

# Enterotoxigenic *Escherichia coli* Prevents Host NF- $\kappa$ B Activation by Targeting I $\kappa$ B $\alpha$ Polyubiquitination

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**The NF- $\kappa$ B pathway regulates innate immune responses to infection. NF- $\kappa$ B is activated after pathogen-associated molecular patterns are detected, leading to the induction of proinflammatory host responses. As a countermeasure, bacterial pathogens have evolved mechanisms to subvert NF- $\kappa$ B signaling. Enterotoxigenic *Escherichia coli* (ETEC) causes diarrheal disease and significant morbidity and mortality for humans in developing nations. The extent to which this important pathogen subverts innate immune responses by directly targeting the NF- $\kappa$ B pathway is an understudied topic. Here we report that ETEC secretes a heat-stable, proteinaceous factor that blocks NF- $\kappa$ B signaling normally induced by tumor necrosis factor (TNF), interleukin-1 $\beta$ , and flagellin. Pretreating intestinal epithelial cells with ETEC supernatant significantly blocked the degradation of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  without affecting I $\kappa$ B $\alpha$  phosphorylation. Data from immunoprecipitation experiments suggest that the ETEC factor functions by preventing I $\kappa$ B $\alpha$  polyubiquitination. Inhibiting clathrin function blocked the activity of the secreted ETEC factor, suggesting that this yet-uncharacterized activity may utilize clathrin-dependent endocytosis to enter host cells. These data suggest that ETEC evades the host innate immune response by directly modulating NF- $\kappa$ B signaling.**

The NF- $\kappa$ B family plays a central role in controlling the expression of genes involved in proinflammatory immune responses (2). In unstimulated cells, NF- $\kappa$ B is maintained in the cytoplasm in an inactive state by the inhibitory NF- $\kappa$ B chaperone I $\kappa$ B $\alpha$ . Upon microbial infection or stimulation by cytokines such as tumor necrosis factor (TNF) and interleukin-1 $\beta$  (IL-1 $\beta$ ), several receptor-mediated signaling pathways that result in the activation of the I $\kappa$ B kinase (IKK) complex are initiated (19). IKK phosphorylates I $\kappa$ B $\alpha$  on Ser32/36, leading to I $\kappa$ B $\alpha$  polyubiquitination by the  $\beta$ -TrCP E3 ubiquitin ligase. I $\kappa$ B $\alpha$  is then degraded by the 26S proteasome (23, 36), liberating NF- $\kappa$ B for nuclear translocation.

Bacterial pathogens have evolved different strategies to subvert host defense mechanisms to promote their survival and transmission. Intracellular microbial pathogens, such as *Legionella pneumophila* and *Brucella abortus*, prevent phagolysosome maturation and evade the host immune response by residing in host cells (28, 37). Extracellular pathogens, which are exposed to components of the host immune system, use virulence factors, such as NF- $\kappa$ B and mitogen-activated protein kinases (MAPKs) (27, 32, 41), that inhibit critical immune signaling pathways. For example, enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC) deliver virulence factors that target different components of the NF- $\kappa$ B pathway by using a type three secretion system (T3SS) (17, 31, 35, 42, 44).

Enterotoxigenic *E. coli* (ETEC) is a significant source of human morbidity and mortality (13). In addition to causing diarrhea, the ETEC heat-labile enterotoxin (LT) plays multiple roles in modulating host cell function and bacterial adherence (21, 22). Soluble LT, as well as LT associated with outer membrane vesicles (OMVs), initiates host immune responses through distinct pathways (6). LT activates both NF- $\kappa$ B and MAPK pathways, and p38 MAPK activation is involved in LT-induced ETEC adherence (43). Patients infected with ETEC elicit a mild inflammatory response, as evidenced by the presence of fecal leukocytes (29) and fecal lactoferrin (18) in stool samples as well as elevated levels of fecal interleukin-1 $\beta$  (18).

Here we found that ETEC has evolved a means to subvert host

innate immune responses by targeting the NF- $\kappa$ B pathway. We show that ETEC strain H10407 prevents NF- $\kappa$ B activation by disrupting the degradation-associated polyubiquitination of I $\kappa$ B $\alpha$ . An unidentified, secreted, heat-stable protein(s) is responsible for this phenotype after associating with the clathrin-dependent host endocytic machinery.

## MATERIALS AND METHODS

**Reagents.** Poly(ADP-ribose) polymerase (PARP) antibody was obtained from BD Biosciences. Extracellular signal-regulated kinase (ERK), p-ERK1/2, I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$ , Jun N-terminal protein kinase (JNK), p-JNK, p38, and p-p38 antibodies, as well as TNF and IL-1 $\beta$ , were obtained from Cell Signaling. Tubulin and p65 antibodies, as well as brefeldin A (BFA), were obtained from Santa Cruz. Chlorpromazine, cycloheximide, filipin, lipopolysaccharide (LPS) (*E. coli*), methyl- $\beta$ -cyclodextrin, MG132, phorbol 12-myristate 13-acetate (PMA), and clathrin heavy-chain (HC) antibody were obtained from Sigma.

**Bacterial strains and cell culture.** The bacterial strains used in this study are listed in Table 1. The intestinal epithelial cell lines HCT-8 and SW480, as well as the human monocyte-like cell line THP-1, were maintained at 37°C in 5% CO<sub>2</sub> in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). THP-1 cells were cultured in the presence of 50 nM PMA for 48 h to induce their differentiation into macrophage-like cells before their use.

