Cohesin-Dockerin Interactions within and between Clostridium josui and Clostridium thermocellum

BINDING SELECTIVITY BETWEEN COGNATE DOCKERIN AND COHESIN DOMAINS AND SPECIES SPECIFICITY*

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The cellulose components are assembled into the cellulose complex by the interaction between one of the repeated cohesin domains of a scaffolding protein and the dockerin domain of an enzyme component. We prepared five recombinant cohesin polypeptides of the Clostridium thermocellum scaffolding protein CipA, two dockerin polypeptides of C. thermocellum Xyn11A and Xyn10C, four cohesin polypeptides of Clostridium josui CipA, and two dockerin polypeptides of C. josui Aga27A and Cel8A, and quantitatively and qualitatively examined the cohesin-dockerin interactions within C. thermocellum and C. josui, respectively, and the species specificity of the cohesin-dockerin interactions between these two bacteria. Surface plasmon resonance (SPR) analysis indicated that there was a certain selectivity, with a maximal 34-fold difference in the $K_D$ values, in the cohesin-dockerin interactions within a combination of C. josui, although this was not detected by qualitative analysis. Affinity blotting analysis suggested that there was at least one exception to the species specificity in the cohesin-dockerin interactions, although species specificity was generally conserved among the cohesin and dockerin polypeptides from C. thermocellum and C. josui, i.e. the dockerin polypeptides of C. thermocellum Xyn11A exceptionally bound to the cohesin polypeptides from C. josui CipA. SPR analysis confirmed this exceptional binding. We discuss the relationship between the species specificity of the cohesin-dockerin binding and the conserved amino acid residues in the dockerin domains.

Cellulosomes, which have been identified and characterized in cellulolytic clostridia such as Clostridium thermocellum (1), C. cellulosolyticum (2), C. cellulosorans (3), and C. josui (4), ruminal bacteria (5), and anaerobic fungi (6), are defined as multienzyme complexes having high activity against crystalline cellulose and related plant cell wall polysaccharides such as xylan, mannan, and pectin. A common feature of the clostridial cellulosomes is that they consist of a pair of well conserved 22-residue repeats catalytic components arranged around noncatalytic scaffolding proteins. The scaffolding proteins have been identified as CipA (or scaffoldin) in C. thermocellum (7), CipC in C. cellulosolyticum (8), CbpA in C. cellulosorans (9), and CipA in C. josui (4). These proteins fundamentally consist of repetitive noncatalytic domains of about 140 residues, termed cohesin domains, and a carbohydrate-binding module (CBM) (1), e.g. C. josui CipA is composed of an N-terminal family 3 CBM followed by a hydrophilic domain and six cohesin domains (see Fig. 1A); and C. thermocellum CipA contains a CBM between the second and third of nine repeated cohesin domains and a type II dockerin domain at its C terminus (Fig. 1A). The amino acid sequences of all the cohesin domains from these bacteria are strikingly similar to each other, especially within the same species (Fig. 1, B and C). Each cohesin domain is a subunit-binding domain that interacts with a docking domain, called dockerin, of each catalytic component. Pages et al. (10) found that C. thermocellum Cel48A (formerly CelIs) did not interact with the first cohesin domain of C. cellulosolyticum CipC and C. cellulosolyticum Cel5A (formerly CelA) did not recognize the second cohesin domain of C. thermocellum CipA. This phenomenon was recognized as species specificity of cohesin-dockerin interactions. Dockerin domains consist of a pair of well conserved 22-residue repeats spaced by a linker of 8–18 residues (see Fig. 2A). In all cases, the 11th and 12th residues of both segments are conserved within the same type of dockerin domain, but are different between different types, i.e. the 11th and 12th residues are a serine residue in the first position and either a serine or a threonine in the second for C. thermocellum dockerin domains, and an alanine and a hydrophobic residue (usually leucine or isoleucine) for C. cellulosolyticum dockerin domains, suggesting that they are involved in determining the binding specificity (11) between C. thermocellum and C. cellulosolyticum. This was demonstrated by swapping these residues between the dockerin domains of C. thermocellum Cel48A and C. cellulosolyticum Cel5A: each of the mutated dockerins acquired the ability to bind to its noncognate cohesin domain but did not lose the affinity for its cognate cohesin domain, suggesting that the conserved 11th and 12th residues do indeed play a role in biorecognition, whereas additional residues may also contribute to the specificity of the interaction (10).

