Journal of Virology

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J. Virol. **2008, 82(13):6721. DOI: 10.1128/JVI.02250-07. Published Ahead of Print 30 April 2008.**

JOURNAL OF VIROLOGY, July 2008, p. 6721–6733 Vol. 82, No. 13
0022-538X/08/$08.00+0 doi:10.1128/JVI.02250-07

Molecular Basis of Cytotoxicity of Epstein-Barr Virus (EBV) Latent Membrane Protein 1 (LMP1) in EBV Latency III B Cells: LMP1 Induces Type II Ligand-Independent Autoactivation of CD95/Fas with Caspase 8-Mediated Apoptosis▽‖

Christophe Le Clorennec,[1]†‡ Tan-Sothéa Ouk,[1]† Ibtissam Youlyouz-Marfak,[1] Stéphanie Panteix,[1] Catherine-Claude Martin,[1] Julia Rastelli,[2]§ Eric Adriaenssens,[3] Ursula Zimber-Strobl,[2] Jean Coll,[4]¶ Jean Feuillard,[1,5] and Chantal Jayat-Vignoles[1]*

UMR CNRS 6101, Faculté de Médecine de Limoges, Université de Limoges, 2 Rue du Docteur Marcland, 87025 Limoges Cedex, France[1]; Institute of Clinical Molecular Biology and Tumor Genetics, Helmholtz Center Munich German Research Centre for Environment and Health (GmbH), D-81377 Munich, Germany[2]; INSERM ERI-8, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq, France[3]; CNRS UMR 8161, Institut de Biologie de Lille, Equipe cancers viro-induits, BP 447, 59021 Lille Cedex, France[4]; and CHU Dupuytren, Laboratoire d'hématologie, 2 Avenue Martin Luther King, 87042 Limoges Cedex, France[5]

Received 17 October 2007/Accepted 10 April 2008

The Epstein-Barr virus (EBV) oncoprotein latent membrane protein 1 (LMP1) is thought to act as the major transforming protein in various cell types, by rerouting the tumor necrosis factor receptor family signaling pathway. Despite this implication in EBV-associated transformation of cells, LMP1 toxicity is a well-known but poorly studied feature, perhaps because it contradicts its role in transformation. We show that LMP1 physiological levels are very heterogeneous and that the highest levels of LMP1 correlate with Fas overexpression and spontaneous apoptosis in lymphoblastoid cell lines (LCLs). To understand the cytotoxic effect of LMP1 in LCLs, we cloned wild-type LMP1 into a doxycycline double-inducible episomal vector pRT-1, with a truncated version of NGFR as a surrogate marker of inducibility. We found that LMP1 overexpression induced apoptosis in LCL B cells, as shown by annexin V labeling, sub-G_1 peak, and poly(ADP ribose) polymerase cleavage. Knocking down Fas expression by small interfering RNA abolished LMP1-induced apoptosis. The absence of detectable levels of Fas ligand mRNA suggested a ligand-independent activation of Fas. LMP1 induced Fas overexpression with its relocalization in lipid raft microdomains of the membrane. Fas immunoprecipitation detected FADD (Fas-associated death domain protein) and caspase 8, suggesting a Fas-dependent formation of the death-inducing signaling complex. Caspases 8, 9, 3, and 7 were activated by LMP1. Caspase 8 activation was associated with BID cleavage and truncated-BID mitochondrial relocalization, consistent with type II apoptosis. Therefore, our results are in agreement with a model where LMP1-dependent NF-κB activation induces Fas overexpression and autoactivation that could overwhelm the antiapoptotic effect of NF-κB, revealing an ambivalent function of LMP1 in cell survival and programmed cell death.

Epstein-Barr virus (EBV) is a member of the gammaherpesvirus family. It is one of the most common human viruses, since it infects as many as 95% of adults (23). After primary infection, classically during infancy and generally asymptomatic, the latent viral infection is maintained throughout life. The virus remains in resting memory B cells by using the B-lymphocyte differentiation program (56). EBV persistence is possible due to a host/virus equilibrium: the immune system of the host controls infected cells, while the virus diverts cell signaling pathways, via its latent proteins, allowing infected cells to be maintained in an immortalized state (latent state) with a coregulation of viral and cellular genes (3, 56). Rupture of this equilibrium leads to lymphoproliferative syndromes.

The virus has been shown to be closely associated with many human malignancies such as nasopharyngeal carcinoma, lymphoproliferative syndrome of immunocompromised patients, and some lymphomas (Burkitt lymphoma, Hodgkin's lymphoma or T-cell lymphoma) (44). In vitro, EBV infection of resting human B lymphocytes results in cell proliferation and transformation into lymphoblastoid cell lines (LCLs), with cells expressing viral-transformation-associated latent genes (46, 55). EBV persists in cells thanks to the expression of, at most, 11 latency genes, grouped into three families: genes coding for EBNA proteins, genes coding for LMP proteins, EBERs, and BART noncoding RNAs (3, 60). Expression of latent proteins contributes to EBV properties for proliferation and/or transformation.

* Corresponding author. Mailing address: UMR CNRS 6101, Faculté de Medecine, 2 rue du Docteur Marchand, 87025 Limoges Cedex, France. Phone: (33) 5 55 43 58 67. Fax: (33) 5 55 43 58 97. E-mail: chantal.vignoles@unilim.fr.

‖ Supplemental material for this article may be found at http://jvi.asm.org/.

† These authors contributed equally to this study.

‡ Present address: Drug Resistance Group, Centre for Cancer Research and Cell biology, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7AB, Northern Ireland.

§ Present address: Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142.

¶ Deceased.

▽ Published ahead of print on 30 April 2008.

Among latent proteins, latent membrane protein 1 (LMP1) is known to be the major transforming protein of EBV (26, 30). LMP1 is a 386-amino-acid transmembrane protein with functional homology to the TNF superfamily receptors (TNFR), such as CD40 or TNFR1, as well as with the interleukin 1 receptor or Toll-like receptors. They all use similar adaptor proteins (TRAFs, TRADD, and RIP) or cellular signalization pathways (NF-κB, JNK, STAT, and p38/MAPK) (4, 10, 34, 51). TNFR plays, in conjunction with TNF molecules, pivotal roles in cell proliferation, differentiation, and apoptosis (16, 48).

LMP1 has been demonstrated to stimulate or inhibit various signaling pathways, resulting in greater transformation and survival in LMP1-expressing cells, especially in B cells. LMP1 rerouting of TRAFs induces NF-κB activation as well as activation of PI3K/Akt pathways, which promotes cell proliferation and activation of transcriptional factors such as AP-1 or ATF-2, involved in antiapoptotic and cellular transformation processes (39, 54). LMP1 leads to overexpression of antiapoptotic molecules, such as Bcl-2, Mcl-1, and Bcl-2-related protein A1 (Bfl1) (9, 22, 49, 58). It also blocks p53-mediated apoptosis through the induction of the A20 gene (14, 32). Complementary to its proliferating function, LMP1 inhibits proapoptotic factors such as Bax (20).

Even though LMP1 is the major oncoprotein of EBV, well known to induce cell transformation and survival, its transmembrane and N-terminal domains exert cytostatic effects (27, 39). This property is linked to LMP1 expression levels and appears at levels as low as twofold higher than the average for EBV-positive lymphoblasts (50). Upon transfection into EBV-negative B-cell lines, LMP1 induces cytostatic or cytotoxic effects if expressed at high levels (13, 21). EBV has also been reported to induce apoptotic processes in human neutrophils (36) and monocyte precursors of dendritic cells (40). LMP1 by itself can sensitize cells or promote apoptosis. It enhances HeLa cell apoptosis induced by Fas or by cytotoxic compounds, such as etoposide and cisplatin (61, 62). In epithelial cell lines transfected with LMP1, apoptosis is also related to high expression levels of the protein (42). Otherwise, LMP1 can upregulate the expression of the proapoptotic receptor Fas, as demonstrated in LMP1-transfected Burkitt lymphoma cell lines (24) or in LCLs (11, 37). In vivo, EBV-induced Fas overexpression allows T lymphocytes to kill infected cells by apoptosis, as they express Fas ligand (FasL). In neutrophils, EBV induces apoptosis by initiating simultaneous Fas and FasL overexpression (36).

Thus, it seems that LMP1 exhibits a paradoxical behavior. On the one hand, LMP1 is associated with cell transformation and survival in LCLs. On the other hand, the protein constitutively up regulates the proapoptotic receptor Fas and sensitizes cells to induction of apoptosis (11, 37). Moreover, the apoptotic pathway mediated by caspases 8 and 9 is functional in LCLs, as recently demonstrated in experiments using NF-κB inhibitors (37, 63). In this study, we examined the cytotoxic function of LMP1 and found that high physiological levels of LMP1 paralleled Fas expression and spontaneous apoptosis in LCLs. With regard to the underlying molecular process, we demonstrated that LMP1 overexpression in latency III B cells induced apoptosis with involvement of caspase 8 and caspase 9 (resulting in downstream caspase 8 activation and BH3-interacting domain death agonist [BID] cleavage) and that apoptosis initiation occurred via Fas receptor overexpression and autoactivation, independently of FasL.

