IDENTIFICATION OF TUMOR NECROSIS FACTOR SIGNALING-RELATED PROTEINS DURING EPSTEIN-BARR VIRUS-INDUCED B CELL TRANSFORMATION

J.-P. JEON, J.-W. KIM, B. PARK†, H.-Y. NAM, S.-M. SHIM, M.-H. LEE, B.-G. HAN*

Korea BioBank, Center for Genome Science, Korea National Institute of Health, Seoul, Korea, 122-701; †Institute of Life Science and Biotechnology, Yonsei University, Seoul, Korea

Received February 13, 2008; accepted July 8, 2008

Summary. – Epstein-Barr virus (EBV) infection *in vitro* transforms primary B cells into continuously proliferating lymphoblastoid cell lines (LCLs) that have been widely used as a genomic resource for variety of immunological and genetic studies. However, the biochemical and biological characteristics that distinguish LCLs from the B cells have not been thoroughly investigated. Our proteomic approach showed that EBV infection induced changes in the profiles of tumor necrosis factor (TNF) signaling-related proteins in LCLs including heat shock protein family members TNF receptor-associated protein 1 (TRAP-1), heat shock 70-kDa protein 9 (HSPA9)) and superoxide dismutase 2 (SOD2). In addition, our literature co-occurrence study placed TNF at the center of a gene cluster network of differentially expressed proteins in LCLs. This study suggested that deregulation of TNF signaling pathway could contribute to the cellular transformation and immortalization of the EBV-infected B cells.

Key words: Epstein-Barr virus; lymphoblastoid cell line; proteomics; tumor necrosis factor

Introduction

EBV is a B-lymphotropic herpesvirus that infects more than 90% of the human population (Young and Rickinson, 2004). Although the majority of carriers remain asymptomatic, EBV has been implicated in the pathogenesis of B cell malignancies including Burkitt's lymphomas and epithelial carcinomas (Middeldorp *et al*., 2003). EBV infection of primary B cells *in vitro* induces B cell activation and transformation into continuously proliferating LCLs that express all latent EBV genes. In contrast, most EBV-associated B cell malignancies do not express all latent genes, but only particular sets of latent genes (Bishop and Busch, 2002; Rickinson, 2002). With respect to the cell growth, EBV infection mimics the effect of B cell activation signals as interaction CD40-CD40L or presence of IgM and LPS in such a way that EBV-infected B cells exhibit higher proliferation activity than resting B cells (Kilger *et al*., 1998*)*.

Proteomic and cDNA microarray analyses have led to the identification of many cellular genes as direct or indirect targets of EBV infection (Spender *et al*., 2001; Carter *et al*., 2000). For example, Epstein-Barr nuclear antigen 1 (EBNA-1) induces production of recombination-activating genes 1 and 2 (RAG-1 and RAG-2). Aberrant RAG-1 and RAG-2 activity may contribute to a chromosomal translocation in B cell neoplasm and may control chromosomal integration of EBV DNA (Humme *et al*., 2003; Srinivas and Sixbey, 1995).

*Corresponding author. E-mail: bokghee@yahoo.com; fax: +82-2-354-1078.

Abbreviations: EBNA-1,2,3 = EBV nuclear antigen 1,2,3; EBV = Epstein-Barr virus; EEF2 = eukaryotic translation elongation factor 2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; 2-DE = two-dimensional gel electrophoresis; HSPA9 = heat shock 70-kDa protein 9; LCLs = lymphoblastoid cell lines; LMP-1 = latent membrane protein 1; LMNA = lamin A/C; MALDI-TOF/MS = Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry; MALDI-QTOF = Matrix Assisted Laser Desorption Ionization Quadrupole Time-of-Flight.; p.i. = post infection; RAG-1,2 = recombination-activating gene 1,2; ROS = reactive oxygen species; SOD2 = superoxide dismutase 2; TNF = tumor necrosis factor; TRAP-1 = TNF receptor-associated protein 1

EBNA-2 and EBNA-3 regulate the expression of cellular genes and the viral latent membrane protein 1 (LMP-1) gene by interacting with RBP-Jk/CBF1 (Johannsen *et al*., 1996). The cellular gene targets of EBNA-2 include TNF-α, leucotriene (LTß), granulocyte colony-stimulating factor (G-CSF), cyclin D2, c-Myc, IL-16, and acute myeloid leukemia (AML) family (Spender *et al*., 2002). LMP-1 activates the NF-κB/Rel, JKN, p38/MAPK, and JAK/STAT signaling pathways (Zhang *et al*., 2004). It was reported that EBV infection promotes proliferation and survival through autotaxin upregulation in the EBV-negative Hodgkin lymphoma cell line, whereas in the case of gastric cancer, cell proliferation did not differ significantly between EBV-positive and EBV-negative cancers (Baumforth *et al*., 2005; Ishii *et al*., 2004).

