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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 oncogene (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 (NFκB1, the p50 precursor), p100 (NFκB2, the p52 precursor), IκBα, IκBβ, IκBγ, IκBε, 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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(IKK) complex, which is composed of catalytic IKKα and IKKβ subunits and the regulatory IKKγ (NEMO) subunit (1, 5, 17, 29). Activation of the trimeric complex leads to phosphorylation and proteasome-dependent degradation of IκBα and release of bound NF-κB subunits. CTAR1 but not CTAR2 also activates NF-κB through a noncanonical pathway where IKKα is activated by the NF-κB-inducing kinase (NIK) independently of the trimeric complex, and it then phosphorylates p100 and mediates processing of p100 to p52 (1, 5, 17, 29). CTAR1 also has the unique ability to upregulate the epidermal growth factor receptor (EGFR) in an IκBα-dependent manner through its interactions with TRAFs (20–23). Since only LMP1 CTAR1 activates p50/p50 homodimers and p50/p52 heterodimers, it is possible that EGFR upregulation is mediated through activation of one of these forms. Neither p50 or p52 has transactivation domains; however, both can bind the oncoprotein Bcl-3 (2, 26). Bcl-3 is an unusual member of the IκB family in that it is most commonly expressed in the nucleus and it has a transactivation domain that can provide transactivating function to p50- or p52-containing complexes (2, 26).

Previous examination of xenografted NPC tumors detected p50/p50 homodimers and Bcl-3 by chromatin immunoprecipitation (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 bovine 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 manufacturer. Stable cell lines were made by transfecting the pCDNA3 vector control 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-polyacrylamide gel electrophoresis (PAGE) and Western blotting. Primary antibodies used for Western blots include anti-p105/p50, anti-β-actin, anti-Bcl-3, anti-c-Myc, anti-IKKβ (Santa Cruz), anti-p65 (Rockland), and anti-p100/p52 (Upstate). A rabbit antiserum raised against the carboxy-terminal 100 amino acids of the EGFR fused to glutathione *S*-transferase (kindly provided by H. Shelton Earp) was used to detect EGFR. Secondary antibodies used were horseradish peroxidase-conjugated antimouse and antirabbit (Amersham Pharmacia) and antigoat (DAKO). Blots were developed using the Pierce Supersignal West Pico chemiluminescence 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 precleared 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 isotype 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 ml DMEM, and cross-linked in 1% formaldehyde 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% deoxycholic acid) spiked with protease inhibitor cocktail (Sigma) for 30 min at 4°C. Lysates were sonicated, clarified, and precleared with GammaBind Plus Sepharose (Amersham Pharmacia). The supernatant was incubated without antibody, with anti-p105/p50, or with anti-Bcl-3 (Santa Cruz) and nutated 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 proteinase 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′ GGGGACCCGAATAAAGGAGCAGTTT 3′ and 5′ CTGA GGAGTTAATTTCCGAGAGGGG 3′ using Platinum *Pfx* polymerase (Invitrogen).

Nuclear extracts and EMSA. Nuclear extracts were used for electrophoretic mobility shift assays (EMSAs). Nuclear extracts were made as previously described (30). Briefly, cells were scraped, washed once with cold PBS, and lysed by incubation 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 cocktails [Sigma]) for 15 min on ice, followed by addition of Nonidet 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). Nuclei 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 5 M NaCl. Insoluble nuclear material was pelleted at high speed for 10 min. EMSAs were performed as previously described (3). An oligonucleotide (UV21) of the NF-κB site from the H-$2K^b$ gene (CA GGGCTGGGGATTCCCCATCTCCCACAGTTTCACTTC) was labeled with [α-^{32}P]dCTP using the Klenow fragment from DNA polymerase I. Five micrograms 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 RNeasy minikit as directed by the manufacturer (QIAGEN). Total RNA was quantified by spectrophometric measurements. Primer pairs were designed using the Primer Express program (Applied Biosystems). The actin primer set was 5′ TCACCCACACTGTGCCCATCTACGA 3′ and 5′ CAGCG GAACCGCTCATTGCCAATGG 3′. The EGFR primer set was 5′ TGCGTC TCTTGCCGGAAT 3′ and 5′ GGCTCACCCTCCAGAAGC 3′. Quantitative reverse transcription-PCR was performed on DNase-treated RNA using the Quantitect SYBR Green RT-PCR kit (QIAGEN) according to the manufacturer'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 determined 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 ΔC_T ($\Delta\Delta C_T$) was determined by adjusting for the difference in the number of cycles required for actin to reach the C_T. Since each PCR cycle results in a twofold amplification of each product, the *n*-fold difference was determined as $2^{\Delta\Delta CT}$. 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 Lipofectamine 2000 transfection reagent (Invitrogen) as directed by the manufacturer. Cells were harvested approximately 36 h posttransfection and were used for Western blot analysis, quantitative reverse transcription-PCR, or immunoprecipitations.

