Structural basis for the core-mannan biosynthesis of cell wall fungal-type galactomannan in *Aspergillus fumigatus*

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Abstract

Fungal cell walls and their biosynthetic enzymes are potential targets for novel antifungal agents. Recently, two mannosyltransferases, namely core-mannan synthases A (CmsA/Ktr4) and B (CmsB/Ktr7), were found to play roles in the core-mannan biosynthesis of fungal-type galactomannan. CmsA/Ktr4 is an α-(1→2)-mannosyltransferase responsible for α-(1→2)-mannan biosynthesis in fungal-type galactomannan, which covers the cell surface of Aspergillus fumigatus. Strains with disrupted cmsA/ktr4 have been shown to exhibit strongly suppressed hyphal elongation and conidiation alongside reduced virulence in a mouse model of invasive aspergillosis, indicating that CmsA/Ktr4 is a potential novel antifungal candidate. In this study we present the 3-D structures of the soluble catalytic domain of CmsA/Ktr4, as determined by X-ray crystallography at a resolution of 1.95Å, as well as the enzyme and Mn²⁺/GDP complex to 1.90Å resolution. The CmsA/Ktr4 protein not only contains a highly conserved binding pocket for the donor substrate, GDP-mannose, but also has a unique broad cleft structure formed by its N- and C-terminal regions and is expected to recognize the acceptor substrate, a mannan chain. Based on these crystal structures, we also present a 3-D structural model of the enzyme-substrate complex generated using docking and molecular dynamics simulations with α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe as the model structure for the acceptor substrate. This predicted enzyme-substrate complex structure is also supported by findings from single amino acid substitution CmsA/Ktr4 mutants expressed in ΔcmsA/ktr4 A. fumigatus cells. Taken together, these results provide basic information for developing specific α-mannan biosynthesis inhibitors for use as pharmaceuticals and/or pesticides.

Introduction

The number of cases of deep-seated mycoses, including invasive pulmonary aspergillosis, is increasing worldwide and poses a great threat to human health. Such infections are currently treated using antifungal agents such as polyenes and azoles targeting ergosterol, candins targeting β-(1→3)-glucan biosynthesis, and pyrimidines that inhibit nucleic acid synthesis (1-3). However, the emergence of fungi that are resistant to antifungal treatments has become a therapeutic challenge and the development of antifungal drugs with novel mechanisms of action is required (1-3). For instance, it is expected that fungal cell wall components and their biosynthetic enzymes which humans lack could be promising novel antifungal agents (1, 4).

Fungal-type galactomannan (FTGM) is a polysaccharide consisting of α-(1→2)/α- (1→6)-mannosyl and β-(1→5)/β-(1→6)-galactofuranosyl residues located on the outer cell wall of the human pathogenic fungus Aspergillus fumigatus (5-8). FTGM contains a linear α-mannan structure known as core-mannan which consists of 9 or 10 α-(1→2)-mannotetraose units linked by α-(1→6) bonds (5-8). Recently, we and another group identified two mannosyltransferases involved in core-mannan biosynthesis: core-mannan synthase A (CmsA/Ktr4) Afu5g02740/AFUB_051270 and CmsB/Ktr7 Afu5g12160/AFUB_059750 (9, 10). CmsA/Ktr4 is an α-(1→2)-mannosyltransferase responsible for the biosynthesis of FTGM α-(1→2)-mannosyl residues in A. fumigatus (9, 10); indeed, it has been shown that strains with cmsA/ktr4 disruption lack FTGM structures (9). Moreover, recombinant CmsA/Ktr4 can transfer the mannopyranoside of guanosine diphosphate-α-D-mannose (GDP-Man) as the donor substrate to the C-2 position of the non-reducing end of α-mannose in both α-(1→2)-mannobiose and α-(1→6)-mannobiose as acceptor substrates, showing higher specific activity toward α-(1→6)-mannobiose than α-(1→2)-mannobiose (9). Furthermore, strains with cmsA/ktr4 disruption display strongly suppressed hyphal elongation and a reduced ability to form conidia, as well as decreased virulence in a mouse model of invasive aspergillosis, indicating that CmsA/Ktr4 could be a potential target for novel antifungal agents (9, 10).

CmsA/Ktr4 is a member of the Kre2/Mnt1 family of glycosyltransferases (GTs) (9, 10), classified as GT family 15 in the CAZy database (11), which catalyzes the transfer of mannose from the donor substrate GDP-Man to α-mannosides on acceptor substrates, requiring divalent metal ions. Orthologs
of cmsA are also present in many fungi that cause plant and animal diseases, including the animal pathogens *Trichophyton rubrum*, *Paracoccidioides brasiliensis*, and *Ajellomyces capsulatus*, and the phytopathogens *Magnaporthe grisea*, *Fusarium graminearum*, and *Botrytis cinerea* (9). Although there have only been a few studies that have reported on phenotypic changes caused by disruption of the cmsA orthologs, this disruption in *Neurospora crassa* showed caused significant growth inhibition, suggesting a general function for CmsA in filamentous fungi (12). Alternatively, in *Beauveria bassiana*, the disruption of the cmsA ortholog ktr4 did not cause significant growth inhibition (13). Hence, core-mannan synthesized by the CmsA ortholog may not contribute significantly to the growth of *B. bassiana.*

Although there are currently no reports on the 3-D structural analysis of these fungal-type CmsA orthologous proteins participating in FTGM biosynthesis, previous studies have revealed the 3-D structures of two α-(1→2)-mannosyltransferases of the Kre2/Mnt1 family, Kre2/Mnt1 and Ktr4, from *Saccharomyces cerevisiae* (ScKre2/ScMnt1 and ScKtr4, respectively) (14, 15). *ScKRE2/ScMNT1* was the first gene reported to encode an α-(1→2)-mannosyltransferase involved in O-mannosylation (16), with further studies revealing that *ScKRE2/ScMNT1* encodes a medial Golgi α-(1→2)-mannosyltransferase required for addition of the second and third mannose residues to O-mannose-type glycans and also participates in the biosynthesis of N-glycan outer chains in *S. cerevisiae* (17, 18). Moreover, although the physiological role of ScKtr4 remains unclear, it has been reported to rescue N-glycosylation and O-mannosylation defects in *Candida albicans* null mutants lacking members of the MNT1/KRE2 gene family (19).

