation procedure are described in the text.

Molybdate, 0.1% (w/v) Triton X-100, and 4 mM IDP. The amount of phosphate was determined by Phosphar C-Test (Wako) (Mitsui et al. 1994). *N*-acetylglucosaminyltransferase activity was determined by measuring the incorporation of [^{3}H]GlcNAc from UDP-[^{3}H] GlcNAc into ovalbumin oligosaccharide. The reaction mixture consisted of 50 mM glycylglycine-NaOH (pH 7.0), 10 mM $MnCl_2$, 0.1% Triton X-100 (w/v), 5 μM UDP-GlcNAc, 1.5 kBq UDP-[^{3}H] GlcNAc (1.85 TBq/mmol), 25 mg/ml ovalbumin, and 300 to 600 μg enzyme proteins in a total volume of 60 μl. The reaction was started by the addition of enzyme and kept for 30 min at 30 ℃, and

then terminated by addition of 10 μl of 200 mM EDTA and cooling down. To determine the incorporation of [^{3}H] GlcNAc, an aliquot of the reaction mixture was placed on a glass filter (Advantec, GC-50) and washed with 10% TCA for 5 min at 70℃, 10% TCA for 10 min twice, and ethanol for 10 min twice at room temperature, sequentially. The radioactivity remaining in the dried glass filter was measured using a liquid scintillation counter (Aloka, LSC-6100). When bovine serum albumin, denatured bromelin, and desialized porcine mucin were used as the acceptor, the incorporation of [^{3}H] GlcNAc to the polypeptide was not detectable (data not shown) (Mikami et al. 2001). The protein assay was carried out according to the method described by Bradford (1976) with bovine gamma globulin as a standard. The sucrose content was determined with a refractometer (Atago, NAT-1T).

Mass analysis

NDPase-associated Golgi membrane fractions were diluted with equal volume of 25 mM HEPES-KOH (pH 7.0) and centrifuged at 100,000 x *g* for 3 h. The membrane pellets were resuspended with 25 mM HEPES-KOH (pH 7.0) and 2% (w/v) CHAPS, and sonicated for 5 min in ice bath two times. The suspension of membrane proteins was mixed with 1/10 volume of 100% (w/v) trichloroacetic acid, and stood on ice for 20 min. The membrane protein precipitates were rinsed with ethanol two times and dissolved in 7.0 M guanidine-HCl buffered (pH 8.0) containing 10 mM EDTA. The preparations were reduced by the addition of 1mM dithiothreitol and were alkalated with 10 mM iodeacetamide under a nitrogen atmosphere. The S-carbamoylmethylated proteins were dialyzed against 10 mM Tris-HCl (pH 8.0) to remove the excess reagents and then were digested overnight at 37℃ with sequence grade modified trypsin (Promega, Madison, WI, USA) at an enzyme-substrate ratio of 1 : 25 (w/w). The digests were acidified to pH 2 by the addition of an aliquot of concentrated HCl, and the precipitates formed were removed by centrifugation. The supernatant was adjusted to pH 8 with aqueous ammonia and was subjected immediately to the automated multidimensional liquid chromatography-tandem mass spectrometry as described by Mawuenyega et al. (2003).

Cell-free Protein Synthesis of GNTI-LP

A rapid cell-free protein synthesis (RTS) from the full-length GNTI-PL cDNA was performed according to the manufacturer's protocol (Roche, Basel, Switzerland). The first PCR was carried out using forward and reverse primers 5' -CTTTAAGAAGGAGATATACCATGGCGCGGAGCCCCTG CGAC -3' and 5'-TGATGATGAGAACCCCCCCCTTATACC CTAAGCTGACTGAG-3', respectively, and LamdaFLC-GNT-LP as DNA template. The forward primers for truncation were as follows: (Δ1-4 amino acid residues) 5' -CTTTAAGAA GGAGATATACCATGTGCGACCTCCGCATCCTCCTC-3', (Δ 1-14 amino acid residues) 5'-CTTTAAGAAGGAGATATACC ATGGCTGCAGCCTTCATCTACATC-3' (Δ1-32 amino acid residues) 5'-CTTTAAGAAGGAGATATACCATGGCCGACC GCCTCGCACAAGCA-3' (Δ1-91 amino acid residues) 5'-CTT TAAGAAGGAGATATACCATGAGCGTGCAGACCTTAGT AAAC-3' (Δ1-120 amino acid residues) 5'-CTTTAAGAAGGA GATATACCATGACAGTTGAATCTATCCTGAAG-3'. The second PCR was carried out with the first PCR products and RTS Wheat Germ linear Template Generation set. The second PCR product was reacted with RTS 500 Wheat Germ CECF kit in RTS PROTEO-MASTER (Roche) at 900 rpm for 24 h at 24℃ .