LCLs have been widely used as a resource for a variety of genetic and human genome studies. However, the genomic and biological characteristics of LCLs that differ from primary B cells have not been well characterized. We investigated the protein profile changes between B cells and EBV-infected LCLs to identify biomarkers of B cell immortalization and to understand better the molecular mechanism of EBV-mediated B cell transformation.

Materials and Methods

Virus. EBV viral stock was collected from the culture of an EBV-transformed B95-8 marmoset cell line that was maintained at 37°C and 5% CO_2 for 4–7 days and then stored at -80°C until used.

Isolation of B cells. Peripheral blood was obtained from the Jungang Blood Center of the Korean Red Cross. Ficoll-Hypaque gradient centrifugation was performed to isolate peripheral blood mononuclear cells according to the manufacturer's instructions (Amersham Biosciences). Primary resting CD19(+) B cells were then purified by negative selection using a B cell isolation kit (Miltenyi Biotech).

Infection of B cells with EBV was performed as described (Hur *et al*., 2005). In brief, purified B cells were incubated with EBV as B95-8 supernatant in complete medium RPMI-1640 supplemented with 10% FBS and penicillin-streptomycin (100 μg/100 unit). After 2 hrs incubation at 37°C, an equal volume of complete medium containing cyclosporine A (0.5 μg/ml) was added to the treated B cell culture. The culture was incubated for 10–20 days post infection (p.i.) until clumps of EBV-infected B cells were visible. The culture medium was then changed with fresh complete medium without cyclosporine A. We further conducted subculture of the EBV-infected cells for several passages to establish EBV-transformed LCLs that were used for two-dimensional gel electrophoresis (2-DE), Western blot, and RNA analysis.

Activation of B cells. In case of activation of CD19 and IgM antigens on B cells, purified B cells were treated for 1 day with 10 μg/ml of mouse anti-human CD19 (BD Pharmingen) and 40 μg/ml of rabbit anti-human immunoglobulin M (IgM) F(ab')2 monoclonal antibody DK-2600 (DakoCytomation) before EBV infection.

Two-dimensional gel electrophoresis was performed as described (Park *et al*., 2004). Cell lysates were extracted and pooled from the primary B cells of eight healthy donors and from EBV-transformed LCLs. To reduce variations among individual samples, pooled cellular extracts of either B cells or LCLs were used for 2-DE analysis and subsequent Western blot analysis. Total proteins were extracted from both B cells and EBV-transformed LCLs. Briefly, 1 mg of total proteins was resolved by isoelectric focusing in a pH gradient (pH 3–10) in the first dimension, in a 8~18% gradient SDS-PAGE in the second dimension, and then visualized by Coomassie blue staining.

MALDI-TOF/MS. Protein data processing was performed with the MS-FIT peptide mass maps searched against the Swiss-Prot or non-redundant NCBI database using the MASCOT search engine (Gorg *et al*., 1988).

Western blot analysis. CD19(+) B cells were purified from whole blood of two donors and then pooled for B cell activation or EBV infection. Activated or EBV-infected B cells were collected at different time points as indicated in the text. Following the cell lysis, total proteins were resolved by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated with polyclonal rabbit anti-lamin A/C antibody (sc-20681, Santa Cruz Biotechnology), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NB 300-221, Novus Biologicals), anti-SOD2 (sc-30080, Santa Cruz Biotechnology), anti-TRAP-1 (sc-135757), and anti-HSPA9 (sc-13967) monoclonal antibodies followed by an incubation with HRP-conjugated anti-rabbit or anti-mouse IgG. The blot signals were detected using chemiluminiscence detection kit (Amersham Biosciences). Scanned images of the blot were used to quantify protein levels relative to GAPDH using the ImageJ, an open domain image processing and analysis program (http://rsb.info.nih.gov/nih-image/about.html).

Real-time PCR. CD19(+) B cells were purified from whole blood of two donors and then activated with anti-CD19 and anti-IgM antibodies. Total RNA was isolated using Trizol (Invitrogen) and used to synthesize first-strand cDNA using oligo(dT) primer and the Superscript II reverse transcription kit (Invitrogen). RT-PCR amplification mixtures (50 μl) contained 100 ng of first-strand cDNA template, 2 × SYBR Green Master Mix buffer (Invitrogen) with forward and reverse primers (Table 1). GAPDH was included as an internal control. PCR cycles were 95°C for 10 mins followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. Reactions were run on an ABI HT 7900 (Applied Biosystems). An average repression was shown for two donors using the Ct difference of GAPDH and test gene for which three experiments were performed in triplicates.

