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Combining Autodisplay and Flow Cytometry for Functional Analysis of HCN4-CNBD Residues Essential for Ligand Binding

Magdalena Wojciechowski, Sarah Maskri, Oliver Koch, Joachim Jose

University of Münster, Institute of Pharmaceutical and Medicinal Chemistry, PharmaCampus, Corrensstraße 48, 48149, Münster, Germany

* E-mail: Magdalena.Wojciechowski@uni-muenster.de

Abstract

The hyperpolarization and cyclic nucleotide activated ion (HCN) channels have garnered increasing attention due to their association with the pathogenesis of various diseases. Given the heterogeneity in their expression patterns among different HCN channel subtypes, a comprehensive understanding of their function remains elusive and requires further investigation. The ion channel properties such as voltage dependence, are subject to modulation by endogenous cyclic nucleotides binding to the cyclic nucleotide binding domain (CNBD) [1]. In this study, we systematically assess the influence of seven distinct amino acids within the CNBD on ligand binding via the method of autodisplay [2] and flow cytometry [3]. Native HCN4 C-Linker-CNBD and its corresponding mutants were separately presented as fusion proteins on the surface of E. coli cells. Following incubation with 8-Fluo-cAMP, the whole cell fluorescence was quantified via flow cytometry. Perturbations in ligand binding, attributable to specific mutation, led to diminished fluorescence intensity (mFI) relative to the unaltered C-Linker-CNBD. Notably, mutations E660R, R669E and R710E resulted in a near-complete loss of whole cell fluorescence, confirming their importance for ligand binding as described before [4]. Additionally, two amino acid substitutions, V642S and L652S, which were not anticipated to directly interact with the ligand, exhibited a strong reduction in mFI. We showed here for the first time the impact of these residues on ligand binding. Only moderate effects on ligand binding were observed for the mutants T670A and C662A. The presented ligand binding assay offers a rapid means of identifying residues essential for ligand binding. This information can be useful in the targeted drug design.

