Understanding Epstein-Barr Virus Life Cycle with Proteomics: A Temporal Analysis of Ubiquitination During Virus Reactivation

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Abstract

Epstein-Barr virus (EBV) is a human γ -herpesvirus associated with cancer, including Burkitt lymphoma, nasopharyngeal, and gastric carcinoma. EBV reactivation in latently infected B cells is essential for persistent infection whereby B cell receptor (BCR) activation is a physiologically relevant stimulus. Yet, a global view of BCR activation-regulated protein ubiquitination is lacking when EBV is actively replicating. We report here, for the first time, the long-term effects of IgG cross-linking-regulated protein ubiquitination and offer a basis for dissecting the cellular environment during the course of EBV lytic replication. Using the Akata-BX1 (EBV⁺) and Akata-4E3 (EBV⁻) Burkitt lymphoma cells, we monitored the dynamic changes in protein ubiquitination using quantitative proteomics. We observed temporal alterations in the level of ubiquitination at ~ 150 sites in both EBV⁺ and EBV⁻ B cells post-IgG cross-linking, compared with controls with no cross-linking. The majority of protein ubiquitination was downregulated. The upregulated ubiquitination events were associated with proteins involved in RNA processing. Among the downregulated ubiquitination events were proteins involved in apoptosis, ubiquitination, and DNA repair. These comparative and quantitative proteomic observations represent the first analysis on the effects of IgG cross-linking at later time points when the majority of EBV genes are expressed and the viral genome is actively being replicated. In all, these data enhance our understanding of mechanistic linkages connecting protein ubiquitination, RNA processing, apoptosis, and the EBV life cycle.

Keywords: proteomics, big data, Association Study

Introduction

EPSTEIN-BARR VIRUS (EBV) is a human γ -herpesvirus that establishes lifelong persistent infections and is closely associated with several types of cancers, including Burkitt lymphoma, nasopharyngeal, and gastric carcinoma (Tsao et al., 2015; Vockerodt et al., 2015). EBV reactivation in latently infected B cells plays a critical role in lifelong persistent infection and transmission to uninfected populations.

One of the physiologically relevant stimuli for EBV reactivation is antigen-induced B cell receptor (BCR) activation. The cellular response to BCR activation involves the

assembly of BCR signaling proteins that regulate a cascade of downstream signaling events, including protein phosphorylation and ubiquitination (Jayasundera et al., 2014; Matsumoto et al., 2009; Satpathy et al., 2015). System-wide identification of downstream signaling events has revealed important roles for phosphorylation and ubiquitination within minutes of the BCR activation process. Some of these early signaling events have been shown to play a critical role in triggering EBV reactivation (Bryant and Farrell, 2002).

In addition to the early signaling events, BCR activation by IgG cross-linking potentially reprograms the cellular environment during EBV lytic replication (Mattes et al., 2009;

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Yuan et al., 2006). Therefore, to better understand the contribution of the B cell environment to EBV life cycle, it is important to dissect the long-term signaling alterations upon BCR activation.

Ubiquitination, catalyzed by a cascade of three enzymes, plays important roles in the control of key cellular processes, including signal transduction, cell cycle progression, transcriptional regulation, and apoptosis. The EBV life cycle is tightly associated with the ubiquitination and deubiquitination pathways (Shackelford and Pagano, 2004, 2005). For example, the E3 ubiquitin ligase, RNF4, triggers the ubiquitination of the EBV transactivator, RTA, to limit EBV lytic replication and virion production (Yang et al., 2013). EBV latent membrane protein 1 (LMP1) induces K63-linked ubiquitination of p53 to rescue tumor cell apoptosis (Li et al., 2012a).

LMP1 can also stimulate receptor-interacting protein (RIP)-dependent TNFR-associated factor 6 (TRAF6)-triggered K63-linked ubiquitination of IRF7 (Huye et al., 2007; Ning et al., 2008). However, LMP1-stimulated IRF7 ubiquitination is negatively regulated by the cellular deubiquitinase A20 (Ning and Pagano, 2010). Others have shown that LMP1 coordinates with TRAF1-mediated polyubiquitin signaling to promote activation of the cell survival pathway (Greenfeld et al., 2015).