**ETEC culture supernatants.** ETEC strains were grown overnight without shaking at 37°C in LB broth, diluted 1:50 into serum- and antibiotic-free RPMI 1640 medium, and grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.8. The bacterial culture was centrifuged (10,000  $\times$  g, 10 min), and the culture supernatant was filtered through

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TABLE 1 Bacterial strains used in this study

Strain	Relevant genotype or phenotype	Source or reference
ETEC H10407	O78:H11 CFA/I LT <sup>+</sup> ST <sup>+</sup> (ST-H and ST-P)	12
ETEC H10407 $\Delta$ eltA	$\Delta$ eltA mutant of ETEC H10407	9
ETEC H10407-P	Derivative of H10407 cured of the 92-kb pCS1 plasmid	14
ETEC B7A	O148:H28 CS6; LT <sup>+</sup> ST <sup>+</sup> (ST-H and ST-P)	11
ETEC E24377A	O139:H28 CS1 <sup>+</sup> CS3 <sup>+</sup> LT <sup>+</sup> ST-H <sup>+</sup>	39
ETEC 91.1626	O6 ST-P <sup>+</sup> LT <sup>+</sup>	C. DebRoy
ETEC 91.1033	O27 ST-P <sup>+</sup> LT <sup>-</sup>	C. DebRoy
ETEC 3030-2	K88ac LT <sup>+</sup> STb <sup>+</sup>	15
ETEC 1836-2	K88ac <sup>+</sup> EAST1 <sup>+</sup>	45
ETEC 2534-86	O8:K87:NM:F4ac LT <sup>+</sup> STb <sup>+</sup>	4
ETEC B41	Bovine isolate; O101:K99, F41 STa <sup>+</sup>	30
<i>E. coli</i> G58-1	O101:K28:NM	3
<i>Shigella flexneri</i> 2457T	Wild type; serotype 2a	16
<i>S. flexneri</i> 2457T(T3SS <sup>-</sup> ) <sup>a</sup>	Virulence plasmid-cured 2457T derivative	26

<sup>a</sup> Mutant deficient in type III secretion system function.

a 0.22- $\mu$ m filter and stored at 4°C. Where indicated, ETEC culture supernatants were preincubated (37°C, 1 h) with 10.0  $\mu$ g/ml proteinase K, 10.0  $\mu$ g/ml DNase I, 10.0  $\mu$ g/ml RNase A, or 5.0  $\mu$ g/ml polymyxin B. In some cases, culture supernatants were also heat inactivated (100°C, 20 min) and then cooled to room temperature before their incubation with HCT-8 cells. For size fractionation experiments, supernatants were passed through an Amicon Ultra-15 centrifugal filter unit with an Ultracel-3 membrane.

**Bacterial infection.** ETEC strains were grown overnight without shaking at 37°C in LB broth, diluted 1:50 into serum- and antibiotic-free RPMI 1640 medium, and grown to an OD<sub>600</sub> of 0.8. Before infections, cell culture medium was replaced with serum- and antibiotic-free RPMI 1640 medium. HCT-8 cells were infected with ETEC strains at a multiplicity of infection of ~50 for 2 h, after which the cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed for subsequent analyses.

**RNA interference.** SW480 cells were grown to 50% confluence on 6-well plates and transfected with either 200 pmol of negative-control small interfering RNAs (siRNAs) or siRNAs directed against the clathrin heavy chain (HC) (Santa Cruz) using Lipofectamine 2000. The transfection mixture was replaced with complete growth medium, Dulbecco's modified Eagle medium (DMEM), after 5 h, and cells were further incubated for 68 h prior to the performance of other experiments. Knockdown efficiency was quantified using immunoblotting.

**Immunoblotting.** Mammalian cell pellets were resuspended in ice-cold lysis buffer A (10.0 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 10.0 mM KCl, 0.5 mM dithiothreitol [DTT], 0.05% NP-40) supplemented with protease and phosphatase inhibitor cocktails (Thermo Scientific) and incubated on ice for 30 min. Lysates were centrifuged (17,000  $\times$  g, 4°C, 10 min), and the supernatant was retained as the cytosolic fraction. The pellet was washed with lysis buffer A and resuspended in buffer B (5.0 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol, 300 mM NaCl). This resuspended nuclear fraction was passed through a 25-gauge needle to shear genomic DNA and then incubated on ice for 30 min with intermittent vortexing. The supernatant, which contains nuclear proteins, was collected by centrifugation. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with Odyssey blocking buffer (LI-COR) at room temperature for 1 h, followed by incubation with primary antibodies overnight at 4°C. Membranes were washed three times in PBS plus 0.1% Tween and incubated with secondary antibodies for 2 h at room temperature. Immunoblots were developed using the Odyssey infrared imaging system. Poly(ADP-ribose) polymerase (PARP) and tubulin abundance were used to normalize protein loading for nuclear and cytoplasmic fractions, respectively.

**Immunoprecipitation.** I $\kappa$ B $\alpha$  immunoprecipitation was performed using an I $\kappa$ B $\alpha$  (L35A5) mouse monoclonal antibody (MAb)-Sepharose bead conjugate. HCT-8 cells were lysed with ice-cold lysis buffer A (10.0 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 10.0 mM KCl, 0.5 mM DTT, 0.05% NP-40) and centrifuged. The supernatant was incubated with anti-I $\kappa$ B $\alpha$  antibody-conjugated beads overnight at 4°C. After centrifugation, the beads were washed three times with buffer A and then resuspended in SDS-PAGE loading buffer for analysis.