RESULTS

NF-κB p50 binds Bcl-3 and immunoprecipitates with the EGFR promoter. Previous studies revealed that LMP1 upregulates the EGFR at the mRNA level (22). In EBV-positive NPC tumors, NF-κB p50 homodimers and Bcl-3 could be immunoprecipitated 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-

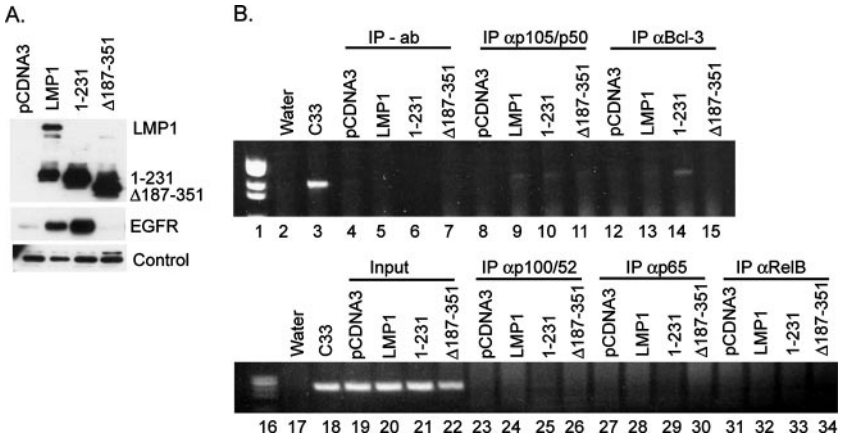

FIG. 1. NF-κB p50 and Bcl-3 immunoprecipitate on the EGFR promoter. (A) EGFR and LMP1 expression was examined by immunoblotting with C33A cells stably expressing Myc-tagged LMP1 or deletion mutant 1-231 or Δ187-351 with anti-c-*myc* and anti-EGFR. The bottom panel is a loading control. (B) A chromatin immunoprecipitation of p50, Bcl-3, p100/p52, p65, and RelB on the *egfr* promoter was performed with C33A cells stably expressing LMP1 or deletion mutant 1-231 or Δ187-351. Lanes 1 and 16 are DNA markers, lanes 2 and 17 are water PCR controls, lanes 3 and 18 are genomic DNA from C33A cells, lanes 4 to 7 are immunoprecipitated in the absence of antibody, lanes 8 to 11 are immunoprecipitated with anti-p105/p50 (α-p105/p50), lanes 12 to 15 are immunoprecipitated with anti-Bcl-3 (α-Bcl-3), lanes 19 to 22 are input DNA from all four stable cell lines, lanes 23 to 26 are immunoprecipitated with anti-p100/p52 (α-p100/p52), lanes 27 to 30 are immunoprecipitated with anti-p65 (α-p65), and lanes 31 to 34 are immunoprecipitated with anti-RelB (α-RelB).

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 Bcl-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 Bcl-3 on the *egfr* promoter.