Product analysis of GNTI-PL enzyme reaction

The enzyme reaction was performed in the mixture consisted of the RTS product of GNTI-PL with or without boiling and 0.16 mM (Man α1-6) (Man α1-3) $(Man)_3$- $(GlcNAc)_2$-PA, 50 mM glycylglycine- NaOH (pH 7.0), 10 mM $MnCl_2$, 0.1 % Triton X-100 (w/v) and 25 mM UDP-GlcNAc in a total volume of 60 μl. The reaction was kept for 2 h at 30℃, and then terminated by addition of 10 μl of 200 mM EDTA and cooling down. The reaction products were analyzed by a combination of reversed-phase (RP-) and size-fractionation (SF-) HPLC, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), and exoglucosidase digestion, as described previously (Misaki et al. 2003). The analysis of PA-sugar chains were performed on a Hitachi HPLC apparatus and monitored at the excitation and emission wavelengths of 310 nm and 380 nm, respectively. The conditions for RP- and SF-HPLC using Cosmosil 5C18-AR (Nacalai Tesque, Kyoto, Japan) and Asahipak NH2P-50 4E (Showa Denko) columns were as described previously (Misaki et al. 2003). For the exoglycosidase digestion of PA-sugar chains, N-acetylglucosaminidase (1 mU; Diplococcus pneumoniae, Roche) and α-mannosidase (10 mU; jackbean, Sigma) were used as described previously (Misaki et al. 2003). The reactions were stopped by boiling the mixtures for 3 min. After centrifugation at 12,000 rpm for 10 min at 4℃, the supernatant was analyzed by SF-HPLC. The elution positions of the components of the supernatant were compared with the elution positions of authentic PA-sugar chains (Takara). The molecular masses of PA-sugar chains were determined by MALDI-TOF MS using an autoflex mass spectrometer (Bruker Daltonics, Billerica, MA).

Expression of Rice GNTI-PL in Escherichia coli

The GNTI-LP cDNA was amplified by PCR from LamdaFLC-GNTI-LP plasmid using forward and reverse primers 5'-ATCATATGATGGCGCGGAGCCCCTGCGA -3' and 5'-TTCTCGAGCTATACCCTAAGCTGACTGAGG-3', respectively. For GNTI-PL expression in E. coli, the NdeI-XhoI fragment from the PCR product was cloned into pET-15b expression vector tocreate pET-GNTI-LP. E. coli Origami B transformed with this plasmid were grown to an absorbance at 600 nm of about 0.6, and then 1 mM isoprophyl-β-D-thiogalactopyranoside was added to the culture medium. These cells were centrifuged at 4,700 x *g* for 20 min and then suspended in HEM buffer (10 mM HEPES,

pH 7.2, 1 mM $MgCl_2$, 1 mM EGTA, 25 mM NaCl, and 1 mM DTT) prior to storage at -80℃ until use. The frozen cells were thawed and suspended in 20 ml lysis buffer for a Ni-column (100 mM Tris-HCl, pH 7.8, 300 mM NaCl, 0.1 mM leupeptin, 0.1 mM PMSF, and 0.1 mM DTT) and then sonicated for 5 min. The sample was clarified by centrifugation at 100,000 x *g* for 1 h at 4℃ . The supernatant was stored at 4℃ until use. The stored supernatant was loaded on a Ni-NTA column, which had been equilibrated with native buffer (300 mM NaCl, 100 mM Tris-HCl, pH 7.5, and 0.2 mM β-mercaptoethanol). The column was washed with native buffer, and then with native buffer containing 50 mM imidazole-HCl, pH 7.5. The desired protein was eluted with 100 mM imidazole-HCl, pH 7.5, in native buffer, and the fractions containing kinesin were pooled. Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which gave a single band on Coomassie-stained gels. Samples were dialyzed against 120 mM NaCl, 30 mM Tris-HCl, pH7.5, and 1 mM DTT, and stored at −80℃ until use.