On the other hand, a lack of specificity in the cohesin-dock-

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† The abbreviations used are: CBM, carbohydrate-binding module; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; SPR, surface plasmon resonance.
erin interactions within the same species was shown by non-denaturing PAGE, e.g. C. thermocellum C9A (formerly CelD) and Cel5C (formerly CelE) bound to the purified CipA at molar ratios up to 9:1 (12, 13), and C. thermocellum Cel48A bound nonspecifically to four cohesin polypeptides from C. thermocellum CipA, which were separately produced by Escherichia coli recombinants. These observations suggested that the dockerin domains of C. thermocellum Cel9A, Cel5C, and Cel48A could bind to any cohesin domains in the cognate CipA with a similar affinity. On the other hand, the second and third cohesin domains of C. thermocellum CipA interacted with all the catalytic subunits, except for the largest catalytic subunit now known as Cel9D-Cel44A (formerly CelJ) (14), of the cellulosome in an affinity blotting analysis (14), suggesting that generally there is no selectivity in the interactions among cohesin and dockerin domains within the same species, but that there may be at least one exception. Because Cel9D-Cel44A is known as a component of the cellulosome, the cohesin domains, except for the second and third ones, should associate with the dockerin domain of Cel9D-Cel44A, i.e. it is possible that the dockerin domains preferentially bind to particular cohesin domains of CipA. However, there have been no reports containing a quantitative analysis of the interaction between a dockerin domain and different cohesin domains within the same species other than C. cellulolyticum (8) or describing an exception to the species specificity of the cohesin-dockerin interactions between different bacteria.

C. josui is closely related to C. cellulolyticum taxonomically (4), although their optimum growth temperatures are different, i.e. 45 °C for the former but 32–35 °C for the latter. The gene cluster of the former, including cipA and a number of genes responsible for catalytic components, is identical to that of the latter with respect to the gene order, and the corresponding genes are highly homologous with each other between the two bacteria. Furthermore, the dockerin domains of C. josui are specified by the two pairs, including an alanine in the first position and a hydrophobic residue (usually leucine or isoleucine) in the second (Fig. 2A) like the dockerin domains of C. cellulolyticum. Therefore, we predicted that the interactions among cohesins and dockers between C. thermocellum and C. josui would be species-specific. In this study, however, we unexpectedly found that the dockerin domain of C. thermocellum Xyn11A exceptionally interacted with all the tested cohesin domains of C. josui CipA, whereas that of C. thermocellum Xyn10C did not, and furthermore those of C. josui Aga27A and Cel8A (formerly CelB) showed no affinity for any of the cohesin domains of C. thermocellum CipA.

