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Characterization of the rapamycin-inducible EBV LMP1 activation system

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Epstein-Barr virus (EBV) latent infection membrane protein 1 (LMP1) is required for EBV-mediated B lymphocyte transformation into proliferating lymphoblastoid cell lines (LCL). LMP1 oligomerizes spontaneously in membrane lipid rafts via its transmembrane domain and constitutively activates signal transduction pathways, including NF-κB, p38 Mitogen-Activated Protein Kinase (MAPK), and c-Jun N-terminal Kinase (JNK). Since LMP1 mimics the tumor necrosis factor receptor (TNFR), CD40, it may be effectively utilized to study the effects of constitutive activation of signal transduction pathways on cellular physiology. On the other hand, LMP1 presents a disadvantage in terms of determining the sequential events and factors involved in signaling pathways. A CD40-LMP1 chimeric molecule has been generated to overcome this limitation but does not represent the authentic and physiological nature of LMP1. In the current study, a ligand-dependent activation system for LMP1 using rapamycin-inducible dimerization was generated to delineate the LMP1 signaling pathway.

Keywords: Epstein-Barr virus, latent membrane protein 1, NF-κB

Introduction

Epstein-Barr virus (EBV), a human γ-herpesvirus with a 172 kb DNA genome, infects more than 90% of the human population and establishes life-long latency. EBV is the causative agent of infectious mononucleosis and associated with several human lymphoid and epithelial malignancies, such as Burkitt's lymphoma, nasopharyngeal carcinoma, T-cell lymphoma and Hodgkin's disease (Grywalska and Rolinski, 2015). The virus mainly infects B cells and uses their system for continuous replication (Thorley-Lawson, 2001). After primary infection, EBV establishes the latent phase to evade host immunity and expresses limited genes. The expression patterns of latent genes change according to tissue, cell, and tumor conditions (Saito *et al.*, 2013). Upon attenuation of host immunity, EBV is reactivated through expression of lytic genes that promote the reproduction of virus particles.

EBV transforms B cells *in vitro* via mechanisms requiring EBV nuclear antigen (EBNA) 1, EBNA2, EBNA3A, EBNA3C, and latent membrane protein 1 (LMP1) (Izumi, 2001). Among these genes, LMP1 has been shown to be essential for transformation of B cells and non-lymphoid cells (Kaye *et al.*, 1993).

LMP1, a 62 kDa transmembrane protein, contains three distinct regions: a 24 amino acid (aa) N-terminus, six hydrophobic transmembrane domains (TM) that induce self-aggregation and a 200 aa C-terminal cytoplasmic signaling domain (CTD) including C-terminal activating regions (CTAR) 1 and 2 that interact with cellular adaptor proteins (Fennewald *et al.*, 1984; Hennessy *et al.*, 1984; Gires *et al.*, 1997). LMP1 displays similarities with the tumor necrosis factor receptor (TNFR), CD40, and can substitute for CD40 signaling in B cells (Mosialos *et al.*, 1995; Rastelli *et al.*, 2008). TNFR-associated factors (TRAF) 1, 2, 3, and 5 bind LMP1 CTAR1, while TNFR-associated death domain protein (TRADD), receptor interacting protein (RIP) and interferon regulatory factor 7 (IRF7) bind CTAR2. In association with these proteins, LMP1 activates NF-κB, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and type 1 interferon (IFN) signaling pathways (Devergne *et al.*, 1996; Izumi *et al.*, 1997, 1999b; Izumi and Kieff, 1997; Song *et al.*, 2008).