Method

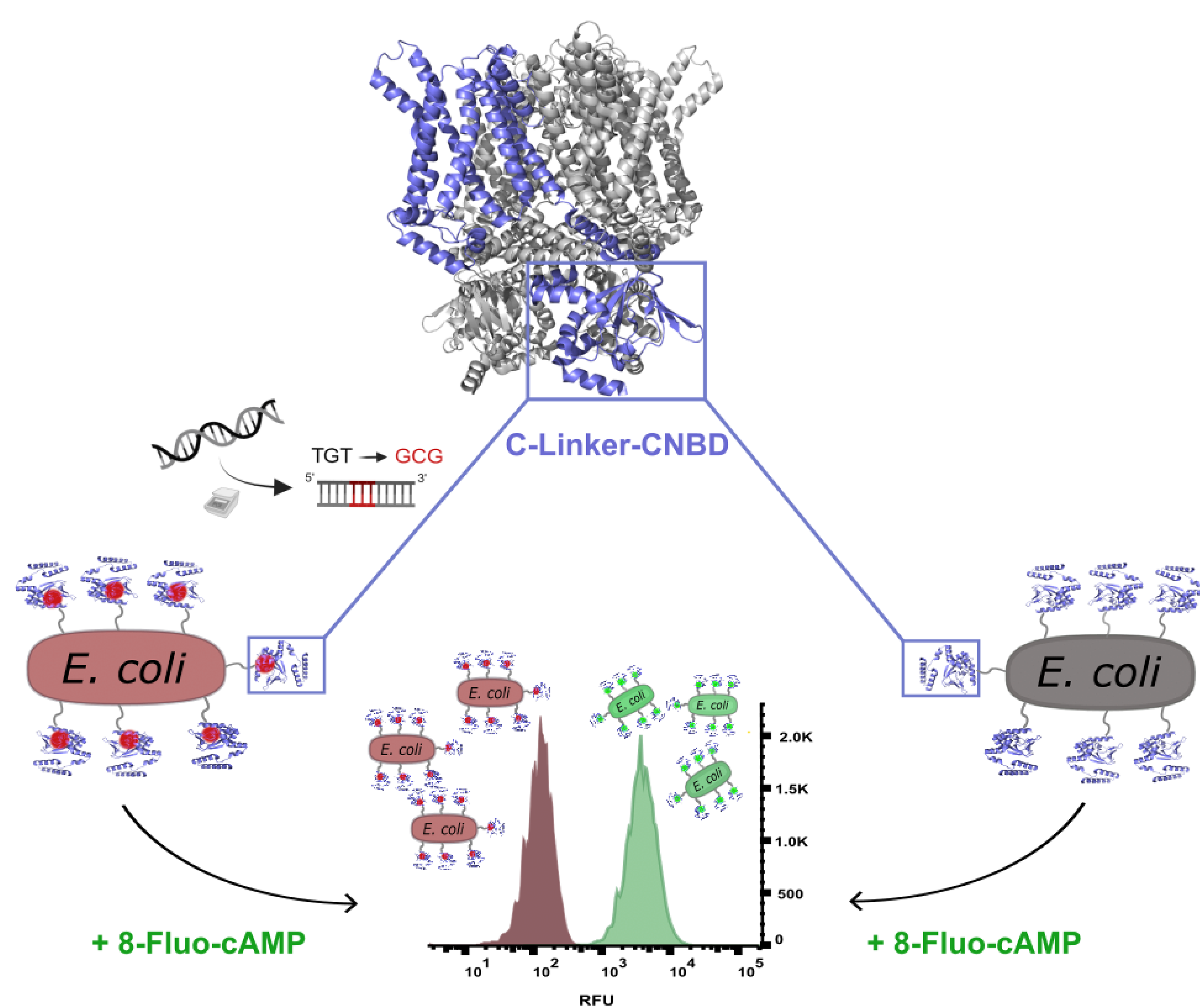

Figure 1. Graphical abstract. Using the autodisplay technique, the native HCN4 (PDB: 6GYN) C-Linker-CNBD (PDB: 3OTF) were presented on the surface of *E. coli* cells. To assess the impact of specific amino acid residues within the CNDB on ligand binding, site-directed mutagenesis was applied to introduce targeted mutations within the CNBD. Cells displaying either the native C-Linker-CNBD or the mutated version were subjected to treatment with 100 nM 8-Fluo-cAMP, followed by flow cytometry analysis. Reduction in ligand binding as compared to the native protein indicates the importance of the specific residue for ligand binding.