Transient expression of p50 and Bcl-3 increase EGFR. In order to determine if expression of p50 and/or Bcl-3 is sufficient to mediate an increase in EGFR protein levels, C33A cells were transiently transfected with vector control, p50, Bcl-3, or p50 and Bcl-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, Bcl-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 Bcl-3 alone had an approximately threefold increase in the EGFR protein. Cells expressing both p50 and Bcl-3 had an approximately fivefold increase in the EGFR

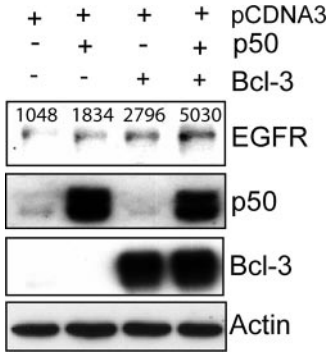

FIG. 2. Transient expression of p50 and Bcl-3 increases EGFR protein. C33A cells were transiently transfected with p50 and Bcl-3, and levels of EGFR were examined by immunoblotting. Equal loading was confirmed with anti-β-actin. EGFR levels were measured by densitometry of the immunoblot in the top panel. 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.

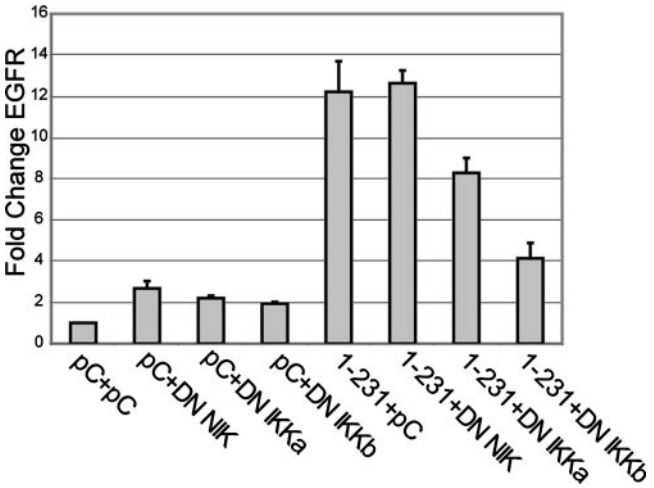

FIG. 3. Increased EGFR expression requires NF-κB activation. Stable cell lines expressing the pCDNA vector control or 1-231 were transiently transfected with the vector control, DN NIK, IKKα, or IKKβ. RNA was harvested and used for quantitative reverse transcription-PCR using primer sets for β-actin 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.

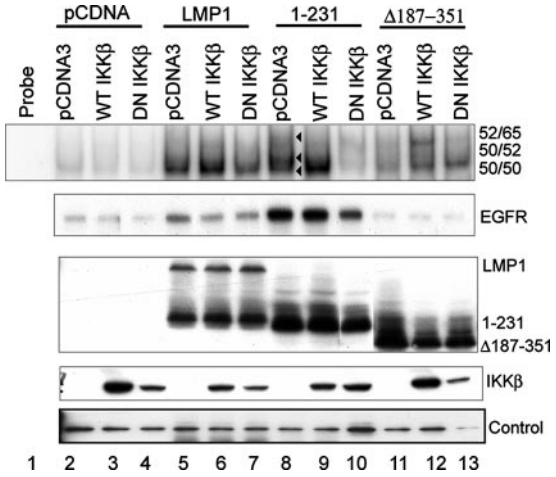

FIG. 4. DN IKKβ decreases nuclear p50 homodimers and EGFR protein. C33A cells transiently transfected with pCDNA3 vector control, wild type IKKβ (WT IKKβ), or double-point-mutant (SS 177, 181 AA) DN IKKβ (DN IKKβ) were tested for nuclear NF-κB complexes by EMSA. Levels of EGFR, LMP1, and IKKβ were examined 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.

protein. These data indicate that both p50 and Bcl-3 contribute to upregulation of EGFR.

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 IKKα but is independent of the trimeric complex (1, 5, 29). A third pathway has been studied, using knockout murine embryo fibroblasts, that was IKKβ dependent and considered atypical canonical (17). To identify the pathways that contribute to CTAR1-induced EGFR expression, dominant-negative forms (DN) of NIK, IKKα, and IKKβ were transiently expressed in cells stably expressing 1-231. EGFR expression was analyzed by quantitative reverse transcription-PCR. Expression of DN NIK, IKKα, and IKKβ did not significantly affect the trace levels of EGFR in vector control cells (Fig. 3). In cells expressing CTAR1 (1-231), DN NIK also did not significantly changes EGFR levels. DN IKKα and DN IKKβ decreased EGFR expression in 1-231 cells, with the most dramatic decrease induced by DN IKKβ. The inhibition of EGFR up-regulation by both DN IKKα and DN IKKβ suggests that CTAR1-mediated effects on EGFR are regulated in part by the canonical pathway. This is consistent with previously published data that demonstrated that expression of a DN IκBα also inhibited LMP1-induced EGFR expression (21). The lack of an effect by the DN NIK indicates that the induction of EGFR by CTAR1 is not due to the noncanonical pathway.

