ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 10, pp. 1134-1139. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © S. V. Diduk, K. V. Smirnova, O. A. Pavlish, V. E. Gurtsevitch, 2008, published in Biokhimiya, 2008, Vol. 73, No. 10, pp. 1414-1421.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-137, September 21, 2008.

Functionally Significant Mutations in the Epstein–Barr Virus *LMP1* Gene and Their Role in Activation of Cell Signaling Pathways

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Received April 15, 2008
Revision received April 25, 2008

Abstract—Latent membrane protein 1 (LMP1) of the Epstein–Barr virus is a constitutively activated analog of the tumor necrosis factor receptor TNF-R1. LMP1 serves as a viral oncogen able to transform human B-lymphocytes and rodent fibroblasts via activation of numerous cellular signal cascades. Two specific motifs within LMP1 are responsible for interaction of this viral protein with the receptor protein β-TrCP/HOS SCF of the ubiquitin ligase E3 complex, playing an important role in degradation of numerous cellular proteins including NF-κB inhibitor IκBα. In this study, we demonstrate for the first time the importance of point mutations affecting HOS-recognizing motifs of LMP1 for activation of NF-κB, AP1, and PI3K/Akt signaling pathways. It has also been shown that rat fibroblast cell lines (Rat-1) expressing different HOS mutants of LMP1 produce different amounts of reactive nitrogen species. Our data confirm the hypothesis that point mutations in the C-terminal region of the LMP1 cytoplasmic domain can influence the transforming potential of the Epstein–Barr virus.

DOI: 10.1134/S0006297908100106

Key words: Epstein–Barr virus, LMP1 protein, HOS mutations, NF-κB, NO

Epstein–Barr virus (EBV) of the herpes family viruses is ubiquitously distributed in humans and is associated with some malignancies of human lymphoid and epithelial origin, such as Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin lymphoma, etc. [1-3]. The *LMP1* gene is one of a few so-called latent infection genes expressed in EBV-associated tumors, which immortalize and transform cells [4, 5]. The LMP1 (latent membrane protein 1) molecule encoded by this gene is a 63-kD integral membrane protein consisting of 386 amino acid residues (aa). The gene includes six transmembane domains (162 aa) and two cytoplasmic domains—a short N-terminal and a long C-terminal domain of 24 and 200 aa, respectively [6]. The C-terminal cytoplasmic domain of LMP1 contains two transactivating regions (CTAR). The proximal transactivating region CTAR1 is located between 194-232 aa and activates the transcription factor NF-κB through the signaling pathway TRAF3/NIK/ IKKα. The distal transactivating region CTAR2 located between 351-386 aa is the main inducer of NF-κB, activating it through the signaling pathway TRAF6/TAK1/ IKKβ [7, 8]. Moreover, both CTAR1 and CTAR2 regions activate other signaling pathways including two MAP-kinase pathways SAPK/JNK and p38 and also a phosphatidylinositol-3-kinase signaling pathway (PI3K/Akt) [9-12]. The LMP1-mediated mechanism of activation of these signaling pathways is not known in detail but is now under intensive study. This interest is caused by the important role of these signaling pathways in regulation of such crucial functions of the cell as growth, survival, aging, and tumor transformation.

The pleiotropic effect of LMP1 on different biological processes is due to its features imitating properties of

Abbreviations: CTAR1) proximal C-terminal activation region of LMP1; CTAR2) distal C-terminal activation region of LMP1; EBV) Epstein–Barr virus; HOS motif) amino acid sequence recognized by HOS receptor; HOS receptor) receptor protein β-TrCP/HOS SCF of the ubiquitin ligase E3 complex; iNOS) inducible NOS; JNK) c-Jun N-terminal kinase pathway; LMP1) latent membrane protein 1; NO) nitric oxide; NOS) NO synthase; PI3K/Akt) phosphatidylinositol-3-kinase signaling pathway.