Results

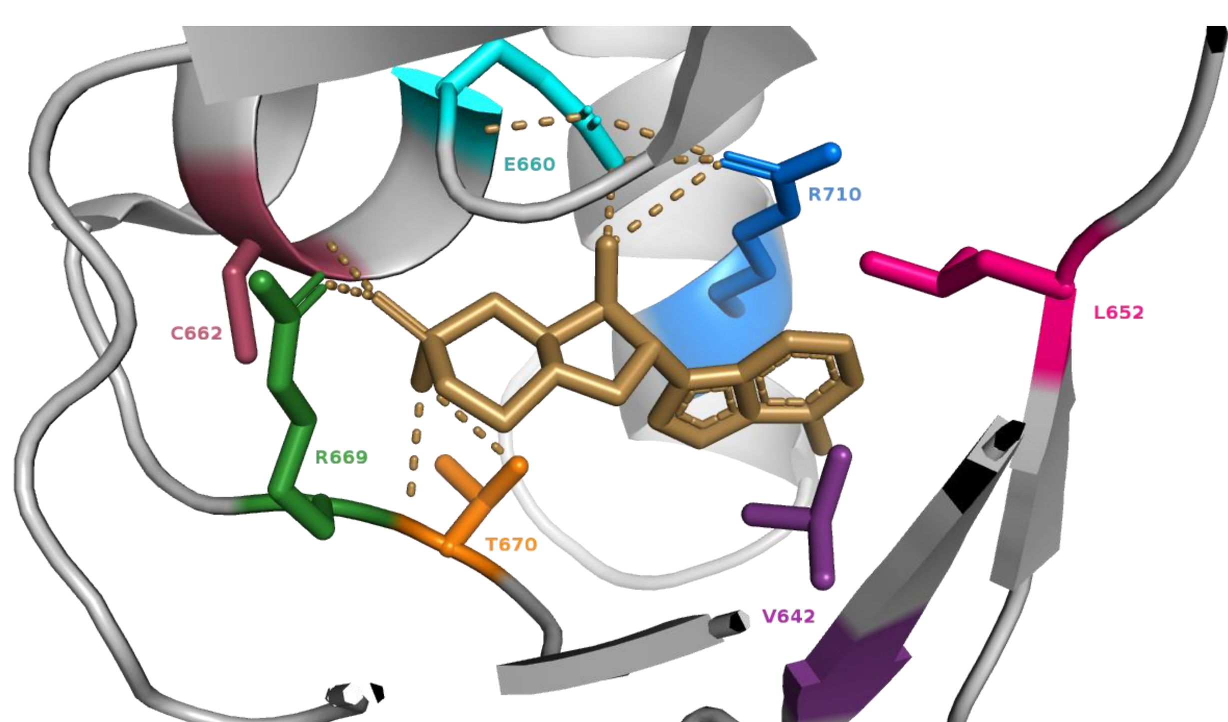

Figure 2. Overview of residues subjected to site-directed mutagenesis. Residues engaging in direct interaction with the ligand (cAMP, gold) include E660 (aqua), C662 (violet), R669 (green), T670 (orange) and R710 (blue), while V642 (purple) and L652 (pink) are residues that do not interact with cAMP.

References

[1] Wahl-Schott, C.; Biel, M. HCN channels: structure, cellular regulation and physiological function. *Cell Mol Life Sci* **2009**, *66*, 470-494, doi:10.1007/s00018-008-8525-0.

[2] Jose, J.; Meyer, T.F. The autodisplay story, from discovery to biotechnical and biomedical applications. *Microbiol Mol Biol Rev* **2007**, *71*, 600-619, doi:10.1128/MMBR.00011-07.

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[4] Zhou, L.; Siegelbaum, S.A. Gating of HCN channels by cyclic nucleotides: residue contacts that underlie ligand binding, selectivity, and efficacy. *Structure* **2007**, *15*, 655-670, doi:10.1016/j.str.2007.04.012.

