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Identification and Characterization of an Intracellular Lectin, Calnexin, from *Aspergillus oryzae* Using *N*-Glycan-Conjugated Beads

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Recently, asparagine-linked oligosaccharides (*N*-glycans) have been found to play a pivotal role in glycoprotein quality control in the endoplasmic reticulum (ER). In order to screen proteins interacting with *N*-glycans, we developed affinity chromatography by conjugating synthetic *N*-glycans on sepharose beads. Using the affinity beads with the dodecasaccharide $Glc_1Man_9GlcNAc_2$, one structure of the *N*-glycans, a 75-kDa protein, was isolated from the membranous fraction including the ER in *Aspergillus oryzae*. By LC-MS/MS analysis using the *A. oryzae* genome database, the protein was identified as one (AO090009000313) sharing similarities with calnexin. Further affinity chromatographic experiments suggested that the protein specifically bound to $Glc_1Man_9GlcNAc_2$, similarly to mammalian calnexins. We designated the gene *AoclxA* and expressed it as a fusion gene with *egfp*, revealing the ER localization of the AoClxA protein. Our results suggest that our affinity chromatography with synthetic *N*-glycans might help in biological analysis of glycoprotein quality control in the ER.

Key words: *N*-glycan; quality control; *Aspergillus oryzae*; calnexin; lectin

Aspergillus oryzae is a filamentous fungus used in traditional Japanese fermentation industries. Due to its safety and ability to secrete large amounts of proteins, *A. oryzae* is exploited in the industrial production of proteins.[1)] The production level of proteins from higher eukaryotes, however, is markedly lower than that of fungal proteins.[2–4)]

Endoplasmic reticulum (ER) quality control is assumed to be one of the bottlenecks limiting the production level of heterologous proteins. Newly synthesized proteins are translocated into the ER, where *N*-glycans are introduced to asparagine residues of nascent polypeptides. ER-resident chaperones and foldases help the proteins to fold correctly. In mammals and yeast, recently, *N*-glycans have been shown to play a key role in glycoprotein quality control, including protein folding, transportation, and degradation.[5)] The calnexin/calreticulin-glucosyltransferase cycle plays a central role in glycoprotein quality control.[5,6)] Calnexin and its soluble homolog, calreticulin, are lectin chaperones that share similar specificities to monoglucosylated *N*-glycans, such as $Glc_1Man_9GlcNAc_2$.[7)] They assist in the folding of glycoproteins by recruiting the protein disulfide isomerase-like protein ERp57.[8)] When protein folding is incomplete, UDP-glucose:glycoprotein glucosyltransferase (UGGT) glucosylates $Man_9GlcNAc_2$ to $Glc_1Man_9GlcNAc_2$, the calnexin/calreticulin ligand.[9)] Misfolded glycoproteins are trapped by MLP (mannosidase-like proteins such as EDEM and Htm1p in mammals and yeast respectively) and, transported to the cytosol.[10,11)] Likewise, Yos9p in yeast, a lectin-like protein involved in the degradation of misfolded glycoproteins, is thought to recognize the *N*-glycan structure.[12)] Nevertheless, the lectin activities and substrate specificities of these lectin-like proteins involved in glycoprotein degradation are yet to be accurately identified.

For precise understanding of the glycoprotein quality control system, an *N*-glycan with a homogeneous structure is required, but it is difficult to extract the homogeneous structure of the *N*-glycan from natural sources consisting of various structures of *N*-glycans. We comprehensively synthesized *N*-glycans associated with the glycoprotein quality control system in the ER.[13–16)] We discovered that synthesized *N*-glycans-methotrexate conjugates can be accepted as a substrate of UGGT.[17)] Moreover, employment of these synthetic

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oligosaccharide probes enabled us to evaluate the activity of glucosidase II[18] and UGGT quantitatively.[17]

Genes related to glycoprotein quality control in the ER are conserved in the *A. oryzae* genome sequence (DOGAN: Database of the Genomes Analyzed at NITE; http://www.bio.nite.go.jp/dogan/Top). Recently, it was suggested that glycoprotein quality control is relevant to protein production efficiency by filamentous fungi,[19,20] but the molecular interactions between *N*-glycans and their recognizing proteins have not been studied in filamentous fungi. In this paper, we report the preparation of beads conjugated with synthetic *N*-glycans and the isolation of an *A. oryzae* protein showing a homology to calnexin, its binding specificity and subcellular localization were determined for the first time in filamentous fungi.

Materials and Methods

Synthesis of N*-glycan-conjugated beads.* One hundred milligrams of thiopropyl sepharose 6B (GE Healthcare Bio-Sciences, Liitle Chalfont, UK) were treated with 500 µl of 100 mM Tris (2-carboxyethyl) phosphine (TCEP) for 10 min at room temperature 3 times, and then washed with 10 mM potassium phosphate buffer (KPB) (pH 7.4). The reduced thiopropyl sepharose 6B was treated with 1 mg of iodoacetamidyl *N*-glycan[21] ($Glc_1Man_9GlcNAc_2$, $Man_9GlcNAc_2$, and $GlcNAc_2$) in 500 µl of 10 mM KPB at 4 °C. After removal of the supernatant, the introduction rate of *N*-glycan to thiopropyl sepharose 6B was calculated by quantification of the thiol group concentration using Ellman's reagent (introduction rate of $Man_9GlcNAc_2$, 16%, theoretical value, 12%).[22] The gel was treated with 500 µl of 100 mM TCEP 3 times for 10 min at room temperature. It was incubated with 100 mM iodoacetamide overnight at room temperature and washed with 25 mM Tris–HCl (pH 7.0) containing 1% Triton X-100 and 10 mM $CaCl_2$. Negative control (NC) beads were prepared by treatment with iodoacetamide to thiopropyl sepharose 6B.

