

RESEARCH ARTICLE

Proteomics Analysis of Gastric Epithelial AGS Cells Infected with Epstein-Barr Virus

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Abstract

Effects of the Epstein-Barr virus (EBV) on cellular protein expression are essential for viral pathogenesis. To characterize the cellular response to EBV infection, differential proteomes of gastric epithelial AGS cells were analyzed with two-dimensional gel electrophoresis (2-DE) followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and liquid chromatography electrospray/ionization ion trap (LC-ESI-IT) mass spectrometry identification. Mass spectrometry identified 9 altered cellular proteins, including 5 up-regulated and 4 down-regulated proteins after EBV infection. Notably 2-DE analysis revealed that EBV infection induced increased expression of heat shock cognate 71 kDa protein, actin cytoplasmic 1, pyridoxine-5'-phosphate oxidase, caspase 9, and t-complex protein 1 subunit alpha. In addition, EBV infection considerably suppressed those cellular proteins of zinc finger protein 2, cyclin-dependent kinase 2, macrophage-capping protein, and growth/differentiation factor 11. Furthermore, the differential expressional levels of partial proteins (cyclin-dependent kinase 2 and caspase 9) were confirmed by Western blot analysis. Thus, this work effectively provided useful protein-related information to facilitate further investigation of the mechanisms underlying EBV infection and pathogenesis.

Keywords: Epstein-Barr virus - gastric epithelial cells - two-dimensional gel electrophoresis - mass spectrometry

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Introduction

Epstein-Barr virus is a member of the herpesvirus family and infects more than 90% of population worldwide (Epstein et al., 1964). It causes infectious mononucleosis and has been reported to be associated with various lymphoid and epithelial malignancies, including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, gastric carcinoma, etc (Young et al., 2004). EBV infection in gastric carcinoma (GC) was first reported by Burke et al. in 1990 (Burke et al., 1990) and 6~16% of GC cases worldwide were EBV-associated GC (EBVaGC). EBVaGC is a unique type of GC, consisting of the monoclonal growth of EBV-infected epithelial cells, and it represents a model of virus-host interactions leading to carcinoma (Fukayama et al., 2008). Nevertheless, the underlying mechanisms of EBV infection and pathogenesis in EBVaGC are not well understood at the present.

The response of a host cell upon viral infection often results in complex and numerous changes in gene expression. Recently the differentially expressed transcripts of EBV-infected AGS cells were analyzed by cDNA microarrays, which provided an overview of the mRNA expression profiles of infected cells (Marquitz et

al., 2012). However, the mRNA abundance is not always consistent with the protein level (Gygi et al., 1999), and viral infection results in post-translational modifications without affecting their transcription rates. Thus, proteomic analyses have also been employed to better understand host alterations to virus infection.

To date, a small but increasing number of studies have used proteomics approaches to reveal the effects of viral infection on the cellular proteome (Maxwell et al., 2007). These include investigations of pseudorabies virus-infected cells (Skiba et al., 2008), coronavirus-infected cells (Emmott et al., 2010), and alterations to the nucleolar proteome in adenovirus- (Lam et al., 2010) and coronavirus- (Emmott et al., 2010) infected cells. The comparative proteomics approaches coupling 2-DE and MS (2-DE/MS) effectively help the study of the molecular profiles of virus-infected cells.

In this study, we utilized a 2-DE/MS proteomics approach followed by Western blot to observe the differentially expressed protein profiles between EBV-infected and mock-infected AGS cells. A total of 9 differentially expressed protein spots were identified. Further analysis of these data provides clues to understanding the pathogenesis of EBV and the virus-host interactions.