NF-κB activation by LMP1 plays an important role in EBV-mediated B cell transformation. The NF-κB transcription factor family consists of p50 (p105), p52 (p100), RelA (p65), RelB, and cRel (Oeckinghaus *et al.*, 2011; Hayden and Ghosh, 2012). NF-κB is crucial for immunity, stress, apoptosis and development and is normally sequestered in the cytoplasm through binding with inhibitor of kappa B (IκB) (Oeckinghaus *et al.*, 2011; Hayden and Ghosh, 2012). NF-κB activation is initiated with IκB phosphorylation by the IκB kinase (IKK) complex composed of IKKα, IKKβ, and IKKγ (NEMO). Phosphorylated IκB, in turn, becomes a target for ubiquitination and proteasome-mediated degradation (Oeckinghaus *et al.*, 2011; Hayden and Ghosh, 2012). After IκB degradation, NF-κB translocates into the nucleus to induce target gene expression. Two NF-κB activation pathways exist, referred to as canonical and non-canonical pathways. In the canonical pathway, IκB is phosphorylated in a IKKβ- and IKKγ-dependent manner, and p65 containing the NF-κB heterodimer translocates into the nucleus. In the non-canonical pathway, IKKα is phosphorylated by NF-κB inducing kinase (NIK). Activated IKKα, in turn, phosphorylates p100, which is subsequently processed into p52. Subsequently, the p52-RelB heterodimer

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translocates into the nucleus and activates target gene expression (Oeckinghaus *et al.*, 2011; Hayden and Ghosh, 2012).

Unlike CD40, LMP1 self-aggregates via six hydrophobic TM and activates the NF-κB signaling pathway in a ligand-independent manner (Gires *et al.*, 1997). Although LMP1 presents an ideal system to analyze the effects of constitutive NF-κB activation on cellular physiology, delineation of the sequential events and factors involved in LMP1 signaling pathways is a difficult task. A recombinant CD40-LMP1 chimeric protein expressing extracellular and transmembrane CD40 domains fused to the LMP1 C-terminal cytoplasmic signaling domain was previously generated to investigate the sequential signaling events of LMP1 in a ligand-dependent manner (Brown *et al.*, 2001). However, the CD40-LMP1 chimeric protein may not represent the natural structure and function of LMP1.

FRB is a 100 aa (E2015–Q2114) domain of mammalian target of rapamycin (mTOR), a serine/threonine kinase that regulates cell growth, proliferation, motility, protein synthesis and transcription (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994; Chen *et al.*, 1995; Hay and Sonenberg, 2004). FK506-binding protein (FKBP), a 12 kDa protein containing 108 aa, exhibits prolyl isomerase activity and belongs to the cyclophilin family (Siekierka *et al.*, 1989). Originally, FKBP was identified as a target of immunosuppressive agents, such as tacrolimus and rapamycin, for organ transplant recipients or patients with autoimmune diseases (Bierer *et al.*, 1990; Wang *et al.*, 1994). Rapamycin is a macrolide anti-fungal antibiotic isolated from *Streptomyces hygroscopicus* that forms a complex with FKBP. The FKBP-rapamycin complex binds to the FRB domain of mTOR to inhibit its activity (Huang *et al.*, 2003; Banaszynski *et al.*, 2005). Since dimerization of proteins fused to FKBP or FRB can be conditionally induced by rapamycin, this system can be utilized to determine the functions of protein interactions (Clemons, 1999; Muthuswamy *et al.*, 1999; Pollock and Clackson, 2002). In this study, a rapamycin inducible LMP1 activation system was constructed using the dimerization system, with a view to delineating sequential events of the LMP1 signaling pathway.

Materials and Methods

Cells, reagents, transfection, and reporter gene assay

Human embryonic kidney 293 (HEK 293) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) containing 10% fetal bovine serum and 1% penicillin/streptomycin. pcDNA3.1-FKBP-myc and pcDNA3.1-FRB-myc were purchased from Addgene. Rapamycin was obtained from Santa Cruz Biotechnology and dissolved in ethanol (EtOH) to achieve a final concentration of 55 nM. Omics-Fect™ for transient transfection was used according to the manufacturer's instructions (Omicsbio), and the luciferase assay performed as described previously (Bari *et al.*, 2011).