Preparation of a membranous fraction from A. oryzae. The *A. oryzae* RIB40 (ATCC42149) strain was used for preparation of the membranous fraction. It was grown in DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$) at 30 °C for 18–20 h. Mycelia were homogenized in liquid nitrogen and suspended in extraction buffer (10 mM Tris–HCl, 50 mM NaCl, 10 mM $CaCl_2$, and protease inhibitor cocktail, pH 7.5, Roche, Penzberg, Germany). The suspension of cell-free extracts was centrifuged at $3{,}000 \times g$ for 10 min at 4 °C, and the supernatant was centrifuged at $25{,}000 \times g$ for 20 min at 4 °C. The pellet was resuspended in extraction buffer including 0.5% Triton X-100, and then this solution was centrifuged at $25{,}000 \times g$ for 20 min at 4 °C. This supernatant was used as a membranous fraction for the subsequent chromatographic experiment.

Screening for proteins interacting with N*-glycan by affinity chromatography.* Approximately 100 µg of solubilized membranous protein were applied to a column with 100 mg $Glc_1Man_9GlcNAc_2$-conjugated beads equilibrated with a binding buffer (25 mM Tris–HCl (pH 7.5), 1% Triton X-100, and 10 mM $CaCl_2$) containing 0.2 mM 1-deoxynojirimycin, 0.2 mM 1-deoxymannojirimycin, and 5% Block Ace (Snow Brand Milk Products, Sapporo, Japan), and then incubated for 30 min at 4 °C. The beads were washed with 4 ml of the binding buffer. After washing with 2 ml of binding buffer containing 100 mM NaCl, elution was conducted with 2 ml of an elution buffer (0.1 M Tris–H_2SO_4 (pH 7.5), 10 mM EDTA, and 5 M guanidine hydrochloride).

LC-MS/MS analysis. The fractions in affinity chromatography were analyzed by SDS–PAGE and stained with Coomassie Brilliant Blue. The bands were subjected to in-gel digestion with 12.5 ng/µl trypsin, as described previously.[23]

The resulting mixture was analyzed by LTQ (Thermo Electron, Waltham, MA) liquid chromatography/linear ion trap mass spectrometry (LC-MS/MS) system, and their corresponding proteins were searched using the program Mascot database-searching software (Matrix Science, London, UK),[24] which accesses protein identification by matching mass spectroscopy data with protein databases, NCBI (http://www.ncbi.nlm.nih.gov), DOGAN (*A. oryzae* genomes database), and Swiss Prot (http://us.expasy.org).

N*-Glycan binding analysis of the AoClxA protein.* Approximately 1 mg of solubilized membranous protein was applied to a column with $Glc_1Man_9GlcNAc_2$-conjugated beads equilibrated with 25 mM Tris–HCl (pH 7.5) containing 1% Triton X-100, 0.2 mM 1-deoxynojirimycin, 0.2 mM 1-deoxymannojirimycin, 10 mM $CaCl_2$, and 5% Block Ace, and then incubated for 30 min at 4 °C. Washing was done with 1.5 ml of the binding buffer. Then the fraction containing the AoClxA protein was obtained by elution with 4 ml of the binding buffer, and concentrated by filtration to approximately 800 µl.

Two hundred µl of the concentrated fraction was applied to $Glc_1Man_9GlcNAc_2$, $Man_9GlcNAc_2$, $GlcNAc_2$, and the NC beads. Washing was done with 500 µl of the binding buffer. Elution was done with 4.5 ml of binding buffer. These fractions were analyzed by SDS–PAGE.

Cloning of the AoclxA *gene and construction of the* egfp*-fused gene.* Primers AoclxA-F and AoclxA-R (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCGTTTCAACGCAGCTGTTGC-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTGGGCAGAAGAACGGGTG-3′ respectively, *att*B sequences required for the MultiSite Gateway™ system are under-

lined) were used to clone the 1.9-kb *AoclxA* ORF from *A. oryzae* genomic DNA. The pgACAE plasmid (Fig. 5A) for expression of the AoClxA-EGFP fusion protein was constructed by the Multisite Gateway™ system.[25] pgACAE carries a 0.6-kb *amyB* promoter followed by a 1.9-kb *AoclxA* ORF, 0.7-kb *egfp*, 0.2-kb *amyB* terminator, and 5.1-kb *niaD* marker.

Localization analysis of the AoClxA protein. *A. oryzae* NS4 (*niaD*$^{-}$ *sC*$^{-}$)[26] was transformed with pgACAE employing the standard transformation method,[1] which generated ClxE strains. Czapek-Dox (CD) medium (0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 2% glucose, pH 5.5) was used for cultivation of the ClxE strain. Approximately 3.0×10^5 of conidia were inoculated in 100 µl of CD medium in glass based dishes (Asahi Techno Glass, Chiba, Japan) and grown at 30 °C for 19 h. ER-Tracker Blue-White DPX (Molecular Probes, Eugene, OR) staining was performed as follows: The media in cultures on cover slips were replaced with CD medium containing 1 µM ER-Tracker Blue-White DPX, and the cultures were incubated for 30 min at 37 °C. Subsequently, the cultures were washed twice with CD media. They were further incubated at 30 °C for 30 min, followed by microscopic analysis with an Olympus System microscope model BX52 (Olympus, Tokyo). A GFP filter (495/20 nm excitation, 510 nm dichroic, 530/35 nm emission) (Chroma Technologies, Brattleboro, VT) was used for observation of EGFP fluorescence. A BH-DMU (330 to 385 nm excitation, 400 nm dichroic, >420 nm emission) UV excitation cube (Olympus) was used to observe the fluorescence of ER-Tracker Blue-White DPX. Images were analyzed with MetaMorph software (Molecular Devices, Sunnyvale, CA).

