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Identification of cholesterol recognition amino acid consensus (CRAC) motif in G-protein coupled receptors

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ABSTRACT

G-protein coupled receptors (GPCRs) are the largest class of molecules involved in signal transduction across membranes, and represent major targets in the development of novel drug candidates in all clinical areas. Membrane cholesterol has been reported to have an important role in the function of a number of GPCRs. Several structural features of proteins, believed to result in preferential association with cholesterol, have been recognized. Cholesterol recognition/interaction amino acid consensus (CRAC) sequence represents such a motif. Many proteins that interact with cholesterol have been shown to contain the CRAC motif in their sequence. We report here the presence of CRAC motifs in three representative GPCRs, namely, rhodopsin, the β_2-adrenergic receptor, and the serotonin$_{1A}$ receptor. Interestingly, the function of these GPCRs has been previously shown to be dependent on membrane cholesterol. The presence of CRAC motifs in GPCRs indicates that interaction of cholesterol with GPCRs could be specific in nature. Further analysis shows that CRAC motifs are inherent characteristic features of the serotonin$_{1A}$ receptor and are conserved over natural evolution. These results constitute the first report of the presence of CRAC motifs in GPCRs and provide novel insight in the molecular nature of GPCR–cholesterol interaction.

1. Introduction

Cholesterol is a major constituent in higher eukaryotic cellular membranes and is crucial in membrane organization, dynamics, function, and sorting. It is often found distributed non-randomly in domains in biological and model membranes [1,2]. Many of these domains (sometimes termed as 'lipid rafts' [3]) are thought to be important for the maintenance of membrane structure and function, although characterizing the spatiotemporal resolution of these domains has proven to be challenging [4]. The concept of such specialized membrane domains gains relevance in cell biology since important functions such as signal transduction [5], and the entry of pathogens [6,7] have been implicated to these putative domains.

The G-protein coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes [8]. They represent major targets for the development of novel drug candidates in all clinical areas [9]. GPCRs are integral membrane proteins with a considerable portion of the protein embedded in the membrane. This raises the obvious possibility that the membrane lipid environment could be an important modulator of receptor structure and function. In case of rhodopsin, for example, it is estimated from molecular dynamics simulation that the lipid–protein interface corresponds to ~38% of the total surface area of the receptor [10]. Interestingly, membrane cholesterol has been shown to modulate the function of a number of GPCRs (reviewed in [11–13]). Previous work from our laboratory has shown the crucial requirement of membrane cholesterol in the organization and function of the serotonin$_{1A}$ (5-HT$_{1A}$) receptor, an important neurotransmitter receptor [12–17]. Serotonin$_{1A}$ receptors represent one of the largest, evolutionarily ancient, and highly conserved families of seven transmembrane GPCRs [18]. Serotonergic signaling plays a key role in the generation and modulation of various cognitive, behavioral and developmental functions. Work from other laboratories has shown that the function of GPCRs such as rhodopsin [19] and the β_2-adrenergic receptor [20] is sensitive to membrane cholesterol.

Two possible mechanisms have been suggested by which membrane cholesterol could influence the structure and function of GPCRs [21]: (i) through a direct/specific interaction with GPCRs, or (ii) through an indirect way by altering membrane physical properties in which the receptor is embedded, or due to a combination of both. Interestingly, recently reported crystal structures

Abbreviations: 5-HT$_{1A}$ receptor, 5-hydroxytryptamine-1A receptor; CRAC, cholesterol recognition/interaction amino acid consensus; GPCR, G-protein coupled receptor; TMD, transmembrane domain.

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of GPCRs have shown structural evidence of cholesterol binding sites [22,23]. Several structural features of proteins that are believed to result in preferential association with cholesterol have been recognized [24,25]. In many cases, proteins that interact with cholesterol have a characteristic amino acid sequence, termed the cholesterol recognition/interaction amino acid consensus (CRAC) motif. The CRAC sequence is defined by the presence of the pattern -L/V-$(X)_{1-5}$-Y-$(X)_{1-5}$-R/K-, in which $(X)_{1-5}$ represents between one and five residues of any amino acid [24,26]. This motif has been shown to be present in caveolin-1 [27], the peripheral-type benzodiazepine receptor [26,28], the HIV-1 transmembrane protein gp41 [29], and the mammalian seminal plasma protein PDC-109 [30].