Results

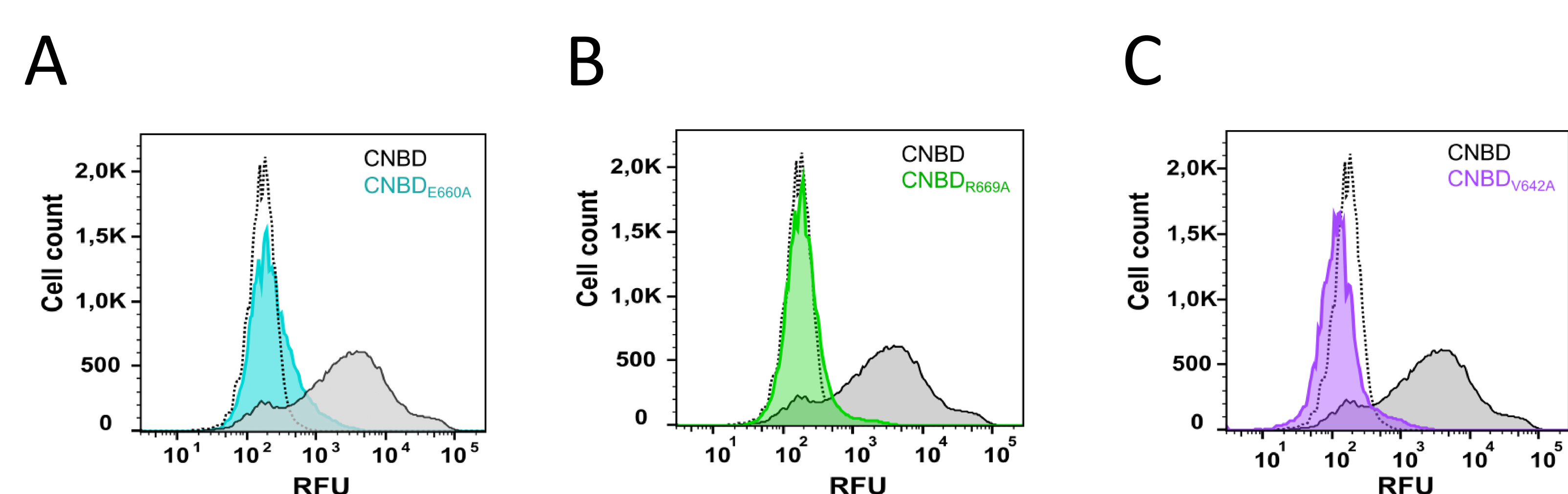
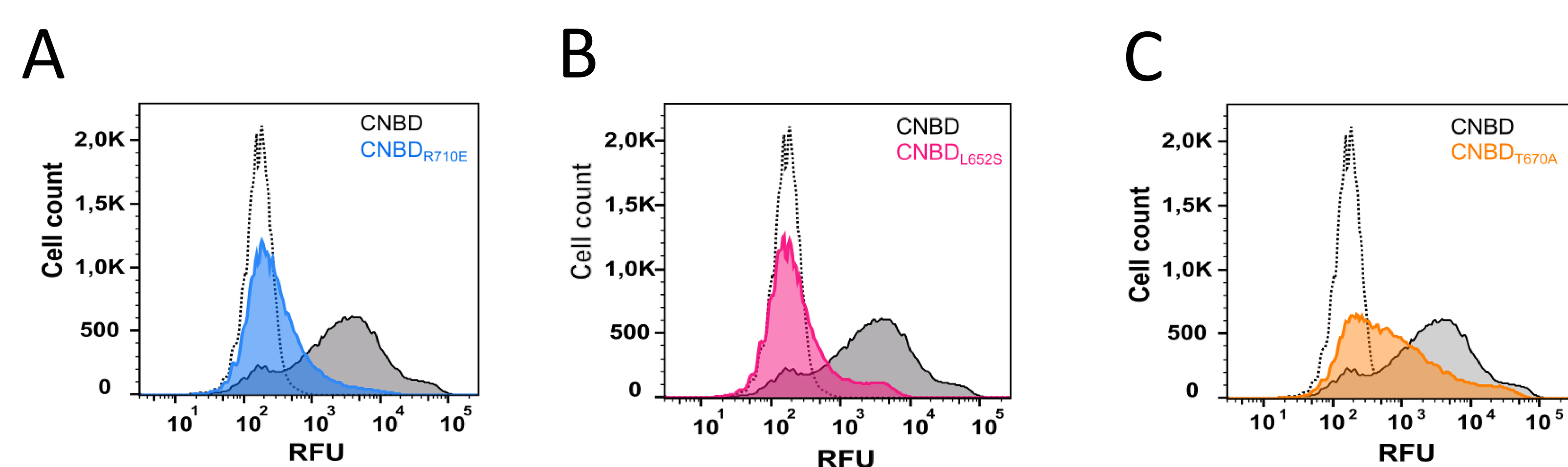
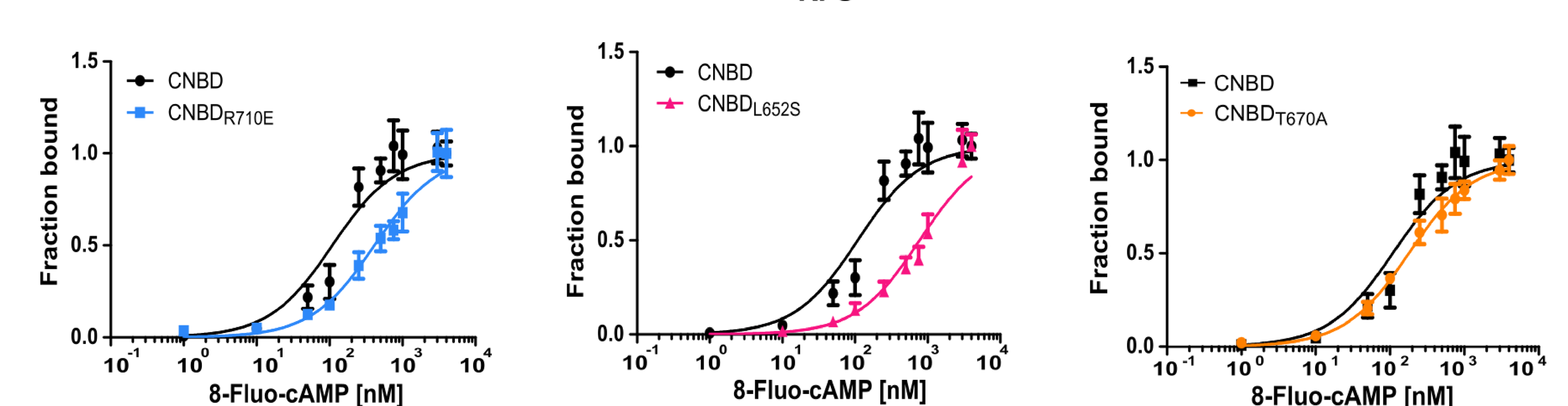
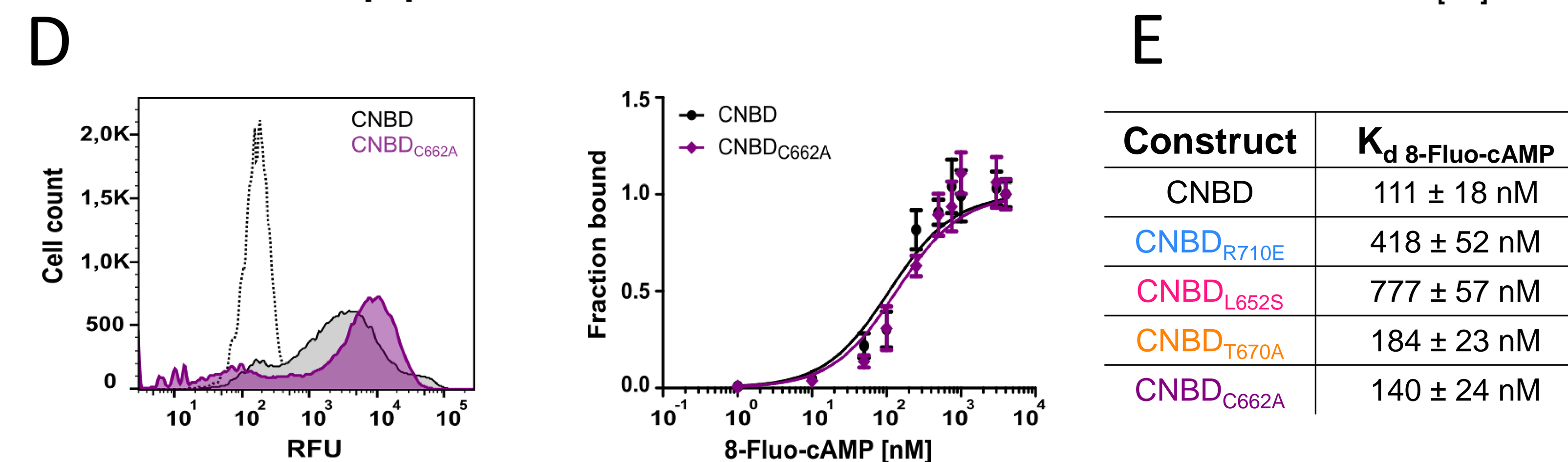