Literature co-occurrence study. We performed a literature association study to annotate the biological relationship and molecular network of differentially expressed proteins in LCLs using the PubGene public services 2.3 package (http://www.pubgene.org). PubGene facilitates annotation of the gene network as well as the biological relationship between genes. For example, signature gene clustering is based on a group of genes that are coordinated or clustered, whereas the literature association studies are used to characterize distinct cell types or biological processes based on the network from the co-occurrence of gene symbols or short gene names in MEDLINE records (Jenssen *et al*., 2001).

Table 1. Primers used for real-time PCR

Gene*	Primer sequence		Amplicon size (bp)
GDI2	Forward	5'-CCAACTCCTGCCAGATCATT-3'	179
	Reverse	5'-CAAGAGCTCCAAAGCTGGTC-3'	
SOD2	Forward	5'-GGAAGCCATCAAACGTGACT-3'	162
	Reverse	5'-CCTTGCAGTGGATCCTGATT-3'	
S100A8	Forward	5'-ATGCCGTCTACAGGGATGAC-3'	223
	Reverse	5'-AGCCTCTGGGCAGTAACTCA-3'	
S100A9	Forward	5'-CAGCTGGAACGCAACATAGA-3'	205
	Reverse	5'-TCAGCTGCTTGTCTGCATTT-3'	
SERPINB1	Forward	5'-GCCTAGGTGTGCAGGATCTC-3'	188
	Reverse	5'-GAAATTTTCTTCGGGCATCA-3'	
HSP90B1	Forward	5'-TTGGAGCTTGCTTCCTCATT-3'	224
	Reverse	5'-GTTCTTGCAAGCCCACTCTC-3'	
YWHAZ	Forward	5'-TTCTTGATCCCCAATGCTTC-3'	211
	Reverse	5'-AGTTAAGGGCCAGACCCAGT-3'	
ACTB	Forward	5'-GGCATCCTCACCCTGAAGTA-3'	280
	Reverse	5'-GGGGTGTTGAAGGTCTCAAA-3'	
LTA4H	Forward	5'-ACTGCTTGGAGGACCAGAGA-3'	157
	Reverse	5'-CCAGGCATTCCAATCAACTT-3'	

*For explanation of acronyms see Table 2.

Results and Discussion

Protein identification

We performed proteomic analysis of LCLs in an attempt to find biomarkers characteristic of EBV-mediated B cell immortalization. The protein spots on a 2-DE image were identified by a comparison with the public proteomic databases and by peptide mass fingerprinting using MALDI-TOF/MS or MALDI-QTOF. Approximately 100 protein spots were expressed differently in primary B cells and LCLs (Fig. 1a). Some of them were selected for peptide tag sequencing by MALDI-TOF. However, because of the low molecular weight and a low amount of peptides, only five spots (TPI1, SOD2, S100A9, STMN1 and UBE2N) were sequenced by MALDI-QTOF (data not shown). Twenty protein sequences were found as expressed differently in LCLs or B cells (Fig. 1b, Table 2). From them, 11 proteins showed the higher expression levels in LCLs (Fig. 1b). The expression level of the remaining 9 proteins was higher in B cells. The majority of identified proteins are involved in a cell cycle and proliferation in accordance with the drastic increase in the proliferation activity of LCLs after EBV

