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Cholesterol sensing by the ABCG1 lipid transporter: Requirement of a CRAC motif in the final transmembrane domain

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ABSTRACT

The ATP-binding cassette (ABC) transporter, ABCG1, is a lipid exporter involved in removal of cholesterol from cells that has been investigated for its role in foam cells formation and atherosclerosis. The mechanism by which ABC lipid transporters bind and recognise their substrates is currently unknown. In this study, we identify a critical region in the final transmembrane domain of ABCG1, which is essential for its export function and stabilisation by cholesterol, a post-translational regulatory mechanism that we have recently identified as dependent on protein ubiquitination. This transmembrane region contains several Cholesterol Recognition/interaction Amino acid Consensus (CRAC) motifs, and its inverse CARC motifs. Mutational analyses identify one CRAC motif in particular with Y667 at its core, that is especially important for transport activity to HDL as well as stability of the protein in the presence of cholesterol. In addition, we present a model of how cholesterol docks to this CRAC motif in an energetically favourable manner. This study identifies for the first time how ABCG1 can interact with cholesterol via a functional CRAC domain, which provides the first insight into the substrate–transporter interaction of an ABC lipid exporter.

1. Introduction

ATP binding cassette (ABC) transporters are proteins that transport substrates across cellular membranes and consume ATP in the process. Humans express 48 ABC transporters that are divided into subfamilies A–G, based on their structural similarities [1]. They transport a diverse range of substrates, including a variety of drugs and lipids. ABC full-transporters comprise at least two ATP binding cassettes and two transmembrane (TM) spanning domains, that contain six TM helices each. The ABCG-subfamily comprises five half-transporters that each contain one ATP binding cassette and one TM spanning domain, and are active as either homo- or heterodimers [1]. The TM spanning domains form a channel where substrate binding and translocation is thought to occur.

The ABCG-subfamily members has altogether five members, of which four (ABCG1, G4, G5 and G8) are involved in the transport of lipids, including cholesterol, oxysterols, cholesterol precursors, plant sterols and phospholipids [2,3]. One of these, ABCG1, is involved in cholesterol and sphingolipid export from cells such as macrophages, to high density lipoprotein (HDL), and has been investigated in the context of atherosclerosis [4]. Apart from transporting cholesterol, the post-translational regulation of ABCG1 is also influenced by the cellular cholesterol status. We have recently shown that when cells overexpressing ABCG1 are starved of cholesterol, ABCG1 is ubiquitinated and degraded via the proteasome. ABCG1 protein was rescued from degradation by addition of cholesterol within two hours [5], indicating a novel post-translational control mechanism for regulating transporter activity.

The mechanism by which ABC transporters operate has been studied in detail for the ABC drug transporters, due to their important role in the development of multidrug resistance and the ongoing search for inhibitors. The current paradigm is that the transporters undergo a conformational change upon substrate binding and ATP-consumption, although there are different models proposed with regards to the exact order of events [6]. The conformational change constitutes a move from an inward facing to an outward facing positioning of the TM domains. For ABC cholesterol transporters, the substrate is also an integral component of the cellular membranes, and hence the transporters' immediate environment. It is well established that cholesterol is not uniformly distributed between membrane leaflets and regions of the plasma membrane [7]. The mechanism by which ABC cholesterol

Abbreviations: ABC, ATP binding cassette; TM, transmembrane; CRAC, Cholesterol Recognition/interaction Amino acid Consensus; CARC, inverted CRAC sequence; HDL, high density lipoprotein

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transporters interact with their substrates is currently unknown, including whether they contribute significantly to plasma membrane lipid asymmetry.

Proteins that interact with and/or bind cholesterol can contain characteristic amino acid sequences that play a role in that interaction [8]. One such sequence, the Cholesterol Recognition/interaction Amino acid Consensus (CRAC) motif, has been identified in proteins that interact with, or are regulated by, cholesterol. CRAC motifs can be located in or near the end of, a transmembrane helix or at a membrane interface, and are defined by the amino acid motif L/V-X(1–5)-Y-X(1–5)-R/K. Considering the somewhat generic sequence definition, these motifs only indicate a potential for being a cholesterol binding/interaction site, with biological data required to confirm their role [9]. In addition to CRAC motifs, an inverted CRAC sequence, termed a CARC motif, has recently been identified in the cholesterol-regulated nicotinic acetylcholine receptor. Modelling studies indicated this receptor accommodates and has high affinity for cholesterol [10]. The CARC sequence is R/K-X(1–5)-Y/F-X(1–5)-L/V, with the tyrosine permissibly replaced by a phenylalanine. CARC motifs are only thought to be present within transmembrane helices, with the R/K amino acid pushing as a "snorkel" out of the hydrophobic region [9].

Considering the lack of understanding regarding the mechanism by which ABCG-lipid transporters operate and bind their substrates, the aim of this paper was to investigate the potential role of CRAC and CARC motifs in the transport function of the ABCG1 cholesterol transporter.

