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Identification of a Key Cholesterol Binding Enhancement Motif in Translocator Protein 18 kDa

Fei Li, Jian Liu, Lance Valls, Carrie Hiser, and Shelagh Ferguson-Miller*

Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824, United States

Supporting Information

ABSTRACT: Translocator protein 18 kDa (TSPO) in the mitochondrial outer membrane has been implicated in cholesterol transport regulating steroidogenesis. A human single polymorphism associated with anxiety disorders (A147T) and reduced pregnenolone production is adjacent to TSPO's cholesterol binding motif. In a mutant mimicking this polymorphism, we observe a lower level of binding of cholesterol. Further, three residues preceding A147 are more hydrophilic in a bacterial TSPO that has an affinity for cholesterol 1000-fold lower than that of the human form. Converting these residues to the human form in the bacterial homologue strikingly increases the affinity for cholesterol. An important role for this extended motif is further supported by covariance analysis.

Translocator protein 18 kDa (TSPO) is a widely distributed integral membrane protein initially discovered as a binding site for anxiolytic benzodiazepine drugs in peripheral tissues[1] and therefore known as the peripheral benzodiazepine receptor (PBR). Recently, it was renamed to reflect evidence of its involvement in a number of complex cellular processes, including cholesterol transport, porphyrin transport, inflammation, tumor progression, Alzheimer's disease, and regulation of apoptosis.[2−8] Ligands of TSPO are also widely used for imaging of brain damage by positron emission tomography (PET)[9] as well as for treatment.[7]

A conserved cholesterol recognition/interaction amino acid consensus sequence [CRAC, L/V-x$_{(1-5)}$-Y-x$_{(1-5)}$-R/K] near the C-terminus of transmembrane helix V (TM-V) was identified as a cholesterol binding site in mammalian TSPO.[10] Despite sharing the majority of this CRAC sequence [L-x$_{(1)}$-F-x$_{(3)}$-R] (Figure S1 of the Supporting Information), the homologue from *Rhodobacter sphaeroides* (*Rs*TSPO)[11] shows an affinity for cholesterol >1000-fold lower than that of mammalian TSPO.[12] A clue about the disparity in binding was provided by recent publications reporting a spontaneous human single polymorphism, giving rise to an altered amino acid sequence, A147T, in the region immediately preceding the CRAC site. This single mutation correlated with anxiety disorders and reduced pregnenolone production,[13,14] as well as a lower affinity for TSPO ligands used in PET imaging.[15] We noted that the three residues preceding the A147T site are highly variable in bacteria but conserved and much more hydrophobic in the mammalian proteins, suggesting that this region adjacent to the CRAC site could also contribute to determining cholesterol affinity. We initially designed a mutant mimicking A147T (*Rs*TSPO-A139T) and showed that this mutant has an affinity for cholesterol and other ligands significantly lower than that of the wild type (WT), consistent with the phenotype exhibited by A147T.[16] Here we report a mutant in which the three preceding residues (A136-T137-A138) were replaced with the human sequence, LAF, creating *Rs*TSPO-LAF.

The triple mutant was well expressed and purified by the same procedure that was used for the WT[12] but had a tendency to aggregate when concentrated, a tendency that could be controlled by maintaining it at a low concentration or by adding cholesterol hemisuccinate (CHS) (Figure S2 and supplementary discussion of the Supporting Information). Figure 1A shows that introduction of the LAF motif causes the affinity for cholesterol to be greatly enhanced, from a K_d of ∼80 μM to ∼20 nM, similar to the affinity of mouse and human TSPO previously reported.[10,17] In addition, the samples purified in the presence of CHS, which was retained by the mutant protein (Figure S2A of the Supporting Information), show a dramatically lower apparent affinity for cholesterol, similar to that of the WT, indicating that CHS is occupying the same site as cholesterol (Figure 1A). These results strongly support a role of the LAF motif in determining the nanomolar affinity for cholesterol observed in mammalian TSPO.