Preparation of anti-GNTI-LP antisera

The pure recombinant rice GNTI-LP expressed in *E. coli* was used to raise antibodies. One mg of the polypeptide mixed with Freund's complete adjuvant was injected into rabbit. After three weeks, three subsequent injections (1 mg for each injection) mixed with incomplete Freund's adjuvant were done at an interval of two weeks. Positive antiserum was collected from the rabbit and stored at −80℃.

Blotting analysis

An aliquot of the NDPase-associated Golgi membranes (30 μl) was subjected to SDS-PAGE. After SDS-PAGE, proteins in the gels were transferred to a nitrocellulose sheet (Advantec, A045A224D) in 25 mM Tris-192 mM glycine- 20% methanol at 0.3 mA cm^{-1} for 3 h using a semi-dry electroblotter (Atto, AE-6677). In immunblotting, anti-GNTI antiserum was diluted to 1 : 4000, and peroxidase-conjugated anti-rabbit IgG IgG was diluted to 1:8000. The blotted nitrocellulose sheet was soaked with PBST (phosphate buffered saline with 0.05% Tween 20) for 15 min three times and incubated with the primary antibody in PBST containing 1% (w/v) skimmed milk for 12 h at 4℃. The sheet was then incubated with the second antibody for 3 h at room temperature as for the primary antibody.

RESULTS AND DISCUSSION

Shotgun proteomic analysis of rice NDPase-associated Golgi membranes

Golgi membranes were isolated from suspension-cultured cells of rice by a discontinuous sucrose density gradient centrifugation under 5 mM $MgCl_2$ supplemented conditions according to the procedure reported previously (Mikami et al. 2001). As shown in Fig 2, the membranes associated with a Golgi marker enzyme NDPase were symmetrically

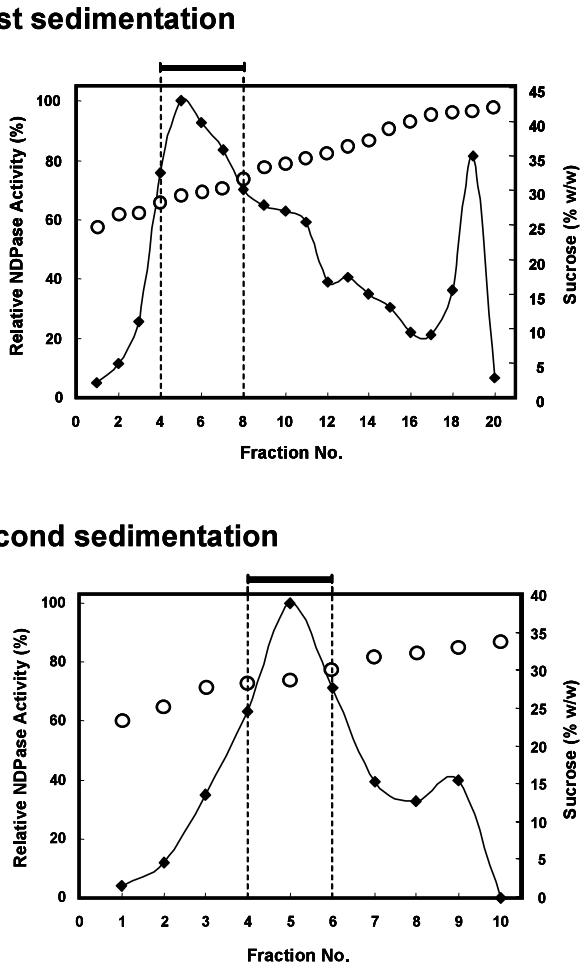

Fig. 2 Isolation of NDPase-associated Golgi membranes from rice cells. Separation profiles of the activity of Golgi marker enzyme NDPase in the first and second sucrose gradients as described in Fig 1.

distributed at around 30% sucrose in the second centrifugation. The isolated Golgi membrane fraction contained little contaminants of cytosol, endoplasmic reticulum, mitochondria, plastid and peroxisome markers (not shown, Mikami et al. 2001). The Golgi membrane proteins were solubilized with lysis buffer containing 9 M urea, 3 % (w/v) IGEPAL CA-630, and 2 % (v/v) 2-mercaptethanol, and completely digested with trypsin. The resultant peptides were subjected to a multidimensional liquid chromatography-tandem mass spectrometry (Kaji et al. 2003). In the shotgun MS/MS spectrometric analysis, reversibly glycosylated polypeptide, putative callose synthase, *N*-acetylglucosaminyl-transferase-I-like protein (GNTI-LP), xylosyltransferase, vacuolar sorting receptor, glyceraldehyde 3-phosphate dehydrogenase, V-ATPase, Ca^{2+}-ATPase, and several membrane traffic-related proteins were detected (Table 1).