Results

Preparation of N*-glycan-conjugated beads*

For screening of proteins interacting with *N*-glycans, we prepared sepharose beads conjugated with *N*-glycans. First, sepharose beads were conjugated with synthetic $Glc_1Man_9GlcNAc_2$, one of the processed structures derived from an *N*-glycan ($Glc_3Man_9GlcNAc_2$) and recognized by lectin-like chaperones in the ER (*viz.*, calnexin and calreticulin).[27] Iodoacetamidyl sugar derivatives were prepared as in our previous report.[21] Iodoacetamidyl $Glc_1Man_9GlcNAc_2$ was introduced to reduced thiopropyl sepharose by alkylation of thiol groups. The rest of the thiol group was capped with iodoacetamide. In order to perform the binding assay, we prepared $Man_9GlcNAc_2$, $GlcNAc_2$ beads too. Amido beads were used as a negative control.

Screening for proteins interacting with the N*-glycan*

In order to find a membranous fraction including the ER, the *A. oryzae* strain expressing BipA-EGFP fusion protein was used. The BipA-EGFP fusion protein was found to localize in the ER.[28] Cell lysate of the strain was subjected to differential centrifugation. Western blot analysis using anti-GFP antibody revealed that the BipA-EGFP fusion protein abundantly resided in the $25{,}000 \times g$ pellet fraction (data not shown). Hence, a $25{,}000 \times g$ pellet from the *A. oryzae* wild-type strain RIB40 was used as a membranous fraction in the subsequent experiments. This fraction also showed ER glucosidase II activity,[29] suggesting that it contained whole sets of the enzymes and lectins related to glycoprotein quality control in the ER.

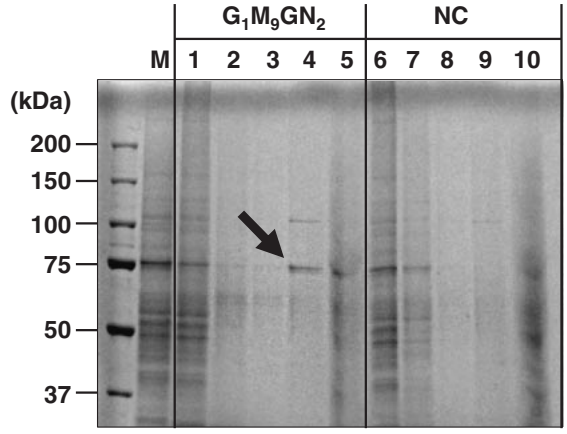

Fig. 1. Affinity Chromatography with $Glc_1Man_9GlcNAc_2$-Conjugated Beads for the Interacting Protein from *A. oryzae* Membranous Fraction.

Lanes: M, membranous fraction from *A. oryzae*; 1 and 6, wash fractions with the binding buffer; 2, 3, 7, and 8, wash fractions with binding buffer containing 100 mM NaCl; 4, 5, 9, and 10, elution fractions with 5 M guanidine solution. Arrow indicates the 75-kDa protein isolated by the $Glc_1Man_9GlcNAc_2$-conjugated beads. $G_1M_9GN_2$, $Glc_1Man_9GlcNAc_2$-conjugated beads; NC, negative control (non-conjugated beads).

Next we screened proteins showing binding affinity to the *N*-glycan from the membranous fraction of *A. oryzae*. The membranous fraction was solubilized by the addition of a detergent (1% Triton X-100), and incubated with $Glc_1Man_9GlcNAc_2$-conjugated sepharose beads in the presence of 10 mM $CaCl_2$. In order to prevent cleavage of *N*-glycan, the glucosidase inhibitor 1-deoxynojirimycin and the mannosidase inhibitor 1-deoxymannojirimycin were added. After washing with a binding buffer containing 100 mM NaCl, the beads were treated with 5 M guanidine hydrochloride solution for elution, since adequate amounts of the synthetic *N*-glycan for elution were not available. Figure 1 shows the result of SDS–PAGE analysis of each fraction. An approximately 75-kDa band was detected specifically in the elution fraction from the $Glc_1Man_9GlcNAc_2$-conjugated beads (lane 4), which was not found in that from the negative control (NC) beads (lane 9). An approximately 100-kDa band was noticed in the elution fractions from both the beads (lanes 4 and 9) and turned out to be translation elongation factor 2 (gi|83769606). The 75-kDa protein was not eluted in similar chromato-

