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Immunology: Proteasome-independent Degradation of Canonical NF κB Complex Components by the NleC Protein of Pathogenic Escherichia coli

Sabrina Mühlen, Marie-Hélène Ruchaud-Sparagano and Brendan Kenny

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The NFκB transcription factor is a key component of immune and inflammatory signaling as its activation induces the expression of antimicrobial reagents, chemokines, cytokines, and anti-apoptotic factors. Many pathogens encode effector proteins that target factors regulating NFκB activity and can provide novel insights on regulatory mechanisms. Given the link of NFκB dysfunction with inflammatory diseases and some cancers, these effectors have therapeutic potential. Here, screening enteropathogenic Escherichia coli proteins for those implicated in suppressing NFκB function revealed that eGFP-NleC, unlike eGFP, strongly inhibited basal and TNFα-induced NFκB reporter activity to prevent secretion of the chemokine, IL-8. Work involving NleC variants, chemical inhibitors, and immunoprecipitation studies support NleC being a zinc metalloprotease that degrades NFκB-IκBα complexes. The findings are consistent with features between residues 33–65 recruiting NFκB for proteasomal-independent degradation by a mechanism inhibited by metalloprotease inhibitors or disruption of a consensus zinc metalloprotease motif spanning NleC residues 183–187. This raises the prospect that mammalian cells, or other pathogens, employ a similar mechanism to modulate NFκB activity. Moreover, NleC represents a novel tool for validating NFκB as a therapeutic target and, indeed, as a possible therapeutic reagent.
that NleC is a zinc metalloprotease that recruits NFκB complexes for degradation.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Transfection*—HeLa cells (ATCC CCL-2) were maintained at 37 °C in DMEM (Invitrogen) supplemented with 10% fetal calf serum in a 5% CO₂ environment. HeLa cells seeded in 12- or 24-well plates (Nunc) were grown for 24 h (to ~80% confluence) prior to introducing mammalian expression vectors by transfection using jetPRIME (PEQLAB Ltd.) following the manufacturer’s recommendations. Transfection efficiency was routinely measured using a FLUOstar Optima 413-3266 (BMG Labtech). Transfection efficiency was routinely measured using a FLUOstar Optima 413-3266 (BMG Labtech).

*Plasmids*—nleC gene constructs, including substitution and truncation variants thereof, were all generated by PCR and cloned into pEFGP-C1 (Clontech) using EcoRI/PstI restriction sites, with desired gene product confirmed by DNA sequencing (GATC Biotech). Studies included plasmids encoding the NleB luciferase reporter protein (22) and IKK pathway components TRAF2, IKKα, IKKβ (23, 24), and p65 (kindly provided by professor Neil D. Perkins, Newcastle University).

*Luciferase and IL-8 Assays*—Approximately 24 h post-transfection, HeLa cells were either left untreated or treated with TNFα (final concentration, 50 ng/ml) for 30 min prior to addition of luciferase cell culture lysis buffer (Stratagene) following the manufacturer’s protocol. Luciferase levels were measured using a FLUOstar Optima 413-3266 (BMG Labtech) plate reader with 96-well plates containing 100 μl luciferase assay solution (Promega) and 25 μl lysis per well. By contrast, IL-8 levels in HeLa supernatants were determined using the OptEIA™ Human IL-8 ELISA (BD Biosciences) kit following the manufacturer’s recommendations. These assays were routinely carried out ~48 h post-transfection with or without an additional 3-h incubation with TNFα (final concentration, 50 ng/ml).

*Co-immunoprecipitation*—For co-immunoprecipitation, cells were transfected with plasmids encoding eGFP or eGFP-fusion proteins as described above. The cells were lysed under native conditions, and proteins were immunoprecipitated using GFP-Trap® beads (Chromotek) according to the manufacturer’s recommendations.

