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Expression of LMP1 in Epithelial Cells Leads to the Activation of a Select Subset of NF-κB/Rel Family Proteins

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This study demonstrates that the Epstein-Barr virus protein LMP1 activates a specific subset of NF-κB/Rel proteins in the C33 epithelial cell line. Western immunoblot analysis used to analyze the intracellular distribution and abundance of the proteins present in these complexes demonstrated that levels of the p50 and p52 proteins were significantly elevated in the nuclei of LMP1-expressing cells. The data also suggest that LMP1 facilitates the translocation of p50 to the nucleus and may affect the processing of the p100 and p105 precursor proteins or the stability of p52 and p50.

Epstein-Barr virus (EBV), a herpesvirus which infects both B lymphocytes and epithelial cells in vivo, causes infectious mononucleosis and posttransplant lymphoma and is associated with both Burkitt's lymphoma and nasopharyngeal carcinoma (NPC). EBV is consistently detected in all examples of NPC regardless of geographic location or ethnic background of the patient (23). Molecular analysis of the genomic structure of EBV found in NPC tumor cells revealed that NPC is a clonal proliferation of a single EBV-infected cell, indicating that EBV infection is an early occurrence in the development of NPC (24, 25).

Latent membrane protein 1 (LMP1) is a viral protein transcribed in most NPCs and is detected at the protein level in 35 to 65% of all NPCs (6, 9, 32). LMP1 is necessary for the transformation of B lymphocytes and transforms rodent fibroblasts (2, 16, 29). LMP1 also affects epithelial cells. LMP1 transgenic mice have altered keratin expression and exhibit hyperplasia of the skin (31). In addition, nontumorigenic epithelial cell lines expressing LMP1 are inhibited in their ability to respond to terminal differentiation signals (8, 33).

Recent work with fibroblasts, B lymphocytes, and T lymphocytic cell lines has demonstrated that LMP1 activates NF-κB/Rel proteins (11, 17). Distinct NF-κB/Rel family complexes, consisting of heterodimeric and homodimeric combinations of the various NF-κB/Rel family proteins—p65 (RelA), RelB, c-Rel, p50 (NFκB1), and p52 (NFκB2)—can be activated in different cell types in response to similar stimuli (18, 20). In addition, different NF-κB family proteins have varied affects on different NF-κB-responsive promoters (10, 19). Therefore, identification of the specific complexes induced by LMP1 in epithelial cells may be an important step in determining a mechanism by which LMP1 alters cellular gene expression and growth regulation in these cells.

To determine whether LMP1 activated NF-κB in epithelial cells, epithelial cell lines stably expressing LMP1 were established by using C33 cells, a human papillomavirus-negative cervical carcinoma cell line (1). LMP1 in these cells was expressed from KLMPneo, a pGem-based vector containing a neomycin resistance gene and LMP1 coding sequences under the control of a keratin 14 promoter (14, 29). Negative control stable clones contained only the neomycin resistance gene in the same vector. The growth rates of the LMP1-expressing and -nonexpressing cells were similar under standard culture conditions; however, in the absence of fetal calf serum LMP1-expressing cells survived longer (reference 21 and data not shown).

