Induction of Epidermal Growth Factor Receptor Expression by Epstein-Barr Virus Latent Membrane Protein 1 C-Terminal-Activating Region 1 Is Mediated by NF-κB p50 Homodimer/Bcl-3 Complexes

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The Epstein-Barr virus (EBV) is associated with the development of numerous malignancies, including the epithelial malignancy nasopharyngeal carcinoma (NPC). The viral oncoprotein latent membrane protein 1 (LMP1) is expressed in almost all EBV-associated malignancies and has profound effects on gene expression. LMP1 acts as a constitutively active tumor necrosis factor receptor and activates multiple forms of the NF-κB family of transcription factors. LMP1 has two domains that both activate NF-κB. In epithelial cells, LMP1 C-terminal activating region 1 (CTAR1) uniquely activates p50/p50-, p50/p52-, and p65-containing complexes while CTAR2 activates canonical p50/p65 complexes. CTAR1 also uniquely upregulates the epidermal growth factor receptor (EGFR). In NPC, NF-κB p50/p50 homodimers and the transactivator Bcl-3 were detected on the EGFR promoter. In this study, the role of NF-κB p50 and Bcl-3 in LMP1-mediated upregulation of EGFR was analyzed. In LMP1-CTAR1-expressing cells, chromatin immunoprecipitation detected p50 and Bcl-3 on the NF-κB consensus sites within the egfr promoter. Transient overexpression of p50 and Bcl-3 increased EGFR expression, confirming the regulation of EGFR by these factors.Treatment with p105/p50 siRNA effectively reduced p105/p50 levels but unexpectedly increased Bcl-3 expression and levels of p50/Bcl-3 complexes, resulting in increased EGFR expression. These data suggest that induction of p50/p50/Bcl-3 complexes by LMP1 CTAR1 mediates LMP1-induced EGFR upregulation and that formation of the p50/p50/Bcl-3 complex is negatively regulated by the p105 precursor. The distinct forms of NF-κB that are induced by LMP1 CTAR1 likely activate distinct cellular genes.

The Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that infects more than 90% of the world’s population and is associated with the development of numerous malignancies, such as nasopharyngeal carcinoma (NPC) (12). Latent membrane protein 1 (LMP1) is expressed in most EBV-associated malignancies, is essential for EBV-induced B-lymphocyte transformation, and is the EBV oncosite (11). LMP1 induces focus formation in rodent fibroblasts, supports anchorage-independent growth of cells in soft agar, and supports tumor formation in nude mice (12). LMP1 is an integral membrane protein that acts as a constitutively active tumor necrosis factor receptor. The C-terminal domain has two signaling regions, CTAR1 and CTAR2, which constitutively associate with tumor necrosis factor receptor-associated factors (TRAFs) (24). Through its association with TRAFs, LMP1 initiates signaling events including activation of the NF-κB signaling cascade (10, 14). The transcriptional up-regulation of multiple cellular genes, such as icam-1, cd80, cd23, cd54, bcl-2, traf1, a20, and egfr, is mediated by LMP1, and many of these genes are known to be regulated by NF-κB (14, 15, 22, 28, 32). In epithelial cells, LMP1 activates at least three distinct types of NF-κB complexes (27).

NF-κB is a family of transcription factors that regulate a broad range of biological processes, including inflammation, angiogenesis, cell cycle regulation, apoptosis, and oncogenesis (7, 19). There are five mammalian NF-κB family members, p50, p52, p65 (RelA), c-Rel, and RelB. The NF-κB family members dimerize and bind NF-κB consensus sequences in cellular and viral promoters through their Rel homology domain. The p65, c-Rel, and RelB family members have transactivation domains that recruit transcriptional machinery to promoters. The activation of NF-κB family members is tightly regulated through interactions with inhibitors of NF-κB (IκB), which sequester NF-κB members in the cytosol. Extracellular stimuli, such as binding of tumor necrosis factor to its receptor, induce a kinase cascade that ultimately results in phosphorylation, ubiquitination, and degradation of an IκB, leading to the release and nuclear translocation of bound NF-κB. The mammalian IκBs include p105 (NFKB1, the p50 precursor), p100 (NFKB2, the p52 precursor), IκBa, IκBβ, IκBγ, IκBe, and Bcl-3.