Plasmid constructs

To generate HA-tagged LMP1 TM-FKBP and FRB-LMP1 CTD fusion genes, cDNA fragments were amplified via polymerase chain reaction (PCR) using pcDNA-LMP1, pCDNA3.1-FKBP-myc or pcDNA3.1-FRB-myc. The following primers were used for PCR: EcoRI-HA-LMP1 TM-BamHI, 5′-GGG GAATTCGGGATGTACCCATACGATGTTCCAGATTA CGCTATGGAACACGACCTTGAGAG-3′ and 5′-GGGG GGGGGGGATCCGTAATACATCCAGATTAAAATCG-3′; BamHI-FKBP-BglII, 5′-GGGGGGGGATCCATGGGAG GCAGGTGGAAAC-3′ and 5′-GGGGGGGGGAGATCTTA TCCAGTTTTAGAAGCTCCAC-3′; EcoRI-FRB-BamHI, 5′-GGGGGGGAATTCGGGATGATCCTCTGGCATGAGA TGGTTC-3′ and 5′-GGGGGGGGATCCCTGCTTTGAGAT CGTCGGA; BamHI-LMP1 CTD-BglII, 5′-GGGGGGGGTC CCATGGACAACGACACAGTGA and 5′-GGGGGGAAT CTGGGTTAGTCATAGTAGCTTAGCTGAACTGGG-3′. After insertion of LMP1 TM and FRB into pSG5, FKBP and LMP1 CTD were inserted into pSG5-HA-LMP1 TM and pSG5-FRB, respectively. For PCR, 5× HOT FIREPol® Blend Master Mix was purchased from Solis Biodyne. Restriction enzymes were purchased from New England Biolabs, and the gel extraction kit from Qiagen. Plasmid DNA isolation kits were obtained from Maestrogen.

SDS-PAGE and western blot analysis

Cell lysis was performed using RIPA lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 0.1% Nonidet-P40; 1 mM EDTA) containing 50 mM NaF, 1 mM PMSF and protease inhibitor cocktail (Roche). After sonication, cell lysates were normalized using DC protein assay reagents (Bio-Rad). Lysates were mixed with 2× SDS-PAGE sample buffer (100 mM Tris-Cl, pH 6.8; 4% SDS; 0.02% bromophenol; 2% β-mercaptoethanol) and boiled at 100°C for 5 min. Prepared cell lysates were separated via SDS-PAGE and transferred to nitrocellulose membrane (EMD Millipore). The membrane was blocked with TBST milk buffer (10 mM Tris, pH 8.0; 100 mM NaCl; 0.05% Tween 20; 5% nonfat dry milk). After treatment with the appropriate primary and secondary antibodies, the membrane was developed using the Immobilon™ Western Chemi-luminescence HRP Substrate kit (EMD Millipore). Antibodies against IκBα, poly (ADP-ribose) polymerase (PARP), and Na^+/K^+ ATPase were purchased from Cell Signaling Technology. Anti-HA antibody (Y-11) was purchased from Santa Cruz Biotechnology. Anti-p100/p52 and anti-tubulin antibodies were purchased from EMD Millipore and Sigma-Aldrich, respectively. Anti-LMP1 CTD antibody (S12) was purchased from KeraFAST. Secondary peroxidase-labeled anti-mouse or anti-rabbit immunoglobulin G antibodies were purchased from Amersham Biosciences.

Immunoprecipitation

Ten million cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 1% NP-40, phosphatase inhibitor cocktail (EMD Millipore) and protease inhibitor cocktail (Roche). Lysates were precleared with protein A/G agarose beads (Santa Cruz Biotechnology) and incubated at 4°C overnight with anti-HA antibody-conjugated agarose beads (Santa Cruz Biotechnology). After washing three times with lysis buffer, protein complexes were eluted with HA (Covance) peptides and subjected to western blot with antibodies against HA and LMP1 CTD (S12).

Subcellular fractionation

The subcellular fractionation kit was employed according to the manufacturer's instructions (Thermo Scientific).