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 (wt) IKKβ, or the IKKβ DN double point mutant (SS 177, 181 AA) (DN IKKβ SSAA). Expression of LMP1, deletion mutants, and IKKβ was confirmed by immunoblotting (Fig. 4, middle two panels). Equal loading was assessed by Western blotting (Fig. 4, bottom panel). NF-κB activation was detected by EMSA with a radiolabeled oligonucleotide probe (UV21) with the NF-κB consensus site from the MHC class I promoter, using nuclear extracts (Fig. 4, top panel). EGFR levels were measured by immunoblotting (Fig. 4, second panel). In C33A epithelial cells, LMP1 and 1-231 activate p50 homodimers, p50/p52 heterodimers, and p52/p65 heterodimers and Δ187-351 activates p52/p65 heterodimers (27). Transfection of wt IKKβ and DN IKKβ into pCDNA3 control cells did not change NF-κB complex levels (Fig. 4, lanes 2 to 4). In both LMP1- and 1-231-expressing cells, three complexes were detected (Fig. 4, upper panel, lanes 5 and 8). The identities of these complexes were confirmed by supershift analysis (data not shown). Expression of DN IKKβ in LMP1 cells reduced all complexes, and that in 1-231 cells almost completely eliminated them. In particular, the strong nuclear p50/p50 homodimer level was greatly diminished (Fig. 4, upper panel, lanes 7 and 10). This decrease in nuclear p50/p50 homodimer levels correlated with a decrease in EGFR levels (Fig. 4, lanes 7 and 10). In the CTAR2 containing Δ187-351 cells, p52/p65

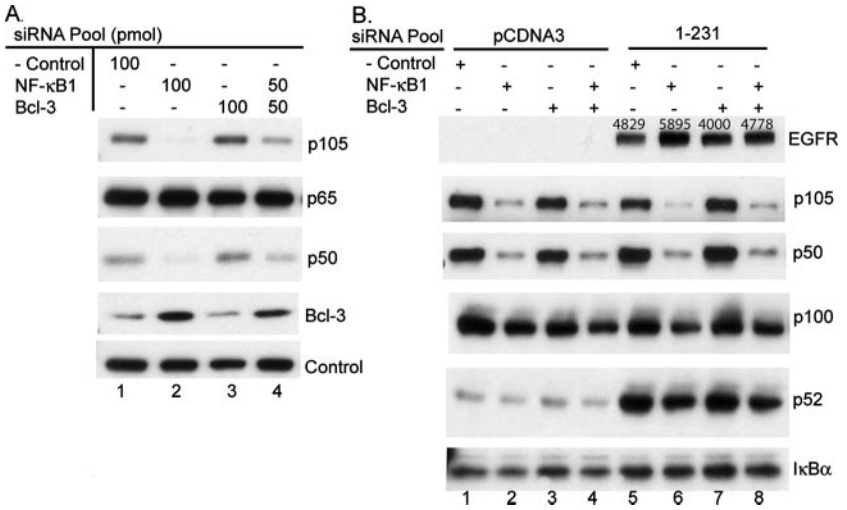

FIG. 5. NF-κB1 siRNA negatively regulates p105/p50 expression and increases Bcl-3 and EGFR expression. (A) C33A cells were transfected with 100 pmol irrelevant siRNA, 100 pmol NF-κB1 siRNA, 100 pmol Bcl-3 siRNA, or 50 pmol NF-κB1 and Bcl-3 siRNAs. Knockdown was confirmed by immunoblotting. (B) C33A cells stably expressing pCDNA3 vector control or 1-231 were transfected with 100 pmol irrelevant siRNA, 100 pmol NF-κB1 siRNA, 100 pmol Bcl-3 siRNA, or 50 pmol NF-κB1 and Bcl-3 siRNAs. NF-κB and EGFR protein levels were examined by immunoblotting. The Image J 1.32j computer program was used to calculate the intensity of each EGFR band from one Western blot. Pixel density values are listed above the corresponding bands. Units are arbitrary. The blot is representative of five 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-κB 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 IκBα, as levels of IκBα 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-κB1 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

