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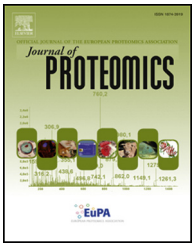

Quantitative proteomic analysis of Huh-7 cells infected with Dengue virus by label-free LC–MS☆

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ABSTRACT

Dengue is an important and growing public health problem worldwide with an estimated 100 million new clinical cases annually. Currently, no licensed drug or vaccine is available. During natural infection in humans, liver cells constitute one of the main targets of dengue virus (DENV) replication. However, a clear understanding of dengue pathogenesis remains elusive. In order to gain a better reading of the cross talk between virus and host cell proteins, we used a proteomics approach to analyze the host response to DENV infection in a hepatic cell line Huh-7. Differences in proteome expression were assayed 24 h post-infection using label-free LC–MS. Quantitative analysis revealed 155 differentially expressed proteins, 64 of which were up-regulated and 91 down-regulated. These results reveal an important decrease in the expression of enzymes involved in the glycolytic pathway, citrate cycle, and pyruvate metabolism. This study provides large-scale quantitative information regarding protein expression in the early stages of infection that should be useful for better compression of the pathogenesis of dengue.

Biological significance
Dengue disease is an important growing public health problem. Dengue infection involves alterations in the homeostasis of the host cell. Defining the interactions between virus and cell proteins should provide a better understanding of how viruses propagate and cause disease. Here, we present for the first time the proteomic analysis of hepatocytes (Huh-7 cells) infected with DENV-2 by label-free LC–MS.
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1. Introduction

Dengue ranks as the most important mosquito-borne viral disease in the world, with an estimated of 67 to 136 million new clinical cases, and many more non-clinical infections, annually [1]. Human infection with dengue virus (DENV) may be either asymptomatic or cause clinical symptoms, ranging from a mild flu-like syndrome known as dengue fever (DF), to the most severe forms of the disease: dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF and DSS can result in death due to leaking plasma, fluid accumulation, respiratory distress, severe bleeding, and organ impairment [2]. Currently, no specific therapies or vaccines are available.

Dengue viruses belong to the *Flaviviridae* family, and exist in nature as four different antigenic groups or serotypes (DENV1 to 4). DENV is an enveloped virus with a positive-sense single-stranded 11Kb RNA genome, coding for a poly-protein precursor of approximately 3400 amino acids, cleaved into three structural (capsid, C; precursor membrane and membrane, prM/M; envelope, E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) [3].

Dengue viruses access the cytoplasm of the host cell through receptor-mediated endocytosis [4]. In hepatocytes, many molecules have been proposed as receptors, such as heparan sulfate [5], 37/67-kDa high-affinity laminin [6], glucose-related protein-GRP78 (BIP) [7], and lymph node-specific ICAM-3-grabbing integrin (L-SIGN) [8]. Once inside the cell, replication of DENV occurs inside virus-induced membrane vesicles in the endoplasmic reticulum (ER) [9], which then bud off the ER and are transported through the trans-Golgi network, where they mature and form infectious particles [3].

The tropism of DENV is widespread. The primary sites of productive infection in humans are immune cells of the myeloid lineage (dendritic cells, monocytes and macrophages) [10] and hepatocytes [11]. The liver is involved in DENV infections in humans and mouse models [12,13]. Patients with severe dengue present hepatomegaly and high levels of serum transaminase. Also, different hepatic cell lines have been shown the relative permissiveness to DENV infection [14].

Different gene expression profiling studies in response to DENV infection have revealed up/down regulation of genes involved in the NF-Kβ immune response, the IFNα/β network, and the ubiquitin proteasome pathway [15,16]. However, few proteomic analyses have been conducted in order to study the cellular response to DENV infection. Studies using two-dimensional electrophoresis combined with mass spectrometry (2D-MS) have shown that some proteins are differentially expressed in the hepatoma cell line HepG2 [17,18], insect cells [19–21], and in blood samples of patients [22]. Yet, these traditional proteomics approaches are limited in their dynamic range and reproducibility [23].

Recently, the interactome of DENV and host proteins was studied using a yeast two-hybrid screening (Y2H) [24–28]. However, the role of these interactions during the infection was only analyzed for 12 proteins, reporting that calreticulin (CALR), ATP-dependent RNA helicase (DDX3X), rab6-interacting protein 2 (ERC1), golgin-95 (GOLGA2), thyroid receptor-interacting protein 11 (TRIP11), and SUMO-protein ligase (UBE2I) caused inhibition of virus replication [24].

The dengue virus genome encodes ten proteins that permit the exploitation of the host cell machinery in order to complete the infectious cycle and generate viral progeny. In recent years, studies have identified many host changes that occur in response to DENV infection, including the induction of different anti-viral pathways such as interferon responses, apoptosis, endoplasmic reticulum stress, autophagy, lipid metabolism, proteasome system, and RNA interference [29,30]. However, it is still poorly understood which proteins are involved in these cellular responses to dengue infection, and the directional changes in protein expression, which may be specific to cell type. In part, this limitation is related to the lack of a suitable animal model and the wide range of cell types capable of being infected by DENV.

In this work, we use a proteomic approach based on label-free MS to identify and quantify the changes in protein expression that result from DENV infection in hepatic cells.

2. Materials and methods

2.1. Cells

Huh-7 cells, from a differentiated hepatocyte-derived carcinoma cell line were kindly provided by Dr. Rosa María del Ángel (CINVESTAV-Mexico). Cells were grown in Advanced Dubelco Modified Eagle Medium (DMEM advance, Invitrogen, Life Technologies), supplemented with 5% fetal bovine serum (FBS), in humidified atmosphere 5% CO_2 at 37 °C. Baby hamster kidney-21 (BHK-21) cells were cultured in Eagle's minimal essential medium (MEM) (Invitrogen, Life Technologies-Thermo Fisher Scientific, USA), containing 10% FBS at 37 °C, 5% CO_2. *Aedes albopictus* C6/36 insect cells were grown in MEM supplemented with 10% FBS at 28 °C.

2.2. Virus growth

The virus used in this study (DENV-2 strain Yuc18110) was isolated from a patient with DHF during a dengue epidemic that occurred in 2007 in Merida, Mexico. Dr. Ma. Isabel Salazar, of the National Polytechnic Institute, Mexico, provided the virus strain. Propagation of DENV-2 was carried out in Balb/c suckling mice brains, as described previously [31]. Viral stock was obtained by inoculating a monolayer of C6/36 cells. Briefly, monolayer of C6/36 was infected at multiplicity of infection (MOI of 0.1); after 5 days, the medium was harvested, cleared from cellular debris by low speed centrifugation, aliquoted, and stored at −80 °C.

2.3. Viral titers

Dengue viral titers were determined by plaque assay on confluent monolayers of BHK-21 cells, grown in 24-well plates, and cultured in MEM, as described previously [32]. Briefly, when the adherent BHK-21 cells reached 80% confluence, 0.25 ml aliquots from mice brain homogenates or cell supernatants from dengue virus-infected C6/36 cells were inoculated with 10-fold serial dilutions. After 2 h of viral adsorption, the BHK-21 cell monolayers were overlaid with MEM containing 2% carboximethil-cellulose (Sigma-Aldrich, St. Louis, USA), 0.5%

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