2. Materials and methods

2.1. Reagents

Primers, protease inhibitor cocktail (P8340) and phosphatase inhibitor cocktail (P5726), BSA (essentially fatty acid free), chloroquine, ammonium chloride and MG132 were purchased from Sigma; Zeocin and all other cell culture media and reagents, including Lipofectamine transfection reagents, were purchased from Life Technologies; BCA protein reagents were purchased from Pierce; Anti-myc polyclonal antibody was from Abcam; anti-tubulin monoclonal antibody were from Sigma; ABCA1 monoclonal antibody was from LifeResearch; secondary anti-mouse and anti-rabbit antibodies were from Sigma; Plasmid purification kits were from Macherey-Nagel. [1α,2α(n)-^{3}H] cholesterol was from Perkin Elmer. ECL reagents were from Amersham. Reagents for SDS-PAGE, including acrylamide, Tris–HCl, glycine, SDS and TEMED were purchased from Amresco.

2.2. Cell culture

CHOK1 cells were cultured in Ham's F12 medium containing 10% (v/v) heat-inactivated foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). ABCG1 overexpressing cells, including cell lines with single point mutations, were cultured as CHOK1 cells with the addition of 200 μg/ml zeocin. In some experiments, cells were cholesterol-enriched by addition of cholesterol/cyclodextrin preparations, which were added to the culture media at concentrations indicated in the figure legends.

2.3. Preparation of constructs and stable cell-lines

Humans express two isoforms of ABCG1, previously described as ABCG1(−12) and ABCG1(+12), that differ in the absence or presence of a 12 amino acid linker between the ATP binding cassettes and the TM domains [11]. Constructs overexpressing either the 666 amino acid isoform ABCG1(−12) or the 678 amino acid isoform ABCG1(+12) were prepared as previously described [11–13]. Single and triple base pair point mutations were introduced in the ABCG1 constructs using a megaprimed PCR method [14] to introduce tyrosine to leucine changes at amino acids 649, 667 and 672 (numbering of amino acids is based on the 678 amino acid isoform as presented in Fig. 1A [11]). Primer sequences are available upon request. Mutations were confirmed by sequence verification.

Cell lines stably overexpressing ABCG1(−12) and ABCG1(+12) in CHOK1 cells have been described previously [11,12]. Cells overexpressing ABCG1 containing point mutations were produced in CHOK1 cells using the same method. After transfection, selection in 1 mg/ml zeocin, and single cell dilution, positive clones were maintained routinely in 200 μg/ml zeocin in Ham's F12 medium containing 10% FBS and additions as specified under "cell culture". Cell lines with comparable basal ABCG1 protein expression between cell lines overexpressing parental and mutated ABCG1 were selected. Cell lines were maintained and handled individually to avoid cross-contamination.

Cells overexpressing triple point mutations were produced in both the ABCG1(−12) and ABCG1(+12) backgrounds. Since the ABCG1(−12) isoform is more ubiquitously expressed amongst species [15], cells overexpressing single point mutations were only produced in the ABCG1(−12) background after results for the triple point mutations were similar in both isoform backgrounds.

2.4. ABCG1 protein expression

ABCG1 protein levels were measured using SDS-PAGE as described previously [13]. Briefly, cells were washed with PBS and lysed in 1% IgePal diluted in 50 mM Tris–HCl, 150 mM NaCl (pH 7.8) with the addition of protease and phosphatase inhibitor cocktails (5 μl/ml). Cell protein levels were measured using the BCA assay [13]. Equal amounts of cell protein per lane were separated using 8% SDS-PAGE. After transfer, membranes were blotted using anti-myc (1:5000), or tubulin (1:3000) antibodies. HRP-conjugated anti-mouse or anti-rabbit secondary antibodies were used at 1:10,000 and blots visualised using enhanced chemiluminescence. Protein expression levels were quantified using Image J software (version 1.45).

2.5. Cholesterol/cyclodextrin complexes

Preparations containing cholesterol complexed to methyl-ß-cyclodextrin (CD) were prepared as described [16]. Briefly, 500 μl of a 5% solution of CD was heated to 80 °C and 10 μl aliquots of cholesterol (15 mg/ml in ethanol) were added every 10 min while gently stirring (50 μl total) until all cholesterol was dissolved. The solution was snap-frozen on dry ice and the liquid evaporated via freeze-drying. Before use, the cholesterol/CD complexes were reconstituted in 1.5 ml sterile Baxter water and filter-sterilised to achieve a final stock concentration of 500 μg/ml cholesterol.

2.6. Cholesterol efflux assay

Cholesterol efflux assays were performed as previously described [13]. Briefly, cells were radio-labelled overnight with [1α,2α(n)-^{3}H] cholesterol at 1 μCi/ml in serum-containing medium. The labelling medium was removed and the cells washed twice with PBS, then incubated for 30 min in equilibration medium (serum-free F12 containing 0.1% essentially fatty acid free BSA). After equilibration, the cells were incubated for 6 h with equilibration media, with or without the addition of HDL_2 at 10 μg/ml (a generous gift from Professor Wendy Jessup, ANZAC Research Institute, Sydney, Australia). Media were collected and spun to remove