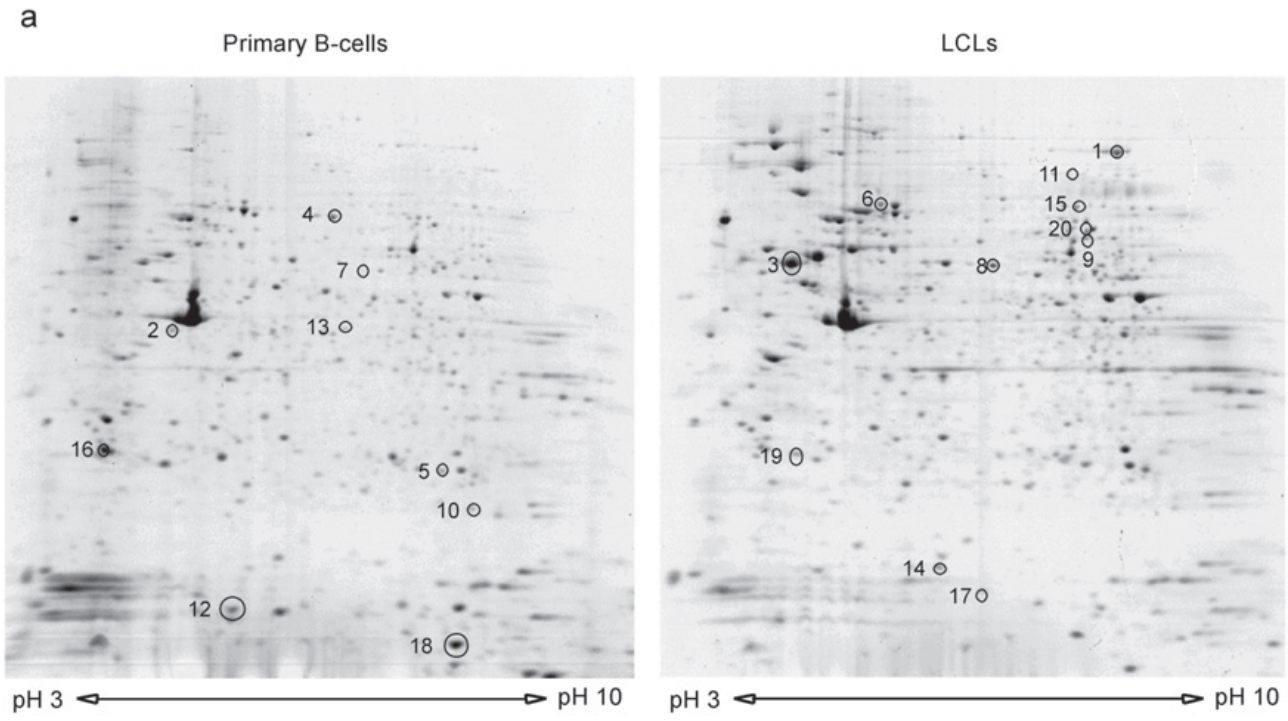

Fig. 1a

Expression level of B cells and LCLs proteins detected by 2-DE

(a) Circled protein spots are marked with the numbers corresponding to their designations in Table 2. (b) Selected protein spots were labeled with the acronyms and their upregulation or downregulation in LCLs is shown.