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Materials and Methods

Cell lines and viral infection

The human gastric epithelial cell line AGS was provided by the Cell Center of Xiangya Medical School (Hunan, China). For EBV infection, the EBV-negative human gastric epithelial cell line AGS was infected with recombinant EBV using the cell-to-cell contact method (Imai et al., 1998). A Burkitt's lymphoma cell line Akata (generous gift from Dr. Jian-pin Liu) was modified to produce recombinant EBV, in which the neomycin resistance gene (Neor) is inserted into the BXLFI site of EBV DNA, and was used as a source of the virus. Following incubation in selective media, drug-resistant cell clones were isolated (Shimizu et al., 1996; Yoshiyama et al., 1997). Establishment of the EBV-infected AGS cells (AGS-EBV) was confirmed by EBER in situ hybridization (ISH). Parallel mock-infected AGS cells were used as control. Both the AGS and AGS-EBV cells were maintained in Ham's F12 medium, supplemented with 10% fetal bovine serum, antibiotics, and G418 at 400 µg/mL (AGS-EBV cells only).

Preparation of the protein samples

The cells were harvested and lysed in lysis buffer of 7 M urea, 2 M thiourea, 100 mM dithiothreitol (DTT), 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris, 2% Pharmalyte (GE Healthcare, Piscataway, USA) and 1 mg/mL DNase I. The lysates were incubated at 37 °C for 1 h, then centrifuged at 10,000 g for 30 min at 4 °C. The supernatant was transferred to a fresh tube, and stored at -80 °C until use. The concentration of the total proteins was determined using a 2-D Quantification kit (Amersham Biosciences).

Two-Dimensional Electrophoresis and Image Analysis

2-DE was carried out as described by the manufacturer (Amersham Biosciences). Protein samples (1000 mg for preparative gels) were diluted to 450 µL with rehydration solution (7 M urea, 2 M thiourea, 0.2% DTT, 0.5% (V/V) immobilized pH gradient buffer, and trace bromophenol blue), and applied to 18 cm pH 3-10 nonlinear immobilized pH gradient (IPG) strips by 24 h rehydration at 30 V. The rehydrated strips were automatically focused successively for 1 h at 100 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, and maintained at 8000 V until 90 kVh were reached in total on an IPGphor. Focused IPG strips were equilibrated for 15 min in a solution (6 M urea, 2% sodium dodecyl sulfate, 30% glycerol, 50 mM Tris-HCl, pH 8.8, and 1% DTT), then for an additional 15 min in the same solution except that DTT was replaced by 2.5% iodoacetamide. After equilibration, second dimension sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on an Ettan DALT II system (Amersham Biosciences). The above processes were performed in triplicate for each sample. The separated proteins were visualized by a sensitive colloidal Coomassie Brilliant Blue G-250 (Bio-Rad) staining described by Candiano and Bruschi (Candiano et al., 2004). After staining, the stained 2-DE gels were scanned with LabScan software on Imagescanner (Amersham Biosciences). ImageMaster

2D Elite 4.01 analysis software (Amersham Biosciences) was used for spot-intensity calibration, spot detection, background abstraction, matching, 1-D calibration, and the establishment of average-gel. To minimize the experimental variations, three separate gels were prepared for each group. The gel spot pattern of each gel was summarized in a standard after spot matching. Thus, we obtained one standard gel for each cell line. These standards were then matched. The following criteria for differential protein expression were used: an increase or decrease in spot intensity of higher than 2 folds in EBV-infected AGS compared with mock-infected AGS cells.

In-gel protein digestion

All the differentially expressed protein spots were manually excised from the gels and transferred to 1.5 ml siliconized Eppendorf tubes. One protein-free gel piece was treated in parallel as a negative control. The gel pieces were washed three times with double distilled water. The fresh solution containing 100 mM NH₄HCO₃ in 50% Acrylonitrile was used to decolor. The gel pieces were dried in a vacuum centrifuge. The dried gel pieces were incubated in 10 ml of digestion solution consisting of 40 mM NH₄HCO₃ in 9% Acrylonitrile solution, and 20 mg/ml proteomics grade trypsin for 10 to 12 h at 37 °C.

