Changes in Rice Golgi Apparatus in Response to Sugar Starvation

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Summary

We investigated changes in rice Golgi apparatus in response to sugar starvation. When rice suspension-cultured cells were incubated under sugar-starved condition, the cells actively secreted the hydrolases, particularly α-amylases. The NDPase-associated Golgi membranes prepared from normal and sugar-starved cells were analyzed by sucrose density gradient centrifugation, indicating that the density of Golgi membranes in sugar-starved cells was shifted to higher density in sucrose gradients compared with that in normal cells. It suggests that the components of Golgi membranes are possibly rearranged for responding environmental condition.

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The Golgi apparatus is a multifunctional organelle, that involved in 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. 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). Furthermore, it has been recently reported 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).

In mammary epithelial cells of rat, proteomic analysis of two functional states of the Golgi apparatus, late pregnancy (basal secretory state) and lactation (maximal secretory state), has been carried out (Wu et al. 2000). This attempt revealed that only a subset of the total protein is up-regulated from steady state during the transition. The proteins identified were several classes of proteins involved in the regulation of membrane fusion and secretion. It has been well established that the secretion of α-amylase from the ER through the Golgi in cereal aleurone layers is stimulated by plant hormone gibberellin (Gubler et al. 1986, Jones and Robinson 1989), and the α-amylase secretion in suspension-cultured cells derived from rice embryo is controlled by sugar (Mitsui et al. 1999). In addition to these, α-amylase glycoproteins were shown to be transported from the ER-Golgi to the chloroplast through the secretory pathway in non-secretory leaf cells overexpressing the glycoproteins (Kitajima et al. 2009). Thus, there might exist different functional states of the Golgi apparatus in plant cells. In the present communication, we report dynamic change in rice Golgi apparatus in response to sugar starvation.

MATERIALS AND METHODS

Plant Materials

Rice seeds (*Oryza sativa* L. cv. Nipponkai) were supplied from the Niigata Agricultural Research Institute (Niigata, Japan). Rice cell culture was carried out according to the procedures described by Mitsui et al. (1990).

Fractionation procedures of Golgi membranes

Rice cells (100 g) 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 a 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

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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 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℃.

Assays

Activities of Triton X-100-stimulated nucleoside diphosphatase (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 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). α-Amylase activity was determined using β-limit dextrin as the substrate. β-limit dextrin was prepared by hydrolyzing potato starch solution (2% (w/v) in 50 mM acetate buffer, pH 5.3), using crystalline potato β-amylase free from α-amylase (24 hr, 30℃). 0.3% β-limit dextrin dissolved in 50 mM acetate buffer (pH 5.3) containing 1 mM $CaCl_2$ served as the substrate. The reaction mixture containing 0.2 ml each of substrate and enzyme solution was incubated at 37℃ for 5 min; the reaction was stopped by adding 0.5 ml of I_2-KI solution, followed by measurement of the decrease in absorbance at 620 nm upon addition of 2 ml H_2O. One enzyme unit was defined as the enzyme activity causing 10% absorbance decrease at 620 nm under the stated assay conditions. 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).

2D-PAGE

The microsomal and Golgi membranes diluted with equal volume of 25 mM HEPES-NaOH (pH 7.0) were centrifuged at 100,000 x *g* for 30 min. The pellet was resuspended with 25 mM HEPES-NaOH (pH 7.0) and 2% (w/v) CHAPS, and sonicated for 10 min in an ice bath. The suspension was mixed with one tenth volume of 100% trichloroacetic acid, and the precipitate was suspended and washed with 100% ethanol three times. The resultant precipitate was suspended with lysis buffer containing 9 M urea, 3% (w/v) IGEPAL CA-630, and 2% (v/v) 2-mercaptethanol at room temperature, and then centrifuged at 100,000 x *g* for 30 min to remove insoluble materials. The supernatant was used as the solubilized membrane proteins.

2D-PAGE was performed using IEF-PAGE and SDS-PAGE according to the procedure of Mikami et al. (2001) with a slight modification. The solubilized membrane proteins (200 μg) with 2% (v/v) ampholine (pH 3.5-10.0) were applied to the cathode side of first-dimensional disc gel (11.5 cm x ϕ 3 mm) composed of 8 M urea, 4% (w/v) acrylamide, 1% IGEPAL CA-630, 2% ampholine (pH 3.5-10), 0.02% ammonium persulfate, and 0.003% N,N,N',N'-tetramethyl ethylenediamine. The electrophoresis was carried out at 300 V for 20 h, followed by 600 V for 30 min at 4℃. After IEF, the gel was placed in an equilibration solution containing 0.06 M Tris-HCl (pH 6.8), 2.5% (w/v) SDS, 5% (w/v) 2-mercaptoethanol and 10% (w/v) glycerol for 10 min at room temperature, and then subjected to SDS-PAGE. Protein spots on the gels were visualized with Coomassie brilliant blue R250 (CBB). Isoelectric point and molecular size of each protein was estimated using 2-D SDS-PAGE standards (Bio-Rad). The localization sites and quantification of individual proteins were evaluated by using image analysis software, Image Master 2D Elite (Amersham Pharmacia Biotech) and PDQuest (Bio-Rad).