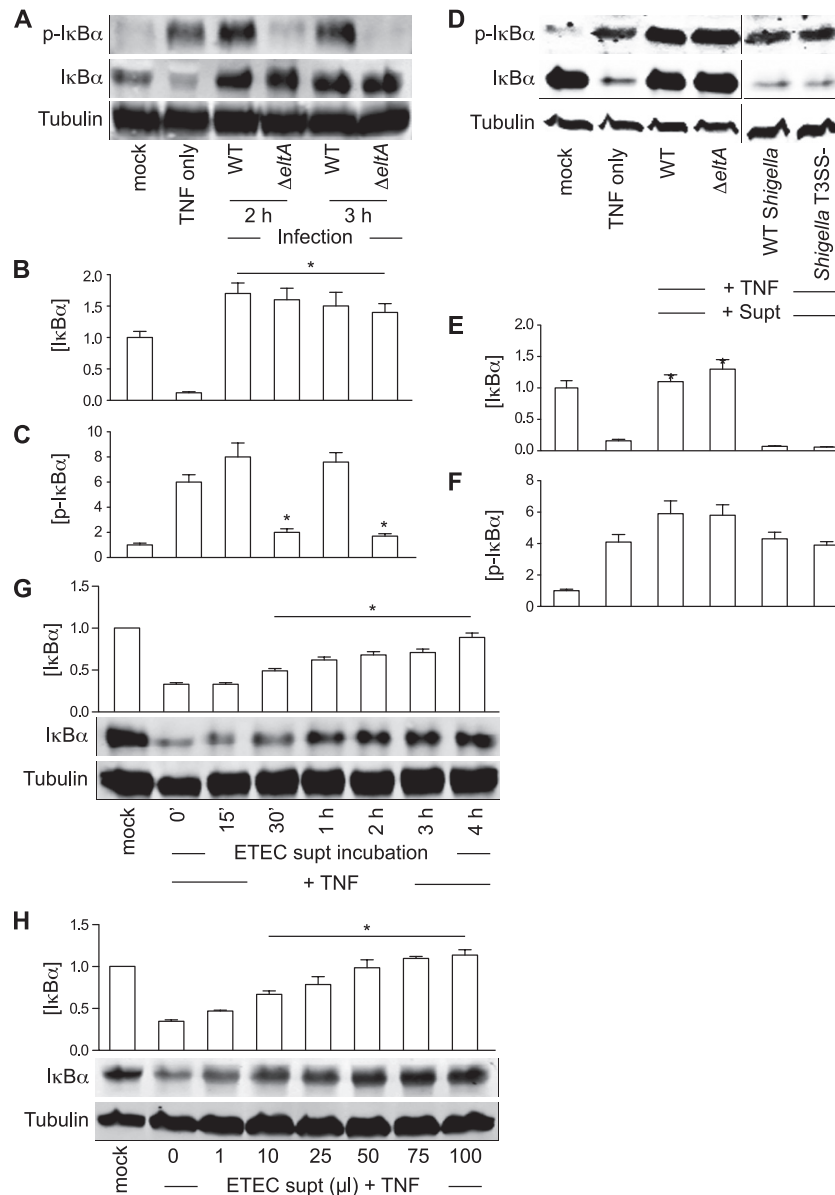
**Real-time quantitative reverse transcription-PCR (RT-PCR).** cDNA from HCT-8 cells was prepared by using a SYBR green cells-to-CT kit by following the manufacturer's instructions (Ambion). Real-time PCR was performed in a SYBR green PCR master mix (Applied Biosystems) with detection in a Fast 7500 system (Applied Biosystems) and with the following primers: for GAPDH, 5'-AC<sub>2</sub>AG<sub>2</sub>TG<sub>2</sub>TCTC<sub>2</sub>TCTGACT<sub>2</sub>C and 5'-G TG<sub>2</sub>TTCGT<sub>2</sub>GAG<sub>3</sub>CA<sub>2</sub>TG; for TNF, 5'-TGCTC<sub>2</sub>TCAC<sub>3</sub>ACAC<sub>2</sub>AT and 5'-G<sub>2</sub>AG<sub>2</sub>T<sub>2</sub>GAC<sub>2</sub>T<sub>2</sub>G<sub>2</sub>TCTG<sub>2</sub>TA. Relative transcription levels were calculated using the threshold cycle ( $\Delta\Delta C_T$ ) method.

**Luciferase assays.** HCT-8 cells were cotransfected at a ratio of 10:1 (2.0  $\mu$ g total DNA) with a firefly luciferase construct driven by a consensus  $\kappa$ B site, together with the *Renilla* luciferase plasmid (Promega). Approximately 48 h after transfection, cells were treated with ETEC supernatants for 2 h and then stimulated with 20 ng/ml TNF, 10 ng/ml IL-1 $\beta$ , or 500 ng/ml flagellin for 30 min. Firefly and *Renilla* luciferase levels were measured using a dual luciferase reporter system kit (Promega) according to the manufacturer's instructions.

**Statistics.** Throughout the manuscript, data are presented as means and standard errors of the means (SEM) ( $n \geq 3$ ), with asterisks used to indicate statistically significant differences ( $P < 0.05$ ; Student's *t* test), as specified in the figure legends.

## RESULTS

**ETEC supernatant inhibits I $\kappa$ B $\alpha$  degradation.** Many pathogens have evolved strategies to prevent NF- $\kappa$ B pathway activation. To determine whether ETEC can also modulate this pathway, we infected HCT-8 intestinal epithelial cells with the commonly studied human ETEC strain H10407 (12). We also infected cells with an H10407 mutant lacking the enzymatic subunit of the heat-labile enterotoxin LT ( $\Delta$ eltA), which exhibits an attenuated ability to induce NF- $\kappa$ B activation (43). Although infection of HCT-8 cells with wild-type (WT) ETEC induced significant I $\kappa$ B $\alpha$  phosphorylation, the subsequent degradation of I $\kappa$ B $\alpha$  was blocked (Fig. 1A to C). The positive control, TNF treatment, resulted in the expected phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$  (Fig. 1A to C).