MALDI-TOF and LC-ESI-IT MS identification of differentially expressed proteins

For matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), the tryptic peptide mixture was extracted and purified with a ZipTip 18C column (Millipore, Billerica, USA). The purified tryptic peptide mixture was mixed with α -cyano-4-hydroxycinnamic acid (CCA) matrix solution, and vortexed gently. A volume (2 µL) of the mixture containing CCA matrix was loaded on a stainless steel plate and air-dried, and analyzed with the Voyager System DE STR 4307 MALDI-TOF mass spectrometer (Applied Biosystems). The parameters of MALDI-TOF were set up as follows: positive ion-reflector mode; accelerating voltage 20 kV; grid voltage 64.5%; mirror voltage ratio 1.12; N₂-laser wavelength 337 nm; pulse width 3 ns; the number of laser shots 50; acquisition mass range 800–3000 Da; delay 100 ns; and vacuum degree 4×10⁻⁷ Torr. In Peptide Mass Fingerprinting (PMF) map database searching, Mascot Distiller was used to get the monoisotopic peak list from the raw mass spectrometry files. The monoisotopic peak list was used to search the NCBI nr or SwissProt databases with the Mascot software (Matrix Science, London, UK). The searching parameter was set up as follows: the taxonomy was selected as Homo sapiens; the mass tolerance was ±200 p.p.m; the missed cleavage sites were allowed up to one; the fixed modifications were selected as carboxymethyl (cysteine); and the variable modification was selected as oxidation (methylation) or none.

MALDI-TOF MS may not be capable of identifying all proteins in this analysis. Unidentified peptide mixtures by MALDI-TOF-MS were analyzed by liquid chromatography electrospray/ionization ion trap mass spectrometry (LC-ESI-IT MS). The tryptic peptide

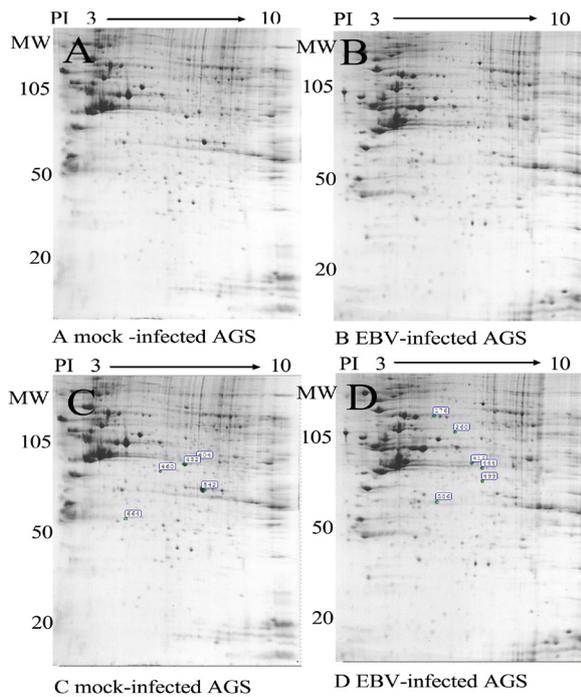


Figure 1. Protein Expression Profiles of the EBV-Infected and Mock-infected AGS Cells: (A) and (B), representative 2-DE gel images of mock-infected and EBV-infected AGS cells. (C) and (D), location map of the differentially expressed protein spots. The differentially expressed protein spots are shown in gels with their unique samplespot protein numbers. Nine spots were identified successfully by mass spectrometry in 11 differentially expressed protein spots (Table 1)

mixture was extracted and injected into a capillary LC system (Agilent 1200). The outlet of the LC system was directly connected to the nanoelectrospray source of an HCT ultra IT mass spectrometer (Bruker Daltonics, Germany). The peptides were detected in the positive ion MS mode or the data-dependent MS/MS mode. For data processing, raw spectra were formatted in Data Analysis 3.4 software (Bruker Daltonics). The produced .mgf files were analyzed using the Mascot software (Matrix Science, London, UK). The searches were performed against the NCBI database; Search parameters were set as follow: Homo sapiens; trypsin enzyme, allowing up to 1 missed cleavage; peptide mass tolerance was 2 Da and MS/MS mass tolerance was 0.8 Da; fixed modification parameter was carbamidomethylation(C), and the variable modification parameter was oxidation (Met) or none.