*Western Blot Analyses*—Cells were either lysed using 1× SDS sample buffer or in PBS with 1% Triton and protease inhibitors for subsequent fractionation as described previously (25). Samples were equalized for protein content, separated via SDS-PAGE (10–12%), and transferred onto nitrocellulose membrane. Membranes were blocked in 5% skimmed milk powder in Tris-buffered saline, pH 7.5, containing 0.1% Tween (TBST) for 1 h followed by an overnight incubation at 4 °C in 5% BSA/TBST solution containing the appropriate antibody. Membranes were washed and incubated in 5% skim milk/TBST containing the respective HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Blots were developed in SuperSignal West Pico chemiluminescent substrate (Pierce) according to the manufacturer’s recommendations, and the signals were detected on Hyperfilm ECL (Amersham Biosciences). Primary antibodies used were NFκB p65, NFκB p50, IκBα, IKKα/β (Santa Cruz Biotechnology), p65, phospho-p65 (Cell Signaling Technologies), actin (Sigma), and GFP (Zymed Laboratories Inc.).

**RESULTS**

*NleC Effector Inhibits NFκB Reporter Activity and IL-8 Secretion*—Bioinformatic analyses of the EPEC genome identified 21 putative effector genes (26, 27), of which one or more are predicted to inhibit NFκB function (25). Our previous work argued against critical roles for the Tir, Map, EspF-H, EspZ, EspG2, NleA, NleF, and NleH effectors (25), so other effector-encoding genes were cloned into mammalian expression vectors for screening. NFκB assays involved co-transfection studies using empty control plasmid (pEFGP-C1) and a vector encoding the luciferase protein under the transcriptional control of NFκB activity in HeLa cells mediated by multiple signaling pathways, as the “NFκB activation inhibitor” (Calbiochem; product no. 481406) reduced luciferase levels by only ~60% (Fig. 1A). Co-transfection studies confirmed basal NFκB activity in pEFGP-transfected cells, with a dramatic reduction (~75%) in cells transfected with the eGFP-NleC-expressing plasmid (Fig. 1B). Western blot analysis revealed similar eGFP and eGFP-NleC expression levels (Fig. 1C). TNFα induction of NFκB activity increased luciferase levels in control cells (~6-fold) but not eGFP-NleC-expressing cells (Fig. 1B). To probe the relationship of this inhibition to NFκB function, cells were assayed for IL-8 secretion levels, as the IL-8 gene is under the transcriptional control of NFκB (28). Consistent with the luciferase assay data, eGFP-expressing cells exhibited low basal levels of IL-8 secretion with reduced levels from eGFP-NleC expressing cells (Fig. 1D). Furthermore, TNFα treatment increased IL-8 secretion levels in control cells (~3-fold) but not eGFP-NleC-expressing cells (Fig. 1D). Thus, the nleC gene product of EPEC has a potent capacity to inhibit NFκB activity when expressed within host cells as an N-terminally tagged eGFP fusion protein.

*NleC Inhibits NFκB Function at Level of Its Constituent Proteins*—Ectopic expression of NleC inhibits basal NFκB luciferase activity by 75–80% compared with ~60% for the NFκB activation inhibitor (Fig. 1B versus 1A), thereby suggesting that NleC inhibits signaling through multiple pathways. As signaling to NFκB converges at the level of IKK complex activation, it was predicted that NleC targets proteins at or below the IKK complex. To examine this, HeLa cells were co-transfected with vectors encoding (i) IKK complex components, (ii) the luciferase reporter protein, and (iii) eGFP or eGFP-NleC. Examination of reporter-gene expression (Fig. 2A) revealed that luciferase activity driven by plasmid expression of IKKα or IKKβ was dramatically reduced (~95%) in pEFGP-NleC, compared with pEFGP transfected cells
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Consistent with this finding was a similar NleC-specific decrease in luciferase activity driven by plasmid expression of an upstream NFκB pathway component; the TNF receptor-associated factor 2 (TRAF2; Fig. 2A). Thus, the data support the contention that NleC interferes with NFκB function by targeting the IKKα/β complex, or downstream components such as 1κB or NFκB itself. Western blot analysis failed to detect NleC-mediated changes in the cellular level or phosphorylation status of IKK (Fig. 2B and data not shown).