Here, we report the results of qualitative and quantitative analyses of the cohesin-dockerin interactions within C. thermocellum and C. josui, respectively, and describe an exception to the species specificity of the cohesin-dockerin interactions between these two bacteria, i.e. the binding of the dockerin domain of C. thermocellum Xyn11A to the cohesin domains of C. josui CipA.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—The C. thermocellum strain F1 (15) and C. josui strain FERM (16) P-9864 were used for the isolation of genomic DNA and cellulosomal proteins. E. coli strains DH5α, JM109, and BL21(DE3)RIL were obtained from Stratagene (La Jolla, CA). Recombinant E. coli strains were cultured in Luria-Bertani broth supplemented with ampicillin (50 µg/ml) and chloramphenicol (25 µg/ml), or ampicillin (50 µg/ml), and then isopropyl-β-D-thiogalactopyranoside was added to the culture to give a final concentration of 0.8 mM. After an additional incubation of 2 h, the cells were centrifuged (3,000 × g, 10 min), and the supernatants were used for cellulosomal protein purification. Cellulosomal proteins that interacted with the His-tagged probe were detected with a peroxidase-conjugated anti-six-His antibody (Anti-His6-Peroxidase; Novagen) and electro-transfer onto PVDF membranes (Bio-Rad, Hercules, CA) using an electro-blotting apparatus (ATTO, Tokyo, Japan). The membranes were blocked with PBS buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.5) containing 0.5% (w/v) BSA and 0.05% Tween 20, followed by a primary wash with PBS buffer containing 0.1% BSA and 0.05% Tween 20. The membranes were incubated with a His-tagged dockerin or cohesin polypeptide (each 10 µg) in PBS buffer containing 15 mM CaCl2, 0.05% (w/v) Tween 20, and 0.1% BSA, and the cellulosomal proteins that interacted with the His-tagged probe were detected with a peroxidase-conjugated anti-six-His antibody (Anti-His6-Peroxidase; Novagen) and electro-transfer onto PVDF membranes (Bio-Rad, Hercules, CA) using an electro-blotting apparatus (ATTO, Tokyo, Japan). The membranes were blocked with PBS buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.5) containing 0.5% (w/v) BSA and 0.05% Tween 20, followed by a primary wash with PBS buffer containing 0.1% BSA and 0.05% Tween 20. The membranes were incubated with a His-tagged dockerin or cohesin polypeptide (each 10 µg) in PBS buffer containing 15 mM CaCl2, 0.05% (w/v) Tween 20, and 0.1% BSA, and the cellulosomal proteins that interacted with the His-tagged probe were detected with a peroxidase-conjugated anti-six-His antibody (Anti-His6-Peroxidase; Novagen) and electro-transfer onto PVDF membranes (Bio-Rad, Hercules, CA) using an electro-blotting apparatus (ATTO, Tokyo, Japan). The membranes were blocked with PBS buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.5) containing 0.5% (w/v) BSA and 0.05% Tween 20, followed by a primary wash with PBS buffer containing 0.1% BSA and 0.05% Tween 20. The membranes were incubated with a His-tagged dockerin or cohesin polypeptide (each 10 µg) in PBS buffer containing 15 mM CaCl2, 0.05% (w/v) Tween 20, and 0.1% BSA, and the cellulosomal proteins that interacted with the His-tagged probe were detected with a peroxidase-conjugated anti-six-His antibody (Anti-His6-Peroxidase; Novagen) and electro-transfer onto PVDF membranes (Bio-Rad, Hercules, CA) using an electro-blotting apparatus (ATTO, Tokyo, Japan). The membranes were blocked with PBS buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.5) containing 0.5% (w/v) BSA and 0.05% Tween 20, followed by a primary wash with PBS buffer containing 0.1% BSA and 0.05% Tween 20. The membranes were incubated with a His-tagged dockerin or cohesin polypeptide (each 10 µg) in PBS buffer containing 15 mM CaCl2, 0.05% (w/v) Tween 20, and 0.1% BSA, and the cellulosomal proteins that interacted with the His-tagged probe were detected with a peroxidase-conjugated anti-six-His antibody (Anti-His6-Peroxidase; Novagen).
RESULTS

Production and Purification of Various Cohesin and Dock-erin Polypeptides—C. thermocellum CipA consists of nine cohe-sin domains, a family 3 CBM, and a type II dockerin domain (Fig. 1A). Among these cohesin domains, Coh3-Ct, Coh4-Ct, Coh5-Ct, Coh6-Ct, Coh7-Ct and Coh8-Ct are highly homologous with each other, and Coh4-Ct, Coh5-Ct, Coh6-Ct, and Coh8-Ct, in particular, show high sequence identity (higher than 99%) among them (Fig. 1B). In contrast, Coh1-Ct, Coh2-Ct, and Coh9-Ct are relatively less homologous with the other cohesin domains. Therefore, we chose Coh1-Ct, Coh2-Ct, Coh3-Ct, Coh4-Ct, Coh7-Ct, and Coh9-Ct to quantitatively measure the cohesin-dockerin interactions, but unfortunately the Coh9-Ct polypeptide could not be purified. On the other hand, C. josui CipA contains six cohesin domains in addition to a family 3 CBM and a hydrophilic domain (Fig. 1A). Among these cohesin domains, Coh2-Cj, Coh3-Cj, Coh4-Cj, and Coh5-Cj are highly homologous with each other, whereas Coh1-Cj and Coh6-Cj are less similar to the other cohesin domains (Fig. 1C). Therefore, rCoh1-Cj, rCoh2-Cj, rCoh5-Cj, and rCoh6-Cj were produced and used for quantitative analysis of the cohesin-dockerin interactions. The dockerin domains of C. thermocellum Xyn11A and Xyn10C, and C. josui Aga27A and Cel8A have typical amino acid residues in their 11th and 12th positions, i.e., “ST” in the first segment and “ST” in the second segment in Xyn11A, “SS” and “ST” in Xyn10C, “AL” and “AL” in Aga27A, and “AL” and “AI” in Cel8A (Fig. 2A). These dockerin domains were produced with three amino acid residues upstream of the first conserved residue of each dockerin domain. All the recombinant proteins used in this study contained a His tag at their N-termini and were found in the soluble fraction of the recombinant E. coli lysate. We obtained homogeneous purified polypeptide preparations by a single step purification using nickel-nitriilorotic acid resin, by keeping the amount of resin lower than that necessary to bind all the His-tagged polypeptides.

