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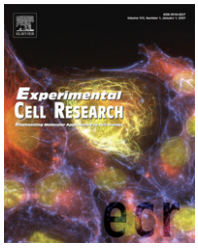

Research Article

Heme carrier protein 1 (HCP1) expression and functional analysis in the retina and retinal pigment epithelium

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ABSTRACT

The retina and retinal pigment epithelium (RPE) are present in the posterior segment of the eye, and the retina is dependent upon the underlying RPE for normal function. The retina is the most oxygenated tissue in the body but is isolated from the blood circulation by blood–retinal barriers. Metabolism of cellular oxygen involves heme but little is known about heme transport in the retina and RPE. Here we report the identification from bovine RPE of a heme transporter bHcp1 (bovine heme carrier protein 1) that is homologous to mouse intestinal HCP1 expressed in duodenal enterocytes. Similar to the mouse protein, bHcp1 exhibited heme uptake ability in *Xenopus* oocytes and localized to the cell membrane in cultured mammalian epithelium. Whereas *bHcp1* expression was detected only in bovine RPE, expression of its human homologue was identified in both retina and RPE. Furthermore, the data revealed low-level wider expression of human *HCP1* transcript in multiple tissues suggesting that it is responsible for heme transport in the body, not the intestine alone. Expression of *HCP1* in the RPE and retina indicates the mechanism of heme transport in these ocular tissues.

Introduction

Heme, ferrous protoporphyrin IX, is absorbed from diet by intestinal enterocytes as well as synthesized in the body from inorganic iron primarily in erythrocytes and hepatocytes [1]. Erythropoietic cells utilize heme for hemoglobin synthesis. In addition, cellular heme is involved in oxygen metabolism, electron transfer and in cell proliferation and differentiation [2]. It also serves as a major source of iron in the body as excess cellular heme is enzymatically degraded by heme oxygenases into iron that is reutilized by the cell [1]. The toxic effects of free heme, involving formation of reactive oxygen species, are reduced by its association with apohemoproteins in the cytosol and with hemopexin and albumin in the plasma. Hemoglobin released due to hemolysis associates with haptoglobin in the serum and is taken up by macrophages that degrade the heme moiety of hemoglobin into iron. Despite its pivotal role in living organisms, mechanisms of heme uptake and export are just beginning to be understood. Recently, heme carrier protein 1 (HCP1), a membrane transporter with heme uptake activity, was identified from duodenal enterocytes and defines the mechanism of intestinal heme absorption [3]. Evidence exists for receptor-mediated uptake of heme-hemopexin in many cell types but

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the identity of the receptor remains elusive [4–6]. Also, membrane transporters FLVCR and ABCG2 have been implicated in heme export from erythroids [7,8]. Whether the aforementioned heme transporters are ubiquitously distributed or are cell-type-specific is as yet unknown. Hence any effort in revealing the identity and/or distribution of the proteins involved in heme transport will be helpful in understanding the mechanisms regulating the homeostasis of this important molecule in various tissues of living organisms.

Transport of heme in tissues that are isolated from the circulating blood because of barrier cells such as brain and retina is vital for their function but poorly understood. The retina is a multilayered neural tissue at the posterior of the eye and lies adjacent to the retinal pigment epithelium (RPE). Tight junctions between the RPE cells form the outer blood–retinal barrier from the underlying vascular bed of choriocapillaris, and similar junctions between endothelial cells of the retinal vasculature form the inner blood–retinal barrier [9,10]. Physiological function and health of the retina are dependent upon continuous biochemical interaction with the RPE [11]. Because of phototransduction (conversion of light impulse into an electrical signal) in the retina, it is the most oxygenated tissue in the body [12,13]. High uptake and metabolism of oxygen in the retina are likely to require higher heme/hemoglobin transport in this tissue. In addition, high oxygenation and light exposure contribute to elevated levels of reactive oxygen intermediates (ROIs) in the retina that can react with free heme giving rise to reactive oxygen species [12]. Thus the importance of heme protective proteins in the retina cannot be overemphasized. As hemoprotein-bound heme is unlikely to cross the blood–retinal barriers [12], heme-binding proteins such as hemopexin, SOUL/HBP and haptoglobin are locally produced by retinal cells [12,14,15]. Moreover, uptake of heme-hemopexin and expression of heme oxygenases in cultured RPE cells indirectly indicate that retinal heme is taken up and catabolized by the RPE [14]. Increased expression of heme oxygenase 1 (HO-1) and ferritin (iron storage protein) in RPE cells in response to heme-hemopexin and heme uptake further suggests that similar to other cell types, the internalized heme is degraded by HO-1 and the released iron is stored by ferritin in the RPE [14]. Presence of heme protective proteins in the retina, degradation enzymes in the RPE and heme-hemopexin uptake by the RPE strongly point to the presence of heme transporter/s in the retina and RPE.

