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Specific effects of BCL10 Serine mutations on phosphorylations in canonical and noncanonical pathways of NF-κB activation following carrageenan

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Bhattacharyya S, Borthakur A, Anbazhagan AN, Katyal S, Dudeja PK, Tobacman JK. Specific effects of BCL10 Serine mutations on phosphorylations in canonical and noncanonical pathways of NF-κB activation following carrageenan. Am J Physiol Gastrointest Liver Physiol 301: G475–G486, 2011. First published June 23, 2011; doi:10.1152/ajpgi.00071.2011.—To determine the impact of B cell leukemia/lymphoma (BCL) 10 on the phosphorylation of crucial mediators in NF-κB-mediated inflammatory pathways, human colonic epithelial cells were exposed to carrageenan (CGN), a sulfated polysaccharide commonly used as a food additive and known to induce NF-κB nuclear translocation by both canonical and noncanonical pathways. Phosphorylations of intermediates in inflammatory cascades, including NF-κB-inducing kinase (NIK) at Thr259, transforming growth factor-β-activating kinase (TAK) 1 at Thr184, Thr187, and Ser192, and inhibitory factor κBα (IkBα) at Ser32, were examined following mutation of BCL10 at Ser138 and at Ser218. Specific phosphoantibodies were used for detection by enzyme-linked immunosorbent assay, immunoblot, and confocal microscopy of differences in phosphorylation following transfection by mutated BCL10. Both mutations demonstrated dominant-negative effects, with inhibition of phosphoSer(Ser)252-IkBα to less than control levels. Both of the BCL10 mutations reduced the CGN-induced increases in nuclear RelA and p50, but only the Ser138 mutation inhibited the CGN-induced increases in nuclear RelB and p52 and in NIK Thr259 phosphorylation. Hence, the phosphorylation of BCL10 Ser138, but not Ser218, emerged as a critical event in the activation of the canonical pathway of NF-κB activation. Either BCL10 Ser138 or Ser219 mutation inhibited the phosphorylation of TAK1 at Thr187 and at Thr184, but not at Ser192. These findings indicate that BCL10 phosphorylations act upstream of phosphorylations of NIK, TAK1, and IkBα and differentially affect the canonical and noncanonical pathways of NF-κB activation.

B cell leukemia/lymphoma; nuclear factor-κB

BCL10 has a caspase recruitment domain (CARD) at its amino terminus (residues 18–102). An intact CARD domain is required for the NF-κB-inducing activity of BCL10, since point mutations (L41Q and G78R) in conserved residues of the CARD resulted in an inactive form of BCL10 (14, 17). BCL10 knockout mice were immunodeficient, and lymphocytes derived from these mice were unable to activate NF-κB following either antigen receptor stimulation or phorbol 12-myristate 13-acetate and ionomycin treatment (25). BCL10 interacts with other CARD-containing cytoplasmic molecules through CARD-CARD domain binding. With the CARD-containing membrane-associated guanylate kinase (MAGUK) known as carma 1 (CARD11), BCL10 and MALT1 form CARMA1-BCL10-MALT1 (CBM) complexes in cells of myeloid origin. The molecular structure and function of the CBM complex in myeloid cells have been presented in several reports (20, 23, 29, 31, 34, 35). CARMA 1, as well as CARMA 2 and CARMA 3, are multidomain proteins that assemble signal transduction complexes by binding to both transmembrane and intracellular signaling proteins, often at sites of cell-cell contact. In addition to the NH2-terminal CARD domain and the COOH-terminal GUK domain, the CARMA1s have a coiled-coil region to which the paracaspase MALT1 can bind, SH3 and PDZ domains, and a linker region. In immune cells, the linker region of CARMA1 can be phosphorylated by protein kinase C (PKC)-θ in T cells or by PKC-θ in B cells (18, 24, 27). In colonic epithelial cells, the recognition of platelet-activating factor by its receptor induced the activation of the CBM complex (9). This complex in turn activated the IKK signalsosome by ubiquitination of NEMO (IKKγ) and stimulated the nuclear translocation of NF-κB (p65) in a series of reactions that parallel those that occur in the T cell- or B cell-induced activation of CARMA1. The phosphorylations required for the activation of the CBM complex in epithelial cells have not yet been clarified, although phosphorylation of BCL10 Ser138 was determined to be required for critical phosphorylations in the inflammatory cascade (1, 15). Phosphorylation of BCL10 Ser218 was reported to be involved in the nuclear translocation of BCL10, thereby enabling subsequent transcriptional events (39).