Qualitative Analysis of Interactions between Cohesin and Dockerin by Affinity Blotting—It was interesting to determine whether the five cohesin polypeptides from C. thermocellum CipA and the four cohesin polypeptides from C. josui CipA interacted nonselectively with cellulosomal proteins within the same species or whether a given cohesin polypeptide interacted specifically with a single or a small number of cellulosomal proteins. Furthermore, we were interested to learn whether the cohesin polypeptides from either C. thermocellum or C. josui interacted specifically with their cognate dockerin domains or whether they interacted with noncognate dockerin domains beyond the species specificity.

To address these questions, an affinity blotting analysis was carried out using various cohesin and dockerin polypeptides as probes. Total cellulosomal proteins from C. thermocellum and C. josui were subjected to SDS-PAGE and transferred onto PVDF membranes. The separated bands were then challenged with the cognate and noncognate cohesin polypeptides rCoh1-Cj, rCoh2-Cj, rCoh5-Cj, rCoh6-Cj, rCoh7-Cj, rCoh8-Cj, and rCoh9-Cj. As shown in Fig. 3A (lane 1), all the cohesin polypeptides from C. thermocellum appeared to interact with almost all of the C. thermocellum cellulosomal proteins. It is noteworthy that these cohesin polypeptides did not detect the largest catalytic component Cel9D-Cel44A. Affinity blotting analysis using a recombinant dockerin polypeptide derived from Cel9D-Cel44A failed to detect the interaction between the dockerin polypeptide and C. thermocellum CipA (data not shown). In all cases, the labeling pattern was almost identical. Similar phenomena were observed in the interactions between the cohesin polypeptides and cellulosomal proteins from C. josui (Fig. 3B). These observations suggested a lack of selectivity in the interactions between cohesin and dockerin domains within the same species. When the cohesin-dockerin interactions between C. thermocellum and C. josui were examined, none of the cohe-sin polypeptides from C. josui CipA interacted with any of the C. thermocellum cellulosomal proteins (Fig. 3A), supporting the previous conclusion that there is species specificity in the co-hesin-dockerin interactions between the two different species. Unexpectedly, in contrast, the cohesin polypeptides from C. josui CipA interacted with two proteins in the C. thermocellum cellulosome, i.e., 75- and 50-kDa proteins (Fig. 3B). We predicted that these two proteins were xylanases Xyn11A and Xyn11B, because the molecular sizes of Xyn11A and Xyn11B were consistent with those of the two proteins that interacted with the cohesin polypeptides, and Xyn11A and Xyn11B have quite similar dockerin domains (Fig. 2A), which were expected to have similar characteristics in the cohesin-dockerin interaction.