Mass analysis

Protein bands excised from 2D-gel were washed with 25 % methanol and 7% acetic acid for 12 h at room temperature, and further destained with 50 mM NH_4HCO_3 in 50% methanol at 40℃ for 1 h. After removing the destained solution carefully, the gels were rinsed with H_2O twice and dried in Speed-Vac. The gels were swollen and incubated with 10 mM dithiothreitol, 100 mM NH_4HCO_3 at 60℃ for 1 h. The dried gels were swollen again and incubated with 40 mM iodoacetamide, and 100 mM NH_4HCO_3 at room temperature for 30 min in the dark. The gels were rinsed twice with H_2O, crashed, and then dried again in Speed-Vac. The gel pieces were re-swollen and incubated with 50 μl of 20 nM trypsin (Promega) and 10 mM Tris-HCl (pH 8.0) at 37℃ for 12 h. Peptide fragments were extracted and collected from the gels with 0.03% (w/v) TFA/ 33% (v/v) acetonitrile, 0.1% TFA, 0.1% TFA/ 50% acetonitrile, 0.1% TFA in acetonitrile, sequentially. The obtained peptides were subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS; Shimadzu AXIMA-CFR, Applied Biosystems Voyager-DE, Bruker Daltonics Autoflex II). The matrix solution was prepared by mixing a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA and 100% acetonitrile (1:1, v/v). The peptides were further analyzed by post-source decay according to the manufacturer's protocol. Based on the peptide mass fingerprints obtained, the protein homologous to each protein was identified by searching the protein databases (DDBJ and NCBI) using MASCOT (http://www.matrixscience.com).

RESULTS AND DISCUSSION

When suspension-cultured cells derived from an embryo of rice seed were incubated in a standard culture condition supplemented with 3% sucrose, the rice cells grew rapidly until 8 days, and the cell growth stopped in the sugar depleted phase. At the time, the cells actively secreted several α-amylase isoforms (Fig. 1).

Microsomal membranes from the rice cells incubated under either sugar-supplemented or starved condition were fractionated and analyzed by a discontinuous sucrose density

gradient centrifugation. As shown in Fig. 2, the Golgi marker enzyme NDPase-associated membranes in sugar-starved cells exhibited higher density compared with that in sugar-supplemented cells.

