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Generation and Application of Polyclonal Antibody Against Replication and Transcription Activator of Kaposi's Sarcoma-Associated Herpesvirus

Yu Qin · Zhao Liu · Tianzheng Zhang · Ying Wang · Xiaomian Li · Jinzhong Wang

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Abstract Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi's sarcoma, the most common neoplasm in untreated HIV-1-infected individuals, and several B cell disorders. KSHV infection goes through lytic and latent phases, and the switch from latency to lytic replication is governed by viral replication and transcription activator (RTA). RTA consists of 691 amino acids, containing an N-terminal DNA-binding and a C-terminal activation domain. In the present study, polyclonal antibody against RTA was generated and evaluated. The C-terminal region of RTA (E482~D691) was expressed in *Escherichia coli*, purified by affinity chromatography, and utilized to raise polyclonal antibody in BALB/c mice. High-affinity antisera were obtained, which successfully detected the antigen at a dilution of 1:13,500 for ELISA and 1:20,000 for Western blot analysis. The antibody can specifically recognize full-length RTA expressed in both *E. coli* and mammalian cells. Furthermore, endogenous RTA can be detected with the antibody in TPA-induced BCBL-1 cells under various conditions. These results suggested that the antibody is valuable for the investigation of biochemical properties and biological functions of RTA.

Keywords Replication and transcription activator · Kaposi's sarcoma-associated herpesvirus · Polyclonal antibody · C-terminal region

Y. Qin · X. Li (✉)
College of Basic Medical Science, Tianjin Medical University, 22 Qixiangtai Road, Tianjin 300070, People's Republic of China
e-mail: lixm@tijmu.edu.cn

Z. Liu · T. Zhang · Y. Wang · J. Wang (✉)
TEDA School of Biological Sciences and Biotechnology, Nankai University, Key Laboratory of Molecular Microbiology and Technology of the Ministry of Education, and Tianjin Key Laboratory of Microbial Functional Genomics, 23 Hongda Street, TEDA, Tianjin 300457, People's Republic of China
e-mail: wangjinzhong@nankai.edu.cn

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is the etiologic agent of Kaposi's sarcoma (KS), the most common neoplasm in untreated HIV-1-infected individuals [1]. KSHV is also closely associated with two B cell disorders: primary effusion lymphoma and multicentric Castleman's disease [2, 3]. These malignancies are more prevalent and invasive in immunosuppressed population, such as HIV-1-positive individuals and transplant patients. With the alarming increase in the number of HIV-1 patients, it is likely that KSHV-associated malignancies will become a serious problem in their disease progresses [4].

KSHV infection goes through lytic and latent phases, and viral lytic replication plays an essential role in the development of KS tumors. The switch from latency to lytic replication is controlled by an immediate-early gene product named replication and transcription activator (RTA) [5]. RTA is encoded by the open reading frame 50 of the viral genome, which contains two exons and one intron. The RTA protein consists of 691 amino acids, containing an N-terminal DNA-binding and a C-terminal activation domain [6]. In the activation domain, four repeated units of a highly hydrophobic region were identified, known as activation domains 1~4 (AD1~AD4) [6, 7]. RTA can be extensively phosphorylated and may be subject to other posttranslational modifications [6, 8].

RTA can transactivate various genes either by direct binding to the promoters or by interacting with cellular or viral cofactors. Transient transfection experiments have demonstrated that RTA activates promoters of a number of viral genes including ORF21 [9], ORF57 [10, 11], ORF59 [12], ORF73 [13], ORF74 [14], K1 [15], K2 [16], K5 [17], K8/K-bZIP [18], K9 [19], K12 [20], K14 [14], K15 [21], polyadenylated nuclear RNA [22], and its own [23]. For proper transactivation and induction of KSHV reactivation, RTA forms a tetramer in solution [24]. RTA can also interact with numerous cellular proteins, including K-RBP [25], RBP-Jκ [26], Oct-1 [27], HMGB1 [28], CBP, p300, HDAC1 [29], and STAT3 [30]. It has been reported that RTA possesses ubiquitin E3 ligase activity that targets IRF-7 for proteosome-mediated degradation [31] and promotes the degradation of K-RBP to regulate viral lytic replication [32].

