A Dictyostelium cellobiohydrolase orthologue that affects developmental timing

ARTICLE *in* DEVELOPMENT GENES AND EVOLUTION · NOVEMBER 2013

Impact Factor: 2.44 · DOI: 10.1007/s00427-013-0460-x · Source: PubMed

4 AUTHORS, INCLUDING:

Takefumi Kawata
Toho University
46 PUBLICATIONS **1,530** CITATIONS

SEE PROFILE

Dev Genes Evol (2014) 224:25–35
DOI 10.1007/s00427-013-0460-x

ORIGINAL ARTICLE

A *Dictyostelium* cellobiohydrolase orthologue that affects developmental timing

Mizuho Kunii · Mami Yasuno · Yuki Shindo · Takefumi Kawata

Received: 6 June 2013 / Accepted: 28 October 2013 / Published online: 16 November 2013

Abstract *Dictyostelium discoideum* is a facultative multicellular amoebozoan with cellulose in the stalk and spore coat of its fruiting body as well as in the extracellular matrix of the migrating slug. The organism also harbors a number of cellulase genes. One of them, *cbhA*, was identified as a candidate cellobiohydrolase gene based on the strong homology of its predicted protein product to fungal cellobiohydrolase I (CBHI). Expression of the *cbhA* was developmentally regulated, with strong expression in the spores of the mature fruiting body. However, a weak but detectable level of expression was observed in the extracellular matrix at the mound — tipped finger stages, in prestalk O cells, and in the slime sheath of the migrating slug — late culminant stages. A null mutant of the *cbhA* showed almost normal morphology. However, the developmental timing of the mutant was delayed by 2–4 h. When a c-Myc epitope-tagged CbhA was expressed, it was secreted into the culture medium and was able to bind crystalline cellulose. The CbhA-myc protein was glycosylated, as demonstrated by its ability to bind succinyl concanavalin A-agarose. Moreover, conditioned medium from the *cbhA-myc*^{oe} strain displayed 4-methylumbelliferyl β-D-cellobioside (4-MUC) digesting activity in Zymograms in which conditioned medium was examined via native-polyacrylamide gel electrophoresis or spotted on an agar plate containing 4-MUC, one of the substrates of cellobiohydrolase. Taken together, these findings indicate that *Dictyostelium* CbhA is an orthologue of CBH I that is required for a normal rate of development.

Keywords Cellulase · Cellobiohydrolase · *Dictyostelium* · Gene expression · Morphogenesis

Communicated by Sureshkumar Balasubramanian

Electronic supplementary material The online version of this article (doi:10.1007/s00427-013-0460-x) contains supplementary material, which is available to authorized users.

M. Kunii · M. Yasuno · Y. Shindo · T. Kawata (✉)
Department of Biology, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan
e-mail: tkawata@bio.sci.toho-u.ac.jp

Present Address:
M. Kunii
Department of Biological Production, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, 3-8-1 Harumi-cho, Fuchu, Tokyo 183-8538, Japan

Introduction

Recent research has revealed that the extracellular matrix (ECM) is not simply the molecules that fill spaces between cells but that it also plays important roles in regulating morphogenesis, via effects on processes such as cell migration, differentiation, tissue organization, and structural support (Brown 2011; Kim and Nelson 2012; Labouesse 2012). Degradation of basement membrane ECM components by specific proteases is also important for the movement of metastatic cancer cells (Friedl and Alexander 2011). However, how the ECM controls the morphogenetic process, particularly in the context of a living organism, has been less well investigated, due mainly to the extreme complexity of ECMs, which complicates attempts to manipulate the components.

The cellular slime mold *Dictyostelium discoideum* is an ideal organism to resolve this problem. In *Dictyostelium*, cellulose is a primary ECM component formed during development. Disruption of *dcsA*, the gene encoding cellulose synthase, results in aberrant morphology and a failure to culminate (Blanton et al. 2000). The *ugpB* null mutant, which cannot synthesize the substrate for cellulose synthesis due to disruption of the uridine diphosphoglucose pyrophosphorylase (UDPGP) gene shows aberrant developmental morphology (Bishop et al. 2002). Further lines of evidence for the involvement of cellulose in slug motility and in cell-type differentiation have been

reported (Freeze and Loomis 1977; Blanton 1993). In addition to cellulose, many proteins with a cellulose-binding domain (CBD) (McGuire and Alexander 1996; Shimada et al. 2004a; Wang et al. 2001), including putative cellulases and expansin-like proteins (Darley et al. 2003; Ogasawara et al. 2009), are found in *Dictyostelium*. Furthermore, some of these genes seem to be under control of the transcription factor Dd-STATa (Shimada et al. 2004a,b), which is necessary for proper development (Kawata 2011). Although biochemical features of two cellulase genes, *celA* and *celB*, have been investigated (Blume and Ennis 1991; Ramalingam and Ennis 1997), less is known about the function of other cellulases, particularly their roles in development.

Cellulases are cellulolytic enzymes and are classified into three groups. The first group is 1,4-β-D-endoglucanase (EC 3.1.2.4) or endo-(1,4)-β-glucanases. Endoglucanases catalyze internal hydrolysis of the cellulose chain and attack the amorphous regions in cellulose, providing new chain ends. The second group is 1,4-β-D-cellobiohydrolase (CBH; EC 3.1.2.91) or exo-(1,4)-β-glucanases. CBHs hydrolyze crystalline cellulose by initiating their action from the ends of the cellulose chains and producing primarily cellobiose or sometimes glucose. The third group is β-glucosidase (EC 3.2.1.21). β-Glucosidases hydrolyze cellobiose to glucose. During degradation of cellulose, these different types of enzymes act synergistically and efficiently hydrolyze the cellulose polymer into glucose (Bhat 2000).

Potent cellulolytic fungi generally produce two different CBHs, CBHI and CBHII; they act at reducing and non-reducing ends, respectively (Teeri 1997). Even in bacteria, similar pairs of cellobiohydrolases act from opposite ends, although some of them belong to different glycoside hydrolase families (Teeri 1997; Teeri et al. 1998). CBHs are key components in the multi-enzyme cellulase complexes as they can attack crystalline areas of cellulose. Therefore, it is important to describe amoebozoan CBH as it has not yet been reported. The expression of two cellobiohydrolase genes, *cbh1* and *cbh2*, in the cellulolytic filamentous fungus *Trichoderma reesei* (*Hypocrea jecornia*) is thought to be regulated by Ras GTPases, which are important for morphogenesis (Zhang et al. 2012). However, the link between cellobiohydrolase proteins and morphogenesis is unknown. In this study, we characterized a gene encoding a cellobiohydrolase homologue in *Dictyostelium*.

Materials and methods

Cells and growth conditions

D. discoideum Ax2 and *cbhA* null cells were cultured axenically in HL5 medium at 22 °C. Cells of the *cbhA* null strain were grown in HL5 supplemented 10 μg/ml blasticidine S (Kaken Pharmaceutical, Tokyo, Japan). Transformants with a Neo^R cassette were selected in HL5 supplemented 10 μg/ml G418 (geneticin; ICN Biochemicals Inc., Ohio, USA) and, in some cases, the concentration of G418 was subsequently increased up to 80 μg/ml.

Genomic DNA cloning and construction of the targeting vector

The genomic sequence of the *Dictyostelium* cellobiohydrolase gene *cbhA* (*DDB_G0268446*) was obtained from the genomic database (http://dictybase.org/db/) using 'glycoside hydrolase family' as a search term. A genomic fragment including promoter, coding (ORF), and terminator regions was amplified by PCR with *LA Taq* polymerase (TaKaRa, Kyoto, Japan) using the oligonucleotides cbhA-G1 and cbhA-G2 as primers and genomic DNA from Ax2 strain as the template. The sequence of each primer used in this manuscript is shown in Table S1. A PCR fragment of approximately 2.5 kbp in length was amplified and subsequently subcloned into the pCR2.1-TOPO vector (Invitrogen) to yield pTOPO[*cbhA*(G1–G2)].