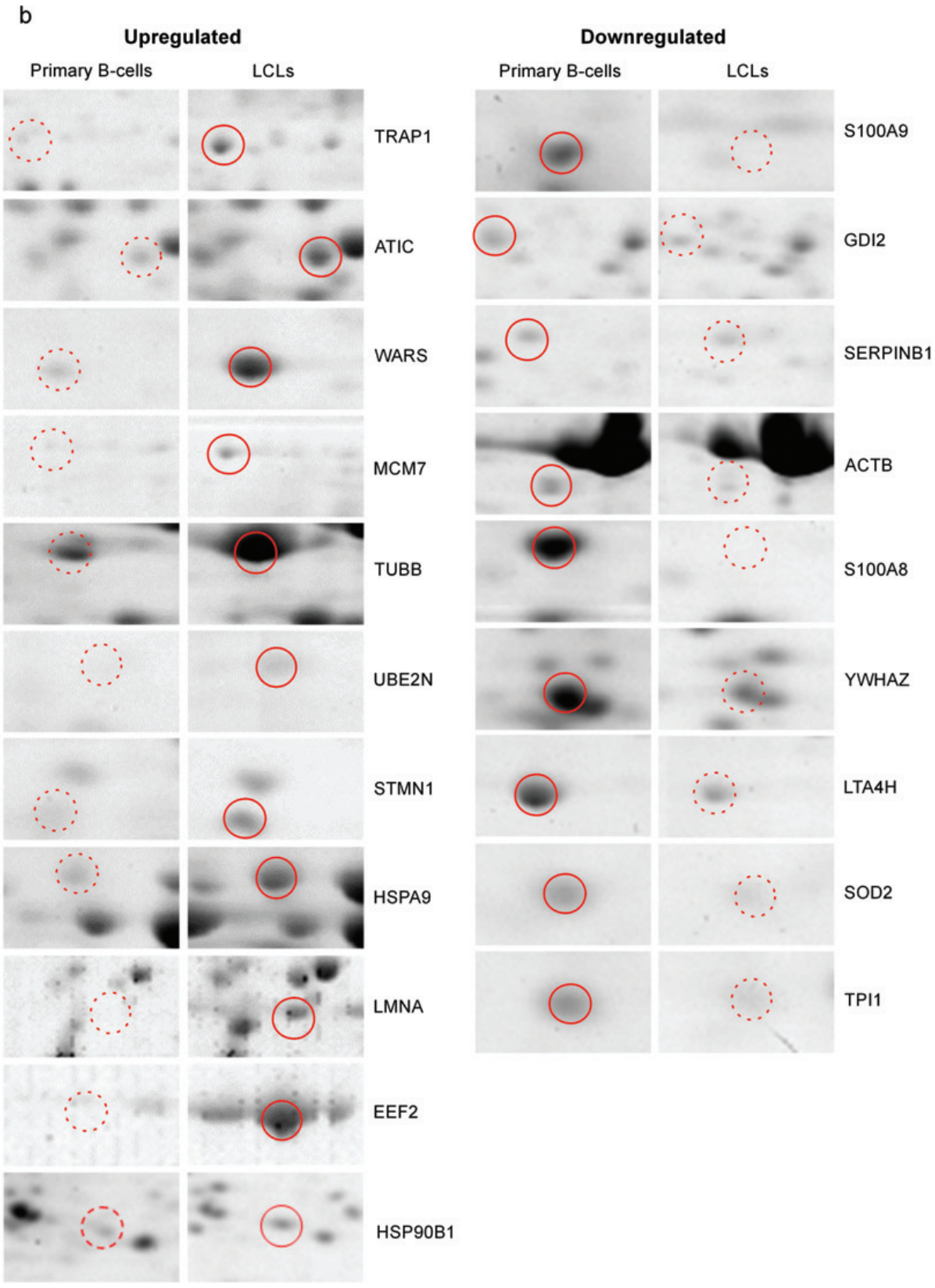
b
Upregulated
Primary B-cells
LCLs
TRAP1
ATIC
WARS
MCM7
TUBB
UBE2N
STMN1
HSPA9
LMNA
EEF2
HSP90B1
Downregulated
Primary B-cells
LCLs
S100A9
GDI2
SERPINB1
ACTB
S100A8
YWHAZ
LTA4H
SOD2
TPI1

Fig. 1b

Table 2. Proteins identified by 2-DE

[a]Spot No.	Identified proteins	Gene	[b]Acc. No	[c]Mr	[c]pI	Upregulated in LCLs or B cells
1	Eukaryotic translation elongation factor 2	EEF2	4503483	96246	6.41	LCL
2	Actin, beta	ACTB	15277503	40536	5.55	B
3	Tubulin, beta	TUBB	18088719	50096	4.75	LCL
4	Chain A, structure of [r563a] leukotriene A4 hydrolase	LTA4H	51247429	69652	5.73	B
5	Chain B, triosephosphate isomerase	TPI1	999893	26807	6.51	B
6	Heat shock 70-kDa protein 9 (mortalin)	HSPA9	24234688	73920	5.87	LCL
7	GDP dissociation inhibitor 2	GDI2	285975	51088	5.94	B
8	Chain A, crystal structure of the aminoacylation catalytic fragment of human tryptophanyl-tRNA synthetase	WARS	50513261	43586	7.12	LCL
9	Lamin A/C isoform 2	LMNA	5031875	65153	6.4	LCL
10	Manganese superoxide dismutase 2	SOD2	34707	24866	8.35	B
11	Minichromosome maintenance complex protein 7	MCM7	33469968	81884	6.08	LCL
12	S100 calcium-binding protein A9	S100A9	4506773	13291	5.71	B
13	Serpin peptidase inhibitor, clade B (ovalbumin), member 1	SERPINB1	13489087	42829	5.9	B
14	Stathmin 1	STMN1	15680064	17326	5.76	LCL
15	TNF receptor-associated protein 1	TRAP-1	17511976	80345	8.3	LCL
16	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	21735625	27899	4.73	B
17	Ubiquitin-conjugating enzyme E2N	UBE2N	4507793	17184	6.13	LCL
18	S100 calcium-binding protein A8	S100A8	29888	10988	9.19	B
19	Heat shock protein 90 kDa beta (Grp94), member 1	HSP90B1	33150616	27563	5.12	LCL
20	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	ATIC	20127454	65089	6.27	LCL

[a]Spot numbers correspond to the protein spots in the 2-DE gels (Fig. 1). [b]Acc. Nos in SWISS PROT. [c]theoretical isoelectric point.

infection. For instance, higher levels of stathmin (STMN1), lamin (LMNA), mortalin (HSPA9), and translation elongation factor 2 (EEF2) were detected in LCLs, when compared to the resting B cells. This result is consistent with our earlier microarray data that detected an increased level of STMN1 in LCLs, what reflected the requirement of higher proliferation activity in LCLs (Baik *et al*., 2007).