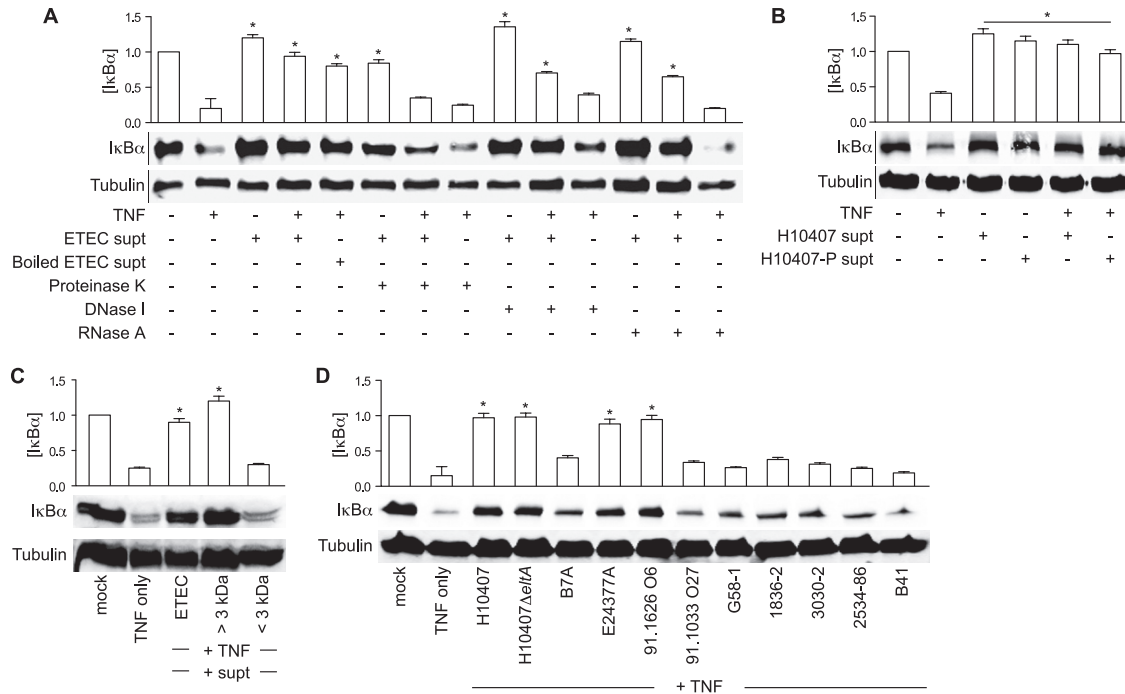


**FIG 1** ETEC supernatants protect I $\kappa$ B $\alpha$  from TNF-induced degradation. (A) Immunoblotting of total and phospho(Ser32/36)-I $\kappa$ B $\alpha$  after TNF stimulation or ETEC (WT or  $\Delta$ eltA mutant) infection. Tubulin immunoblotting was used to normalize protein abundance for quantification. (B and C) Quantification of I $\kappa$ B $\alpha$  and phospho(Ser32/36)-I $\kappa$ B $\alpha$  abundance from data shown in panel A. Asterisks indicate protein abundance significantly different from that in the TNF-only lane. (D) Immunoblotting of total and phospho-I $\kappa$ B $\alpha$  after incubating HCT-8 cells with ETEC or *Shigella* supernatants (supt) and then stimulating the cells with TNF. (E and F) Quantification of I $\kappa$ B $\alpha$  and phospho(Ser32/36)-I $\kappa$ B $\alpha$  abundance from data shown in panel D. Asterisks indicate protein abundance significantly different from that in the TNF-only lane. (G) I $\kappa$ B $\alpha$  protection as a function of incubation time with the ETEC supernatant. Asterisks indicate protein abundance significantly different from that in the TNF-only lane. (H) I $\kappa$ B $\alpha$  protection as a function of ETEC supernatant volume. Asterisks indicate protein abundance significantly different from that in the TNF-only lane.

We speculated that ETEC H10407 secretes a virulence factor that blocks I $\kappa$ B $\alpha$  degradation. To test this idea, we incubated HCT-8 cells with cell-free ETEC supernatants for 2 h and then stimulated the cells with TNF. For comparison with an enteric pathogen that induces significant host inflammation, we also used WT *Shigella flexneri* and an *S. flexneri* mutant deficient in type III secretion system (T3SS) function (24). Pretreating HCT-8 cells with ETEC H10407 supernatants (both the WT and the  $\Delta$ eltA mutant) completely blocked TNF-induced I $\kappa$ B $\alpha$  degradation but not I $\kappa$ B $\alpha$  phosphorylation (Fig. 1D to F). In

contrast, pretreating HCT-8 cells with *S. flexneri* supernatants did not block I $\kappa$ B $\alpha$  degradation (Fig. 1D to F). I $\kappa$ B $\alpha$  abundance after TNF treatment increased as a function of both the time (Fig. 1G) and the volume of the ETEC supernatant (Fig. 1H) incubated with HCT8 cells.

**ETEC secreted factor has heat-stable, protein-like qualities and is expressed by multiple human ETEC strains.** To determine the biological characteristics of the ETEC secreted factor (ESF), we treated ETEC supernatants with proteinase K, DNase I, or RNase A and then tested these treated supernatants for



**FIG 2** ESF has heat-stable, proteinaceous qualities. (A) I $\kappa$ B $\alpha$  immunoblotting after treating cells with TNF or with ETEC supernatants first incubated with either proteinase K, boiling, DNase I, or RNase A. (B) I $\kappa$ B $\alpha$  immunoblotting after treating cells with TNF and supernatants derived from ETEC H10407 possessing or lacking the major virulence plasmid (H10407-P). (C) I $\kappa$ B $\alpha$  immunoblotting after treating cells with ETEC supernatant subjected to size fractionation (3-kDa cutoff). (D) I $\kappa$ B $\alpha$  immunoblotting after treating cells with TNF and supernatants derived from the indicated human, porcine, and bovine ETEC strains. For all quantitative data in this figure, asterisks indicate I $\kappa$ B $\alpha$  abundance significantly different from that in the TNF-only lane.

their ability to inhibit TNF-induced I $\kappa$ B $\alpha$  degradation. Proteinase K treatment completely abrogated the ability of ESF to block TNF-induced I $\kappa$ B $\alpha$  degradation. In contrast, DNase I or RNase A had no impact on ESF activity, suggesting that the secreted factor is a protein (Fig. 2A). We also incubated ETEC supernatants at 100°C for 20 min and then retested their activity. Boiled ETEC supernatant still retained an inhibitory activity against TNF-induced I $\kappa$ B $\alpha$  degradation, suggesting that the active protein is heat stable (Fig. 2A).

We tested whether a strain of ETEC cured of the large pCS1 virulence plasmid (H10407-P) retained the inhibitory activity. This CFA/I-encoding plasmid also encodes the heat-stable toxin H (ST-H/ST-1b) and other recently identified ETEC adhesins (14). We found that H10407-P supernatants still blocked TNF-induced I $\kappa$ B $\alpha$  degradation (Fig. 2B), suggesting that ESF is not encoded on this virulence plasmid. By fractionating the ETEC supernatant by size exclusion, using Amicon filtration devices with a 3-kDa cutoff, we determined that ESF is larger than 3 kDa (Fig. 2C), suggesting that the activity is unlikely to be attributable to the small ETEC enterotoxins (34).