The resultant vector was digested with *Bam*HI and blunt-ended by a fill-in reaction with Klenow enzyme to eliminate the *Bam*HI site, yielding pTOPOΔ*Bam*HI[*cbhA*(G1–G2)]. A new, unique *Bam*HI site was introduced by inverse PCR using cbhA-Bam1 and cbhA-Bam2 primers. The resultant vector was digested with *Bam*HI, treated with calf intestinal alkaline phosphatase (TOYOBO, Tokyo, Japan), and then ligated with *Bam*HI digested blasticidine S resistant cassette (Bs^R) to yield the knockout construct pTOPO[*cbhA:*Bs^r(KO)] (see Fig. 3).

Screening of the *cbhA* null strains

The target vector, pTOPO[*cbhA:*Bs^r(KO)], was digested with *Not*I and *Spe*I. After phenol/chloroform extraction followed by ethanol precipitation of the vector DNA, Ax2 cells were transformed by electroporation as described previously (Shimada and Kawata 2007). Transformants were selected in 96-well plates containing HL5 medium supplemented with 10 μg/ml blasticidine S. To screen the clones for those in which the *cbhA* had been successfully targeted, genomic DNA was isolated from the Bs^R clones and subjected to PCR analysis using two pairs of primers Bsr-1/cbhA-KO and cbhA-G1/cbhA-G2 (see Fig. 3).

Analysis of gene expression by semi-quantitative RT-PCR

Ax2 cells, axenically grown in shaking culture, were washed twice with KK_2 phosphate buffer (16.5 mM KH_2PO_4 and 3.8 mM K_2HPO_4, pH 6.2) and allowed to develop on nitrocellulose filters (black gridded, 0.45 mm pore size, HABG04700; Millipore) at 22 °C. Every 3 h, total RNA was extracted from the developing Ax2 cells using the RNeasy

Extraction kit (Qiagen, Germany). cDNA synthesis and RT-PCR were performed with the RNA LA PCR Kit (AMV) version 3 (TaKaRa) using a pair of primers, cbhA-RT and cbhA-G5-Bam. For loading normalization, the product of the *IG7* (*rnlA*) gene was detected using the IG-7 forward and IG-7 reverse primer pair.

Construction of CbhA expression vectors

For an expression construct to rescue the *cbhA* null strain, a DNA fragment corresponding to the promoter, ORF including stop codon, and terminator regions of the *cbhA* was amplified by PCR with KOD polymerase Neo (TOYOBO) using the oligonucleotides cbhA-G1-Xba and cbhA-G2-Xho as primers and genomic DNA from the Ax2 strain as the template. A PCR fragment of ~2.5 kbp in length was digested with *Xba*I and *Xho*I and gel-purified. Then, the fragment was ligated with *Xba*I and *Xho*I digested pDd-gal17(H+) (Harwood and Drury 1990) to yield pDd-neo^R[*cbhA*/*cbhA*].

To produce a GFP-tagged CbhA expression vector, a DNA fragment containing the 2.3-kbp *cbhA* promoter and entire coding region (ORF) without a stop codon was amplified by PCR with *LA Taq* polymerase using cbhA-G1 and cbhA-G5Bam primers and pTOPOΔ*Bam*HI[*cbhA*(G1–G2)] as the template. This DNA fragment was subcloned into pCR2.1-TOPO vector (Invitrogen) to yield pTOPO[*cbhA*/*cbhA*(G1–G5Bam)]. After digestion of the resultant vector with *Xba*I and *Bam*HI, the DNA fragment corresponding to *cbhA* promoter and entire ORF without a stop codon was extracted from the gel. The GFP containing vector, pLD1ΔBX-GFP (Y. Yamazaki, N. Sasaki and T.K., unpublished), was digested with *Bam*HI and *Xba*I and gel-purified. These gel-purified DNA fragments were ligated to yield pLD1ΔBX[*cbhA*/*cbhA*-GFP].

To produce an ectopic over-expression vector of CbhA with a myc-tag, the entire ORF region without a stop codon was amplified by PCR with KOD polymerase Neo (TOYOBO) using the oligonucleotides cbhA-G3-Bam2 and cbhA-G5-Xho2 primers and genomic DNA from the Ax2 strain as the template. A PCR amplified DNA fragment of size ~1.37 kbp was digested with *Bam*HI and *Xho*I and gel-purified. The fragment was then ligated with *Bam*HI and *Xho*I digested pLD1ΔBX-myc (N. Sasaki and T.K., unpublished) to yield pLD1ΔBX[*act15*/*cbhA*-myc].

Construction of a β-galactosidase reporter and detection of its activity

To create the *cbhA/lacZ* reporter construct, the promoter region of the *cbhA* was amplified by PCR with *LA Taq* polymerase (TaKaRa) using the oligonucleotides cbhA-G4Bam and M13 forward (F) as primers and pTOPO[*cbhA*(G1–G2)] as the template. The PCR fragment of ~0.9 kbp in length was digested with *Xba* I and *Bam*HI and gel-purified. Then, the fragment was ligated with *Xba*I and *Bgl*II digested pDd-gal17(H+) (Harwood and Drury 1990) to yield pDd-neo^R[*cbhA*/*lacZ*].

To detect promoter activity, cells transformed with pDd-neo^R[*cbhA*/*lacZ*] were grown and developed on filters (OMNIPORE membrane filters, 1.0 μm pore size; Millipore). Fixation and staining were performed as described previously (Shimada et al. 2005).

Microscopic analysis of CbhA-GFP fusion protein

For detection of the CbhA-GFP fusion protein, aggregation stage and various multicellular stage structures were allowed to settle on Teflon coated slides and visualized with a fluorescence microscope (model IX71; Olympus, Tokyo, Japan). Microscope images were captured using the Olympus DP71 Digital Camera System (Olympus) and processed with LuminaVison software (Mitani Corporation, Tokyo, Japan). For detection of the fusion protein in spores, the sorus of the fruiting body was squeezed between a slide and cover glass and observed at 100× objective lens using immersion oil.

Western blot analysis of CbhA fusion proteins

Cells were solubilized in SDS sample buffer containing proteinase inhibitors (EDTA-free cOmplete Mini; Roche, Penzberg, Germany). Proteins were analyzed on 5–20 % SDS-polyacrylamide gels (Miniquick Gel, Anatech, Tokyo, Japan) and blotted onto Hybond™-C Extra filters (Amersham Biosciences, Little Chalfont, UK). Filters were blocked and detected using Promega's Proto Blot II AP System with Stabilized Substrate according to the manufacturer's protocol (Promega Corp., Wisconsin, USA). For detection of the GFP fusion protein, an anti-GFP mouse monoclonal antibody (1:2,000 dilution; Roche, Germany) was used as the primary antibody; for detection of the c-Myc fusion protein, an anti-c-Myc mouse monoclonal antibody (9E10) (1:2,000 dilution; Wako, Tokyo, Japan) was used. In both cases, an alkaline phosphatase-conjugated goat anti-mouse IgG(H+L) antibody (1:10,000–20,000 dilution; Promega Corp.) was used as the secondary antibody.

Cellulose-binding assay

The cellulose-binding ability of the CbhA-myc fusion protein was tested according to the procedure described by Gilbert et al. (1990) with some modifications. HL5 medium containing cells over-expressing the CbhA-myc protein (*cbhA-myc*^{OE} strain) was concentrated by ultrafiltration (Amicon ® Ulutra Ultracel-10 K and Microcon Ultracel YM-10; Millipore, Massachusetts, USA). Buffer exchange into either KK_2 buffer or binding buffer (100 mM Tris–HCl, pH 8.0) was

effected by repeated dilution of the concentrated solution into new buffer.

Microcrystalline cellulose beads (Avicel PH-101; Sigma) were suspended in binding buffer at a final concentration of 5 % (w/v). Then, 500 μl of the Avicel solution was added to 300 μl of buffer-replaced CM and allowed to bind with gentle rotation at 4 °C for 1 h. The Avicel was pelleted by centrifugation at 20,000×*g* for 2 min and washed three times each with 1 M NaCl/50 mM phosphate buffer (pH 7.5) and with 50 mM phosphate buffer (pH 7.5). Bound protein was eluted by heating at 105 °C for 10 min in 1× SDS sample buffer. Unbound and wash fractions were concentrated by ultrafiltration (Microcon Ultracel YM-10), mixed with 1/3 volume of 3× SDS sample buffer, and heated as above. The fusion protein in each fraction was detected by Western blot analysis as described above.