In epithelial cells, CTAR1 activates at least three different dimeric forms of NF-κB, including p50/p50 homodimers, p50/p52 heterodimers, and complexes containing p65 (27). In contrast, CTAR2 induces only one complex that contains p65. The distinct forms of NF-κB induced by CTAR1 or CTAR2 are in part mediated by different signaling pathways. Both CTAR1 and CTAR2 can activate NF-κB through the canonical pathway. This pathway is activated through the trimeric IκB kinase

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The growth factor receptor (EGFR) in an I
CTAR1 also has the unique ability to upregulate the epidermal
ependently of the trimeric complex, and it then phosphorylates
IKK
/H9251
also activates NF-
B subunits. CTAR1 but not CTAR2
/H9260
5, 17, 29). Activation of the trimeric complex leads to phos-
/H9252
function to p50- or p52-containing complexes (2, 26).
Previous examination of xenograft NPC tumors detected
p50/p50 homodimers and Bcl-3 by chromatin immunoprecipita-
tion (ChIP) on the NF-κB sites within the egfr promoter. In this
study, the unique ability of CTAR1 to activate p50/p50
homodimers and upregulate the EGFR was examined. The
data indicate that the induction of p50/p50 homodimers by
CTAR1 and the increased formation of complexes containing
Bcl-3 induce EGFR expression.