In this study, polyclonal antibody against RTA was generated and evaluated. The C-terminal region (E482~D691) containing the RTA activation domain was expressed in *Escherichia coli*, purified by affinity chromatography, and utilized to raise polyclonal antibody against RTA in BALB/c mice. The antibody can specifically recognize the full-length RTA in both *E. coli* and mammalian cells. Furthermore, endogenous RTA can be detected with the antibody in TPA-induced BCBL-1 cells by Western blotting and immunofluorescence assay. These results suggested that the antibody is useful in biochemical and functional studies on KSHV RTA.

Materials and Methods

Cell Culture and Transfection

The human embryonic kidney fibroblast cell line 293T was cultured in Dulbecco's modified Eagle medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO_2. The KSHV-positive B cell line BCBL-1 was cultured in RPMI-1640 medium (HyClone) containing 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2. Transfection was conducted with Lipofect-AMINE reagent (Invitrogen, Carlsbad, CA, USA) as described previously [33].

Plasmid Construction

The DNA fragment in KSHV genome (GenBank accession number NC_003409, nt 73997 to nt 74629), which encodes E482~D691 of RTA carboxy-terminal region (RTAc), was amplified from plasmid pCMV-Tag50 [33] using forward primer 5′-cgaattcgaagcctcgggc gaaga-3′ and reverse primer 5′-actcgagtcagtctcggaagtaattacgccattg-3′ (*EcoR* I and *Xho* I sites were underlined, respectively). The polymerase chain reaction product was ligated into the pGEM-T vector (Promega, Madison, WI, USA). The *EcoR* I/*Xho* I fragment from the resulting plasmid was subcloned into the pET28a vector (EMD Biosciences, San Diego, CA, USA) to generate pET28a-RTAc for expression of His_6-tagged protein in *E. coli*. The plasmid was confirmed by DNA sequencing.

Protein Expression and Purification

E. coli BL21 (DE3) cells harboring the plasmid pET28a-RTAc were induced with 0.1 mM isopropylthio-β-D-galactoside (IPTG) for 4 h at 37°C then harvested and resuspended with lysis buffer (50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, and 10 mM imidazole) supplemented with 0.5 mM phenylmethylsulfonyl fluoride. Total soluble protein was applied to HIS-Select® Nickel Affinity Gel (Sigma–Aldrich, Saint Louis, MO, USA), and the His_6-tagged protein was purified according to the manufacturer's instructions. Protein was eluted with 50 mM sodium phosphate, pH 8.0, containing 0.3 M sodium chloride and 250 mM imidazole, and was dialyzed overnight against phosphate-buffered saline (PBS). Protein concentration was determined using a bicinchoninic acid assay (Pierce, Rockford, IL, USA).

Antibody Production

Polyclonal antibody was raised against purified RTAc in four female BALB/c mice (8 weeks old). For the first injection, 50 μg of purified protein in 50 μl PBS was emulsified with equal volume of complete Freund's adjuvant (Sigma–Aldrich) and injected subcutaneously. Three booster injections of 25 μg protein emulsified with incomplete Freund's adjuvant were performed at 2-week intervals. Ten days after the last immunization, the antisera were collected, aliquot, and stored at −70°C.

SDS-PAGE and Western Blot Analysis

For electrophoresis and Western blot analysis, protein samples were mixed with an equal volume of 2× SDS sample buffer (125 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol, and 0.001% bromophenol blue). Proteins were heat-denatured and resolved by SDS-PAGE [34], using 10% polyacrylamide separation gels and 4% polyacrylamide stacking gels. For Western blotting, proteins were transferred onto PVDF membranes (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions and Towbin's method [35]. The membranes were blocked in TBST (50 mM Tris, pH 7.5, 0.15 M sodium chloride, 0.5‰ Tween 20) containing 5% nonfat milk powder and probed with antibodies diluted in blocking buffer. Detection employed horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Super Signal West Pico chemiluminescence (Pierce). The monoclonal antibodies against 6× histidine, 1× FLAG, and β-tubulin were purchased from Clontech Laboratories (Mountain View, CA, USA), Stratagene (La Jolla, CA, USA), and Sigma–Aldrich, respectively.