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the family of tumor necrosis factor receptors (TNF-R). Activation by the viral protein of inducible NO-synthase (iNOS) synthesis and production of nitric oxide (NO) [13, 14] is associated with just these properties. Nitric oxide is a free short-living biologically important radical generated in various cells and involved in regulation of intra- and intercellular signaling systems as a cellular messenger [15, 16]. Being a reactive nitrogen species, NO together with cytokines influences cellular systems responsible for proliferation, apoptosis, and differentiation of cells, as well as their resistance to stress. Intracellular NO is synthesized by NO-synthase (NOS). Three NOS isoforms are known: neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III). Both nNOS and eNOS are constitutively expressed in cells, while iNOS is produced *de novo* as a cell response to stress or as a result of action of different cellular cytokines [17, 18]. The iNOS expression and NO synthesis in cells correlate with tumorigenicity of LMP1 variants supposed to be associated with a Cao-specific deletion in the CTAR2 region of LMP1 [19].

Accumulation of a number of significant mutations has been found in CTAR regions of the LMP1 molecule in Epstein–Barr virus isolates from tumor tissue of patients with EBV-associated diseases in different geographical regions. These mutations change the cytostatic/cytotoxic status of this protein and also promote its transforming effect on cells [20-23]. These frequent mutations include the replacement of glycine by serine in position 212 (G212S), replacement of serine by threonine in position 366 (S366T), and also a 30-bp Cao-deletion located in the C-terminal cytoplasmic domain. All these mutations have been shown to disturb LMP1 binding with the receptor protein β-TrCP/HOS SCF of the ubiquitin ligase E3 complex (HOS receptor) that enhances the *in vitro* transforming activity of LMP1 and also disturbs regulation of the signaling function of NF-κB protein [24].

This work was designed to look for a possible influence of a set of HOS mutations in EBV LMP1 on activation of some signaling pathways (NF-κB, AP1, PI3K/Akt) and generation of NO. We used a highly sensitive model based on Rat-1 rat fibroblast cells and a collection of HOS mutants created by us based on a retroviral vector. It is been shown that some amino acid replacements play a role in the activation of transcription factors NF-κB, AP1, and the PI3K/Akt signaling pathway. These mutations are shown to influence the intracellular production of NO and kinetics of its accumulation in the Rat-1 cell culture.

MATERIALS AND METHODS

Plasmids. Vector constructions pSG5-LMP1-B95-8 and pSG5-LMP1-Cao were kindly provided by F. Grasser (Homburg, Germany). *LMP1* gene variants with single (G212S, S350A, S366T), double (G212S/S350A, G212S/S366T), and triple (G212S/S350A/S366T) replacements in HOS sites inside the pSG5 vector were obtained from S. Fuchs (Pennsylvania, USA). We recloned all these variants of the full-size *LMP1* from the eukaryotic expressing vector pSG5 into the retroviral vector pBabe-puro (pBabe). The prototypical variant *LMP1-B95-8* and *LMP1* variants with mutations in HOS sites were cloned into the pBabe vector at EcoRI restriction sites. A highly tumorigenic variant *LMP1-Cao* was cloned at the BamHI restriction site. The transcription factor NF-κB activation was analyzed using a reporter plasmid κB-ConA-Luc kindly presented by F. Grasser. Activation of the JNK signaling pathway was analyzed using a *jun2*-luciferase reporter plasmid (AP1-Luc) kindly provided by M. Rowe (Cardiff, Wales).

Cell cultures, preparation of retroviral stock, and transduction. All cell lines were maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco), L-glutamine (2 mM), and gentamycin (100 U/ml) at 37°C in the presence of 5% CO_2. Cells of the line Phi-NX-Ampho Phoenix-A (a derivate of HEK293 cells) were transfected with genetic constructions created on the base of pBabe-puro using LipofectAMINE Plus (Invitrogen, USA) according to the producer's instructions. The supernatant containing viral particles was collected 48 h after the transfection and centrifuged at 3000 rpm for 5 min at 4°C. The cell line Rat-1 was transduced with the supernatant purified of the cellular debris in the presence of polybrene (4 mg/ml). Cell selection was performed with puromycin (5 mg/ml) (Sigma, USA).