Western blot analysis of cyclin-dependent kinase 2 (CDK2) and caspase 9 (CASP9)

After the analysis of selected proteins, CDK2 and CASP9 were selected to validate the 2-DE spot protein content results. Samples of EBV-infected and mock-infected AGS cells were lysed as described above and protein extracts (50 µg) were separated on a 12% SDS-polyacrylamide gel. Proteins were then transferred to a poly-vinylidene difluoride membrane (Bio-Rad). After blocking, membranes were incubated with a polyclonal rabbit anti-CDK2 antibody (1:1000 dilutions, Abzoom, USA) and a polyclonal rabbit anti-CASP9

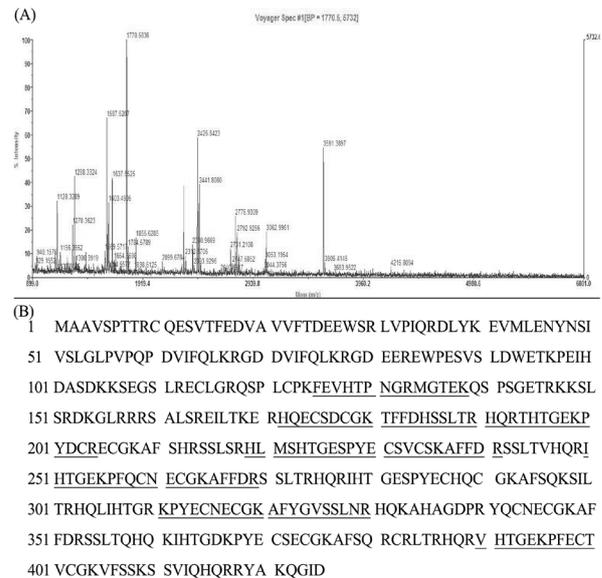


Figure 2. Matrix-assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) Analysis of Differential Protein Spot No. 542 (A) The MALDI-TOF MS mass spectrum of spot No. 542, identified as the zinc finger protein 2 (ZNF2) according to the matched peaks. (B) Protein sequence of ZNF2 and matched peptides are indicated underlined

antibody (1:1000 dilution; Abzoom, USA) for 16 h at 4 °C. Subsequently the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit ,Abzoom, USA) for 2 h at room temperature. The specific proteins were visualized with an enhanced chemiluminescence detection reagent (Abzoom, USA). As a control for equal protein loading, blots were restained using anti-actin antibody (1:1000 dilution, Abzoom, USA). The band intensity was analyzed with the PDQuest software version 7.1. The amount of relative expression was calculated as the intensity ratio of CDK2 and CASP9 to that of actin.

Results

Two-dimensional gel electrophoresis profiles of EBV-infected AGS cells

To examine the effects of EBV infection on the host cell proteome, the cellular proteins of EBV-infected and mock-infected AGS cells were extracted for 2-DE analysis. Representative gels of EBV-infected and mock-infected AGS cells extracts are shown in Figure 1A and B. In the 2-DE analysis, 891±32 protein spots were detected on the 2-DE gels in EBV-infected AGS cells, which localized in the ranges of pI 3-10 and MW 10-100 kDa. In the mock-infected AGS cells, 886±23 protein spots were detected on the 2-DE gels, which localized in the ranges of pI 3-10 and MW 10-100 kDa. A total of 824 spots were matched each other. On the basis of the average intensity ratios of protein spots, differentially stained proteins were evaluated by the ImageMaster 2D Elite 4.01 analysis software. The protein spots that showed 2-fold greater differential expression were considered to be differentially expressed candidates (Figure 1C and D). In this study, 11 significantly different candidate protein