The ubiquitin ligase Itch regulates EBV maturation through BFRF1-mediated nuclear envelope modification (Lee et al., 2016). EBV EBNA3C regulates the ubiquitin ligase activity of Mdm2 to facilitate p53 ubiquitination and degradation (Forte and Luftig, 2009; Saha et al., 2009). The EBV EBNA1 protein recruits deubiquitinase USP7 to the EBV latent origin of replication to alter histone modification (Sarkari et al., 2009). EBV LMP1 induces the expression of a cellular deubiquitinase UCH-L1 during viral latency (Bentz et al., 2014), while EBV EBNA3 family proteins target a USP46/USP12 deubiquitinase complex for virus-induced growth transformation (Ohashi et al., 2015).

In addition, EBV also encodes a deubiquitinase BPLF1, which regulates the deubiquitination of viral and cellular proteins to foster viral replication (Gastaldello et al., 2010; Saito et al., 2013; van Gent et al., 2014; Whitehurst et al., 2009, 2012). BPLF1 also coordinates with Rad6/18 ubiquitin complex to regulate virus infectivity (Kumar et al., 2014). Taken together, these studies indicate that ubiquitination plays an important role in the EBV life cycle. However, a global view of BCR activation-regulated protein ubiquitination when EBV is being actively replicated is still lacking.

In this study, we employed stable isotope labeling by amino acid in cell culture (SILAC)-based quantitative proteomics (Li et al., 2015; Pinto et al., 2015; Zhong et al., 2012) to monitor the dynamic changes in protein ubiquitination in EBV⁺ and EBV⁻ B cells upon IgG cross-linking-mediated BCR activation. For the first time, this report describes the long-term effects of BCR activation on protein ubiquitination, which offers a basis for dissecting the cellular environment during the course of EBV lytic replication.

Materials and Methods

Cell culture and treatment

The Akata-BX1 (EBV⁺) and Akata-4E3 (EBV⁻) cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) (Gibco) in 5% CO₂ at 37°C (Li et al.,

2011, 2012b). To label cells with stable isotopic amino acids, EBV⁺ and EBV⁻ cells were propagated in RPMI 1640 SILAC media deficient in both L-lysine and L-arginine (Cat. no. 26140079; Thermo Fisher Scientific) and supplemented with light lysine (¹²C₆¹⁴N₂-K) and arginine (¹²C₆¹⁴N₄-R) for light state (Cat. nos. L-9037 and A-8094; Sigma), ¹³C₆¹⁴N₂-K and ¹³C₆¹⁴N₄-R for medium state (Cat. nos. CLM-2247-H-1 and CLM-2265-H-1; Cambridge Isotope Laboratories), and ¹³C₆¹⁵N₂-K and ¹³C₆¹⁵N₄-R for heavy state labeling (Cat. nos. CNLM-291-H-1 and CNLM-539-H-1; Cambridge Isotope Laboratories) (Li et al., 2015). Cells were cultured for at least six doubling times for complete incorporation. The light-labeled EBV⁺ and EBV⁻ cells were untreated (0 h) and the medium- and heavy-labeled EBV⁺ and EBV⁻ cells were treated with goat anti-human IgG (1:200; Cat. No. 55087; MP Biomedicals) for 24 and 48 h, respectively.