ConA agarose-binding assay

To test glycosylation of CbhA, CM from the *cbhA-myc*^{OE} strain was prepared as for the cellulose binding assay. The ability of the CbhA-myc fusion protein to bind a ConA-agarose column was tested according to the procedure as previously described (Ramalingam et al. 1992; Ramalingam and Ennis 1997) with a slight modification. The concentrated CM was applied repeatedly to a 1-ml packed volume ConA-agarose column (J-Oil Mills, Inc., Tokyo, Japan) and washed with 15 ml of buffer (10 mM Tris–HCl/20 mM NaCl, pH 7.5). The column was then eluted with 15 ml of buffer containing 500 mM α-methyl-mannoside (Sigma, St. Louis, MO, USA). The second–fourth fractions (1 ml each) were combined to form the bound fraction. The washes and bound fractions were concentrated, and the buffer exchanged for phosphate-buffered saline (PBS), pH 7.2, containing proteinase inhibitors (EDTA-free cOmplete), by ultrafiltration (Microcon Ultracel YM-10; Millipore). The presence of the fusion protein in each fraction was detected by Western blot analysis as described above.

Cellulase assay

A volume of 100 μl HL5 medium including CM and 1×10^5 cells of Ax2, *cbhA* null, or *cbhA-myc*^{OE} cells was spotted on an agar plate containing 0.2 mM 4-methylumbelliferyl β-D-cellobioside (4-MUC) and incubated at room temperature for 24 h. After incubation, 4-MUC digesting activity was assayed by detecting fluorescence of the product 4-methylumbelliferone (4-MU) under UV light.

Cellulase (cellobiohydrolase) activity was also detected in a non-denaturing polyacrylamide gel electrophoresis (native PAGE). To do this, 1× sample buffer without SDS containing proteinase inhibitors (EDTA-free cOmplete Mini) was added to concentrated CM from the *cbhA-myc*^{OE} strain. After electrophoresis, the gel was immersed in a solution containing 0.2 mM 4-MUC and shaken gently at room temperature for 2 h. The activity was detected under exposure to the UV light. At the same time, half of the gel was subjected to Western blot analysis as described above.

Results

A *Dictyostelium* homologue of cellobiohydrolase

As *Dictyostelium* harbors a number of genes coding for proteins that contain CBD, we searched the whole genome against the term 'glycoside hydrolase family' and found several uncharacterized cellulase genes (Y. Yamazaki et al., unpublished data). One of them is *DDB_G0268446* gene, which encodes for a protein composed of 457 amino acids that has a predicted molecular weight of 49.5 kDa. A homology search using the BLAST program (http://blast.ddbj.nig.ac.jp/blast/blastn?lang=ja) showed that the *DDB_G0268446* gene encodes a protein with homology to fungal cellobiohydrolase I (CBHI) (Fig. S1). CBHI is an exo-(1,4)-β-glucanase (EC 3.1.2.91), which hydrolyzes crystalline cellulose by initiating its action from the reducing end of cellulose chains and produces primarily cellobiose. The *Dictyostelium* DDB_G0268446 protein has the strongest homology to the cellobiohydrolase of the thermophilic fungi *Thermoascus aurantiacus* var. *levisporus* (aligned score 64 on multiple sequence alignment ClustalW program; http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja) and *Talaromyces emersonii* (aligned score 63), then to that of the filamentous ascomycete fungus *Aspergillus niger* (aligned score 64), *Penicillium oxalicum* (aligned score 61), and *Neurospora crassa* (aligned score 54) (Fig. 1a). Most fungal CBHs belong to family 6 or 7 of glycosyl hydrolases. The *Dictyostelium* representative is predicted by the *Dictyostelium* database (http://dictybase.org/gene/DDB_G0268446; King et al. 2010) to belong to the glycoside hydrolase family 7. Hence, we designated the gene as *cbhA* (cellobiohydrolase A).

We focused on functional analysis of the *cbhA* as the genomic database showed *Dictyostelium* has a unique cellosiohydrolase gene. The existence of a single cellobiohydrolase gene contrasts with fungal genomes where generally two different types of CBHs, CBHI and CBHII, exist, as judged by sequence identity and their functions. Phylogenetic analysis revealed that CbhA showed less homology to *Talaromyces emersonii* CBHII (aligned score 7) and belongs to the same clade to CBHI (Fig. 1a); therefore, CbhA must be a CBHI homologue in *Dictyostelium*.

cbhA expression is developmentally regulated

To characterize *cbhA*, we first examined its temporal expression pattern in the wild-type Ax2 strain via a semi-quantitative RT-PCR experiment. After 26 cycles of amplification, *cbhA* transcripts were barely detectable until 12 h (tip), and weakly

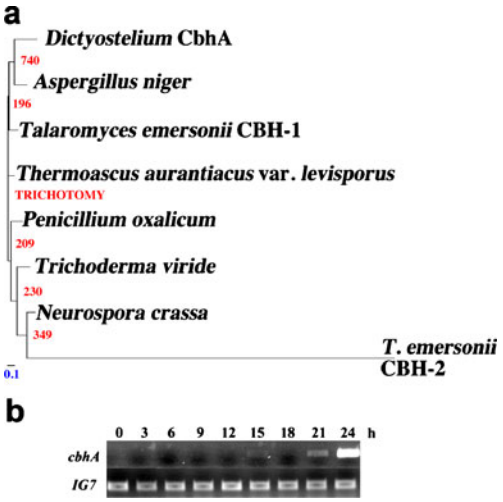

Fig. 1 **a** Phylogenetic tree (phylogram) of *Dictyostelium* CbhA and representative fungal CBHI proteins. The phylogenetic tree was created using the TreeView program after ClustalW multiple-sequence alignment (http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja). *Numbers* at the branch points indicate percentage bootstrap support calculated from 1,000 trees. The scale bar represents genetic distance; it is equivalent to 10 % amino acid diversity. Genes investigated in this work are CbhA, *Dictyostelium discoideum* cellobiohydrolase CbhA (accession number XP_647587), *Aspergillus niger* CBHI (accession number AF156269), *Talaromyces emersonii* CBHI (accession number AY081766), *Thermoascus aurantiacus* var. *levisporus* CBHI (accession number AY840982), *Penicillium oxalicum* CBHI (accession number EU727171), *Trichoderma viride* CBHI (accession number GQ927756), *Neurospora crassa* CBHI (accession number X77778) and *Talaromyces emersonii* CBHII (accession number AF439936). **b** Developmental time course of *cbhA* gene expression detected by semi-quantitative RT-PCR. Total RNA was extracted from Ax2 cells at every 3-h interval and used as a template for amplification of a specific *cbhA* DNA fragment as described in Materials and methods. The *upper row* shows the RT-PCR results for *cbhA* after 26 cycles of amplification. *IG7* was detected in the same reactions as a normalization control. The *IG7* DNA product (*lower row*) was detectable after 20 cycles of amplification before the PCR reaction saturated. Stages are loose aggregate (6 h), mound (9 h), tipped aggregate (12 h), slug (15 h), Mexican hat (18 h), and culminant (21 h), mature fruiting body (24 h), respectively