Table 1. List of the Differentially Expressed Proteins in EBV-infected AGS Cells Identified by MALDI-TOF and LC-ESI-IT MS

| Spot no. ^a | Protein identified | Gene identified | Accession number ^b | MW/PI ^c | Protein Coverage(%) ^d | Identification method | Fold change | GO analysis(molecular function) |
|-------------------------|-----------------------------------|-----------------|-------------------------------|--------------------|----------------------------------|-----------------------|-------------|--|
| 176 | Heat shock cognate 71 kDa protein | HSPA8 | gil5729877 | 71082/5.37 | 20 | LC-ESI-IT | 2.89 | ATP binding; ATPase activity, coupled. |
| 412 | Actin cytoplasmic 1 | ATCB | gil15277503 | 40536/5.55 | 20 | MALDI | 3.12 | ATP binding; structural constituent of cytoskeleton |
| 444 | Pyridoxine-5'-phosphate oxidase | PNPO | gil8922498 | 30311/6.62 | 6 | LC-ESI-IT | 2.57 | Catalyzes the oxidation of either pyridoxine |
| 499 | Caspase 9 | CASP9 | gil54300706 | 17528/5.93 | 19 | LC-ESI-IT | 3.92 | 5'-phosphate (PNP) or pyridoxamine 5'-phosphate (PMP) into pyridoxal 5'-phosphate (PLP) Involved in the activation cascade of caspases; |
| 260 | T-complex protein 1 subunit alpha | TCP1a | gil57863257 | 60819/5.80 | 29 | MALDI | 2.47 | responsible for apoptosis execution.cysteine-type endopeptidase activity;enzyme activator activity Molecular chaperone; assists the folding of proteins upon ATP hydrolysis |
| Down-regulated proteins | | | | | | | | |
| 542 | Zinc finger protein 2 | ZNF2 | gil124053426 | 49930/8.86 | 32 | MALDI | -2.08 | DNA binding; zinc ion binding |
| 432 | Cyclin-dependent kinase 2 | CDK2 | gil312803 | 34077/8.80 | 4 | LC-ESI-IT | -3.67 | ATP binding; cyclin-dependent protein kinase activity; metal ion binding |
| 460 | Macrophage-capping protein | CAP-G | gil187456 | 38779/5.88 | 29 | MALDI | -2.79 | Actin capping |
| 664 | Growth/differentiation factor 11 | GDF11 | gil13236528 | 54990/7.05 | 3 | LC-ESI-IT | -2.51 | Transferase activity |

^a Spot no. is the unique sample spot protein number that refers to the labels in Figure 1; ^b NCBI database accession number; ^c Theoretical molecular mass (kDa) and theoretical Pi; ^d Sequence coverage of MALDI and LC-ESI-IT MS analysis

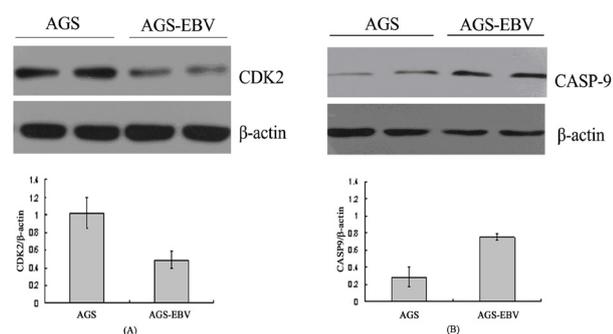


Figure 3. Western Blotting Analysis of CDK2 and CASP9 in EBV-infected AGS Cells (AGS-EBV) and Mock-infected AGS Cells (AGS). (A) Down-regulation of CDK2 in AGS-EBV compared to AGS. The specific proteins were visualized with a chemiluminescent reagent. As a control for equal protein loading, blots were restained with antiactin antibody. Immuno-signals were quantified by densitometry scanning. The relative quantification was calculated as the ratio of CDK2 to actin as shown in the bar graph. (B) Up-regulation of CASP9 in AGS-EBV compared to AGS. The relative quantification was calculated as the ratio of CASP9 to β -actin as also shown in the bar graph. Error bar reveals standard deviation

spots were found. Six protein spots were up-regulated and 5 down-regulated in EBV-infected AGS cells compared to mock-infected AGS cells.