To determine whether the secreted protein is conserved among other ETEC strains, we prepared culture supernatants from different ETEC isolates and tested their activity. Supernatants from the sequenced human ETEC isolate E24377A (39) as well as a relatively uncharacterized human isolate (91.1626; C. DeRoy, personal communication) also inhibited TNF-induced I $\kappa$ B $\alpha$  degradation (Fig. 2D). In contrast, the human ETEC isolate 91.1033 and ETEC strains of porcine (1836-2, 3030-2, 2534-86) and bovine (B41) tropism did not block TNF-induced I $\kappa$ B $\alpha$  degradation (Fig. 2D). Another sequenced hu-

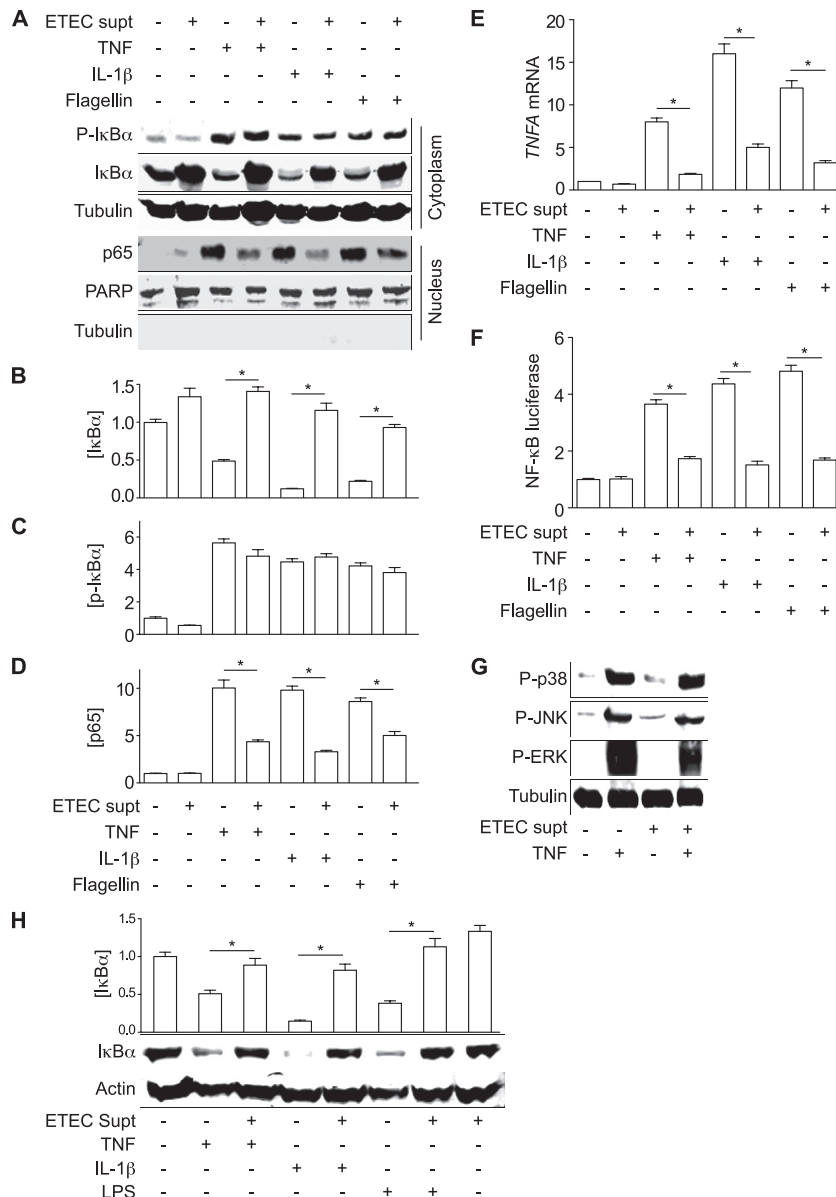
man ETEC isolate, B7A (11), had no significant impact on TNF-induced I $\kappa$ B $\alpha$  degradation. We failed to detect the inhibitory activity in both the laboratory *E. coli* strain BL21 (not shown) and a porcine commensal *E. coli* isolate (G58-1) (3) (Fig. 2D). Thus, some strains of ETEC of significance to human health appear to encode the ability to inhibit TNF-induced I $\kappa$ B $\alpha$  degradation while others do not.

**ESF inhibits NF- $\kappa$ B nuclear translocation and transcriptional activity.** I $\kappa$ B $\alpha$  degradation is required to activate NF- $\kappa$ B. We next examined whether ESF can block NF- $\kappa$ B nuclear translocation and subsequent transcriptional activity. NF- $\kappa$ B can be activated by different stimuli, including TNF, IL-1 $\beta$ , and microbial products such as flagellin (1, 10). We found that ETEC H10407 supernatants blocked the degradation of phospho-I $\kappa$ B $\alpha$  induced by TNF, IL-1, and flagellin (Fig. 3A to D).

We next determined whether ESF-mediated blockage of I $\kappa$ B $\alpha$  degradation was sufficient to inhibit the nuclear translocation of the NF- $\kappa$ B p65 subunit. Stimulation of HCT-8 cells with TNF, IL-1 $\beta$ , or flagellin induced NF- $\kappa$ B p65 nuclear translocation, whereas pretreating cells with ETEC supernatant significantly blocked p65 nuclear translocation (Fig. 3A to D).

We also examined the impact of ESF on NF- $\kappa$ B-dependent transcriptional activity induced by different stimuli. HCT-8 cells incubated with ETEC supernatants exhibited a lower level of TNF- $\alpha$  gene induction, irrespective of the stimulus (Fig. 3E). Data from luciferase reporter assays also showed that pretreating HCT-8 cells with ETEC supernatant significantly suppressed NF- $\kappa$ B transcriptional activity (Fig. 3F).