In this report, we extend findings from our previous studies about how activation of canonical and noncanonical signaling of NF-κB is mediated by BCL10 following stimulation by carrageenan (CGN) or lipopolysaccharide in human colonic epithelial cells (2–6, 10). The sulfated polysaccharide CGN has been used for decades to induce inflammation in animal models to study mediators of inflammation and pharmacological agents that reduce inflammation. Recent studies of the mechanisms by which CGN induces inflammation in colonic epithelial cells have demonstrated that CGN stimulation of

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BCL10 and NF-κB is attributable to its distinctive chemical structure, and, in particular, its unusual α-α-Gal(1→3)α-Gal link (7, 13). Because of its resemblance, yet its difference, it can mimic to some extent the naturally occurring sulfated glycosaminoglycans, yet can induce the innate immune response mediated by Toll-like receptor (TLR) 4 and BCL10. CGN exposure leads to NF-κB activation by three distinct approaches, including 1) a reactive oxygen species (ROS)-mediated pathway that involves Hsp27-IKKβ-inhibitory factor κBα (IkBα), but does not require BCL10; 2) a TLR4-BCL10-IKKγ-IKKβ-IkBα-NF-κB (RelA) pathway; and 3) a TLR4-BCL10-NF-κB-inducing kinase (NIK)-IKKα-NF-κB (RelB) pathway (2–6, 10) (Fig. 1).

Elucidation of the interactions between BCL10 phosphorylations and other critical phosphorylations of mediators in the canonical and noncanonical pathways of NF-κB activation, including transforming growth factor (TGF)-β-activating kinase (TAK) 1, NIK, and IkBα, can provide new insights into upstream signaling mechanisms in the inflammatory cascades. TAK-1 is regarded as a critical mediator of the canonical pathway of NF-κB activation upstream of the IKK signalosome (26, 40) and responds to multiple stimuli, including TGF-β, interleukin (IL)-1, and tumor necrosis factor (TNF)-α. The activation loop of TAK1 includes NH2-terminal phosphorylation sites, whereas regulatory phosphorylation sites are present at the COOH-terminus. In this report, we present the effects of specific dominant-negative BCL10 serine mutations (S138G and S218G) on phospho-NIK (Thr559), phospho-TAK1 (Thr184, Thr187, and Ser192), and phospho-IkBα (Ser32) and NF-κB components (RelA, RelB, p50, and p52) following exposure to CGN.

Because the concentrations of CGN used in the cell-based experiments that follow are less than the exposure anticipated in the average diet, the effects of CGN on inflammation in mammalian cells are highly relevant to the potential clinical impact of CGN. The CGN concentration of 1 μg/ml in cell culture represents an exposure of 1 mg/l, far less than anticipated from an average intake of ∼250 mg/day in intestinal contents of ∼5 liters (250 mg/5,000 ml = 50 μg/ml) (12, 36). In unpublished studies, a 10 μg/ml concentration of CGN in the mouse water presents a total intake of ∼50 μg/day in a 30-g animal that consumes 5 ml/day and is comparable to 100 mg/day in a 60-kg person and significantly less than the anticipated average intake of 250 mg/day. Hence, the impact of BCL10 on the inflammatory cascades in the experiments that follow may be highly relevant to human intestinal disease.

MATERIALS AND METHODS

Cell culture. NCM460 is a nontransfected, human colonic epithelial cell line, originally derived from the normal colon mucosa of a 68-yr-old Hispanic male. NCM460 cells were procured and expanded in M3:10A media (INCELL, San Antonio, TX) (21). HT-29 cells (HTB-38; ATCC, Manassas, VA) were cultured in McCoy’s 5a medium with 10% FBS as recommended. The cells were maintained at 37°C in a humidified 5% CO2 environment with media renewal at 2- to 3-day intervals. Confluent cells in T-25 flasks (Costar, Cambridge, MA) were harvested by trypsinization and subcultured in multiwell tissue culture clusters (Costar). Cells were treated with 1 μg/ml of λ-CGN (SIGMA-Aldrich, St. Louis, MO) for 24 h. At the end of the treatment, spent media were collected from control and treated wells and stored at −80°C until further analysis. These cells were harvested by scraping, and cell lysates were prepared and stored at −80°C. Total cell protein was measured by the BCA protein assay kit (Pierce, Rockford, IL), using BSA as the standard. The TAK1 inhibitor (5Z)-oxozeaenol (Tocris Bioscience, Ellisville, MO) was used at a concentration of 0.05 mM for 1 h before addition of λ-CGN and then in combination with λ-CGN (1 μg/ml) for 24 h. The free radical scavenger 2,2,6,6-tetramethylpiperidinyloxy (Tempol; Axxora Life Sciences, San Diego, CA) was used at a concentration of 100 mM for 1 h preincubation and then in the presence of λ-CGN (1 μg/ml) for 24 h.

Site-directed mutagenesis. Site-directed mutagenesis was performed to generate mutant BCL10 proteins in which serine residues were changed to glycine (S138G or S218G), using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) (25). Mutations were confirmed by DNA sequencing. The mutant oligonucleotides were, for S138G 5′-CTCTCCAGATCAATGGAGAC-3′ and for S218G 5′-GGACCTTGTGCACACCGTGATGAGATTTCTT-3′ (underlined letters indicate mutated residues).