Mass Spectral identification of differentially expressed proteins

To identify the differentially expressed proteins in EBV-infected AGS cells in 2-DE gels, all 11 differentially-expressed protein spots were excised from these 2-DE gels and subjected to in-gel trypsin digestion and subsequent analyzed by MALDI-TOF MS. Four of the selected 11 protein spots were identified successfully by MALDI-TOF MS and database search. Figure 2 shows an example of a MALDI-TOF peptide mass fingerprint of protein spot No.542. Since it is possible that several protein spots have very low abundance, MALDI-TOF MS may not be capable of identifying all proteins in this analysis. Therefore, complementary identification was obtained by using LC-ESI-IT MS for the undetected 7 protein spots. Five protein spots were identified successfully by LC-ESI-IT MS and NCBI database search. For example, the peptide sequence of protein spot No. 432 by LC-ESI-IT MS was matched to the cyclin-dependent

kinase 2. We failed to detect 2 protein spots. In EBV-infected AGS cells, the up-regulated spots corresponded to the following 5 proteins: heat shock cognate 71 kDa protein, actin cytoplasmic 1, pyridoxine- 5'-phosphate oxidase, caspase-9, and t-complex protein 1 subunit alpha; the down-regulated spots were found to correspond to the following 4 proteins: zinc finger protein 2, cyclin-dependent kinase 2, macrophage-capping protein, and growth/differentiation factor 11. Table 1 summarizes the results, with 5 proteins found to be up-regulated and 4 down-regulated in response to EBV infection.

Validation of CDK2 and CASP9 by Western blot

To validate a selection of the 2-DE proteomic data, the two proteins CDK2 and CASP9 were selected for Western blot analysis. Equal amounts of cell lysates of EBV-infected and mock-infected AGS cells were examined by Western blot analysis with specific antibodies to CDK2 and CASP9. Figure 3 shows the representative Western blot result of CDK2 and CASP-9 expression in EBV-infected AGS cells. In Western blot analysis, the expression of CDK2 was shown to be up-regulated in the EBV-infected AGS, and the expression of CASP-9 was shown to be down-regulated in the EBV-infected AGS. These data agreed with the expression changes shown by the 2-DE analysis.

Discussion

Increasing evidence emphasizes comparative proteomics to screen the differentially expressed proteins associated with host cellular pathophysiological processes of virus infection (Maxwell et al., 2007). The goal of this study was to analyze the effects of EBV infection on cellular protein expression in order to gain further insight into EBV pathogenesis. In our study, we used the 2-DE approach coupled with MS to study the differentially expressed proteins of gastric epithelial AGS cell, with and without EBV infection. The same system has previously been used to analyze differences in gene expression using cDNA microarrays (Marquitz et al., 2012). In the current study, we obtained an overview of the altered protein expressions of host cells responding to EBV infection. According to annotations from the UniProt Knowledgebase and Gene Ontology Database,

the 9 identified cellular proteins could be classified into several functional classes: cell cycle regulation protein (cyclin-dependent kinase 2), apoptosis (caspase 9), chaperones (heat shock cognate 71 kDa protein, T-complex protein 1 subunit alpha), cytoskeletal protein (actin cytoplasmic 1), metabolic enzyme (pyridoxine-5'-phosphate oxidase), transcription factor (zinc finger protein 2), and others (macrophage capping protein, growth/differentiation factor 11). Two of the nine identified cellular proteins, CDK2 and CASP-9, were selected for Western blot analysis. The two proteins were selected since they represent some important functional classes, including cell cycle regulation and apoptosis, and because they had not been reported previously in relation to EBV infection.