Table 1. Mass Spectrometric Identification of Lectin-Like Protein

Sequence position	Matched sequence	Ion score
48–61	APFLEQFTDDWESR	67
62–68	WTPSHAK	19
69–93	KDDSQTEEDWAYVGEWSVEEPTVFK	57
70–93	DDSQTEEDWAYVGEWSVEEPTVFK	51
94–99	GIDGDK	17
105–115	NPAAHHAISAK	25
119–123	KIDNK	22
126–147	TLVQYEVKPQNSLVCGGAYLK	44
154–174	KLHAEEFSNATPYVIMFGPDK	71
155–174	LHAEEFSNATPYVIMFGPDK	109
181–186	VHFIFR	30
192–198	TGEYEEK	26
208–235	TNKVTSLYTLIVRPDQSFQILIDGEAVK	47
211–235	VTSLYTLIVRPDQFQILIDGEAVK	74
236–251	NGTLLEDFNPPVNPEK	45
260–269	KPDDWVDDVK	82
337–351	CNDVSGCGPWSAPMK	75
337–352	CNDVSGCGPWSAPMKK	28
360–371	WTAPMIDNPAYK	34
372–377	GPWAPR	19
378–388	KIANPAYFEDK	66
379–388	IANPAYFEDK	67
429–435	KEFDVK	17
436–449	HPVEVAEEEASKPK	67
450–470	KEETAPATSVSFQEDPITFVR	90
471–481	EKVDHFVGLAK	46
473–481	VDHFVGLAK	61
482–489	QDPVNAVK	30
490–514	QAPEVAGTLGALVLSMVLIIVGAIK	79
490–524	QAPEVAGTLGALVLSMVLIIVAIKASSPAPAPVK	58
515–524	ASSPAPAPVK	50
535–549	EKVSEAVSSSADTGK	72
537–549	VSEAVSSSADTGK	77

Probability of Based Mowse Scores > 40 indicates identity or extensive homology ($p < 0.05$).

graphic experiments using other subcellular fractions, such as the $100{,}000 \times g$ pellet and supernatant from *A. oryzae* (data not shown).

Identification of the 75-kDa protein by LC-MS/MS analysis

For identification of the 75-kDa protein eluted specifically from the $Glc_1Man_9GlcNAc_2$-conjugated sepharose beads, the band was excised from the SDS–PAGE gel, and in-gel digestion with trypsin was carried out. The resulting peptides were analyzed by LC-MS/MS, and the data for mass and internal amino acid sequences were analyzed using Mascot database-searching software. The 75-kDa protein was identified as *A. oryzae* unnamed protein product (gi|83764647 in DDBJ) and *A. oryzae* calnexin (AO090009000313 in DOGAN: the *A. oryzae* genome database). Thirty three of the predicted tryptic fragments were matched (Table 1), and the sequence coverage of the matched peptides was 59.4% (Fig. 2).

The gene consisted of 1,924 bp, including 5 exons and 4 introns, and was deduced to encode 562 amino acid residues (molecular mass, 61,976 Da) showing similarities in amino acid sequence to calnexins from other organisms, 83.6%; *Aspergillus niger* ClxA (gi|1856-4807), 39.1%; *Homo sapiens* (gi|543920), 39.1%; *Arabidopsis thaliana* (gi|231683), 43.2%; *Schizosaccharomyces pombe* (gi|543923), and 24.5%; *Saccharomyces cerevisiae* (gi|115549) (Fig. 3). Hence, the gene was designated *AoclxA*.

Calnexin has a signal sequence in the N-terminus and one transmembrane domain in the C-terminus.[30–32] The signal peptide in the AoClxA protein was predicted from 1st to 23rd amino acids using the programs SignalP (http://www.cbs.dtu.dk/services/SignalP/) and SIGCLEAVE (http://bioweb.pasteur.fr/seqanal/interfaces/sigcleave.html) (Fig. 3). A putative transmembrane domain was found between amino acid residues 498 and 515 using the program SOSUI signal (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html) for prediction of the membrane protein. LC-MS/MS data for the 75-kDa protein included tryptic fragments from the deduced transmembrane domain, but not from the signal peptides (Fig. 2). This implies that the signal peptide of the AoClxA protein was removed by signal peptidase. The lectin sites in calnexin recognize the terminal glucose residue and three underlying mannose residues in the $Glc_1Man_9GlcNAc_2$ oligosaccharide.[33–35] The AoClxA protein has two conserved residues related to lectin sites (Fig. 3). Calnexin and calreticulin bind to calcium with their KPEDWDE-like repeat motif representing high affinity to calcium.[31,32,36] There were four calcium binding motifs (KPEDWDE-like repeats) in the AoClxA protein (Fig. 3). These results indicate that the AoClxA protein contains common features of calnexins from other eukaryotes.

In the *AoclxA* promoter, three CT-rich regions were found near positions [−50, −110, −160 bp] (Fig. 2). CT-rich regions have been identified in the upstream region of the transcriptional start points in many fungal genes, including *A. niger clxA*.[32,37] The binding sequence, 5′-CAN(G/A)NTGT/GCCT-3′, of the HacA protein,[38] a UPR (unfolded protein response) transcription factor, was not present in the *AoclxA* promoter. CreA (a transcriptional repressor in carbon catabolite repression) binding sites are found in the *AoclxA* promoter, as reported for *A. niger clxA* gene.[32]

Specific binding of the AoClxA protein to $Glc_1Man_9GlcNAc_2$

Although mammalian calnexins bind to $Glc_1Man_9GlcNAc_2$,[27,33] calnexins from yeast and filamentous fungi have not been studied for their specific binding to $Glc_1Man_9GlcNAc_2$ *in vitro*. We performed a binding assay of the 75-kDa protein (AoClxA) using *N*-glycan-conjugated beads. For this experiment, $Man_9GlcNAc_2$ and $GlcNAc_2$-conjugated beads were also prepared. The 75-kDa protein was eluted with a large volume of the binding buffer from the $Glc_1Man_9GlcNAc_2$-conjugated beads incubated with the membranous fraction. After the