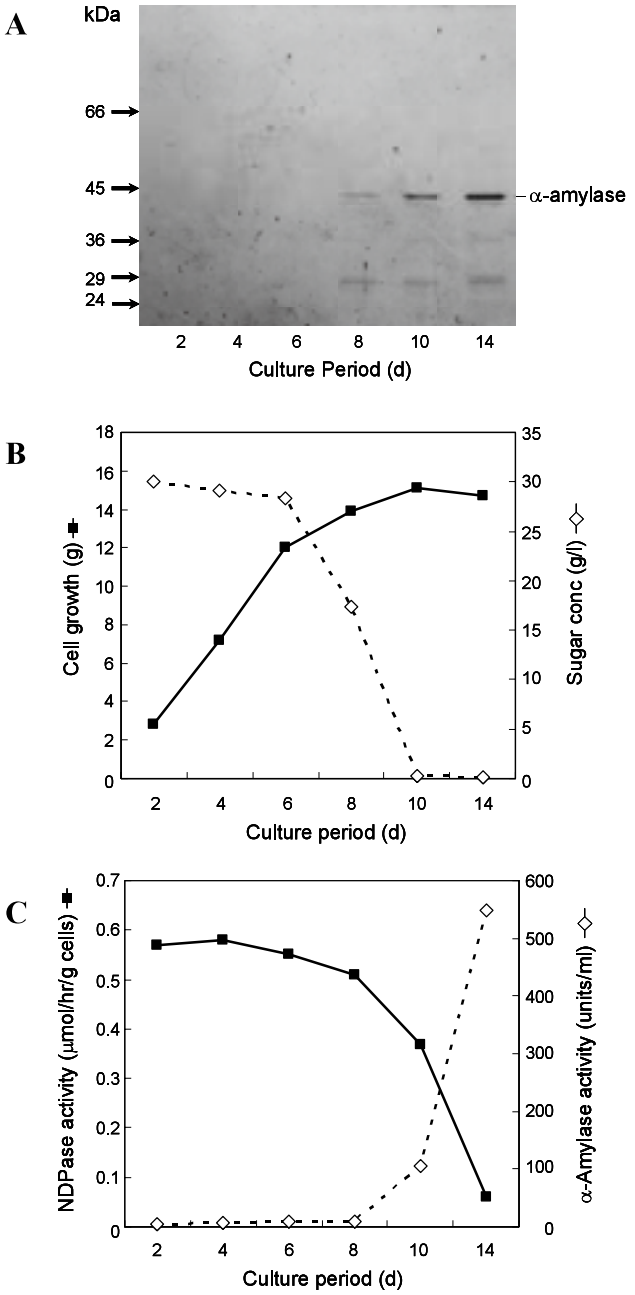

Fig. 1 Changes in secretion of α-amylase, sugar concentration, cell growth, and NDPase activity during cell culture of rice. Rice suspension cells were cultured in MS medium with 3% sucrose at 28℃ for the time indicated. (A) SDS-PAGE pattern of proteins secreted in culture media. Arrow indicates the α -amylase band. (B) Fresh weight of cells (cell growth) and sugar concentration in media. (C) α-Amylase activity in media and NDPase activity in microsomes.

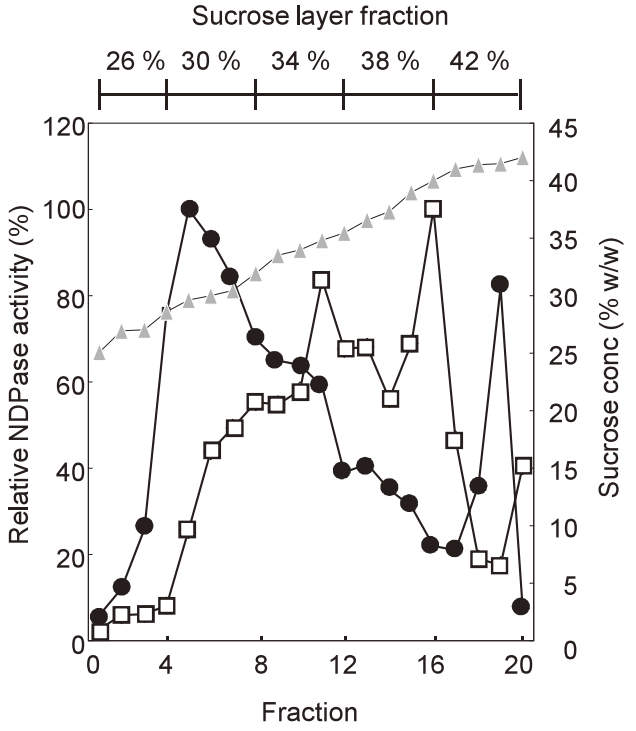

Fig. 2 Sedimentation behavior of NDPase-associated Golgi membranes in sucrose gradients during the functional transition from non-secretory (sugar-supplemented) to secretory (sugar-starved) state. Rice cells cultured for 6 days were further incubated in culture media with or without 3% sucrose for 18 h at 28℃. Microsomes prepared from the sugar-supplemented and -starved cells were subjected to discontinuous density gradient centrifugation-sedimentation. Experimental details are described in the text. Closed circle, sugar-supplemented condition; open square, sugar-starved condition; shaded triangle, sucrose concentration. The highest activity of NDPase in sugar-supplemented condition was normalized to 100%.

To determine sedimentation behavior of the other proteins in sucrose gradient, 30, 34, and 38% sucrose fractions from the two different functional states of cells were subjected to 2D-PAGE analysis. Comparative display of protein spots in 2D-gels revealed that distribution of some Golgi-related proteins such as vacuolar H+-ATPase (V-ATPase), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and voltage-dependent anion channel (VDAC) were shifted in the sucrose gradients under the two states, those are similar to that of the Golgi NDPase (Fig. 3).

Under the sugar-starved condition, protein content in rice cells actually decreased in comparison with the wild-type. By contrast, the density of the Golgi membranes increased, assuming that the ratio of protein/lipid in membrane increased. It has been reported that VDAC localizes in the secretory pathway in addition to the outer mitochondrial membrane (Buettner et al. 2000). GAPDH was