The *Arabidopsis* Golgi proteins assigned by LOPIT using putative galactosyltransferase gtl6 as the Golgi marker

Table 1 List of rice Golgi proteins identified in NDPase-associated Golgi membranes

FUN. CAT	database	gi(ID)	protein
1.05	oryza	gi|4158221	reversibly glycosylated polypeptide
1.05	oryza	gi|34915190	reversibly glycosylated polypeptide
1.05	oryza	gi|3646373	RGP1 protein
1.05	oryza	gi|3646375	RGP2 protein
1.05	oryza	gi|30017489	putative reversibly glycosylated polypeptide
1.05	oryza	gi|34394574	putative reversibly glycosylated polypeptide
1.05	oryza	gi|34902210	putative callose synthase
6.07	oryza	gi|20372917	N-acetylglucosaminyltransferase I-like protein
6.07	oryza	gi|37535628	putative xylosyltransferase
6.07	oryza	gi|34896984	putative xylosyltransferase
7.01	oryza	gi|9743458	rice EST AU030811, similar to rice Ca+2-ATPase (U82966)
7.01	oryza	gi|24796816	putative calcium ATPase
7.01	oryza	gi|7436378	T04172 Ca2+-transporting ATPase
7.22	oryza	gi|34908022	putative vacuolar proton-ATPase subunit 1
7.22	oryza	gi|14150751	vacuolar ATPase B subunit
8.07	oryza	gi|30017578	putative cop-coated vesicle membrane protein
8.99	oryza	gi|7678766	zeta1-COP
8.07	oryza	gi|7670066	epsilon1-COP
8.07	oryza	gi|34902322	putative Nonclathrin coat protein gamma - like protein
8.07	oryza	gi|7259350	nonclathrin coat protein zeta2-COP
8.07	oryza	gi|13324786	putative alpha-coat protein
8.07	oryza	gi|13324789	putative alpha-coat protein
8.07	oryza	gi|34908014	coatomer delta subunit
8.07	oryza	gi|34903382	putative coatmer beta subunit (beta-COP)
8.07	oryza	gi|34394800	putative coatomer protein gamma 2-subunit
8.07	oryza	gi|34911188	putative dynamin-like protein
8.07	oryza	gi|37534770	putative endoplasmic reticulum membrane fusion protein
8.07	oryza	gi|34906964	putative gamma-adaptin 1
8.07	oryza	gi|42761389	coated vesicle membrane protein-like
8.07	oryza	gi|34896970	putative transport protein particle component
8.07	oryza	gi|29150380	synaptobrevin-like protein
8.07	oryza	gi|37806416	putative alpha-soluble NSF attachment protein
8.07	oryza	gi|29150373	putative vacuolar protein sorting-associated protein
8.07	oryza	gi|37533174	putative vacuolar sorting receptor protein
8.07	oryza	gi|15217311	putative vacuolar sorting receptor protein homolog
8.07	oryza	gi|34897136	putative vacuolar sorting-associated protein
8.07	oryza	gi|13195452	GTP-binding protein
8.07	oryza	gi|34913186	putative GTP-binding protein
8.07	oryza	gi|40539018	putative GTP-binding protein
8.07	oryza	gi|41469625	putative GTP-binding protein
8.07	oryza	gi|34894960	putative GTP-binding protein
8.07	oryza	gi|34906164	putative GTP-binding protein
8.07	oryza	gi|34909538	putative GTP-binding protein
8.07	oryza	gi|34910940	putative GTP-binding protein
8.07	oryza	gi|34912576	putative GTP-binding protein
8.07	oryza	gi|14140133	putative GTP-binding protein
8.07	oryza	gi|33146858	putative GTP-binding protein
8.07	oryza	gi|34900600	putative GTP-binding protein GTP6
8.07	oryza	gi|29647488	putative GTP-binding protein(RAB11G)
8.07	oryza	gi|7438437	GTP-binding protein rab2
8.07	oryza	gi|29293694	small GTP binding protein
8.07	oryza	gi|34913324	putative Rab GTP-binding protein Rab11a
8.07	oryza	gi|27261084	putative RAB24 protein
8.07	oryza	gi|34910572	putative RAB7A protein
8.07	oryza	gi|1710078	REHY_ORYSA RAB24 protein
8.07	oryza	gi|34908216	rac-GTP binding protein -like
8.07	oryza	gi|5360230	Ran
8.07	oryza	gi|34897394	Ras-related GTP-binding protein
8.07	oryza	gi|34908298	Ras-related GTP-binding protein
8.07	oryza	gi|38175435	putative ras-related protein
8.07	oryza	gi|3024552	Ras-related protein
8.07	oryza	gi|34914060	putative RIC1
8.07	oryza	gi|34911282	guanine nucleotide-binding protein beta subujit-like protein
8.07	oryza	gi|34904236	RAS-related GTP-binding protein Rab7 family
8.16	oryza	gi|3643271	33 kDa secretory protein
8.16	oryza	gi|23450951	33-kDa secretory protein
8.16	oryza	gi|38636830	putative 33 kDa secretory protein
8.16	oryza	gi|38636827	putative 33-kDa secretory protein
1.05	oryza	gi|100654	alpha-amylase precursor
2.01	oryza	gi|29150193	putative glyceraldehydes 3-phosphate dehydrogenase
2.01	oryza	gi|42407702	glyceraldehyde 3-phosphate dehydrogenase
8.99	oryza	gi|34894094	putative Rer1A protein (AtRer1A)