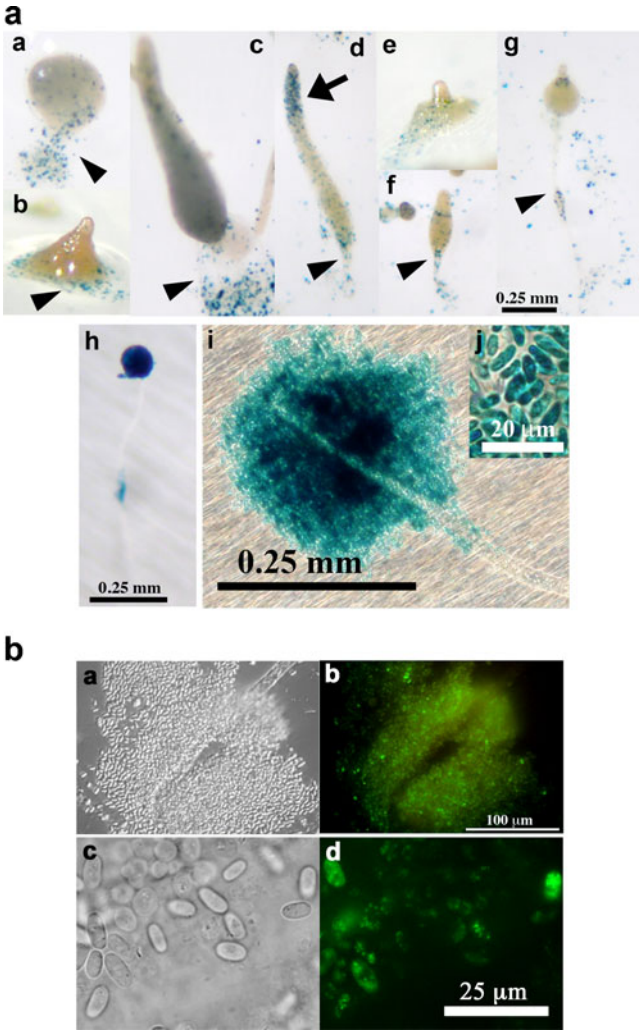

Fig. 2 Expression pattern of the *cbhA* and CbhA-GFP fusion protein. **a** Spatial expression pattern of *cbhA* promoter activity as detected by a *lacZ* reporter construct. Ax2 cells transformed with *cbhA/lacZ*, which contains a 920-bp upstream promoter region fused to the β-galactosidase (*lacZ*) gene (subcultured in the presence of 80 µg/ml G418), were allowed to develop on filters to the stages of mound (*a*), tip (*b*), first finger (*c*), slug (*d*), Mexican hat (*e*), early culminant (*f*), late culminant (*g*) and fruiting body (*h*). A sorus was squeezed between a slide and cover glasses (*i*); a magnified image (*j*) is inserted at the top right corner. The scale bar indicates 0.25 mm for (*a*)–(*i*) and 20 µm for (*j*). The staining was performed at 37 °C overnight for (*a*)–(*g*) and at 37 °C for 1 h for (*i*). **b** Localization of the CbhA-GFP fusion protein in spores. In the strain expressing the fusion protein Ax2 / [cbhA]:cbhA-GFP (subcultured in the presence of 80 µg/ml G418), the sorus of the fruiting body was squeezed as described above prior to detection of the GFP signal using a fluorescent microscope (IX-71; Olympus, Tokyo, Japan). The *left column* (*a*, *c*) shows bright field images and the *right column* (*b*, *d*) the corresponding fluorescent images. Bars represent 100 µm for the *upper row* and 25 µm for the *lower row*

detectable at 15 h (slug). The transcripts were moderately up-regulated at 21 h (early culminant) and highly up-regulated at 24 h of development (fruiting body) (Fig. 1b). This pattern indicates that expression of *cbhA* occurs predominantly at the late stages of development, particularly in the fruiting body.

Next, to observe the spatial expression pattern of the *cbhA*, we amplified a promoter fragment of the *cbhA* by PCR and subcloned it into pDd-gal17(H+) (Harwood and Drury 1990) to make a *cbhA/lacZ* fusion reporter construct. Promoter activity was assayed in transformants carrying the *cbhA/lacZ* reporter via detection of β-galactosidase activity. Other than in the fruiting body, activity was very weak. However, it was detectable after overnight incubation at 37 °C in structures developed from cells that had been cultured in the presence of 80 µg/ml of G418 (Fig. 2a(*a*–*g*)). Until the first finger stage, expression of *lacZ* was restricted to cells at the periphery of the basal region and to cells outside the multicellular structure, assumed to be the surface sheath (Fig. 2a(*a*–*c*), arrowheads). At the slug stage, prestalk O (pstO) and rear guard prestalk B (pstB) cells, as well as cells in the slime trail, expressed *cbhA/lacZ* (Fig. 2a(*d*), arrow for

pstO and arrowhead for pstB). During culmination, cells of the upper cup and slime trail expressed the *lacZ* reporter. There was also scattered staining in the stalk of the culminant (Fig. 2a(*f* and *g*), arrowheads). The fruiting body showed very strong staining in sorus (Fig. 2a(*h*)). This staining was visible even after a short incubation (less than 30 min) at 37 °C. When the sorus was squeezed between a slide and cover glass, strong staining was detected in individual spores (Fig. 2a(*i*) and (*j*)).

A similar spatial pattern was also observed in Ax2/[cbhA]:cbhA-GFP transformants, where expression of the CbhA-GFP fusion protein was driven by the *cbhA* promoter (Fig. S2b). Again, a strong GFP signal was observed in spores (Fig. 2b(*b*)). The fusion protein showed uneven granular distribution in spores (a magnified view is shown in Fig. 2b(*d*)). Presumably, as mentioned later, the GFP tag might have affected the CbhA protein function. However, CbhA-GFP tissue localization is not affected.

Phenotype of the *cbhA* null mutant

To determine whether *Dictyostelium* cellobiohydrolase has a role in development, particularly in morphogenesis, two mutant strains (#7 and #15) lacking the *cbhA* were created by homologous recombination (Fig. 3). The potential clones were screened by PCR, using genomic DNA as the template (Fig. 3b, c). In addition, semi-quantitative RT-PCR (Fig. 4a) analysis showed that the *cbhA* transcript was undetectable.

Because the two mutant clones displayed the same phenotype, only one on them (#15) was used for later experiments and is shown in the associated figures. When the *cbhA* null strain was developed under various conditions (on filter placed on phosphate buffered filter pads, on filters on water agar plates, or directly on an agar plate), its morphology was almost normal (Fig. 4b). However, developmental timing was delayed by 2–4 h until the onset of culmination. The degree of delay varied in some experiments, and, in few experiments, was even not observed (data not shown). The average delay at the Mexican hat stage was approximately 4 h. Overall, the extent of developmental delay shown in Fig. 4b is representative of repeated experiments. When the *cbhA* null mutant cells were transformed with a plasmid to express *cbhA* driven by its own promoter, the above phenotype was largely rescued (Fig. 4b). Thus, the delayed development phenotype can be attributed to the loss of *cbhA*.

CbhA binds to cellulose and is secreted

If CbhA is a cellulase, it must have a CBD and possess cellulose-binding activity. However, CbhA lacks a representative CBD consensus as described previously (Linder and Teeri 1996; Birch 1998). To test the cellulose-binding ability of the CbhA, we first created two CbhA-GFP expressing strains, Ax2/[*act15*]:*cbhA*-GFP and Ax2/[*cbhA*]:*cbhA*-GFP. When the latter strain was developed, expression of CbhA-GFP fusion protein was detected during all multicellular stages of development (Fig. S2a,b), similar to our observations in the *cbhA/lacZ* β-galactosidase assay (Fig. 2a). In both strains, an ~80-kDa CbhA-GFP band was detected; this size was almost equal to the sum of that predicted for CbhA (~49 kDa) and GFP (~27 kDa) (Fig. S2c).

We used the Ax2/[*act15*]:*cbhA*-GFP strain for a cellulose-binding assay using microcrystalline cellulose beads, Avicel PH-101 (ESM Fig. 2c). In that assay, the fusion protein was observed in the unbound fraction. Thus, CbhA-GFP is not capable of binding to cellulose. A similar result was observed for a bacterially expressed GST-CbhA recombinant fusion protein (data not shown).

The inability of the CbhA-GFP and GST-CbhA fusion proteins to bind cellulose may be caused by the large size of the epitope tag relative to the CbhA protein (~27 or 26 vs. ~49 kDa). To eliminate this possibility, we next tried use the c-Myc epitope as a smaller tag. First, we repeatedly tried to generate *cbhA*-myc expression construct driven by its own promoter, but we did not succeed due to unknown reason. Therefore, we created an over-expression strain, Ax2/[*act15*]:*cbhA*-myc and used it in the experiments described below. When axenically growing cells expressing this fusion protein were harvested, a single band of ~60 kDa was detected with an anti-c-Myc antibody (9E10), whereas no band was detected in samples derived from the Ax2 strain (Fig. 5a). Notably, a larger amount of the protein was secreted into the culture medium (conditioned medium; CM) than was retained in cells (Fig. 5a, lanes 3 and 4). Thus, the majority of the CbhA fusion protein was secreted. When the fusion protein was tested for binding to Avicel, although a faint band was detected in the unbound and washed fractions, a larger amount of the fusion protein bound to Avicel (Fig. 5b). This result indicates that CbhA is capable of binding to cellulose.