Sample preparation

The untreated and IgG-treated cells (5 × 10⁸ cells each condition) were harvested by centrifugation at 400 g for 5 min. Pellets were washed twice by resuspending in 250 mL of prechilled Dulbecco's PBS (Cat. no. 10010-049; Thermo Fisher Scientific). The cell pellets were resuspended in 20 mL freshly prepared Urea Lysis Buffer [20 mM HEPES pH 8.0, 9 M urea, and protease inhibitors (Cat. no. 05892791001; Roche) for sonication. The lysate was centrifuged at 15,000 g for 10 min at 18°C. The supernatant was stored at -80°C for proteomic analysis. Protein concentration was measured by BCA assay (Cat. no. 23227; Pierce). Peptides were prepared by an in-solution tryptic digestion protocol (Li et al., 2015). Trypsin digestion efficiency was assessed by SDS-PAGE and Coomassie Brilliant Blue staining before further processing.

Protein digests were acidified by 1% (v/v) trifluoroacetic acid (TFA) and subjected to centrifugation at 2000 g at 25°C for 5 min. The supernatant of the protein digests was loaded onto a Sep-Pak C₁₈ column (Cat. no. WAT051910; Waters, Columbia, MD) equilibrated with 0.1% (v/v) TFA. Columns were washed with 6 mL of 0.1% (v/v) TFA twice and peptides were eluted in 2 mL of 40% (v/v) acetonitrile (ACN) with 0.1% (v/v) TFA three times. Eluted peptides were lyophilized and subjected to enrichment for ubiquitinated peptides by immunoprecipitation using anti-K-ε-GG antibody-conjugated beads (Cat. no. 5562; Cell Signaling Tech), according to the manufacturer's instructions.

In brief, anti-K-ε-GG antibody beads were washed four times with 1 mL of the ice-cold immunoprecipitation buffer (IAP) (50 mM MOPS, pH 7.2, 10 mM sodium phosphate, 50 mM NaCl). For each of the biological replicates, 15 mg lyophilized peptides were resuspended in 1.5 mL IAP buffer and incubated for 2 h with the anti-K-ε-GG antibody beads on a rotator at 4°C. Anti-K-ε-GG antibody beads were washed three times with 1 mL of cold IAP buffer and twice with 1 mL of cold H₂O before elution with 65 μL of 0.15% TFA solution twice. Eluted peptide supernatants were desalted using C₁₈ STAGE tips. STAGE tips were packed with a C₁₈ Empore disk (Cat. no. 2315, 3M; St. Paul, MN) and conditioned with 50 μL of 100% ACN and two applications of 50 μL of 0.1% TFA before the peptide solution was loaded. Peptides were washed twice with 50 μL of 0.1% TFA and eluted with three times 20 μL of 50% ACN (0.1% TFA). Desalted peptides were dried and kept at -80°C in a freezer until further analysis.

Liquid chromatography–tandem mass spectrometry analysis

The enriched peptides were analyzed on an LTQ-Orbitrap Elite mass spectrometer interfaced with Easy-nLC II nano-flow LC system (Thermo Scientific) using the same parameters as previously described (Li et al., 2015).

MS data analysis

The MS derived data were screened using MASCOT (version 2.2.0) and SEQUEST search algorithms against a human RefSeq database (version 59) using Proteome Discoverer 1.4 (Thermo Scientific). The search parameters for both algorithms included carbamidomethylation of cysteine residues as a fixed modification and N-terminal acetylation, oxidation at methionine, GlyGly addition to lysine, and SILAC labeling $^{13}\text{C}_6$ $^{15}\text{N}_2$ -K, $^{12}\text{C}_6$ $^{14}\text{N}_2$ -K, $^{12}\text{C}_6$ $^{14}\text{N}_4$ -R, and $^{13}\text{C}_6$ $^{15}\text{N}_4$ -Ras variable modifications. MS/MS spectra were searched with a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.05 Da. Trypsin was specified as the protease and a maximum of two missed cleavages were allowed.

The data were screened against a target decoy database and the false discovery rate (FDR) was set to 1% at the peptide level. The SILAC ratio for each peptide-spectrum match (PSM) was calculated by the quantitation node. The wrongly assigned ubiquitination sites (the C-terminal lysine in the peptide) were manually corrected. Because BCR activation triggers a dramatic downregulation in ubiquitination at later time points, it is not suitable to obtain a cutoff value using a normal distribution-based method for regulated ubiquitination. Therefore, we applied a commonly used cutoff value of twofold change for differentially regulated ubiquitination upon BCR activation (Satpathy et al., 2015; Tong et al., 2014). Peptides with ratios greater than twofold change were used for further analysis.