Legend of Table 1

Functional category. 1.05: Metabolism (Sugars and polysaccharides); 2.01: Energy (Glycolysis); 6.07: Protein destination and storage (Modification); 7.01: Transporters (Ions); 7.22: (Transport ATPases); 8.07: Intracellular traffic (Vesicular); 8.16: (Extracellular); 8.99: (Others)

contained several predicted glycosyltransferases (Dunkley et al. 2006). The proteomic analysis of rice cis-Golgi membranes labeled with GFP-SYP31 revealed that the cis-Golgi membranes contains no glycosyltransferases, although several membrane traffic-related proteins including a set of RAB (YPT1) family and ER resident proteins were frequently detected (Asakura et al. 2006). Thus, the NDPase-associated Golgi membranes isolated in the present study assumed to be a whole Golgi largely including the medial/trans-Golgi residents.

Characterization of *N*-acetylglucosaminyltransferase-I-like protein

The protein predicted from the full-length cDNA of GNTI-LP comprised 442 amino acid residues with a calculated molecular mass of 51.5 kDa. Its deduced amino acid sequence was identical to *N*-acetylglucosaminyltransferase-I (CAD30022) reported by Léonard et al. (2004). Rice *N*-acetylglucosaminyltransferase-I contained no potential glycosylation site, though the other *N*-acetylglucosaminyltransferase-I found in tobacco, *Arabidopsis thaliana, Caenorhabditis elegans, Drosophila melanogaster, Xenopus laevis* had the *N*-glycosylation site, and the rabbit and human enzyme proteins had the *O*-glycosylation site. To determine whether or not GNTI-LP protein exhibited the activity of *N*-acetylglucosaminyltransferase-I, the protein synthesized in vitro by a cell-free transcription/translation system with wheat germ lysate from the cDNA clone was subjected to the assay of transferase reaction. When the synthesized proteins were incubated with (Manα1-6) (Manα1-3) $(Man)_3$-$(GlcNAc)_2$-PA and UDP-GlcNAc, (GlcNAcβ1-2) (Manα1-6) (Manα1-3) $(Man)_3$-$(GlcNAc)_2$-PA was actually produced, and the enzyme reaction was perfectly stopped by heat treatment (Fig. 3). The recombinant enzyme showed no activity toward UDP-GalNAc and UDP-Glc (Table 2). The optimum pH and temperature for enzyme reaction was 8.0 and 30℃, respectively, but the enzyme activity was expressed at a broad pH range between 5 and 8 (Fig. 4). The enzyme required divalent cation, Mn2+ or Mg2+ and the activity was inhibited with EDTA (Table 2). These results indicate GNTI-LP is *N*-acetylglucosaminyltransferase-I.