In this study, we present the crystal structure of the soluble catalytic domain of *A. fumigatus* CmsA/Ktr4 to a resolution of 1.95 Å and its complex with Mn<sup>2+</sup>/GDP to 1.90 Å resolution. In addition to a highly conserved binding pocket that recognizes the GDP-Man donor substrate, we identified a broad cleft structure that recognizes the mannan chain acceptor substrate. Based on this crystal structure, we modeled the complex structure using molecular docking and molecular dynamics simulations with α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe as the model acceptor substrate. This is the first report to present the 3-D structure and enzyme-substrate complex model of the enzyme responsible for FTGM biosynthesis. In addition, we determined the functionally relevant amino acids in CmsA using single amino acid substitution mutants. Together, these results provide basic information for developing specific α-mannan biosynthesis inhibitors as pharmaceuticals and/or pesticides.

**Results**

**Structural determination of apo-form and Mn<sup>2+</sup>/GDP CmsA complexes**

CmsA/Ktr4 protein crystals suitable for X-ray data collection appeared after 1 week at 4°C. The apo-form crystal underwent X-ray diffraction to 1.95 Å resolution, indexed in the space group C222<sub>1</sub> with the unit cell dimensions a = 154.7 Å, b = 274.3 Å, and c = 186.0 Å. Molecular replacement with the polyalanine structural model generated using ScKtr4 (PDB code 5A07) as a search model successfully placed six monomers with a Matthews' coefficient of 3.64 and a solvent content of 66%. The final R and R<sub>free</sub> values were 0.166 and 0.187, respectively, and the root mean square deviation (RMSD) of bond length and angle from ideal values were 0.008 Å and 1.31°, respectively (Table S2).

CmsA/Ktr4 crystals with the same space group and the unit cell dimensions a = 154.5 Å, b = 273.0 Å, and c = 186.3 Å were obtained from a solution containing Mn<sup>2+</sup> as the metal ion and GDP-Man as the donor substrate, with X-ray diffraction data measured to a resolution of 1.9 Å. The structure of the complex was determined using the refined apo structure as the search model, yielding final R and R<sub>free</sub> values of 0.163 and 0.181 and the RMSD from ideal values for bond length and angle of 0.008 Å and 1.34°, respectively (Table S2). In the complex, the electron densities assignable to Mn<sup>2+</sup> and the GDP moiety of GDP-Man were visible around Arg87, Glu205, and His354; therefore, the molecular models of Mn<sup>2+</sup> and GDP were positioned and refined.
Overall CmsA structure

The crystal structure of CmsA/Ktr4 contained six monomers in the asymmetric unit. Both the apo-form and the Mn\(^{2+}\)/GDP complex structures were well-defined in the electron density map and the proteins were continuously modeled, except for residues 35 to 39 in chains A, B, and D. These residues are visible in chains C, E, and F and correspond to a loop structure connecting β1 and β2 sheets. The RMSD for the Cα atom positions between protein molecule pairs in the asymmetric units of the apo-form or complex structures were 0.09–0.21 Å.

The monomeric structure of CmsA/Ktr4 (Figure 1A) was comprised of a mixed α/β-fold containing fifteen α-helices, eight 3α-helices, and fourteen β sheets, as determined by DSSP (Figure 1A and Supporting Figure 1A) (20). All monomer molecules in the asymmetric unit of the crystals had identical structures except for two loop regions that consisted of residues 35-39 and 169-174, respectively (Supporting Figure 1B). The core of the structure contained a seven-stranded β-sheet comprised of β-strands (β5, β4, β3, β6, β11, β8, and β12) that were all parallel, except for β11. This β-sheet was surrounded by α-helices and flanked by two small β-sheets, one consisting of two short antiparallel strands (β9 and β10) and one of three short antiparallel strands (β7, β13, and β14). Three disulfide bonds formed between residues Cys212-Cys366, Cys288-Cys386, and Cys360-Cys369.

