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論文審査委員	（主査）　教授　妹尾春樹 （副査）　教授　大森泰文　　教授　後藤明輝

学 位 論 文 内 容 要 旨

Characterization of glycosylphosphatidylinositol-anchored ceruloplasmin enriched in the apical plasma membrane of rat hepatocytes

（ラット肝細胞の頂端面側細胞膜に局在するＧＰＩ－セルロプラスミンの特性に関する研究）

申請者氏名　フローレスマリアジョリナルー

Purpose

Ceruloplasmin (Cp) is an acute-phase protein and a member of the multicopper oxidase family of enzymes. It has been implicated in iron metabolism because of its ferroxidase activity. It is expressed as soluble (sCp) or glycosylphosphatidylinositol-anchored ceruloplasmin (GPI-Cp) form; the former is primarily synthesized in the liver, and the latter is primarily found in the brain. Although recent studies reported GPI-Cp expression on hepatocytes, little is known regarding its presence in specific liver cell compartments and its possible involvement in liver pathophysiology. This study aimed to characterize the distribution of GPI-Cp in liver cells and specifically in the apical part of the plasma membrane.

Methods

Rat liver samples were preperfused, fixed, PIPLC treated and immunohistochemically stained with a polyclonal goat anti-Cp antibody. While, frozen liver and brain tissues were homogenized and lysed with RNAiso Plus. CDNA was synthesized with oligo(dT) primers according to the PrimeScript RT reagent Kit procedure (Takara Bio Inc.). The resulting cDNA strand was used as a template for PCR with specifically designed primers: Rat Cp, GPI-Cp, β-actin. The total-, apical- and basolateral- liver plasma membrane (tLPM), of both SD and LEC rats, were isolated as described previously（Cefaratti et al., 2000) and characterized using Alkaline Phosphatase, Na/K-ATPase and 5′-nucleotidase assays. In order to release GPI-Cp from the cell surface, isolated samples were incubated with bacterial enzyme PIPLC, and to prove Cp has a covalent linkage to GPI anchor, triton X114 extraction and phase partitioning was done on all samples. （Cefaratti et al., *J. Biol. Chem.*, 2000: 275, 3772-3780）

Results

GPI-anchored Cp expression on the surface of rat liver cells

We confirmed the presence of GPI-Cp at both mRNA and protein levels in the rat liver. GPI-Cp localization in the liver was demonstrated here by following results: (i) tissue-surface staining was markedly decreased after treatment with PI-PLC; (ii) the presence of mRNA corresponding to GPI-Cp was detected using RT-PCR; and (iii) Western blot analysis demonstrated that PI-PLC-treated membranes released a 148-kDa band that was immunoprecipitated by a polyclonal anti-Cp antibody.

Detection of GPI-Cp preferentially in aLPM more than bLPM

As presented, membrane-bound Cp was preferentially localized in aLPM of the plasma membrane. A significant difference was evident in the presence of GPI-Cp between the two membrane domains ($p < 0.001$). Furthermore, when both membranes were incubated with PIPLC, aLPM supernatant fractions revealed higher concentrations of released GPI-Cp compared with untreated ones, whereas treated aLPM pellets revealed lower immunoreactive Cp signals. Moreover, significant relevant bands were not detected in the basolateral membrane fraction.

Cp in membrane domains was covalently attached to GPI

We confirmed that Cp expressed in membrane fractions was covalently linked to GPI; immunoblotting of TX-114-treated samples was performed. All Cp from untreated membrane fractions primarily partitioned into the detergent phase because the GPI-anchored protein (containing an intact GPI anchor) partitioned into the detergent phase after TX-114 extraction because of the presence of hydrophobic lipid chains on the GPI anchor. As, PI-PLC digestion removes GPI-anchored proteins from the hydrophobic portions of the GPI anchor and causes these proteins to partition into the aqueous phase; these results support our data showing that PI-PLC-treated membranes exhibited higher Cp concentrations in the aqueous phase. These findings confirmed that Cp is directly anchored to the liver cell membrane by GPI rather than through association with some other GPI-anchored proteins.

Oxidase activity of GPI-Cp

The evidence that GPI-Cp has ferroxidase activity in the total and apical but not the basolateral membrane was proven using SDS-PAGE under nondenaturing conditions. Gels were stained with *p*-phenylenediamine, which produces a purple precipitate when oxidized.