MATERIALS AND METHODS
Cell culture and reagents. C33A cervical carcinoma cells were cultured in
Dulbecco’s modified Eagle medium (Gibco) supplemented with 10% fetal bo-
vine serum (Sigma) and antibiotics at 37°C with 5% CO
2. Cells were transfected
using the Fugene 6 transfection reagent (Roche) as directed by the manufac-
turer. Stable cell lines were made by transfecting the PCDNA3 vector carrier or
Myc-tagged LMP1 vectors into C33A cells. Forty-eight hours posttransfection,
cells were trypsinized, replated, and selected with 0.6 mg/ml G418-supplemented
medium. Stable cell lines were passaged in the presence of G418.
Cell extracts and Western blots. Cells were scrape harvested, washed once
with cold phosphate-buffered saline (PBS), and lysed with RIPA buffer (10 mM
Tris-HCl [pH 8.0], 140 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate
[SDS], 1% deoxycholic acid) supplemented with protease and phosphatase
inhibitor cocktails (Sigma). Equal amounts of protein were used for SDS-poly-
acrylamide gel electrophoresis (PAGE) and Western blotting. Primary antibod-
ies used for Western blots include anti-p105/p50, anti-β-actin, anti-Bcl-3, anti-c-
Myc, anti-βCatenin (Santa Cruz), anti-p65 (Rockland), and anti-p65/p50 (Upstate).
A rabbit antiserum raised against the carboxy-terminal 100 amino acids of the
EGFR fused to glutathione S-transferase (kindly provided by H. Sheltos Earl)
was used to detect EGFR. Secondary antibodies used were horseradish peroxi-
dase-conjugated antimouse and antirabbit (Amersham Pharmacia) and antib-
goat (DAKO). Blots were developed using the Pierce Supernisal West Pico chemi-
luminescence system.
Immunoprecipitations. Cells were scraped, washed with cold 1× PBS, and
lysed in IP buffer (1% Triton X-100, 0.5% of Nonidet P-40, 150 mM NaCl, 1 mM
EDTA, 1 mM EGTA) supplemented with protease inhibitor cocktail (Sigma).
 Seventy-five micrograms of cellular extracts were preincubated with GammaBind
Plus Sepharose (Amersham Pharmacia) for 2 h at 4°C. Precleared lysates were
immunoprecipitated with 1 µg anti-p105/p50, anti-Bcl-3, anti-p100/p52, or iso-
type control antibody overnight. Immunoprecipitations were incubated with
GammaBind Plus Sepharose for 2 h at 4°C, washed two times, resuspended in
SDS-PAGE sample buffer, boiled, and used for SDS-PAGE and Western blots.
ChIP. ChIPs were performed as previously described (30). Briefly, 1 × 10
7 cells were scraped, resuspended in 50 mM DMEM, and cross-linked in 1% form-
alddehyde for 15 min at room temperature, followed by quenching with 120 mM
glycine. The cell pellet was washed with 1× PBS and lysed in RIPA buffer (10
mM Tris-HCl [pH 8.0], 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxy-
cholic acid) spiked with protease inhibitor cocktail (Sigma) for 30 min at 4°C.
Lysates were sonicated, clarified, and precleared with GammaBind Plus Sepha-
rose (Amersham Pharmacia). The supernatant was incubated without antibody,
with anti-p105/p50, or with anti-Bcl-3 (Santa Cruz) and mutated overnight at 4°C.
Lysates were immunoprecipitated with GammaBind Plus Sepharose and washed
four times, 5 min each, and DNA/protein was eluted from the beads with 1% SDS,
1× Tris-EDTA at 65°C. The cross-linking was reversed overnight at 65°C and samples treated with protease K at 37°C for 2 h. Sample DNAs were
purified with a QIAGEN PCR purification kit (QIAGEN) as directed by the
manufacturer. The NF-κB sites in the egfr promoter were amplified by PCR with
the primer set 5′ GGAGCCCGGAATTAAGGACGAGGT 3′ and 5′ CTGA
GGAGTTAATTTCCGAGAGGGG 3′ using Platinum Pfur polymerase (Invitro-
gene).
Nuclear extracts and EMSA. Nuclear extracts were used for electrophoretic
mobility shift assays (EMSA). Nuclear extracts were made as previously de-
scribed (30). Briefly, cells were scraped, washed once with cold PBS, and lysed
in a hypotonic buffer (20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, protease, and phosphatase inhibitor cock-
tails [Sigma]) for 15 min on ice, followed by addition of Nonident P-40 to a final
concentration of 1%. Nuclei were pelleted by low-speed centrifugation at 1,200
rpm for 10 min at 4°C. The nuclei were purified using the Optiprep reagent
[Sigma] as directed by the manufacturer, as previously described (30). Nuclear
were lysed with nuclear extraction buffer (20 mM Tris [pH 8.0], 420 mM NaCl,
1.5 mM MgCl
2, 0.2 mM EDTA, 25% glycerol, protease inhibitor cocktail
[Sigma], and phosphatase inhibitor cocktail [Sigma]) with the salt concentration
adjusted to 400 mM with 3 M NaCl. Insoluble nuclear material was pelleted at
high speed for 10 min. EMSAs were performed as previously described (30).
An oligonucleotide (UV21) of the NF-
B consensus sites in the
egfr promoter was synthesized (CA
GGGCTGGGAATTTCCGAGAGGGG 3′). Nuclear extracts were incubated with
radiolabeled probe. For supershift assays, 1 µg antibody was incubated with extracts. Antibodies used were anti-
p105/p50, anti-Rel-B, anti-cRel (Santa Cruz), anti-p65 (Rockland), and anti-
p100/p52 (Upstate).
Quantitative reverse transcription-PCR. Total RNA was isolated from cells, using
an RNaseasy minikit as directed by the manufacturer (QIAGEN). Total RNA
was quantified by spectrophotometric measurements. Primer pairs were
designed using the Primer Express program (Applied Biosystems). The activin
primer set was 5′ TCACCCACACTGTGCCCAATCTCGA 3′ and 5′ CAGCC
GAACCGCTCATTGCCAATGG 3′. The EGFR primer set was 5′ TGCCG
CTCTTGGCGGAT 3′ and 5′ GCCGGCCACCTGGAGAGG 3′. Quantitative
reverse transcription-PCR was performed on DNase-treated nuclear RNA using
the Quantitect SYBR Green RT-PCR kit (QIAGEN) according to the manufactur-
er’s directions. Amplification of target sequences was detected using an ABI
7900HT sequence detection system (Applied Biosystems) and analyzed using
SDS 2.0 software (Applied Biosystems). The cycle threshold (C
T) was deter-
mined as the number of PCR cycles required for a given reaction to reach an
arbitrary fluorescence value within the linear amplification range. The change in
C
T (ΔC
T) was determined between the same gene primer sets and different
samples, and the change in C
T (ΔΔC
T) was determined by adjusting for the
difference in the number of cycles required for action to reach the C
T. Since each
PCR cycle results in a twofold amplification of each product, the n-fold differ-
ence was determined as 2–ΔΔC
T. Each sample was analyzed in triplicate, and the
standard error was determined.
siRNA. Chemically synthesized small interfering RNA (siRNA) pools were
purchased from Dharmacon, targeting an irrelevant RNA, NF-κB1, and Bcl-3.
 Either 100 or 50 pmol siRNA was transfected into stable cells using a Lipo-
fectamine 2000 transfection reagent (Invitrogen) as directed by the manufac-
turer. Cells were harvested approximately 36 h posttransfection and were used
for Western blot analysis, quantitative reverse transcription-PCR, or immuno-
precipitations.