Preparation of cell lysates and Western-blotting. The Rat-1 line cells expressing different LMP1 variants were washed thrice in PBS, suspended in 200 μl of lysing buffer (Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% β-mercaptoethanol), ultrasonicated with an Ultrasonic Amplifier (England), and boiled (100°C) for 5 min. The resulting cellular lysate was cleared by centrifugation at 12,000 rpm for 10 min at 4°C. Protein concentration in the resulting extract was determined by the Bradford method [25], and optical density of the extract was determined with a Junway 6305 spectrophotometer (England). The proteins were separated in 12.5% polyacrylamide gel in buffer for electrophoresis (25 mM Tris-HCl, 200 mM glycine, 0.1% SDS) at 20 mA. The proteins were transferred for 30 min onto a nitrocellulose membrane using a Fastblot B43 apparatus for semi-dry transfer (Biometra, Germany) at 2.5 mA/cm, 4°C. The protein transfer efficiency was determined by staining the membrane with Ponceau S solution. The dye was removed by washing in 0.01 M Tris-HCl. Blockage was performed for 1 h in 5% solution of defatted dry milk in PBS. Then, depending on the purpose of the experiment, the protein-carrying membrane was incubated for 12 h at 4°C with monoclonal antibodies as follows: PKBα/Akt1, PKB-pSer473, and

also with antibodies to β-catenin (Sigma) and S12 to LMP1, which were obtained from F. Grasser.

The antibody-treated membrane was washed in PBS and incubated with secondary anti-mouse and anti-rat antibodies conjugated with horseradish peroxidase for 1 h at 4°C. The membrane was washed five times in PBS, and then the antigen–antibody complex was detected by staining with solution of 3,3′-diaminobenzidine and H_2O_2. To measure the protein amount, the stained bands were scanned, and the staining intensity was determined with an ImageJ software.

Luciferase analysis. Activities of transcription factors NF-κB and AP1 were quantitatively measured by expression of luciferase from reporter plasmids κB-ConA-Luc and AP1-Luc co-transfected into the HEK293 cells together with vector constructions containing full-size LMP1 variants [24]. Twenty-four hours after the transfection, the cells were washed in PBS and lysed in 1× CCLR buffer (Promega, USA). The expression of luciferase was measured as recommended by the producer. The cellular extract (30 μl) was supplemented with 100 μl of luciferin solution, and the luminescence intensity was determined with a Tuner BioSystem luminometer (USA). The luciferase activity was measured for 10 sec. In experiments, three independent samples were taken from each specimen.

Nitrate/nitrite analysis. Accumulation of NO was determined by formation of its stable end products (nitrate/nitrite) in supernatant of the cultured Rat-1 cells. The total amount of nitrate/nitrite was determined with a kit of reagents for nitrate/nitrite analysis (Cayman Chemical Co., USA) as recommended by the producer. The supernatant (80 μl) was incubated with 20 μl of nitrate reductase at room temperature for 1 h and then supplemented with 100 μl of Griess-reagent (1% sulfanilic acid, 0.1% naphthylethylenediamine dihydrochloride, 2.5% phosphoric acid). The optical density of each sample was analyzed with a microplate spectrophotometer at 540 nm using sodium nitrite for the calibration curve. The same number of cells ($5 \cdot 10^5$ cell/ml) was passaged into each culture flask, and measurements were performed in three independent experiments.