To prove this prediction, an affinity blotting analysis was performed with rXyn11ADoc as the probe. As shown in Fig. 4, rXyn11ADoc bound to the scaffolding protein CipA of C. josui in addition to C. thermocellum CipA, although rXyn10CDoc, rAga27ADoc, and rCel8ADoc interacted with their cognate scaffolding proteins but not with noncognate scaffolding pro-teins. Furthermore, the antisera raised against Xyn11A (21) interacted with two proteins, Xyn11A and Xyn11B, whose sizes were in good agreement with those of the two proteins that interacted with the cohesin polypeptides from C. josui CipA (Fig. 5). The results obtained by these qualitative analyses clearly indicated that species specificity in the cohesin-dockerin interactions was generally conserved between C. thermocellum and C. josui, but that there was at least one exception taking...
the dockerin domain of *C. thermocellum* Xyn11A as an example. However, because the affinity blotting analysis includes a step of protein denaturation for SDS-PAGE, the results from this method may not necessarily reflect the cohesin-dockerin interactions in the native state. Therefore, these cohesin and dockerin polypeptides were subjected to quantitative analysis of their interactions by SPR.

**Interactions between Cognate Cohesin and Dockerin Polypeptides**—The SPR method was used to study the real-time interactions of cohesin polypeptides from *C. thermocellum* and *C. josui* with the cognate dockerin polypeptides. All the cohesin polypeptides were covalently immobilized to carboxymethyl groups of the dextran on the surface of the CM5 sensor chip via the amine coupling method. The running buffer (23) contained 10 mM CaCl$_2$, because calcium ions are required for the binding of dockerin domains to cohesin domains (26). The surface of the sensor chip could be efficiently regenerated by injection of 10 mM HCl without any apparent damage to the immobilized polypeptides. Tables I and II show the affinity constants for the binding of cohesin and dockerin polypeptides from *C. josui* and *C. thermocellum*, respectively. Sensorgrams from which the affinity constants were calculated for the binding of cohesin...
and dockerin polypeptides from C. josui are shown in Fig. 6. In the case of the cohesin-dockerin interactions of C. josui, rAga27ADoc, which specifically recognized C. josui CipA in the affinity blotting analysis, associated with rCoh1-Cj, rCoh2-Cj, rCoh5-Cj, and rCoh6-Cj with $k_d$ values of 1.8 x $10^5$ to 6.9 x $10^5$ and $k_{on}$ values of $3.3 \times 10^{-3}$ to $3.4 \times 10^{-3}$, leading to $K_D$ values of $1.9 \times 10^{-9}$ to $2.4 \times 10^{-9}$, i.e. it showed the highest

**FIG. 2.** Alignment of the amino acid sequences of dockerin domains and PCR primers used for the amplification of some DNA fragments encoding one of dockerin domains. A, alignment of dockerin domains of Aga27A, Cel8A and Cel48A of C. josui (Cj); and Xyn11A, Xyn11B, Xyn10C, Cel5A, Cel6A, Cel9A, Cel26A-Cel5E (formerly CelH), Cel9D-Cel44A, Cel48A, and Lic16A (formerly LicB) of C. thermocellum (Ct). Asterisks indicate amino acid residue involved in calcium binding. Residues suspected of serving as selectivity determinants are indicated by pound signs (#). Amino acids that have conserved similar chemical properties (I, L, M, V, K, R, S, and T) are presented in white on black or black on gray. B, PCR primers used for the amplification of some DNA fragments encoding one of dockerin domains. Artificial NdeI and SalI sites are underlined.

**FIG. 3.** Recognition of cellulosomal components by recombinant cohesin polypeptides. A, cellulosome preparations from C. thermocellum (lane 1) and C. josui (lane 2) were subjected to SDS-PAGE, the separated proteins were blotted onto PVDF membranes, and probed with three recombinant cohesin polypeptides, rCoh1-Ct, Coh4-Ct, and rCoh7-Ct, from C. thermocellum. The His-tagged probes were detected with anti-His$_6$-peroxidase (Roche Applied Science) and 3,3'-diaminobenzidine tetrahydrochloride on the blots. B, the blotted cellulosomal proteins of C. thermocellum (lane 1) and C. josui (lane 2) were probed with four recombinant cohesin polypeptides, rCoh1-Cj, rCoh2-Cj, rCoh5-Cj, and rCoh6-Cj from C. josui, and the His-tagged probes were detected as described in A.