Figure 3. Impact of E660, R669 and V642 mutations on ligand binding. Mutation E660A (A, aqua), R669A (B, green) and V642A (C, purple) resulted in a complete abolition of ligand binding in comparison to the native CNBD (gray). Dotted line represents cells without the plasmid, serving as negative control to rule out non-specific ligand binding.







Construct	$K_{d\ 8\text{-Fluo-cAMP}}$
CNBD	111 ± 18 nM
$CNBD_{R710E}$	418 ± 52 nM
$CNBD_{L652S}$	777 ± 57 nM
$CNBD_{T670A}$	184 ± 23 nM
$CNBD_{C662A}$	140 ± 24 nM

Figure 4. Impact of R710, L652, T670 and C662 on ligand binding. The mutation R710E (A, blue) and L652S (B, pink) resulted in a substantial reduction in mean fluorescence intensity. To determine the binding affinity of 8-Fluo-cAMP to the mutated CNBD, cells were incubated with increasing concentrations of 8-Fluo-cAMP ranging from 1 nM to 4 µM, followed by subsequent analysis *via* flow cytometry. Comparative evaluation of the binding curves obtained for the mutant and the native CNBD revealed a rightward shift indicative of higher dissociation constants (E). In contrast, minimal effect on ligand binding was observed with the T670A (C, orange) or C662A (D, violet) mutations.

Conclusion

- Autodisplay based ligand binding assay is suitable for assessing the significance of specific amino acid residues in ligand binding, eliminating the need for protein purification
- E660, R669 and V642 are crucial for ligand binding
- Binding affinity is strongly reduced upon the replacement of R710 and L652
- Substitution of T670 and C662 is well tolerated
- The importance of V642 and L652 on ligand binding was shown for the first time
- Further analysis of CNBD residues could be used for targeted drug design

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