It has been demonstrated that *N*-acetylglucosaminyltransferase-I exhibits an ordered 'Bi Bi' kinetic mechanism (Nishikawa et al. 1988). The enzyme binds first Mn^{2+}/UDP-GlcNAc and then $Man_5GlcNAc_2$ acceptor; the oligosaccharide product is then released, followed by UDP. We determined the K_m value for UDP-GlcNAc of rice recombinant enzyme synthesized in vitro to be 0.58 μM (Table 2). Human *N*-acetylglucosaminyltransferase-I expressed in *E. coli* was reported to be an apparent K_m value of 0.483 mM for (Manα1-6) (Manα1-3) $(Man)_3$-$(GlcNAc)_2$-PA (Fujiyama et al. 2001). The K_m value of rice *N*-acetylglucosaminyltransferase-I (CAD30022) expressed in yeast for was 50 μM (Léonard et al. 2004). We considered that these discrepancies come from different translation systems and their combinations.

Furthermore, we produced and determined the enzyme

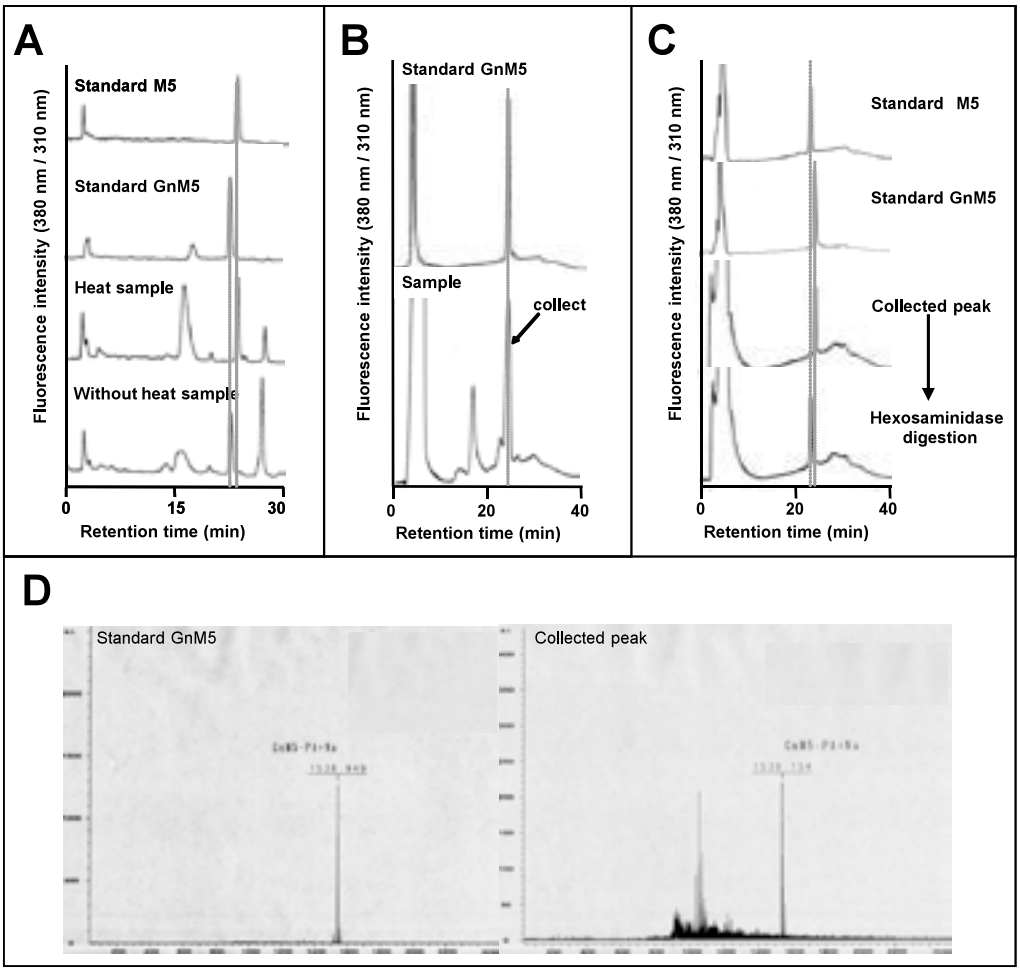

Fig. 3　Structural analysis of reaction product of GNTI-PL. (A) Separation profiles of the reaction product by reverse phase (Cosmosil 5C18-AR)-HPLC. (B) Separation profiles by size fractionation (Asahipak NH2P-50 4E)-HPLC. (C) Separation profiles of *N*-acetylglucosaminidase digested product by size fractionation-HPLC. (D) MALDI-TOFMS spectrum of the reaction product. Details of experiments are described in the text.