Data availability

The MS data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) through the PRIDE partner (Vizcaino et al., 2016) repository with the dataset identifier PXD004562.

Immunoblotting

Cells were harvested and lysed in 2×SDS-PAGE sample buffer and boiled for 5 min. The samples were separated

on 4–20% TGX gels (Biorad), transferred to PVDF membranes, and probed with primary and horseradish peroxidase-conjugated secondary antibodies. Primary antibodies purchased from Cell Signaling Technology were anti-cleaved PARP (Cat. no. 5625), anti-cleaved caspase substrate motif (Cat. no. 8698), and anti-K63-linkage-specific polyubiquitin (D7A11) Rabbit mAb (Cat. no. 5621). Rabbit anti- β -actin polyclonal antibody (Cat. no. A5441) was obtained from Sigma Aldrich. Rabbit anti-Bcl-2 was bought from Bethyl (Cat. no. A303-675A). Mouse anti-ZTA antibody (Cat. no. 11-007) was purchased from Argene. Mouse anti-BGLF4 antibody has been described previously (Wang et al., 2005).

Bioinformatic analysis

The gene ontology (GO) annotation and enrichment of differentially regulated ubiquitinated proteins were obtained through Cytoscape (V3.2.0) plugin BINGO following the methods described in Maere et al. (2005). Statistical significance was determined by hypergeometric test after FDR correction.

Results and Discussion

Strategy for analysis of BCR activation-regulated ubiquitination during EBV reactivation

Previously, proteomic studies were performed to examine the ubiquitination events shortly after IgG cross-linking of mouse or human B cell lines (Jayasundera et al., 2014; Satpathy et al., 2015). To obtain a global view of the long-term effects on protein ubiquitination, we utilized SILAC-based proteomics to survey the dynamic changes in ubiquitination in EBV⁺ B cells upon IgG cross-linking (Harsha et al., 2008; Ong et al., 2002). To better understand the effects of BCR activation on ubiquitination, we used both Akata-BX1 (EBV⁺) (Molesworth et al., 2000) and Akata-4E3 (EBV[−]) B cells (Shimizu et al., 1994). The Akata-BX1 (EBV⁺) cell line was derived from Akata-4E3 (EBV[−]) B cells with recombinant EBV. Therefore, both cell lines have the same genetic background, allowing for a direct comparison.

These Burkitt lymphoma cells express surface immunoglobulin receptors of the G(κ) (IgG) class, which is suitable to investigate BCR activation-regulated changes in ubiquitination (Daibata et al., 1990; Shimizu et al., 1994; Takada, 1984; Takada et al., 1991). As shown in Figure 1, both EBV⁺ and EBV[−] cells were isotopically labeled using light ($^{12}\text{C}_6$,

FIG. 1. Schematic illustration of the SILAC-based quantitative proteomic approach. Akata-BX1 (EBV⁺) and Akata-4E3 (EBV[−]) cells were cultured in light, medium, and heavy media, as indicated. EBV, Epstein-Barr virus; SILAC, stable isotope labeling by amino acid in cell culture.

$^{14}\text{N}_2$ -lysine and $^{12}\text{C}_6$, $^{14}\text{N}_4$ -arginine), medium ($^{13}\text{C}_6$, $^{14}\text{N}_2$ -lysine and $^{13}\text{C}_6$, $^{14}\text{N}_4$ -arginine), and heavy ($^{13}\text{C}_6$, $^{15}\text{N}_2$ -lysine and $^{13}\text{C}_6$, $^{15}\text{N}_4$ -arginine) amino acids, respectively. IgG was added into the medium- and heavy-labeled cells for 24 and 48 h before harvesting. An equal amount of cell lysate was mixed and then digested by trypsin.

