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Subcellular quantitative proteomic analysis reveals host proteins involved in human cytomegalovirus infection

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ABSTRACT

Viral replication requires host cell macromolecules and energy, although host cells can alter their protein expression to restrict viral replication. To study the host cell response to human cytomegalovirus (HCMV) infection, a stable isotope labeling by amino acids in cell culture (SILAC)-based subcellular quantitative proteomic study of HCMV-infected human embryo lung fibroblast (HEL) cells was performed, and a total of 247 host proteins were identified as differentially regulated by HCMV. Western blotting and immunofluorescence confocal microscopy were performed to validate the data sets. Gene Ontology analysis indicated that cellular processes involving the metabolism, localization and immune system were regulated as a result of HCMV infection. Functional analysis of selected regulated proteins revealed that knockdown of HNRPD, PHB2 and UB2V2 can increase HCMV replication, while knockdown of A4 and KSRP resulted in decreased HCMV replication. Our study may improve our understanding of the dynamic interactions between HCMV and its host and provide multiple potential targets for anti-HCMV agent research.

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1. Introduction

Human cytomegalovirus (HCMV) belongs to the β-herpesvirinae family. As one of the largest animal viruses, a mature HCMV virion has a diameter of ~200 nm. With a linear double-stranded DNA genome ~230 kb in length, the HCMV genome encodes at least 166 open reading frames (ORFs). HCMV genes are expressed in a temporally coordinated manner, and they regulate a cascade of transcriptional events that lead to the synthesis of three categories of viral proteins that can be separated based on the timing of their expression, namely immediate-early (IE), early (E), and late (L) proteins. The transcription of E and L genes depends on the presence of functional IE proteins [1]. The IE genes begin to be expressed immediately after viral entry, while E genes begin to be expressed thereafter [2]. The products of E genes are mostly non-structural proteins, including viral DNA replication factors and proteins involved in immune evasion. L proteins mainly have structural roles and primarily contribute to the assembly and morphogenesis of virions [3]. HCMV is ubiquitous, infecting the majority of the world's population [2]. In immune-competent subjects, HCMV is usually asymptomatic [4]. However, in human immunodeficiency virus (HIV)-infected patients or organ transplant patients, HCMV can result in primary or recurrent infection [5]. HCMV is not only the leading infectious agent causing neonatal mental retardation and congenital malformation, as it is also linked to certain cardiovascular diseases, cancer and immunosenescence [6].

HCMV enters host cells via direct membrane fusion or receptor-mediated endocytosis [7] and replicates in the nuclei of infected cells [8]. During infection, the virus requires macromolecular precursors and energy derived from the host cell to replicate [9], and the antiviral response of the host cell becomes activated [10]. A comprehensive understanding of host–virus interactions could lead to the identification of potential drug targets and promote the development of novel antiviral agents [11]. Because host–virus interactions are complex, many recent studies have used large-scale screening approaches to study host–virus interactions [12], such as cDNA and siRNA library screening [13], haploid genetic screening [14], small molecule screening [15], and proteomics analysis [16]. To identify host cell metabolic enzymes that are required for the efficient production of HCMV progeny, Koyuncu et al. performed an siRNA screen targeting 401 cellular enzymes, including many enzymes involved in fatty acid and lipid metabolism, and concluded that multiple long chain acyl-CoA synthetases and fatty acid elongases are required for HCMV infection [17]. The limitation of siRNA screening is that off-target effects result in false-positive

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hits, while inefficient siRNA-mediated depletion can produce false-negative results [12]. Zhu et al. employed DNA array technology to monitor the expression levels of 6600 human mRNAs in HCMV-infected human foreskin fibroblast (HFF) cells and determined that the abundance of 258 mRNAs changed as a result of HCMV infection [18].

However, because the final executers of essentially all of the biological processes are proteins, and because mRNA and protein-level inconsistencies are well documented [19], systematic analysis at the proteome level is also very important for studies of virus–host interactions. Subcellular quantitative proteomics analysis, which aims to profile protein changes in a targeted subcellular fraction, has been used to effectively identify proteins involved in HCMV/host interactions. For example, Gudleski-O'Regan et al. performed a semi-quantitative analysis of the cell surface proteome in HCMV-infected MRC5 fibroblasts and determined that LRP1, a protein that is up-regulated during HCMV infection, can decrease the infectious virus yield [20]. Weekes et al. also performed quantitative analysis on the plasma proteins of HCMV-infected cells and monitored over 8,000 host proteins (including 1200 cell surface proteins) at different time points post-infection [21]. However, quantitative proteomics analysis of the cytoplasmic and nuclear fractions of HCMV-infected cells was not performed.

Here, a SILAC-based subcellular quantitative proteomics analysis was performed to profile the proteome of HCMV-infected human embryonic lung (HEL) cells. The HEL cell line was used in this study because it was reported to be highly permissive for infection by the HCMV Towne strain [22], and it is widely used as an in vitro model for the study of HCMV infection [23]. Immunofluorescence and Western blotting (WB) were performed to verify the mass spectrometry (MS) data. Gene Ontology (GO) analysis indicated that cellular processes involving metabolism, localization and immune system were dysregulated as a result of HCMV infection. To explore the associations between HCMV-regulated host proteins, Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) 9.1 analysis was performed on these proteins. An RNAi-based functional study on selected proteins identified Heterogeneous nuclear ribonucleoprotein D0 (HNRPD), Prohibitin-2 (PHB2) and ubiquitin-conjugating enzyme E2 variant 2 (UB2V2) as proteins that could be involved in anti-HCMV activities, while amyloid beta A4 protein (A4) and far upstream element-binding protein 2 (KSRP) could play roles in promoting HCMV replication. This is the first study to report the antiviral function of UB2V2 and PHB2, which promotes us to perform further investigation of these host factors and provides potential targets for anti-HCMV research.