RESULTS
NF-κB p50 binds Bcl-3 and imaging was precipitated with the
EGFR promoter. Previous studies revealed that LMP1 upregu-
lates the EGFR at the mRNA level (22). In EBV-positive NPC
neuroblastomas, NF-κB p50 homodimers and Bcl-3 could be induc-
emuno-
precipitated with NF-κB consensus sites in the egfr promoter
(30). In order to determine if LMP1 can induce the binding of
p50 and Bcl-3 to the egfr promoter, C33A cells stably express-

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ing the pCDNA3 vector control, full-length LMP1, LMP1(1-231) (LMP1 that retains CTAR1 but not CTAR2; hereafter referred to as 1-231), and LMP1(Δ187-351) (LMP1 that retains CTAR2 but not CTAR1; hereafter referred to as Δ187-351) were examined by ChIP using primers that flank the NF-κB sites in the egfr promoter. In C33A stable cells, LMP1 expression and EGFR upregulation were confirmed by immunoblotting (Fig. 1A). As described previously, LMP1 activated EGFR through CTAR1. The deletion mutant that retains CTAR1, 1-231, strongly increased EGFR, while the deletion mutant that retains CTAR2, Δ187-351, did not upregulate EGFR (Fig. 1A). By ChIP, precipitation with anti-p105/p50 weakly detected the NF-κB sites in the egfr promoter in cells expressing LMP1 and 1-231 (Fig. 1B, lanes 9 and 10) but not in pCDNA3 cells or Δ187-351 cells (Fig. 1B, lanes 8 and 11). Bcl-3 was detected on the egfr promoter in 1-231-expressing cells (Fig. 1B, lane 14) and weakly detected on the egfr promoter in LMP1-expressing cells (Fig. 1B, lane 13). Bcl-3 was not detected on the egfr promoter in pCDNA3- or Δ187-351-expressing cells (Fig. 1B, lanes 12 and 15). This ChIP correlated with EGFR expression. In LMP1- and 1-231-expressing cells, EGFR was upregulated and p50 and Bcl-3 were detected on the egfr promoter. A very low level of EGFR was detected in vector control cells and Δ187-351-expressing cells, and Bel-3 and p50 were not detected on the egfr promoter. The other NF-κB family members activated by LMP1, p65, p52, and RelB were not detected on the egfr promoter (Fig. 1B, lanes 23 to 34). The absence of p52 on the egfr promoter indicates that the unique activation of the noncanonical pathway by CTAR1 does not activate EGFR expression and that EGFR expression is specifically correlated with the presence of p50 and Bel-3 on the egfr promoter.

**Transient expression of p50 and Bel-3 increase EGFR.** In order to determine if expression of p50 and/or Bel-3 is sufficient to mediate an increase in EGFR protein levels, C33A cells were transiently transfected with vector control, p50, Bel-3, or p50 and Bel-3. Expression of each was confirmed by immunoblotting, and equal loading was confirmed with a β-actin immunoblot (Fig. 2). EGFR expression levels were also detected by immunoblotting. Transient expression of p50, Bel-3, or both detectably increased EGFR compared to results with the vector control (Fig. 2). The intensities of the EGFR bands shown in Fig. 2 were quantified using the Image J 1.32j computer program and were listed immediately above the corresponding bands. This experiment has been repeated five times; however, the values were calculated from the representative Western blot shown in Fig. 2. Cells expressing p50 alone had an approximately twofold increase in the EGFR protein. Cells expressing Bel-3 alone had an approximately threefold increase in the EGFR protein. Cells expressing both p50 and Bel-3 had an approximately fivefold increase in the EGFR protein.