Transfection with BCL10 mutants. NCM460 and HT-29 cells at 60–70% confluency were grown in 24-well tissue culture plates and were transiently transfected with 1.5 μg/well of wild-type (WT) or mutated BCL10 untagged cDNA in a pCMV6-XL6 vector (Origene; sc101447) with Fugene (Roche). Medium was exchanged at 24 h after transfection with fresh NCM460 or McCoy’s 5a medium with or without 1 μg/ml CGN. After 24 h of treatment, the spent media were collected, and the cells were harvested for further studies.
Western blots of phospho-BCL10, BCL10, and phospho(Thr<sup>184</sup>)-TAK1. Western blots were performed on 10% SDS gel using standard methods with antibodies to phospho-BCL10 [Ser<sup>138</sup>, Santa Cruz Biotechnology (SCBT), Santa Cruz, CA], BCL10 (SCBT), and phospho-Tak1(Thr<sup>184</sup>) (Cell Signaling) to probe for the proteins of interest and compared with density of β-actin from the same specimens.

ELISAs for measurements of secreted IL-8 and cellular BCL10 and phospho-IκBα. The secretion of IL-8 in the spent media of control and treated NCM460 cells was measured by the DuoSet enzyme-linked immunosorbent assay (ELISA) kit for human IL-8 (R&D Systems, Minneapolis, MN), as previously reported (4, 5). Hydrogen peroxide-tetramethylbenzidine (TMB) chromogenic substrate was used to develop the color, and the intensity of color was measured at 450 nm in an ELISA plate reader (FLUOstar; BMG). IL-8 concentrations were extrapolated from a standard curve, and the sample values were normalized with total protein content (BCA Protein assay kit; Pierce).

Expression of BCL10 protein in control and CGN-treated NCM460 was determined by a solid-phase ELISA, developed for quantitative determination of BCL10 (8). Control or treated cells were lysed in RIPA buffer (50 mM Tris containing 1:5 M NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS, pH 7.4), and the cell extracts were stored at −80°C until assayed by the ELISA.

Phosphorylation of IκBα following CGN treatment of NCM460 cells was determined using a PathScan Sandwich ELISA kit (Cell Signaling), as described previously (3, 10). Briefly, the IκBα in the cell extracts was captured in a 96-well ELISA plate that was precoated with mouse monoclonal antibody anti-IκBα. Phospho-IκBα was determined by a specific phospho-IκBα (Ser<sup>152</sup>) antibody and detected by horseradish peroxidase (HRP)-conjugated secondary antibody and hydrogen peroxide-TMB chromogenic substrate.

Oligonucleotide-based ELISAs for measurement of nuclear RelA, p50, RelB, and p52. Nuclear extracts were prepared from CGN-treated and control NCM460 and HT-29 cells by use of a nuclear extraction kit (Active Motif, Carlsbad, CA). Activated NF-κB, including RelA, p50, RelB, and p52, were determined by oligonucleotide-based ELISA (Active Motif), as described previously (1, 3). Treated and control samples were incubated in 96-well microtiter wells that were coated with the NF-κB consensus nucleotide sequence 5′-GGGACTTTCC-3′. NF-κB from the samples attached to the wells and was determined using specific antibody to either RelA, p50, RelB, or p52. The extent of binding of the antibody was detected by anti-rabbit-IgG and color was developed with hydrogen peroxide-TMB chromogenic substrate. The intensity of developed color was proportional to the quantity of either RelA, p50, RelB, or p52 in the sample. Specificity of the NF-κB binding to the nucleotide sequence was determined by premixing of either free consensus nucleotide or mutated consensus nucleotide to the nuclear extract sample before adding the sample to the well. The optical density values were normalized using the total cell protein determined by a protein assay kit (Pierce), and results were expressed as percent unexposed control.

Measurement of phospho-BCL10 and phospho-NIK by FACE. Phospho-BCL10 in control and CGN-treated NCM460 cells transfected with WT or mutant BCL10 vector was measured by a fast-activated cell-based ELISA (FACE; Active Motif), as described previously (1, 3). Control and treated NCM460 cells were fixed by 4% formaldehyde and washed, endogenous cellular peroxidase activity was quenched, and the nonspecific binding was blocked. Phospho-BCL10 (Ser<sup>138</sup>) and total BCL10 were assessed by specific monoclonal antibody to either RelA, p50, RelB, or phospho-BCL10 antibody (SCBT) or BCL10 monoclonal antibody (SCBT) and detected by anti-mouse-IgG-HRP (SCBT) and hydrogen peroxide-TMB substrate.

Total and phospho-NIK in control and CGN-treated NCM460 cells were also measured by FACE (Active Motif). Primary goat antibodies for NIK against the epitope 700–947 (sc-7211; SCBT) and for phospho-NIK that recognized Thr<sup>559</sup> phosphorylation (sc-12957; SCBT) were used to assess differences in the extent of NIK phosphorylation by comparing measurements of optical density between control and treated samples.

Phospho-TAK1 assays by FACE. Total and phospho-TAK1 were measured by FACE (Active Motif). Primary antibodies for TAK1 (SCBT) and for three different TAK1 phosphorylation sites [Thr<sup>184</sup> and Thr<sup>187</sup> (Cell Signaling) and Ser<sup>192</sup> (SCBT)] were obtained and used at a concentration of 1:250. NCM460 cells were grown in 96-well plates and treated with CGN (1 μg/ml) for 24 h. The cells were washed and fixed with 4% formaldehyde, quenched for endogenous cellular peroxidase activity, and blocked with blocking buffer. The fixed cells were then challenged with TAK1 and phospho-TAK1 antibodies to capture the total TAK1 and phospho-TAK1. The captured TAK1 and the three phospho-TAKs were then detected by HRP-conjugated secondary antibody (either goat anti-rabbit or goat anti-mouse), and the color was developed with hydrogen peroxide-TMB chromogenic substrate. The intensity of developed color was proportional to the content of TAK1 or phospho-TAK1. Optical densities were measured at 450 nm and compared between control and treated wells.