MATERIALS AND METHODS

Plasmid constructs and cDNA. The inducible vector pRT-1 was derived from the previously described vector CKR516 (1), in which the Eμ-respiratory syncytial virus promoter was replaced by an Eμ-chicken β-actin promoter (Eμ-CAG promoter). The EBNA1 gene was added to favor maintenance of the vector as an episome also in EBV-negative cells. The enhanced green fluorescent protein marker was replaced by an inactive truncated version of NGFR lacking the cytoplasmic domain nerve growth factor (NGFRt). The bidirectional tetracycline-inducible promoter drives the expression of both NGFRt and the cDNA of interest (LMP1wt or luciferase). A complete description of these vectors is given elsewhere (2). The cDNA coding for LMP1wt or luciferase was cloned into the Sfi-I sites.

Cell culture, transfection, and cell sorting. The LCLs PRI, 1602, EMICA, TSOB, TSOC, and EREB were cultured in RPMI 1640 medium (Gibco BRL-Life Technologies, Cergy-Pontoise, France) supplemented with 10% decomplemented fetal calf serum (Dutscher, Brumath, France), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL-Life Technologies), and 2 mM L-glutamine (Gibco BRL-Life Technologies) at 37°C in a fully humidified 5% CO_2 incubator.

Stable transfection of cells and hygromycin selection (Calbiochem, La Jolla, CA) were performed as described elsewhere (1, 12). Induction was stable after 4 weeks of selection. After 24 h treatment with 1 μg/ml doxycycline (Sigma Aldrich, Saint Louis, MO), the cells became NGFRt positive, with transfection rates varying from 60 to 90%.

Where indicated, NGFRt-expressing cells were purified with Macs microbeads following the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, viable cells were isolated by Ficoll-Paque Plus density gradient centrifugation (Eurobio, Les Ulis, France). Cells were divided into two batches, one treated with 1 μg/ml doxycycline for 24 h and one without doxycycline. Both batches were then subjected in parallel to cell purification. Cells were incubated for 30 min with 1 μg anti-NGFR monoclonal antibody (MAb) (BD Pharmingen, San Diego, CA) for 10^6 doxycycline-treated cells or without primary antibody for untreated cells. Cell separation was performed with 20 μl goat anti-mouse immunoglobulin G magnetic beads (Miltenyi Biotec) for 10^7 cells. Purified cells were lysed in Blue Laemmli lysis buffer (Bio-Rad, Hercules, CA) or resuspended at 10^6 cells/ml for fluorescence-activated cell sorting analysis.

Annexin V labeling and DNA content analysis. Induction of NGFRt expression was assessed by flow cytometry on a FACSCalibur (Becton Dickinson) apparatus after direct labeling with a phycoerythrin (PE)-conjugated NGFR MAb (BD Pharmingen) diluted 1/20.

The apoptosis rate was measured by using annexin V–fluorescein isothiocyanate (FITC) or annexin V-PE (BD Pharmingen) according to the manufacturer's instructions. To assess cell membrane permeability, the following DNA-specific impermeant dyes were added to samples just before flow cytometric analysis: 1 μM TOPRO-3 (Invitrogen) and 50 μg/ml propidium iodide (Sigma). For simultaneous Fas staining, cells were incubated with 10 μl of PE-conjugated Fas MAb (BD Pharmingen).

The DNA content of cells was assessed with a DNA Prep kit (Beckman Coulter Immunotech SA, Marseille, France), according to standard procedures recommended by the manufacturer.

Luciferase assay. Analysis were performed with a luciferase assay system (Promega, Charbonniéres, France), according to the manufacturer's instructions. Briefly, 10^5 cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended in 10 μl cell culture lysis reagent. Samples were stored at −20°C. For relative light unit measurements, 10 μl of sample was incubated with 50 μl luciferase assay reagent. Analyses were performed on a $20/20^n$ luminometer (Turner Biosystems, Sunnyvale, CA).

siRNA experiment and transfection of siRNA. RNA interference mediated by duplexes of 21-nucleotide RNA was performed in LCLs (PRI, TSOB, and TSOC) or LMP1wt-transfected PRI cells. Small interfering RNA (siRNA) duplex 1 and 2 sequences were designed to target exon 1 of the Fas gene (GenBank accession no. NM_000043). Duplex 3 was chosen according to the literature (8). Fas siRNA and control siRNA sequences were as indicated in Table 1. The 21-nucleotide RNAs for silencing were chemically synthesized by Eurogentec (Seraing, Belgium).

siRNA transfection used the Amaxa cell optimization kit V (Amaxa, Köln, Germany) and followed the Amaxa guidelines. Briefly, cells were resuspended in Nucleofector V solution. Cell suspension (100 μl, density of 5×10^6/ml) with 5

TABLE 1. Sequences of irrelevant nonspecific and Fas-specific siRNAs duplexes

siRNA	Sequence
Irrelevant, sense	5′-CAUGUCAUGUGUCACAUCUCdTdT-3′
Irrelevant, antisense	5′-GAGAUGUGACACAUGACAUGdTdT-3′
Fas specific duplex 1, sense	5′-CCAAGGUUCUCAUGAAUCUdTdT-3′
Fas specific, duplex 1, antisense	5′-AGAUUCAUGAGAACCUUGGdTdT-3′
Fas specific duplex 2, sense	5′-CAGUUGAGACUCAGAACUUdTdT-3′
Fas specific, duplex 2, antisense	5′-AAGUUCUGAGUCUCAACUGdTdT-3′
Fas specific duplex 3, sense[a]	5′-GGACAUUACUAGUGACUCAdTdT-3′
Fas specific, duplex 3, antisense[a]	5′-UGAGUCACUAGUAAUGUCCdTdT-3′

[a] Sequences from reference 8.

μg of 100 μM siRNA was transferred into a cuvette and nucleofected with an Amaxa Nucleofector apparatus. Cells were transfected using the O-017 pulsing parameter and were immediately transferred into flask cultures containing 37°C prewarmed culture medium. At 24 h after siRNA transient transfection, cells were induced with doxycycline.

Fas expression was assessed with an IntraPrep kit (Beckman Coulter Immunotech) according to the manufacturer's instructions. Fas expression was detected by direct labeling with a PE-conjugated Fas MAb (BD Pharmingen) and analyzed by flow cytometry.

Real-time quantitative-PCR. Extraction of RNA and real-time quantitative-PCR were performed as described elsewhere (1, 15). We defined, as reference RNA, a pool of RNA extracted from tonsils, lymph nodes, and spleens with benign reactive follicular hyperplasia. RNA levels for the Fas, FasL, and A20 genes were quantified in parallel with the different RNA extracts and the RNA pool on an ABI Prism 7000 automat using the TaqMan R assay on-demand gene expression reference system (Applied Biosystems). The Abl1 gene was used as a reference gene for the control of amplification. All amplification steps were performed in duplicate. The calculated relative gene expression level was equal to $2^{-\Delta\Delta C_T}$, where C_T is the cycle threshold, as previously described (1, 15).

GM1 staining by cholera toxin. GM1 staining was performed as previously described (33). Briefly, cells were plated on polylysine-coated coverslips 24 h after doxycycline induction. Cells were washed twice with cold PBS and incubated at 4°C with 2.5 μg/ml AlexaFluor 594-conjugated recombinant cholera toxin subunit B (CTB; Invitrogen, Cergy-Pontoise, France) for 30 min. Unbound cholera toxin was removed by washing cells twice in cold cell culture medium. The cells were fixed with 4% paraformaldehyde (PFA) for 30 min and processed for immunostaining.

To obtain the mean cell fluorescence from confocal pictures, the fluorescence histogram of each cell was extracted with the free software ImageJ, a public domain Java image processing program available from the National Institutes of Health (http://rsb.info.nih.gov/ij/).

Immunofluorescent staining. Concerning confocal microscopy, for GM1, LMP1 and Fas staining, after fixation with 4% PFA, cells were washed twice with cold PBS. Fixed cells were permeabilized with 0.05% Triton X-100 in PBS. For truncated BID (tBID) and Tom20 experiments, cells were washed in cold PBS, fixed for at least 1 h in methanol at −20°C, and washed in cold PBS before staining in hemolysis tubes. Cells were incubated with primary antibodies for 30 min and then stained with secondary antibodies for 30 min. Cells were washed twice with PBS after incubation with both primary and secondary antibodies. The following dilutions were used for each primary antibody: mouse MAb anti-LMP1 (Dako; CS1-4), 1/25; mouse MAb anti-Fas (Santa Cruz; B-10), 1/50; rabbit polyclonal anti-Fas (Santa-Cruz; C-20), 1/50; goat polyclonal antibody against tBID (Santa Cruz), 1/25; and rabbit polyclonal antibody against Tom20 (Santa Cruz), 1/200. All secondary antibodies were used at a 1/100 dilution. For double staining, AlexaFluor 488- and AlexaFluor 594-conjugated secondary antibodies (Invitrogen) were used. Cells were mounted on slides with SlowFade Gold antifade reagent (Invitrogen) and examined with an LSM 510 laser scanning confocal microscope (Carl Zeiss, LePecq, France).

