Versatile derivatives of carbohydrate-binding modules for imaging of complex carbohydrates approaching the molecular level of resolution

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The innate binding specificity of different carbohydrate-binding modules (CBMs) offers a versatile approach for mapping the chemistry and structure of surfaces that contain complex carbohydrates. We have employed the distinct recognition properties of a double His-tagged recombinant CBM tagged with semiconductor quantum dots for direct imaging of crystalline cellulose at the molecular level of resolution, using transmission and scanning transmission electron microscopy. In addition, three different types of CBMs from families 3, 6, and 20 that exhibit different carbohydrate specificities were each fused with either green fluorescent protein (GFP) or red fluorescent protein (RFP) and employed for double-labeling fluorescence microscopy studies of primary cell walls and various mixtures of complex carbohydrate target molecules. CBM probes can be used for characterizing both native complex carbohydrates and engineered biomaterials.

INTRODUCTION

Carbohydrates make up a vital and ubiquitous class of essential nutrients and play important roles in innumerable biological processes, including cell recognition, intercellular signal transduction, cell growth, and cell differentiation (1–3). However, progress toward understanding their structure and function lags behind other biomolecules, such as nucleic acids and proteins. This is primarily due to the high degree of complexity and heterogeneity displayed by carbohydrate-containing materials. Today, methods available for characterizing complex carbohydrates include spectroscopy [mass spectroscopy (MS), Fourier transform infrared, Raman, and nuclear magnetic resonance (NMR) spectroscopies], immunochemical labeling, electron microscopy, and X-ray crystallography. These approaches often have low spatial resolution (micron level or above) or require extensive sample preparation that may alter the molecular structure of the polysaccharide sample.

The plant cell wall is an intricate conglomerate that includes numerous polysaccharides as major components (e.g., cellulose, xyloglucan, xylans, mannans, arabinans, and pectins). The synthesis and incorporation of the various wall polysaccharides into a durable fibrous network has been the subject of numerous recent investigations (4). For this purpose, various fluorescence and electron microscopic labeling methods have proved especially effective. In this context, immunocytochemical (5–8), enzyme- (9–14), and lectin-based (9,15) labeling techniques have been developed to examine the in situ distribution of plant cell wall polysaccharides.

Carbohydrate-binding modules (CBMs) are noncatalytic protein modules found in many carbohydrate-hydrolyzing enzymes, such as the cellulases and hemicellulases. They function as recognition modules that convey the catalytic modules of these enzymes to the target substrate. Several hundred CBMs have been identified to date, and these have been grouped into

45 families using amino acid sequence similarity algorithms (afmb.cnrs-mrs.fr/CAZY/index.html). The structures and ligand specificities of many CBMs have also been determined experimentally (16). Three types of CBMs have now been proposed based on their structure and function; these include type-A, which bind to specific surfaces of insoluble, crystalline cellulose and/or chitin; type-B, which bind to individual glycan chains; and type-C, or small sugar-binding CBMs, which have lectin-like properties and bind to mono-, di-, or trisaccharides.

The fine-tuned polysaccharide recognition capacity of the CBMs has prompted their development as precise molecular probes for mapping the chemistry and structure of carbohydrate-containing materials (16–19). CBMs have thus been used for fluorescence labeling of plant cell walls (20–22) and for ultrastructural labeling of *Valonia* cellulose crystals (23). Notably, following the original submission of this paper, differential recognition of plant cell walls in different tissues by different xylan-specific CBMs was reported (22). However, these published descriptions of CBM probes require complex labeling procedures or the development of monoclonal antibodies. In addition, imaging at the nanometer scale with high resolution has not been described.

In the present study, we describe an approach that simplifies the labeling procedures, requires minimal sample preparation, and heightens substantially the resolution power. Three CBMs from three different families that possess three distinctive polysaccharide specificities were engineered with dual His-tags to facilitate their coordination to highly luminescent quantum dots for correlative imaging using fluorescence and electron microscopy. In addition, the CBMs were each expressed as fusion proteins, with either green fluorescent protein (GFP) or red-fluorescent protein (RFP), to allow direct imaging using fluorescence microscopy at the near-molecular level of resolution. This approach complements newly developed techniques for visualizing the interaction of CBMs with carbohydrate composites and is potentially capable of single molecule detection.