RESULTS AND DISCUSSION

LMP1 is known to have two so-called HOS motifs: a canonical DSGxxS motif in position 210-215 (CTAR1 region) and a cryptic DSGxx in position 349-351 (CTAR2 region). These motifs serve as a signal for ubiquitination of some cell proteins such as IκB inhibitor, β-catenin, I-type α-interferon receptor, etc. A prototypical LMP1-B95-8 variant lacking mutations in HOS sites has been shown to interact with receptor protein β-TrCP/HOS that results in a "squelching" phenomenon, i.e. inhibition of the NF-κB signaling pathway activation associated with exhaustion in the cell of a component of the ubiquitin-proteosomal system (HOS receptor). Such effect is absent in cells expressing LMP1 variants with mutations affecting HOS motifs, and this enhances the transforming effect of corresponding LMP1 proteins on the cell [24]. To exclude the well-known *LMP1* hyperexpression observed on transfection of this gene into cells, which is often responsible for wrong results, we recloned full-size variants of the gene with single (G212S, S350A, S366T), double (G212S/S350A, G212S/S366T), and triple (G212S/S350A/S366T) mutations located in the HOS sites, as well as its control variants (LMP1-B95-8 and LMP1-Cao) from eukaryotic expressing vector pSG5, into retroviral vector pBabe-puro. On packing of the resulting genetic constructions in HEK293 cells (Phoenix), the retroviral stocks were used for transduction of Rat-1 rat fibroblasts. Then, cells carrying the desired vector constructions were subjected to selection in the presence of puromycin for two weeks. Expression of LMP1 in all resulting stable cell lines was confirmed by Western blotting.

Mutations in canonical and cryptic HOS motifs of LMP1 protein are required for activation of transcription factor NF-κB. The ability to influence activation of the transcription factor NF-κB and *c-jun* N-terminal kinase pathway (JNK) was analyzed in cell lines constitutively expressing LMP1 with single, double, and triple muta-

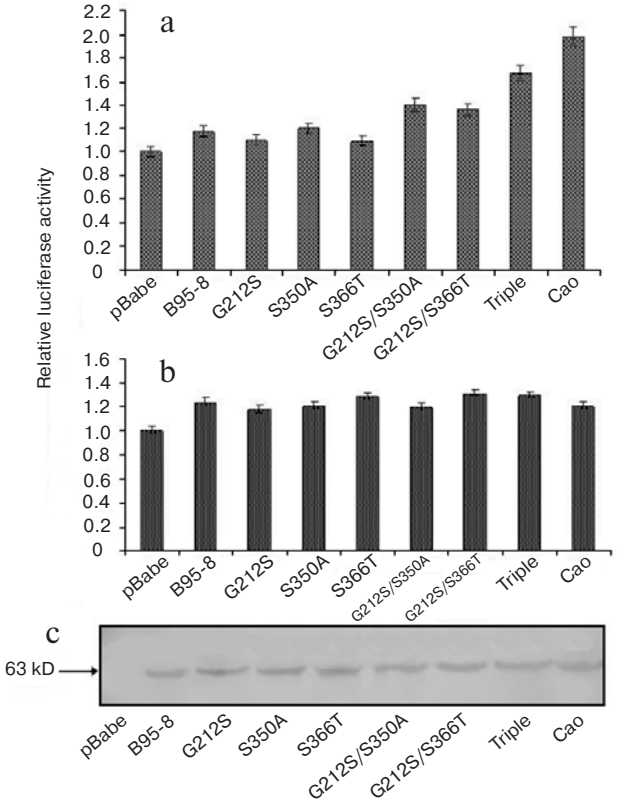

Fig. 1. Activation of NF-κB (a) and AP1 (b) signaling pathways in rat fibroblast line Rat-1; expression of LMP1 variants (c).

tions and also in corresponding control variants of the gene. Full-size LMP1 variants carrying double mutations in HOS sites (G212S/S350A, G212S/S366T) were found to increase NF-κB activation by 10-20% as compared to the prototype, whereas the Triple variant activated NF-κB similarly to its activation by the tumorigenic variant LMP1-Cao (Fig. 1a). However, NF-κB activation in variants with single mutations (G212S, S350A, S366T) in CTAR regions of LMP1 was only slightly higher than in the prototypical variant LMP1-B95-8. Thus, all LMP1 variants under study could activate NF-κB. Nevertheless, activation of this transcription factor by LMP1 variants with double and triple mutations responsible for disorder in the HOS receptor with both HOS motifs was significantly higher than the activation induced by the control variant LMP1-B95-8.