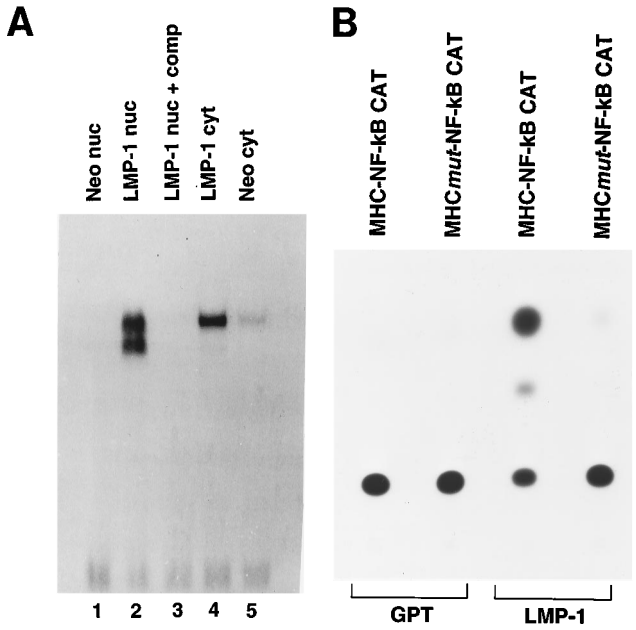

FIG. 1. (A) LMP1 activation of NF-κB/Rel in epithelial cells. EMSA were performed by using a probe containing the NF-κB site in the H-2K^b major histocompatibility complex class I gene. Portions (4 μg) of protein from cytoplasmic extracts from LMP1-expressing (LMP-1 cyt) and control (Neo cyt) cells and from nuclear extracts from LMP1-expressing (LMP-1 nuc) and control (Neo nuc) cells were used in each assay. A 10-fold excess of cold probe was added to show specific competition (LMP-1 nuc + comp). (B) Transcriptional activity of NF-κB/Rel family proteins activated by LMP1 in epithelial cells. A plasmid containing a CAT reporter gene expressed from a minimal fos promoter with three intact NF-κB sites upstream (MHC-NFκB CAT) was cotransfected into C33 cells with either an LMP1-expressing plasmid (LMP-1) or a control plasmid (GPT). The LMP1-expressing plasmid and the control were also cotransfected with a similar reporter construct containing mutant NF-κB sites (MHC*mut*-NF-κB CAT).

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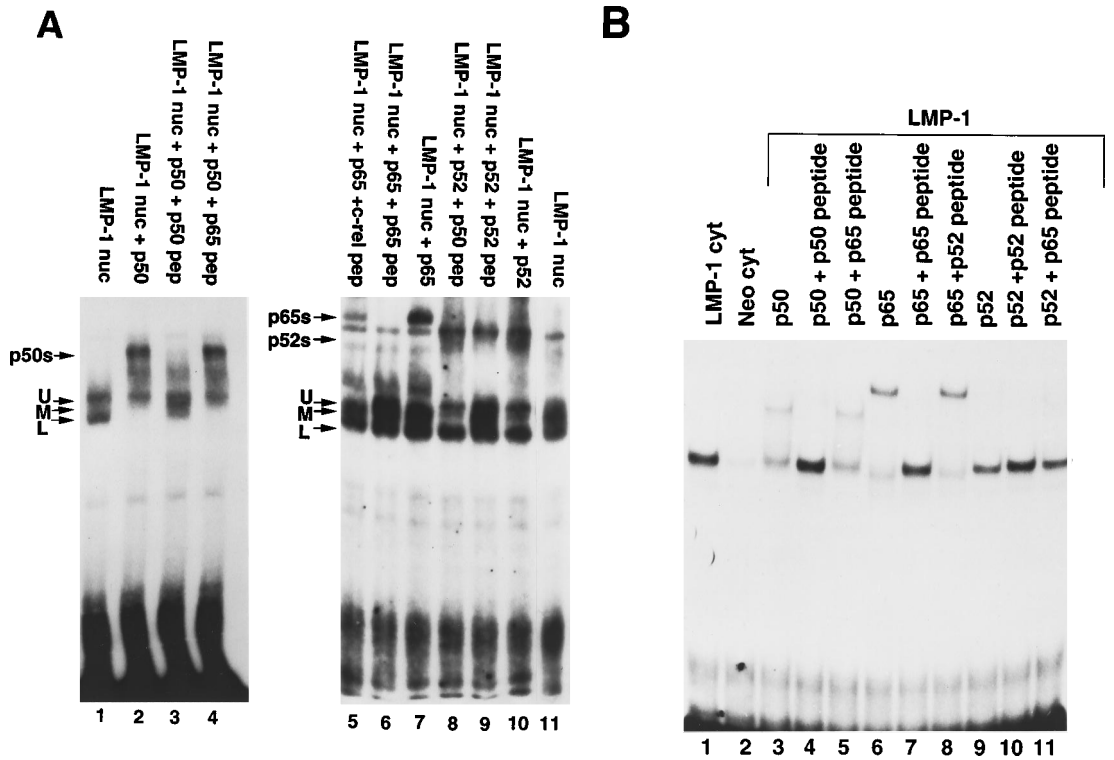

FIG. 2. Identification of the NF-κB/Rel components of LMP1-activated complexes. (A) Supershift assays were performed with nuclear extracts from LMP1-expressing cells by using antibodies to p52, p50, and p65 (Santa Cruz). Where indicated (+), extracts were preincubated for 5 min with the indicated antibody or antibody plus specific or nonspecific peptide (pep), prior to addition of the probe. U, M, and L refer to the upper, middle, and lower complexes as described in the text. p50s, p52s, and p65s refer to the shifted bands resulting from addition of p50, p52, and p65 antibody. (B) Supershift assays were performed with cytoplasmic extracts from LMP1-expressing cells preincubated with deoxycholate. Cytoplasmic extracts were preincubated with p52, p50, or p65 antibody, or antibody plus the designated specific or nonspecific peptide, 5 min prior to the addition of the labelled probe. Portions (4 μg) of protein from cytoplasmic extracts (cyt) from LMP1-expressing (LMP-1) and control cells (Neo) were used in each assay.