In spite of the importance of membrane cholesterol in GPCR function [11–13], specific motifs for binding of cholesterol to GPCRs have not been identified yet. We report here the presence of CRAC motifs in three representative GPCRs, namely, rhodopsin, the β_2-adrenergic receptor, and the serotonin$_{1A}$ receptor. As mentioned above, all three receptors have been shown to have cholesterol dependence for their function. Interestingly, we show that CRAC motifs are inherent characteristic features of the serotonin$_{1A}$ receptor and are conserved over natural evolution. Our results constitute the first report of the presence of CRAC motifs in GPCRs.

2. Materials and methods

2.1. Identification of CRAC motif in representative human GPCRs: rhodopsin, the β_2-adrenergic receptor and serotonin$_{1A}$ receptor

The putative CRAC motifs in these GPCRs (with the conserved tyrosine (Y) along with leucine (L)/valine (V) toward its amino terminus and lysine (K)/arginine (R) toward carboxy terminus, within a distance of 5 residues on either side), were manually identified (see Fig. 1). The amino acid sequences of GPCRs were from NCBI database. The transmembrane helices of the serotonin$_{1A}$ receptor were predicted using the program TMHMM2 (see Fig. 2). These predictions were confirmed by other programs such as MEMSTAT, SPLIT4 and HMMTOP2 (it has been earlier reported that these programs were among the best and perform equally well in predicting transmembrane helices [31]). Since the structure of human rhodopsin is not available, we aligned it to bovine rhodopsin [32] using ClustalW and mapped the transmembrane regions based on their similarity.

2.2. Sequence alignment of the serotonin$_{1A}$ receptor and identification of CRAC motif

The evolution of CRAC motif(s) in the serotonin$_{1A}$ receptor over various phyla was analyzed by examining amino acid sequences of the serotonin$_{1A}$ receptor obtained from NCBI and Expasy databases (see Figs 2 and 3). Partial, duplicate and other non-specific sequences were removed from the set of sequences obtained. Initial alignment of sequences was carried out using ClustalW. After eliminating the relatively divergent parts of the receptor, the sequence was realigned using the same program. The putative CRAC motifs in the serotonin$_{1A}$ receptor were manually identified. The amino acid sequences used for the analysis belong to diverse taxa that include insects, fish and other marine species, amphibians and extending up to mammals. The sequences for *Trichoplax adhaerens*, *Manduca sexta* and *Anopheles gambiae* are putative serotonin$_{1A}$ receptors whereas those of *Strongylocentrotus purpuratus*, *Bos taurus*, *Ornithorhynchus anatinus*, *Danio rerio*, *Monodelphis domestica*, *Macaca mulatta* and *Taeniopygia guttata* are predicted by homology. The sequence for *Caenorhabditis elegans* belongs to the serotonin receptor family.

3. Results and discussion

In the overall context of cholesterol sensitivity of GPCR function [11–13], we examined whether the sequence of these GPCRs contains any CRAC motif(s). Interestingly, we identified the presence of CRAC motifs in all three GPCRs, namely, rhodopsin, the β_2-adrenergic receptor and the serotonin$_{1A}$ receptor (see Fig. 1). Our analysis shows that while the sequences of rhodopsin and the serotonin$_{1A}$ receptor contain three CRAC motifs, the β_2-adrenergic receptor sequence shows two CRAC motifs. Rhodopsin sequence contains CRAC motifs in putative transmembrane helices I (residues 57–66), III (residues 131–141) and VII (residues 304–311), while the serotonin$_{1A}$ receptor sequence is characterized by CRAC motifs in putative transmembrane helices II (residues 90–101), V (residues 208–219) and VII (residues 394–405). The β_2-adrenergic receptor sequence, on the other hand, exhibits CRAC motifs in putative transmembrane helices V (residues 213–221) and VII (residues 324–328).