For flow-cytometric analysis, total Fas and LMP1wt were assessed with an IntraPrep kit (Beckman Coulter Immunotech). Fas was detected with a PE-conjugated MAb (BD Pharmingen) and LMP1wt with a mouse MAb (clone CS 1-4; DakoCytomation) revealed by an AlexaFluor 633–goat anti-mouse immunoglobulin secondary antibody (Invitrogen). The use of two independent lasers for excitation (argon laser at 488 nm for PE and HeNe laser at 633 nm for AlexaFluor 633) avoided emission spectrum overlap between the two dyes, allowing independent fluorescence measurements. Anti-BID (full-length and cleaved large fragment) antibody (Cell Signaling, Danvers, MA) was visualized by AlexaFluor 594 goat anti-rabbit immunoglobulin secondary antibodies (Invitrogen).

Immunoprecipitation experiments. After cell sorting, NGFRt-positive and -negative cells were lysed in radioimmunoprecipitation assay buffer (Santa Cruz) with 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 mM protease inhibitor cocktail added prior to use. The lysates were clarified by centrifugation at 13,000 rpm for 10 min. The supernatant was precleared under gentle agitation with protein A/G–agarose beads (Santa Cruz) overnight to eliminate the primary anti-NGFR antibody and the secondary goat anti-mouse immunoglobulin G antibody magnetic beads used in cell sorting. The samples were then centrifuged at 3,000 rpm for 5 min, and the supernatant was transferred to another microcentrifuge tube. Total cellular protein (500 to 1,000 μg) was incubated for 6 h under gentle agitation at 4°C with 2 μg primary anti-Fas antibody (rabbit polyclonal antibody C-20; Santa Cruz). Then, 20 μl protein A/G–agarose beads was added to the total cellular protein (Santa Cruz) and incubated overnight under gentle agitation. Beads were pelleted by centrifugation for 5 min at 3,000 rpm, washed four times in radioimmunoprecipitation assay buffer, and eluted by boiling in 100 μl Blue Laemmli lysis buffer (Bio-Rad). The eluate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blotting to detect proteins of interest.

Western blotting. Total protein extracts were obtained as follows: 5 million cells were resuspended in 100 μl Blue Laemmli lysis buffer (Bio-Rad) and 5% β-mercaptoethanol (Bio-Rad). The lysates were sonicated and stored at −20°C.

Western blotting was performed as described elsewhere (1). Equal loading of proteins was assessed by Ponceau red staining of the membrane after transfer of proteins from the gel. The following antibodies were used: anti-LMP1 (S12 from hybridoma) at 1/100, anti-Fas (C-20 rabbit polyclonal or B-10 mouse MAb; Santa Cruz) at 1/500, anti-caspase 8 (rabbit polyclonal, BD Pharmingen; mouse MAb, clone 1C12; Cell Signaling, Beverly, MA) at 1/500, anti-caspase 9 (rabbit polyclonal antibody; Cell Signaling) at 1/500, anti-caspase 3 (rabbit polyclonal antibody; Cell Signaling) at 1/500, anti-caspase 7 (rabbit polyclonal antibody; Cell Signaling), anti-poly(ADP ribose) polymerase (anti-PARP) (rabbit polyclonal antibody; Cell Signaling) at 1/500, anti-FADD (Fas-associated death domain protein) (rabbit polyclonal antibody, clone H-181; Santa Cruz) at 1/500, anti-α-tubulin (mouse MAb, clone B5.2.1; Sigma-Aldrich) at 1/10,000, anti-Tom20 (FL-145 rabbit polyclonal antibody; Santa Cruz) at 1/1,000, anti-BID (rabbit polyclonal antibody; Cell Signaling) at 1/500. After 1 h washing in Tris-buffered saline–0.1% Tween, the corresponding horseradish peroxidase-conjugated secondary antibodies, goat anti-mouse immunoglobulin (Bio-Rad) at 1/5,000 and goat anti-rabbit immunoglobulin (Bio-Rad) at 1/5,000, were added for 1 h. Proteins were visualized after washing for 2 h in Tris-buffered saline–0.1% Tween using an ECL Plus kit (Amersham, Orsay, France) before autoradiography (X-Omat R films; Kodak).

Caspase activity assay (fluorochrome-labeled inhibitors of cleaved caspase [FLICA] test). The carboxyfluorescein (FAM)-labeled caspase inhibitors FAM-LETD-FMK, FAM-LEHD-FMK, and FAM-DEVD-FMK were designed to detect the presence of active caspases 8, 9, and 3, respectively, within apoptotic cells. These labeled caspase inhibitors were obtained from Serotec (Immunochemistry Technologies LLC, Bloomington, MN).

FAM-labeled reagents were dissolved in dimethyl sulfoxide to obtain a 150×-concentrated stock solution aliquoted and stored at −20°C. Prior to use, a 30× working solution of FLICA was prepared by diluting the stock solution (1:5 in PBS) and mixed until it became transparent. Further dilutions were made in cell culture medium.

The LCLs PRI, EMICA, and 1602 were transfected by the inducible vector pRT-1, which contains the cDNA coding for LMP1wt, and divided into two batches, one which received doxycycline treatment for 24 h and one without doxycycline treatment. Each cell sample that was subjected to FLICA labeling was redistributed into 300-μl aliquots containing 3×10^5 cells, and FAM-LETD-FMK, FAM-LEHD-FMK, or FAM-DEVD-FMK was added to these cultures to obtain a 10 μM final concentration. Cells were incubated for 1 h at 37°C under 5% CO_2 prior to harvesting. Direct labeling was performed with a PE-conjugated NGFR MAb (BD Pharmingen) diluted 1/20 for 20 min. Following incubation with FLICA, cells were pelleted, washed twice with 1× wash buffer solution, and resuspended in 400 μl 1× PBS containing 10 μl propidium iodide (Serotec) before analysis by flow cytometry.

Mitochondrial transmembrane potential analysis. After cell sorting, NGFRt-positive and -negative fractions were stained in parallel with JC1 (5,5′,6,6′-

tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide/chloride), as previously described (64). Briefly, cells adjusted to a density of 10^6/ml in PBS were stained for 20 min at room temperature with 1 μg/ml JC1. The dye accumulates as aggregates in mitochondria with high membrane potential ($\Delta\psi$), resulting in orange fluorescence, whereas in apoptotic and necrotic cells, JC1 exists in a green monomeric form. Cell membrane permeability was assessed by adding 1 μM TOPRO-3 (Invitrogen) to samples, just before flow-cytometric analysis.

Mitochondrial and cytoplasmic extract fractionation. Mitochondrial purification was performed with a mitochondrion isolation kit for cultured cells (Perbio Science France SAS, Brebières, France), according to the manufacturer's instructions. For flow-cytometric analysis, isolated mitochondria were incubated with a primary antibody directed against BID (FL-195 rabbit polyclonal antibody; Santa Cruz Biotechnology, San Diego, CA) at 1/50 for 15 min at room temperature and revealed with a donkey-anti-rabbit immunoglobulin AlexaFluor 488-conjugated secondary antibody (Invitrogen, Cergy-Pontoise, France) at 1/200 for 15 min at room temperature. Cytoplasmic fractions were obtained during cell fractionation, according to the mitochondrion isolation kit indication, and analyzed by Western blotting.

RESULTS

Spontaneous apoptosis in LCLs is associated with high levels of LMP1 and Fas expression. As already demonstrated by us and others, LMP1wt expression leads to Fas overexpression in LCLs (11, 37, 62). Here, we show that LMP1wt levels are very heterogeneous from cell to cell and that Fas expression parallels LMP1 expression (Fig. 1A). The use of independent lasers for excitation (argon laser at 488 nm for Fas-PE and HeNe laser at 633 nm for LMP1-AlexaFluor 633) avoided emission spectrum overlap between the two dyes and allowed independent fluorescence measurements. Also, latency III LMP1-positive cells (PRI, EMICA, TSOB, TSOC, and EREB), particularly TSOB cells, showed some basal levels of spontaneous apoptosis, as demonstrated by PARP cleavage (Fig. 1B). The subpopulation that underwent apoptosis and had exposed phosphatidylserine on the external surface of the plasma membrane (3, 21, and 10% for PRI, TSOB, and TSOC, respectively) exhibited higher levels of Fas than the nonapoptotic cells (Fig. 1C). These results were consolidated using an ImageStream imaging flow cytometer (Amnis, Seattle, WA); analysis, cell by cell, showed a decrease in nuclear integrity for cells expressing high levels of LMP1 (see the supplemental material). Thus, even though LMP1wt is primarily known as the EBV major transforming oncoprotein, high physiological levels of the protein paralleled Fas overexpression and spontaneous apoptosis in LCLs.