**FIG. 2. Transient expression of p50 and Bel-3 increases EGFR protein.** C33A cells were transiently transfected with p50 and Bel-3, and levels of EGFR were examined by immunoblotting. Equal loading was confirmed with anti-β-actin. EGFR levels were measured by densitometry of the immunoblot in the top panel. The Image J 1.32j program was used to calculate the intensity of each band from one Western blot. Pixel densities are listed above their corresponding bands. The blot is representative of five independent experiments.
Increased EGFR expression requires canonical NF-κB activation. Several studies have determined that CTAR2 activates NF-κB via the canonical pathway that is dependent upon the trimeric IKK complex composed of IKKα, IKKβ, and IKKγ (NEMO). CTAR1 activates the NF-κB canonical pathway and the noncanonical pathway that induces processing of p100 to p52. This processing is dependent on NIK and IKK. These data indicate that both p50 and Bcl-3 contribute to upregulation of EGFR.

DN IKKβ decreases nuclear p50 and EGFR protein. To further characterize the forms of NF-κB that contribute to LMP1-mediated EGFR up-regulation, cells stably expressing the pCDNA3 vector control, LMP1, 1-231, and Δ187-351 were transiently transfected with pCDNA3 vector control, wild-type IKKβ (wt IKKβ), or double-point-mutant (SS 177, 181 AA) DN IKKβ (DN IKKβ) were tested for nuclear NF-κB complexes by EMSA. Levels of EGFR, LMP1, and IKKβ were examined by immunoblotting. The LMP1 expression panel is a composite from two different gels. Equal loading was confirmed by using a loading control (bottom panel). Lane 1 is a probe-alone control for the EMSA. Lanes 2 to 4 are pCDNA3 stable cells transiently transfected with pCDNA3, wt IKKβ, or DN IKKβ; lanes 5 to 7 are LMP1 stable cells transfected with pCDNA3, wt IKKβ, or DN IKKβ; lanes 8 to 10 are 1-231 stable cells transfected with pCDNA3, wt IKKβ, or DN IKKβ, and lanes 11 to 13 are Δ187-351 stable cells transfected with pCDNA3, wt IKKβ, or DN IKKβ. Arrows identify the complexes. The EMSA and Western blots are each representative of four independent experiments.

FIG. 3. Increased EGFR expression requires NF-κB activation. Stable cell lines expressing the pCDNA vector control or 1-231 were transiently transfected with the vector control, DN NIK, IKKα, or IKKβ. RNA was harvested and used for quantitative reverse transcription-PCR using primer sets for β-actin and EGFR. The graph shows the n-fold change from the level for pCDNA3 stable cells transfected with the vector control and represents the average from separate experiments. All samples are normalized to vector control results, and EGFR levels were additionally normalized to the sample’s corresponding β-actin level. The bars represent the n-fold change in EGFR levels from levels for pCDNA3 cells transfected with vector control. Each sample was performed in triplicate, and the standard error is indicated. The graph shown is representative of three independent experiments.