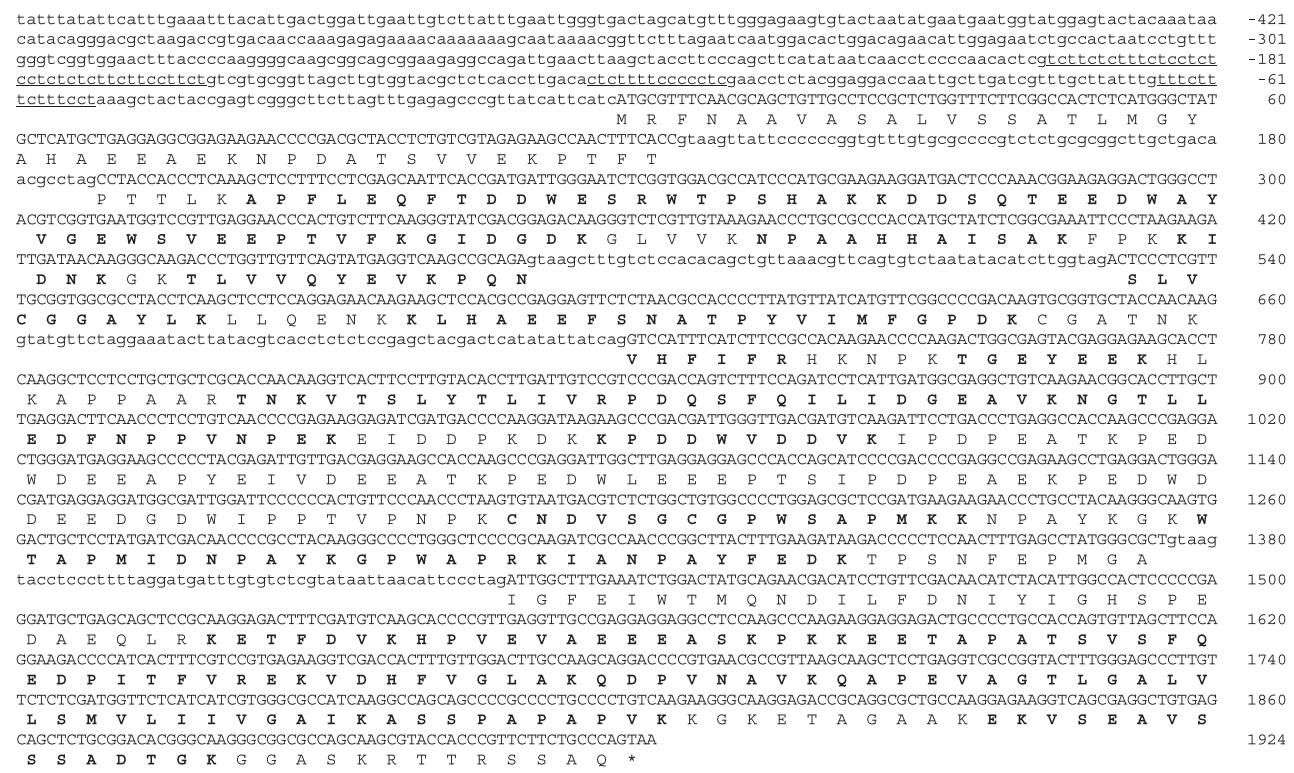

Fig. 2. Nucleotide and Deduced Amino Acid Sequences of the *AoclxA* Gene.

Intron and promoter sequences are written in lower case letters. CT-rich regions are underlined. The amino acids sequences matched by LC-MS/MS analysis are shown in bold letters.

non-specifically binding proteins were removed by washing with the binding buffer, the 75-kDa protein was applied to the $Glc_1Man_9GlcNAc_2$, $Man_9GlcNAc_2$, $GlcNAc_2$, and NC beads. The incubated beads were washed, and then the eluted fractions were analyzed by SDS–PAGE (Fig. 4). The 75-kDa protein bound only to the $Glc_1Man_9GlcNAc_2$-conjugated beads (Fig. 4, lane 2), strongly suggesting that the binding target of the AoClxA protein was $Glc_1Man_9GlcNAc_2$.

Localization analysis of the AoClxA protein

In order to confirm the localization of the 75-kDa protein (AoClxA) isolated from the membranous fraction, the *AoclxA* gene was fused with *egfp* and expressed in *A. oryzae*. In the strain expressing the AoClxA-EGFP fusion protein, EGFP fluorescence was observed as a tubular network containing ring-like structures (Fig. 5). We stained the strain with ER-Tracker Blue-White DPX, an ER-specific dye, resulting in co-localization of the two colors of fluorescence. The results indicated that the AoClxA protein was distributed in the ER. Therefore, it was revealed at the cellular level that the membranous fraction contained the ER with a lectin binding to $Glc_1Man_9GlcNAc_2$, one structure of the *N*-glycans.

Discussion

Recently, the function of *N*-glycans in glycoprotein quality control in the ER has drawn much attention,[5] but a major obstacle to molecular investigation of them is the difficulty in preparing homogeneous substrates of *N*-glycans. Chemical synthesis of *N*-glycans related to glycoprotein quality control in the ER provides sufficient quantities of homogeneous substrates,[13–16,39] enabling detailed analysis at the molecular level.[17,18,40] In this paper, we report the development of *N*-glycan-conjugated beads, and isolation and characterization of a protein interacting with $Glc_1Man_9GlcNAc_2$.

Although genes related to glycoprotein quality control in the ER are found in the *A. oryzae* genome database, knowledge of their molecular functions is limited. In this study, by employing beads conjugated with synthesized *N*-glycans, we isolated an AoClxA protein interacting with $Glc_1Man_9GlcNAc_2$ from the membranous fraction of *A. oryzae*. Using beads conjugated with other structures, we are attempting to screen proteins binding to various structures of *N*-glycans. LC-MS/MS analysis raised the determination efficiency of unidentified proteins, since it gives information not only on the mass of peptides, but also on internal amino acid sequences. The use of LC-MS/MS should contribute effectively to comprehensive identification of proteins in *A. oryzae*.