The monomeric CmsA/Ktr4 structure was compared to those of known proteins in PDB using DALI (http://ekhidna2.biocenter.helsinki.fi/dali/). The proteins with the highest Z score were ScKtr4 (Z score = 52.1 and RMSD = 1.5 Å for the alignment of 353 residues with 41% sequence identity, and PDB code 5A08, chain A (14)) and ScKre2/ScMnt1 (Z score = 41.8 and RMSD = 1.4 Å for the alignment of 304 residues with 45% sequence identity, and PDB code 1S4N, chain A (15)). The amino acid alignments based on the 3-D structures of CmsA/Ktr4 with these proteins are shown in Figure 2. Other proteins in PDB showed distinctly lower structural similarity with CmsA/Ktr4. Moreover, the core structure of CmsA/Ktr4 is similar to those of ScKre2/ScMnt1 and ScKtr4 (Supporting Figure 2A). Specifically, the core seven-stranded β sheet surrounding alpha helices, and two small β-sheets, consisting of two short antiparallel strands and three short antiparallel strands, were particularly well conserved, with the greatest differences found at the N- and C-termini of the proteins (Figure 2 and Supporting Figure 2A). ScKtr4 has two long α-helices at its N-terminus preceding the terminal β-strand, β1, whereas the same region of ScKre2/ScMnt1 contains only five residues forming a loop structure, meanwhile that of CmsA/Ktr4 contains a β sheet composed of two antiparallel β strands. Therefore, the secondary structural terminal β strand element (β1) in ScKtr4 and ScKre2/ScMnt1 corresponds to β3 in CmsA/Ktr4. Further, the additional small β-sheet present in the N-terminal region of CmsA/Ktr4 was close to the C-terminal helices (η8 and η15) that contact its well-conserved core region. Many insertions and deletions were also observed in the primary structure of their terminal regions (Figure 2), indicating a certain level of structural diversity within these regions. Indeed, these terminal regions were found to form a characteristic cleft structure adjacent to its GDP-Man recognition site in CmsA/Ktr4 structure (see below).

CmsA active center

In the complex, the electron densities assignable to the Man moiety of GDP-Man were not visible like the crystal structures of ScKre2/ScMnt1 and ScKtr4 (14, 15). The positions of Mn\(^{2+}\) and GDP were clearly observed using the electron density map (Figure 1B) and were bound to the cleft of the active site located on the surface of the protein (Figure 1A). Furthermore, the complex structure revealed only minor structural changes in CmsA/Ktr4 upon ligand binding, as its monomeric structure in the presence or absence of Mn\(^{2+}\) and GDP could be superimposed with a maximum RMSD of 0.21 Å over 372 Cα atoms. Mn\(^{2+}\) was coordinated to six ligands with a hexahedral geometry, namely Glu205 OE1, His354 NE2, two phosphate oxygens in GDP (O1α and O2β), and two water molecules (Figure 1C). Several GT families possess a DXD motif (X, any amino acid) which coordinates the divalent cation at the active center. CmsA/Ktr4 did not contain the archetypal DXD motif; however, it has been reported that the
EPK sequence (residues 205–207) serves the same purpose, as do EPD and EPN in ScKre2/ScMnt1 and ScKtr4, respectively (14, 15). As also observed in ScKre2/ScMnt1 and ScKtr4, the CmsA/Ktr4 Glu205 residue bound to Mn$^{2+}$ at the active center but Lys207 had no direct interaction with the metal atom.

The GDP phosphate group also interacted with protein residues as well as coordinating Mn$^{2+}$ (Figure 1C), with its O3β and O1β being hydrogen bonded to Tyr172 OH and Tyr178 OH, respectively. The GDP ribose group also formed two hydrogen bonds, wherein its O2' and O3' atoms interacted with the main chain oxygen of Leu85 while forming van der Waals interactions with Leu85, Met181, and Pro206 in CmsA/Ktr4. The guanine group of GDP formed hydrogen bonds with Asp118 OD1 and Asn117 OD1 via its nitrogen atom and many van der Waals interactions with the side chains of Leu85, Arg87, Trp147, and Met181. The amino acid residues and their interactions with GDP were similar to those observed for ScKre2/ScMnt1 and ScKtr4 (14, 15), suggesting that the molecular mechanisms underlying GDP recognition are highly conserved in this protein family.

**Acceptor substrate-binding site of CmsA**

As previously mentioned, many insertions and deletions were detected in their N- and C-terminal regions when comparing the primary structures of CmsA/Ktr4 with ScKre2/ScMnt1 and ScKtr4 structures (Figure 2). Specifically, the terminal regions of CmsA/Ktr4 were well-folded and oriented near to the GDP-Man recognition site in its well-conserved core region. Further, the molecular surface of CmsA/Ktr4 confirmed the formation of a large, wide cleft adjacent to the GDP-Man recognition site (Figure 3A). Figure 3B illustrates the arrangement of amino acid residues forming the cleft, including Pro356, Tyr357, Phe380, Leu381, and Glu384 in the C-terminal region. A similar cleft was found in ScKtr4, while ScKre2/ScMnt1 appears to have a smaller pocket in the same region (Supporting Figures 2B and 2C). ScKre2/ScMnt1, ScKtr4, and fugal-type CmsA/Ktr4 are all α-(1→2)-mannosyltransferases that catalyze the transfer of the α-Man of GDP-Man to their respective acceptor substrates to synthesize α-Man-(1→2)-α-Man; however, each is believed to have a different physiological acceptor substrate, although that of ScKtr4 remains unclear (14, 19). The structural differences observed in the clefts near the GDP-Man recognition site likely correspond to differences between acceptor substrates. ScKre2/ScMnt1 catalyzes the extension of the second and third mannose residues to O-mannose-type and N-glycans (16, 22); while A. fumigatus CmsA/Ktr4 transfers a mannose residue to facilitate the elongation of FTGM core-mannan composed of α-(1→2)-α-(1→6)-mannosyl and β-(1→5)/β-(1→6)-galactofuranosyl residues. Moreover, in vitro, ScKre2/ScMnt1 was reported to recognize only α-(1→2)- and not α-(1→6)-mannobiose, for mannose transfer (23); meanwhile, A. fumigatus CmsA/Ktr4 recognized both (9, 10) with 31-fold higher specific activity toward α-(1→6)-mannobiose (9). The large cleft observed close to the GDP-Man recognition site of CmsA/Ktr4 would be suitable for binding its physiological acceptor substrate, the terminal region of the growing FTGM.