Confocal microscopy of phospho-TAK1 (Thr<sup>184</sup> and Thr<sup>187</sup> and Ser<sup>192</sup>) in NCM460 cells. NCM460 cells were grown in compartment slides for 24 h until 50% confluent and then transiently transfected with 1.5 μg/well of either WT or mutated BCL10 DNA using Fugene (Roche) (1). Medium was exchanged at 24 h after transfection with either fresh NCM460 medium or medium containing 1 μg/ml CGN or control IgG antibody for 24 h. Methods for staining and examination of cells by confocal microscopy were described previously (3, 6). Cells were washed one time in 1× PBS containing 1 mM calcium chloride (pH 7.4), fixed for 1.5 h with 2% parafomaldehyde, and then permeabilized with 0.08% saponin. Preparations were washed with PBS, blocked in 5% normal goat serum, incubated overnight with phospho-BCL10 antibody, phospho-TAK1, or control anti-rabbit IgG antibody for negative staining at 4°C, and then washed and stained with either Alexa Fluor 594 rabbit-anti-mouse or Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1:100; Invitrogen). Slides were coverslipped using 4′,6-diamidino-2-phenylindole-mounting medium (Vectorshield; Vector Laboratories, Burlingame, CA) for nuclear staining. Slides were washed, mounted, and observed using a Zeiss LSM 510 laser-scanning confocal microscope with excitation at 488 and 534 nm from an Ar/Kr laser and at 361 nm from an ultraviolet laser. Green [phospho(Thr<sup>184</sup>, Thr<sup>187</sup>, or Ser<sup>192</sup>)-TAK1] and red [phospho(Ser<sup>138</sup>) BCL10] fluorescence were detected through LP505 and 585 filters and scanned sequentially. Images were exported with Zeiss LSM Image Browser software as TIFF files for analysis and reproduction using Adobe Photoshop.

Inhibitor of calcium/calcmodulin kinase. KN93 (Sigma) is the inhibitor of calcium/calmodulin kinase (CAMKII) reported to inhibit BCL10 phosphorylation following stimulation by phorbol ester in Jurkat and 293T cells (15). NCM460 cells were exposed to 40 μM KN93 for 30 min before treatment with λ-CGN (1 μg/ml × 1 h or 24 h). The effect on phospho(Ser<sup>138</sup>)-BCL10 was tested by FACE assay, and IL-8 secretion was tested by ELISA, as described above.

Statistics. Data presented are means ± SD of at least three independent biological experiments with technical replicates of each measurement. Unless stated otherwise in the text or legends to Figs. 1–8, statistical significance was determined by one-way ANOVA, followed by a post hoc Tukey-Kramer test for multiple comparisons using Instat (GraphPad) software. A P value ≤0.05 is considered statistically significant. Significance is represented by asterisks in Figs. 1–8.

RESULTS

Effect of BCL10 mutations on CGN-induced increases in BCL10 and phospho-BCL10. CGN treatment of NCM460 cells produced an increase in BCL10 from 1.3 ± 0.1 to 3.9 ± 0.2
ng/mg protein compared with untreated control cells ($P < 0.001$, 1-way ANOVA with Tukey-Kramer posttest). Following transfection with either WT BCL10 or BCL10 with Ser138Gly or Ser218Gly mutations, CGN induced increases in total BCL10 to ~5.3 ng/mg protein ($P < 0.001$) (Fig. 2A).

The phospho(Ser138)-BCL10 in the untreated, NCM460 control cells increased to ~4.0 times the baseline value following exposure to CGN (1 μg/ml × 24 h) (Fig. 2B). When NCM460 cells were transfected with WT BCL10 and then exposed to CGN, phospho(Ser138)-BCL10 further increased to ~5.0 times the baseline value. Similarly, following transfection with the Ser218 mutant BCL10 and exposure to CGN, the phospho-(Ser138)-BCL10 increased to ~5.3 times the control. Following transfection with the Ser138 mutant BCL10, decline in phospho-BCL10 to less than the control value occurred ($P < 0.001$), demonstrating the dominant-negative effect of transfection with the mutant BCL10. Representative Western blot presenting phospho-BCL10 in untreated control, and following exposure to CGN and transfection with WT BCL10, mutated BCL10 (S138G), or the vector control demonstrated the increase in phospho-BCL10 in the control and WT transfected cells but not in the BCL10 mutant 138 transfected cells (Fig. 2C).

When HT-29 cells were transfected with WT BCL10 or with Ser138 and Ser218 mutant BCL10s, total BCL10 was measured by ELISA and was increased significantly, demonstrating effectness of the transfection ($P < 0.001$) (Fig. 2D). CGN exposure further increased the total BCL10 in the transfected cells to a maximum of 6.6 ± 0.2 ng/mg protein in the cells transfected with the Ser138 mutant construct. BCL10 Western blot confirms the effectness of the transfections with the WT and mutant BCL10 constructs (Fig. 2E).