CbhA is a glycoprotein and has cellulase activity

In the above experiment, the apparent molecular weight of the c-Myc tagged protein was approximately 60 kDa, which is larger than the predicted one of ~49 kDa. This difference in molecular size must be attributed to post-translational modification. Because cellulases are known to be commonly glycosylated, we investigated this possibility by passing conditioned medium from Ax2/[*act15*]:*cbhA*-myc cells through a succinyl concanavalin A (ConA)-agarose column. The CbhA-myc fusion protein bound to the ConA (Fig. 5c). Therefore, the CbhA is a glycoprotein.

Because CbhA is a cellulose-binding glycoprotein, the next question is whether the protein possesses cellulase activity. To evaluate this possibility, we used 4-MUC as a substrate, since cellobiohydrolase digests it preferentially (Boschker and Cappenberg 1994; Tuohy et al. 2002). First, we prepared a water agar plate containing 4-MUC and placed droplets of

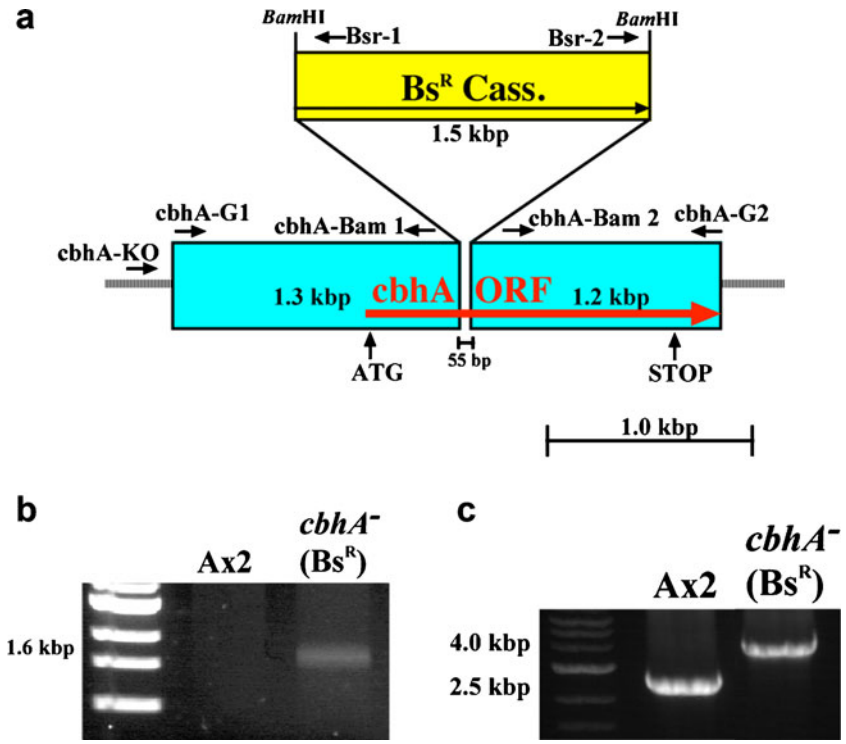

Fig. 3 Creation of the *cbhA* null mutant. **a** Knockout construct of the *cbhA* used for isolation of the null mutant. The *boxed region* is subcloned in the vector and the *long arrows* at the base of the *blue and yellow boxes* indicate the orientation of *cbhA* and Bs^R cassette, respectively. *Solid bars* represent the flanking regions of the *cbhA* and are not included in the knockout construct. *Short arrows* indicate the positions and orientations of primers used for screening the null mutant. **b** Screening of the *cbhA* null mutant. PCR was carried out using genomic DNA extracted from Ax2 or the *cbhA* null mutant [$cbhA^-$(Bs^R)] as the templates and the primer pair cbhA-KO / Bsr-1. A fragment containing the Bs^R cassette (1.6 kbp long) was amplified only in the mutant cells. **c** Confirmation of targeting of the *cbhA* gene. PCR was carried out using genomic DNA extracted from Ax2 or the *cbhA* null mutant [$cbhA^-$(Bs^R)] as the templates and the primer pair cbhA-G1 / cbhA-G2. A *cbhA* fragment containing the Bs^R cassette (4.0 kbp long) was amplified in the mutant cells, while a fragment corresponding to the *cbhA* gene alone (2.5 kbp long) was amplified in the Ax2 cells

equal volume of CM (HL5 medium), containing an equal number of cells for each strain, and incubated the plate for 24 h. In this experiment, Ax2 and *cbhA* null strain showed barely detectable or trace amounts of 4-MUC digesting activity. In contrast, the CbhA-myc over-expressing strain (*cbhA-myc*^{OE}) displayed clear 4-MUC digesting activity (Fig. 6a). Next, we separated proteins from concentrated CM from the *cbhA-myc*^{OE} strain via non-denaturing polyacrylamide gel electrophoresis (native PAGE). After electrophoresis, the gel was soaked in a solution containing 4-MUC for 2 h. Doublet bands showing 4-MUC digesting activity that is assumed to be CbhA were detected (Fig. 6b). These lines of evidence suggest that CbhA has cellulase activity, most likely that of a cellobiohydrolase.

Discussion

We have investigated the role of cellulases in *Dictyostelium* morphogenesis. In this study, we focused on the *Dictyostelium* cellobiohydrolase homologue *cbhA* and investigated its function during development.

As described above, CBHs are key components in cellulase complexes and potent cellulolytic fungi generally produce two different CBHs, CBHI and CBHII. In contrast, *Dictyostelium* genome sequence data reveals the presence of a single copy CBH (Eichinger et al. 2005), which we have designated CbhA. We conclude that CbhA is an active cellulase and is the CBHI orthologue in *Dictyostelium*. This assertion is based on several observations: (1) that CbhA has strong homology to fungal CBHI (Fig. S1); (2) that phylogenetic analysis revealed that CbhA belongs to the same clade as CBHI but not CBHII (Fig. 1a); (3) that CbhA bound to crystalline cellulose Avicel (Fig. 5b) in spite of lacking a typical CBD consensus as described previously (Linder and Teeri 1996; Birch 1998); (4) that CM from a *cbhA-myc*^{OE} strain showed 4-MUC degradation activity (Fig. 6). Release of 4-methylumbelliferone (4-MU) from 4-MUC by CBHII is very inefficient; instead CBHII prefers to digest 4-methylumbelliferyl β-D-cellotrioside to release 4-MU (Tuohy et al. 2002). Thus, it is unlikely that CbhA is a CBHII homologue. Although endoglucanases also can digest 4-MUC, *Dictyostelium* amoeboid cells are known to have very low cellulolytic activity (Rosness 1968). This is also true for 4-MUC digesting

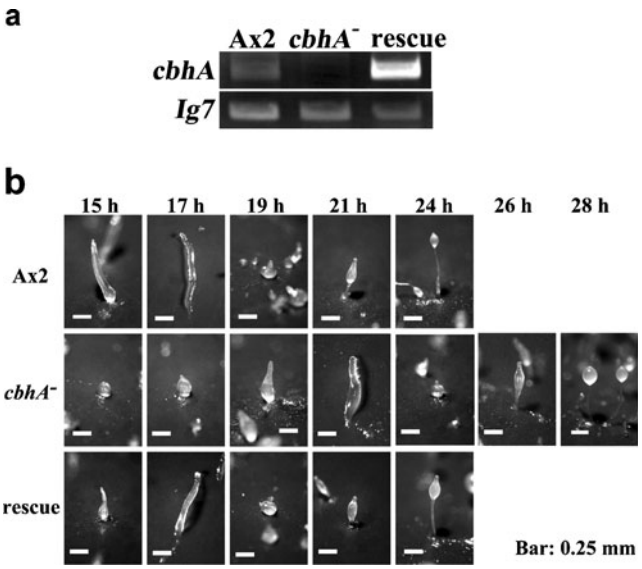

Fig. 4 Phenotype of the *cbhA* null mutant. **a** The expression of the *cbhA* in Ax2, the *cbhA* null mutant, and in the rescued strain was detected by semi-quantitative RT-PCR. Total RNA was extracted from each strain and analyzed as described in Materials and methods. After synthesis of cDNAs from 0.15 μg total RNA, 30 cycles of PCR were performed with the primer pair cbhA-RT and cbhA-G5-Bam. The strain name is indicated at the top of each lane. *IG7* was again used as a normalization control (see Fig. 1 legend). **b** Delay of development in the *cbhA* null strain. Cells were allowed to develop on nitrocellulose filters placed on buffered filter pads for the length of time indicated above each column. The strains are indicated to the left of each row and include Ax2 (the wild-type strain), *cbhA-*, (the *cbhA* null strain clone #15; another *cbhA* null strain, clone #7, displayed the identical phenotype), and rescue (*cbhA* null strain transformed with pDd-neo^{R}[*cbhA*/*cbhA*]; the resultant strain is the rescue strain, *cbhA*$^{-}$/[*cbhA*]:*cbhA*). The developmental stage during aggregation and mound formation for each strain is shown in Fig. S3. The bar represents 0.25 mm