ELISA

Indirect ELISA was conducted according to Engvall's method [36] with minor modifications. Briefly, flat bottom ELISA plates (Corning, Lowell, MA, USA) were coated with 100 μl/well purified RTAc (5 μg/mL) diluted with carbonate buffer (0.15 M sodium carbonate, 0.35 M sodium bicarbonate, pH 9.6), and were incubated overnight at 4°C. The plates were blocked for 1 h at 37°C with PBS containing 0.5‰ Tween-20 (PBST) and 2% bovine serum albumin (BSA). The antisera were diluted at 1:500, 1:1,500, 1:4,500 1:13,500, 1:40,500, or 1:121,500 with PBST containing 0.1% BSA. After incubation for 1 h at 37°C, the plates were washed with PBST, probed with HRP-conjugated goat anti-mouse IgG (Santa Cruz), and incubated at 37°C for 1 h. Then, 3,3′,5,5′-tetramethylbenzidine substrate (Tiangen, Beijing) was added, and the color reaction was allowed to develop for 10 min at room temperature without light exposure. Finally, 2 M sulfuric acid was added to terminate the reaction and the absorbance at 450 nm was read.

Immunofluorescence Assay

The immunofluorescence assay was conducted as described by He et al. [37]. For KSHV lytic cycle induction, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was added to the medium at a final concentration of 20 ng/ml 24 h before harvested. An aliquot of 15 μl of 1×10^7 cells/ml was spotted onto a well of an 8-well slide and allowed to dry. Fixation was conducted by plunging the slide into cold acetone and incubating at −20°C for 10 min. The anti-RTA antibody and the cyanine-3-conjugated goat anti-mouse antibody (Proteintech Group Inc., Chicago, IL, USA) were diluted 1:100, respectively, and incubated at 37°C for 30 min each. Images were photographed using an Olympus FV1000S laser scanning confocal microscope. BCBL-1 cells without TPA treatment were used as a control.

Results

Expression and Purification of RTAc

The 0.6 kb DNA fragment encoding the C-terminal region of RTA (E482~D691) was amplified and cloned into pET28a expression vector. The RTAc was expressed in *E. coli* and purified (Fig. 1). SDS-PAGE analysis revealed that the RTAc was about 40 kDa. The IPTG-induced RTAc was detected in the soluble fraction almost exclusively (Fig. 1, lanes 2 and 3). Fusion with 6× histidine tag at the N-terminus allowed purification of RTAc by affinity chromatography. Most of the recombinant protein bound to the HIS-Select gel effectively, only a small portion appeared in the flow-through (Fig. 1, lane 4). RTAc can be eluted with 250 mM imidazole (Fig. 1, lanes 5–9), with a peak appeared at the second elution fraction (Fig. 1, lane 6). Imidazole was removed by overnight dialysis against 1× PBS. At least 0.5 mg purified RTAc can be obtained from 200 ml bacterial culture by using 0.5 ml HIS-Select gel.

Generation of an Anti-RTA Antibody

Polyclonal antibodies were generated in mice by using the purified RTAc. The RTAc antigen can be detected with the antisera at the dilution of 1:13,500 by indirect ELISA. Western blot analyses were conducted with purified RTAc and anti-RTAc antiserum at

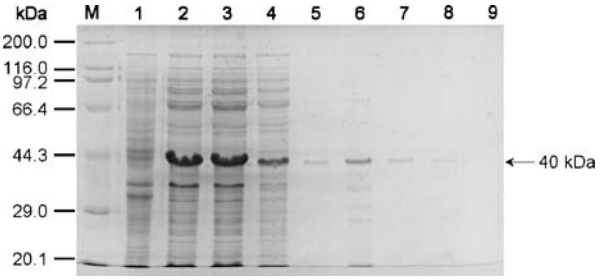

Fig. 1 Expression and purification of RTAc; the plasmid pET28a-RTAc was transformed in *E. coli* BL21 (DE3), and His_6-tagged RTAc was purified by affinity chromatography. *Lane M* protein molecular weight marker broad (TaKaRa Biotech., Dalian); *lane 1* total protein from uninduced *E. coli* BL21 (DE3) harboring pET28a-RTAc; *lanes 2 and 3* total and soluble proteins from *E. coli* BL21 (DE3) harboring pET28a-RTAc, induced with IPTG; *lane 4* flow-through; *lanes 5–9* elution fractions 1–5 from affinity chromatography. The 40-kDa recombinant RTAc was indicated with an *arrow*

different dilutions (Fig. 2A, lanes 1–4). A single band at about 40 kDa can be detected even the antibody was 1:20,000 diluted (Fig. 2A, lane 4). When total proteins were analyzed by Western blotting with the antiserum, the 40 kDa band was observed, whereas almost no cross-reactions with other bacterial proteins can be detected (Fig. 2B, lanes 1 and 2). These results demonstrated that the antibody can recognize the antigen specifically. No obvious difference in affinity and specificity was found among antisera from the four mice.