Effect of BCL10 mutations on IL-8, phospho-IκBα, and phospho-NIK. CGN treatment of NCM460 cells transfected with WT BCL10 increased the IL-8 secretion from 808 ± 28

Fig. 2. Effects of BCL10 mutations on total BCL10 and phospho-BCL10. A: CGN treatment (1 μg/ml × 24 h) of NCM460 cells increased BCL10 from 1.26 ± 0.11 to 3.92 ± 0.15 ng/mg protein ($P < 0.001$, 1-way ANOVA with Tukey-Kramer posttest) when quantified by enzyme-linked immunosorbent assay (ELISA). After transfection with either wild-type (WT) BCL10 or BCL10 with S138G or S218G mutations, CGN increases to ~4.2 times the baseline level of total BCL10. All increases in total BCL10 following CGN were statistically significant ($P < 0.001$). Following transfection with either WT or the mutant BCL10s, the CGN-induced increases were significantly higher than following CGN stimulation of the control cells ($P < 0.001$). (Unless stated otherwise, statistical significance was determined by 1-way ANOVA with Tukey-Kramer posttest for multiple comparisons.) B: increases in phospho-BCL10 were measured using a phospho-BCL10 (Ser138) antibody and cell-based ELISA assay. CGN (1 μg/ml × 24 h) caused significant increases, except in the cells transfected with the S138G mutation, demonstrating the effectiveness of the transfection in reducing phospho(Ser138)-BCL10. The S138G BCL10 mutant appears to act as a dominant negative and suppresses the phosphorylation of the endogenous BCL10. C: representative Western blot demonstrates the increase in phospho-BCL10 (Ser138) that followed transfection with WT BCL10 and exposure to CGN in the NCM460 cells. Following transfection with the S138G mutant, no increase in phospho-BCL10 occurred. m138, S138G BCL10 mutant; m218, S218G BCL10 mutant. D: when HT-29 cells were transfected with BCL10 WT, S138G, and S218G mutations, BCL10 increased significantly ($P < 0.001$), suggesting greater transfection efficiency in the HT-29 cells than in the NCM460 cells. CGN exposure produced further increase in total BCL10 in the transfected cells ($P < 0.001$). E: in HT-29 cells, immunoblot also demonstrated increases in BCL10 following transfection with the WT and mutant BCL10 constructs. m1, m138=S138G BCL10 mutant; m2, m218=S218G BCL10 mutant; Vcon, empty vector control. *** $P \leq 0.001$. 

AJP-Gastrointest Liver Physiol • VOL 301 • SEPTEMBER 2011 • www.ajpgi.org
to 1,520 ± 54 pg/mg of protein (Fig. 3A). Following transfection with the BCL10 mutants, the increases in IL-8 were significantly reduced to 1,031 ± 41 pg/mg protein (S138G) and 1,141 ± 48 pg/mg protein (S218G) (P < 0.001). Similar effects occurred in the transfected HT-29 cells, with IL-8 peaking at 2,511 ± 286 pg/mg protein following transfection with WT BCL10 and declining by >1,000 pg/mg protein following transfection with the mutant BCL10 constructs (P < 0.001) (Fig. 3B). These results confirm a requirement for these BCL10 phosphorylation sites to achieve the maximum CGN-induced increase in IL-8 secretion.

CGN-exposed NCM460 cells transfected with WT BCL10 demonstrated a significant increase in phospho-IκBα (Ser32), to 2.3 ± 0.2 times the baseline (P < 0.001). Following transfection with the mutated BCL10, the increases were reduced to ~1.6 times the control value (Fig. 3C).

When NCM460 cells were transfected with either WT BCL10 or BCL10 mutated at Ser218 (m218), phospho-NIK increased to 2.7 ± 0.07 and 2.4 ± 0.09 times the control values, respectively (P < 0.001) (Fig. 3D). In contrast, CGN treatment of the cells following transfection with the S138G mutation produced no increase in phospho-NIK. These results are consistent with the dominant-negative impact of the mutation and the selective impact of the Ser138 mutation on the noncanonical pathway of NF-κB activation.