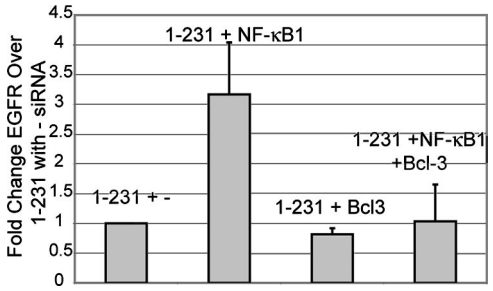

FIG. 6. NF-κB1 siRNA increases EGFR RNA. C33A cells stably expressing vector control or 1-231 were transfected with 100 pmol irrelevant siRNA (+−), 100 pmol NF-κB1 siRNA (NF-κB1), 100 pmol Bcl-3 siRNA (Bcl-3), or 50 pmol NF-κB1 and Bcl-3 siRNAs (NF-κB1+Bcl-3). β-Actin and EGFR levels were examined with quantitative reverse transcription-PCR. All samples were normalized to results for 1-231 stable cells transfected with irrelevant siRNA, and each EGFR PCR was further normalized to the β-actin level in its corresponding sample. The graph shows *n*-fold change in EGFR RNA level over that for 1-231 with irrelevant siRNA and is a representative result from five independent experiments.

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 EGFR protein and RNA levels and that this correlated with 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-κB1 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 consequential increase in Bcl-3 resulted in more Bcl-3