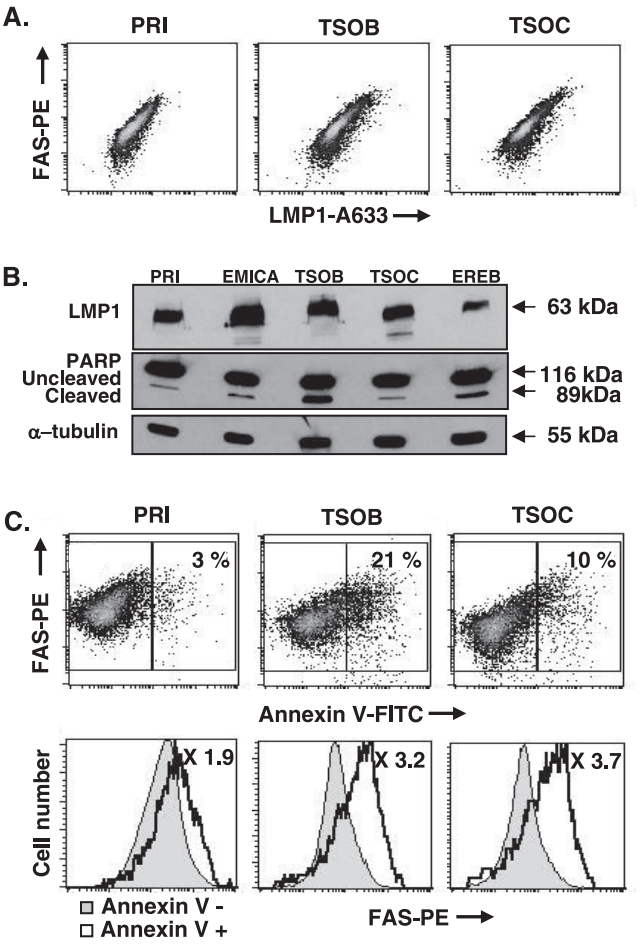


FIG. 1. In LCLs, cells with high LMP1wt and Fas expression levels are more sensitive to induction of apoptosis, (A) LMP1 and total Fas (intracellular and cytoplasmic membrane) expression levels of three LCLs (PRI, TSOB, and TSOC) were analyzed by flow cytometry after immunostaining, as described in Materials and Methods. The use of two lasers for excitation allowed independent fluorescence analysis for Fas and LMP1. (B) Five LCLs (PRI, EMICA, TSOB, TSOC, and EREB) were analyzed by Western blotting with anti-LMP1, anti-PARP, and anti-α-tubulin antibodies, as described in Materials and Methods. (C) PRI, TSOB, and TSOC cells were stained with annexin V-FITC and PE-conjugated Fas MAb and analyzed by flow cytometry. For each cell line, histograms of cytoplasmic membrane Fas expression levels were reported for annexin V-negative against annexin V-positive cells.

LMP1 overexpression induces apoptosis. LMP1 overexpression has been shown to sensitize cells to apoptosis induction in an NF-κB-dependent manner (37, 62). To study LMP1wt apoptotic features and to understand the underlying molecular process, the previously obtained LCL named PRI was stably transfected with the bidirectional Tet-on inducible vector pRT-1, which conditionally expresses both NGFRt and LMP1wt after doxycycline treatment (37). The main advantage of this cellular model is the simplicity of the negative control, which consists of the same cells without doxycycline treatment. In order to study LMP1 cytotoxicity in terms of apoptotic response, we first labeled cells with annexin V-FITC and TOPRO-3 dye for DNA after 24 h induction with increasing doses of doxycycline (leading to NGFRt as well as LMP1wt protein overexpression) (37). The percentage of cells undergoing apoptosis progressively increased for the NGFRt-positive (LMP1wt overexpression) compared to the NGFRt-negative (endogenous LMP1wt) subpopulation (Fig. 2A). These results were confirmed in two other LCLs transfected with the inducible vector pRT-1-LMP1wt, EMICA and 1602 (results not shown). After 24 h doxycycline induction, LMP1wt protein-induced expression was correlated with PARP protein cleavage, demonstrated by the appearance of the carboxy-terminal catalytic domain (89 kDa) on Western blotting, as a hallmark of apoptosis (Fig. 2B). Cell DNA content analysis by flow cytometry after 24 h doxycycline induction showed the emergence of a cell fraction containing subdiploid amounts of DNA (2 to 31% of events). This sub-G_1 peak is commonly associated with DNA fragmentation in terminal events of

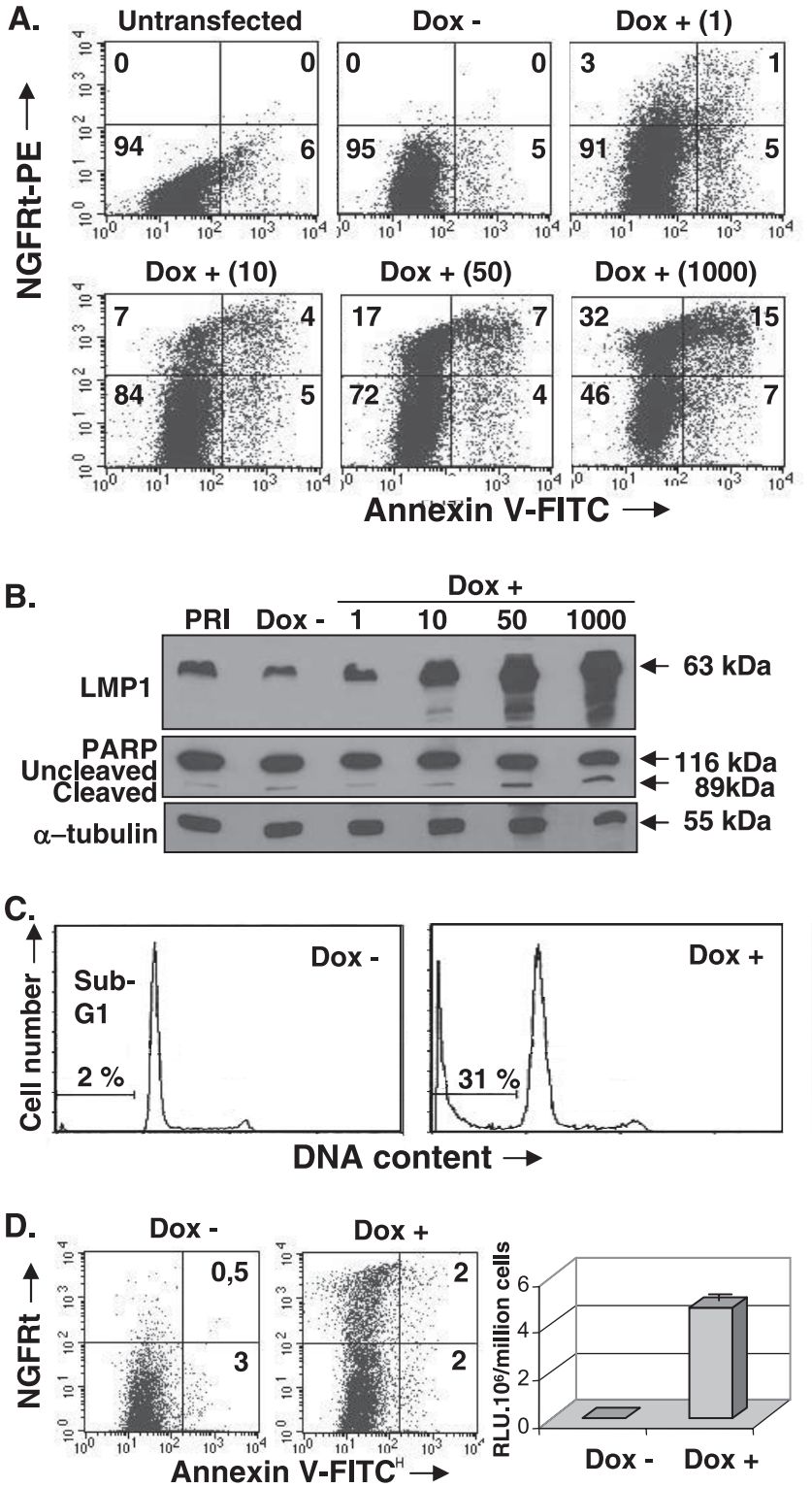


FIG. 2. LMP1wt overexpression induces apoptosis in PRI-LCLs. (A) Latency III LMP1-positive PRI-LCLs were stably transfected with the inducible vector pRT-1, which contains cDNA coding for LMP1wt and NGFRt as a reporter gene. After 24 h doxycycline induction, untreated cells (Dox −) and doxycycline (1 to 1,000 ng/ml)-treated cells (Dox +) were stained with annexin V-FITC and PE-conjugated NGFR MAb and analyzed by flow cytometry. (B) Whole-cell extracts were analyzed in parallel for LMP1 and PARP expression by Western blotting. (C) LMP1-transfected PRI-LCLs were stained with propidium iodide to analyze cell DNA content before (Dox −) or after (Dox +) doxycycline induction at 1 μg/ml by flow cytometry. (D) PRI-LCLs were stably transfected with the inducible vector pRT-1, which contains cDNA coding for luciferase instead of LMP1. Annexin V-FITC and PE-conjugated NGFR MAb staining (left) were analyzed by flow cytometry, and luciferase activity (right) was assessed by luciferase assay, as described in Materials and Methods.