MATERIALS AND METHODS

Sample Preparation

Crystalline cellulose was prepared from the algae, *Valonia ventricosa*, as described previously (24,25). *V. ventricosa* vesicles were harvested and rinsed with distilled water, then were boiled in 2% NaOH for 6 h (changing fresh 2% NaOH every 2 h). The purified vesicles were neutralized with dilute HCl and washed with distilled water. For the preparation of cellulose crystals, purified vesicles were homogenized for 10 min at 10,000 rpm. The resulting suspensions were hydrolyzed by stirring for 5 h in 3.5 M HCl and then neutralized with a few drops of NaOH solution and washed with distilled water. The suspensions of *Valonia* cellulose crystal were stored at 4°C in distilled water containing 0.01% (w/v) NaN_3 . Small aliquots of cellulose crystal stock suspension were diluted in 20 mM Tris buffer, pH 8.0, to make the final concentration of 1 mg/mL used for this study.

Phosphoric-acid-treated cellulose was produced by preparing a cellulose slurry (1 mg/mL) in dilute (2% v/v) phosphoric acid overnight at room temperature, followed by extensive washing with distilled water to neutral pH.

Xyloglucan-bound cellulose was prepared by adding 30 μL xyloglucan solution (10 mg/mL) to a 50- μL cellulose suspension, followed by vortex mixing, centrifugation at 4000 $\times g$ for 1 min, and washing steps. This substrate was finally suspended in 50 μL Tris buffer.

Starch granules were purchased from Sigma-Aldrich (St. Louis, MO, USA). The granules were suspended in distilled water (10 mg/mL), and 0.5 mL was mixed with an equivalent suspension of *Valonia* cellulose (1 mg/mL). The mixture was washed with distilled water three times by centrifugation (4000 $\times g$ for 1 min) and suspended in 1 mL 20 mM Tris buffer.

The glycoprotein, cellobiohydrolase (Cel7A), was purified from the fermentation broth of induced *Trichoderma reesei* (26). Cel7A is a naturally glycosylated protein, which

contains a catalytic and a family-1 CBM that mediates attachment to the cellulose substrate. Thirty microliters Cel7A (1 mg/mL in 50 mM acetate buffer, pH 5.0) were added to 50 μL 1 mg/mL cellulose suspension, gently mixed for 30 min, followed by centrifugation (4000 $\times g$ for 1 min) and three additional washes with acetate buffer. The final pellet was suspended in 20 μL acetate buffer.

Maize (*Zea mays* L.) was grown in the lab greenhouse. Parenchyma tissue was carefully separated from stem pith of a mature plant and immediately pulverized in liquid nitrogen. Small pieces of primary cell walls were washed and finally suspended in distilled water.

Sample Labeling

CBMs were cloned, expressed, and purified (see the supplementary material available online at www.BioTechniques.com). CBM labeling was performed in 1.5-mL Eppendorf® tubes, with 50 μL sample (suspension of *Valonia* cellulose or amorphous cellulose, starch/cellulose mixture, xyloglucan-bound-cellulose, or Cel7A-bound-cellulose, prepared above) and 20 μL desired CBM solution (1 mg/mL) in 20 mM Tris buffer (pH 8.0). The sample was brought to a total slurry volume of 100 μL with Tris buffer, mixed gently for 30 min, and washed three times by centrifugation (4000 $\times g$ for 1 min) using Tris buffer. The final pellet was suspended in 20 μL 20 mM Tris buffer. These samples were used for fluorescence microscopy measurement.

Colloidal Quantum Dots

(CdSe)ZnS core-shell quantum dots (QDs) were prepared as described previously (27). The surface capping groups were exchanged for mercaptopropionic acid (MPA) to confer solubility in 20 mM Tris buffer, pH 6.5.