To detect activated NFκB/Rel proteins in extracts from LMP1-expressing and -nonexpressing clones, electrophoretic mobility shift assays (EMSA) were performed as previously described by using a labelled oligonucleotide probe containing an NF-κB site that is recognized by most NF-κB/Rel complexes (3, 26). In EMSA performed with nuclear extracts from the LMP1-expressing C33 cell lines, two shifted bands were present (Fig. 1A; compare lane 2 and lane 1). Further analysis revealed that the upper band could be resolved into two shifted bands (Fig. 2A). These shifted bands could be eliminated by the addition of excess cold probe (Fig. 1A, lane 3). Similar results were obtained with two LMP1-expressing clones derived from BALB/MK cells, a mouse keratinocyte cell line (30) (data not shown).

EMSA were also performed with cytoplasmic extracts and revealed a predominant shifted band in extracts from both LMP-expressing and non-LMP-expressing cells. This band was significantly greater in the lane showing results for the assay performed with extracts from LMP1-expressing cells (Fig. 1A, lanes 4 and 5).

These EMSA results demonstrated an increase in the amount of protein complexes available for binding to the NF-κB probe in the cytoplasm and the new appearance of complexes in the nuclei of LMP1-expressing cells.

That the protein binding demonstrated by the shifts seen in the experiments described above had a functional effect on promoters containing an NF-κB site was verified by the fact that a chloramphenicol acetyltransferase (CAT) reporter gene (MHC-NF-κB CAT) expressed from an NFκB-responsive promoter was transactivated in cells transiently expressing LMP1 (3, 29). LMP1 increased CAT expression approximately 30-fold over the levels seen in non-LMP1-expressing cells. The specificity of this transactivation was demonstrated by the observation that a reporter plasmid containing point mutations in the NF-κB sites was transactivated at only trace levels (Fig. 1B).

To demonstrate that the proteins binding to the probe were NF-κB/Rel proteins, and to identify the specific NF-κB/Rel proteins that are activated by LMP1, antibodies to p50 (NFκB-1), p52 (NFκB-2), and p65 (RelA) were preincubated with nuclear extracts prior to addition of the probe. These supershift assays demonstrated that the lower complex (L) was supershifted only by the p50 antibody (Fig. 2A, lane 2). The upper complex (U) was supershifted by both p52 and p65 antibodies (Fig. 2A, lanes 7 and 10). Although it is less clear, the middle complex (M) was diminished by both p50 and p52 antibodies (Fig. 2A, lanes 2 and 10). c-Rel and RelB antibodies were unable to shift any of the complexes present in the nuclear extracts (data not shown).

Complex L comigrates with the band known to represent the p50/p50 homodimer in extracts from HeLa cells activated with tumor necrosis factor alpha (TNF-α). Cross-linking experiments with a bromodeoxyuridine-labelled probe also indicated that this complex is a p50/p50 homodimer (4) (data not shown).

These data demonstrated that complex U contains p65 and p52 and that complex L contains p50 and suggest that complex M contains p50 and p52.