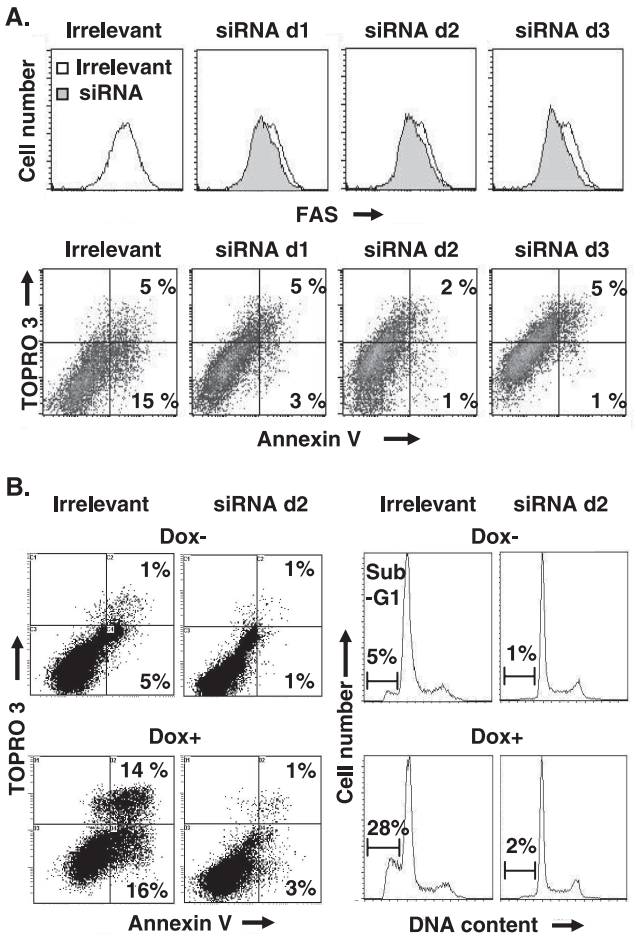


FIG. 3. Downregulation of Fas expression decreases apoptosis in LCLs and LMP1wt-induced cells. Downregulation of Fas expression was optimized using siRNA against Fas gene, as described in Materials and Methods. (A) Downregulation of Fas expression in the LCL TSOB, after transient transfection with an irrelevant siRNA or with Fas-specific siRNA duplexes 1 to 3 (d1, d2, and d3), were analyzed by flow cytometry at 24 h by staining Fas with a PE-conjugated Fas MAb for 15 min (top). siRNA-transfected TSOB cells were stained with annexin V-FITC and TOPRO-3 as an impermeant DNA dye (bottom). (B) LMP1wt-transfected LCL PRI cells, 24 h after transient transfection with irrelevant siRNA or Fas-specific siRNA d2, were induced with doxycycline for 24 h. Then, untreated cells (Dox−) and doxycycline-treated cells (Dox+) were stained with annexin V-FITC and with TOPRO-3 as an impermeant DNA dye. Induced cells were previously identified with PE-conjugated NGFR MAb and analysis performed by flow cytometry (left). LMP1wt-transfected PRI cells, 24 h after transient transfection with irrelevant siRNA or Fas-specific siRNA d2, were stained with propidium iodide to analyze cell DNA content by flow cytometry, before (Dox−) or after (Dox+) doxycycline induction at 1 μg/ml (right).

apoptosis (Fig. 2C). Control experiments with cells transfected with a luciferase/NGFRt doxycycline double-inducible vector showed virtually no variation of apoptosis induction after 24 h doxycycline treatment (Fig. 2D).

Thus, LMP1wt overexpression in PRI-LCL initiates apoptosis induction, shown by phosphatidylserine externalization and a sub-G_1 DNA peak observed by flow cytometry, as well as by PARP cleavage shown on Western blotting.

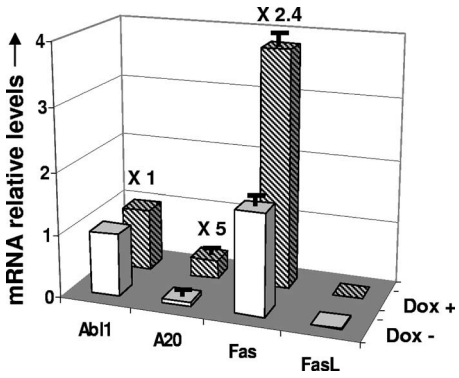


FIG. 4. Absence of FasL induction in LMP1wt-induced PRI-LCLs cells. After 24 h doxycycline induction, cells were harvested and mRNA from untreated cells (Dox −) and doxycycline cells (Dox +) was extracted. A20 mRNA, Fas mRNA, and FasL mRNA were analyzed by real-time RT-PCR. The Abl1 gene was used as a reference gene. Error bars correspond to the standard deviations of three experiments.

LMP1 apoptosis requires the Fas receptor. Since LMP1wt was reported to markedly up regulate Fas expression (11, 24, 37), we examined whether apoptosis could be initiated from this death receptor. Fas-specific siRNAs, named duplexes 1, 2, and 3 (sequences in Table 1), were used to knock down Fas expression in LCLs and in LMP1wt-transfected PRI cells before and after doxycycline treatment. An irrelevant siRNA was used as a control (sequence in Table 1).

For TSOB (the LCL with the highest spontaneous apoptosis percentage), a reduction of total Fas protein (intracellular plus cytoplasmic membrane) was detected by flow cytometry after siRNA transfection with duplex 1, 2, or 3, compared to the irrelevant siRNA (Fig. 3A). The effect of Fas siRNA transfection on the apoptotic response was studied by using the annexin V binding assay. When cells were transfected with the irrelevant siRNA, 20% of them showed an externalization of phosphatidylserine, compared to only 8, 3, and 6%, respectively, for duplexes 1, 2 and 3 (that is, decreases of about 60, 85 and 80%, respectively) (Fig. 3A). Fas-specific siRNA also led to the abolition of spontaneous apoptosis in PRI cells (data not shown).

For LMP1wt-transfected PRI cells, transfection with Fas-specific siRNA duplexes 1, 2, and 3 led to a significant reduction of total Fas protein, compared to levels in cells transfected with the irrelevant siRNA (data not shown). Regarding the apoptotic response, for irrelevant siRNA, doxycycline induction of LMP1wt led to the externalization of phosphatidylserine on the surface of the cell plasma membrane for 30% of NGFRt-positive cells, compared to 6% of uninduced cells (Fig. 3B). In contrast, the more effective siRNA duplex 2 prevented LMP1-induced apoptosis. The percentages of annexin V-positive cells before and after induction were about 2 and 4%, respectively (that is, a decrease of about 87% for doxycycline-induced cells). Decrease of apoptosis in LMP1wt-transfected PRI cells after transfection with Fas siRNA was confirmed by sub-G_1 peak analysis (Fig. 3B). Apoptotic-cell percentages were also decreased by using duplexes 1 and 3, even though the decreases were less extensive (decreases of about 33 and 50%,

