行政院國家科學委員會專題研究計畫　成果報告

以標籤蛋白吸附純化法研究與D型肝炎抗原作用之細胞蛋白

計畫類別：個別型計畫
計畫編號：NSC91-2315-B-002-011-
執行期間：91年08月01日至92年07月31日
執行單位：國立臺灣大學醫學院內科

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 10 月 31 日

中文摘要及

D 型肝炎病毒錄製二種同類型的 D 型抗原。小 D 型抗原為 D 型肝炎病毒複製所必須，而大 D 型抗原比小 D 型抗原多了 C 端 19 個胺基酸，不但可抑制病毒複製且為病毒組合所必須。目前已知 D 型抗原可與 RNA 聚合脢 II、核仁磷化蛋白 B23 及 nucleolin 結合，且經由此結合而調節病毒複製。但是 D 型抗原參與病毒複製的詳細機轉仍有待探討。大部分的蛋白質交互作用皆用酵母菌二雜交法來研究。此法只能探知二種蛋白質的交互作用。最近標籤蛋白吸附純化法加上質譜儀分析被成功應用於酵母菌的研究。有上千的多蛋白質複合體的個別蛋白被詳細區分出來。我們試了幾種標籤載體來測試與 D 型抗原形成複合體的細胞蛋白。結果以 pCMV-TAP 為最理想。 目前已找到幾種蛋白，正做質譜儀分析
關鍵詞：D 型抗原，標籤蛋白吸附純化法，質譜儀

英文摘要

Hepatitis delta virus (HDV) encodes two isoforms of delta antigens (HDAgs). The small form of HDAg (SHDAg) is required for HDV RNA replication, while the large form of HDAg (LHDAg), with additional C-terminal 19 amino acids, inhibits the viral replication and is required for viral assembly. Although HDAg was found to bind to RNA polymerase II, nucleolar phosphoprotein B23, and nucleolin and modulate HDV RNA replication, the detail mechanism and the role of HDAg in viral replication remain to be elucidated. Most protein interaction has been studied with yeast two-hybrid assay, which only detects interaction between two proteins. Recently, tagged-protein purification combined with mass spectrometry was applied successfully to characterize hundreds of distinct multiprotein complexes in the yeast. We had tried several tagged-vector and found pCMV-TAP, which contains proteinA and calmodulin was the best. we had found some proteins, which are characterized by mass spectrometry.

keywords;delta antigen, tagged-protein affinity purification, mass spectrometry

前言、

Infection with hepatitis delta virus (HDV), a satellite virus of hepatitis B virus (HBV), is associated with severe and sometimes fulminant hepatitis. HDV encodes two isoforms of delta antigens (HDAgs). The small form of HDAg (SHDAg)is required for HDV RNA replication, while the large form of HDAg (LHDAg), with additional C-terminal 19 amino acids, inhibits the viral replication and is required for envelopment of the HDV genomic RNA by hepatitis B virus surface antigens (HBsAg). The

HDV RNA is replicated via RNA-dependent RNA synthesis, which is thought to be mediated by host DNA-dependent RNA polymerase II (RNAPII) (1, 2). SHDAg binds RNAPII directly and stimulates transcription by displacing negative elongation factor NELF and promoting RNAPII elongation (3). The nucleolar phosphoprotein B23, involved in disparate functions including nuclear transport, cellular proliferation, and ribosome biogenesis, interacts with HDAg and modulates the HDV RNA replication (4). Nucleolin binding activity of HDAg is critical for its nucleolar targeting and is involved in the modulation of HDV replication (5). . The C-terminal 19 aa of LHDAg contains a neclear export signal (6), and is required for interaction with HBsAg (7). LHDAg is sufficient to inhibit HDV genomic RNA synthesis from the antigenomic RNA template. However, the synthesis of antigenomic RNA, including both the 1.7-kb HDV RNA and the 0.8-kb HDAg mRNA, from the genomic-sense RNA is resistant to inhibition by LHDAg (8). Despite these findings, the detailed mechanism and the role of HDAg in viral replication remain to be elucidated.

Most proteins function within complicated cellular pathways, interacting with other proteins either in pairs or as components of larger complexes. Protein interaction has been studied with yeast two-hybrid assay, which only detects interaction between two proteins. Recently, Gavin et al. (9) and Ho et al. (10) used approaches in which individual proteins are tagged and used to pull down associated proteins, which are then analyzed by mass spectrometry. The first group used tandem affinity purification (TAP) tag, which included a fusion cassette encoding calmodulin-binding peptide CBP, a TEV cleavage site, and protein A. The second group use single FLAG tag. They have characterized hundreds of distinct multiprotein complexes in the budding yeast Saccharomyces cerevisiae with this approach.

In this project, we will apply the same methodology to characterize cellular proteins that complexes with HDAg. Hopefully, this will give us a better understanding of how HDAg exerts its effect in HDV replication.

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研究方法、

Cloning and transfection

LHDAg and SHDAg were cloned into pcDNA4His/Max vector (Invitrogen), which adds 6xHis and Xpress epitope tags to N-terminus of HDAgs.
LHDAg and SHDAg were cloned into pCMV-TAP vector (EMBL), which adds calmodulin and protein A epitope tags to N-terminus of HDAgs.
To study SHDAg protein complex, HuH7 cell line was transfected with tagged-SHDAg and replication-deficient HDV dimer.
To study LHDAg protein complex, HuH7 cell line was transfected with tagged-LHDAg and replication-competent HDV trimer.

Capture of protein complexes

Fro pcDNA4 construct, transfected cell was lyzed under native condition, and purified sequencially using Probond Xpress resin and Anti-Xpress affinity column (Invitrogen) according manufacturer's manual.

For pCMV-Tap construct, transfected cell was lyzed under native condition, purified with IgG-Sepharose, cleaved with TEV protease, and purifyied with calmodulin affinity resin.

Mass Spectrometry

The purified proteins was concentrated, fractionated 10% SDS-PAGE gels and stained. The visible bands will be excised and sent for MALDI-TOF peptide mapping and bioinformatic analysis

結果與討論

The expressed tagged-HDAg of pcDNA4 constrcut was poorly purifyied either with Probend resin for 6xHis tag or anti-Xpress affinity resin for Xpress tag. The poor recovery of tagged protein probably due to non-exposure of 6XHis epitope or X-press epitope in nature condition or HDAg contains two many basic amino acids (arginine and lysine) which interferes with the action of histidine. After several trials we decided to seek for other vector. We applied from EMBL(izaurralde@embl-heidelberg.de) a pCMV-TAP vector , which contains calmodilin, protein A tags and a TEV protease site between the tags. However, EMBL sent us with a wrong clone twice, and later with a clone with wrong restriction map of multiple cloning sites. After working out all these hassles, we successfully constructed plasmids expressing tagged small and large HDAg. After transfecting these plasmids into Huh7 cells, cellular proteins were purified with IgG-sepharose and calmodulin resin, and subjected to SDS-PAGE. Several clear bands of proteins were noted, which did not appear in control group. Currently they are sent for MALDI-TOF analysis.

計畫成果自評部份

Although this project was delayed due to mistakes of EMBL and some laboratory trial and error, we finally get some cellular proteins that possibly interact with HDAg. Hopefully, after mass spectrometry analysis we can find out what those proteins are, and do further research to determine their roles in HDV replication.