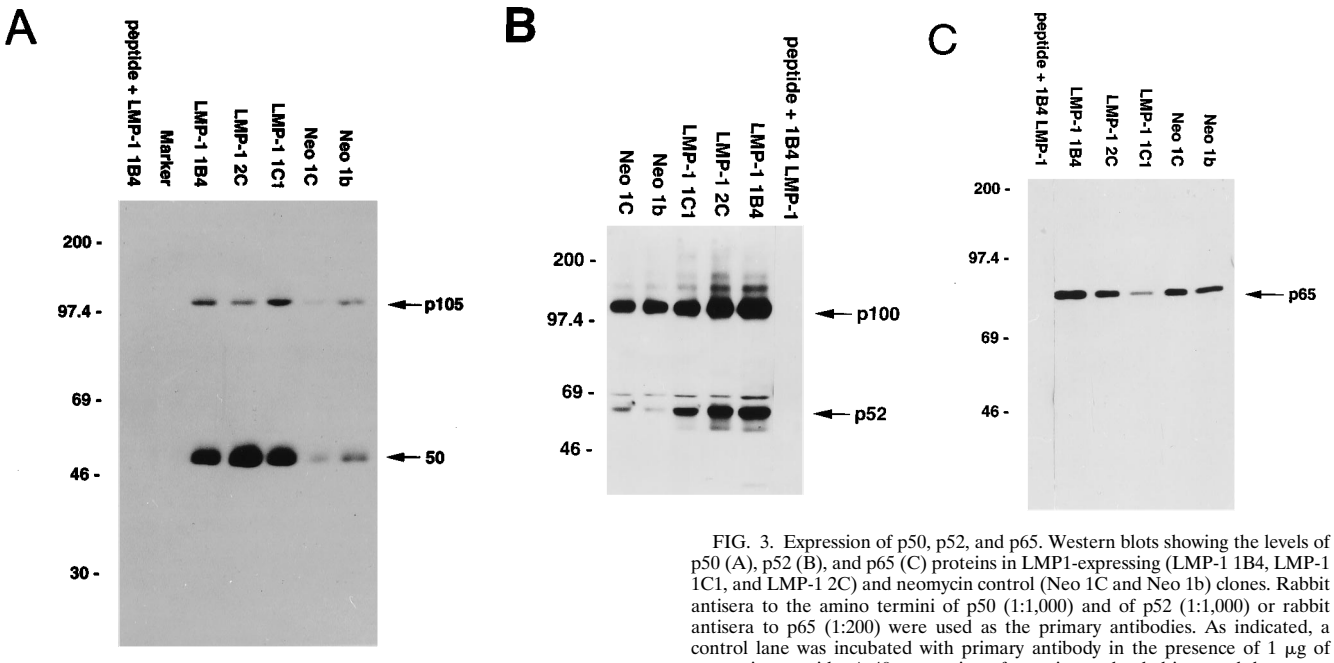

FIG. 3. Expression of p50, p52, and p65. Western blots showing the levels of p50 (A), p52 (B), and p65 (C) proteins in LMP1-expressing (LMP-1 1B4, LMP-1 1C1, and LMP-1 2C) and neomycin control (Neo 1C and Neo 1b) clones. Rabbit antisera to the amino termini of p50 (1:1,000) and of p52 (1:1,000) or rabbit antisera to p65 (1:200) were used as the primary antibodies. As indicated, a control lane was incubated with primary antibody in the presence of 1 μg of competing peptide. A 40-μg portion of protein was loaded into each lane.

To identify the NF-κB proteins present in the cytoplasm, supershifts were performed with the cytoplasmic extracts from both LMP1-expressing and non-LMP1-expressing cells. Results from supershifts done with LMP1-expressing cells were identical to those obtained with extracts from non-LMP1-expressing cells. The results from assays done with the LMP1-expressing cells are shown. The predominant complex found in the cytoplasmic extracts was supershifted by both p65 and p50 antibodies, but not by a p52 antibody (Fig. 2B, lanes 6, 3, and 9). A very faint supershift was obtained with antibodies to c-Rel and RelB (data not shown). These results indicated that the predominant cytoplasmic complex contains p65 and p50.

In summary, these experiments suggest that the nuclear NF-κB/Rel complexes activated by LMP1 include a p52/p65 dimer, a p50/p50 homodimer, and a possible p50/p52 heterodimer. The predominant complex in the cytoplasm of both LMP1-expressing and non-LMP1-expressing cells is most likely a p50/p65 dimer. These data indicate that the p50/p65 complexes are retained in the cytoplasm even as other complexes are activated by LMP1.

Western immunoblot analysis was performed to determine the effects of LMP1 on the abundance and intracellular location of the components of the specific complexes. Analysis of whole-cell extracts from LMP1-expressing and non-LMP1-expressing cells was performed by using antibodies to p50, p52, and p65. Equal amounts of protein were loaded into each lane as determined by a Bradford assay. While the levels of p105 and p100 precursor proteins were variable, levels of p50 and p52 were consistently elevated in all three LMP1-expressing clones (LMP1 1B4, LMP1 1C1, and LMP1 2C) compared with the levels found in two control clones (Neo 1C and Neo 1b) (Fig. 3A and B). The average increases in the levels of p50 and p52 in LMP1-expressing cells over several experiments were 4.4- and 5.1-fold, respectively. Median increases were 3.4- and 4.3-fold, respectively. Similar analysis of p65, RelB, and c-Rel levels in whole-cell extracts detected no consistent differences in the levels of these proteins in the LMP1-expressing clones versus the nonexpressing clones (Fig. 3C and data not shown).