By contrast, the analysis revealed a dramatic decrease in the cellular levels of the NFκB components, p65 and p50, relative to the actin loading control (Fig. 2B), thereby suggesting that NleC targets the transcription factor for degradation. In support of this premise, luciferase activity induced by plasmid expression of p65 was, in essence, abolished in pEGFP-NleC-transfected cells (Fig. 2C).

Critical Role for NleC Residues 237–266 in Cellular Loss of p65 and p50—Bioinformatic analyses of the 330-amino acid residue NleC protein failed to provide clues on how it might induce the cellular loss of NFκB components. Hence, a series of C-terminal truncations were generated in an attempt to define regions critical for the process. Fig. 3A provides a schematic of the variants used in co-transfection studies with the NFκB luciferase reporter construct. These studies confirmed the potent inhibitory activity of full-length NleC, whereas similar findings with the NleC1–298 and NleC1–266 variants revealed that the C-terminal 64 residues are dispensable (Fig. 3B). However, removing another 29 (NleC1–237 variant) or more residues (Fig. 3A) abolished the inhibitory activity of NleC (Fig. 3B). Western blot analysis confirmed the expression of each variant (Fig. 3C) and also unlinked inhibitory defects from reduced protein levels, as one of the poorest expressed proteins (pEGFP-NleC1–266) inhibited luciferase activity nearly as effectively as NleC (Fig. 3, A and C). This suggests that feature(s) located between residues 237 and 266 are required, directly or indirectly, for the inhibitory process. However, the C-terminal NleC domain is not sufficient for the inhibitory process. This work implies that feature(s) located between residues 237 and 266 play a critical, direct or indirect, role in enabling NleC to induce the targeted loss of NFκB complex components from the host cell.
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*NleC Induces Proteasome-independent Degradation of p65 and p50—Several mechanisms have been described for regulating NFκB function, including proteasome-dependent degradation and p65 processing by caspase and serine proteases (7–9). To examine whether NleC is exploiting or mimicking these processes, Western blot analyses were carried out with antibodies against N- or C-terminal p65 domains using extracts from cells treated, or not, with the MG132 proteasome inhibitor. This drug reduced basal NFκB activity of control and pEGFP-NleC transfected cells by ~50% (Fig. 4A) suggesting that the NleC inhibitory process occurs independently of the proteasomal degradation system. Moreover, the decrease in luciferase activity implies that proteasomal degradation antagonizes the NleC inhibitory process. Western blot analysis confirmed that MG132 treatment failed to prevent NleC-mediated cellular loss of p65 or p50 (Fig. 4B) and verified the ability of the drug to inhibit IκB degradation as it allowed phospho-IκB to accumulate within TNFα-treated control cells (Fig. 4C). Interestingly, probing for IκB in extracts from non-stimulated HeLa cells revealed its cellular loss, unlike upstream IKK, from cells expressing eGFP-NleC, but not eGFP or the NleC1–237 variant that does not induce cellular loss of p50 or p65 (Fig. 4D). Thus, NleC appears to induce the proteasome-independent loss of p50, p65, and IκBα. Furthermore, the inhibitory process appears to be independent of known p65 processing events, as probing cellular extracts with antibodies that specifically recognize the N- or C-terminal domains failed to identify p65 processed forms (Fig. 4B and data not shown). Collectively, these data suggest that NleC targets p65, p50, and IκBα for proteasome-independent degradation.