Furthermore, orthologs of *cmsA* are found in many fungi including animal pathogens and phytopathogens. Specific orthologs determined to be involved in the biosynthesis of the FTGM core-mannan as in *Neurospora crassa* (12) and *Beauveria bassiana* (13), exhibited high sequence similarities with *A. fumigatus* CmsA/Ktr4 in their terminal regions (Figure 2). Although, there have been no reports describing their 3-D structures, the terminal regions of the fugal-type CmsA orthologous proteins may be important for their binding of acceptor substrates for the elongation of FTGM.

**In silico analyses of CmsA binding its acceptor substrate**

To further investigate the binding of the acceptor substrate to *A. fumigatus* CmsA/Ktr4, we performed *in silico* analyses using α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe as a model compound for the terminal region of the growing mannan chain. The structure of CmsA in complex with the donor substrate GDP-Man, and acceptor substrate α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe, was generated using docking and MD simulations in AutoDock and GROMACS. Unrestrained 100 ns
Molecular dynamics (MD) simulations were performed to accommodate the substrates in the active site and test the stability of the prepared complex. We also applied the same simulation procedures to the system of ScKre2/ScMnt1 and the artificial acceptor substrate, methyl-α-Man, and obtained the results consistent with the theoretical enzyme-substrate complex model (27) and the crystal structure (15) (Supporting Figure 3), indicating that the simulations in this study have sufficient quality and robustness.

The RMSD of the main protein chain, Mn\(^{2+}\), and substrates between each frame and the initial structure are shown in Figure 4A. During the first 0.4 ns, the RMSD of α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe increased rapidly before fluctuating around 3.0 Å, suggesting that its position and structure deviated rapidly, however, only slightly from the initial docking structure forming a more stable structure. Conversely, the RMSDs of the main chain atoms, Mn\(^{2+}\) ion, and GDP-Man were all lower than 2.0 Å. In anticipation of sufficient equilibration, further analyses were performed using the last 85 ns of the MD simulation. To examine the flexibility of each residue in the polypeptide chain, the root mean square fluctuations (RMSF) of the main chain atoms were calculated over the final 85 ns. Results show that the RMSF was below 1.6 Å, save for in the terminal region, indicating that the helices and β-sheets were stable, as expected, with peaks in the plot indicating that the loop regions exhibited slight flexibility (Figure 4B). These results suggest that the system is adequately equilibrated and that the modeled complex exhibits sufficient stability.

Next, we analyzed the fluctuations in distance between protein residues in the complex and α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe for the final 85 ns of the MD simulations to predict hydrogen bonding. The distances between the heavy atoms of the Gln171, Asp237, Trp291, Arg324, and Tyr357 side chains and α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe were nearly all below 3.5 Å, suggesting that the interactions persist during MD simulation (Supporting Figure 4). Overall, there were six putative hydrogen bonds identified: four between the reacting terminal α-Man and Asp237, Trp291, and Arg324; as well as two between the middle α-Man moiety and Gln171 and Tyr357 (Table 1). The four hydrogen bonds to the terminal α-Man residue were consistent with previous studies on ScKre2/ScMnt1, which reported that the corresponding residues in ScKre2/ScMnt1 [Glu279 (Asp237 in CmsA/Ktr4), Trp325, and Arg358] directly participate in binding of the acceptor substrate, methyl-α-Man, with mutation of Glu279 to Gln leading to complete loss of activity (15).

Next, cluster analysis based on the RMSD of the main protein chain atoms was performed to obtain their representative conformations and population from MD simulations, using MD trajectories of the final 85 ns and a RMSD cut-off of 1.0 Å. The analysis yielded 35 clusters, with representative snapshots taken of each (Figure 5A). Figure 5B and 5C show the representative structure of the most populated cluster (87% population), with α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe bound to the wide cleft close to the GDP-Man recognition site via the hydrogen bond described above, and van der Waals interactions with Ala378, Phe380, and Leu381 in the CmsA/Ktr4 C-terminal region.

GTs are classified as "inverting" or "retaining", containing α-(1→2)-mannosyltransferases, depending on whether the anomeric configuration of the C1 carbon of the monosaccharide moiety transferred from the donor to the acceptor is inverted or retained. Although the general reaction mechanisms of the retaining GTs have been debated and are believed to vary between families (24-26), the docking features and MD structures of the CmsA/Ktr4 are consistent with the proposed catalytic mechanisms. That is, in the complex, the reactive C2-hydroxyl group (2-OH) of the terminal α-Man residue in α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe was positioned closest to the GDP-Man reaction site. The mean distance between the 2-OH oxygen of the terminal α-Man and the dissociable phosphate oxygen bridging the GDP and mannose moieties of GDP-Man was 3.59 ± 0.30 Å during the MD simulation, indicating a direct interaction between the donor and acceptor substrates. The Tyr178 hydroxyl group also interacted with the 2-OH of the terminal α-Man and the dissociable phosphate oxygen with mean distances of 3.66 ± 0.42 Å and 4.62 ± 0.55 Å, respectively. Tyr178 corresponds to Tyr220 in ScKre2/ScMnt1, whose hydroxyl group has been demonstrated to be...
essential for its possible catalytic mechanisms consisting of either a double-displacement mechanism with the hydroxyl group as the potential nucleophile, or an $S_{	ext{N}2}$-like mechanism involved in positioning the donor and acceptor substrates (13). The hybrid quantum mechanics/molecular mechanics study on ScKre2/ScMnt1 strongly supports the hypothesis of an $S_{	ext{N}2}$-like mechanism (27). The docking features and MD structures of the CmsA/Ktr4 are reasonable for the enzyme-substrate complex.