LMP1 variants carrying mutations in HOS motifs do not activate signaling pathways AP1 and PI3K/Akt. The signaling cascade *c-jun* of N-terminal kinase (JNK/AP-1) is initiated by many different factors, such as temperature shock, ionizing radiation, growth factors, etc. Expression of LMP1 protein in the cell also results in induction of stress-activated protein kinase (SAPK) [26]. Using a *jun2*-luciferase reporter vector, we compared activations of AP-1 by LMP1 variants carrying HOS mutations in CTAR regions of the molecule, a prototypical variant LMP1-B95-8, and a highly tumorigenic variant LMP1-Cao (Fig. 1b). All LMP1 variants carrying HOS mutations induced nearly the same activation of the *jun2*-luciferase reporter as LMP1-B95-8. On determining the luciferase activity, the LMP1 expression in the studied cell lines was confirmed by immunoblotting with S12 antibodies specific to the region of the protein repeats (Fig. 1c).

The phosphatidylinositol-3-kinase signaling pathway (PI3K/Akt) was recently shown to be activated by the LMP1 protein CTAR1 region [12, 27]. Considering that HOS mutations studied by us are also located in the LMP1 CTAR regions, we analyzed their possible influence on the PI3K/Akt activation. By densitometry of immunoblotting conducted with specific antibodies to protein kinase B (Akt/PKB) and its phosphorylated form (p-Akt, Ser473), an increased content of p-Akt was detected in cells expressing a tumorigenic variant LMP1-Cao, and this was 1.5-fold higher compared with the studied LMP1 variants which carried one, two, or three amino acid replacements (Fig. 2a). The latter variants induced phosphorylation of Akt similarly to the prototypical variant LMP1-B95-8. In these studies, monoclonal antibodies to β-catenin were used as a control. Therefore, it is concluded that the studied amino acid substitutions in the LMP1 protein do not influence the phosphatidylinositol-3-kinase activity in the transduced cells.

Decrease in NO synthesis upon expression of LMP1 variant with inactivated HOS motifs. As mentioned above, LMP1 can activate synthesis of inducible NO-synthase and production of NO. The prototypical LMP1-B95-8 variant and highly transforming variant LMP1-Cao were shown to differ in the levels of iNOS activation and NO production [19]. These differences seemed to be associated with the so-called Cao-deletion of 10 aa in the LMP1-Cao CTAR2 region. Therefore, it was supposed that other frequently observed mutations in this region of the gene, namely G212S and S366T, could also be involved in enhancement of the intracellular generation of NO. To determine the effect of these amino acid replacements on NO generation in Rat-1 cell lines constitutively expressing LMP1 variants with HOS mutations under study and control variants (LMP1-B95-8 and LMP1-Cao), the nitrate/nitrite concentration was measured in 3-day-old culture medium. Figure 3a shows that the cell lines expressing LMP1 with single and double substitutions in HOS sites release into the culture medium virtually the same amount of NO as the control prototypical variant LMP1-B95-8 (12-12.5 and 13.1 nmol/ml of medium, respectively). However, the NO content in the culture medium of the triple variant and of the control highly tumorigenic variant LMP1-Cao was significantly decreased (9.7 and 6.4 nmol/ml of medium, respectively). Accumulation of NO by the cell lines expressing differently tumorigenic variants LMP1-B95-8, LMP1-Cao, and also LMP1-Triple was linear during three days and flattened out by the fourth day. Figure 3b also shows that