*N-terminal NleC Features Are Required to Recruit NFκB—* To test whether NleC interacts with NFκB, GFP-Trap beads were used to isolate eGFP, eGFP-NleC, and the eGFP-NleC1–237 fusion protein that does not induce NFκB degradation. Probing immunoprecipitates revealed that p65 and p50 interact, directly or indirectly, with NleC as they can be isolated with eGFP-NleC1–237 but not eGFP (Fig. 5A). The absence of p65 or p50 from eGFP-NleC co-immunoprecipitates (Fig. 5A) presumably reflects their rapid degradation. Thus, features located between NleC residues 1 and 237 are involved in recruiting NFκB components for degradation. To define domain(s) required in this interaction process, a series of N-terminal truncations was generated and screened in the luciferase reporter assay. Fig. 5B provides an illustration of the generated variants, with screening data revealing a dispensable role for the first 32 residues (Fig. 5C). By contrast, features located between residues 33 and 64 (NleC65–330) are critical for the inhibitory process (Fig. 5C) and, thus, all remaining variants failed to inhibit luciferase activity (data not shown). Western blot analyses confirmed variant expression, with the ability of eGFP-NleC65–330 to induce the cellular loss of a NFκB component illustrated (Fig. 5D and data not shown). Importantly, p65 and p50 could be co-immunoprecipitated with the eGFP-NleC1–66 but not eGFP-NleC65–330 variant (see Fig. 6C) revealing that N-terminal features (apparently located between residues 33–64) play a critical and sufficient role in recruiting NFκB.

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**FIGURE 3. Key role for NleC residues 237–266 in inducing cellular loss of p65 and p50.** A, schematic of NleC C-terminal truncation variants constructed and screened in the NFκB luciferase reporter assay. B, relative luciferase activity of cells expressing NleC, and variants thereof, relative to pEGFP-transfected cells. NleC1–266, but not NleC1–237, inhibits NFκB luciferase reporter activity revealing a critical role for residues between 237–266. Data shown are mean (±S.D.) of three experiments done in triplicate with level of significance (Student’s t test) indicated. *, p ≤ 0.05; ***, p ≤ 0.005 as compared with empty vector control. C, representative immunoblot probed for p65 (and phosphorylated activation-associated form; pp65), p50, IKK (α and β), actin, and GFP, that links loss of NFκB luciferase reporter activity with an inability to deplete p65 and p50 from HeLa cells. aa, amino acids.
NleC Acts as a Zinc Metalloprotease—To provide insight into the degradation process a variety of nonspecific and specific inhibitors (including those of calpain, caspase, and autophagy proteases) were screened for their ability to prevent NleC-mediated cellular loss of p65. Importantly, only one reagent, a commercial inhibitor mixture (Sigma; product no. P8340) led to the detection of NleC and p65 in the same cellular extract and hence prevented degradation (data not shown). Interestingly, this inhibition was replicated by EDTA as illustrated in NFκB reporter luciferase assay (Fig. 6B) and Western blot analyses (Fig. 6A), thereby indicating a key role for a metalloprotease. Indeed, p65 and p50 could be co-immunoprecipitated with NleC if the cells were pretreated with EDTA revealing that the drug reduces basal NFκB activity and increases the inhibitory activity of NleC. Data shown are mean (± S.D.) of three experiments done in triplicate with level of significance (Student’s t test) indicated. **, p < 0.01; ***, p < 0.005 as compared with pEGFP-transfected cells. B–D, representative immunoblots probing for p65, p50, IκB, pIκB (phosphorylated form targeted for proteasomal degradation), actin, and IKK(α and β) proteins in cellular extracts from cells transfected with pEGFP, pEGFP-NleC, or pEGFP-NleC1–237 incubated, or not, with MG132. Data verify the inhibitory activity of MG132 on TNFα-treated cells (prevents proteasomal-dependent degradation of pIκB; see C) and ability of eGFP-NleC, but not eGFP or eGFP-NleC1–237 proteins, to deplete p50, p65, and/or IκBα from untreated and MG132-treated cells.