The levels of EEF2 and human tryptophanyl-tRNA synthetase (WARS) were higher also in LCLs when compared to the resting B cells. B-lymphocytes increase in size when activated by B cell receptor signaling or EBV infection, what promotes changes in the expression of genes encoding the proteins involved in protein synthesis or in cytoskeleton (Fruman *et al*., 2002). Thus, the elevated protein levels of EEF2 combined with increased levels of WARS reflected the higher rate of protein synthesis required for the proliferation of EBV-infected B cells. The expression profiles of individual ribosomal protein genes are closely related to the differentiation or proliferation state of the cell (Bevort and Leffers, 2000). However, our LCL protein profiles did not identify many ribosomal proteins, what could be due to the low number of selected protein spots that were used for the protein identification.

It was reported that immortalization of human epithelial cells mediated by a viral oncogene or by Simian virus 40 (SV40) was required for the inactivation of tumor suppressors and telomere stabilization (Macera-Bloch *et al*., 2002). Compared to the B cells, tumor suppressors and oncoproteins were not identified in our LCL protein profiles. This outcome suggested that the protein spots did not include these types of proteins or the mechanism of B cell immortalization differed from that of other cell types. Short-term cultured LCLs (4–5 weeks p.i.) might still be at the pre-transformation stage, although they were highly proliferative. We suppose that long-term cultured LCLs (160 passages) might express oncoproteins, tumor suppressors, or high telomerase activity.

Functional significance of identified proteins

The identity of most proteins recognized by MALDI-TOF was confirmed either by Western blotting or by quantitative real-time PCR. Increase of LMNA expression levels was detected as early as 1 day p.i., whereas the levels of SOD2, HSPA9, and TRAP-1 were changed as late as 6–30 days p.i. (Fig. 2a,b). These results suggested that LMNA was the early marker of EBV-mediated B cell transformation, whereas SOD2, TRAP-1, and HSPA9 were the late markers.

We found that upregulation of SOD2 was sustained for 2 days p.i. but after this initial increase the expression level was dramatically downregulated (Fig. 2a,b). SOD2 gene encodes a mitochondrial manganese superoxide dismutase