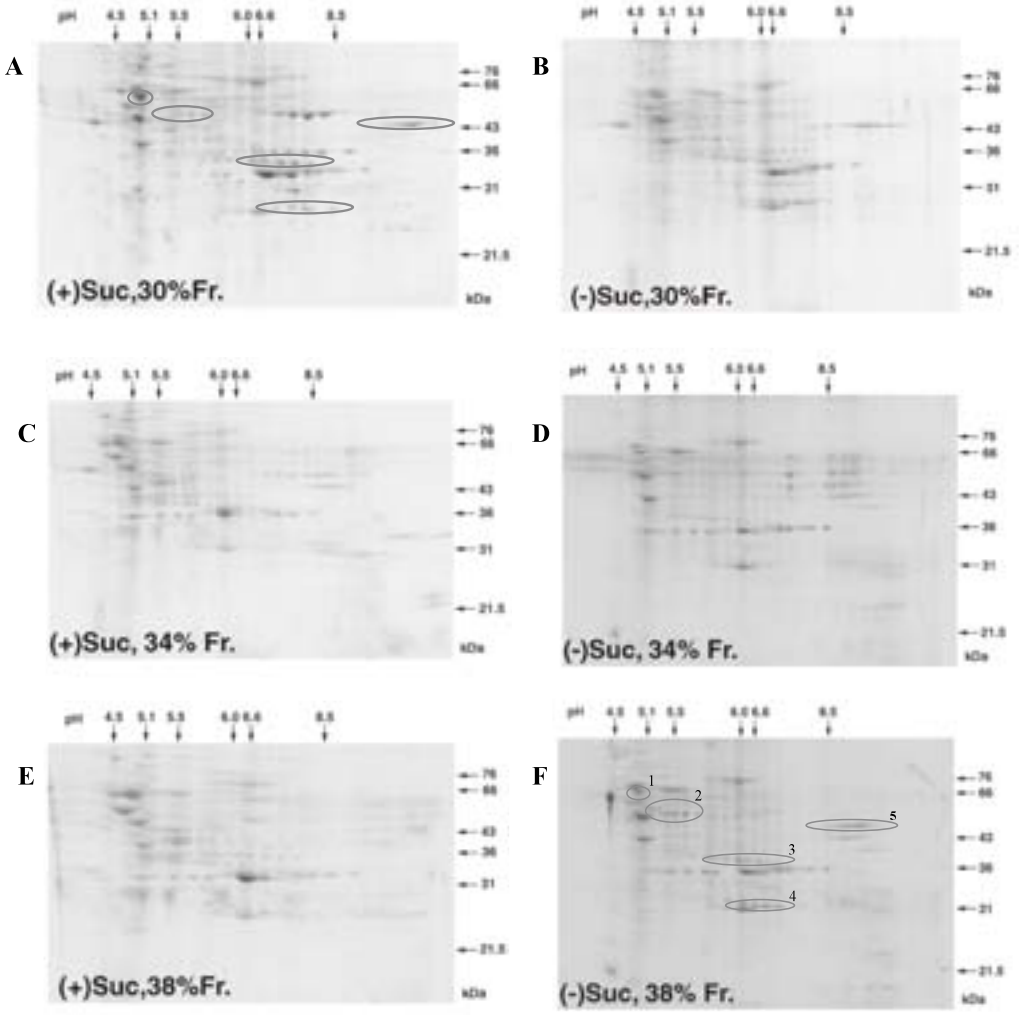

Fig. 3 Sedimentation behavior of Golgi membrane proteins in sucrose gradients during the functional transition from non-secretory (sugar-supplemented) to secretory (sugar-starved) state. Comparison of protein composition in 30 (A, B), 34 (C, D), and 38% (E, F) sucrose fractions from the sugar-supplemented and -starved cells obtained in Fig. 2 was carried out by 2D-PAGE. Density shifted protein spots during the functional transition were circled. (+)Suc, sucrose-supplemented cells (A, C, E); (-)Suc, sugar-starved cells (B, D, F). 1, V-ATPase (accession no. P49087); 2, H+-ATPase (accession no. CAA35787); 3, GAPDH (accession no. T02722); 4, VDAC (accession no. CAB82853); 5, Unknown protein.

involved in the vesicular transport in the early secretory pathway in mammalian NRK cell (Tisdale et al. 2004). Recently, V-ATPase was shown to be required for endocytic and secretory trafficking in Arabidopsis cells (Dettmer et al. 2006). It seems that the sugar starvation concentrates secretory machinery in the Golgi membranes in rice cells, and the components of Golgi membranes are possibly rearranged for responding environmental stimuli.

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糖欠乏に応答するイネゴルジ体の動的変化

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要　約

我々は糖欠乏に応答したイネゴルジ体の変化を見出した。イネ懸濁培養細胞は糖欠乏条件下で培養された時、加水分解酵素、特にα - アミラーゼを活発に分泌する。正常および糖欠乏条件下で培養した細胞より調製した、NDPase 結合ゴルジ膜をショ糖密度勾配遠心分離により分析したところ、糖欠乏条件下では正常条件下より高密度にシフトすることが分かった。このことは環境要因に対してゴルジ膜構成成分が動的に再編成されることを示唆する。

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