Western blot analysis of the nuclear and cytoplasmic extracts demonstrated that while p50 was present in the cytoplasm of both LMP1-expressing and non-LMP1-expressing cells, it was present in the nuclei of only LMP1-expressing cells. In contrast, while p52 was present in both the nuclei and the cytoplasm of LMP1-expressing and non-LMP1-expressing cells, it was considerably more abundant in both the nuclei and the cytoplasm of LMP1-expressing cells. This suggests that LMP1 interferes with some mechanism that retains p50, but not p52, in the cytoplasm (Fig. 4). No consistent difference in the intracellular distribution of p65 in LMP1-expressing versus non-LMP1-expressing cells was found (data not shown).

Mutated or abnormally regulated NF-κB/Rel proteins have been shown to contribute to oncogenesis (15). LMP1 may regulate NF-κB/Rel proteins through cleavage of the p105 and p100 precursors. The three complexes activated by LMP1 all contain p50 or p52, whose total levels are increased by LMP1. The increase in the total amount of p50 and p52 seen here suggests that LMP1 affects the processing of the precursor proteins or increases the synthesis or stability of the processed or unprocessed forms. The degradation and resynthesis of IκB, a process which has been found to be involved in NF-κB/Rel activation, and which has recently been shown to play a role in the LMP1 activation of NF-κB/Rel in B-lymphocytic cell lines, could also contribute to NF-κB/Rel activation in these cells (5, 7, 12, 13, 27, 28).

This study reveals that a specific subset of NF-κB/Rel complexes are activated by LMP1, including p52/p65, p50/p50, and p50/p52. These forms of NF-κB/Rel activated by LMP1 are different from the p52/p65, p65/p50, and p50/p50 complexes believed to be activated in LMP1-expressing B-lymphocytic cell lines (13). In epithelial cells, through the action of these different complexes, NF-κB could specifically affect cellular gene expression in a way that is distinct from that seen in B lymphocytes. Complexes containing p52/p65 and p50/p52 have been identified as a subset of the complexes activated by TNF-α in HeLa cells, another epithelial cell line. Recent work by Mosialos et al. has demonstrated that LMP1 interacts with

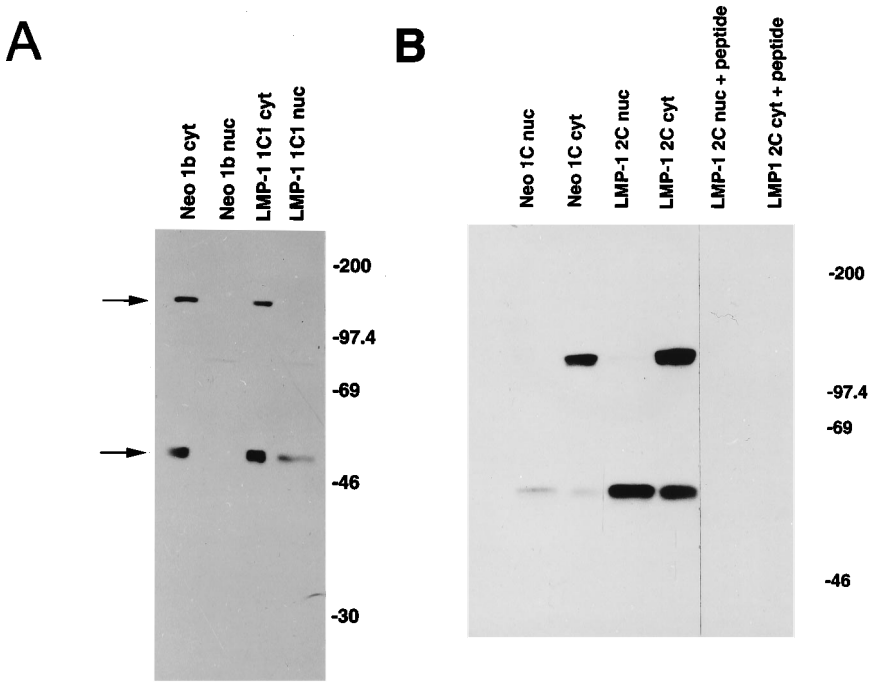

FIG. 4. Distribution of p50 and p52. Western blots were performed as described in the legend to Fig. 3 to examine the distribution of p50/p105 (A) and p52/p100 (B) in nuclear (nuc) and cytoplasmic (cyt) extracts of LMP1-expressing (LMP-1 1C1 or 2C) and control (Neo 1b or 1C) cells.

two signalling proteins which interact with members of the TNF-α receptor family (22). Through these proteins, LMP1 may activate a subset of the NF-κB complexes activated by TNF-α.

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