**FIG. 4.** Recognition of scaffolding proteins of C. thermocellum and C. josui by recombinant dockerin polypeptides. Cellulosomal proteins from C. thermocellum (lane 1) and C. josui (lane 2) were separated by SDS-PAGE, blotted onto PVDF membranes, and probed with four recombinant dockerin polypeptides, rXyn11ADoc, rXyn10C-Doc from C. thermocellum rAga27A-Doc and rCel8A-Doc, from C. josui. The His-tagged probes were detected as described in Fig. 3. An arrow in the upper position indicates C. thermocellum CipA (210 kDa), and another arrow in the lower position indicates C. josui CipA (120 kDa).
affinity for rCoh2-Cj ($K_D = 2.4 \times 10^{-10}$) and the lowest affinity for rCoh1-Cj ($1.9 \times 10^{-9}$), meaning that there is about an 8-fold difference in the affinity between the cohesin domains in CipA and the dockerin domain from A. xylosoxidans. rCelBDoc also showed preference of rCoh2-Cj over rCoh1-Cj, i.e. rCelBDoc associated with rCoh2-Cj with the $K_D$ value of $1.3 \times 10^{-10}$ and rCoh1-Cj with the $K_D$ value of $4.4 \times 10^{-9}$, meaning 34-fold difference in the affinity between the cohesin domains in CipA and the dockerin domain from C. thermocellum.

On the other hand, when SPR analysis was carried out for the binding of rXyn10CDoc and the cohesin polypeptides (rCoh1-Ct, rCoh2-Ct, rCoh3-Ct, rCoh4-Ct, and rCoh7-Ct) from C. thermocellum, $k_{on}$ values for these interactions were obtained from their sensorgrams (data not shown), i.e. $1.8 \times 10^{5}$ to $6.9 \times 10^{5}$, but $k_{off}$ values exceeded the quantitatively measurable range ($10^{-4}$) of the BIAcore system (Table II). The affinities of the interactions between rXyn10CDoc and the cohesin polypeptides appear to be comparable to those obtained for the combinations of cohesin and dockerin polypeptides from C. josui.

**Interactions of rXyn11ADoc and the Cohesin Polypeptides from C. josui**—Because the affinity blotting analysis indicated that rXyn11ADoc bound to C. josui CipA as described above, the affinity constants for the binding of rXyn11ADoc to the C. josui cohesin polypeptides were determined by SPR (Fig. 6). As shown in Table III, rXyn11ADoc interacted with all the cohesin polypeptides with affinities high enough to detect the interactions ($K_D = 6.4 \times 10^{-9}$ to $1.7 \times 10^{-8}$) but with relatively low affinities compared with those observed in combinations of cognate cohesin and dockerin domains. Although the $k_{on}$ values of rXyn11ADoc for the cognate and noncognate cohesin polypeptides were similar to each other, the $k_{off}$ values of rXyn11ADoc for the C. josui cohesin polypeptides were larger than those for the C. thermocellum cohesin polypeptides, resulting in the increased $K_D$ values. On the other hand, SPR analysis confirmed that rXyn10CDoc from C. thermocellum and rAga27ADoc and rCel8ADoc from C. josui did not interact with their noncognate cohesin polypeptides.

**DISCUSSION**

Although some earlier qualitative analyses using affinity blotting (10, 26) and nondenaturing PAGE (11, 17) techniques in C. thermocellum suggested a lack of selectivity in the binding of cohesin domains to dockerin domains (26), this suggestion has not been tested by quantitative analysis until now. Before the quantitative analysis, we carried out an affinity blotting analysis and confirmed at a qualitative level that there was no selectivity in the cohesin-dockerin interactions within a given species, in both C. thermocellum and C. josui (Figs. 3A and B). Simultaneously, however, we found that rXyn11ADoc bound to C. josui CipA and the recombinant cohesin polypeptides (Figs. 3B and 4) beyond the species specificity, which was observed in the cohesin-dockerin interactions between the dockerin polypeptides from C. thermocellum Cel48A and C. cellulolyticum Cel5A, and one cohesin polypeptide from each of C. thermocellum CipA and C. cellulolyticum CipC.