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using the RNeasy® kit, and reverse-transcribed into cDNA using the QuantiTect® Reverse Transcription kit as recommended by the manufacturer (Qiagen). The corresponding cDNAs were amplified and quantified in the MxPro3000P QPCR System (Agilent Technologies) using HOT FIREPol® EvaGreen qPCR Mix Plus (Solis BioDyne) and the following primers: TNFAIP3 (A20), 5′-CTGCCCA GGAATGCTACAGATAC-3′ and 5′-GTGGAACAGCTCG GATTTCAG-3′; PTGS2 (COX-2), 5′-CACCCATGTCAA AACCGAGG-3′ and 5′-CCGGTGTTGAGCAGTTTTCTC-3′; NFKBIA (IκBα), 5′-GATCCG CCAGGTGAAGGG-3′ and 5′-GCAATTTCTGGCTGGTT GG-3′; IL-6, 5′-AATT CGGTACATCCTCGACGG-3′ and 5′-GGTTGTTTTCTGC CAGTGCC-3′; β-actin, 5′-ATCATGTTTGAGACCTTCAA C-3′ and 5′-CAGGAAGGAAGGC TGGAAGAG-3′.

Results

Construction and characterization of rapamycin-inducible LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer

Since rapamycin had a negligible effect on LMP1-induced NF-κB activation (data not shown), the rapamycin-induced dimerization system was applied to generate a rapamycin-inducible LMP1 activation system. LMP1 was divided into the N-terminus and transmembrane domain (LMP1 TM) and the C-terminal cytoplasmic signaling domain (LMP1 CTD) regions which were fused with FKBP and FRB, respectively, to generate LMP1 TM-FKBP and FRB-LMP1 CTD (Fig. 1). LMP1 TM-FKBP and FRB-LMP1 CTD were cloned into the pSG5 mammalian expression vector. To examine rapamycin-induced dimerization of LMP1 TM-FKBP and FRB-LMP1 CTD, HEK293 cells were co-transfected with vectors expressing both constructs and treated with rapamycin (Fig. 2). Cell lysates were immunoprecipitated with an anti-LMP1 antibody (S12) that recognizes an epitope com-

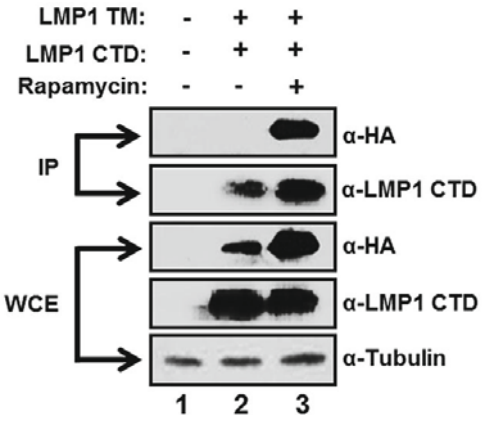

Fig. 2. Rapamycin induces dimerization of LMP1 TM-FKBP and FRB-LMP1 CTD. HEK 293 cells were transfected with pSG5 (lane 1) or pSG5-HA-LMP1 TM-FKBP plus pSG5-FRB-LMP1 CTD (lanes 2 and 3). At 24 h after transfection, cells were treated with equal volumes of EtOH (lanes 1 and 2) or rapamycin at 55 nM (lane 3). Anti-LMP1 CTD immunoprecipitation was performed 24 h after treatment. LMP1 CTD immunoprecipitates and whole cell extracts were subjected to western blot with antibodies against HA and LMP1 CTD (S12). (EtOH, ethanol; WCE, whole cell extracts; IP, immunoprecipitation).