DISCUSSION

Here, it is demonstrated that the nleC gene of enteropathogenic E. coli encodes a protein whose expression as an eGFP-fusion protein within HeLa cells potently inhibits the basal- and TNFα-stimulated activity of the NFκB transcription factor. This finding has applications for understanding and controlling the function of this critical component of mammalian inflammatory, immune modulation, and anti-apoptotic responses as its dysfunction is linked to inflammatory diseases and some cancers (10, 11). Although a variety of proteins delivered into host cells by bacterial pathogens have been described to inhibit NFκB activity (12, 13), this study reveals that NleC represents a novel mechanism. Thus, ectopic ex-
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Expression of NleC is shown to lead to a dramatic decrease in the cellular levels of both p65 (RelA) and p50 that comprise the most abundant form of the dimeric transcription factor in the canonical NFκB signaling pathway (3). Although most pathogen-encoded proteins target upstream kinases, ubiquitinases, and adaptor molecules (12, 13), some target NFκB components as illustrated by chlamydia infection leading to p65 processing, whereas another EPEC effector, NleH, binds RPS3 to inhibit transcription of genes under the control of a NFκB (p65, RelB, Bcl3), Bcl3), NFκB-associated cofactors (e.g. RPS3, CBP/p300) and/or co-functional transcription factors (e.g. AP-1, STAT).

The idea, that NleC employs a novel mechanism for inhibiting NFκB function was evidenced by the finding that loss of transcription activity not only involves proteasome-independent degradation of p65, p50, and IkBα in the absence of p65 processing intermediates but was linked to NleC being a metalloprotease. The metalloprotease premise was supported by multiple lines of evidence. First, the NFκB inhibitory activity of NleC was dramatically reduced by pretreating cells with metalloprotease inhibitors (DTA or GM6001) unlike chemicals that interfere with caspase, calpain, or autophagosome-related proteases. Moreover, NleC carries a consensus zinc metalloprotease motif spanning residues 183–187 whose disruption of NleC was dramatically reduced by pretreating cells with metalloprotease inhibitors (DTA or GM6001) unlike chemicals that interfere with caspase, calpain, or autophagosome-related proteases. Moreover, NleC carries a consensus zinc metalloprotease motif spanning residues 183–187 whose disruption of NleC targets NFκB-IκBα complex. Proteasome-independent, not dependent, degradation of NFκB components represents a novel inhibitory mechanism.

Fluorescent microscopy studies revealed a pool of eGFP-NleC within the nucleus, unlike the similarly sized eGFP-NleH protein that accumulates at the cell periphery (21) (data not shown). As NleC is significantly larger than the cut-off size for free nuclear entry (<50 kDa), this suggests that it may carry a novel nuclear translocation signal (not evident by bioinformatic analysis; data not shown) or enters by associating with nuclear-targeted protein(s). Of interest, the EPEC EspF effector protein enters the nucleus by a process dependent on a domain with no recognizable signal sequence (32). Given that NleC is normally delivered into the cytoplasm and degrades p65, p50, and IkBα, it is likely that its target relates to the cytoplasmic NFκB-IκBα complex. Given the multifunctional nature of EPEC effectors (26), it is possible that nuclear import represents a distinct function or perhaps relates to targeting NFκB-IκBα complexes being shuttled to the cytoplasm. Time course studies (epifluorescent microscopy and Western blot analysis) failed to resolve whether NleC has a preference for cytoplasmic or nuclear pools of NFκB (data not shown). The mechanism and role of nuclear NleC import in the NFκB inhibitory process deserves further investigation.

