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Expression and characterization of N-terminal domain of Epstein–Barr virus latent membrane protein 2A in *Escherichia coli*

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Abstract

Latency of Epstein–Barr virus (EBV) is maintained by the transmembrane protein latent membrane protein (LMP) 2A, which mimics the B-cell receptor (BCR) and perturbs BCR signaling. LMP2A contains a cytoplasmic N-terminal domain composed of 119 amino acids, which provides signals that are responsible for the association with various signal molecules, resulting in negative regulation of B-cell signaling and the EBV lytic cycle. In the present study, to obtain N-terminal domain of LMP2A (LMP2A NTD, 13 kDa) in *Escherichia coli* for structural analysis, a strategy for obtaining the unfused form of LMP2A NTD without any fusion partners was proposed. Recombinant LMP2A NTD has previously been expressed using the GST fusion system in *E. coli* [Virology 268 (2000) 178, J. Virol. 71 (1997) 4752, Mol. Cell. Biol. 20 (2000) 8526]. However, we were unable to obtain untagged LMP2A NTD from this construct because of rapid proteolysis by thrombin. To overcome the proteolysis by thrombin, C-terminal His-tagged LMP2A NTD and intein-fused LMP2A NTD were prepared. As a result, LMP2A NTD without a fusion partner could be successfully obtained using non-enzymatic cleavage. The secondary structure of the recombinant LMP2A NTD was analyzed using circular dichroism. In aqueous solution, LMP2A NTD adopts an unordered structure, which was not affected by varying pH and salt concentration. In addition, any secondary structural components of LMP2A NTD were not induced in the membrane–mimicking environments, suggesting that LMP2A NTD may intrinsically have a random coil-like structure. The biological activity of recombinant LMP2A NTD was monitored by chemical shift perturbation in HSQC spectra of LMP2A NTD with or without WW domains, which result supports that the structural change induced by WW domains is restricted within narrow region.

Keywords: EBV; LMP2A; Proteolysis; Intein; NMR; WW domain

Epstein–Barr virus (EBV) is classified as γ-herpesviruses and establishes latent infection in human B lymphocytes [4]. Latency of EBV is maintained by the latent membrane protein (LMP) 2A, which mimics the B-cell receptor (BCR) and perturbs BCR signaling [5–8]. This mimicking enables EBV to escape from host immunity [9]. Normal BCR signaling associates with the Src familiy protein tyrosine kinases (PTKs), Lyn, Fyn, and Blk, followed by Syk PTK activation [10]. These signal molecules also interact with LMP2A [11,12].

LMP2A contains a cytoplasmic N-terminal domain composed of 119 amino acids, 12 potential membrane-spanning domains, and a cytoplasmic C-terminal domain composed of 27 amino acids [13]. The N-terminal domain of LMP2A (LMP2A NTD) may provide signals

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that are responsible for the association with the protein tyrosine kinases Lyn and Syk, resulting in negative regulation of B-cell signaling and the EBV lytic cycle [5,8,14]. Lyn PTK binds to phosphorylated tyrosine 112 of LMP2A NTD [12], followed by phosphorylation of the LMP2A immunoreceptor tyrosine-based activation motif (ITAM) at tyrosines 74 and 85. Syk PTK binds to this phosphorylated ITAM of LMP2A [11,12]. In addition, the other tyrosine residues of LMP2A NTD seem to be involved in binding sites for SH2 domains of signal molecules like the docking protein Shc, the 85 kDa regulatory subunit of PI3-kinases, and Csk [5,15]. The bound Lyn PTK is rapidly digested in LMP2A-expressing cells, which results in modulation of B-cell signal transduction. This process occurs by ubiquitination of the bound Lyn PTK and LMP2A NTD contains two PY motifs (PPPPY) that interact with WW domains of Nedd4 family ubiquitin–protein ligases [1,3,16].

While the biological function of LMP2A has been investigated by several groups of investigators, the structural information is completely insufficient to clarify the mechanism of LMP2A function. To obtain enough protein for structure analysis, it is necessary to develop an optimized expression system. Recombinant LMP2A NTD has previously been expressed using the GST fusion system in *Escherichia coli* [1–3], and was used to identify interaction partners of LMP2A NTD. However, we were unable to obtain untagged LMP2A NTD because of digestion by thrombin (refer this paper), although the intact LMP2A NTD is required for structure analysis.

In this study, a strategy for obtaining the unfused form of LMP2A NTD was proposed. The secondary structure of the recombinant LMP2A NTD was examined by circular dichroism (CD) and the capacities of AIP4 WW2 and WW3 peptides to interact with LMP2A NTD were monitored by chemical shift perturbation in HSQC spectra.

Materials and methods

Materials

Restriction endonucleases were purchased from New England Biolabs (Beverly, MA, USA), Böehringer–Mannheim GmbH (Mannheim, Germany), and Promega (Madison, WI, USA). Cloned *Pfu* DNA polymerase was obtained from Stratagene (La Jolla, CA, USA). Glutathione–Sepharose 4B resin was supplied by Amersham–Pharmacia Biotech (Uppsala, Sweden). Oligonucleotides used for DNA sequencing and PCR amplification reaction were obtained from Bioneer (Dae-Jun, Korea). The pET-21a vector was obtained from Novagen (Darmstadt, Germany). The plasmid pTWIN1 and chitin–binding resins were supplied by New England Biolabs (Beverly, MA, USA). Dodecyl phosphocholine (DPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Sodium dodecyl sulfate (SDS) was obtained from Sigma (St. Louis, MO, USA). All materials were of reagent or biotechnological grade.

Construction of plasmids

Construction of the plasmid for the expression of GST–LMP2A NTD fusion protein has been described elsewhere [1]. The other LMP2A NTD constructs were prepared using pET21a and pTWIN1. Before subcloning the LMP2A NTD gene into pTWIN1 and pET21a, silent mutations are introduced in the first few codons of each forward primer to stabilize the secondary structure of the transcribed mRNA and to facilitate expression of LMP2A NTD (Table 1). That is, the third and fourth codons, TCC and CTA, were substituted for AGT and TTA, respectively. This was conducted using the web-program ProteoExpert (www.proteoexpert.com). The plasmids were generated by inserting the PCR fragment containing the LMP2A NTD region into the appropriately digested vectors. The LMP2A NTD coding gene was inserted between the *Nco*I and *Pst*I sites in pTWIN1 for N-terminal *Ssp Dna*B intein fusion, and the *Nde*I and *Xho*I sites in pET21a. The primer pairs used in PCR amplification are shown in Table 1. The constructed plasmids were confirmed by restriction analysis and DNA sequencing.

Expression of recombinant LMP2A NTD

Each LMP2A NTD construct was transformed into the appropriate *E. coli* host cells: pET21a/BL21(DE3)

Table 1
Primers used for plasmid construction

Plasmid	PCR primers used for construction	Endonuclease sites
pET-21a	5′-GGAATTCCATATGGGGAGTTTAGAAATGGT-3′	*Nde*I
	5′-CCGCCGCTCGAGACTTCCTCTGCCCGCTTCTTC -3′	*Xho*I
pTWIN1	5′-GGTGGTCCATGGGGAGTTTAGAAATGGTA-3′	*Nco*I
	5′-GGTGGTCTGCAGTTAACTTCCTCTGCCCGC-3′	*Pst*I

Note. Restriction sites are underlined. The boxed sequences are silent mutations to stabilize the secondary structure of the transcribed mRNA and to facilitate expression of LMP2A NTD.