To facilitate the identification of ubiquitination events, we employed a di-Gly capture method to enrich ubiquitinated peptides (Tong et al., 2014). The enriched ubiquitinated peptides were then analyzed on an LTQ-Orbitrap Elite mass spectrometer (Fig. 1). The acquired data were processed and searched using MASCOT and SEQUEST search algo-

ritms through the Proteome Discoverer platform and stringently filtered for an FDR of 1% at the peptide level. A good correlation was observed between the replicated analyses for both EBV⁺ and EBV⁻ cells (Supplementary Fig. S1).

Global view of proteomic data

To obtain a global picture of the regulated ubiquitination events, we first performed a distribution analysis. Interestingly, we found that there were widespread changes in protein ubiquitination at the 48-h time point compared with the

FIG. 2. Widespread changes in protein ubiquitination upon IgG cross-linking. **(A)** IgG cross-linking triggers widespread changes of protein ubiquitination. *Scatter plot* showing the SILAC ratio (24 h/0 h and 48 h/0 h) and signal intensity of ubiquitinated peptides identified from EBV⁺ and EBV⁻ B cells. *Red dots*: peptides with ubiquitination downregulated or upregulated; *Blue dots*: peptides not regulated by IgG cross-linking. **(B)** IgG cross-linking triggers minimal changes of nonubiquitinated peptides. *Scatter plot* showing the SILAC ratio (24 h/0 h and 48 h/0 h) and signal intensity of non-ubiquitinated peptides identified from EBV⁺ and EBV⁻ B cells. *Red dots*: downregulated or upregulated peptides; *Blue dots*: peptides not regulated by IgG cross-linking. See also Supplementary Tables S1 and S2 and Supplementary Figure S1.

FIG. 3. Analysis of IgG-triggered protein ubiquitination in EBV⁺ and EBV⁻ B cells. **(A)** Overlap of identified ubiquitinated sites in EBV⁺ and EBV⁻ B cells. **(B)** Heat map analysis of ubiquitinated sites with significant changes in EBV⁺ B cells and the corresponding changes of these sites in EBV⁻ B cells. *Red*: highest abundance; *Blue*: lowest abundance. See also Supplementary Table S3.

24-h time point (Fig. 2A). To rule out any systematic bias on the observed changes, we also analyzed the nonubiquitinated peptides captured in our analysis. We found that the majority (>98%) of SILAC ratios of these peptides were changed less than twofold after both 24 and 48 h (Fig. 2B). These results demonstrate that the large change in protein ubiquitination was not simply due to alteration in protein levels.

We identified 314 and 291 unique ubiquitination events for EBV⁺ and EBV⁻ cells, respectively, with 210 sites identified in both cell lines (Fig. 3A). To identify ubiquitination events that were differentially regulated by IgG

cross-linking, we used a cutoff value of twofold change for both increased and decreased ubiquitination. As summarized in Table 1, in EBV⁺ cells, ubiquitination at 7 and 29 sites showed a twofold increase at 24 and 48 h post-IgG cross-linking, respectively (Supplementary Table S1). In contrast, ubiquitination at 86 and 127 sites was downregulated by twofold at 24 and 48 h post-IgG cross-linking, respectively (Table 1 and Supplementary Table S1). Similarly, in EBV⁻ cells, we observed 15/15 hyperubiquitination sites and 122/142 hypoubiquitination sites at 24/48 h post-IgG treatment, respectively (Table 1 and Supplementary Table S2).

Because EBV reactivation has been shown to regulate cellular gene expression (Yuan et al., 2006), we then asked whether the general ubiquitination is also regulated by EBV reactivation. We first used a hierarchical clustering method to analyze the regulated ubiquitination events for EBV⁺ cells (Eisen et al., 1998), and then we matched the same ubiquitination events to those detected in the EBV⁻ cells. Surprisingly, we found that changes in ubiquitination were highly similar for both cell lines (Fig. 3B), suggesting that IgG cross-linking plays a dominant role in regulation of protein ubiquitination regardless of EBV reactivation. In addition, we also noticed that the majority of ubiquitination gradually decreased from the 24-h time point to 48-h time point. Taken together, these global analyses suggested that there were dramatic changes in ubiquitination at later time points post-BCR activation and these changes were largely not affected by the presence of EBV.