Table 2　Characteristics of rice GNTI-PL

Molecular mass (kDa)	51
Substrate specificity	
Donor (Relative activity, %)	UDP-GlcNAc (100), UDP-GlaNAc (22), UDP-Glc (b.d.l.)
acceptor	(Man α1-6) (Man α1-3) $(Man)_3$- $(GlcNAc)_2$
K_m (UDP-GlcNAc, μM)	0.58
Cation requirement (Relative activity, %)	Mn^{2}+ (100), Mg^{2+} (77), Co^{2+} (21), Ca^{2+} (15)
Optimum pH	8.0
Optimum temperature (℃)	30
Sugar chain	No

b.d.l., below detection limit

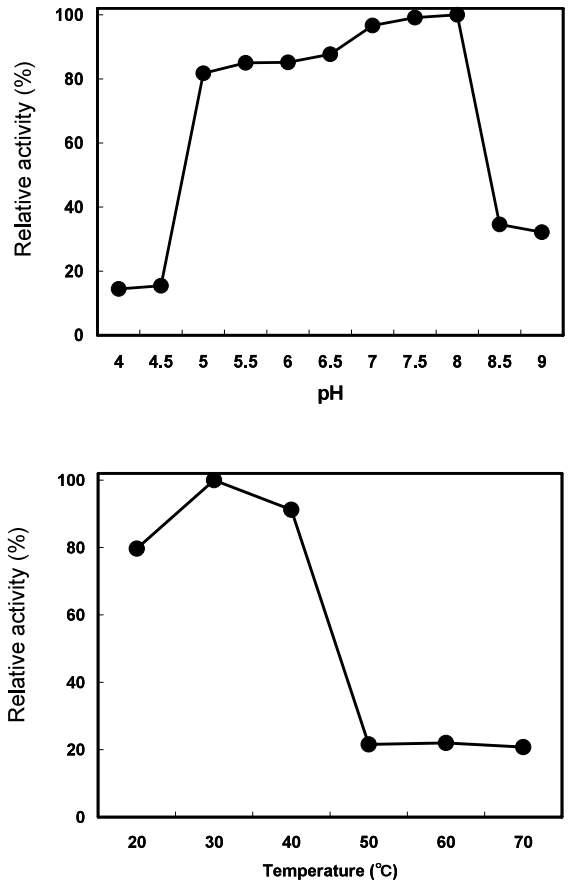

Fig. 4 Optimal pH (A) and temperature (B) for the enzyme reaction of rice GNTI-PL.

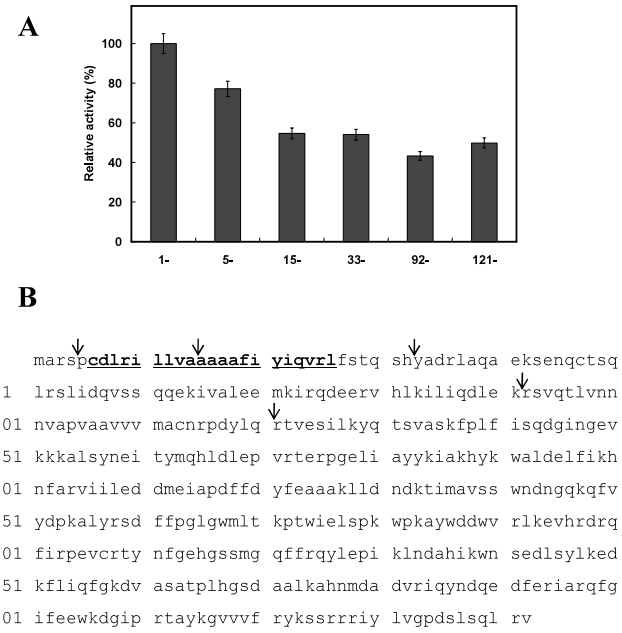

Fig. 5 Activity expression of *N*-terminal truncated GNTI-PL. (A) Activities of the truncated enzymes. (B) Diagram of the truncated GNTI-PL. Underlined bold type, transmembrane domain; arrows, the truncated sites.

activity of a series of *N*-terminal truncated rice GNTI-LP, showing that the *N*-terminal region including a transmembrane domain (M1 to Q120) was not essential for the enzyme activity (Fig. 5). Removal of 106 amino acid residues from the N-terminus of rabbit *N*-acetylglucosaminylt ransferase-I (447 residues) also did not inactivate the enzyme (Sarkar et al. 1998). The study of X-ray crystal structure of rabbit *N*-acetylglucosaminyltransferase-I further supported that residues critical for substrate binding and catalysis exists in the domain 1 (107 to 317 residues) (Ünligil et al. 2000). Role of the *C*-terminal domain 2 remains to be determined.