**Essential amino acid residues in CmsA/Ktr4**

Next, we determined whether single amino acid substitutions would affect the enzymatic activity of CmsA/Ktr4 by evaluating the importance of 13 amino acids conserved between ScKre2/ScMnt1, ScKtr4, and CmsA/Ktr4: Glu102, Tyr172, Ser177, Tyr178, Met181, Cys182, Arg203, Glu205, His289, Trp291, Arg324, Ala328, and His354 (Figure 2 and 6A). These residues were all substituted to alanine, save for Ala328 which was mutated to phenylalanine, after which the wild-type CmsA/Ktr4 and 13 CmsA/Ktr4 mutants were expressed in $\Delta$cmsA/ktr4 A. fumigatus (Figure 6B). Although Glu102 is conserved in ScKre2/ScMnt1, ScKtr4, and CmsA/Ktr4, it is located far from the active center and thus, expected to exert minimal effects via mutation. Alternatively, the mycelial growth of the $\Delta$cmsA/ktr4 cells expressing the M181A, C182A, H289A, W291A, and E102A mutants recovered completely compared to the wild-type strain. Although Met181 and Cys182 are located at the GDP-Man binding site, while His289 and Trp291 are expected to interact with the acceptor substrate, single mutations of these residues had negligible effect on the function of CmsA.

Conversely, the strains expressing Y178A, E205A, R324A, and A328F CmsA/Ktr4 mutants displayed similar colony growth to $\Delta$cmsA/ktr4 cells, indicating that these amino acids are essential for mannosyltransferase activity. Glu205 in CmsA/Ktr4 corresponds to Glu247 in ScKre2/ScMnt1, which is an essential amino acid that coordinates its manganese ion and activity (15). As mentioned above, Tyr220 in ScKre2/ScMnt1, which corresponds to Tyr178 of CmsA/Ktr4, are believed to have an essential role in positioning the donor and acceptor sugars for catalysis (15, 27). In this study, the results of the Y178A and E205A CmsA/Ktr4 mutants support the findings of these previous reports. Furthermore, the docking and MD simulations suggest that Arg324 in CmsA/Ktr4 strongly recognizes 3-OH and 4-OH in the terminal mannose of the acceptor substrate (Figure 5B). The markedly reduced R324A mutant activity was consistent with the enzyme-substrate complex structure suggested by the simulations. Moreover, the Ala328 residue of CmsA/Ktr4 was located at the bottom of the binding pocket for the manno moity of GDP-Man; thus, introducing a bulky Phe residue at this position may disrupt the binding site structure and subsequent catalytic activity.

The mycelial growth of the strains expressing Y172A, S177A, R203A, and H354A CmsA/Ktr4 mutants was moderately restored compared to the $\Delta$cmsA/ktr4 strain. Especially, the Mn$^{2+}$ ion was coordinated by His354 NE2, as well as the oxygen atoms of two phosphates and Glu205. The E205A mutant had no activity, but the same situation did not occur in His354A. This would indicate that the contribution of His354 NE2 to the binding of Mn$^{2+}$ atom is lower than Glu205 OE1 and the Mn$^{2+}$ affinity of H354A mutant was not completely lost, which is not inconsistent with the Hard and Soft Acid and Base (HSAB) principle. It was suggested that these amino acids are not essential but are important for the enzymatic activity of CmsA/Ktr4.

**Discussion**

In this study, we clarified the 3-D structure of CmsA/Ktr4, an enzyme essential for the core-mannan biosynthesis of FTGM and mycelial growth of A. fumigatus. Although CmsA/Ktr4 has a similar core fold to those of $\alpha$-(1→2)-mannosyltransferases, ScKre2/ScMnt1 and ScKtr4, function in the biosynthesis of N- and O-mannose-type glycans, CmsA/Ktr4 contains a unique wide cleft structure formed by its N- and C-terminal regions. A previous study has reported that ScKtr4 also has a large pocket that may recognize its large acceptor substrate, however, shows higher activity values for methyl-$\alpha$-mannose than for $\alpha$-(1→2)-mannobiose (14). CmsA/Ktr4 can recognize $\alpha$-(1→2)- and $\alpha$-(1→6)-mannobiose as acceptor substrate with 31-fold higher specific activity toward $\alpha$-(1→6)-mannobiose (9). Although the
physiological acceptor substrate of ScKtr4 is unknown and the direct comparison is difficult, the difference of fungal CmsA/Ktr4 and ScKtr4 in recognition of their acceptor substrates would be due to their cleft structures formed by their terminal regions. Previous reports also demonstrated that Ktr1, Ktr3, and Kre2/Mnt1 of the Kre2/Mnt1 family have overlapping roles and collectively add most of the second and the third α(1→2)-mannosyl residues on O-mannose-type glycans in S. cerevisiae (18, 28). However, their enzymatic activities have been significantly varied depending on the acceptor substrate used in the in vitro assays, e.g., the activity of Ktr1 was at the background level by using methyl-α-mannose as an acceptor substrate (18). Although these proteins show sufficient similarity with over 50% identities at the amino acid level, more mutations are observed at their N- and C-terminal regions than their core-fold region. The recognition of acceptor substrate of the Kre2/Mnt1 family may depend on the pocket structure formed by the terminal regions in general.