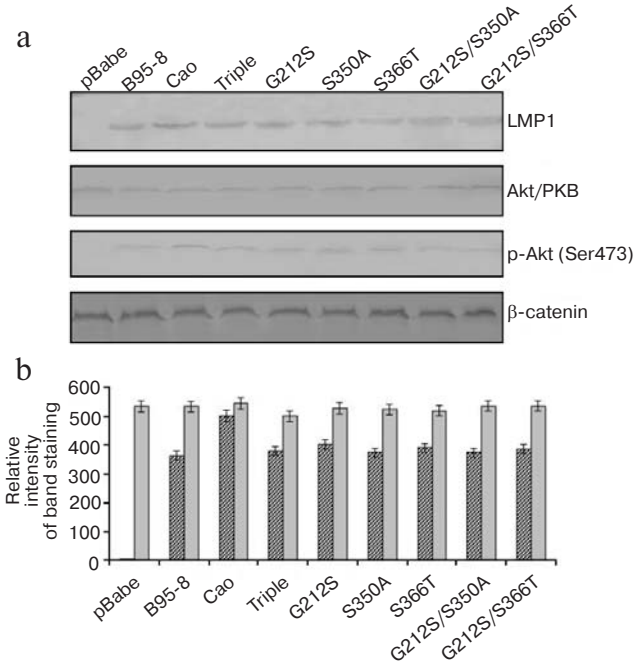

Fig. 2. Activation of Akt/PKB protein kinase by LMP1 variants carrying mutations in HOS sites: a) Western blotting with antibodies specific to LMP1 (S12), Akt/PKB, phosphorylated form of Akt (p-Akt), and β-catenin. β-Catenin was used as a control of application; b) quantitative analysis of total and phosphorylated form of Akt performed with the ImageJ software. Gray and hatched columns present Akt/PKB and p-Akt, respectively.

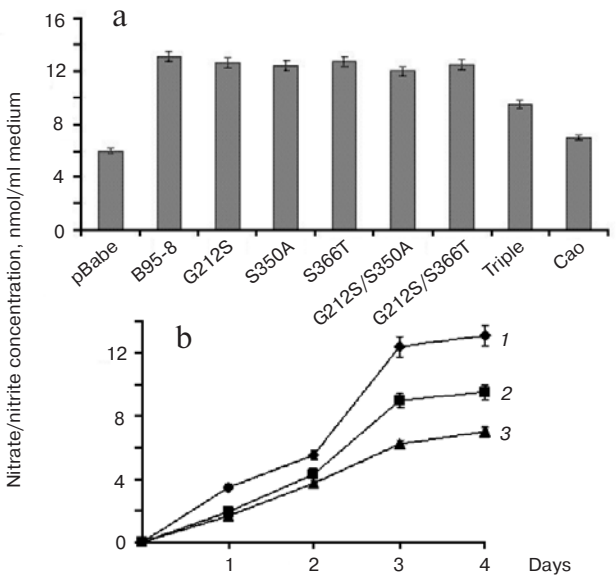

Fig. 3. Accumulation of nitrate/nitrite in the culture medium of Rat-1 cell lines expressing HOS mutants of LMP1: a) determination of NO three days after plating; b) accumulation of NO during four days. *1*) B95-8; *2*) Triple; *3*) Cao.

in the culture medium of cell lines expressing the LMP1-Triple and LMP1-Cao variants, NO was produced more slowly than in the case of the LMP1-B95-8 variant. Thus, the findings suggested that mutations inactivating HOS motifs of LMP1 should influence both the rate of NO generation in the cell and its accumulation in the culture medium.

Biological properties of full-size EBV gene *LMP1* variants of different origin characterized by specific mutation sets are now under intensive study. Nevertheless, there are only a few studies concerning the role of individual *LMP1* mutations in the regulation of cell signaling pathways determining the cell life. Therefore, the purpose of our work was to elucidate the role of single, double, and triple mutations in the gene in the functional activity of LMP1.

Thus, LMP1 variants with double mutations (G212S/S350A, G212S/S366T) leading to disorders in binding of the β-TrCP/HOS receptor in the protein canonical and cryptic sites increased activation of the transcription factor NF-κB (Fig. 3). And these variants, as well as the triple variant, activated NF-κB stronger than the LMP1-B95-8 prototype and were close to the activation by the Cao variant of the protein (Fig. 1a). However, the activation levels of NF-κB by LMP1 variants with different single substitutions (serine by alanine and serine by threonine in the positions 350 and 366, respectively) were the same and corresponded to the activation level of NF-κB by the LMP1-B95-8 prototypical variant. These findings are in agreement with data of other researchers indicating that LMP1 with three amino acid substitutions activated NF-κB stronger than the prototypical molecule [24]. This effect recorded by us and other authors can be caused by different abilities of the studied LMP1 variants to bind with HOS receptor. Note also that although another substitution studied by us, that of glycine by serine in position 212, affects the destructive motif of LMP1, it seems to have no significant influence on phosphorylation of serines located, respectively, in the positions 211 and 215. Phosphorylated state of these two amino acids located close to the protein-binding site TRAF with LMP1 does not directly influence the interaction of these proteins, but it is likely to influence this process indirectly [28].