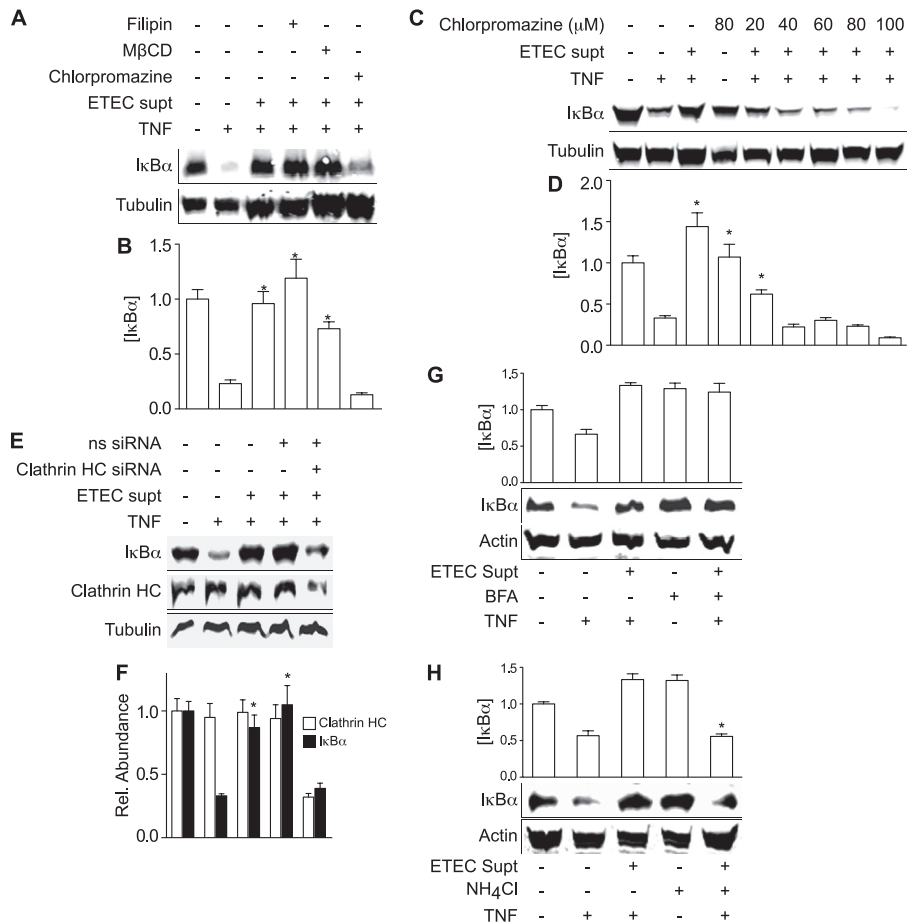
MAPK signaling also plays important roles in regulating host innate immunity (8, 38). We also examined whether ESF also



**FIG 3** ESF inhibits NF- $\kappa$ B nuclear translocation and transcriptional activity. (A) Immunoblotting of total and phospho(Ser32/36)-I $\kappa$ B $\alpha$ , as well as nucleus-localized NF- $\kappa$ B p65, after pretreating HCT-8 cells with ETEC supernatant for 1.5 h and then stimulating the cells with either TNF (20 ng/ml), IL-1 $\beta$  (10 ng/ml), or flagellin (200 ng/ml) for 30 min. PARP and tubulin immunoblotting were used to normalize nuclear protein concentrations and to demonstrate the absence of contaminating cytoplasmic proteins in the nuclear fractions, respectively. (B to D) Quantification of I $\kappa$ B $\alpha$ , phospho(Ser32/36)-I $\kappa$ B $\alpha$ , and p65 abundance from data shown in panel A. Asterisks indicate significantly different protein abundance between the specified pairwise comparisons. (E) RT-PCR analysis of TNF- $\alpha$  gene (*TNFA*) transcription after treating cells with TNF, IL-1 $\beta$ , or flagellin, with or without the ETEC supernatant. Asterisks indicate significantly different protein abundance between the specified pairwise comparisons. (F) NF- $\kappa$ B luciferase activity after treating cells with TNF, IL-1 $\beta$ , or flagellin, with or without the ETEC supernatant. Asterisks indicate significantly different protein abundance between the specified pairwise comparisons. (G) Immunoblotting of phospho-p38, phospho-JNK, and phospho-ERK1/2 after ETEC supernatant and TNF treatment. (H) I $\kappa$ B $\alpha$  immunoblotting after treating differentiated THP-1 cells with TNF (20 ng/ml), IL-1 $\beta$  (10 ng/ml), or LPS (1  $\mu$ g/ml) for 30 min, with or without the ETEC supernatant. Asterisks indicate significantly different protein abundance between the specified pairwise comparisons.

targets the host MAPK signaling pathway. Pretreatment of HCT-8 cells with ETEC supernatant did not inhibit TNF-induced MAPK activation (Fig. 3G). Similar to its impact on intestinal epithelial cells, ESF also inhibited the degradation of I $\kappa$ B $\alpha$  in the macrophage-like THP-1 cell line when these cells were stimulated with TNF, IL-1 $\beta$ , or LPS (Fig. 3H). Taken together, these data indicate that one or more ETEC H10407 secreted factors subvert NF- $\kappa$ B activation by blocking I $\kappa$ B $\alpha$  degradation.

**ESF utilizes a clathrin-dependent endocytosis pathway.** We next examined whether ESF enters intestinal epithelial cells through an endocytic pathway. Treating HCT-8 cells with the clathrin-dependent endocytosis inhibitor chlorpromazine, but not with the lipid raft-dependent endocytosis inhibitors filipin or methyl- $\beta$ -cyclodextrin (M $\beta$ CD), prevented the ETEC supernatant from blocking TNF-induced I $\kappa$ B $\alpha$  degradation (Fig. 4A and B). The ability of chlorpromazine to prevent the ETEC superna-