We produced anti-GNTI-LP antisera by using recombinant GNTI-LP expressed in *E. coli*. Western blot analyses with anti-GNTI-LP antisera revealed that the rice Golgi membranes accumulate a protein that migrates as a 51 kDa band in denaturing SDS gels (Fig. 6). A data base search indicated that rice *N*-acetylglucosaminyltransferase-I is encoded by single gene (Léonard et al. 2004). From these information, the 51 kDa protein band detected with anti-GNTI-LP antisera was concluded to be available as a Golgi marker in rice cell.

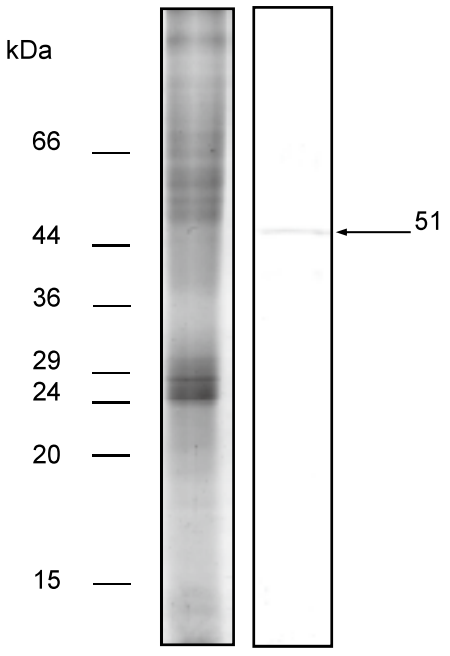

Fig. 6 Immunochemical detection of 51 kDa protein band in SDS-gel with anti- GNTI-PL antisera. The isolated NDPase-associated Golgi membranes were subjected to SDS-PAGE, followed by immunoblotting with anti-GNTI-PL antisera. Details of experiments are described in the text.

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イネゴルジ体プロテオーム：*N*-アセチルグルコサミニルトランスフェラーゼ - Ⅰ様タンパク質の同定と機能解析

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要　約

イネ培養細胞から単離したNDPase-結合ゴルジ体膜のショットガンプロテオミック解析を多次元液体クロマトグラフタンデム質量分析装置を用いて行った。ゴルジ体膜には可逆性グリコシル化ポリペプチド、キシロシルトランスフェラーゼ、液胞選別レセプターに加えて、*N*-アセチルグルコサミニルトランスフェラーゼⅠ様タンパク質（GNTI-LP）が含まれていた。無細胞転写・翻訳系を用いてcDNAクローンから合成したGNTI-LPは、（Man α1-6）（Man α1-3）（Man）$_{3}$-（GlcNAc）$_{2}$-PAとUDP-GlcNAcから（GlcNAc β1-2）（Man α1-6）（Man α1-3）（Man）$_{3}$-（GlcNAc）$_{2}$-PAを形成する反応を触媒し、*N*-アセチルグルコサミニルトランスフェラーゼⅠであることが明らかになった。酵素反応の至適pHは8.0であるが、酵素活性はpH 5からpH 8の広い範囲で維持された。また、至適温度は30℃であった。酵素はMn^{2+}とMg^{2+}を要求し、EDTAにより活性が阻害された。UDP-GlcNAcに対する*K*m値は0.58 μMを示した。さらに、膜貫通ドメインを含むN末端領域（M1からQ120）は酵素活性に必須ではないことが分かった。抗GNTI-LP抗体を用いたウエスタンブロット解析によりゴルジ膜は確かにSDSゲル中で51 kDaのバンドとして泳動されるタンパク質を蓄積することが明らかになった。

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キーワード：糖タンパク質、*N*-アセチルグルコサミニルトランスフェラーゼ-I、イネ、ゴルジ体、プロテオーム

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