Cellular processes affected by BCR activation

To gain insight into the cellular pathways regulated by BCR activation, we performed a GO enrichment analysis of the proteins with upregulated or downregulated ubiquitination sites at either 24 or 48 h post-IgG treatment in the two cell lines by Cytoscape plugin BiNGO. The commonly enriched biological process items in both cell lines were extracted and are presented in Figure 4. Interestingly, we found that for the proteins containing upregulated ubiquitination sites, RNA splicing and mRNA-processing factors were highly enriched. For the proteins containing downregulated ubiquitination sites, factors regulating the apoptotic process, ubiquitin-dependent protein catabolic process, and DNA repair were statistically enriched. The percentage of proteins in each GO term was similar between EBV⁺ and EBV⁻ cells (Fig. 4), further suggesting that IgG cross-linking played a major role in protein ubiquitination regardless of EBV status.

TABLE 1. SUMMARY OF IDENTIFIED UBIQUITINATED SITES FOR EBV⁺ AND EBV⁻ B CELLS

	Akata-BX1 (EBV ⁺)				Akata-4E3 (EBV ⁻)			
	Unique ubiquitinated site no.		Unique ubiquitinated protein no.		Unique ubiquitinated site no.		Unique ubiquitinated protein no.	
α -IgG treatment, h	24	48	24	48	24	48	24	48
Upregulated	7	29	7	24	15	15	13	13
Downregulated	86	127	53	81	122	142	78	86
All quantified	311	235	193	151	284	212	166	129

EBV, Epstein-Barr virus.

FIG. 4. GO enrichment of hyper- and hypoubiquitinated proteins from EBV⁺ and EBV⁻ B cells. Significantly over-represented ($p < 0.05$) biological processes are shown by *line chart*. p values were determined by hypergeometric test after FDR correction. The percentage of each GO item is shown by *bar chart*. FDR, false discovery rate; GO, gene ontology.

FIG. 5. The overlap of proteins involved in three major GO items between EBV⁺ and EBV⁻ B cells. **(A)** The overlap of proteins with upregulated ubiquitination sites between EBV⁺ and EBV⁻ B cells. Proteins highlighted by *red* and *bold* belong to the enriched GO item of RNA splicing. **(B)** The overlap of proteins (containing downregulated ubiquitination sites) involved in ubiquitin-dependent protein catabolic process between EBV⁺ and EBV⁻ B cells. **(C)** The overlap of proteins (containing downregulated ubiquitination sites) involved in regulation of apoptotic process between EBV⁺ and EBV⁻ B cells.

TABLE 2. THE DYNAMIC CHANGES OF DIFFERENT UBIQUITINATION SITES ON UBIQUITIN (RPS27A) IN EBV⁺ AND EBV⁻ B CELLS UPON IgG CROSS-LINKING

	<i>Akata-BX1</i> (EBV ⁺)		<i>Akata-4E3</i> (EBV ⁻)	
	24 h/0 h	48 h/0 h	24 h/0 h	48 h/0 h
Ubiquitin (RPS27A)				
K6	0.55	0.13	0.39	0.10
K11	0.67	0.29	0.57	0.20
K27	0.58	0.17	0.76	0.24
K63	0.92	0.23	0.44	0.05

Among the hyperubiquitinated proteins, our analysis revealed that 8 of these 29 proteins in EBV⁺ cells and 5 of 15 proteins in EBV⁻ cells were directly involved in RNA splicing (Fig. 5A and Supplementary Tables S1 and S2). All these RNA splicing-related proteins belong to the heterogeneous ribonucleoprotein family. Interestingly, many members of this family have been shown to play a role in the

EBV life cycle. For example, HNRNPC interacts with the EBV SM splicing protein to regulate viral mRNA processing (Key et al., 1998). HNRNPK was reported to coordinate with EBNA2 to enhance viral latent membrane protein 2A (LMP2A) expression (Gross et al., 2012). We observed a dramatic increase in ubiquitination at Lys-8 of HNRNPC and Lys-198 of HNRNPK at 48 h for both EBV⁺ and EBV⁻ Akata cells (Supplementary Table S3).