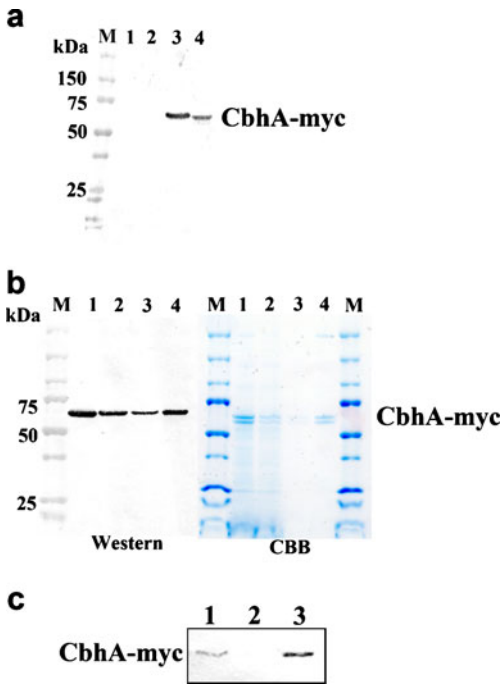

Fig. 5 Biochemical properties of the CbhA. **a** Expression and secretion of the CbhA-myc fusion protein. Axenically grown the Ax2 (*lanes 1 and 2*) and the CbhA-myc overexpressing cells, Ax2/[*act15*]:*cbhA*-myc (*cbhA-myc*^{OE}) (*lanes 3 and 4*), were harvested by centrifugation. *Lanes 1 and 3* represent concentrated supernatant (CM) prepared as described in Materials and methods. *Lanes 2 and 4* represent total cellular proteins from the pellets. The solubilized proteins were subjected to SDS-PAGE, followed by Western blot analysis using anti-c-Myc antibody as a primary antibody, as described in Materials and methods. *Lane M* denotes protein size markers. **b** Binding of the CbhA-myc fusion protein to the microcrystalline cellulose Avicel. Binding ability of the CbhA-myc fusion protein to the Avicel was examined as described in Materials and methods. The *left panel* shows the result of Western blot analysis probed with anti-c-Myc antibody. The *right panel* shows Coomassie brilliant blue R-250 (*CBB*) staining after SDS-PAGE using the same samples as tat for the Western blot analysis. *Lanes M* contain protein size markers. The samples in each lane were: *1*, input concentrated total CM protein from the *cbhA-myc*^{OE} strain; *2*, Avicel unbound fraction; *3*, wash fraction; *4*, Avicel bound fraction. Input, unbound and wash fractions were concentrated using ultrafiltration as described in Materials and methods. The apparent molecular weight of CbhA-myc fusion protein is ~60 kDa. Note that doublet bands are visible after CBB staining. Although both of them apparently have cellulose binding and cellulase activity (see Fig. 6b), only the upper band was detected by the anti-c-Myc antibody. Most likely, the lower band may represent the protein without the c-Myc tag, which has been removed during secretion or sample preparation. **c** Glycosylation of the CbhA-myc fusion protein. Glycosylation was tested by binding to a ConA-agarose column as described in Materials and methods. The samples used in each lane were: *1*, input concentrated total CM protein from the *cbhA-myc*^{OE} strain; *2*, ConA-agarose unbound fraction; *3*, ConA-agarose bound fraction. All fractions were concentrated using ultrafiltration as described in Materials and methods

activity, as it was hardly detectable in either Ax2 or *cbhA* null strains in a 4-MUC agar plate assay (Fig. 6a). Therefore, the cellulase activity detected in the CM from *cbhA-myc*^{OE} strain via native PAGE (Fig. 6b) is likely to be due to CbhA, thereby further supporting the above conclusion. In these experiments, we used semi-constitutive *actin15* promoter instead of *cbhA* promoter to express CbhA-myc fusion protein. When the *act15/cbhA*-myc construct was transformed into the *cbhA* null strain, the developmental delay was rescued (data not shown). Therefore, we believe CbhA-myc is functional and the use of *actin15* promoter will not affect the conclusion.

The reason why *Dictyostelium* lacks an apparent CBHII is currently unknown. Other uncharacterized enzymes in *Dictyostelium* may substitute for the activity of CBHII. Indeed, in some species, such as most brown rot fungi and a wood-rotting basidiomycete, cellobiohydrolase is apparently absent and is substituted for by processive endoglucanases with properties of both endo- and exoglucanases (reviewed by Baldrian and Valášková 2008). Alternatively, *Dictyostelium* may not require CBHII activity and, hence, has lost CBHII during evolution. To address this issue, the biochemical properties of CbhA and other cellulases have to be examined.

The active CbhA detected by Western blot analysis as a c-Myc fusion protein was larger than expected, ~60 versus ~49 kDa (Fig. 5a,b). This difference was due to the addition of glycosyl chain(s) as confirmed by binding to a ConA-agarose

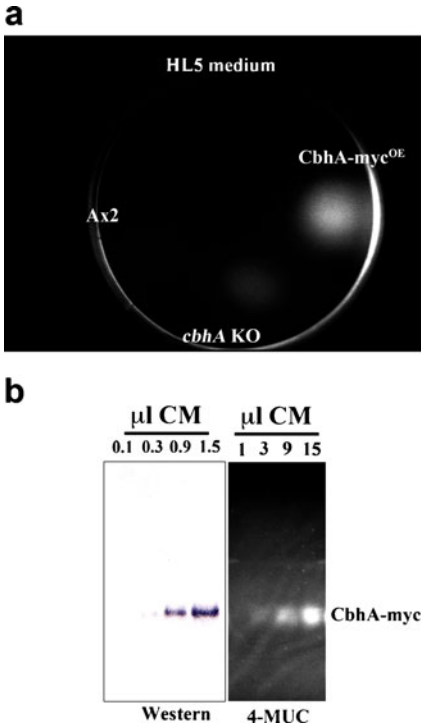

Fig. 6 Detection of the cellulase activity of the CbhA-myc fusion protein. **a** Cellulase activity in the strain overexpressing CbhA-myc. For detection of cellulase activity, 100 μl of HL5 medium, including an equal number of cells (2.7×10^4 cells), from each strain was spotted onto a 4-methylumbelliferyl β-D-cellobioside (*4-MUC*) containing agar plate. 4-MUC digesting activity (which is assumed to be cellobiohydrolase activity) was detected as described in Materials and methods. The strains used were: Ax2, wild type strain; CbhA-myc^{OE}, Ax2/[*act15*]:*cbhA*-myc strain (*cbhA-myc*^{OE}); *cbhA* KO, *cbhA* null strain. As a negative control, HL5 medium alone was spotted. Note that, because cells were spotted in the presence of HL5 medium, the assay was not initially carried out under starvation conditions and hence limited development into multi-cellular structures occurred. **b** A Zymogram stained with 4-MUC. The concentrated CM used for the cellulose-binding assay from the *cbhA-myc*^{OE} strain was fractionated on a non-denaturing polyacrylamide gel. The *left panel* shows the result of Western blot analysis probed with anti-c-Myc antibody. The *right panel* shows the Zymogram using the same sample and 4-MUC as a substrate. The volume of the CM applied is shown at the top of each lane. Note that the major protein in the CM is CbhA-myc (Fig. 5b) and that activity was detected as a doublet, similar to that seen in the stained gel after SDS-PAGE (Fig. 5b). However, only the upper band was detected by Western blot analysis