We suggest that the AoClxA protein specifically bound to $Glc_1Man_9GlcNAc_2$, as previously reported for mammalian calnexins.[27,33] Although in filamentous fungi the calnexin gene (*clxA*) has been cloned from

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                    Signal Peptide
A. oryzae       MRFNAAVASALVSSATLMGYAHAEEAEKNPDATSVVEKPTFTP--------------------TTLKAPFLEQFTDDWESRWTPSHAKKDDSQTEEDWAYVGEWSVEEPTVFKGIDGDK  99
A. niger        MRFNAALTSALVSSASIMGYAHAEETEKKPETTSLAEKPTFTP--------------------TSIEAPFLEQFTDDWDSRWTPSHAKKEDSKSEEDWAYVGEWSVEEPTVLKGMEGDK  99
H. sapiens      MEGKWLLCMLLVLGTAIVEAHDGHDDDVIDIEDDLDDVIEEVEDSKPDTTAPPSSPKVTYKAPVPTGEVYFADSFDRGTLSGWILSKAKKDDTD-DEIAKYDGKWEVEEMKESK-LPGDK 118
A. thaliana     MRQRQLFSVFLLLLAFVSFQKLCYGDDQTVLYESFDEPFDGR-----------------------------------WIVSKN----------SDYEGVWKHAKSEG----HEDY  66
S. pombe        MKYGKVSFLALLCSLYVRGSLADPESEQEPLVFNPTEVKAPLV-----------------------------EQFQGAWSERWIPSHAKRFVNG-IEEMSYVGEWTVEESSGPGALKGEA  90
S. cerevisiae   MKFSAYLWWLFLNLALVKGTSLLSN--VTLAEDSFWEHFQAYTN-------------------------------TKHLNQEWITSEAVNNEGS----KIYGAQWRLSQGRLQG-SAWDK  82
                *.       ::    :              .  :                                    *  *.           * . *   :        :

                                                                                                  L1
A. oryzae       GLVVKNPAAHHAISAKFPKKIDN-KGKTLVVQYEVKPQNSLVCGGAYLKLLQ-ENKKLHAEEFSNATP-YVIMFGPDKCG-ATNKVHFIFRHKNPKTGEYEEKHLKAP----PAARTNKV 211
A. niger        GLVVKNVAAHHAISAKFPKKIDN-KDKTLVVQYEVKPQNSLVCGGAYLKLLQ-DNKQLHLDEFSNASP-YVIMFGPDKCG-ATNKVHFIFRHKNPKTGEYEEKHLKAP----PAARTSKV 211
H. sapiens      GLVLMSRAKHHAISAKLNKPFLF-DTKPLIVQYEVNFQNGIECGGAYVKLLS-KTPELNLDQFHDKTP-YTIMFGPDKCG-EDYKLHFIFRHKNPKTGIYEEKHAKRPDADLKTYFTDKK 234
A. thaliana     GLLVSEKARKYGIVKELDEPLNL-KEGTVVLQYEVRFQEGLECGGAYLKYLRPQEAGWTPQGFDSESP-YSIMFGPDKCG-GTNKVHFILKHKNPKSGEYVEHHLKFP----PSVPYDKL 179
S. pombe        GLVMKDEAAHHAISYEFDEPINE-PEKDLVVQYEVNPEEGLNCGGAYLKLLA----EPTHGEMSNSID-YRIMFGPDKCG-VNDRVHFIFKHKNPLTGEYSEKHLDSRP---ASLLKPGI 200
S. cerevisiae   GIAVRTGNAAAMIGHLLETPINVSETDTLVVQYEIKLDNSLTCGGAFIKLMSGFMNVEALKHYAPDTEGVELVFGPDYCAPEINGVQFAINKVDKITHESKLRYLQEMP--LSKLTDTSQ 200
                *: :       *   :   :       :::***:. ::.: ****::* :                  ::**** *.     ::* :.: :  :     :: .

                                                                                                  Ca1             Ca2             Ca3             Ca4
A. oryzae       TSLYTLIVRP-DQSFQILIDGEAVKNGTLLE-----DFNPPVNPEKEIDDPKDKKPDDWVDDVKIPDPEATKPEDWDEEAPYEIVDEEATKPEDWLEEEPTSIPDPEAEKPEDWDDEEDG 325
A. niger        TSVYTLVVNP-DQTFQILIDGESVKEGSLLE-----DFIPPVLPPVEIYDPEDIKPADWVDEAKIPDPEATKPEDWDEEAPFEIVDEEATIPEDWLEDEPTSIPDPEAEKPEDWDDEEDG 325
H. sapiens      THLYTLILNP-DNSFEILVDQSVVNSGNLLN-----DMTPPVNPSREIEDPEDRKPEDWDERPKIPDPEAVKPDDWDEDAPAKIPDEEATKPEGWLDDEPEYVPDPDAEKPEDWDEDMDG 348
A. thaliana     SHVYTAILKP-DNEVRILVDGEEKKKANLLSGE---DFEPALIPAKTIPDPEDKKPEDWDERAKIPDPNAVKPEDWDEDAPMEIEDEEAEKPEGWLDDEPEEVDDPEATKPEDWDDEEDG 296
S. pombe        TNLYTLIVKP-DQTFEVRINGDVVRQGSLFY-----DFIPPVLPPVEIYDPEDIKPADWVDEPEIPDPNAVKPDDWDEDAPRMIPDPDAVKPEDWLEDEPLYIPDPEAQKPEDWDDEEDG 314
S. cerevisiae   SHLYTLIIDESAQSFQILIDGKTVMVREHIEDKKKVNFEPPITPPLMIPDVSVAKPHDWDDRIRIPDPEAVKLSDRDERDPLMIPHPDGTEPPEWNSSIPEYILDPNAQKPSWWKELEHG 320
                : :** ::    : ..: :: .       :      :: *.: *   * * .  ** ** :  .****:*.* .* **  *  * . :.  *  * .. *  : **:* **. *.:  .*