The serotonin$_{1A}$ receptor is an important member of the GPCR superfamily and is estimated to have differentiated ~650 million years ago from the serotonin$_1$ receptor subfamily in the time period during which vertebrates diverged from invertebrates [33]. In the context of the presence of CRAC motifs in the human serotonin$_{1A}$ receptor, we further analyzed whether the motif(s) is conserved during the natural evolution of the receptor. In order to examine the evolution of CRAC motif(s) in the serotonin$_{1A}$ receptor over various phyla, we analyzed amino acid sequences of the serotonin$_{1A}$ receptor from available databases (see Fig. 3). Partial, duplicate and other non-specific sequences were removed from the set of sequences obtained. The amino acid sequences used for the analysis belong to diverse taxa that include insects, fish and other marine species, amphibians and extending up to mammals. Initial alignment carried out using ClustalW showed that CRAC motif(s) are conserved in most species. Realignment with ClustalW (after eliminating the relatively divergent parts of the receptor) exhibited conservation of CRAC motif(s) across all phyla analyzed (see Fig. 3). It therefore appears that CRAC motif(s) represents an inherent characteristic feature of the serotonin$_{1A}$ receptor and is conserved during the course of natural evolution. It appears from Fig. 3 that the CRAC motif(s) is present even in organisms that are not capable of biosynthesis of cholesterol. Organisms which lack cholesterol biosynthesis could, however, acquire cholesterol through diet [34]. Organisms such as insects possess sterols that are different from cholesterol which have diverged from cholesterol during the sterol evolution pathway [35]. The presence of

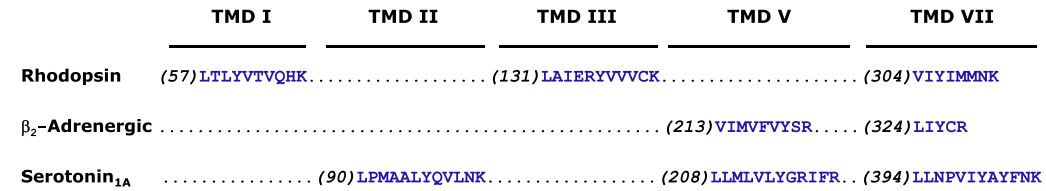

Fig. 1. Putative cholesterol recognition/interaction amino acid consensus (CRAC) motifs in representative human G-protein coupled receptors. The CRAC motifs in transmembrane domains (TMD) of rhodopsin, the β_2-adrenergic receptor, and serotonin$_{1A}$ receptor are shown. The numbers corresponding to the starting amino acid position in the respective sequences are mentioned in parentheses.