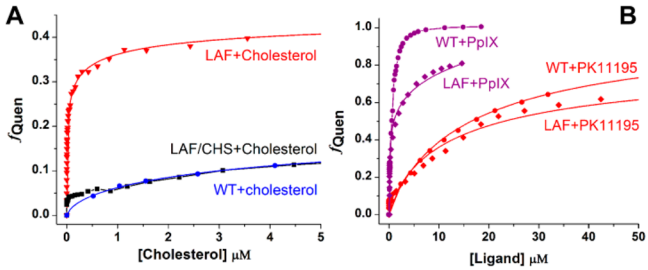

Figure 1. LAF significantly increases the affinity for cholesterol (A) but slightly decreases it for PpIX and PK11195 (B).

We also tested the binding of PK11195 and protoporphyrin IX (PpIX) with *Rs*TSPO-LAF. Figure 1B shows that the *Rs*TSPO-LAF mutant has some influence on the binding of PK11195 (10 ± 1 μM for the WT vs 15 ± 4 μM for LAF) and PpIX (0.31 ± 0.01 μM for the WT vs 1.1 ± 0.1 μM for LAF), an ∼1.5−3-fold decrease in affinity, unlike the dramatic increase in cholesterol affinity. Notably, the distinctive emission

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spectrum previously seen in PK11195 quenching curves with the *Rs*TSPO-WT protein[12] was also seen in the fluorescence quenching of the mutant, suggesting that the binding site of PK11195 was not strongly influenced by the mutation. Importantly, the modest effects of the three-amino acid substitution in *Rs*TSPO-LAF upon binding of ligands other than cholesterol indicate that it is causing a quite specific effect on the cholesterol binding site.

To improve our understanding of the structural basis of cholesterol binding, we analyzed ~2000 sequences of TSPO with the covariance method,[18] an independent test of residues of structural and functional importance, complementary to evidence from sequence conservation (supplementary discussion in the Supporting Information). Our analysis identified a number of high-scoring interactions between TM-IV and TM-V, while there were very few between other helices (Figure 2), suggesting that these two helices may be critical for the

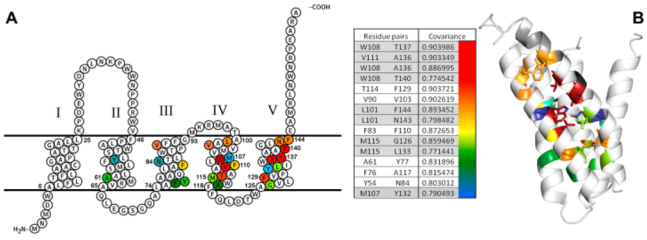

Figure 2. Interacting residues are predicted by covariance analysis (A) and agree with the crystal structure (B).

stability and function of TSPO. In fact, in an early effort to obtain nuclear magnetic resonance structures of TSPO fragments, Jamin and colleagues showed that TM-IV itself could not form a stable helix except when stabilized by strong interactions with TM-V.[19]

Interestingly, the two highest-scoring interactions predicted by covariance analysis are exactly within the ATA/LAF region (*Rs* A136 and T137), providing strong evidence supporting the importance of this region. Independently, the hydrogen/deuterium exchange analysis with mouse TSPO shows that these residues on TM-V paired with TM-IV are the most stable core in the whole protein.[20] In our *Rs*TSPO-LAF mutant, we created a better binding site for cholesterol but perhaps at the expense of weakening some of these critical interactions, which may account for the somewhat lower stability. Remarkably, interactions predicted by the covariance analysis completely agree with the crystal structure of *Rs*TSPO[16] (Figure 2B and Figure S3 of the Supporting Information). In particular, side chains of T137 and W108, the pair with the highest covariance score, form a hydrogen bond. The second strongest predicted pair, A136 and V111, is also close in the crystal structure (4.1 Å), as are N143 and L101 (3.5 Å). The outstanding agreement among the covariance analysis, the mutational analysis, and the crystal structure gives much credence to the importance of this extended motif in cholesterol binding.