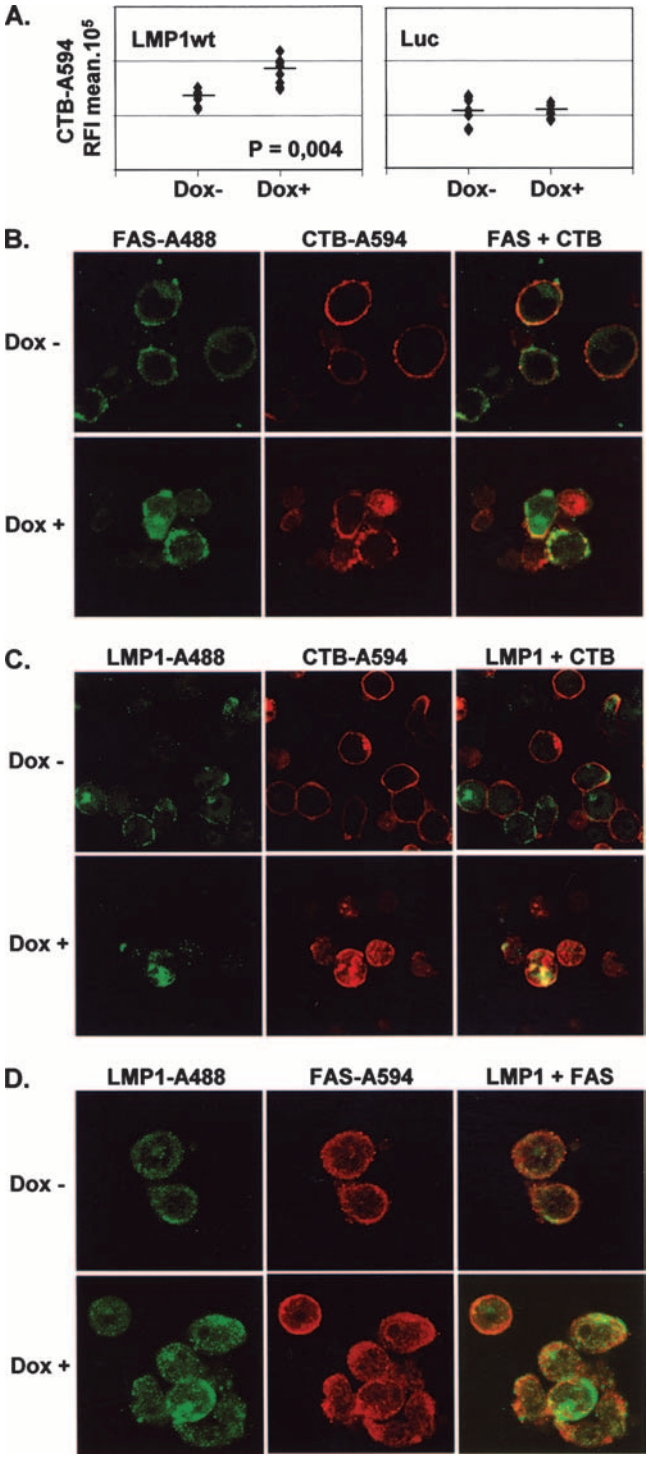


FIG. 5. LMP1 and Fas are recruited in dissimilar GM1 lipid rafts. (A) GM1 levels in untreated cells (Dox−) or in doxycycline-treated cells (Dox+) were analyzed after 24 h doxycycline induction, by staining with 2.5 μg/ml AlexaFluor 594 CTB, fixation with 4% PFA, and permeabilization with PBS–0.05% Triton X-100. LMP1wt/NGFRt or luciferase/NGFRt vector-transfected cells were analyzed by confocal microscopy, and mean cell fluorescence was extracted with ImageJ software (A). Colocalization of Fas and CTB (B) or of LMP1 and CTB (C) was detected as described in Materials and Methods. After 24 h doxycycline induction, cells were incubated with 2.5 μg/ml AlexaFluor 594 CTB (red) at 4°C for 30 min. Cells were washed twice with cold PBS and fixed with 4% PFA. Then, cells were permeabilized with PBS–0.05% Triton X-100 and incubated with primary antibodies (anti-LMP1 antibody CS1-4 or anti-Fas antibody B-10) for 30 min, and proteins were visualized with AlexaFluor 488-conjugated goat anti-mouse immunoglobulin secondary antibodies (green) for 30 min. Colocalization between LMP1 and Fas (D) was detected by incubation with primary antibodies (CS1-4 [anti-LMP1] and C-20 [anti-Fas]) for 30 min, revealed with AlexaFluor 488-conjugated goat anti-mouse immunoglobulin and AlexaFluor 594-conjugated goat anti-rabbit immunoglobulin secondary antibodies, respectively, for 30 min. Colocalization experiments were performed by confocal microscopy.

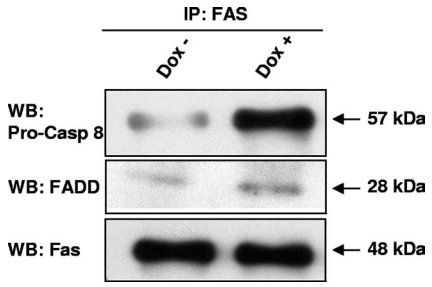


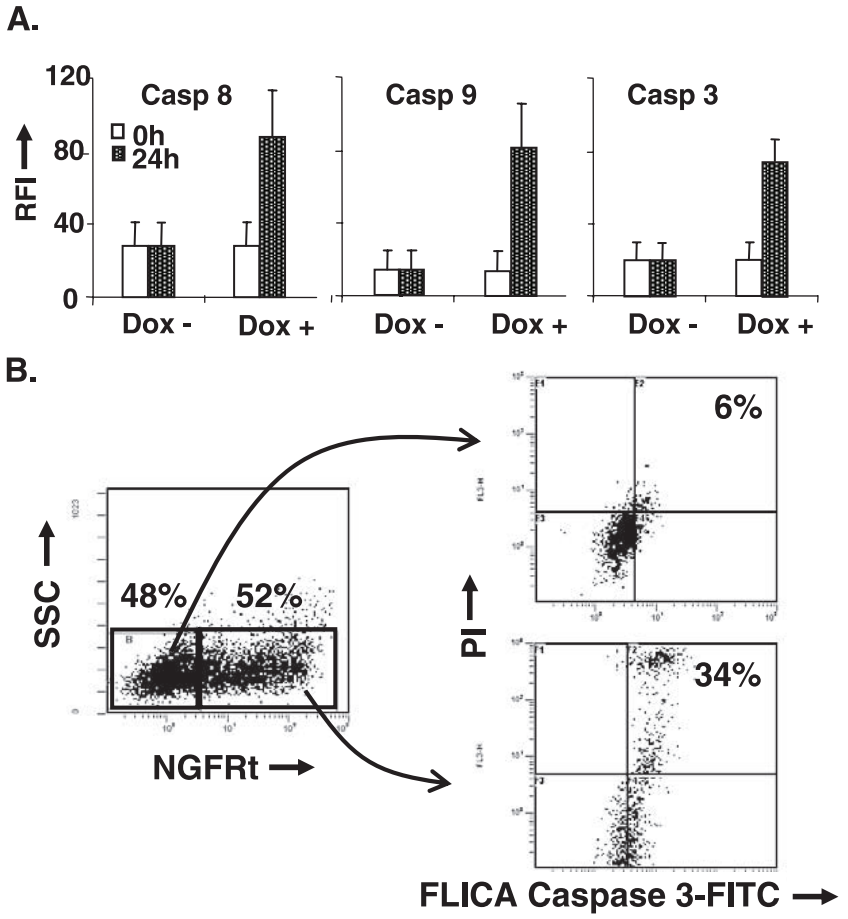
FIG. 6. Apoptosis involves Fas-dependent DISC formation. After 24 h doxycycline induction, untreated cells (Dox −) and doxycycline-treated cells (Dox +) were harvested, and whole-cell extracts were immunoprecipitated with an anti-Fas (C-20) MAb. Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis using anti-caspase 8, anti-FADD, and anti-Fas antibodies.

respectively, were observed for doxycycline-induced cells) (data not shown). This was probably due to the lower efficiency of these duplexes, exacerbated by the fact that LMP1 simultaneously carried out Fas overexpression.

In summary, the results show that decreasing Fas expression prevents spontaneous apoptosis in LCLs as well as apoptosis initiated by LMP1wt overexpression, which suggested a Fas-mediated cell death in cells expressing high levels of LMP1.

Apoptosis induction is FasL independent. Apoptosis inhibition after Fas expression knock-down demonstrated the central role of this receptor in the initiation of the process. Since the extrinsic Fas death receptor apoptosis pathway can be activated by interactions with its ligand, FasL, we studied the expression of FasL and Fas, as well as A20 as a control. As expected, we observed an increase in A20 and Fas mRNA levels after 24 h doxycycline induction (5- and 2.4-fold increases in mRNA levels, respectively) (Fig. 4). No FasL mRNA was detected before or after doxycycline induction. Thus, it is unlikely that FasL could play a role in LMP1-induced apoptosis, since this molecule is not expressed in our EBV-infected cells and is not induced by LMP1. Furthermore, FasL-neutralizing antibodies had no effect on LMP1-induced apoptosis (not shown).

LMP1 and Fas are recruited in dissimilar GM1 lipid rafts. Fas-mediated cell death is known to require the formation of apoptotic membrane microdomains enriched in ganglioside GM1 lipid (lipid rafts). We determined whether GM1 lipid rafts could be associated with apoptosis. Studies were carried



out with Alexa-labeled CTB binding protein, a GM1 lipid marker.

As a preliminary result, CTB mean fluorescence intensity was extrapolated for each cell from confocal pictures, to estimate GM1 expression before and after doxycycline induction. We showed that apoptosis induction by LMP1wt overexpression was associated with GM1 lipid synthesis, as shown by a 3.3-fold CTB fluorescence increase ($P < 0.05$) (Fig. 5A). A control with luciferase/NGFRt vector-transfected cells confirmed that NGFRt alone did not lead to GM1 lipid synthesis (Fig. 4A).

Further analyses were then carried out to visualize GM1 lipid raft organization. Before LMP1wt induction, CTB staining was strictly limited to the cytoplasmic membrane, whereas after doxycycline treatment it was concentrated in cytoplasmic microdomains or present at the intracellular level (Fig. 5B and C). Fas expression was clearly increased by doxycycline treatment (Fig. 5B). Moreover, Fas/GM1 costaining indicated that the death receptor became concentrated in GM1 lipid raft microdomains located at the cytoplasmic membrane.

For untreated cells, LMP1wt protein was preferentially located in plasma membrane microdomains, different from GM1 lipid rafts (Fig. 5C). As expected, doxycycline induction was associated with a significant increase in oncoprotein expression, particularly at the intracellular level. In addition, LMP1wt and CTB were colocalized.

Therefore, doxycycline induction markedly increased LMP1wt and Fas death receptor localization into GM1 rafts. However, no significant colocalization of these two proteins was seen. LMP1wt and CTB were mainly colocalized at the cytoplasmic level, whereas Fas and CTB were essentially colocalized at the plasma membrane (Fig. 5D).