column (Fig. 5c). Such a difference between the apparent molecular weight and the predicted one was not observed for either CbhA-GFP (Fig. S2b) or GST-CbhA (data not shown). Since neither of these fusion protein could bind cellulose (Fig. S2b; data not shown for GST-CbhA), it is probable that they are not functional. Although whether glycosylation is necessary for the function of CbhA is not yet known, addition of a large tag, such as GFP, at least prevented glycosylation of CbhA. Both *O*-linked and *N*-linked glycosylations are prevalent in fungal CBHs. There are reports suggesting that *N*-glycosylation is important not only for CBH activity but also for stability (Jeoh et al. 2008; Adney et al. 2009; Beckham et al. 2010; Gao et al. 2012). There are three *N*-glycosylation consensus sequence motifs (NXS/T) in *Dictyostelium* CbhA (Fig. S1, shown as bold letters). One of them, Asp-131, is the equivalent site to Asp-137 of *Penicillium decumbens* CBHI; *N*-glycosylation at this site improved CBH activity (Gao et al. 2012). Therefore, *N*-glycosylation of CbhA may be necessary for its function and full activity.

Expression of *cbhA* was tightly developmentally regulated, late stage-specific, and especially strong in the spores of the fruiting body (Figs. 1b and 2a). This finding was confirmed by observation of a CbhA-GFP fusion protein whose expression was driven by the *chbA* promoter (Fig. 2b). This observation implies that CbhA may have a function in spores. So far, however, we have not detected any obvious difference among spores from wild-type (Ax2), *cbhA* null, rescued, and *cbhA-myc*^{OE} strains, with respect to spore maturation (as judged by Calcofluor staining), spore viability (as judged by resistance to NP-40 and EDTA) or germination efficiency in HL5 medium (data not shown). These are unexpected results given the abundant expression of *cbhA* in spores. In spite of discrete localization of the CbhA-GFP fusion protein in the spores (Fig. 2b), this fusion protein is assumed to be non-functional, since it was unable to bind to crystalline cellulose (Fig. S2b). The endogenous CbhA protein in spores may be secreted, as observed for amoebae of the *cbhA-myc*^{OE} strain (Fig. 5a). Alternatively, at least after germination, the preexisting CbhA in spores may be secreted from the amoebae. Or, ChbA may be involved in feeding activity, acting to digest the cell walls of food microorganisms. However, under laboratory experimental conditions using *Klebsiella planticola* as a food source, there was no difference in the feeding activity among various strains (data not shown), although this result may not be directly applicable to natural environmental conditions. Alternatively, CbhA may have an auxiliary function during germination, when it could act synergistically with other germination-specific cellulolytic enzymes, such as CelA and CelB, which are the major cellulases at this stage (Giorda et al. 1990; Blume and Ennis 1991; Ramalingam and Ennis 1997).

The phenotype of the *cbhA* null mutant is a developmental delay (Fig. 4b). The delay was minimal during aggregation, until ~9 h (Fig. S3), but it occasionally expanded to up to 4–5 h during multicellular stages (see Fig. 4b). These results imply that CbhA is necessary for efficient morphogenetic cell movement during multicellular development. Expression of *cbhA/lacZ* (Fig. 2a) and CbhA-GFP (Fig. S2a and b) was detected from the early stages of development onward. Specifically, expression of *cbhA/lacZ* was observed in the basement region of multicellular structures and in the extracellular sheath. These observations indicate that CbhA may have a role in digesting the ECM, which is composed mainly of cellulose. Conceivably, therefore, insufficient digestion of

cellulose due to a lack of cellobiohydrolase might produce an extra sticky substratum for cell locomotion that could cause a delay in developmental timing (Figs. 4b, Fig. S3). In accordance with this hypothesis, production of the surface sheath is known to begin increasing at 8 h of development (Freeze and Loomis 1977), when the developmental delay of *cbhA* null strain began (Fig. S3). The amount of sheath increases until the tip formation stage (~12 h) (Freeze and Loomis 1977) as does the crystallinity of cellulose (Grimson et al. 1996). In almost all cases, the developmental delay of the *cbhA*$^-$ mutant expanded at this phase (Fig. 4b). A similar but stronger phenotype was observed for mutants deficient in uridine diphosphoglucose (UDPG)-pyrophosphorylase activity, which cannot synthesize the cellulose substrate UDPG; those mutants form slugs that migrate inefficiently and fail to culminate (Dimond et al. 1976).

We have not observed any phenotype of the *cbhA* null mutant other than delayed development. However, there may be a difference in intercellular ultrastructures, particularly in ECMs such as the slime sheath. Indeed, the slime trails of the *cbhA* null mutant seem to be thicker than those of the parental strain (data not shown). There is evidence for involvement of the surface sheath, whose major component is cellulose, in the control of morphogenesis (Loomis 1972; Freeze and Loomis 1977). In addition, loss of the cellulose synthase gene, *dcsA*, causes aberrant morphology during multicellular development (Blanton et al. 2000). CbhA may act synergistically with other endoglucanases and with β-glucosidase, so that the effect of loss of a single gene on the developmental morphology would be minor.

One of the most important findings of this research is the detection of cellobiohydrolase activity in the amoebozoan microorganism *Dictyostelium*. The cultivation of *Dictyostelium* is easier and cheaper than other eukaryotic model organisms. In addition, manipulation of genes is also easy in *Dictyostelium* cells. As shown in this study, the major protein in the CM of growing amoebae is CbhA in cell lines in which it is overexpressed (Fig. 5b). This relative abundance makes it easier to characterize the biochemical properties of CbhA. Mutational analyses of fungal CBHs shows that an increase in enzyme activity is possible when the *N*-glycosylation sites are properly manipulated, regardless of whether the CBH expression system is heterologous or homologous (Adney et al. 2009; Gao et al. 2012). If this is the case for *Dictyostelium* expression system, then it would be a very useful system for the production of the CBH enzyme on an industrial scale.

Acknowledgments This work was supported by a Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) to T. Kawata (no. 2150230, 24510307), and a Faculty of Science Special Grant for Promoting Scientific Research at Toho University to T. Kawata (301–12). We thank Dr. Tamao Saito, Sophia University, Japan for her helpful comments on the manuscript. We are grateful to Dr. Margaret K. Nelson, Allegheny College, PA, USA, for proofreading the manuscript.

References

Adney WS, Jeoh T, Beckham GT, Chou Y-C, Baker JO, Michener W, Brunecky R, Himmer ME (2009) Probing the role of N-linked glycans in the stability and activity of fungal cellobiohydrolase by mutational analysis. Cellulose 16:699–709

Baldrian P, Valášková V (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 32:501–521

Beckham GT, Bomble YJ, Matthews JF, Taylor CB, Resch MG, Yarbrough JM, Decker SR, Bu L, Zhao X, McCabe C, Wholert J, Bergenstråhle M, Brady JW, Adney WS, Himmer ME, Crowley MF (2010) The *O*-glycosylated linker from the *Trichoderma reesei* family 7 cellulase is a flexible, disordered protein. Biophys J 99: 3773–3781

Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383

Birch PR (1998) Targeted differential display of abundantly expressed sequences from the basidiomycete *Phanerochaete chrysosporium* which contain regions coding for fungal cellulose-binding domains. Curr Genet 33:70–76

Bishop JD, Moon BC, Harrow F, Ratner D, Gomer RH, Dottin RP, Brazill DT (2002) A second UDP-glucose pyrophosphorylase is required for differentiation and development in *Dictyostelium discoideum*. J Biol Chem 277:32430–32437

Blanton RL (1993) Prestalk cells in monolayer cultures exhibit two distinct modes of cellulose synthesis during stalk cell differentiation in *Dictyostelium*. Development 119:703–710

Blanton RL, Fuller D, Iranfar N, Grimson MJ, Loomis WF (2000) The cellulose synthase gene of *Dictyostelium*. Proc Natl Acad USA 97: 2391–2396