Unfortunately, we could not determine the structure of the enzyme-substrate complex for CmsA/Ktr4 with both donor and acceptor substrates via X-ray crystallography, presumably since the enzymatic reaction took place in the crystals like previous reports on ScKre2/ScMnt1 and ScKtr4 (14, 15). Hence, to further investigate the binding of the acceptor substrate to A. fumigatus CmsA/Ktr4, we performed in silico analyses. GTs generally have the Michaelis constants of the micromolar order for the donor substrate but show the values of the millimolar order for the acceptor substrate (15, 29). In many cases, their low affinities for their acceptor substrate would be explained by results of the artificial acceptor substrates used. In the docking and MD simulations, the physiological compound of GDP-Man was used for donor substrates, while an α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe molecule, as a model compound for the terminal region of the growing mannan chain, was used for the acceptor. The fluctuation of α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe was high (Figure 4 and 5), which would not be inconsistent with CmsA/Ktr4 showing low affinity for the acceptor substrate as well as the general GTs. The docking features and MD structures of the CmsA/Ktr4 are reasonable for the enzyme-substrate complex and consistent with the catalytic activity of CmsA/Ktr4 in vitro, indicating that CmsA/Ktr4 recognizes its physiological acceptor substrate via a specific mechanism involving the cleft structure formed by its terminal regions.

As mentioned above, the residues in the C-terminal region are highly diverse among the Kre2/Mnt1 family, while the fungal-type CmsA orthologous proteins demonstrate significant similarity (Figure 2). Hence, the similar characteristic mechanism for recognizing receptor substrates through their C-terminal region of CmsA orthologs may be partially conserved in filamentous fungi. Furthermore, specific catalytically inactive mutants of CmsA/Ktr4 could not recover the mycelial growth ability of the ΔcmsA/ktr4 cells, indicating that the inhibitors of CmsA/Ktr4 may prove effective as antifungal agents. This report provides the structural basis for the in-silico screening and the rational designs of A. fumigatus-specific inhibitors capable of competing for the acceptor substrate, having the potential to suppress A. fumigatus core-mannan biosynthesis and growth.

**Experimental Procedures**

**Microorganisms and growth conditions**

The *Aspergillus fumigatus* strain A1151 was obtained from the Fungal Genetics Stock Center (Kansas City, MO, USA) and the ΔcmsA strain was constructed previously (9). Both strains were grown on MM composed of 1% glucose, 0.6% NaNO₃, 0.052% KCl, 0.052% MgSO₄·7H₂O, and 0.152% KH₂PO₄ with Hunter’s trace elements (in w/v; pH 6.5). VinoTaste Pro (Novozymes, Bagsværd, Denmark) was used to prepare A. fumigatus protoplasts. *A. fumigatus* was transformed using standard procedures. Plasmids were amplified in *Escherichia coli* DH5α and proteins expressed in *E. coli* strain Rosetta-gami B (DE3; Merck Millipore, Darmstadt, Germany).

**Construction of CmsA/Ktr4 expression vectors and single amino acid CmsA/Ktr4 substitution mutants**

The *cmsB/ktr4* gene containing promoter and terminator regions was amplified by PCR and cloned into pHSG396. Briefly, the whole *cmsB/ktr4* gene was amplified by PCR using
genomic DNA from *A. fumigatus* A1151 as a template for the pHSG396-AfCmsA-F and pHSG396-AfCmsA-R primers (Table S1). The amplified fragment was inserted into the Bam HI site of pHSG396 using an in-fusion HD cloning kit (TAKARA, Japan) to yield pHSG396-AfCmsA. The following single point mutations were introduced into the putative CmsA catalytic domain via site-directed mutagenesis: E102A, Y172A, S177A, Y178A, M181A, C182A, R203A, E205A, H289A, W291A, R324A, A328F, and H354A. PCR was performed with pHSG396-AfCmsA as a template and the following primer pairs: AfCmsA-E102A-F and AfCmsA-E102A-R, AfCmsA-Y172A-F and AfCmsA-Y172A-R, AfCmsA-S177A-F and AfCmsA-S177A-R, AfCmsA-Y178A-F and AfCmsA-Y178A-R, AfCmsA-M181A-F and AfCmsA-M181A-R, AfCmsA-C182A-F and AfCmsA-C182A-R, AfCmsA-R203A-F and AfCmsA-R203A-R, AfCmsA-E205A-F and AfCmsA-E205A-R, AfCmsA-H289A-F and AfCmsA-H289A-R, AfCmsA-W291A-F and AfCmsA-W291A-R, AfCmsA-R324A-F and AfCmsA-R324A-R, AfCmsA-A328F-F and AfCmsA-A328F-R, AfCmsA-H354A-F and AfCmsA-H354A-R. The amplified DNA fragments were purified using a Fast Gene Gel/PCR Extraction Kit (NIPPON GENE, Japan) and circulated using an in-fusion HD cloning kit according to the manufacturers’ protocols. The following plasmids were constructed as described above: pHSG396-AfCmsA(E102A), pHSG396-AfCmsA(Y172A), pHSG396-AfCmsA(Y178A), pHSG396-AfCmsA(M181A), pHSG396-AfCmsA(C182A), pHSG396-AfCmsA(R203A), pHSG396-AfCmsA(E205A), pHSG396-AfCmsA(W291A), pHSG396-AfCmsA(A328F), and pHSG396-AfCmsA(H354A). All PCR reactions were performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, USA). DNA sequences were confirmed by sequence analysis using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster, CA, USA).