The Antibody Recognizes the Full-length RTA

E. coli BL21 (DE3) was transformed with pET28b-RTA [33] containing the full-length coding region of RTA and induced with 0.1 mM IPTG for 4 h. Total proteins were detected by Western blotting (Fig. 3A). A single band at about 90 kDa can be detected with either

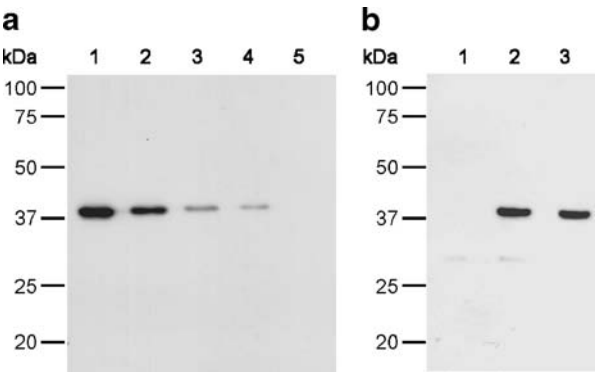

Fig. 2 Evaluation of the antibody by Western blotting; **A** The purified RTAc was analyzed by Western blotting with antiserum at dilutions of 1:2,500 (*lane 1*), 1:5,000 (*lane 2*), 1:10,000 (*lane 3*) and 1:20,000 (*lane 4*), or with pre-immune serum at 1:2,500 dilution (*lane 5*). Precision Plus Protein standards (Bio-Rad, Hercules, CA, USA) are shown at the *left*. **B** The *E. coli* BL21 (DE3)-expressed RTAc was detected in Western blot analysis with antiserum at 1:2,500 dilution. *Lane 1* total protein from uninduced *E. coli* BL21 (DE3) harboring pET28a-RTAc; *lane 2* total protein induced with IPTG; *lane 3* purified RTAc

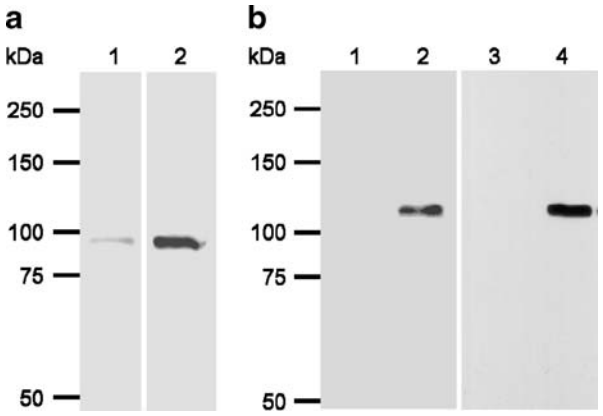

Fig. 3 Detection of full-length RTA in *E. coli* and mammalian cells; **A** Full-length RTA expressed in *E. coli* was detected by Western blotting. *E. coli* BL21 (DE3) harboring pET28b-RTA was induced with IPTG. Total proteins from 15 μl bacteria culture were loaded in each lane, resolved in SDS-PAGE, and detected by Western blotting with anti-His antibody (*lane 1*) or anti-RTA antibody (*lane 2*). **B** Full-length RTA expressed in transfected 293T cells was detected by Western blotting. 293T cells were transfected with pCMV-Tag50 (*lanes 2 and 4*) or empty vector pCMV-Tag2A (*lanes 1 and 3*). Total proteins were analyzed by Western blotting with anti-RTA antibody (*lanes 1 and 2*) or anti-Flag antibody (*lanes 3 and 4*)

anti-His_6 antibody (Fig. 3A, lane 1) or anti-RTA antibody (Fig. 3A, lane 2), demonstrating that the antibody generated in this study can recognize the His_6-tagged full-length RTA specifically.

The full-length RTA expressed in transfected mammalian cells can also be detected with the anti-RTA antibody. The plasmid pCMV-Tag50 was transfected into the 293T cells. Cells were harvested 48 h post transfection, and total proteins were resolved in SDS-PAGE and detected by Western blotting (Fig. 3B). A single band at about 110 kDa was detected only in the transfected cells (Fig. 3B, lane 2), demonstrating that the antibody generated against the C-terminal region of RTA can recognize the full-length RTA expressed in mammalian cells specifically. This band can also be detected with the anti-FLAG monoclonal antibody (Fig. 3B, lane 4).