Blume JE, Ennis HL (1991) A *Dictyostelium discoideum* cellulase is a member of a spore germination-specific gene family. J Biol Chem 256:15432–15437

Boschker HTS, Cappenberg TE (1994) A sensitive method using 4-methylumbelliferyl-β-cellosiose as a substrate to measure (1,4)-β-glucanase activity in sediments. Appl Environ Microbiol 60:3592–3596

Brown NH (2011) Extracellular matrix in development: insights from mechanisms conserved between invertebrates and vertebrates. Cold Spring Harb Perspect Biol 3:a005082

Darley CP, Li Y, Schaap P, McQueen-Mason SJ (2003) Expression of a family of expansin-like proteins during the development of *Dictyostelium discoideum*. FEBS Lett 546:416–418

Dimond RL, Farnsworth PA, Loomis WL (1976) Isolation and characterization of mutations affecting UDPG pyrophosphorylase activity in *Dictyostelium discoideum*. Dev Biol 50:169–181

Eichinger L, Pachebat JA, Glockner G, Rajandream MA, Sucgang R, Berriman M, Song J, Olsen R, Szafranski K, Xu Q, Tunggal B, Kummerfeld S, Madera M, Konfortov BA, Rivero F, Bankier AT, Lehmann R, Hamlin N, Davies R, Gaudet P, Fey P, Pilcher K, Chen G, Saunders D, Sodergren E, Davis P, Kerhornou A, Ni X, Hall N, Anjard C, Hemphill L, Bason N, Farbrother P, Desany B, Just E, Morio T, Rost R, Churcher C, Cooper J, Haydock S, van Driessche N, Cronin A, Goodhead I, Muzny D, Mourier T, Pain A, Lu M, Harper D, Lindsay R, Hauser H, James K, Quiles M, Madan Babu M, Saito T, Buchrieser C, Wardroper A, Felder M, Thangavelu M, Johnson D, Knights A, Loulseged H, Mungall K, Oliver K, Price C, Quail MA, Urushihara H, Hernandez J, Rabbinowitsch E, Steffen D, Sanders M, Ma J, Kohara Y, Sharp S, Simmonds M, Spiegler S, Tivey A, Sugano S, White B, Walker D, Woodward J, Winckler T, Tanaka Y, Shaulsky G, Schleicher M, Weinstock G, Rosenthal A, Cox EC, Chisholm RL, Gibbs R, Loomis WF, Platzer M, Kay RR, Williams J, Dear PH, Noegel AA, Barrell B, Kuspa A (2005) The genome of the social amoeba *Dictyostelium discoideum*. Nature 435:43–57

Freeze H, Loomis WF (1977) The role of the fibrillar component of the surface sheath in the morphogenesis of *Dictyostelium discoideum*. Dev Biol 56:184–194

Friedl P, Alexander S (2011) Cancer invasion and the microenvironment: plasticity and reciprocity. Cell 147:992–1009

Gao L, Gao F, Wang L, Geng C, Chi L, Zhao J, Qu Y (2012) N-glycoform diversity of cellobiohydrolase I from *Penicillium decumbens* and synergism of nonhydrolytic glycoform in cellulose degradation. J Biol Chem 287:15906–15915

Gilbert HJ, Hall J, Hazlewood GP, Ferreira LMA (1990) The N-terminal region of an endoglucanase from *Pseudomonas fluorencens* subspecies cellulosa constitutes a cellulose-binding domain that is distinct from the catalytic centre. Mol Microbiol 4:759–767

Giorda R, Ohmachi T, Shaw DR, Ennis HL (1990) A shared internal threonine–glutamic acid–proline repeat defines a family of *Dictyostelium discoideum* spore germination specific proteins. Biochemistry 29:7264–7269

Grimson MJ, Haigler CH, Blanton RL (1996) Cellulose microfibrils, cell motility, and plasma membrane protein organization change in parallel during culmination in *Dictyostelium discoideum*. J Cell Sci 109:3079–3087

Harwood AJ, Drury L (1990) New vectors for expression of the *E. coli lacZ* gene in *Dictyostelium*. Nucleic Acids Res 18:4292

Jeoh T, Michener W, Himmer ME, Adney WS (2008) Implications of cellobiohydrolase glycosylation for use in biomass conversion. Biotechnol Biofuels 1:10

Kawata T (2011) STAT signaling in *Dictyostelium development*. Develop Growth Differ 53:548–557

Kim HY, Nelson CM (2012) Extracellular matrix and cytoskeletal dynamics during morphogenesis. Organogenesis 8:56–64

King AJ, Cragg SM, Li Y, Dymond J, Guille MJ, Bowles DJ, Bruce NC, Graham IA, McQueen-Mason SJ (2010) Molecular insight into lignocellulose digestion by a marine isopod in the absence of gut microbes. Proc Natl Acad USA 107:5345–5350

Labouesse M (2012) Role of the extracellular matrix in epithelial morphogenesis. A view from *C. elegans*. Organogenesis 8:65–70

Linder M, Teeri T (1996) The cellulose-binding domain of the major cellobiohydrolase of *Trichoderma reesei* exhibits true reversibility and a high exchange rate on crystalline cellulose. Proc Natl Acad USA 93:12251–12255

Loomis WF (1972) Role of the surface sheath in the control of morphogenesis in *Dictyostelium discoideum*. Nat New Biol 240:6–9

McGuire V, Alexander S (1996) PsB multiprotein complex of *Dictyostelium discoideum*. Demonstration of cellulose binding activity and order of protein subunit assembly. J Biol Chem 271: 14596–14603

Ogasawara S, Shimada N, Kawata T (2009) Role of an expansin-like molecule in *Dictyostelium* morphogenesis and regulation of its gene expression by the signal transducer and activator of transcription protein Dd-STATa. Develop Growth Differ 51:109–122

Ramalingam R, Ennis HL (1997) Characterization of the *Dictyostelium disicoideum* cellulose-binding protein CelB and regulation of gene expression. J Biol Chem 272:26166–26172

Ramalingam R, Blume JE, Ennis HL (1992) The *Dictyostelium discoideum* spore germination-specific cellulase is organized into functional domains. J Bacteriol 174:7834–7837

Rosness PA (1968) Cellulolytic enzymes during morphogenesis in *Dictyostelium discoideum*. J Bacteriol 96:639–645

Shimada N, Kawata T (2007) Evidence that noncoding RNA *dutA* is a multicopy suppressor of *Dictyostelium* STAT protein, Dd-STATa. Eukaryot Cell 6:1030–1040

Shimada N, Nishio K, Maeda M, Urushihara H, Kawata T (2004a) Extracellular matrix family proteins that are potential targets of Dd-STATa in *Dictyostelium discoideum*. J Plant Res 117:345–353

Shimada N, Maeda M, Urushihara H, Kawata T (2004b) Identification of new modes of Dd-STATa regulation of gene expression in *Dictyostelium* by *in situ* hybridisation. Int J Dev Biol 48:679–682

Shimada N, Maruo T, Maeda M, Urushihara H, Kawata T (2005) Evidence that the *Dictyostelium* STAT protein Dd-STATa plays a role in the differentiation of inner basal disc cells and identification of a promoter element essential for expression in these cells. Differentiation 73:50–60

Teeri TT (1997) Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol 15:160–167

Teeri TT, Koivula A, Linder M, Wohlfarhrt G, Divne C, Jones TA (1998) *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? Biochem Soc Trans 26:173–178

Tuohy MG, Walsh DJ, Murray PG, Claeyssens M, Cuffe MM, Savage AV, Coughlan MP (2002) Kinetic parameters and mode pH action of the cellobiohydrolases produced by *Talaromyces emersonii*. Biochim Biophys Acta 1596:366–380

Wang Y, Slade M, Gooley AA, Atwell BJ, Williams KL (2001) Cellulose-binding modules from extracellular matrix proteins of *Dictyostelium discoideum* stalk and sheath. Eur J Biochem 268: 4334–4345

Zhang J, Zhang Y, Zhong Y, Qu Y, Wang T (2012) Ras GTPases modulate morphogenesis, sporulation and cellulase gene expression in the cellulolytic fungus *Trichoderma reesei*. PLoS One 7:e48786