**BCL10 mutations have differential effects on NF-κB components.** NCM460 cells were transfected with either WT or mutated BCL10 and exposed to λ-CGN (1 μg/ml × 24 h).
Fig. 4. BCL10 mutations have differential effects on NF-κB family members. A: NCM460 cells were transfected with either WT or mutated BCL10 and exposed to λ-CGN (1 μg/ml × 24 h), and NF-κB components were measured by an NF-κB oligonucleotide assay. Nuclear RelA increased to 2.52 ± 0.03 times the baseline value following CGN and transfected by WT BCL10, but declined significantly, to ~1.5 times the baseline value (P < 0.001), following transfection with either of the BCL10 mutants. B: similar to the changes in RelA, p50 increased to 2.67 ± 0.22 times the baseline value when transfected with WT BCL10 and treated with CGN, but to only ~1.60 ± 0.05 (S138G) and 1.56 ± 0.12 (S218G) times the baseline values when transfected with either of the mutants (P < 0.001). C: following transfection with either the WT BCL10 or the S218G mutant, RelB increased to ~1.5 times the baseline value. When transfected with the S138G BCL10, this increase in RelB was inhibited (P < 0.001), consistent with the requirement for BCL10 Ser138 for activation of the noncanonical pathway of NF-κB. D: corresponding to the changes in RelB, p52 increased to ~1.65 times the baseline value following transfection with either WT BCL10 or the S218G mutant and CGN exposure. However, following transfection with the S138G mutation, no increase in p52 occurred (P < 0.001), consistent with the requirement of BCL10 Ser138 for the noncanonical pathway and the dominant-negative effect of the mutant BCL10. E: in the HT-29 cells, RelA increased to 2.5 ± 0.3 times the baseline value following exposure to CGN. Following transfection with WT BCL10 and CGN exposure (1 μg/ml × 24 h), RelA increased to 3.0 ± 0.2 times the baseline but declined to 1.6 times baseline when transfected with either of the mutant BCL10 constructs (P < 0.001) ***P ≤ 0.001.
Nuclear RelA increased to 2.29 ± 0.17 times the baseline value with CGN exposure and to 2.52 ± 0.09 times the baseline value when transfected with WT BCL10 and exposed to CGN (P < 0.001) (Fig. 4A). When transfected with the mutated BCL10 (S138G and S218G), the increase declined to only 1.52 ± 0.05 times the baseline. Similarly, p50 increased to 2.67 ± 0.22 when transfected with WT BCL10 and treated with CGN, but to only 1.60 ± 0.05 and 1.56 ± 0.12 times the baseline value when transfected with the mutant BCL10 (S138G and S218G, respectively; P < 0.001) (Fig. 4B).

In contrast, different effects were evident on RelB and p50 following transfection with the S138G vs. the S218G BCL10 mutants. CGN treatment produced an increase to

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Fig. 5. BCL10 mutations inhibit phosphorylation of transforming growth factor-β-activating kinase (TAK) 1 Thr184 and Thr187 but not Ser192. A–C: CGN treatment (1 μg/ml × 24 h) of NCM460 cells yielded an increase to 4.2 times the baseline level in phospho-TAK1 (Thr184, Thr187, or Ser192) in control, untransfected cells. Following transfection by WT BCL10 and CGN treatment, these increases were 5.0, 5.0, and 5.3 times the baseline level, respectively (P < 0.001). Following transfection by the mutated BCL10 and CGN treatment, no increases in phospho-TAK1 (Thr184 or Thr187) occurred, but the increase in phospho-TAK1 (Ser192) was not inhibited. Following CGN, phospho-TAK1(Ser192) increases ranged from 4.2 to 5.3 times the baseline levels. D: representative Western blot of phospho-TAK1(Thr184) demonstrates the CGN-induced increase in phospho-TAK1(Thr184) in the control and WT transfectant, but not following transfection with the BCL10 mutant constructs. E: total TAK1 was not affected by treatment with CGN or transfection with WT or mutated BCL10. ***P < 0.001.
1.49 ± 0.14 times the baseline in the nuclear RelB following transfection with WT BCL10 (Fig. 4C). RelB increased to 1.65 ± 0.05 times the baseline following transfection with the Ser\textsuperscript{218}Gly mutation but did not increase significantly following the Ser\textsuperscript{138}Gly mutation. Similarly, p52 increased to 1.67 ± 0.03 times the baseline following transfection with WT BCL10 and CGN and to 1.63 ± 0.07 times the baseline following transfection with the Ser\textsuperscript{218} mutated BCL10, but did not increase significantly following transfection with the S138G mutant (Fig. 4D). These findings indicate a dominant-negative effect of the S138G mutation and a requirement for Ser\textsuperscript{138} of BCL10 for the nuclear translocation of RelB and p52 by the noncanonical pathway of NF-κB activation.

Findings in the HT-29 cells were consistent with the results in the NCM460 cells, since transfection with the WT BCL10 produced significant increases in RelA following CGN exposure (Fig. 4E). The increases in RelA were significantly reduced following transfection with either the S138G or the S218G mutant vectors (P < 0.001).