**CmsA expression in *E. coli* and recombinant protein purification**

The catalytic domain of *A. fumigatus* CmsA/Ktr4 was expressed in an *Escherichia coli* system and purified by Ni-affinity chromatography as described previously (30). Protein concentration was determined using a Qubit Protein Assay Kit (Thermo Fisher Scientific, USA) while purity and molecular weight were analyzed by SDS-PAGE. CmsA/Ktr4 protein solution was collected and further purified using a HiLoad 16/600 Superdex 75 pg gel filtration column (GE Healthcare, USA) equilibrated with Buffer A (50 mM HEPES-NaOH (pH 7.0), 30 mM KCl, 100 mM NaCl, 1 mM MnCl2, 5% (w/v) glycerol, and a complete EDTA-free protease inhibitor cocktail; Roche Diagnostics, Basel, Switzerland). Fractions containing CmsA/Ktr4 were concentrated using a Centriprep YM10 (Millipore, USA) and stored in a deep freezer for the crystallization screening experiments.

**Crystallization, data collection, and structural determination of CmsA**

Crystallization conditions were screened using the hanging-drop vapor diffusion method with a Crystal Screen kit (Hampton Research, USA) and ProPlex (Molecular Dimensions, USA). CmsA/Ktr4 was concentrated to ~ 13 mg/mL in Buffer A. Each droplet containing 0.8 μL of CmsA/Ktr4 solution and 0.8 μL of screening solution was equilibrated against 300 μL of reservoir solution. After several rounds of optimization, CmsA/Ktr4 was crystallized in a droplet containing 0.9 M sodium citrate and 100 mM HEPES-Na (pH 7.0). For the enzyme and
donor substrate complex, CmsA/Ktr4 was premixed with 50 mM of MnCl₂ and 20 mM of GDP-Man and crystallized using the same procedure.

The crystals were equilibrated in a solvent identical to the reservoir solution with a final concentration of 30% glycerol (v/v), after which they were frozen and stored in liquid nitrogen. X-ray data collection was carried out at 100 K and a wavelength of 0.9000 Å using a beamline 44XU at SPring-8 (Harima, Japan). Diffraction data were integrated and scaled using XDS (31), while the crystal structure was determined by molecular replacement using MOLREP (32) from the CCP4 suite (33). A polyalanine structural model generated from the structure of mannosyltransferase ScKtr4 (PDB code 5A07 (14)) was used as an initial search model. Model building was carried out using the automated program ARP/wARP (34), and several rounds of manual building, as well as further refinement, were performed using REFMAC5 (35) and Coot (36). In the enzyme and donor substrate complex, the electron density of the Mn²⁺ ion and the GDP moiety of GDP-Man were clearly observed and their atomic model was built and refined using Coot and REFMAC5. The quality of the final models was assessed using PROCHECK (37) and MolProbity (38). Atomic coordinates and structural factors were deposited in the Protein Data Bank (http://www.rcsb.org/pdb) under the ID codes 7BOO and 7BOP for the apo-form structure and Mn²⁺/GDP complex, respectively. The data collection and refinement statistics are summarized in Table S2. Figures were generated using PyMOL (39).

Docking simulation of the complex and acceptor substrate

In the enzyme and donor substrate complex, only the electron density map for the GDP moiety of GDP-Man was observed, suggesting that GDP-Man had been hydrolyzed during crystallization. First, the missing mannose moiety was manually modeled by superimposing the GDP-Man molecule onto the GDP moiety of our Mn²⁺/GDP complex structure based on data from a previous report on ScKre2/ScMnt1 (15). After the model had been energetically minimized using the GROMACS 5.1 package (40), we performed a docking simulation with α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe as the acceptor substrate for the Mn²⁺/GDP-Man complex structure using AutoDock 4.2 (41). The ideal α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe structure was built using the GLYCAM-Web server. The search space was defined as a grid of 38, 46, and 40 points in the x, y, and z directions, with 0.375 Å grid spacing centered between the Arg324 and Tyr357 side chains, and encompassing the entire active site cavity. Searches were performed using the Lamarckian Genetic Algorithm, with 100 runs, a population size of 150, and 25,000,000 energy evaluations. A total of 27,000 generations were analyzed and clustered using an RMS tolerance of 2.0 Å per cluster.

Molecular dynamics (MD) simulation of the complex with the acceptor substrate

To evaluate the dynamic properties of the modeled structure of the CmsA/Ktr4 enzyme-substrate complex, we carried out MD simulations with the CmsA/Ktr4 complex structure of α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe, one Mn²⁺ ion, one GDP-Man, and the highest AutoDock docking score as the initial model. All simulations were performed using GROMACS 5.1 with the Amber ff14SB force field (42) using the previously reported topology and parameters of GDP-Man (43). The topology and parameter files for α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe were generated using AmberTools17’s LEaP (44) with the GLYCAM06-j force field (45). The system was explicitly solvated in a cubic box with TIP3P water models and eight Na⁺ ions added to neutralize the charge of the system. Amber topologies were created using AmberTools17’s LEaP and exported to GROMACS topologies using ACPYPE (46) before being subjected to energy minimization by steepest descent. Following minimization, position-restrained MD simulations were carried out and the system was equilibrated in NVT ensemble for 200 ps at 300 K using V-rescale temperature coupling, followed by NPT ensemble for 200 ps at 1 atm using Parrinello–Rahman pressure coupling. A 14 Å cut-off was applied for short-range neighbors, electrostatic, and van der Waals interactions, while long range electrostatic interactions were measured using the Particle Mesh Ewald method. The
equilibrated system was then subjected to MD simulation for 100 ns, with each simulation performed three times. MD trajectories were analyzed using AmberTools17 and visualized using PyMOL and VMD (47). To validate the procedures and the results of the simulations, we performed the same docking and MD simulations for the complex structure of ScKre2/ScMnt1 with one Mn$^{2+}$ ion, one donor substrate, GDP-Man, and the artificial acceptor substrate, methyl-α-Man. The monomer structure of ScKre2/ScMnt1 was used for calculations and the results were compared with the reported structures, i.e., the proposed enzyme-substrate complex model by the theoretical study (27) and the crystal structure (15).