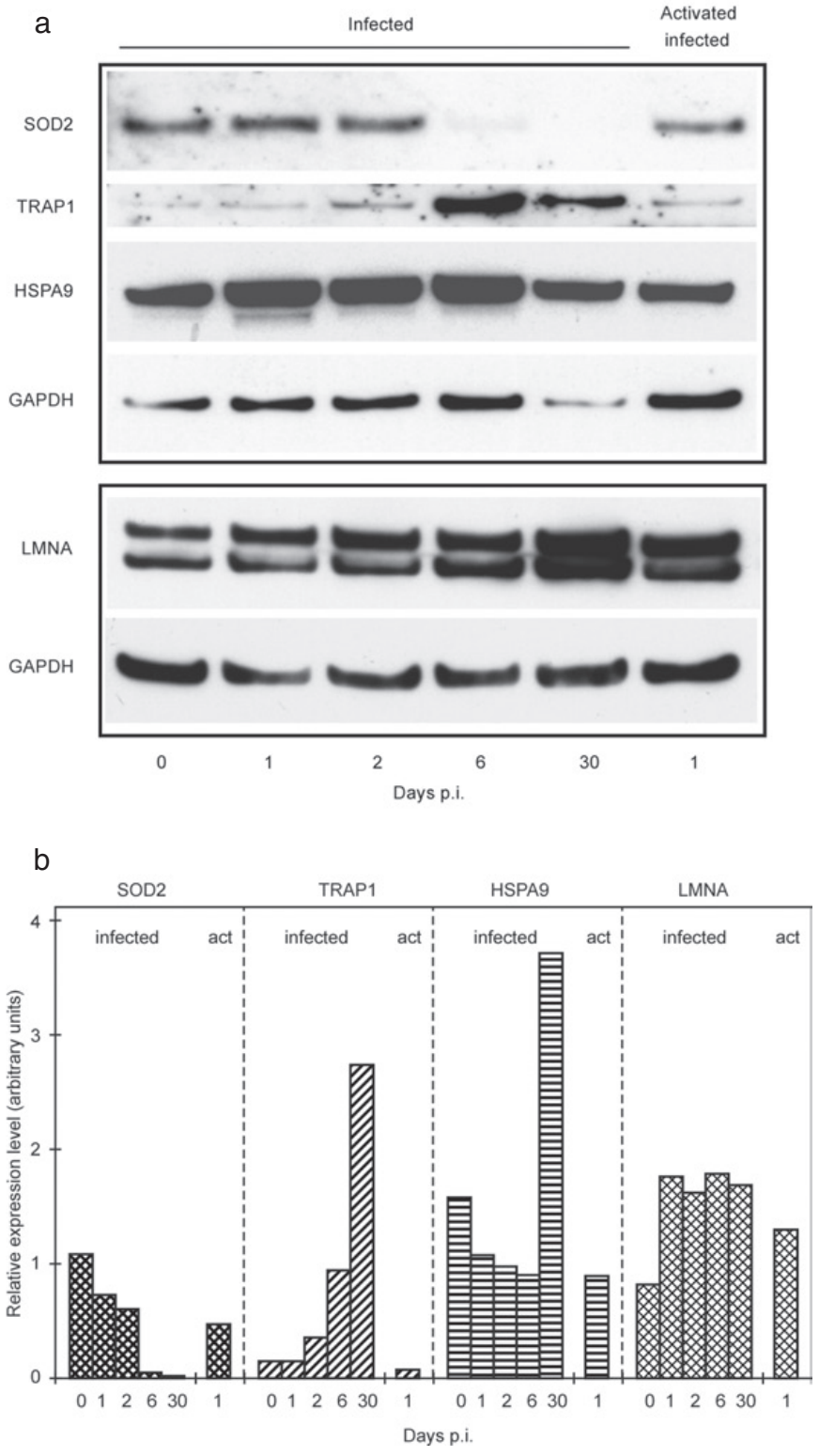

Fig. 2

Expression level of four deregulated proteins SOD2, TRAP-1, HSPA9, and LMNA in LCLs detected by Western blot analysis

(a) B cells were infected with EBV for 30 days (infected) or activated only (act). GAPDH = control protein. (b) Relative expression levels evaluated from signals shown in (a).

(SOD2), an intramitochondrial free radical scavenging enzyme that is required for normal biological function of tissues by maintaining the integrity of mitochondrial enzymes susceptible to direct inactivation by superoxide. Thus, the downregulation of SOD2 suggested that oxidative stress contributed to the transformation of EBV-infected B cells in the earlier stages of immortalization. HSPA9 was initially downregulated for 6 days p.i., but its expression level was dramatically increased at day 30 p.i. Usually, B cells become established as LCLs about 1 month after EBV infection. Thus, this result suggested that the transition between downregulation and upregulation for HSPA9 was a marker for the establishment of EBV-infected lymphoblastoid cell lines. Indeed, HSPA9 suppression induced senescence-like growth arrest in human papillomavirus 16-immortalized fibroblasts and conversely, HSPA9 overexpression reduced the survival of NIH3T3 cells (Wadhwa *et al.*, 2004; Kaul *et al.*, 1998). HSPA9 is frequently overexpressed in many cancers including colorectal adenocarcinoma (Dundas *et al.*, 2005). Therefore, upregulation of HSPA9 could contribute to the B cell transformation possibly by blocking the cellular senescence in LCLs at the late stage of EBV-induced B cell transformation.

We also found that TRAP-1 expression level gradually increased in the transformation process of EBV-infected cells reaching the highest level at day 30 p.i. (Fig. 2a,b). TRAP-1 is a representative mitochondrial heat shock protein with homology to heat shock protein 90 (HSP90). TRAP-1 overexpression decreases levels of reactive oxygen species (ROS) and SOD2 in deferoxamine-treated Chang cells, where deferoxamine induces mitochondrial dysfunction (Im *et al.*, 2007). TRAP-1 expression was decreased in apoptosis-induced HL60 cells by β-hydroxyisovalerylshikonin that produces ROS (Masuda *et al.*, 2004). EBV infection resulted in the induction of ROS in Burkitt's lymphoma cell lines through an EBNA-2-dependent pathway (Cerimele *et al.*, 2005). The increased level of TRAP-1 found in EBV-infected B cells suggested that high-level of TRAP-1 expression might play a role in the protection of the EBV-infected B cells against ROS and apoptosis. Thus, TRAP-1 likely performed a function similar to the antioxidant SOD2 and protected EBV-infected B cells against ROS-mediated apoptosis.