These experiments show that LMP1wt induction was associated with lipid raft formation and with GM1 synthesis. Fas mobilization and capping into GM1 lipid rafts support its implication in apoptosis. LMP1wt and Fas did not colocalize, despite being located in lipid rafts. This suggests that any Fas signaling was topographically separated from LMP1 signaling in apoptosis induction.

DISC formation occurs at the Fas receptor level. The adaptor FADD and the caspase initiator procaspase 8 are both required for the formation of the death-inducing signaling complex (DISC). To confirm that the apoptotic process requires the Fas signaling pathway, we evaluated recruitment of these two molecules at the receptor level.

Protein coimmunoprecipitations were carried out with Fas polyclonal antibodies. Subsequent Western blotting with FADD and procaspase 8 antibodies revealed a weak recruitment of both proteins by the Fas receptor before doxycycline treatment, indicating the formation of a DISC at the receptor level of some cells (Fig. 6). After LMP1wt induction, the recruitment of both proteins by the Fas death receptor was considerably increased (Fig. 6). Recruitment of procaspase 8 protein into DISC was significantly higher than for FADD. As a control, coimmunoprecipitation with anti-FADD or anti-caspase 8 antibodies followed by LMP1 Western blotting did not reveal any interaction between these two proteins and LMP1 (not shown), confirming the independence of the two signaling pathways.

Altogether, these results suggest that Fas overexpression induced by LMP1, associated with Fas autoactivation, is responsible for LMP1-induced apoptosis in EBV-infected B cells.

LMP1-triggered apoptosis is caspase dependent. Fas-mediated apoptosis implies procaspase 8 activation. We first studied caspase 8, 9, and 3 activation by flow cytometry, using FLICA (18, 53). In this technique, specificity of the labeling is based on the cleavage site of the active form of each caspase type. Whatever the caspase studied, relative fluorescence intensity was considerably increased for the doxycycline-induced NGFRt-positive population, compared to that in untreated cells (Fig. 7A and B). To confirm flow cytometry results, we performed Western blotting for these three caspases. Results show that 24 h doxycycline treatment of cells was associated with the cleavage of caspases 8, 9, and 3 (Fig. 7C). Caspase 7 was also cleaved, albeit at a lower level (Fig. 7C). Results confirmed that PRI LCL apoptosis initiated by LMP1wt was associated with the activation of initiator caspases 8 and 9 and effector caspases 3 and 7.

The mitochondrial intrinsic apoptotic pathway is activated downstream from caspase 8 activation, via BID cleavage. Caspase 9 activation during LMP1 overexpression might imply intrinsic apoptotic pathway involvement. To confirm mitochondrial involvement, mitochondrion $\Delta\Psi$ was analyzed by flow cytometry in whole cells, using the JC1 probe. The physiological status of cells stained by JC1 and TOPRO-3 (intact, apoptotic, or necrotic) was assessed with a control consisting of cells treated with H_2O_2 (1 mM, 3 h), a reactive oxygen species (ROS) which activates the intrinsic apoptotic pathway leading to mitochondrial $\Delta\Psi$ loss (data not shown). After 24 h doxycycline induction, the subpopulation corresponding to apoptotic features (low JC1 orange fluorescence due to dye efflux in TOPRO-3-negative cells

FIG. 7. LMP1wt-induced apoptosis involves caspase activation. (A) After doxycycline induction, cells were subjected to FLICA labeling by adding labeled-caspase inhibitor FAM-LETD-FMK, FAM-LEHD-FMK, FAM-DEVD-FMK, respectively, to detect the presence of active caspases 8, 9 and 3. Induced cells were previously identified with PE-conjugated NGFR MAb and analyses were performed by flow cytometry. Propidium iodide was used as a DNA impermeant dye. Fluorescence unit was reported as RFI (Relative Fluorescence Intensity). (B) Example of graphs for caspase 3 analysis after FLICA labeling. After 24 h of doxycycline induction, NGFRt positive subpopulation selected on the cytogram NGFRt versus SSC (side scatter) showed an increase in the percentage of cells with positive fluorescence (caspase active form), compared to NGFRt negative cells (from 6 to 34%). For the NGFR positive subpopulation, caspase 3 activated cells were not, partially or highly stained by the permeability marker propidium iodide (PI), corresponding to the different stages seen during apoptosis (intact, plasma membrane disturbed and late apoptotic or necrotic cells, respectively). (C) After 24 h doxycycline induction, untreated cells (Dox −) and doxycycline-treated cells (Dox +) were harvested, and whole-cell extracts were analyzed by Western blotting with anti-caspase 8, anti-caspase 9, anti-caspase 3, anti-caspase 7, and anti-α-tubulin antibodies, as described in Materials and Methods.

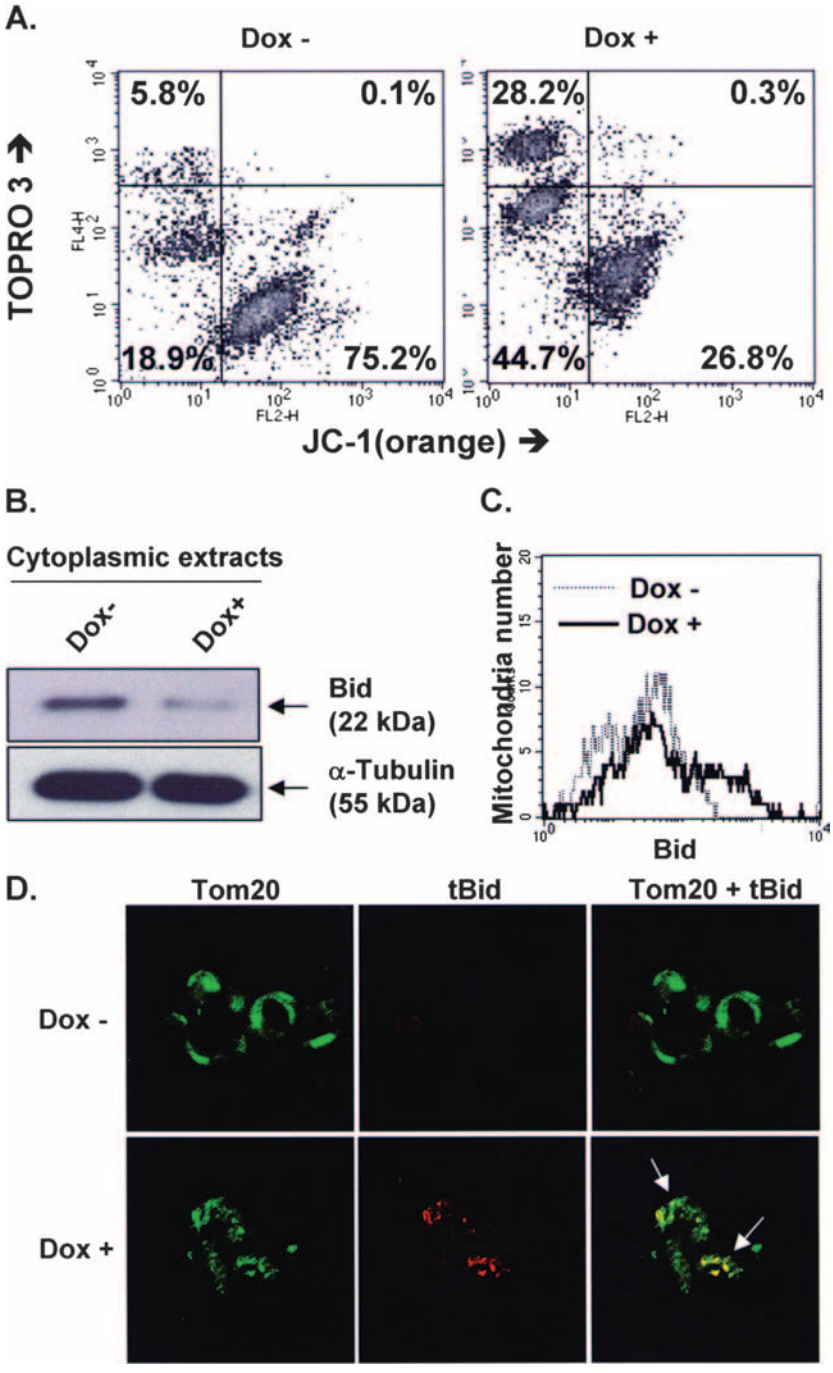


FIG. 8. LMP1wt induction induces mitochondrial transmembrane potential loss and tBID relocalization at the organelle level. (A) After 24 h doxycycline induction, untreated cells (Dox−) and doxycycline-treated cells (Dox+, NGFRt-expressing sorted cells) were stained by JC-1 (1 μg/ml, 20 min) and the impermeant DNA dye TOPRO-3 (1 μM) before analysis by flow cytometry. Cell physiological status was defined according to transmembrane $\Delta\psi$ and cytoplasmic membrane permeability. Three stages were identified: intact ($JC\text{-}1^{+}/TOPRO\text{-}3^{-}$), apoptotic ($JC\text{-}1^{-}/TOPRO\text{-}3^{-}$), and late apoptotic or necrotic ($JC\text{-}1^{-}/TOPRO\text{-}3^{+}$) cells. (B) Mitochondrion-depleted cytoplasmic extracts were subjected to Western blot analysis with anti-Bid and anti-α-tubulin (cytoplasm-specific) antibodies. (C) Isolated mitochondria were incubated with anti-Bid antibodies, visualized with AlexaFluor 594, and analyzed by flow cytometry. (D). tBID expression and its colocalization with Tom20 were assessed by incubation with primary antibodies (anti-Tom20 and anti-tBID) for 30 min and visualization with AlexaFluor 488-conjugated (green) and AlexaFluor 594-conjugated (red) antibodies, respectively, for 30 min. Analyses were carried out by confocal microscopy. Arrows indicate cells with colocalized tBID and Tom20.

that had not yet lost their membrane integrity) was indeed markedly increased (from about 20 to 45%) in doxycycline-treated LMP1/NGFRt-positive cells (Fig. 8A).