                                                                                                  L2
A. oryzae       DWIPPTVPNPKCNDVSGCGPWSAPMKKNPAYKGKWTAPMIDNPAYKGPWAPRKIANPAYFEDKTPSNFEPMG-AIGFEIWTMQNDILFDNIYIGHSPEDAEQLRKETFDVKHPVE--VAE 442
A. niger        DWVPPTVPNPKCQDASGCGPWSPPMKKNPDYKGKWSAPLIDNPAYKGPWAPRKIANPAYFEDKTPSNFEPMG-AIGFEIWTMQNDILFDNIYVGHSAEDAEKLRQETFDVKHPIE--LAE 442
H. sapiens      EWEAPQIANPRCESAPGCGVWQRPVIDNPNYKGKWKPPMIDNPSYQGIWKPRKIPNPDFFEDLEPFRMTPFS-AIGLELWSMTSDIFFDNFIICADRRIVDDWANDGWGLKKAAD--GAA 465
A. thaliana     MWEAPKIDNPKCEAAPGCGEWKRPMKRNPAYKGKWSSPLIDNPAYKGIWKPRDIPNPDYFELDRPD-YEPIA-AIGIEIWTMQDGILFDNILIAKDEKVAETYRQTTWKPKFDVE--KEK 412
S. pombe        DWIPSEIINPKCIEGAGCGEWKPPMIRNPNYRGPWSPPMIPNPEFIGEWYPRKIPNPDYFDDDHPSHFGPLY-GVGFELWTMQPNIRFSNIYVGHSIEDAERLGNETFLPKLKAERELLS 433
S. cerevisiae   EWIPPMIKNPLCTAERGCGQQIPGLINNAKYKGPGELNEIINPNYMGEWHPPEIENPLYYEEQHPLRIENVISGVILEFWSGSPNMLISNIYVGKNVTEAQIIGNKTWLMRDRAFR---- 436
                 * .. : ** *    ***     :  *. *:*      * ** : * * * .* ** :::   *     .  .: :*:*:   .: :.*: : .   .:   :  :  :

                                                                                                  TM domain
A. oryzae       EEASKPKKEETAPAT---SVSFQEDPITFVREKVDHFVGLAKQDPVNAVKQAPEVAGTLGALVLSMVLIIVGAIKASSPAPAPVKKGKETAGAAKEKVSEAVSSSADTGKG----GASKR 555
A. niger        EEANKPKPEEKAAEP---SVSFKEDPVGHIKEKVDNFVRLSKQDPINAVKQVPDVAGGLAAVLVTMILVIVGAVGASTPAPAPAKKGKEAAGATKEKTGAASSSSADTGKG----GATKR 555
H. sapiens      EPGVVGQMIEAAEERPWLWVVYILTVALPVFLVILFCCSGKKQTSGMEYKKTDAPQPDVKEEEEEKEEEKDKGDEEEEGEEKLEEKQKSDAEEDGGTVSQEEEDRKPKAEEDEILNRSPR 585
A. thaliana     QKAEEEAAGSADGLKSYQKVVFDLLNKVADLSFLSAYKSKITELIEKAEQQPNLTIGVLVAIVVVFFSLFLKLIFGGKKAAAPVEKKKPEVAE-----SSKSGDEAEKKEE----TAAPR 523
S. pombe        KQESMEKQSMHVDEESNQILEKFLDVYDIIKAKLPPNVAEKVDYYVETIIETPEIGIAIVAVLGSLTAVILTCYFYFFASSSPASLSTGTTEAEKEQQEKFKQETETEKIDVSYAPETES 553
S. cerevisiae   ----------GSDGPTERKFMNSRLGNLQTTFHNERESPNPFDRIIDRILEQPLKFVLTAAVVLLTTSVLCCVVFT---------------------------------------- 502
                              .                  :       :