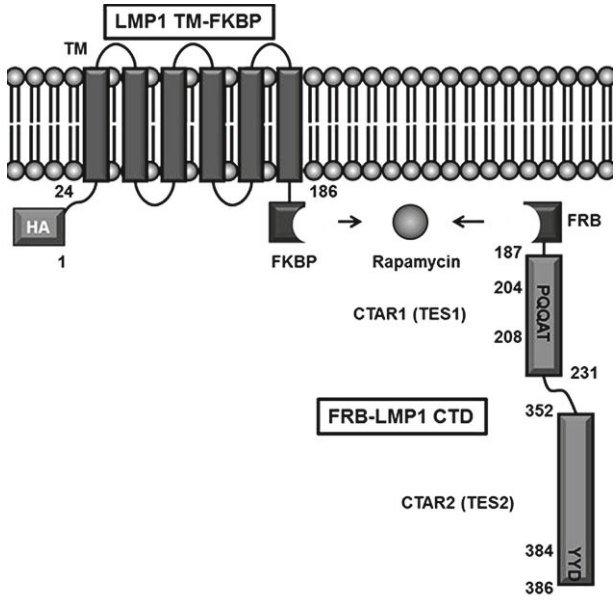

Fig. 1. Schematic representation of the rapamycin-inducible LMP1 TM-FKBP and FRB-LMP1 CTD dimerization system (TM, transmembrane domain; CTD, C-terminal cytoplasmic signaling domain; FKBP, FK506 binding protein; FRB, FKBP-rapamycin binding domain).

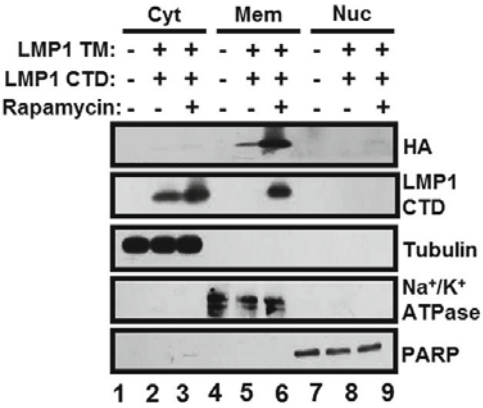

Fig. 3. Subcellular localization of the rapamycin-induced LMP1 TM-FKBP: FRB-LMP1 CTD heterodimer. HEK 293 cells were transfected with pSG5 (lanes 1, 4, and 7) or pSG5-HA-LMP1 TM-FKBP plus pSG5-FRB-LMP1 CTD (lanes 2, 3, 5, 6, 8, and 9). At 24 h after transfection, cells were treated with equal volumes of EtOH (lanes 1, 2, 4, 5, 7, and 8) or rapamycin at 55 nM (lanes 3, 6, and 9). Cytoplasmic (lanes 1 to 3), membrane (lanes 4 to 6) and nuclear (lanes 7 to 9) extracts were harvested 6 h after treatment and subjected to western blot with antibodies against HA, LMP1 CTD (S12), tubulin, Na^+/K^+ ATPase or PARP. Tubulin, Na^+/K^+ ATPase, and PARP were used as markers for cytoplasmic, membrane and nuclear fractions, respectively (Cyt, cytoplasmic fraction; Mem, membrane fraction; Nuc, nuclear fraction).

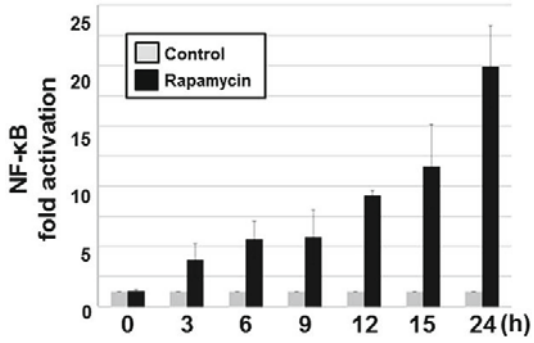

Fig. 4. NF-κB activation by the rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer. HEK293 cells were co-transfected with vectors expressing LMP1 TM-FKBP and FRB-LMP1 plus NF-κB-dependent firefly luciferase and control *Renilla* luciferase plasmids. At 24 h after transfection, cells were treated with equal volumes of EtOH (control) or rapamycin at 55 nM, and luciferase activity was measured using a dual-luciferase assay system at different time points. NF-κB dependent luciferase activity was expressed in relative luciferase units (RLU) by normalizing firefly luciferase activity with constitutive *Renilla* luciferase activity. Luciferase assay data shown here represent three independent experiments, and significant differences between control and rapamycin-treated samples were determined based on the *P* values obtained from the two-sample *t* test ($P < 0.05$).