FIG. 4. DN IKKβ decreases nuclear p50 homodimers and EGFR protein. C33A cells transiently transfected with pCDNA3 vector control, wild-type IKKβ (WT IKKβ), or double-point-mutant (SS 177, 181 AA) DN IKKβ (DN IKKβ) were tested for nuclear NF-κB complexes by EMSA. Levels of EGFR, LMP1, and IKKβ were examined by immunoblotting. The LMP1 expression panel is a composite from two different gels. Equal loading was confirmed by using a loading control (bottom panel). Lane 1 is a probe-alone control for the EMSA. Lanes 2 to 4 are pCDNA3 stable cells transiently transfected with pCDNA3, wt IKKβ, or DN IKKβ; lanes 5 to 7 are LMP1 stable cells transfected with pCDNA3, wt IKKβ, or DN IKKβ; lanes 8 to 10 are 1-231 stable cells transfected with pCDNA3, wt IKKβ, or DN IKKβ, and lanes 11 to 13 are Δ187-351 stable cells transfected with pCDNA3, wt IKKβ, or DN IKKβ. Arrows identify the complexes. The EMSA and Western blots are each representative of four independent experiments.
heterodimers and p50 homodimers were detected (Fig. 4, upper panel, lanes 2 and 11, upper panel). Expression of wt IKKβ in Δ187-351 cells greatly increased the amount of the p65-containing complex, indicating activation of the canonical pathway. In addition, the p50/p50 homodimer was increased, but this did not affect the trace levels of EGFR (Fig. 4, lanes 11 and 12, top two panels). Expression of the DN IKKβ in Δ187-351 cells eliminated the p52/65 form but surprisingly increased the p50/50 homodimer. This increase did not increase the level of EGFR. The effects of expression of the wt IKKβ and DN IKKβ on the complexes detected by EMSA in CTAR1 suggest that the IKKβ subunit contributes to p50/p50 homodimer activation and LMP1-mediated EGFR up-regulation. The absence of EGFR up-regulation in the Δ187-351 cells by either the wt IKKβ or DN IKKβ, both of which increased the levels of p50 homodimers, suggests that p50 homodimers are not sufficient to activate EGFR expression and that an additional activity is required that is not induced by Δ187-351.

NF-κB1 precursor negatively regulates p50/50 complexes and EGFR expression. In order to determine if p50 and Bcl-3 are necessary for CTAR1-mediated EGFR upregulation, siRNAs targeting NF-κB1 and Bcl-3 were utilized to decrease p50 and Bcl-3 expression. Cells were transfected with siRNA directed against an irrelevant RNA, the p50 precursor, NF-κB1 (p105), Bcl-3, or both, and the effect on the cellular proteins was determined by immunoblotting (Fig. 5). Transfection with NF-κB1 siRNA effectively decreased expression of the p50 precursor protein, p105, and p50 (Fig. 5A, lane 2), and the decrease was dose dependent (Fig. 5A, lanes 2 and 4). In contrast, transfection with Bcl-3 siRNA did not affect the levels of cellular Bcl-3 (Fig. 5A, lanes 3 and 4). Interestingly, transfection with NF-κB1 siRNA significantly increased the amount of Bcl-3, suggesting that p105 or p50 may negatively regulate Bcl-3. Transfection with NF-κB1 siRNA or Bcl-3 siRNA did not alter the other NF-κB family member, p65 (Fig. 5A).

The regulation of the processing of p105 to p50 is distinct from that of the processing of p100 to p52. In the pcDNA3 control cells, processed p50 was constitutively abundant while processed p52 was detected at very low levels (Fig. 5B). LMP1 CTAR1 induces processing of p100 to p52 (1, 5, 17, 29). This effect was apparent with greatly increased p52 in the 1-231-expressing cells (Fig. 5B). In contrast, the relative amount of p50/p105 over the pcDNA3 control was slightly increased by LMP1 CTAR1.

To determine the effect of NF-κB1 siRNA treatment on EGFR upregulation, the siRNAs were transfected into vector control cells or cells expressing the LMP1 mutant, 1-231, which has the strongest activation of EGFR. NF-κB1 p105 and p50 were effectively decreased by siRNA transfection in both pcDNA3- and 1-231-expressing cells (Fig. 5B). The NF-κB1 siRNA or Bcl-3 siRNA had a very slight effect on the total amount of p100 and p52 and did not affect the LMP1-induced processing of p100 (Fig. 5B). Surprisingly, siRNA-mediated decrease of NF-κB1 either alone or in combination with Bcl-3 increased EGFR expression compared to the irrelevant siRNA control in 1-231-expressing cells (Fig. 5B, lanes 5, 6, and 8). The effects of EGFR expression were quantified using the Image J 1.32j computer program, and pixel intensities were listed immediately above the corresponding bands. This experiment was repeated three times; however, the values were calculated from one representative Western blot. The increased EGFR protein was not due to a decrease in the NF-κB-regulated inhibitory protein IkBα, as levels of IkBα were not affected by the siRNA-mediated decrease of NF-κB1 or the increased Bcl-3 (Fig. 5B, bottom panel). However, the increase in EGFR correlated with the increased Bcl-3 in the NF-κB siRNA-transfected 1-231 cells (Fig. 5A). It is known that the precursors for p52 and p50 contain ankyrin repeats and can function as repressors. The increase in EGFR induced by the siRNA-mediated decrease in p105 likely reflects the...
inhibitory properties of p105 for the availability of the p50 homodimer complex.