The increased expression of LMNA was of particular interest regarding the higher mitotic activity of LCLs that was confirmed by Western blot analysis (Fig. 2a,b). LCLs exhibited a higher level of LMNA expression as compared to the B cells. A previous report suggested that LMNA provided a proper platform for anchoring a particular chromatin locus 4q31.2 to the nuclear envelope (Masny *et al.*, 2004). DNA synthesis is typically initiated in the perinuclear foci overlapped with lamin structures as a tightly regulated event. However, immortalized cells initiate DNA replication in a more distributed pattern throughout the nucleus (Kennedy *et al.*, 2000). Increased LMNA expression in LCLs could result from the EBV-directed deregulation or subsequent events during the immortalization process. However, Western blot analysis showed that there was no significant change in LMNA expression in EBV-infected Ramos cells (data not shown). Thus, the increased LMNA expression in LCLs might be due to the B cell activation accompanied by EBV-mediated cellular changes in gene expression suggesting that LMNA overexpression could be an indirect consequence of EBV infection.

To expand the protein profiling of EBV-infected B cells to the transcriptional level, we investigated the expression patterns of protein-coding genes that were downregulated in EBV-infected LCLs as detected by 2-DE (Fig. 2b). Quantitative real-time PCR confirmed the protein profiling of differentially expressed genes including GDP dissociation inhibitor 2 (GDI2), SOD2, and S100 calcium-binding protein A9 (S100A9) that were repressed in LCLs (Fig. 3). Interestingly, the β-actin gene (ACTB) was repressed about 5-fold in LCLs as compared to the B cells, what might be associated with the morphological changes during EBV-mediated B cell transformation.

Literature co-occurrence study

According to our literature association study, TNF was placed at the center of a gene cluster network of certain

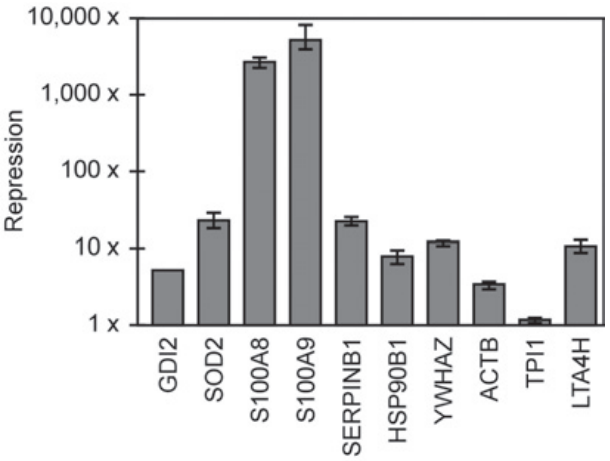

Fig. 3

Real-time PCR of 10 repressed genes in LCLs

Ordinate y: 10, 100, 1,000, and 10,000 multiple repression of the corresponding transcript level detected in LCLs. For explanation of acronyms see Table 2.

differentially expressed proteins in LCLs. Indeed, heat shock protein family members, HSPA9, TRAP-1, and SOD2 are known to play protective roles against apoptosis or oxidative stress through TNF signaling. TNF-related proteins have been individually identified as cellular targets of EBV infection in different experiments: TNF-α as an EBNA-2 target, and TNF receptor–associated factors, TNF-associated death domain protein, and TNF-interacting protein as the LMP-1 targets (Izumi *et al*., 1999). Thus, combining the literature co-occurrence study and protein profiling allowed us to identify TNF as the central target of EBV-mediated B cell transformation. In addition, since most of the proteins identified in this study were known as stress-related proteins, deregulation of these proteins did not have to be a primary event, but a secondary consequence accompanied by the modulation of TNF signaling pathway in EBV-infected B cells.

To better understand the EBV-induced B cell transformation, previous research has focused on the importance of LMP-1-mediated NF-κB activation. However, our findings of the protein profiles of EBV-infected B cells and our literature co-occurrence study suggested that the TNF signaling pathway also played an important role in the EBV-infected B cell transformation. Therefore, the TNF signaling pathway would be an excellent target for drugs to control EBV-induced B cell transformation as well as EBV-positive cancers. In addition, we previously reported that EBV infection raised the mitochondrial DNA copy number and mitochondrial biogenesis (Jeon *et al*., 2007). Taken together, EBV-induced B cell transformation may result in various mitochondrial dysfunctions including elevated mitochondrial biogenesis and mitochondrial deregulation of the TNF signaling pathway.

Acknowledgement. This work was supported by an intramural grant from the National Institute of Health, Korea.

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