Interestingly, ectopic NleC expression inhibited the basal NFκB activity in immortalized HeLa cells to a greater extent than the NFκB activation inhibitor from Calbiochem (product no. 481406). This suggests that this basal activity involves additional non-canonical pathways, of which some may be sensitive to NleC. It is likely that such pathways relate to other NFκB subunits composed of RelB, c-Rel, and/or p52 proteins. Moreover, as NleC expression depletes cellular p50, this implies that it would also alter the expression of genes regulated by p50 homodimers (33). Studies are underway to define whether NleC degrades specific isoforms of NFκB (p65, RelB, c-Rel, p105/p50, p100/p52), IkBα (e.g. IkBβ, IkBγ, IkBe, Bc3), NFκB-associated cofactors (e.g. RPS3, CBP/p300) and/or co-functional transcription factors (e.g. AP-1, STAT).

The idea that NleC employs a novel mechanism for inhibiting NFκB function was evidenced by the finding that loss of transcription activity not only involves proteasome-independent degradation of p65, p50, and IkBα in the absence of p65 processing intermediates but was linked to NleC being a metalloprotease. The metalloprotease premise was supported by multiple lines of evidence. First, the NFκB inhibitory activity of NleC was dramatically reduced by pretreating cells with metalloprotease inhibitors (DTA or GM6001) unlike chemicals that interfere with caspase, calpain, or autophagosome-related proteases. Moreover, NleC carries a consensus zinc metalloprotease motif spanning residues 183–187 whose disruption had a similar effect as adding metalloprotease inhibi-
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A minor inhibitory activity for metalloprotease-treated NleC and the NleC metalloprotease motif-disrupted variant probably relates to the recruitment of NFκB, directly or indirectly, interfering with its transcription activity. Indeed, p65 and p50 could only be isolated with full-length NleC when it lacked its protease function. Importantly, residues within the first 66 residues of NleC were shown to be sufficient and essential to recruit p65 and p50. Interestingly, the first 32 residues were dispensable for the inhibitory process thereby suggesting that features residing between residues 33–66, directly or indirectly, recruit canonical NFκB components. Further studies are necessary to define the residues and mechanism (direct or indirect) by which p65, p50 and IκBα are recruited to NleC for degradation. The requirement for residues 237–266 (upstream of the zinc metalloprotease motif) but not residues 267–330 in the inhibitory process presumably reflects an indirect deleterious consequence on a NleC protein structure needed for its protease activity. Collectively, the data is consistent with a model (see Fig. 7) whereby NleC, directly or indirectly, interacts with NFκB-IκBα complexes via features residing between residues 33 and 64 for degradation though its function as a zinc metalloprotease.

Little is known about the role of NleC in the context of pathogenesis, as deleting the nleC gene does not impact on virulence, at least in interrogated strains and models (34, 35). Studies using a disease-relevant small intestinal model suggest that NFκB cellular levels remain relatively unchanged following EPEC infections where NFκB function has been inhibited (25). Recent work suggests that such inhibition is due to the activity of the NleB and NleE effectors predicted to act at the level of IKK or the upstream kinase TGF-β-activated kinase 1 (19, 20). Nevertheless, these studies are consistent with contributions for other effectors, a premise illustrated by the documented role for NleH (21). Although EPEC can deliver plasmid-expressed NleC into host cells, an interrogated EPEC strain displayed little evidence of native nleC gene transcription or NleC production under the examined conditions (36). Preliminary studies support EPEC delivery of plasmid-expressed NleC in host cells where it decreases cellular levels of p65 and p50 (data not shown). Why EPEC have evolved mechanisms to limit the level of NleC within host cells and/or the ability of the effectors to degrade NFκB remains to be defined. Despite the apparent subtle role for NleC in inhibiting NFκB function within the context of an infection, the protein has a potent NFκB-specific degradation activity that may be useful for inhibiting the function of this transcriptional factor whose dysfunction is linked to inflammatory diseases and some cancers (10, 11). Thus, the novel NFκB-specific protease property of NleC has interesting applications. Firstly, as proteins (such as the >120-kDa functionally active β-galactosidase) can be delivered into cells in culture and whole animal models (37), NleC may be useful not only as a NFκB research tool but also in validating NFκB as a therapeutic target in disease samples or, indeed, as a therapeutic reagent.

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