Because cholesterol is a major component of the membranes of higher animals[21] and plays a critical role in membrane structure and as the source of all steroid hormones, conservation of high affinity for cholesterol might be expected in higher animals for a protein such as TSPO, insofar as it is involved in regulating cholesterol transport. To address the question of conservation, we analyzed the sequences of TSPO proteins from different species previously described.[3,22] The analysis shows that the cholesterol binding enhancement motif associated with CRAC is very well conserved within mammals as Leu-Ala-Phe [LAF (Figure 3A and Figures S1 and S4 of the

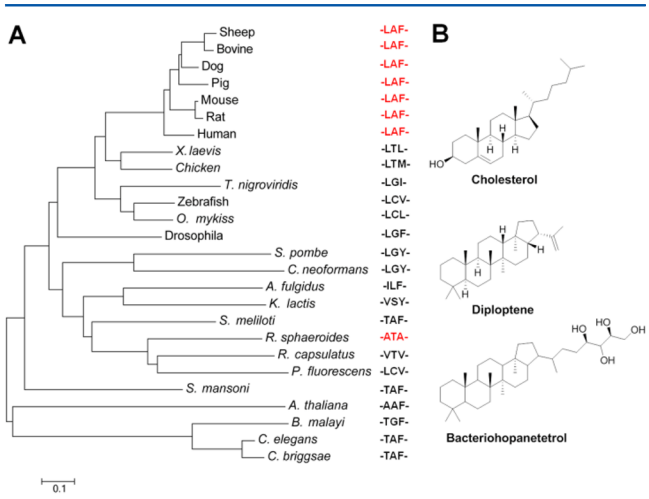

Figure 3. Cholesterol binding enhancement motif, LAF, (A) well conserved in mammalian TSPOs and variable in bacteria, to accommodate different tails of steroid-like ligands (B).

Supporting Information)], including human and mouse forms for which extensive studies have shown that TSPO could play important[23,24] but still controversial[25] roles in the transport of cholesterol into mitochondria and steroidogenesis. While bacteria and archaea have a similar CRAC sequence with the central Y replaced by F, their LAF motifs are very diverse. Hopanoids, which have a ring structure similar to that of cholesterol, have been identified in bacteria[26,27] as serving a function analogous to that of cholesterol in the higher animals. However, they can have quite different tails as shown by two representative hopanoids in Figure 3B. The more variable sequence in bacteria and other lower orders may reflect the need to accommodate the distinctive tails of the hopanoids in different bacteria or to accommodate alternative ligands. In fact, a high-resolution crystal structure recently determined by our group confirms that lipidic ligands indeed bind in this region (Figure S5 of the Supporting Information). Taken together with the covariance evidence that TM-IV and TM-V co-evolve, our results are consistent with the increasing functional importance of cholesterol and its metabolic products in higher animals, supplying a selection pressure for the evolution of high-affinity cholesterol binding involving the enhancement motif.

We also addressed the question of whether there is any general significance of this high-affinity-conferring motif in other human membrane proteins by surveying 5183 human membrane proteins from the database. A total of 28627 CRAC sites were identified in 4684 human membrane proteins (~6 per protein), while only 66 LAF-CRAC sites were identified (1 one protein), including members from all major membrane protein families (Table S1 of the Supporting Information). Cholesterol binding sites with the enhancement motif are thus fairly widespread and could be critical for their involvement in transport or regulation. The results indicate relatively low predictive ability of the CRAC motif alone, as concluded by Song et al.,[28] but in contrast, the much smaller subset found with the extended motif suggests more predictive value. It also seems likely that they represent binding modes with functional

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significance beyond that conferred by cholesterol as a solvent. The location of TSPO in mitochondria, which are low in cholesterol,[21] and the proposed function of TSPO as part of a cholesterol transport system are consistent with a requirement for the enhancement motif we have identified to provide significantly increased affinity for cholesterol. Indeed, a LAF-CRAC sequence is identified in several membrane proteins that are related to cholesterol transport and metabolism (supplementary discussion in the Supporting Information).

In summary, biochemical and mutational studies of the purified *Rs*TSPO identify a cholesterol binding enhancement motif that is highly conserved in mammalian but not bacterial TSPO. High-resolution crystal structures also confirm that lipidic ligands bind at this site. This motif is adjacent to the previously identified CRAC sequence in the C-terminus of TM-V and to a human single polymorphism associated with anxiety disorders. Mutations in either location strongly affect cholesterol binding. The altered properties of a purified mutant form *Rs*TSPO-LAF are consistent with genetic covariance analysis showing this region to be critically important to the structure and function of TSPO. The cholesterol binding enhancement motif is also identified in a number of human membrane proteins, suggesting that this motif may be useful for identifying high-affinity cholesterol binding regions with functional and regulatory significance.

■ ASSOCIATED CONTENT

Supporting Information

Methods, supplementary discussion, tables, and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: fergus20@msu.edu.

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Notes

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