prising aa 232 to 351 within LMP1, and the interactions between LMP1 TM-FKBP and FRB-LMP1 CTD analyzed via western blot (Fig. 2) (Izumi *et al.*, 1999a). In rapamycin-treated cells, LMP1 TM-FKBP immunoprecipitated with FRB-LMP1 CTD indicating dimerization of the fusion proteins (Fig. 2; compare lanes 3 with 2). Owing to the presence of the NF-κB motif within the SV40 promoter of the pSG5 vector, expression of LMP1 TM-FKBP or FRB-LMP1 CTD in rapamycin-treated cells was induced, possibly via heterodimer-stimulated NF-κB activation (Fig. 2; compare lanes 3 with 2). Our data indicate that rapamycin treatment facilitates dimerization of LMP1 TM-FKBP and FRB-LMP1 CTD fusion proteins.

The rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer is translocated into the membrane compartment

LMP1 translocates into and signals primarily from intracellular compartments (Lam and Sugden, 2003a, 2003b; Liu *et al.*, 2006). To determine whether rapamycin stimulates translocation of the LMP1 TM-FKBP:FRB-LMP1 CTD heterodimers into membrane compartments, HEK293 cells expressing both LMP1 TM-FKBP and FRB-LMP1 CTD were treated with rapamycin, and subjected to subcellular fractionation (Fig. 3). LMP1 TM-FKBP was located in the membrane compartments of cells treated with or without rapamycin (Fig. 3; compare lanes 6 with 5). On the other hand, FRB-LMP1 CTD was located in the membrane compartments only in cells treated with rapamycin (Fig. 3; compare lanes 6 with 5). The cytoplasmic FRB-LMP1 CTD monomer was additionally detected in rapamycin-treated cells (Fig. 3; lane 3). Our data indicate that the LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer induced by rapamycin translocates into membrane compartments, similar to wild-type LMP1.

The rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer activates NF-κB

To determine whether the rapamycin-induced heterodimer is functionally intact, NF-κB activation was assessed using an NF-κB-dependent luciferase reporter (Fig. 4). At 3 h after rapamycin treatment, NF-κB-dependent luciferase activity was elevated 3-fold in cells expressing both LMP1 TM-FKBP and FRB-LMP1 CTD (Fig. 4). NF-κB-dependent luciferase activity was further increased to 22-fold at 24 h after rapamycin treatment (Fig. 4). Clearly, the rapamycin-induced heterodimer promotes NF-κB activation.

The rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer promotes both canonical and non-canonical NF-κB activation

To determine whether the rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer activates the canonical and/or non-canonical NF-κB pathways, IKKα and IKKβ activities were analyzed (Fig. 5). For determining IKKβ activity, HEK293 cells expressing both LMP1 TM-FKBP and FRB-LMP1 CTD were treated with either control or rapamycin, and equal amounts of cell extracts were subjected to western blot at 0, 30, 60, 90, and 120 min after treatment (Fig. 5A). At 30 min after rapamycin treatment, IκBα degradation was induced, indicative of IKKβ activation (Fig. 5A;