Caspase 8 is known to carry out BID protein cleavage (yielding tBID) and its relocalization at the mitochondrial level, where tBID mediates opening of mitochondrial voltage-dependent anion channels, resulting in $\Delta\Psi$ loss. Western blot results showed a marked decrease in full-size BID protein in mito-

chondrion-depleted cytoplasmic extracts, after 24 h doxycycline induction. Sample purity was assessed with Tom20 (mitochondria) and α-tubulin (cytoplasm) antibodies (Fig. 8B). At the same time, BID protein became detectable on about 30% of isolated mitochondria, as analyzed by flow cytometry (Fig. 8C). This suggests a relocalization of tBID at the mitochondrial level. Results were confirmed by confocal microscopy observations. After 24 h doxycycline induction, the tBID protein was observable at the mitochondrial level (colocalization with Tom20 staining), whereas it was undetectable in untreated cells (Fig. 8D).

DISCUSSION

Homeostasis of the EBV-infected-cell compartment, involving death and proliferation control, is essential in host-virus equilibrium, and a rupture in this system can lead to lymphoproliferation. Among the viral proteins responsible for B-cell transformation, LMP1, the major EBV oncoprotein, contributes to in vivo and in vitro B-cell transformation and proliferation, leading to lymphomas and LCLs, respectively, particularly due to NF-κB signalization (39, 54). Paradoxically, this latent viral protein has been reported to induce, or sensitize cells of various types (such as neutrophils, monocytes, epithelial cells, and LCLs) to induction of, apoptosis (36, 40, 42, 61, 63). LMP1 is clearly implicated in the balance of life and death of infected cells by regulating antiapoptotic (Bcl-2, Mcl-1, and Bfl1) (9, 22, 49, 58) and proapoptotic factors (Bax and Fas receptor) (11, 20, 24, 37). However, the associated molecular basis of LMP1 cytotoxicity remains unclear.

We show here that LMP1 overexpression leads to apoptosis in B cells, as demonstrated in epithelial cells (42). This apoptotic process was characterized by typical associated hallmarks, such as plasma membrane phosphatidylserine externalization, DNA fragmentation, and PARP cleavage (preventing DNA repair). We demonstrated, through siRNA experiments, that the core molecule responsible for the initiation of the apoptotic process was the Fas receptor and that this process occurred independently of its ligand, FasL. We and others showed that the Fas promoter was activated via the NF-κB pathway, which up-regulated the expression of Fas protein (7, 11, 37). Furthermore, we demonstrated that caspases 3, 8, and 9, which belong to the class of proteases involved in apoptosis, were activated.

Lipid raft formation is an essential step for cell signaling by both LMP1 and Fas (19, 33, 43). We showed that these proteins were both relocated into GM1 lipid rafts after induction of LMP1 overexpression, which indicated cell signaling initiation from the two molecules (33, 38, 41, 45). Interestingly, Fas and LMP1 were not colocalized. LMP1 was relocated in intracellular GM1 lipid rafts, in agreement with the fact that it principally signals from the intracellular compartment (33). Fas receptor was recruited in membrane-specific GM1-rich microdomains, a prerequisite for signaling (38, 45). The absence of colocalization of LMP1 with Fas indicated the existence of distinct GM1 lipid rafts, probably with different functional properties.

Coimmunoprecipitation experiments demonstrated the interaction of Fas with FADD and procaspase 8. These results revealed that LMP1 induces the formation of a DISC at the Fas death receptor. LMP1 was not found in the DISC (data not shown), confirming its indirect role in the apoptotic process.

The fact that apoptosis was abolished after Fas siRNA transfection implied that the mitochondrial intrinsic apoptotic pathway, characterized by caspase 9 activation, was not directly activated. We demonstrated that caspase 9 activation was due to cleavage of BID, resulting in downstream caspase 8 activation by Fas receptor involvement. tBID mediates the formation of the mitochondrial cytochrome *c* release channel and activation of caspase 9 in a complex with dATP, cytochrome *c*, and Apaf-1 (31, 59). Mitochondrial contribution was validated by mitochondrial transmembrane potential loss during apoptosis. Signaling from the Fas receptor appeared to be dependent on the mitochondrial amplification loop, which is consistent with type II apoptosis.

Even though the central role of the Fas death receptor in apoptosis was clearly demonstrated by our work, the molecular signals responsible for its activation and leading to its autoaggregation in lipid rafts remain to be defined. Fas receptor overexpression by itself may lead to apoptosis, as observed in other models (28, 35, 52). LMP1 has also been shown to induce ROS in LCL (6). This could promote Fas activation, as demonstrated for chemotherapeutic and DNA-damaging molecules (25, 43). Mitochondrion-derived ROS could moreover potentialize Fas aggregation. However, preliminary results obtained after DCF-DA staining and flow cytometry analysis indicated a slight increase in ROS production after LMP1 induction (data not shown).

Fas overexpression by LMP1 is due to the activation of NF-κB signaling pathways (7, 11, 37). NF-κB has already been shown to be required for the induction of caspase-dependent apoptosis by LMP1 in epithelial cells and fibroblasts (47). NF-κB is necessary for the induction of apoptosis in various EBV-negative cellular models (29, 57). Numerous results, including ours, clearly evidence the role of LMP1-dependent NF-κB activation in protection against apoptosis. The fact that increased levels of LMP1 are able to induce cell proliferation arrest (13) and/or apoptosis is reminiscent of other cellular oncogenes, such as c-*myc*. Both c-*myc* and LMP1 are able to induce p53 expression (37). c-*myc* induction of p53 is associated with induction of apoptosis, with the consequence that c-*myc* proliferating potential is expressed in tumor cells only if additional secondary oncogenic events such as p19/ARF inactivation have occurred (5). These features are interpreted as security mechanisms to prevent inappropriate proliferation of normal cells. Our results suggest that the LMP1 gene is an ambivalent oncogene functionally closer to cellular oncogenes than to classical viral oncogenes, such as v-*src*, v-*rel*, and the TAX gene, or chimeric oncogenes, such as the Bcr-Abl gene. In this view, sensitizing cells to induction of apoptosis by the immune system at normal levels of LMP1 expression or inducing apoptosis of cells at high LMP1 expression levels would participate in the panoply of security mechanisms of EBV, a virus very well adapted to its host, to avoid inducing inappropriate lymphoproliferation. This is certainly related to the fact that LMP1 acts as an active receptor that targets the cellular transcription factor NF-κB, which has its own negative retroactive regulation loops. Also, we previously showed that LMP1 can activate its own promoter (pLMP1) through the JNK signaling pathway, whereas the LMP1 promoter is repressed by

NF-κB activation (17). Thus, LMP1 expression levels would be regulated so that NF-κB activation levels would be sufficient to protect the majority of infected cells against apoptosis and would not be strong enough to induce Fas autoactivation. This retroactive feedback of LMP1 regulation mediated by NF-κB would prevent EBV-infected B cells from deleterious consequences of NF-κB overactivation. Apoptosis could, however, occur in cells with higher LMP1 expression levels, contributing to the homeostatic control of an LMP1-infected-B-cell compartment.

ACKNOWLEDGMENTS

This work was supported by Cancéropôle Grand-Sud-Ouest, Ligue Nationale contre le Cancer, Conseil Régional du Limousin, and Association pour la Recherche sur le Cancer. UMR CNRS is a Ligue Nationale contre le Cancer laboratory. C.L.C. is supported by Ligue Nationale contre le Cancer, Comité de la Corrèze, and Fondation pour la Recherche Médicale. T.-S.O. is supported by Institut National du Cancer. I.Y.-M. is supported by Conseil Régional du Limousin. S.P. is supported by the Ministère de la Recherche et de l'Enseignement Supérieur. J.R. was supported by Boehringer Ingelheim Fonds. U.Z.-S. was funded by the Deutsche Krebshilfe.

We thank A. Allegraud and M. Bathias from Laboratoire d'Hématologie CHU Dupuytren for technical assistance in cell culture. We thank J. Cook-Moreau for English rereading.

We dedicate this report to the memory of our colleague Jean Coll.

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