CDK2 is a member of a highly conserved family of protein kinases that regulate the eukaryotic cell cycle (Manning et al., 2002). CDK2 plays a critical role in the transition through S phase and the S to G2 transition. A number of viruses have been reported to interact with the cell cycle to create the optimal conditions for viral replication. Coronavirus infectious bronchitis virus arrests cells in G2/M to increase viral protein translation and progeny virus production (Dove et al., 2006). Measles virus can arrest cells in the G0 phase to prevent an antiviral response (Naniche et al., 1999). In the present study, the proteomic analysis and subsequent validation with Western blot, confirmed that CDK2 expression was down-regulated in EBV-infected AGS cells. Hence, we speculate that the inhibition of CDK2 expression in EBV-infected cells may create conditions more favorable for viral replication.

CASP9 is one of the 12 cysteine-dependent aspartate-specific proteases (caspases) that have been identified in humans. Caspases are instrumental to the execution of apoptosis. CASP9 is the initiator caspase and plays a central role in the mitochondrial or intrinsic apoptotic pathway of apoptosis (Li et al., 1997; Budihardjo et al., 1999). In our study, the proteomic analysis and subsequent validation with Western blot analysis, confirmed that expression of CASP9 was up-regulated in EBV-infected AGS cells. Caspases are implicated in virus infection and host defense response against virus (Shi, 2002). Numerous signals, including virus infection, can induce host cell apoptosis by activating initiator caspase to initiate the execution of cell death program (Lee et al., 2011). In insect, apoptosis has already been shown to be an effective defense response against baculovirus infection (Clem, 2005). However, the role of CASP9 in EBV-infected AGS cells remains unexplored. This study suggests that regulation of apoptosis could be one of the defense mechanism against EBV infection.

Of the 7 other differentially expressed proteins, 4 were up-regulated and 3 down-regulated in EBV-infected AGS cells. The up-regulated cellular proteins contain chaperones, cytoskeletal protein, and metabolic enzyme. Heat shock cognate 71 kDa protein (HSPA8) is a constitutively expressed chaperone that is also inducible by heat shock stress. It binds to nascent polypeptides to facilitate correct folding and also participates in the formation and repair of higher order protein structures such

as that of ATPase (Dworniczak et al., 1987; Egerton et al., 1996; Tsukahara et al., 2000). T-complex protein 1 subunit alpha (TCP1a) is a selective molecular chaperone in tubulin biogenesis and assists the folding of proteins upon ATP hydrolysis (Schuller et al., 2001). Actin cytoplasmic 1 (ATCB) belongs to the actin family and helps to maintain cell shape and enable intracellular transport (Procaccio et al., 2006). Pyridoxine-5'-phosphate oxidase (PNPO) is an enzyme that catalyzes several reactions in the vitamin B6 metabolism pathway (Musayev et al., 2003). The down-regulated cellular proteins include transcription factor, calcium-sensitive protein, and growth factor. Zinc finger protein 2 (ZNF2) is one of the most important transcription factors in eukaryotes, playing a very important role in regulating the genes (Rosati et al., 1991). Macrophage-capping protein (CAP-G) is a calcium-sensitive protein. It may play an important role in macrophage function (Pellieux et al., 2003). Growth/differentiation factor 11 (GDF11) is a transforming growth factor beta family member and plays a role in regulation of development and differentiation (Esquela et al., 2003). Clearly further large scale studies are necessary to understand the roles of these differentially expressed cellular proteins in EBV infection.

In conclusion, this study adopted a gel-based proteomics approach to probe the changed proteins in EBV-infected AGS cells. It is noteworthy that the comparative proteomics approach allowed for the initial identification of 9 altered cellular proteins during EBV infection and showed that partial of the altered cellular proteins appear to have roles in revealing the viral pathogenesis. Thus, this work contributes to uncover the underlying mechanism of EBV infection and pathogenesis.

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