In this study, we used cohesin and dockerin polypeptides

![Image](http://www.jbc.org/)

**TABLE I**

Association and dissociation constants for the binding of dockerin polypeptides to immobilized cohesin polypeptides of C. josui

| Cohesin   | Cj-rCelBDoc | Cj-rAga27ADoc |
|-----------|-------------|---------------|
| $k_{on}$  | $k_{off}$   | $K_D$         | $k_{on}$  | $k_{off}$   | $K_D$         |
| Coh1-Cj   | $1.8 \times 10^5$ | $3.4 \times 10^{-4}$ | $1.9 \times 10^{-9}$ (7.9) | $2.1 \times 10^5$ | $9.0 \times 10^{-4}$ | $4.4 \times 10^{-9}$ (34.3) |
| Coh2-Cj   | $6.9 \times 10^5$ | $1.7 \times 10^{-4}$ | $2.4 \times 10^{-10}$ (1.0) | $1.1 \times 10^6$ | $1.4 \times 10^{-4}$ | $1.3 \times 10^{-10}$ (1.0) |
| Coh5-Cj   | $3.9 \times 10^5$ | $2.5 \times 10^{-4}$ | $6.3 \times 10^{-10}$ (2.6) | $1.4 \times 10^6$ | $4.7 \times 10^{-4}$ | $3.5 \times 10^{-10}$ (2.7) |
| Coh6-Cj   | $2.1 \times 10^5$ | $1.3 \times 10^{-4}$ | $8.5 \times 10^{-10}$ (2.7) | $1.2 \times 10^6$ | $7.4 \times 10^{-4}$ | $6.5 \times 10^{-10}$ (5.0) |

**TABLE II**

Association and dissociation constants for the binding of dockerin polypeptides to immobilized cohesin polypeptides of C. thermocellum

| Cohesin   | Ct-rXyn11ADoc | Ct-rXyn10CDoc |
|-----------|---------------|---------------|
| $k_{on}$  | $k_{off}$     | $K_D$         | $k_{on}$  | $k_{off}$     | $K_D$         |
| Coh1-Ct   | $5.4 \times 10^5$ | $<10^{-4}$ | $<10^{-9}$ | $5.2 \times 10^4$ | $<10^{-4}$ | $<10^{-9}$ |
| Coh2-Ct   | $6.9 \times 10^5$ | $<10^{-4}$ | $<10^{-9}$ | $5.1 \times 10^4$ | $<10^{-4}$ | $<10^{-9}$ |
| Coh3-Ct   | $3.2 \times 10^5$ | $<10^{-4}$ | $<10^{-9}$ | $5.3 \times 10^4$ | $<10^{-4}$ | $<10^{-9}$ |
| Coh4-Ct   | $4.8 \times 10^5$ | $<10^{-4}$ | $<10^{-9}$ | $8.0 \times 10^4$ | $<10^{-4}$ | $<10^{-9}$ |
| Coh7-Ct   | $1.8 \times 10^5$ | $<10^{-4}$ | $<10^{-9}$ | $3.8 \times 10^4$ | $<10^{-4}$ | $<10^{-9}$ |
isolated from other functional domains such as the CBM, hydrophilic domain, and catalytic domain, to examine the cohesin-dockerin interactions. Previously, Fierobe et al. (23) reported that the CBM, hydrophilic domain, and linker sequence adjacent to the first cohesin domain of \textit{C. cellulolyticum} CipC were not critical for binding of the cohesin domain to the dockerin domain of \textit{C. cellulolyticum} Cel5A but that removal of the linker sequence from the dockerin domain reduced the affinity for a cohesin domain by $10^6$-fold compared with the dockerin containing a linker of 11 amino acids. All the dockerin polypeptides used in this study contained a linker sequence of only three amino acids. All the cohesin polypeptides were coupled to a carboxymethyl-dextran layer on the surface of the sensor chip in this study, whereas biotinylated recombinant proteins derived from \textit{C. cellulolyticum} CipA were used for coupling to a streptavidin-dextran layer in the previous experiments (23). Notwithstanding the differences in the experimental conditions for the SPR analysis, the $K_D$ values (1.3 to 4.4) for the interactions between cohesin and dockerin polypeptides from \textit{C. josui} were comparable with the value (2.5) for the interaction between the first cohesin domain of CipC and the dockerin domain of Cel5A from \textit{C. cellulolyticum}, suggesting that the experimental conditions adopted here were suitable for SPR analysis.