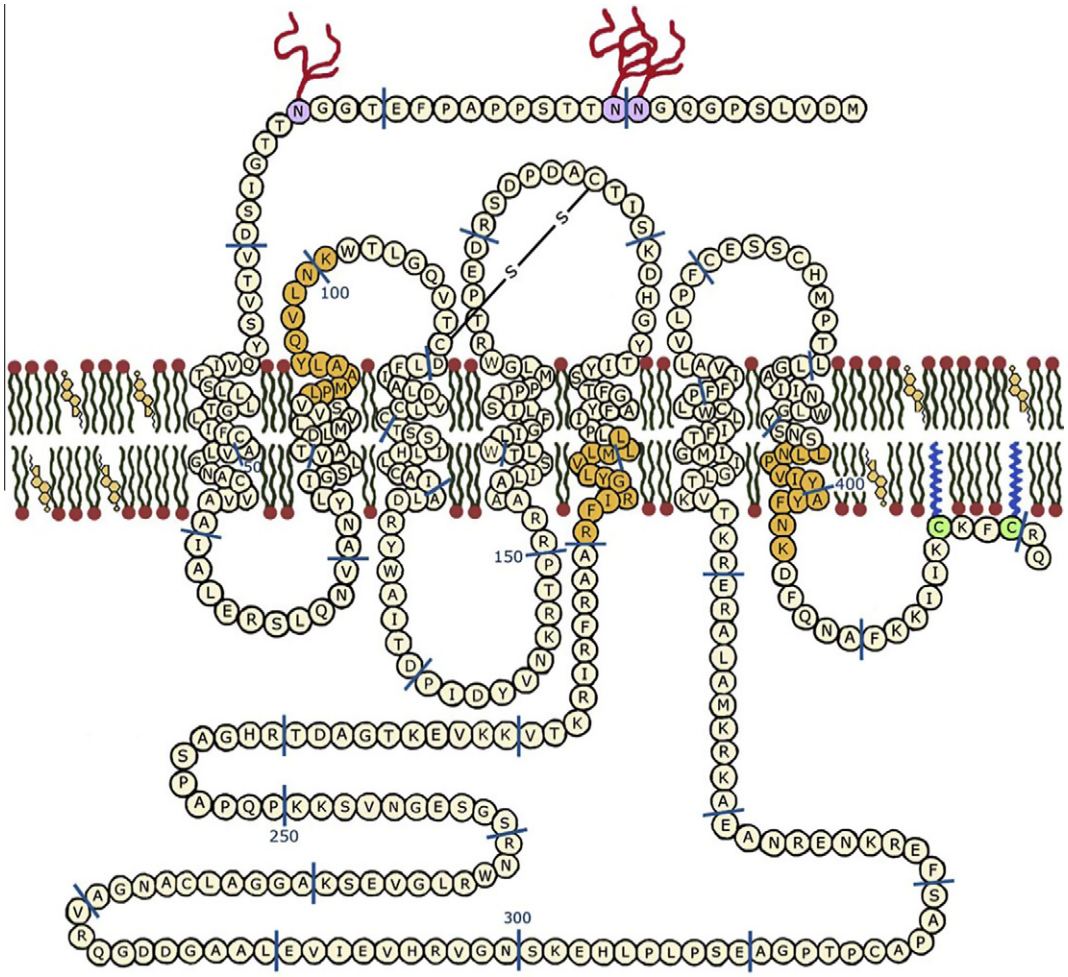

Fig. 2. A schematic representation of the membrane embedded human serotonin$_{1A}$ receptor showing its topological and other structural features. The membrane is shown as a bilayer of phospholipids and cholesterol, representative of typical eukaryotic membranes. The transmembrane helices of the receptor were predicted using the program TMHMM2 (see Section 2 for details). Seven transmembrane stretches, each composed of ~22 amino acids, are depicted as putative α-helices. The exact boundary between the membrane and the aqueous phase is not known and therefore the location of the residues relative to the membrane bilayer is putative. The putative CRAC motifs are highlighted (in yellow). The amino acids in the receptor sequence are shown as circles. Further structural details of the receptor are available in [12,18,44]. Adapted and modified from [36]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CRAC motif(s) in these organisms could be due to binding of closely related sterols or dietary cholesterol to the CRAC sequence.

The mechanism underlying the effect of cholesterol on the structure and function of integral membrane proteins and receptors is complex and no general consensus has evolved yet [11–13,21,36]. As mentioned above, it has been proposed that cholesterol can modulate GPCR function in two ways: (i) by a direct/specific interaction with the GPCR, which could induce a conformational change in the receptor [11,37–41], or (ii) through an indirect way by altering the membrane physical properties in which the receptor is embedded [42,43] or due to a combination of both. In this overall scenario, the presence of CRAC motifs in GPCRs lends support to specific interaction of cholesterol with GPCRs. This is further corroborated by our recent results from receptor modeling studies in which we showed that the serotonin$_{1A}$ receptor is more compact in the presence of cholesterol [44].

The molecular details of specific receptor–cholesterol interaction through CRAC motifs merit comment. It has been recently proposed that cholesterol binding motif should contain at least one aromatic amino acid which could interact with ring D of cholesterol [23] and a positively charged residue [45,46] capable of participating in electrostatic interactions with 3β-hydroxyl group of cholesterol. In addition, the aromatic amino acid side chain of CRAC motif has been suggested to interact with cholesterol since there could be interaction between the planar aromatic ring and the near planar tetracyclic fused steroid ring of cholesterol [27]. We recently proposed that cholesterol binding sites in GPCRs could represent nonannular binding sites [36]. Nonannular sites are characterized by lack of accessibility to the annular lipids, *i.e.*, these sites cannot be displaced by competition with annular lipids. It has been suggested that the possible locations for the nonannular sites could be either inter or intramolecular (interhelical) protein interfaces, characterized as deep clefts (or cavities) on the protein surface [47,48]. Interestingly, it has been suggested that cholesterol binding by CRAC motif is induced by a cleft located at the membrane interfacial region [45].

GPCRs are involved in a multitude of physiological functions and represent important drug targets. Although pharmacological and signaling features of GPCRs have been extensively studied, aspects related to their interaction with membrane lipids are only beginning to be addressed. The presence of CRAC motifs in GPCRs therefore represents an important milestone in this context. With further progress in understanding molecular details on the nature of GPCR–lipid interaction, our overall understanding of GPCR function in health and disease would improve significantly, thereby enhancing our ability to design better therapeutic strategies to combat diseases related to malfunctioning of these receptors.