**Data availability:** The structures presented in this paper have all been deposited in the Protein Data Bank (PDB) with the codes 7BOO and 7BOP.
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Table 1. Predicted hydrogen bonds existing between α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe and CmsA/Ktr4.

| CmsA residue atom | Acceptor substrate atom | Distance (Å)\(^a\) |
|-------------------|-------------------------|-------------------|
| Gln171 NE2        | 2ʹ-OH (middle mannose)  | 3.3 ± 0.5         |
| Asp237 OD2        | 4-OH (terminal mannose)| 2.9 ± 0.2         |
| Trp291 NE1        | 5-O (terminal mannose) | 3.0 ± 0.2         |
| Arg324 NH1        | 3-OH (terminal mannose)| 2.9 ± 0.1         |
| Arg324 NH2        | 4-OH (terminal mannose)| 3.0 ± 0.2         |
| Tyr357 OH         | 4ʹ-OH (middle mannose) | 3.1 ± 0.5         |

\(^a\)The mean distances and their standard deviations between two heavy atoms.
Figure 1. Crystal structure of CmsA/Ktr4. A. Ribbon representation of the CmsA/Ktr4 structure. The GDP molecule is displayed as a ball-and-stick and the Mn\(^{2+}\) atom is shown as a sphere. The helix and sheet structures, as well as the N-termini and C-termini are labeled. B. 2\(F_{\text{obs}}\)–\(F_{\text{calc}}\) electron density map of the GDP binding site contoured at 1.0 \(\sigma\). The Mn atom and its ligands, GDP, Glu205, and His354, are labeled. Red non-bonded spheres represent water molecules coordinating to the Mn atom. C. The residues and water molecules around the GDP binding site are shown by ball-and-stick models and labeled. The dashed lines represent hydrogen bonds.
Figure 2. Structure-based sequence alignment of CmsA/Ktr4 with known orthologous proteins. The sequence of CmsA/Ktr4 is aligned to its fungal orthologous proteins from *Neurospora crassa* (N. crassa), *Beauveria bassiana* (B. bassiana), and *S. cerevisiae* Kre2/Mnt1 and Ktr4 (ScKre2/ScMnt1 and ScKtr4), whose 3-D structures have been reported. The secondary structure elements are drawn above the sequences. Filled triangles and circles under the sequences represent the amino acid residues coordinating to the Mn$^{2+}$ atom and interacting with the GDP molecule, respectively. Green numbering indicates the Cys-pair residues forming three conserved disulfide bonds. The figure was produced by ESPript 3.0 (48).
Figure 3. Large and wide cleft structure adjacent to the GDP-Man recognition site of CmsA/Ktr4. A. Molecular surface representation of CmsA/Ktr4. The Mn atom and GDP are shown as spheres. Dashed circle and the enlarged picture indicate a large cleft formed at the adjacent regions to the GDP-Man recognition site. B. The GDP molecule and the amino acid residues forming the cleft are shown by white and green ball-and-stick models and labeled.
Figure 4. Conformation analyses of the MD simulation of CmsA/Ktr4 in complex with GDP-Man and α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe. A. Root mean square deviation (RMSD) analysis of the protein main chain (black), Mn²⁺ ion (magenta), GDP-Man (green) as donor substrate, and acceptor substrate model compound, α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe (blue) during a 100-ns MD simulation. B. Root mean square fluctuation (RMSF) of the main chain atoms against the starting structure were calculated over the final 85 ns time. Save for the terminal region, RMSF is below 1.6 Å. Some peaks in the plot indicate that the loop regions marked by the dashed circles were a little flexible.
Figure 5. Representative structure of CmsA/Ktr4 in complex with GDP-Man and α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe from the most populated cluster. A. Representative structures of 35 clusters. Clustering analysis based on the RMSD of the main protein chain atoms was performed using MD trajectories of the final 85 ns and a RMSD cut-off of 1.0 Å. The Mn atoms are shown as spheres. The GDP-Man and α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe molecules are shown by stick. B. Molecular surface representation of CmsA/Ktr4 in complex. The Mn$^{2+}$ ion, GDP-Man, and α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe are shown as spheres. The model of physiological acceptor substrate, α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe was bound at the cleft close to the GDP-Man recognition site. C. The binding of α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe by the hydrogen bond and van der Waals interaction. The α-Man-(1→6)-α-Man-(1→2)-α-Man-OMe molecule is shown by thick ball-and-stick model. The residues around its binding site are shown by thin stick models and labeled. The dashed lines represent hydrogen bonds.
Figure 6. Mycelial growth of the ΔcmsA/ktr4 cells of A. fumigatus. A. The positions of residues investigated by mutational analysis. Thirteen mutated residues, Glu102, Tyr172, Ser177, Tyr178, Met181, Cys182, Arg203, Glu205, His289, Trp291, Arg324, Ala328, and His354 are shown by ball-and-stick and labeled. Each mutant was expressed in ΔcmsA/ktr4 A. fumigatus. B. Wild-type and 13 mutants (E102A, Y172A, S177A, Y178A, M181A, C182A, R203A, E205A, H289A, W291A, A328F, and H354A) of CmsA/Ktr4 were expressed in ΔcmsA/ktr4 cells of A. fumigatus.
Structural basis for the core-mannan biosynthesis of cell wall fungal-type galactomannan in *Aspergillus fumigatus*
Daisuke Hira, Takuya Onoue and Takuji Oka

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