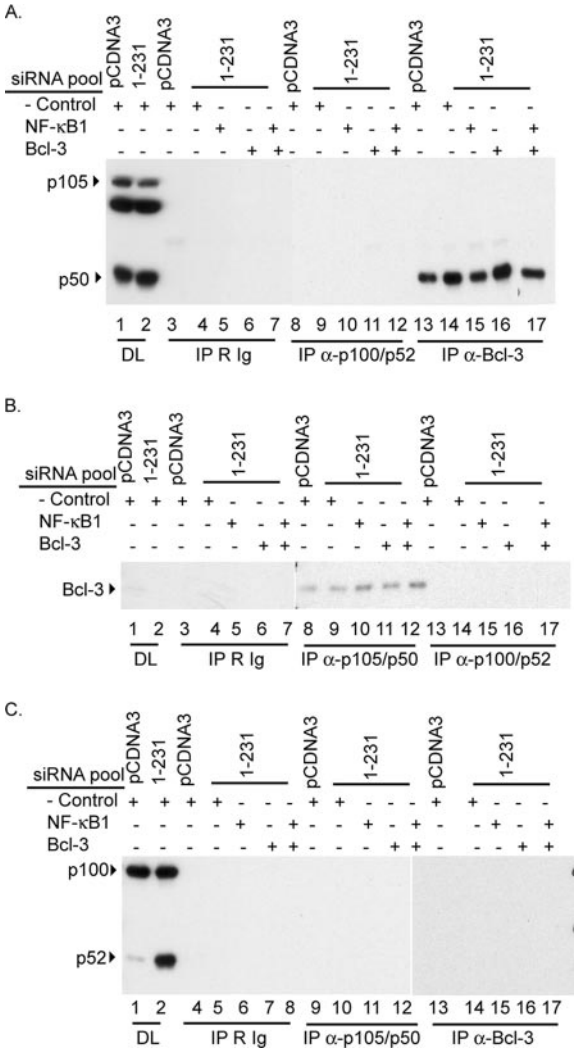

FIG. 7. Increased Bcl-3 coimmunoprecipitates with NF-κB1 after transfection with NF-κB1 siRNA. (A) Coimmunoprecipitation of p105/p50 with p100/p52 and Bcl-3 was measured after knockdown with 100 pmol irrelevant siRNA, 100 pmol NF-κB1 siRNA, 100 pmol Bcl-3 siRNA, or 50 pmol NF-κB1 and Bcl-3 in C33A cells stably expressing pCDNA3 or 1-231. Stable cells were transfected with the siRNA indicated above the immunoblot. Lanes 1 and 2 are a direct load of protein lysates from pCDNA3 and 1-231 stable cells. Lanes 3 to 7 are immunoprecipitated with rabbit immunoglobulin G (IgG) isotype control, lanes 8 to 12 are immunoprecipitated with anti-p100/p52 (α-p100/p52), and lanes 13 to 17 are immunoprecipitated with anti-Bcl-3 (α-Bcl-3). All immunoprecipitates were immunoblotted with anti-p50. (B) Coimmunoprecipitation of Bcl-3 with anti-p105/p50 (α-p105/p50) and anti-p100/p52 was measured after knockdown with siRNAs as described for panel A with C33A cells stably expressing pCDNA3 or 1-231. Stable cells were transfected with the siRNA indicated above the immunoblot. Lanes 1 and 2 are direct loads of protein lysates from pCDNA3 and 1-231 stable cells. Lanes 3 to 7 are immunoprecipitated with goat IgG isotype control, lanes 8 to 12 are immunoprecipitated with anti-p105/p50, and lanes 13 to 17 are immunoprecipitated with anti-p100/p52. The complexes were immunoblotted with anti-Bcl-3. (C) Coimmunoprecipitation of anti-p100/p52 with anti-p105/p50 and Bcl-3 was measured after siRNA knockdown as described for panel A in pCDNA3 and 1-231 stable C33A cells. Stable cells were transfected with the siRNA indicated above the immunoblot. Lanes 1 and 2 are a direct load of protein lysates from pCDNA3 and 1-231 stable cells. Lanes 3 to 7 are immunoprecipitated with mouse IgG isotype control, lanes 8 to 12 are immunoprecipitated with anti-p105/p50, and lanes 13 to 17 are immunoprecipitated with anti-Bcl-3. The immunoprecipitates were immunoblotted with anti-p52.

in complexes immunoprecipitated with anti-p105/p50 (Fig. 7B, lanes 10 and 12). Immunoprecipitation with anti-p100/p52 confirmed that in C33A cells expressing 1-231, p100/p52 did not bind p50 (Fig. 7A, lanes 8 to 12) or Bcl-3 (Fig. 7B, lanes 13 to 17). NF-κB p100/p52 also did not immunoprecipitate with either anti-p105/p50 or anti-Bcl-3 (Fig. 7C, lanes 8 to 17). These data indicate that transfection of NF-κB1 siRNA did not induce formation of p52/Bcl-3 complexes. These results reveal that despite a significant decrease in the overall pool of p105/p50, the remaining p50 binds more efficiently to Bcl-3 in the absence of p105 and that the increased EGFR expression correlated with increased complex formation between p50 and 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/p50 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 LMP1, can be transcriptionally regulated by p50 homodimers (13, 28). In EBV-positive samples of NPC, p50/p50 homodimers preferentially bound Bcl-3 and did not bind 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, IKKα and IKKβ, and a regulatory domain, IKKγ (NEMO). IKKβ is the dominant kinase in phosphorylation of IκB (7). NIK can also phosphorylate and activate IKKα, which can phosphorylate the p52 precursor, p100, in an IKKγ-independent manner to produce p52. LMP1 CTAR1 activates NF-κB through both IKKγ-dependent and noncanonical IKKγ-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 IKKγ-dependent IKK complex.

The role of NF-κB was confirmed in this study, since transfection of DN IKKα and IKKβ 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 IKKβ decreased EGFR RNA threefold, while transient transfection of DN NIK did not change EGFR and DN IKKα decreased EGFR RNA by approximately one-third. The noncanonical processing and activation of p52 by CTAR1 is dependent upon NIK and IKKα; therefore, the minimal effects of DN NIK and DN IKKα and the more-pronounced inhibition by DN IKKβ 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 IKKβ is a significant factor in the regulation of EGFR by CTAR1.

However, the increased levels of p50 homodimers induced by wt IKKβ and DN IKKβ 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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