Increased GPI-Cp expression during copper accumulation

We also showed increased GPI-Cp presence in liver cell membranes from LEC rats. LEC rat plasma membranes showed no specific localization of GPI-Cp between both membrane domains, in contrast to the apical preference of the normal SD membrane samples. In addition, the same 148-kDa band was present in LEC rat cellular membranes, although the amount of this protein was almost identical in aLPM and bLPM fractions. Furthermore, treatment of LEC membranes with PI-PLC allowed us to confirm that the attachment of Cp to both aLPM and bLPM occurs through its GPI anchor, after we observed a robust signal in the supernatant corresponding to the released GPI-Cp. Band signals of LEC rats were stronger compared with those of GPI-Cp bands from healthy SD rats. After PI-PLC treatment, supernatant fractions of LEC rats revealed higher immunoreactive Cp compared with corresponding pellet fractions, although LEC rat membranes still demonstrated higher Cp concentrations compared with those in membranes from samples from SD rats.

Conclusions

In conclusion, our findings extend previously reported data on localization of not only secretory Cp but also the membrane form of Cp (GPI-Cp) in the liver. Moreover, we were able to further pinpoint the location of GPI-Cp, which turned out to be the apical membrane of liver cells. The apical membrane has short transit on the basolateral side, suggesting that GPI-Cp, like other GPI-APs, follows the indirect or transcytosis pathway during protein sorting. More detailed research into its possible relevance to copper and iron metabolism in the liver is a subject of an upcoming project.

論文審査結果の要旨

主査：妹尾春樹

申請者：フローレス　マリア　ジョリナルー

Characterizationof glycosylphosphatidylinositol-anchored ceruloplasmin enriched in the apical plasma membrane of rat hepatocytes

（ラット肝細胞の頂端面側細胞膜に局在するＧＰＩ－セルロプラスミンの特性に関する研究）

Ceruloplasmin(Cp)は急性期炎症に出現するタンパク質で、多数の銅を含有する酸化酵素ファミリーの一つである。Cpはferroxidase活性を持つことから鉄代謝に関連している。分泌型 Cp (sCp) あるいは glycosylphosphatidylinositol をアンカーにもつ Cp (GPI -Cp) の２種類が知られており、前者は肝臓で生合成され、一方後者は脳に主に見いだされている。最近の研究で GPI-Cp が肝実質細胞で発現していることが報告されているが、肝臓細胞のどの部位に存在しているかという問題や肝臓の病態にどのように関与しているかについてほとんど知られていない。本研究では GPI-Cp が肝実質細胞のどの細胞膜に分布しているか、そしてその性質を検討したものである。

本論文の斬新さ、重要性、実験方法の正確性、表現の明瞭さは以下の通りである

1)　斬新さ

肝臓には先に報告された分泌型 Cp だけではなく膜型の Cp(GPI-Cp)が存在していることを報告している。さらに GPI-Cp の存在場所は肝実質細胞の頂端面膜であることを正確に示した。GPI-Cp は間接的あるいは transcytosis により基底側面膜から頂端面膜へ移送することを初めて提唱した点で斬新性がある。

2)　重要性

GPI - Cp が基底側面膜ではなく、頂端面膜で ferroxidase 活性示すことから、鉄代謝に何らかの重要な役割を担っているのではないかと推測している。そこで、肝臓の銅代謝と鉄代謝との関連性についての研究に大きく貢献できる可能性を示した。

3)　実験方法の正確性

ラット肝臓実質細胞の頂端面膜と基底側面膜は収量が極端に低いために、実験に供する量を得ることは大変困難である。しかし、それぞれの膜マーカーを用いて純度の高い膜を調整することに成功している。また、Western blotting, RT-PCR、免疫組織染色などの分子生物学的手法はよく熟練しており、正確性が高いと思われる。

4)　表現の明瞭さ

GPI-Cp の細胞膜での局在性やその性質に関して、その背景や研究戦略などを含めて明快に表現されていた。考察も熟慮の後が見られ、全体として簡潔・明瞭に記載された論文である。

以上に述べたように、本論文は学位を授与するに十分値する研究と判定された。