The Antibody Recognizes both Native and Denatured RTA in BCBL-1 Cells

BCBL-1 cells were used to determine whether the antibody was suitable for the detection of endogenous RTA. It has been known that incubation of BCBL-1 cells with TPA will lead to the expression of RTA and the switch from latent to lytic [6]. BCBL-1 cells were collected at 0, 2, 4, 8, 24, 48, or 72 h after TPA treatment, and the expression of RTA and β-tubulin was monitored by Western blotting (Fig. 4). At 2 h after induction, enhanced RTA expression above background (0 h) was detected (Fig. 4, lanes 1 and 2). The accumulation of endogenous RTA can be observed from 4 h after TPA treatment, with the highest expression level detected at 24 h after TPA treatment (Fig. 4, lanes 3–5). At 48 or 72 h, the expression level of RTA decreased (Fig. 4, lanes 6 and 7), and the cells started to be lysed, as indicated by the expression level of β-tubulin (Fig. 4, lower panel).

To evaluate the application of anti-RTA antibody on native RTA, BCBL-1 cells were collected for immunofluorescence assay after being treated with TPA for 24 h. The anti-RTA antibody can recognize native RTA and is probed by a cyanine-3-conjugated

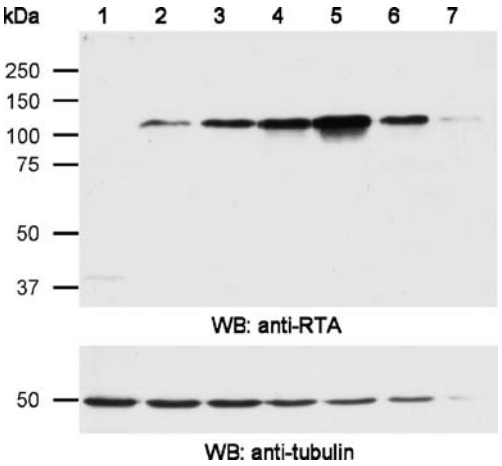

Fig. 4 Detection of endogenous RTA in BCBL-1 cells by Western blotting. BCBL-1 cells were collected at 0 h (*lane 1*), 2 h (*lane 2*), 4 h (*lane 3*), 8 h (*lane 4*), 24 h (*lane 5*), 48 (*lane 6*), or 72 h (*lane 7*) after treated with TPA, and total proteins were analyzed by Western blotting with anti-RTA antibody (*upper panel*) or anti-β-tubulin antibody (*lower panel*)

secondary antibody that emits a red fluorescence signal under illumination with light at 570 nm (Fig. 5E). However, the TPA-untreated BCBL-1 cells did not exhibit any red fluorescence (Fig. 5B). These results demonstrated that the anti-RTA antibody can recognize endogenous RTA specifically under native condition.

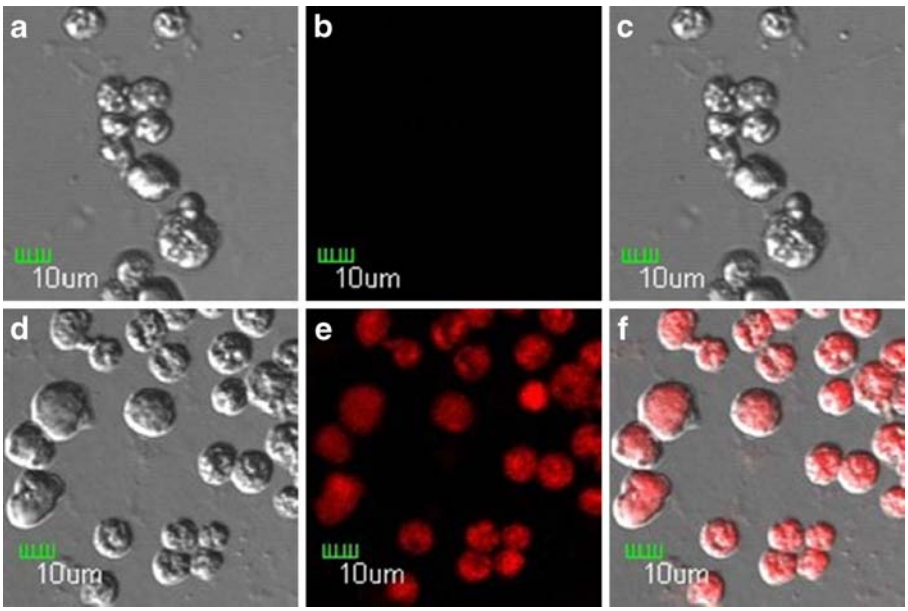

Fig. 5 Immunofluorescence assay of native RTA in BCBL-1 cells. BCBL-1 cell treated without (**A**–**C**) or with TPA (**D**–**F**) were examined by immunofluorescence microscopy with the anti-RTA antibody, which is recognized by cyanine-3 conjugated goat anti-mouse antibody (**B**, **E**). Images under transmitted light (**A**, **D**) and confocal images (**C**, **F**) are also shown. The *bar* indicates 10 μm

