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RESEARCH PAPER

Analysis of ISG15-Modified Proteins from A549 Cells in Response to Influenza Virus Infection by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract: Interferon stimulated gene 15 kDa protein (ISG15) is the first ubiquitin-like protein identified, which plays vital roles in a variety of fields including viral infection and immunological regulation. In this study, liquid chromatography-tandem mass spectrometry was used to analyze ISG15-modified proteins in A549 cells in response to infection by influenza virus, which were enriched by immunoprecipitation. A total of 22 cellular host proteins were identified in A549 cells infected by influenza virus, including ubiquitin-like ISG15 protein, cyclin-T1, heat shock protein 71 kDa, caldesmon, eukaryotic translation initiation factor, and so on. Besides, non-structural protein (NS1) from influenza virus was also identified. Among the 22 host proteins identified, 6 were also identified in the control non-infected A549 cells, including annexin A1, fructose-bisphosphate aldolase A, ATP synthase subunit g, enolase, actin, and tubulin. Bioinformatics analysis revealed that the identified ISG15-modified host proteins induced by influenza virus infection could be classified into 9 protein classes: chaperone, oxidoreductase, enzyme modulator, transferase, nucleic acid binding, transcription factor, kinase, cytoskeletal protein, and structural protein. This study provides a specific and effective tool for analyzing ISG15-modified proteins at proteome level.

Key Words: Influenza virus; Ubiquitin-like protein; Interferon stimulated gene 15 kDa protein; Liquid chromatography-tandem mass spectrometry

1 Introduction

Influenza viruses and other microorganisms can effectively replicate and circumvent the immune response in host cells through complex biological processes, including several important intracellular signaling pathways, such as WNT[1], NF-κB[2], MAPK[3], PI3K/Akt[4], etc. Post-translational modifications of proteins such as ubiquitination and ubiquitin-like modifications usually play a very important role during these processes, and they belong to some of the most inducible and reversible protein modifications[5]. The interferon stimulated gene 15 protein (ISG15) is one of these ubiquitin-like modifiers, and plays key roles in a variety of processes, such as innate immune response of host cells, regulation of infection and reproduction of pathogenic microorganism, etc[6,7]. ISG15, a 15-kDa protein induced by interferon, comprises two ubiquitin-like small subunits linked by a short hinge[8]. The modification of proteins by ISG15 is reversible and covalent, and can regulate the stability, functional activity and cellular localization of the substrate proteins[9]. In addition, it has been shown that the mice with deletion of ISG15 are often more susceptible to many kinds of

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viruses, including influenza, than the wild-type mice[10]. Further studies indicated that ISG15 modification can dramatically inhibit viral gene expression and replication in the cells of human origin, whereas a similar inhibitory effect was not observed in fibroblast cells from mice[11]. This finding indicates that ubiquitin-like modification, involving ISG15, plays an extremely important role during interaction between influenza virus and its infected human cells. Therefore, the analysis of the differentially expressed proteins with ubiquitin-like ISG15 modification in human cells upon influenza virus infection is very important for a better understanding of the molecular mechanism of interaction between human host and influenza virus.

Modern mass spectrometry has been extensively used in the field of protein structure analysis and large scale proteome analysis. In comparison with other analytical techniques, mass spectrometry has high resolution and high sensitivity, and has become one of the most important techniques in the analysis of the molecular structure of peptides and proteins[12–15]. Based on the high specific reaction between an antibody and an antigen, an immunoprecipitation technique was developed for the sample preparation and specific enrichment of the antigen from a complicated mixture using its antibody. At present, the combination of immunoassay and mass spectrometry has become a powerful tool for qualitative, semi-quantitative and quantitative analysis of complex biological samples. This technology combines the high specificity of traditional immunoassay with the high sensitivity and high resolution of mass spectrometry, enabling both detection and identification of proteins with a variety of post-translational modifications[16,17].

Currently, large-scale analysis of ubiquitin or ubiquitin-like modification is mainly focused on the characterization of ubiquitin (Ubiquitin) and ubiquitin like (SUMO, ISG15)-modified proteins in a variety of biological systems[18–21]. Large-scale analysis of ISG15-modified proteins in human host cells infected with the influenza virus has not been reported. In the present study, we used immunoprecipitation and liquid chromatography tandem mass spectrometry for the identification of the ISG15 modified proteins in the host cells upon influenza virus infection. First, the influenza virus H3N2 strain was inoculated into the human lung adenocarcinoma epithelial cell line A549. The ubiquitin-like ISG15 modified proteins were enriched and purified by immunoprecipitation, and were then analyzed by liquid chromatography coupled with mass spectrometry. Up to 22 host cellular proteins, including the ubiquitin-like ISG15 protein, were identified. Actually, a protein from influenza virus, a non-structural protein, was identified as the target of ISG15 modification. The results indicate that influenza virus infection can cause ubiquitin-like ISG15 modification on a variety of host proteins, suggesting that the immunoprecipitation coupled with liquid chromatography-mass spectrometry can be very effective to analyze and identify ubiquitin-like ISG15 modification of proteins.

2 Experimental

2.1 Instruments and reagents

The protein identification was carried out on a LTQ Orbitrap mass spectrometer (Thermo). The sample separation was performed on a BIO Wide Pore C_{18} HPLC column (150 mm × 0.18 mm, 5 μm, Sigma). Mini-PROTEAN Tetra Cell vertical electrophoresis system and Mini Trans-Blot Cell electrotransfer system (Bio-Rad), Allegra TM X-22R centrifugator (Beckman Coult), Microchemi Chemiluminescence system 4.2 (DNR Bio-Imaging Systems), Varioskan Flash microplate reader (Thermo), and Vertical Rotators (Qilin Medical Instrument Factory) were also used in the experiment.

Bradford protein assay kit, urea, ammonium bicarbonate, dithiothreitol, iodoacetamide were purchased from Bio-Rad. Sequencing-grade TPCK-modified trypsin was from Promega. Classic IP Kit was from Pierce. Formic acid, acetonitrile (ACN) and β-mercaptoethanol were from Sigma. Anti-ISG15 monoclonal antibody and HRP-labeled secondary antibody were from Santa Cruz. DMEM medium was from Hyclone. Fetal bovine serum (FBS) was from Gibco. BSA and PVDF were from Millipore. Enhanced Chemiluminescence kit (ECL Prime) came from GE Healthcare. All other chemicals with analytical grade were made in China. Human lung adenocarcinoma A549 cell line and human influenza virus H3N2 strain were kept in our laboratory. Ultra-pure water was prepared with a Milli-Q purification system (Millipore).

2.2 Virus infection

A549 cells were cultured in DMEM medium containing 10% FBS until the cell density reached 80%–90%, followed by washing with PBS three times. After the cells were incubated with FBS-free DMEM medium containing 0.2% trypsin for 2 h, a properly diluted influenza A virus H3N2 strain was inoculated onto the monolayers of the A549 cells. The infected cells were collected at 24 h post infection (hpi), and lyzed with the IP lysis buffer (pH 7.4, 25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP40, 5% glycerol). The lysate was cleared by centrifugation at 4 °C, 12000 rpm for 20 min, and the supernatant was then collected, aliquoted and kept at –80 °C until used. The protein concentration was determined with the Bradford protein assay kit.

2.3 Enrichment of ISG15-modified proteins by immunoprecipitation

The classic IP Kit from Pierce was used to enrich the ISG15-modified proteins. Prior to immunoprecipitation, the agarose beads, whose surface was covered with protein A/G, without antibody were added into the supernatant of the cell