The CBM-HIS probe was first bound to cellulose crystals as described above. Ten microliters QDs solution were then added to the CBM-HIS-bound cellulose, followed by three

centrifugation (at 4000 $\times g$ for 1 min)/wash steps. The QD-labeled, CBM-bound cellulose was then suspended in 20 μL Tris buffer.

Atomic Force Microscopy

Five microliters *Valonia* cellulose suspension (1 mg/mL in water) were spin-coated on a fresh-cut mica, which was precoated by polyhistidine (1 mg/mL in water). Atomic force microscopy (AFM) [MultiMode™ Scanning Probe Microscope (SPM); Veeco Instruments, Woodbury, NY, USA] was operated in tapping mode underwater using a flow cell.

Fluorescence Microscopy

One microliter freshly prepared sample was placed between two glass coverslips. Direct imaging was performed by fluorescence microscopy using an Olympus IX71 inverted microscope (Olympus America, Center Valley, PA, USA), equipped with a DP70 charge-coupled device (CCD) camera, mercury lamp as excitation source, fluorescence cubes including U-MNU (Ex = 360–370 nm and Em $\geq$ 420), U-MNB (Ex = 470–490 nm, BA = 515 nm, and DM = 500), U-MNG (Ex = 530–550 nm and Em $\geq$ 570 nm), objective lens (150 $\times$ Uapro, oil immersion, and N.A. = 1.45).

Electron Microscopy

Freeze-dried samples were prepared by the wet-film method described in Reference 28. Samples were embedded in a very thin (<1 μm) layer of water, deep-frozen, and slowly freeze-dried while warming from -80°C. Transmission electron microscopy (TEM) images were captured with a CCD camera using DigitalMicrograph™ software (Gatan, Pleasanton, CA, USA) on a Tecnai G2 20 microscope (FEI, Hillsboro, OR, USA) operating at 200 kV. Scanning transmission electron microscopy (STEM) was performed at 40 keV with a cold field-emission gun, a 0.3-nm probe, and two annular detectors subtending 15–40 and 40–200 mRadian

at the specimen (STEM, Brookhaven National Laboratory STEM facility, Upton, NY, USA; www.biology.bnl.gov/stem/stem.html). A specimen changer air lock permitted vacuum transfer of frozen or freeze-dried specimens from a separate ion-pumped freeze dryer or between instruments.

RESULTS AND DISCUSSION

Three different CBMs (Table 1) were selected as examples in this study to demonstrate the variety in their binding specificities for different carbohydrate-containing materials and their application as selective labeling agents for high-resolution microscopy. *CtCBM3* was originally cloned from a *Clostridium thermocellum* CipA protein and has been reported to be a type A surface-binding CBM with high affinity for crystalline cellulose (29,30). *SlCBM20* is from a *Streptomyces lividans* α -amylase (AmlA) and is a putative chain-binding CBM that recognizes starch. *CtCBM6* is cloned from a *C. thermocellum* xylanase (XynZ). Based on experimental studies and X-ray structures (31–33), members from this particular family of CBMs exhibit two potential binding sites with distinct ligand recognition for glucan and xylan oligosaccharides. Indeed, the *CtCBM6*

used for these studies is highly similar in sequence and conserved binding residues to the well-studied Xyn11A CBM6 from the same bacterium. The Xyn11A CBM6 was previously demonstrated to bind primarily to xylan, but also interacts weakly with β -glucan, soluble forms of cellulose, cellohexaose, and xylooligosaccharides (31). The XynZ CBM6 exhibits similar preference for xylan but also binds to xyloglucan and noncrystalline cellulose (Table 1).

Each CBM was engineered in three different recombinant forms with different functional tags: CBM-HIS (containing two 6 $\times$ -His-tags, at the N and C termini), CBM-GFP (peak emission 509 nm), and CBM-RFP (peak emission 585 nm). Six carbohydrate-containing materials, including crystalline and amorphous cellulose, starch, xyloglucan, glycoproteins, and plant primary cell walls, were used in this study for demonstrating the specific binding of the CBMs and their use in correlative labeling studies.