2. Materials and methods

2.1. Materials

The HCMV Towne strain was kindly provided by Dr. Fenyong Liu (University of California-Berkeley, Berkeley, CA). HEL and HFF cells were gifts from Dr. Min-Hua Luo (Wuhan Institute of Virology, CAS). Both cell lines were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT) supplemented with 1% penicillin/streptomycin (Beyotime, Shanghai, China) and 10% fetal bovine serum (FBS, GIBCO, Waltham, MA).

For the SILAC analysis, HEL cells were grown in SILAC DMEM (Thermo Scientific, Waltham, MA) containing either L-$^{13}C_6^{15}N_4$-arginine and L-$^{13}C_6^{15}N_2$-lysine (R10K6, for "heavy" labeling) or L-$^{13}C_6$-arginine and L-$^{2}H_4$-lysine (R6K4, for "medium" labeling) supplemented with 1% penicillin/streptomycin (Beyotime, Shanghai, China) and 10% dialyzed FBS (Thermo Scientific, Waltham, MA). After six doublings, cells cultured in "R10K6" medium were infected with HCMV at a multiplicity of infection (MOI) of 1, while cells in "R6K4" medium were mock-treated. At 96 hpi, HCMV and mock-infected cells were collected, and mixed at a ratio of 1:1. Four biological replicates were performed.

2.2. Enrichment of cytoplasmic and nuclear proteins

Subcellular fractionation was performed with a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime, Shanghai, China). The cell pellets from 5×10^7 cells were washed with PBS (Hyclone, Logan, UT), re-suspended in pre-chilled Nuclear and Cytoplasmic Protein Extraction kit Buffer A, and incubated at 4 °C for 15 min. Then, Nuclear and Cytoplasmic Protein Extraction kit Buffer B was added, and the mixture was vortexed and centrifuged at 500 *g* for 5 min. The supernatant was collected as the cytoplasmic fraction. The pellet was re-suspended in Nuclear and Cytoplasmic Protein Extraction kit Buffer C, incubated at 4 °C for 15 min, and centrifuged at 13,200 *g* for 15 min. The supernatant was then collected as the nuclear fraction. For whole-cell protein extraction, cell pellets were re-suspended in RIPA buffer and centrifuged at 13,200 *g* for 15 min. The supernatant was collected as the whole-cell lysate fraction (WCL).

2.3. In-solution digestion and peptide fractionation

The proteins from the nuclear or cytoplasmic fractions in Nuclear and Cytoplasmic Protein Extraction kit Buffer were precipitated with 3 volume of 50% acetone/50% methanol/0.1% acetic acid, and centrifuged at 8000 *g* for 20 min. The pellets were re-suspended in 8 M urea/4 mM $CaCl_2$/0.2 M Tris–HCl, pH 8.0, reduced with 10 mM DTT at 50 °C for 30 min and alkylated with 40 mM IAA in the dark for 30 min. Then, the proteins were kept at room temperature for 60 min, and the protein concentration was measured with the Bradford assay. A total of 200 μg of protein was digested with trypsin (Sequencing Grade, Promega, Madison, WI) at a ratio of 1:50 (trypsin/protein, w/w) overnight. The digested peptides were desalted with a SepPak C18 cartridge (Waters, Milford, MA) and stored at −80 °C.

Desalted peptides were fractionated by strong cation exchange chromatography (SCX). SCX fractionations were performed on a PolySULFOETHYL Aspartamide column (2.1 mm × 50 mm, 5 μm, PolyLC, Columbia, MD). A 60 min gradient elution with SCX Buffer A (5 mM KH_2PO_4, pH 2.7, in 20% acetonitrile/80% ddH_2O) and SCX Buffer B (5 mM KH_2PO_4 and 0.5 M KCl, pH 2.7, in 20% acetonitrile/80% ddH_2O) was performed, and 10 fractions were collected. Each fraction was desalted with a ZiptipC18 micropipette tip (Millipore, Billerica, MA) and stored at −80 °C.

2.4. Mass spectrometry analysis

Fractionated peptides from replicates 1 and 2 were analyzed using a QStar Elite mass spectrometer (ABSCIEX, Mississauga, ON) coupled with an Eksigent Tempo nano MDLC system as described previously [24]. Peptides from replicates 3 and 4 were analyzed using a TripleTOF 5600+ mass spectrometer (ABSCIEX, Mississauga, ON) coupled with a splitless Ultra 1D Plus (Eksigent, Dublin, CA) system. Peptides were dissolved in 0.1% formic acid/2% acetonitrile/98% H_2O, loaded onto a C18 trap column (5 μm, 5 x 0.3 mm, Agilent, Santa Clara, CA) at a flow rate of 5 μL/min, and subsequently eluted from the trap column onto the C18 analytical column (75 μm × 150 mm, 3 μm particle size, 100 Å pore size, Eksigent, Dublin, CA) at a flow rate of 300 nL/min using a 100 min gradient (Fig. S1). The mobile phase consisted of two solutions: solution A was 3% DMSO/97% H_2O with 0.1% formic acid, and solution B was 3% DMSO/97% acetonitrile with 0.1% formic acid. DMSO was included in the mobile phase because it has been reported that a DMSO concentration equal to or below 5% can enhance the electrospray ionization of peptides [25]. Information-dependent acquisition (IDA) mode was used to acquire MS/MS spectra. Survey scans were acquired in 250 ms, and 40 product ion scans were collected in 50 ms per scan. The precursor ion range was set from m/z 350 to m/z 1500, and the product ion range was set from m/z 100 to m/z 1500.

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