A. oryzae       TTRSSAQ- 562
A. niger        TTRSSAE- 562  83.6%
H. sapiens      NRKPRRE- 592  39.1%
A. thaliana     KRQPRRDN 531  39.1%
S. pombe        PTAKNED- 560  43.2%
S. cerevisiae   -------- 502  24.5%
```

Fig. 3. Alignment of Amino Acid Sequences of AoClxA and Calnexins from *A. niger*, *H. sapiens*, *A. thaliana*, *S. pombe*, and *S. cerevisiae*.

The alignment was generated with the ClustalW program. The predicted signal peptide sequences are shown in the open box. The vertical arrowheads indicate the deduced *N*-glycosylation site. Two conserved calnexin lectin sites, L1 and L2,[35] are shaded in gray. Four calcium binding motifs (Ca1–Ca4) are shown in gray. The TM domain indicates the transmembrane domain (open boxes enclosed by dashed lines).

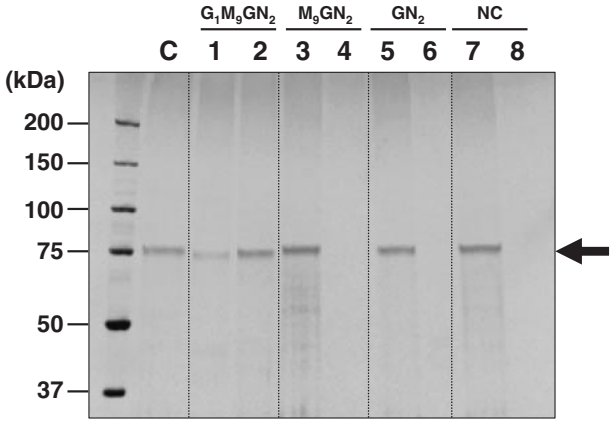

Fig. 4. Binding Analysis of the AoClxA Protein with *N*-Glycan-Conjugated Beads.

The 75-kDa protein captured in the $Glc_1Man_9GlcNAc_2$-conjugated beads (lane C) was subjected to another affinity chromatographic experiment using beads conjugated with $Glc_1Man_9GlcNAc_2$ ($G_1M_9GN_2$, lanes 1 and 2), $Man_9GlcNAc_2$ (M_9GN_2, lanes 3 and 4), $GlcNAc_2$ (GN_2, lanes 5 and 6), and NC (non-conjugated, lanes 7 and 8). Lanes: 1, 3, 5, and 7, wash fractions; 2, 4, 6, and 8, elution fractions.

A. niger,[32] its binding specificity to *N*-glycans has not been studied. This paper provides the first evidence for lectin activity and the specific binding of calnexin to $Glc_1Man_9GlcNAc_2$ from filamentous fungi. On the other hand, though it has been suggested that yeast calnexin plays a part in glycoprotein quality control,[41–44] its binding specificity to *N*-glycan has not yet been reported. Using the $Glc_1Man_9GlcNAc_2$-conjugated beads, calnexin could not be isolated from *S. cerevisiae* (data not shown). The ER in *A. oryzae* develops a large network as compared with yeast,[28] and the capacity of protein secretion in filamentous fungi such as *A. oryzae* is higher than in yeast.[4] Therefore, it is suggested that *A. oryzae* may be a good model for biological analysis of glycoprotein quality control in the ER.

In filamentous fungi, a correlation between glycoprotein quality control and protein production has been suggested. In a cellulolytic filamentous fungus, *Trichoderma reesei*, impairment in glucosidase II activity caused efficient protein secretion.[20] Overexpression of calnexin in *A. niger* resulted in higher production of a fungal protein, manganese peroxidase, from the white rot basidiomycete *Phanerochaete chrysosporium*.[19] These results suggest that glycoprotein quality control

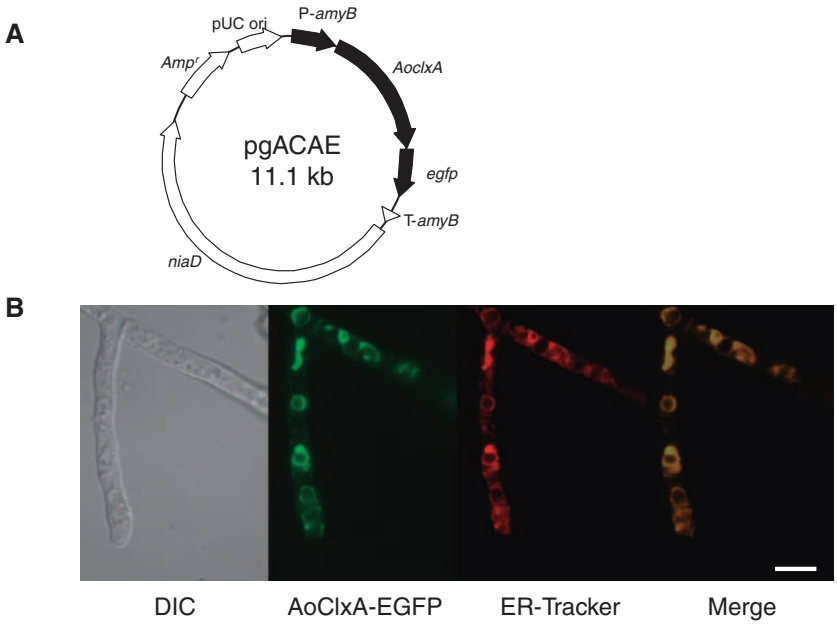

Fig. 5. Localization Analysis of the AoClxA Protein in *A. oryzae*.

A, Expression plasmid for the AoClxA-EGFP fusion protein. B, Localization of the AoClxA-EGFP fusion protein in *A. oryzae*. Hyphae expressing the AoClxA-EGFP fusion protein were observed by fluorescence microscopy. The strain was stained with ER-Tracker Blue-White DPX. The two fluorescent images were merged (AoClxA-EGFP: green, ER-Tracker: red), showing their co-localization in yellow. Bar, 10 μm.

can affect protein production, but the molecular machinery of glycoprotein quality control is largely unknown in filamentous fungi. In this study, the use of the *N*-glycan-conjugated beads allowed us to isolate a lectin from the membranous fraction, including the ER of *A. oryzae*. Hence it is suggested that the use of synthetic *N*-glycan-conjugated beads is a powerful strategy to screen for proteins with lectin activity from the ER, one that might provide not only a breakthrough in the improvement of heterologous protein production by filamentous fungi, but also new knowledge of the lectin-like proteins involved in glycoprotein degradation.

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