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Fig. 6. Confocal images of phospho-BCL10 and phospho(Thr\textsuperscript{184},Thr\textsuperscript{187},Ser\textsuperscript{192})TAK1 demonstrate colocalization following transfection by WT BCL10. A-I: the phospho-TAK1 findings are supported by confocal images acquired using specific phospho-TAK1 and phospho-BCL10 (Ser\textsuperscript{138}) antibodies. In images A-I, NCM460 cells were transfected with the WT BCL10. Specific phospho-TAK1 antibodies were used, showing phosphorylated (p)-Thr\textsuperscript{184} (A), p-Thr\textsuperscript{187} (D), and p-Ser\textsuperscript{192} (G) as green. Phospho-BCL10 (Ser\textsuperscript{138}) antibody was used as described in MATERIALS AND METHODS and appears red (B, E, and H). Phospho-BCL10 (Ser\textsuperscript{138}) and phospho-TAK1 colocalized and stained yellow, as seen in C, F, and I. Nuclei are stained blue by 4',6-diamidino-2-phenylindole (DAPI). Marker = 10 μm.
BCL10 mutations inhibit phosphorylation of TAK1 at Thr^{184} and Thr^{187} but not at Ser^{192}. CGN treatment (1 μg/ml × 24 h) of NCM460 cells yielded an increase in the phospho-TAK1(Thr^{184}) to 4.2 ± 0.1 times the baseline value (P < 0.001), as detected by FACE (Fig. 5A). Following transfection with WT BCL10, CGN treatment produced a further increase to 5.0 ± 0.2 times the baseline value. When transfected with the mutated BCL10 constructs, no increase in phospho-TAK1(Thr^{184}) occurred. Similarly, transfection with the mutated BCL10s inhibited the increase in phospho-TAK1(Thr^{187}) that occurred following CGN, whereas transfection with WT BCL10 produced an increase to 5.0 ± 0.2 times the baseline value (Fig. 5B). These effects are consistent with the dominant-negative effect of the mutant BCL10s.

In contrast, the CGN-induced increase in TAK1 Ser^{192} phosphorylation was unaffected by either of the BCL10 mutations (Fig. 5C). Following transfection with the WT BCL10 construct and CGN treatment, phospho(Ser^{192})-TAK1 increased to 5.3 ± 0.2 times the baseline value, and comparable increases occurred with the mutated BCL10 constructs (to 4.7 ± 0.5 for S138G and to 4.7 ± 0.3 for S218G). These findings indicate a requirement for BCL10 Ser^{138} or Ser^{218} for the TAK1 phosphorylations at Thr^{184} or Thr^{187} but not for the Ser^{192} phosphorylation. Representative Western blot indicates that the increase in phosphorylation of Tak1 Thr^{184} that followed exposure to CGN and transfection with WT BCL10 was reduced following transfection with either the BCL10 Ser^{138} or Ser^{218} mutant (Fig. 5D). Total TAK1 was not increased by

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**Fig. 7. Confocal images following transfection with mutated BCL10 (S138G).** A–F: NCM460 cells were transfected with mutated BCL10 (S138G), and staining was repeated as above. Phospho-TAK1 (Thr^{184} and Thr^{187}) are absent in images A–B and C–D, respectively. In contrast, images E and F demonstrate the presence of phospho-TAK1 (Ser^{192}) (green). Distinct phospho-BCL10 (Ser^{138}) immunostaining (red) is absent, and merged images show no yellow immunostaining. Nuclei stain blue using DAPI. Marker = 10 μm.
either CGN or transfection with WT or mutated BCL10 (Fig. 5E).

Confocal images of phosho-TAK1 following transfection with WT BCL10. Confocal images (Fig. 6, A–F) of phospho-TAK1 using specific antibodies to phospho-TAK1 for Thr184, Thr187, or Ser192 support the findings demonstrated by the cell-based ELISA presented above. Phospho-TAK1 (Thr184, Thr187, and Ser192) (Fig. 6, A, D, and G) appears green, and phospho-BCL10 (Ser138) (Fig. 6, B, E, and H) appears red. Phospho-BCL10 and phospho-TAK1 appear to colocalize in the perinuclear region and appear yellow in the merged images (Fig. 6, C, F, and I).

Confocal images of phospho-TAK1 following transfection with BCL10 m138. In confocal images (Fig. 7, A–F) following transfection with the BCL10 S138G mutant, neither phospho-BCL10 nor phospho-TAK1 (Thr184, Thr187, or Ser192) (Fig. 6, A, D, and G) appears green, and phospho-BCL10 (Ser138) (Fig. 6, B, E, and H) appears red. Phospho-BCL10 and phospho-TAK1 appear to colocalize in the perinuclear region and appear yellow in the merged images (Fig. 6, C, F, and I).

Effect of TAK1 inhibitor and Tempol on CGN-induced increase in phospho-IκBα. NCM460 transfected with either WT or mutant BCL10 were exposed to λ-CGN with or without the TAK1 inhibitor (5Z)-7-oxozeaenol (Fig. 8A). In the presence of the TAK1 inhibitor, the λ-CGN-induced increase in phospho-IκBα(Ser32) was reduced. However, following BCL10 Ser138 or Ser218 mutation, the TAK1 inhibitor had no effect on the CGN-induced increase in phospho-IκBα, indicating that the presence of the BCL10 phosphorylation sites was required for the TAK1 effect on phosphorylation of IκBα.

In contrast, when the NCM460 cells were transfected with WT or mutant BCL10 and pretreated with Tempol, a scavenger of ROS, the CGN-induced increase in phospho-IκBα (Ser32) was completely inhibited in the presence of the mutant BCL10 but only partially inhibited in the WT (Fig. 8B). This result is distinct from the effect of the TAK1 inhibitor and consistent with the occurrence of both BCL10- and ROS-mediated effects of CGN.

Lack of effect of KN93 inhibitor of CAMKII on phospho-(Ser138)-BCL10 or IL-8. When NCM460 cells were exposed to KN93, an inhibitor of CAMKII, for 30 min before exposure to λ-CGN (1 μg/ml × 1 h and 24 h), no decline in the CGN-induced increase in phospho(Ser138)-BCL10 was detected by cell-based ELISA. Secreted IL-8 also did not decline following exposure to this inhibitor (data not shown), implying that the effects of CGN do not require CAMKII.