**FIG 4** Cellular uptake of ESF. (A) IκBα immunoblotting after pretreating HCT-8 cells with ETEC supernatant for 1.5 h and then stimulating the cells with TNF (20 ng/ml) for 30 min. Where indicated, HCT-8 cells were first treated with filipin (7 μM), MβCD (15 mM), or chlorpromazine (50 μM) for 30 min before adding ETEC supernatant. (B) Quantification of data shown in panel A (mean ± SEM; *n* = 3). (C) IκBα protection as a function of chlorpromazine treatment. (D) Quantification of data shown in panel C (mean ± SEM; *n* = 3). (E) IκBα and clathrin heavy chain (HC) immunoblotting. Where indicated, HCT-8 cells were first transfected with an siRNA duplex targeting clathrin HC or a nonspecific (ns) siRNA duplex. Tubulin immunoblotting was used to normalize protein concentrations. (F) Quantification of data shown in panel E (mean ± SEM; *n* = 3). For all quantitative data in this figure, asterisks indicate IκBα abundance significantly different from that in the TNF-only lane. (G) IκBα immunoblotting after pretreating SW480 cells with BFA (500 ng/ml) for 6 h and then with ETEC supernatant for 2 h, followed by TNF for 20 min. (H) IκBα immunoblotting after pretreating SW480 cells with NH<sub>4</sub>Cl (20 mM) for 6 h and then with ETEC supernatant for 2 h, followed by TNF for 20 min.

tant from protecting against TNF-induced IκBα degradation was dose dependent (Fig. 4C and D), suggesting that ESF may enter intestinal epithelial cells through clathrin-dependent endocytosis.

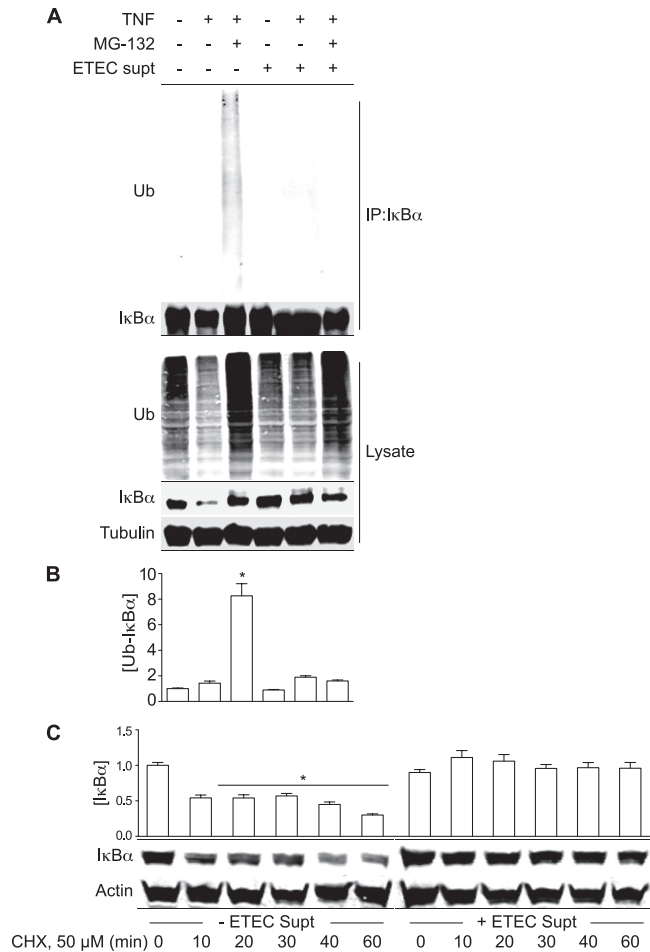
To substantiate further the role of clathrin in the uptake of ESF, we also used siRNA to knock down clathrin heavy chain (HC) abundance in intestinal epithelial cells. We used the SW480 cell line for siRNA transfection, as this cell line has high transfection efficiency. Knocking down clathrin HC levels by ~65% significantly abrogated the ability of ETEC supernatant to block TNF-induced IκBα degradation (Fig. 4E and F).

We also performed a preliminary investigation of how ESF might traffic within intestinal epithelial cells. Pretreating cells with brefeldin A (BFA), an inhibitor of Golgi-endoplasmic reticulum (ER) trafficking (25), did not abrogate the ability of ESF to block TNF-induced IκBα degradation (Fig. 4G). In contrast, preincubating cells with NH<sub>4</sub>Cl, an inhibitor of endosomal acidification (7), significantly reduced the inhibitory activity of ESF (Fig. 4H). Thus, these data suggest that endosomal acidification but not Gol-

gi-ER trafficking is important for ESF translocation after its delivery into host cells.

**ESF inhibits IκBα polyubiquitination.** Because ESF blocks IκBα degradation, but not its phosphorylation, we hypothesized that it might disrupt the polyubiquitination of phosphorylated IκBα, thus affecting the subsequent recognition and degradation of IκBα by the 26S proteasome. We used immunoprecipitation to examine the levels of polyubiquitinated IκBα in treated intestinal epithelial cells. Pretreatment of HCT-8 cells with MG132, a potent inhibitor of the 26S proteasome, significantly blocked TNF-induced IκBα degradation and thus resulted in the accumulation of polyubiquitinated IκBα (Fig. 5A and B, lane 3). In contrast, pretreating the HCT-8 cells with ETEC supernatant did not increase IκBα polyubiquitination, although IκBα degradation was significantly blocked (Fig. 5A and B, lane 5). Coincubation with both ESF and MG132 failed to result in the accumulation of polyubiquitinated IκBα (Fig. 5A and B, lane 6).

We also considered the possibility that ESF might act by in-



**FIG 5** ESF prevents I $\kappa$ B $\alpha$  ubiquitination. (A) HCT-8 cells were pretreated with ETEC supernatant and then stimulated with TNF (20 ng/ml) for 30 min. Cell lysates were immunoprecipitated using an I $\kappa$ B $\alpha$  antibody and immunoblotted for both ubiquitin (Ub) and I $\kappa$ B $\alpha$ . Where indicated, HCT-8 cells were first treated with MG132 (50  $\mu$ g/ml). (B) Quantification of ubiquitinated I $\kappa$ B $\alpha$  from immunoprecipitated samples (mean  $\pm$  SEM;  $n = 3$ ). Ubiquitinated I $\kappa$ B $\alpha$  intensity was normalized to immunoprecipitated I $\kappa$ B $\alpha$  abundance. Asterisks indicate I $\kappa$ B $\alpha$  abundance significantly different from that in the TNF-only lane. (C) I $\kappa$ B $\alpha$  immunoblotting after pretreating SW480 with 50  $\mu$ M cycloheximide (CHX) for the indicated times. Where indicated, cells were first incubated with ETEC supernatant (2 h) before the addition of CHX.