The SPR analysis of the cohesin-dockerin interactions revealed the following facts: there is a certain selectivity, with a maximal 34-fold difference in the $K_D$ values, in the cohesin-dockerin interactions within a combination of \textit{C. josui}, although this was not detected by qualitative analysis; there is at least one exception to the species specificity in the cohesin-dockerin interactions.

First, when the $K_D$ values were compared in \textit{C. josui}, a 34-fold difference was observed in the combination of rCel8ADoc and the cognate cohesin polypeptides (Table I). Both the two dockerin polypeptides, rCel8ADoc and rAga27ADoc, from \textit{C. josui} showed the highest affinity for rCoh2-Cj and the lowest affinity for rCoh1-Cj, suggesting that a particular cohesin domain has a higher affinity for the cognate dockerin domains.

| Cohesin |.s| k_{on} | k_{off} | K_D |
|-------|-----------------|-----------------|-----------------|-----------------|
| Coh1-Cj| $8.0 \times 10^{5}$ | $5.5 \times 10^{-3}$ | $6.9 \times 10^{-9}$ | (1.1) |
| Coh2-Cj| $2.5 \times 10^{5}$ | $2.3 \times 10^{-3}$ | $9.2 \times 10^{-9}$ | (1.4) |
| Coh5-Cj| $9.3 \times 10^{5}$ | $5.9 \times 10^{-3}$ | $6.4 \times 10^{-9}$ | (1.0) |
| Coh6-Cj| $2.6 \times 10^{5}$ | $4.4 \times 10^{-3}$ | $1.7 \times 10^{-9}$ | (2.7) |

Numbers in parentheses indicate values relative to the smallest $K_D$ value for each dockerin polypeptide. The smallest $K_D$ value was defined as 1.0.
than the other cohesin domains. Although the extreme difference in the $k_{on}$ and $k_{off}$ values was not observed between rAga27ADoc and rCel8BDoc of C. josui toward respective cohesin polypeptides, the product of relatively small differences in both of the $k_{on}$ and $k_{off}$ values resulted in the 34-fold difference. This phenomenon is not ascribable to artifacts, e.g. artificial change in the tertiary structure of the cohesin domains by chemical immobilization to the sensor chips, because rXyn11ADoc showed a higher affinity for rCoh1-Cj than for rCoh2-Cj and the lowest affinity for rCoh6-Cj (Table III). In the combinations of C. thermocellum, the $k_{on}$ values of rXyn11A toward the respective cohesin polypeptides were 5- to 14-fold larger than those of rXyn10C toward the corresponding cohesin polypeptides, suggesting the possibility that there is the binding selectivity of cohesin and dockerin domains within C. thermocellum. In C. josui CipA, five cohesin domains, Coh1-Cj to Coh6-Cj, are highly homologous with each other, but Coh6-Cj is less homologous with the others (Fig. 1). It is interesting that the less homologous cohesin polypeptide, rCoh6-Cj, did not show the lowest affinity for their cognate dockerin polypeptides.

Second, we found for the first time that rXyn11ADoc as a wild-type dockerin bound to noncognate cohesin domains. In the affinity blotting analysis, we confirmed that full-length wild-type dockerin bound to noncognate cohesin domains. In fact, none of the cohesin polypeptides from C. thermocellum CipA reacted with Cel9D-Cel44A in the cellulose fraction, suggesting that Cel9D-Cel44A is incorporated into the cellulose by interaction between its dockerin domain and cohesin domain(s) other than those tested in this study or a novel scaffolding protein. From the observations for Xyn11A and Cel9D-Cel44A, it is likely that the species specificity of the cohesin-dockerin interaction is caused by subtle differences in their tertiary structures rather than a small number of conserved residues.

In conclusion, this study indicated that there is a certain binding selectivity in the cohesin-dockerin interactions within a given species and species specificity between C. thermocellum and C. josui but with an apparent exception. The precise molecular determinants responsible for the binding selectivity and species specificity of the cohesin-dockerin interactions should be resolved by tertiary structure analysis of cohesin-dockerin complexes from a given species and from different species.

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