To determine if the increase in EGFR protein levels after siRNA treatment of NF-κB1 and Bcl-3 occurred at the RNA level, quantitative reverse transcription-PCR was performed on vector control cells and cells expressing 1-231 transiently transfected with siRNA of irrelevant RNA, NF-κB1, and Bcl-3. PCR was performed with actin-specific primers for RNA integrity control and EGFR-specific primers. Each sample was normalized to RNA from cells expressing 1-231 transfected with irrelevant siRNA. The graph in Fig. 6 shows the n-fold change over the level for 1-231 with irrelevant siRNA and represents results from four independent experiments. The actin levels were unchanged and therefore confirm the integrity of the RNA (data not shown). Cells expressing 1-231 and transfected with NF-κB1 siRNA had an approximately threefold increase of EGFR RNA over that for 1-231-expressing cells. Bcl-3 siRNA expression alone or in combination with NF-κB1 siRNA did not significantly change EGFR RNA (Fig. 6). These results confirmed the immunoblot analysis and suggested that siRNA degradation of cellular NF-κB1 increased the increase in Bcl-3.

Increased Bcl-3 coimmunoprecipitates with NF-κB1 after transfection with NF-κB1 siRNA. To determine the effect of an siRNA-mediated decrease of NF-κB1 and Bcl-3 on formation of the p50/Bcl-3 complex, coimmunoprecipitation of p105/p50, p100/p52, and Bcl-3 was analyzed. P105 and p50 did not immunoprecipitate with anti-p100/p52 (Fig. 7A, lanes 8 to 12). A considerable level of NF-κB p50 immunoprecipitated with anti-Bcl-3 in vector control pCDNA3 cells (Fig. 7A, lane 13), and increased p50 immunoprecipitated with anti-Bcl-3 in 1-231 cells treated with irrelevant siRNA (Fig. 7A, lane 14). Although the amount of p50 was greatly reduced by transfection with NF-κB siRNA (Fig. 5A), the amount of p50 that coimmunoprecipitated with Bcl-3 was only slightly affected (Fig. 7A, lanes 15 and 17). The siRNA-mediated decrease of p105/p50 and the consequent increase in Bcl-3 resulted in more Bcl-3.
DISCUSSION

The data presented in this study indicate that the LMP1 CTAR1 domain induces the binding of NF-κB p50 and Bcl-3 to the NF-κB sites in the egfr promoter in C33A cells and this correlated with EGFR upregulation (Fig. 1). It has previously been determined that egfr has five NF-κB consensus binding sites in its promoter, and p50 specifically binds to four of those sites (25). Furthermore, it was determined that in xenografted NPCs, both p50 and Bcl-3 coimmunoprecipitate with three of those sites in the egfr promoter (30). The data in this paper support the in vivo model in which p50 and Bcl-3 are present on the egfr promoter when CTAR1 is present. Of note, anti-p100/p52, anti-p65, and anti-RelB did not immunoprecipitate the NF-κB sites in the egfr promoter, indicating that in C33A cells, these family members do not promote EGFR upregulation. The absence of p52 or RelB confirms that the upregulation of EGFR by CTAR1 was not mediated by the effects of CTAR1 on the noncanonical NF-κB pathway.

Expression of p50 and Bcl-3 in the absence of LMP1 also increased EGFR expression (Fig. 2). NF-κB p50/p52 and p52/p52 homodimers have been thought of as being transcriptionally inhibitory because neither p50 nor p52 has transactivation domains. NF-κB p50 can bind the transcriptionally inhibitory histone deacetylase 1 (34). However, if bound to Bcl-3, p50/p50 and p52/p52 homodimers may be transcriptionally active (2, 33). It has been shown that p52/Bcl-3 complexes transcriptionally upregulated cyclin D1 and promoted cell cycle progression more efficiently than other NF-κB family members (33). NF-κB p50 may also directly activate cellular promoters, and the antiapoptotic protein Bcl-2, which is also upregulated by NF-κB p50 may also directly activate cellular promoters, and the antiapoptotic protein Bcl-2, which is also upregulated by NF-κB p50 can bind the transcriptionally inhibitory histone deacetylase 1, and both p50 and Bcl-3 were detected by ChIP on the EGFR promoter (30).