Another RNA-binding protein, PCBP2, was recently identified as a negative regulator of EBV lytic replication (Koganti et al., 2015). The hyperubiquitination on Lys-115 observed in our screen (Supplementary Table S3) suggested that BCR activation may regulate the function of PCBP2 to facilitate the EBV lytic replication. Because HNRNPK Lys-198 and PCBP2 Lys-115 are located within the conserved RNA binding K homology (KH) domains (Teplova et al., 2011), ubiquitination on these sites may regulate their RNA binding ability and impact viral mRNA processing.

Among the hypoubiquitinated proteins detected in either EBV⁺ and EBV⁻ cells, 19 proteins are involved in ubiquitin-dependent protein catabolic process, including the ubiquitin

FIG. 6. IgG cross-linking reduces ubiquitin chain formation. (A) MS spectra showing the relative abundance of ubiquitin K63 containing ubiquitinated peptide at 24 and 48 h after IgG cross-linking of both EBV⁺ and EBV⁻ Akata cells. (B) Validation of SILAC results. EBV⁺ and EBV⁻ B cells were treated with IgG cross-linking as indicated. Western blot analysis of K63 ubiquitination on ubiquitin (RPS27A) was performed by using K63-linkage-specific antibody. β -Actin was included as loading control.

precursor protein (RPS27A), three ubiquitin conjugation enzymes (UBE2C, UBE2T, and UBE2E1), four E3 ubiquitin ligases/ligase complex subunits (RNF5, CUL3, KCTD10, and RBCK1), three proteasome subunits (PSMD1, PSMD2, and PSMA4), two deubiquitinases (USP5 and USP13), and four ubiquitin-associated proteins (RAD23A, RAD23B, UBXN1, and VCP) (Fig. 5B and Supplementary Table S3).

Consistent with previous reports (Berard et al., 1999; Donjerkovic and Scott, 2000; Yoshida et al., 2000), we also found that proteins involved in the regulation of apoptotic process were significantly enriched for both cell lines. As shown in Figure 5C, most of these proteins were identified for the two cell lines, including VCP, TUBB, MAGED1, RPS3, RPL11, CD74, YWHAB, CD27, YWHAZ, and YWHAZ. Because the ubiquitin-proteasome system is highly connected with apoptosis induction (Orlowski, 1999), it is not surprising that some proteins were enriched in both ubiquitin-dependent protein catabolic process and regulation of apoptotic process, including VCP and CUL3 (Fig. 5B, C). For ubiquitin (RPS27A) itself, we detected 4 hypoubiquitination sites (K6, K11, K27, K63) with all of the levels significantly decreased at 48 h post-IgG cross-linking (Table 2).

To validate the SILAC-MS results, we performed western blot analysis using K63-linkage-specific antibody. Consistent with the MS results (Fig. 6A), we found a gradual decrease in K63-linkage-specific polyubiquitination level for both cell lines upon IgG cross-linking (Fig. 6B). Because linkage-specific polyubiquitination plays different roles in protein turnover and signaling (Adhikari and Chen, 2009; Li and Ye, 2008; Xu et al., 2009), the misregulation of ubiquitination may affect multiple aspects of normal cell function during EBV replication. Together, these results suggest that BCR activation plays a major role in the regulation of ubiquitination of proteins involved in RNA processing and apoptosis among others.