Cellulose is a linear polymer of D-glucose linked by the β -1,4-glucosidic bond. The glucan chains pack side-by-side to form an insoluble macromolecule—the microfibril—by a combined intra- and intermolecular hydrogen bonding network. Subtle changes in hydrogen bonding between adjacent

polymer chains of native cellulose may generate crystalline, subcrystalline, and amorphous celluloses (34). From any given cellulose source, these regions are not particularly discrete, and there is a gradation among these fractions along the length of a single microfibril. *Valonia* cellulose is among the most crystalline of all celluloses. The *Valonia* cellulose 1 α crystals selected for this study were about 20 nm in diameter and usually 10–15 μ m in length (23,35), which was confirmed by AFM using a MultiMode SPM imaged in water (36) (Figure 1, A and B). The length of *Valonia* cellulose crystals varies within a given preparation, and the crystals sometimes associate to form bundles. The cellulose crystals exhibit distinct hydrophobic faces (the 1, 1, 0 or planar face) and two hydrophilic faces (the 1, 0, 0 and 0, 0, 1 or step faces) (24). Amorphous cellulose, in which portions of the cellulose crystal are disrupted, was produced by mixing *Valonia* cellulose with dilute (2% v/v) phosphoric acid. The resultant product is believed to emulate, to some extent, the amorphous regions dispersed in crystalline cellulose (37). The *CtCBM3*-GFP and *CtCBM3*-RFP probes bind strongly to crystalline and phosphoric acid-treated cellulose, and the single cellulose crystal could be visualized using fluorescence microscopy (Figure 1, C–E). Unlike *CtCBM3*, the *CtCBM6* probes demonstrated no detectable binding to crystalline *Valonia* cellulose. Upon correlative binding using the *CtCBM3*-RFP and *CtCBM6*-GFP probes however, we have confirmed that *CtCBM6*-GFP binds to amorphous regions of phosphoric acid-treated cellulose (Figure 2A). To identify the preferred face of the cellulose crystal for *CtCBM3* binding, *CtCBM3*-HIS was first introduced to the sample of *Valonia* cellulose, followed by the addition of semiconductor (CdSe)ZnS QDs. The highly luminescent colloidal QDs (peak emission at 571 nm) were previously treated with mercaptopropionic acid to confer water solubility (27). In this way, we were able to label the bound *CtCBM3*-HIS using the strong coordinate covalent interaction between the His-containing peptides and the ZnS shell of the QDs (38). In this context, the His-tags at the N and C termini of

Figure 1. Micrographs of single *Valonia* cellulose crystals and CBM3 binding. Atomic force micrograph of cellulose crystals (panel A, scale bar = 500 nm) and higher magnification of the hydrophobic face of a single crystal (panel B, scale bar = 50 nm). Fluorescence microscope imaging of cellulose crystals labeled with (C) *CtCBM3*-RFP, (D) *CtCBM3*-GFP, and (E) *CtCBM3*-HIS/QDs, scale bars, 5 μ m. (F) TEM and (G) STEM of cellulose crystal bound with *CtCBM3*-HIS/QDs. Quantum dots (QDs) are visible as dark spots in the TEM image, and white dots in dark-field STEM image. Scale bars, 50 nm. TEM, transmission electron microscopy; STEM, scanning transmission electron microscopy.

the CBH3 molecule are positioned distally to the cellulose-binding surface (29), thus providing oriented labeling of the molecule by the QDs. The resulting QD-labeled sample was imaged using fluorescence microscopy (Figure 1E), TEM (Figure 1F), and STEM (Figure 1G). The latter image clearly shows that *CtCBM3*-HIS binds to only one face of the cellulose crystal. This face is about 2 nm wide and was previously reported by Lehtio et al. to be the planar hydrophobic (1, 1, 0) face (23) as predicted earlier (29). The resolution afforded by this approach is far superior to indirect immunogold-based labeling, which was evaluated statistically in the previous study (23). The extensive labeling along the entire (1, 1, 0) face of the crystal is unequivocal in Figure 1G (compared with figure 4 in Reference 23), requiring no additional mapping of the label, thus verifying the results of the Lehtio et al. and demonstrating the utility of the method described in this communication.