Induction of transcription factor AP-1 leads to expression of important genes crucial for cell proliferation, differentiation, and transformation [29]. JNK/AP-1 can be activated by various stress factors, including protein synthesis inhibitors, UV, and heat shock [30, 31]. This process occurs with involvement of TNFR family members (TNFRI, TNFRII) and CD40 [32, 33]. Activation of JNK by LMP1 also can be a response of the cell to stress induced by this protein. Note that some authors have shown that constitutive expression of LMP1-B95-8 and LMP1-Cao variants in different cell lines is accompanied by relatively low constitutive activation of AP-1 [26, 34]. We found approximately the same activation of JNK in Rat-1 cells constitutively expressing the LMP1 variants with HOS mutations (Fig. 1b). The absence of significant differences between the studied LMP1 HOS mutants and the control variants LMP1-B95-8 and LMP1-Cao indicate independence of JNK activation of disorders in HOS sites of this protein. Although AP-1 seems to be activated through the TRAF2 domain, there are natural LMP1 variants capable of activating this transcription factor independently of any amino acid replacement in LMP1 domains, including those in CTAR2 [35, 36]. Nevertheless, an increase in the AP-1 level recorded in tumor cells of patient with Hodgkin's lymphoma seems to be associated with protection of these cells against apoptosis.

The ability of LMP1 to activate the PI3K/Akt signaling pathway allows us to explain many phenotypic genetic changes associated with LMP1 expression in various cell cultures. The LMP1 domain CTAR1 seems to play a leading role in the activation of PI3K/Akt by LMP1 protein. This activation results in changes in the cell cytoskeleton accompanied by a significant increase in actin filaments [12, 37]. Activation of the PI3K/Akt signaling pathway concerns not only epithelial cells, but such activation has also been detected in B-lymphocytes [38]. Our data by densitometry indicate that all LMP1 variants under study equally activate PI3K/Akt in Rat-1 cells (Fig. 2, a and b). This might have been due to a high conservativeness of the CTAR1 domain in all LMP1 variants. On the other hand, although a substitution in posi-

tion 212 of the CTAR1 domain affected the canonic HOS site, it seemed to be insufficient for critical changes resulting in disorders of the PI3K/Akt signaling pathway.

The mechanism of activation of iNOS in LMP1 expressing cells is still unclear. The LMP1-B95-8 and LMP1-Cao variants were earlier found to differ in the intracellular generation of NO, and these differences correlated with cell survival [19]. The well-known cytotoxic/cytostatic effect of LMP1-B95-8 in different cell lines seems to be mediated through an increase in induction of iNOS and elevation of the amount of intracellular NO [19, 39, 40]. In contrast, a decrease in NO production in cells expressing the highly tumorigenic variant LMP1-Cao was accompanied by abolishment of cytotoxic effect that seemed to be associated with the 10-aa deletion in this variant of the protein. The decreased synthesis of NO found by us in rat fibroblasts on expression of LMP1-Triple also suggested that these substitutions could be involved in regulation of NO production (Fig. 3, a and b). Nevertheless, the cytostatic effect of LMP1 could also be regulated through the HOS-dependent transcription factor NF-κB. This process is associated with abolishment of HOS receptor squelching caused by mutations in the canonical and cryptic sites of LMP1. This could also reduce the cytostatic effect of the prototypical variant LMP1-B95-8 [24].

Thus, in the present work we have shown for the first time the effect of individual frequently occurring mutations in the EBV *LMP1* gene on activation of signaling pathways crucially important for cell functioning and also on induction in the cell of reactive nitrogen species. More extended and detailed studies seem promising for elucidation of mechanisms of EBV-associated carcinogenesis and creation of a basis for using the data for diagnosis and treatment of tumors associated with this virus.

This work was supported by the Russian Foundation for Basic Research (project No. 07-04-00604-a).

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