Discussion

RTA regulates numerous viral and host gene expression, controls the life cycle of KSHV, and plays an important role in the pathogenesis of KSHV [6, 7]. Since RTA is sufficient for the switch from latency to lytic replication, it is anticipated to be a target to control KSHV infection and reactivation. To demonstrate the expression pattern and biochemical properties of RTA, several antibodies against RTA have been generated by using full-length RTA, the DNA-binding domain of RTA, or selected synthetic peptides conjugated to keyhole limpet hemocyanin as immunogens [6, 25, 38–40]. In this study, we report the generation and evaluation of polyclonal antibody against RTA by using a C-terminal region, which comprises the intact activation domain of RTA. High-avidity antisera were obtained, which are functional at least in three different assays: ELISA, Western blots, and immunofluorescence microscopy. The crude antisera were applied directly without further purification, but almost no cross-reaction was observed with other bacterial or mammalian proteins, demonstrating that the antibody we raised is highly specific.

The RTA protein can be divided into an N-terminal DNA-binding and dimerazation domain (1~530) and a C-terminal activation domain (486~691) [7, 41]. Choosing the activation domain of RTA to generate antibody is resulted from two considerations. Firstly, it has been reported the limited expression level of full-length soluble RTA in bacteria and difficulties in the purification process [38], a shortened form of RTA may provide a better chance to obtain large amounts of pure protein for antibody production. Indeed, the RTAc was induced almost exclusively in soluble fraction and was purified to near homogeneity (Fig. 1). Secondly but more importantly, considering its application, an antibody raised against the activation domain of RTA will have less interference on its binding to DNA, comparing with the disturbance of RTA from binding with other viral or cellular factors. Therefore, the antibody against RTAc may provide a new tool to block the interaction of RTA with other coactivators and subsequently block the function of RTA. The usage of the antibody in other experiments including chromatin immunoprecipitation and EMSA needs to be evaluated in the future.

For the full-length RTA expressed in *E. coli,* a band at about 90 kDa was recognized by the antibody (Fig. 3A), about 13 kDa higher than the predicted molecular mass of 73.7 kDa. This size is in agreement with that expressed by other groups [6, 38]. Song et al. reported the recombinant DNA-binding domain of RTA migrated in SDS-PAGE at approximately 37 kDa, similar to its predicted molecular mass, 36.7 kDa [38]. In the present study, the recombinant RTAc migrated in SDS-PAGE and Western blot analysis at approximately 40 kDa, which is also 13 kDa higher than the predicted molecular weight of 27 kDa (Figs. 1, 2). Therefore, it is clear that the 13-kDa size retardation is resulted from the C-terminal region of RTA. This is probably because of the unusual amino acid composition of the RTAc. It is known that on SDS-PAGE, the net charge of a protein will affect its mobility; highly charged proteins will tend to bind less SDS and will have altered mobility. Proteins rich in proline may also exhibit dramatically slower mobility in SDS-PAGE [42]. In RTA, a proline-rich region has been identified at amino acid 385 to 600 [41]. Within the activation domain we expressed, 27 proline residues were found which is more than 12% of the total 210 amino acids and may cause the aberrant migration. In BCBL-1 cells and the transfected 293T cells, a band at about 110 kDa was detected, which is 36.3 kDa larger than was predicted and 20 kDa larger than was expressed in *E. coli*, may represent a post-translationally modified form of RTA. The size of 110 kDa that full-length RTA expressed in mammalian cells is actually in agreement with what was expressed in Cos-7, 293T and BCBL-1 cells by other groups [6, 38].

In conclusion, sensitive and specific polyclonal antibody against RTAc was generated and applied to examine the expression pattern of RTA. Since KSHV RTA is a key transcriptional regulator governing the switch from viral latency to lytic replication, the antibody generated in the present study will provide a powerful tool for the detection of RTA under various conditions and the investigation of biochemical properties and biological functions of RTA.

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