NF-κB activity is controlled by a kinase cascade that begins with an extracellular signal that leads to activation of the IKK complex and phosphorylation of IκB. The IKK complex is a trimeric complex consisting of two catalytic domains, IκKα and IκKβ, and a regulatory domain, IκKγ (NEMO). IκKβ is the dominant kinase in phosphorylation of IκB (7). NIK can also phosphorylate and activate IκKα, which can phosphorylate the p52 precursor, p100, in an IκKγ-dependent manner to produce p52. LMP1 CTAR1 activates NF-κB through both IκKγ-dependent and noncanonical IκKγ-independent pathways, mediates NF-κB release from IκB, and induces phosphorylation of p100 followed by processing of p100 to p52 (1, 5, 17, 29). In contrast, CTAR2 induces canonical NF-κB activation through the IκKγ-dependent IKK complex.

The role of NF-κB was confirmed in this study, since transfection of DN IκKα and IκKβ decreased EGFR in cells expressing 1-231 (Fig. 3 and 4). These findings confirm the previous inhibition of LMP1-mediated EGFR upregulation by a DN IκBα (20). In 1-231 cells, transient transfection of DN IκKβ decreased EGFR RNA threefold, while transient transfection of DN NIK did not change EGFR and DN IκKα decreased EGFR RNA by approximately one-third. The noncanonical processing and activation of p52 by CTAR1 is dependent upon NIK and IκKα; therefore, the minimal effects of DN NIK and DN IκKα and the more-pronounced inhibition by DN IκKβ further indicate that the CTAR1 upregulation of EGFR is not mediated by p52 and the noncanonical pathway. The data presented here indicate that the unique CTAR1 upregulation of EGFR is linked in part to its induction of p50 homodimers and that IκKβ is a significant factor in the regulation of EGFR by CTAR1.

However, the increased levels of p50 homodimers induced by wt IκKβ and DN IκKβ in cells expressing CTAR2-containing Δ187-351 LMP1 were not sufficient to induce EGFR expression. This suggests that other signaling pathways activated by LMP1 CTAR1 contribute to EGFR upregulation in conjunction with NF-κB. A recent publication indicated that GSK3 phosphorylates Bcl-3 and mediates its degradation (31). LMP1 activates Akt through CTAR1, and phosphorylated, inactive GSK3β is elevated in EBV-infected cells (4, 18). The activation of Akt and inactivation of GSK3β by LMP1 may affect the activity of Bcl-3.

The siRNA-mediated decrease in p105/p50 surprisingly increased levels of Bcl-3 and p50/Bcl-3 complexes and correlated with increased levels of EGFR (Fig. 6 and 7). Although the siRNA-mediated decrease in p50 could affect the amount of IκB proteins, a change in IκBα was not detected (Fig. 5B). NF-κB p105 is the p50 precursor and is also considered an IκB since it can act as an inhibitory molecule by binding and sequestering p50 in the cytoplasm (6, 8, 9, 16). The decrease in the inhibitory p105 likely increases the availability of the remaining processed p50, which may complex with Bcl-3 more efficiently in the absence of p105 (Fig. 7). Bcl-3 can also interact with p52 to form a transcriptionally active complex; however, p52 was not detected on the egfr promoter in C33A cells (Fig. 1) and p52 was not detected in a complex with p50 or Bcl-3 by coimmunoprecipitation in C33A cells (Fig. 7). These findings support the previous detection of only p50 and Bcl-3 on the egfr promoter in xenografted NPC and indicate that p50/Bcl-3 more likely mediates EGFR upregulation (5, 30). The data presented in this article suggest a new role for NF-κB p50/Bcl-3 complexes as transcriptional activators and indicate that this complex likely functions in the transcriptional regulation of EGFR.

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