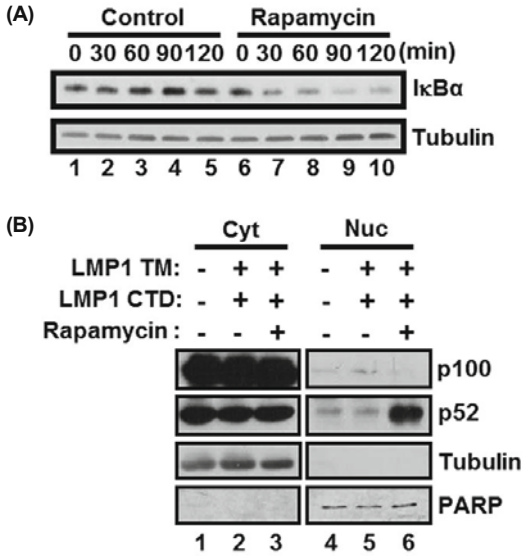

Fig. 5. Canonical and non-canonical NF-κB activation by the rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer. (A) HEK 293 cells were co-transfected with pSG5-HA-LMP1 TM-FKBP and pSG5-FRB-LMP1 CTD. At 24 h after transfection, cells were treated with equal volumes of EtOH (control) (lanes 1 to 5) or rapamycin at 55 nM (lanes 6 to 10). Equal amounts of cell extracts were harvested at 24 h after treatment and subjected to western blot with antibodies against IκBα or tubulin. (B) HEK 293 cells were transfected with pSG5 (lanes 1 and 4) or pSG5-HA-LMP1 TM-FKBP plus pSG5-FRB-LMP1 CTD (lanes 2, 3, 5, and 6). At 24 h after transfection, cells were treated with equal volumes of EtOH (lanes 1, 2, 4, and 5) or rapamycin at 55 nM (lanes 3 and 6). Cytoplasmic or nuclear extracts were harvested 6 h after treatment and subjected to western blot with antibodies against p100/p52, tubulin or PARP antibody. Tubulin and PARP were used as markers for cytoplasmic and nuclear fractions, respectively (Cyt, cytoplasmic fraction; Nuc, nuclear fraction).

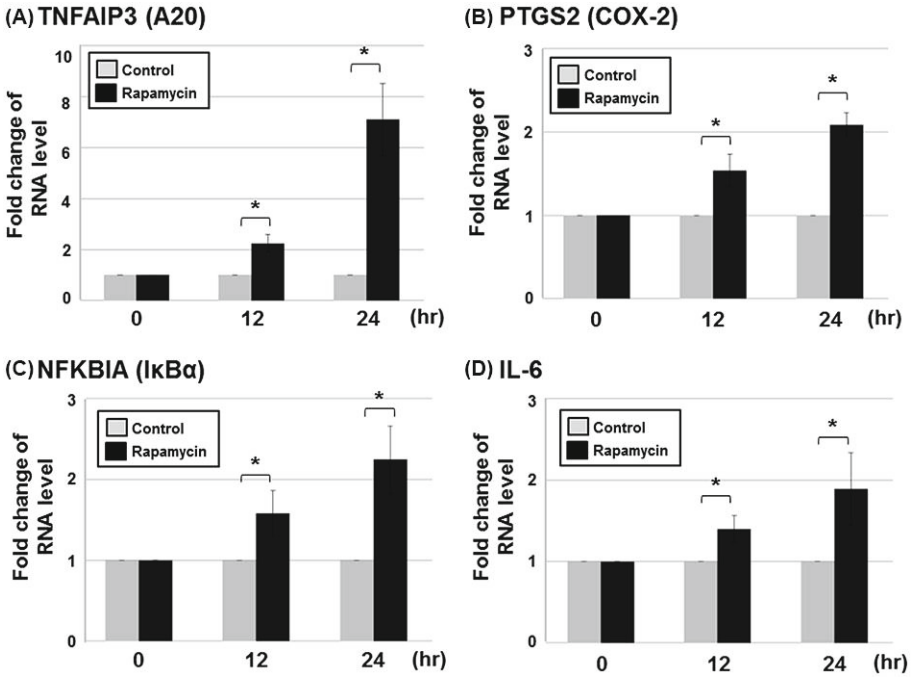

Fig. 6. Effects of the rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer on NF-κB responsive gene expression. HEK 293 cells expressing both LMP1 TM-FKBP and FRB-LMP1 CTD were treated with equal volumes of EtOH (control) or rapamycin at 55 nM. At 0, 12, and 24 h after treatment, mRNAs were isolated, and the levels of (A) TNFAIP (A20), (B) PTGS2 (COX-2), (C) NFKBIA (IκBα) and (D) IL-6 gene transcripts were analyzed by qRT-PCR. To calculate relative changes in gene expression, mRNA levels of NF-κB target genes in control-treated cells at 0 h were set at 1. Significant differences between control and rapamycin-treated samples were determined based on the *P* values obtained from the two-sample *t* test. * Significant at $P < 0.05$.