The versatility of employing different CBM-bearing GFP- and RFP-fusion proteins for double-labeling fluorescence-microscopy studies was further demonstrated using mixtures of complex carbohydrate-containing materials. Native starch granules contain a mixture of amylose (α -1,4-D-glucan) and amylopectin (α -1,4-D-glucan backbone with α -1,6-glucose linked branch points approximately every 20 backbone residues). Correlative binding of *CtCBM3*-GFP and *S/CBM20*-RFP to a mixture of starch granules and crystalline cellulose showed distinct and unique recognition for these two CBMs. *CtCBM3*-GFP bound only to the cellulose crystals, whereas *S/CBM20*-RFP bound only to starch (Figure 2B). These results demonstrate that the *CtCBM3* and *S/CBM20* probes specifically label the β - and α -1,4-glycosidic linkages, respectively.

Tamarind xyloglucan (Megazyme, Wicklow, Ireland) used in this study is a branched polysaccharide, which has a molecular weight of 202 kDa, comprising Ara:Gal:Xyl:Glc at a ratio of 3:16:36:45. Soluble xyloglucan was layered on cellulose crystal surfaces by simple mixing, followed by extensive centrifugation and washing steps. Correlative binding of the

Figure 2. Fluorescence micrographs showing distinct recognition of correlative binding of two different carbohydrate-binding molecules (CBMs) to various carbohydrate-containing materials. Micrographs in column 1 show red fluorescent protein (RFP) labeling of the respective samples; those in column 2 show green fluorescent protein (GFP) labeling; and those in column 3 the two combined. (A) Phosphoric acid-treated preparations of *Valonia* cellulose, co-labeled with *CtCBM3*-RFP and *CtCBM6*-GFP. Note the noncrystalline patches in the cellulose fiber associated with the *CtCBM6*-based probe in (panel A, column 2) and (panel A, column 3). (B) Correlative binding of a mixture of *Valonia* cellulose and starch granules, labeled with *S/CBM20*-RFP and *CtCBM3*-GFP. Note the explicit discrimination between the *S/CBM20*-mediated labeling of the starch granule and the *CtCBM3*-induced labeling of the cellulose crystal. (C) Xyloglucan-cellulose mixture, labeled as in panel A. (D) Selective labeling of a cellulose-bound glycoprotein (*Trichoderma reesei* Cel7A), labeled as in panel A. (E) Correlative mapping of the maize parenchyma cell wall. *CtCBM3*-GFP highlights the cellulose macrofibrils versus the *CtCBM6*-RFP that labels the primary cell wall matrices. Scale bars, 5 μ m.

CtCBM3-RFP and *CtCBM6*-GFP probes to this substrate showed that *CtCBM6*-GFP bound only to the adsorbed xyloglucan, whereas the *CtCBM3*-RFP selectively labeled the *Valonia* cellulose crystals in the sample (Figure 2C).

The glycoprotein cellobiohydrolase I (Cel7A) from *T. reesei* is a processive β -1,4-exo-glucanase that contains a glycoside hydrolase family-7 catalytic

module, a family-1 CBM that binds to crystalline cellulose, and a heavily glycosylated linker peptide connecting the two. The CBM1 of *T. reesei* Cel7A has been reported to display high affinity for crystalline cellulose (39). Similar to *CtCBM3*, *TtCBM1* binds to the narrow planar (1, 1, 0) faces of the cellulose crystal (23). In the present study, Cel7A was first bound to *Valonia* cellulose crystals, followed by correlative binding