creasing I $\kappa$ B $\alpha$  synthesis rather than by blocking I $\kappa$ B $\alpha$  degradation. To test this idea, we treated HCT-8 cells with the protein synthesis inhibitor cycloheximide (CHX) in the presence or absence of ESF. As expected, treating cells with 50  $\mu$ M CHX significantly reduced I $\kappa$ B $\alpha$  protein abundance as a function of time (Fig. 5C, left). However, in the presence of ESF, I $\kappa$ B $\alpha$  protein levels were maintained over the course of CHX treatment, suggesting that ESF prevented the degradation of previously synthesized I $\kappa$ B $\alpha$  (Fig. 5C, right) rather than promoting I $\kappa$ B $\alpha$  synthesis. Overall, these data suggest that ESF affects the NF- $\kappa$ B pathway at a point involving I $\kappa$ B $\alpha$  polyubiquitination.

## DISCUSSION

We have discovered that ETEC suppresses host innate immune responses by targeting the NF- $\kappa$ B signaling pathway. An ETEC

secreted factor (ESF) with heat-stable, protein-like qualities blocks NF- $\kappa$ B activation by disrupting the polyubiquitination and subsequent degradation of I $\kappa$ B $\alpha$ . NF- $\kappa$ B plays a crucial role in mediating intestinal immune response to enteric pathogens (32). Activation of this immune signaling pathway by bacterial infection elicits a large number of protective mechanisms against infection (5). Many pathogens have evolved strategies to modulate NF- $\kappa$ B signaling (32), and our data provide evidence that ETEC has also evolved a strategy to evade innate responses by targeting NF- $\kappa$ B.

ETEC H10407 is a highly virulent strain originally isolated from a patient with severe diarrhea in Bangladesh (12) that has been commonly used in challenge experiments for studying ETEC pathogenesis (20). ETEC H10407 inhibition of innate responses might contribute to the high virulence of this strain. We also found that two other human ETEC isolates, but not any porcine or bovine isolates, block TNF-induced I $\kappa$ B $\alpha$  degradation. Although we examined a limited number of ETEC isolates, including some that have not been extensively characterized, we suggest that the factor responsible for suppressing NF- $\kappa$ B activation is not conserved in all ETEC strains. However, our data do not distinguish between the possibilities that ESF is either unique to ETEC or encoded by other enteric pathogens.

The boiling of ETEC supernatants did not abrogate their ability to inhibit TNF-induced I $\kappa$ B $\alpha$  degradation, suggesting that ESF is a heat-resistant factor. Most human ETEC isolates encode colonization factor antigens (CFAs), LT, and heat-stable toxins, such as ST-H (ST-1b) and ST-P (ST-1a). Most of these characterized virulence factors are encoded on multiple plasmids (12, 13), although some virulence determinants, such as the type two secretion system (T2SS), are chromosomally encoded. Curving the large pCS1 virulence plasmid from H10407 (H10407-P) did not affect the ability of ETEC to block TNF-induced I $\kappa$ B $\alpha$  degradation, suggesting that ESF is not encoded on this virulence plasmid. In addition, some ETEC isolates tested in our study that did not block TNF-induced I $\kappa$ B $\alpha$  degradation express ST-1a/ST-P, suggesting that this heat-stable toxin may not be involved in producing the activity we measured. Furthermore, fractionating the supernatant to remove small proteins (<3 kDa) also failed to abrogate ESF activity. Taken together, our data suggest the possibility that ESF may be a previously uncharacterized ETEC protein. However, they do not definitively rule out the possibility that some of the phenotypes we observe are due to the activity of one or more previously characterized heat-stable toxins.

Not only ETEC infection but also cell-free ETEC culture supernatants blocked TNF-induced I $\kappa$ B $\alpha$  degradation. Further investigation showed that clathrin-mediated endocytosis is involved in the uptake of ESF into intestinal epithelial cells. Although ETEC lacks a T3SS, this pathogen has evolved other mechanisms to deliver virulence proteins into host cells. ETEC uses a T2SS to secrete LT and other virulence factors (40). However, deleting T2SS function from ETEC had no effect on its ability to inhibit I $\kappa$ B $\alpha$  degradation (data not shown).

Many bacterial toxins enter host cells by receptor-mediated endocytosis and then translocate to the cytoplasm by exploiting intracellular trafficking pathways to reach their cytosolic targets (33). Disrupting Golgi-ER trafficking with BFA did not alter the ability of ESF to block TNF-induced I $\kappa$ B $\alpha$  degradation, suggesting that ESF trafficking is independent of Golgi-ER trafficking. However, inhibiting endosomal acidification

with NH<sub>4</sub>Cl significantly reduced ESF activity, suggesting that endosomal acidification may be involved in ESF trafficking. These studies are limited by the fact that we have not yet identified the source(s) of the ESF activity.

EPEC supernatants blocked TNF-, IL-1β-, and flagellin-induced IκBα degradation but not IκBα phosphorylation, suggesting that the secreted protein is a general inhibitor of the NF-κB pathway that prevents IκBα ubiquitination. Additionally, EPEC supernatants were active against NF-κB pathway agonists (TNF, IL-1β, LPS) in multiple cell types, including the macrophage-like THP-1 cell line.

Upon stimulation with microbial products or cytokines, phosphorylated IκBα is recognized by the β-TrCP E3 ubiquitin ligase and is polyubiquitinated by the host ubiquitination machinery (23, 36). It is possible that ESF disrupts IκBα polyubiquitination by interacting with the host ubiquitination machinery. The *Shigella* virulence protein OspG also blocks IκBα polyubiquitination by interacting with multiple ubiquitin-conjugating enzymes (24). However, we cannot rule out the possibility that ESF might instead affect IκBα ubiquitination indirectly. Identifying and characterizing the biochemical mechanism of action of ESF in future studies will undoubtedly clarify these issues. Dysregulated NF-κB signaling is involved in the pathogenesis of numerous chronic inflammatory diseases. Ultimately, one might envision utilizing bacterial or viral proteins as starting points for developing novel anti-inflammatory compounds.

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