compare lanes 7 with 2). The heterodimer-induced IKKβ activation was confirmed with an *in vitro* kinase assay (data not shown). We further analyzed the processing of p100 into p52 signifying IKKα activation (Fig. 5B). Since the rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer did not promote p100 processing to p52 until 120 min after treatment (data not shown), we analyzed IKKα activation at later time points. In rapamycin-treated cells expressing both LMP1 TM-FKBP and FRB-LMP1 CTD, processing of p100 into p52 and nuclear localization of p52 were detected at 6 h (Fig. 5B; compare lanes 6 with 5). Thus, LMP1 CTAR1-induced IKKα activation may be induced at later time point than CTAR2-induced IKKβ activation. These findings collectively indicate that the rapamycin-induced heterodimer activates both canonical and non-canonical NF-κB activation pathways, similar to wild-type LMP1.

The rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer activates NF-κB responsive gene expression

HEK293 cells expressing both LMP1 TM-FKBP and FRB-LMP1 CTD were treated with rapamycin, and the mRNA levels of four NF-κB responsive genes, TNFAIP (A20), PTGS2 (COX-2), NFKBIA (IκBα) and IL-6, were determined using qRT-PCR at 0, 12, and 24 h after treatment (Fig. 6). Compared to the control, rapamycin treatment elevated TNFAIP mRNA to 7-fold at 12 h after treatment (Fig. 6A). This finding is consistent with the previous report that LMP1 CTAR2 strongly activates A20 expression (Gewurz *et al.*, 2011). Interestingly, compared to the control, PTGS2, NFKBIA, and IL-6 mRNA levels were only slightly increased in rapamycin-treated cells (Fig. 6B, C, and D). Overall, our findings suggest that the rapamycin-induced LMP1 TM-FKBP:FRB-LMP1 CTD heterodimer activates NF-κB responsive gene expression.

Discussion

Chemically-induced dimerization system has been used as an effective tool in cell biology research for 20 years (DeRose *et al.*, 2013). Numerous biological reactions in cells are mediated by protein interactions (Austin *et al.*, 1994). Since protein interactions can be artificially regulated by chemical treatment, the chemically-induced dimerization system is widely used in the medical and biological fields to investigate functions of protein-protein interactions in gene expression, signal transduction and cellular physiology (Spencer, 1996).

In this research, a rapamycin-inducible LMP1 TM and LMP1 CTD dimerization system was constructed with the aim of determining sequential events in the LMP1 signaling pathway. Rapamycin treatment triggered interactions between LMP1 TM-FKBP and FRB-LMP1 CTD, and NF-κB activation. Subcellular fractionation analysis revealed that LMP1 CTD localizes into the membrane compartment, similar to wild-type LMP1. The rapamycin-induced LMP1 TM:LMP1 CTD heterodimer activates both canonical and non-canonical NF-κB pathways. Based on these findings, we propose that the rapamycin-inducible LMP1 TM:LMP1 CTD dimerization system mimics the physiological function of wild-type LMP1 and can therefore be effectively used to study the sequential events of LMP1 signaling pathway in a ligand-dependent manner.

Using the dimerization system, we found that LMP1 activates the canonical NF-κB pathway much earlier than the non-canonical NF-κB pathway. The mechanisms underlying functional crosstalk between canonical and non-canonical NF-κB activation in LMP1 signaling are currently unclear. It is possible that some characteristics of LMP1 signaling mechanisms may not be reproduced in the rapamycin-inducible LMP1 TM and LMP1 CTD dimerization system. Although rapamycin did not interfere with LMP1-induced

NF-κB activation (data not shown), it may negatively affect specific functions of LMP1 (Lambert and Martinez, 2007). Nonetheless, the rapamycin-inducible LMP1 activation system generated in this study presents a powerful tool to investigate the LMP1 signaling pathway in detail.

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