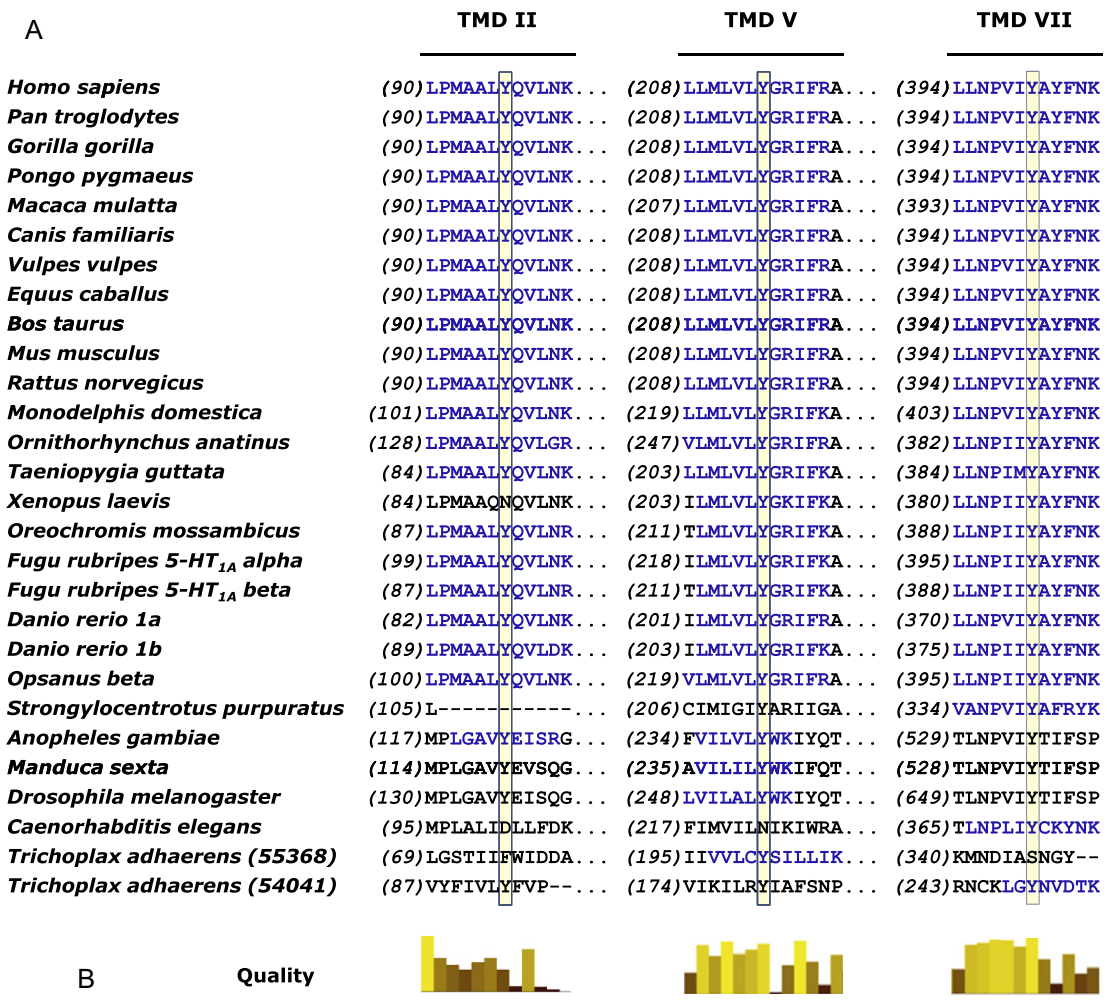

Fig. 3. Multiple alignment of the putative CRAC motifs in the serotonin$_{1A}$ receptor over various phyla across natural evolution. The conserved tyrosine (Y) residue along with leucine (L)/valine (V) toward its amino terminus and lysine (K)/arginine (R) toward carboxy terminus, within a distance of 5 residues on either side (the CRAC motif), are highlighted in blue. As evident from panel (A), the putative CRAC motifs are conserved from fish to human. Interestingly, the motif in the transmembrane helix II is not present in frog (*Xenopus laevis*), whereas the motifs in the transmembrane helices V and VII are found even in *T. adhaerens*. The numbers corresponding to the starting amino acid position in the respective sequences are mentioned in parentheses. Amino acid sequences of serotonin$_{1A}$ receptors are from NCBI and Expasy databases. Panel (B) is a graphical representation displaying the quality of alignment, with lighter shades representing higher quality. See Section 2 for other details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

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