Table 1. Carbohydrate-Binding Molecules and Their Ligands Used in This Study

CBM	Parent Protein (Swiss-Prot Accession No.)	Organism	Specificity	Reference
CtCBM3	CipA (Q06851)	<i>Clostridium thermocellum</i>	Crystalline cellulose	(29,30)
CtCBM6	XynZ (P10478)	<i>C. thermocellum</i>	Strong interaction with xylan and xylohex- trins; weak interaction with cellobextrins, noncrystalline cellulose, xyloglucan	(41); this study
S/CBM20	AmlA (O86876)	<i>Streptomyces lividans</i>	Starch	This study

CBM, carbohydrate-binding molecule.

of CtCBM3-RFP and CtCBM6-GFP. The character of the label in each case is very different—while CBM3 labels relatively broad regions of the cellulose crystals in a comparatively uniform manner, the interaction of CBM6 with the Cel7A-bound substrate results in a punctate labeling pattern (Figure 2D), possibly indicating single Cel7A molecules or small clusters of molecules. It is currently unclear whether the latter label is due to the interaction of CBM6 with Cel7A oligosaccharides, or whether the observed label reflects enzymatic action on the substrate that could produce or expose noncrystalline regions on the cellulose surface.

The primary cell wall is an example of a natural higher-order complex of carbohydrate-containing materials, which comprises networks of cellulose microfibrils embedded into colloidal matrices containing various branched polysaccharides (e.g., xyloglucans, arabinoxylans, and pectins), lignins, and glycoproteins (4,40). To visualize the cellulose microfibrils that are as small as 5–10 nm in diameter, EM techniques and consequent extraction and dehydration processes are often required. However, these extensive sample preparation steps may alter the native molecular structure of cell walls. We thus employed correlative binding of the CBM3 and CBM6 probes to map cell wall structure. Parenchyma tissue was carefully cut from living *Z. mays* L. stem, pulverized in liquid nitrogen, and small pieces of primary cell walls were then collected, washed, and suspended in distilled water prior to interaction with the CBM probes. Fluorescence microscopy imaging showed distinct binding patterns of CtCBM3-GFP versus CtCBM6-RFP (Figure 2E). Notably, it appears that CtCBM3-GFP binds to the fiber network, whereas CtCBM6-RFP binds to the primary cell wall matrices,

as shown clearly in Figure 2E. As noted in Table 1, the recognition specificity of CtCBM3 is for crystalline cellulose and that of CtCBM6 for soluble polysaccharides or oligosaccharides. Using the CBM probes, we were therefore able to label and image directly plant cell wall cellulose microfibrils at the molecular level of resolution. This approach supports our recently proposed model for the primary cell wall in which the microfibrils, containing primarily crystalline cellulose, appear only on the uppermost surface (the plasma membrane side) of the dissected parenchyma cell wall (36).

In summary, we have successfully demonstrated the use of CBMs as molecular probes for mapping the chemistry and structure of various complex carbohydrate-containing materials, which then permits direct imaging of these macromolecules using fluorescence microscopy, TEM, and STEM correlatively at the molecular level of resolution. Numerous CBMs with finely tuned recognition specificities have been characterized; many are currently available but await characterization, and many more are undoubtedly yet to be discovered. An intriguing possibility yet to be explored is that the CBMs are essentially devices designed by nature that reflect the microheterogeneity believed to exist in most natural glycans. Thus, once fully characterized, these probes may be even more relevant as probes for examining natural carbohydrate-containing surfaces than antibodies elicited against distinct epitopes (8). Many of these CBMs are relatively stable molecules; therefore they are easily cloned and expressed in prokaryotic systems with GFP or other functional tags useful for further chemical and/or biological labeling studies (e.g., His-tag/QD labeling as demonstrated in this study). In addition,

CBMs are small polypeptides, usually containing 30–150 amino acids. The size of most CBM proteins is about 3–4 nm in diameter, which is much more compact than most antibodies. This method thus simplifies the labeling procedure and provides higher spatial resolution than that provided by immunochemical and spectroscopic methods. The new methodology should be useful for applications in biomass characterization, pulp and paper, food and feed, biomedicine, biomaterials, and design of new hybrid bionanostructures (35,36).

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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