In an attempt to identify genes important in the retina and RPE, we earlier reported the isolation, through subtractive hybridization, of genes abundantly or predominantly expressed in the bovine retina and/or RPE [16]. Further investigation of one of these genes, GenBank accession number AF451172, specifically expressed in the bovine RPE, revealed it to be an orthologue of the newly identified mouse and human intestinal HCP1. Here we report the membrane localization and heme uptake ability of this RPE-specific bovine Hcp1. We also show, for the first time, ocular expression of the human intestinal HCP1 and its wider expression in non-ocular tissues. The data presented here suggest that HCP1 mediates heme transport in the retina and RPE, and that HCP1-mediated heme transport in mammalian species is not confined to intestinal cells but is shared by many other tissues in the body.

Materials and methods

DNA manipulation

To clone the full-length bovine *Hcp1* cDNA, the λ uniZap XR bovine eyecup cDNA library was screened using AF451172 cDNA as probe [17]. The isolated clones were sequenced and found to be longer than AF451172 but lacked the translation initiation codon and the 5′ untranslated region. Therefore 5′ RACE (rapid amplification of cDNA ends) was performed with the SMART RACE cDNA amplification kit (Clontech). For 5′ RACE, first strand bovine RPE cDNA was synthesized with gene-specific primer S812-2 (5′-ATGCCATGTGGCCAGTTCTG) and PCR was performed with nested gene-specific primer S812-RACE (5′-GCAAGAAGCCACCAATGAAGCTCGCCAG) and the universal primer mix included in the SMART RACE cDNA amplification kit. The amplified 5′-end cDNA was verified by sequencing and cloned upstream of the longest library clone to generate a full-length cDNA. The entire coding region of *bHcp1* was amplified from the full-length cDNA using bS812-GFP-F (5′-CCGCGGATCCTTGCGCCAACTCTG) and bS812-GFP-R2 (5′-TGCCGGTACCCTCTGGGAAAACTCCTG) primers, incorporating *Bam*HI and *Kpn*I restriction sites at the ends, and cloned at *Bgl*II/*Kpn*I sites in pEGFP-N1 to generate bHcp1-GFP expressing fusion construct.

Sub-cellular localization of the protein

MDCK (Madin Darby Canine Kidney) cells were grown in a humidified atmosphere at 37 °C and 5% CO_2. Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. For microscopy, $2–3\times10^5$ cells were seeded onto glass coverslips in 6-well plates and transfected on the third day with bHcp1-GFP construct or pEGFP-N1 using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Approximately 48 and 72 h post-transfection Golgi staining was performed in culture with 2 μM BODIPY-TR-ceramide for 2 h after which the cells were fixed in 4% paraformaldehyde, mounted on slides in buffered glycerol and viewed in an Olympus AX70 microscope attached to a Bio-Rad 1024 MRC scanning confocal system equipped with an Argon Ion and a Helium Neon laser using LaserSharp version 3.1 software. GFP was excited with 488 nm laser line and detected at 522 nm. BODIPY-TR-ceramide was excited with 589 nm laser line and detected at 617 nm.

Oocyte injection, surface expression and heme uptake assay

Oocyte preparation and maintenance were performed as described previously [18]. bHcp1-GFP cRNA was synthesized *in vitro* using linearized bHcp1-GFP cDNA cloned into oocyte expression vector pGem-He-Juel. Following the day of isolation, *Xenopus laevis* oocytes were injected with 20 ng bHcp1-GFP cRNA. After an expression period of 4–5 days, uptake of ^{55}Fe-heme (RI Consultants LLC, Hudson, NH, USA) was measured over a time period of 120 min. Because ^{55}Fe-heme is essentially insoluble in aqueous solutions, the dried ^{55}Fe-heme was dissolved in 100 μl methanol/chloroform/acetic acid