Differential regulation of apoptosis

Due to the similar trend of ubiquitination for both EBV⁺ and EBV⁻ cells, we tested whether apoptosis was similarly induced in these cells upon BCR activation. We first monitored the level of cleaved PARP, a marker for caspase activation and apoptosis. Interestingly, the level of cleaved PARP slightly increased in EBV⁺ cells, but dramatically increased in EBV⁻ cells following IgG cross-linking (Fig. 7, top panel). To further confirm these results, we checked the level of cleaved Bcl-2, another marker of apoptosis (Kirsch et al., 1999). We found that Bcl-2 cleavage was evident in EBV⁻ cells, but not in EBV⁺ cells (Fig. 7, the second panel). Furthermore, we examined the general caspase-mediated cleavage of cellular substrates by a motif-specific antibody.

As expected, the cleavage signals were weaker in EBV⁺ cells than those seen in EBV⁻ cells (Fig. 7, the third panel). Taken together, these results demonstrate that BCR activation-mediated apoptosis induction is partially blocked by EBV replication, which is likely due to the expression of multiple EBV proteins with antiapoptosis function, including EBNA3C and BHRF1 (a viral homology of Bcl-2) (Cai et al., 2011; Desbien et al., 2009; Kvasnakul et al., 2010; McCarthy et al., 1996; Yuan et al., 2006). Future studies should focus on the ubiquitination system and its functional importance in apoptosis, RNA processing, and EBV reactivation upon BCR activation.

FIG. 7. IgG cross-linking triggers apoptosis induction. EBV⁺ and EBV⁻ B cells were treated with IgG cross-linking as indicated. Western blot analysis showing the cleavage of PARP, Bcl-2, and general caspase substrates. Asterisk denotes uncleaved Bcl-2 and arrow denotes cleaved Bcl-2. EBV lytic transactivator ZTA and protein kinase BGLF4 blots were included to indicate lytic induction efficiency. β -Actin was included as loading control.

Conclusions and Expert Outlook

In this study, we applied quantitative proteomics to dissect the long-term effects of BCR activation in protein ubiquitination during the course of EBV lytic replication. Our study revealed that BCR activation initiates widespread changes in ubiquitination of proteins involved in multiple cellular processes, such as mRNA processing and apoptosis. Although EBV gene expression leads to dramatic changes in cellular gene expression, we found that the trend of individual protein ubiquitination was highly similar between EBV⁺ and EBV⁻ cells. For hyperubiquitinated proteins, only those involved in RNA splicing and mRNA processing were highly enriched.

Interestingly, the ubiquitination sites at HNRNPK, HNRNPM, HNRNPH1/H3, PCBP1, and PCBP2 are located within their conserved RNA binding domains, indicating that ubiquitination may affect their RNA binding ability. Because EBV genes are tightly regulated by the RNA-processing machinery, ubiquitination of these RNA-processing proteins could affect viral gene processing.

Importantly, we demonstrated that IgG cross-linking also led to downregulation of ubiquitination on many proteins

involved in apoptosis and that apoptosis was triggered in both EBV⁺ and EBV⁻ Akata cells. However, apoptosis induction in EBV⁺ cells was partially suppressed compared with that seen in EBV⁻ cells. This is consistent with the fact that EBV has evolved multiple strategies to counteract apoptosis to promote viral replication.

In summary, these comparative and quantitative proteomic observations represent the first analysis on the effects of IgG cross-linking at later time points when the majority of EBV genes are expressed and the viral genome is actively being replicated. Our study offers a global view of ubiquitination upon BCR activation and contributes new knowledge on mechanistic linkages among protein ubiquitination, RNA processing, apoptosis, and the EBV life cycle.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Abbreviations Used

BCR = B cell receptor
EBV = Epstein-Barr virus
FDR = false discovery rate
GO = gene ontology
LC = liquid chromatography
MS = mass spectrometry
PSM = peptide-spectrum match
RIP = receptor-interacting protein
SILAC = stable isotope labeling by amino
acid in cell culture
TFA = trifluoroacetic acid