DISCUSSION

CGN has been widely used for decades to induce inflammatory responses in animal models, to study chemical and cellular mediators of inflammation, and to determine the effectiveness of pharmacological agents on reduction of inflammation. Recent studies of the mechanisms by which CGN exposure stimulates an inflammatory response have elucidated three pathways by which CGN can activate NF-κB: increases in IL-8 secretion in human colonic epithelial cells (Fig. 1) (3, 4, 6). These pathways include an ROS-mediated response that does not require BCL10 and two BCL10-mediated pathways, leading to either canonical or noncanonical activation of NF-κB. By effects on both RelA/p50 and RelB/p52, the

BCL10-associated effects can potentially produce a fuller range of NF-κB-mediated effects than some other well-studied activators of inflammation, such as TNF-α, which activates the noncanonical pathway (RelB/p52), or dextran-sodium sulfate, which activates the ROS-mediated pathway leading to increased nuclear RelA/p50.

Because translocations involving BCL10 activation are of etiological significance in development of the MALT lymphomas in myeloid cells, other mechanisms that activate BCL10 may also be critical in induction of constitutive activation of NF-κB and in perpetuating inflammation in epithelial cells. The presence of an NF-κB-binding site in the BCL10 promoter suggests an underlying, essential mechanism for the constitutive activation of BCL10 by NF-κB (9, 3). In turn, BCL10 can continue to activate NF-κB through effects on upstream mediators, including TAK1, NIK, TRAF6, and NEMO. Malignancies of epithelial origin that arise in association with chronic inflammation, such as ulcerative colitis, exemplify the provocative effects of constitutive activation of BCL10 and NF-κB.

The experimental evidence regarding phosphorylations of BCL10 and of the other upstream mediators of NF-κB activa-
tion in cells of myeloid origin provides insights that may help to clarify the role of phosphorylations in the inflammatory cascade in colonic epithelial cells. The proximity of the Ser\(^138\) site to the CARD domain (amino acids 18–102) suggests that it may be uniquely positioned to interact with the guanylate kinase domain of the MAGUK, when there is a CARD-CARD interaction between BCL10 and CARMA3 (CARMA1 in myeloid cells). However, an earlier study demonstrated that the GUK domain of CARMA1 was not involved in BCL10 phosphorylation (32). The requirement for CAMKII for phosphorylation of Ser\(^138\) of BCL10 in the Jurkat and the 293T cells (15) does not appear applicable to the NCM460 cells, since there was no effect of the CAMKII inhibitor KN93 on the CGN-induced increase in BCL10 Ser\(^138\) phosphorylation or in IL-8 secretion. In the 293T cells, BCL10 activation of NF-κB was associated with apoptosis, rather than inflammation. Hence, this discrepancy in the role of CAMKII signaling may coincide with specific cellular responses related to inflammation vs. apoptosis. The source of BCL10 phosphorylation in the colonic epithelial cells remains undefined at this time.

The specificity of the phospho-TAK1 responses to the BCL10 Ser\(^138\) and Ser\(^218\) mutations (reduced phosphorylations of Thr\(^184\) and Thr\(^187\), but no effect on phosphorylation of Ser\(^192\)) is intriguing, but the specific mechanisms and the functional implications of this specificity are not yet elucidated. The specificity of the BCL10 Ser\(^138\) mutation for the NIK phosphorylation site (Thr\(^859\)) presents an opportunity to differentiate the effects of BCL10 attributable to the noncanonical pathway of NF-κB activation, vs. the canonical pathway. The dominant-negative effect of the BCL10 Ser\(^138\) mutation produced significant and distinct inhibition of the noncanonical pathway of NF-κB activation.

In contrast to an intracellular signal transduction mediated by receptor tyrosine kinases, such as the epidermal growth factor (EGF) receptor, the intracellular phosphorylations activated in the BCL10-mediated inflammatory pathways are serine-threonine phosphorylations, with multiple potential phosphorylation sites for each of the phosphoproteins. In the phosphosite analysis reported for EGF, most of the proteins contained multiple phosphorylation sites, although with different kinetics (22). It is possible that the reported observations at 24 h might be specific for this interval, and other TAK1 phosphorylation sites might be involved at different time points. Clarification of functionally relevant phosphorylations might depend on the timing of interactions with other possible activating cascades, such as might be initiated by TNF-α or TGF-β. Also, specific phosphorylation sites may be regulated differently and interact differently with the available phosphatases.

NIK and TAK1 likely serve as integrating platforms for stimuli from TNF-α, TGF-β, and other cytokines, as well as CGN. CGN, through TLR4, BCL10, and ROS, may elicit a multicomponent response, characteristic of the B cell receptor antigenic response to a foreign carbohydrate. Further investigation of the molecular mechanisms associated with BCL10 may improve understanding of innate immunity and inflammatory responses and enable interventions to either terminate uncontrolled inflammation or to induce limited inflammation and thereby eliminate abnormal cells.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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