NleB/SseK effectors from Citrobacter rodentium, Escherichia coli, and Salmonella enterica display distinct differences in host substrate specificity

Received for publication, April 10, 2017, and in revised form, May 9, 2017. Published, Papers in Press, May 18, 2017, DOI 10.1074/jbc.M117.790675

Samir El Qaidi†, Kangming Chen‡, Adnan Halim§, Lina Siukstaite§, Christian Rueter‡, Ramon Hurtado-Guerrero**, Henrik Clausen&, and Philip R. Hardwidge††

From the †College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506, the ‡Copenhagen Center for Glycomics, Departments of Cellular and Molecular Medicine, Faculty of Health Sciences, University of Copenhagen, DK-2200 Copenhagen N, Denmark, the §Laboratory of Cellular and Structural Biology, Rockefeller University, New York, New York 10065, the ¶Institute of Infectiology, Center for Molecular Biology of Inflammation, University of Muenster, 48149 Muenster, Germany, and the **Fundacion AIRAID, Edificio CEEI ARAGON and Institute of Biocomputation and Physics of Complex Systems, University of Zaragoza, Instituto de Quimica Fisica Rocsolano, Consejo Superior de Investigaciones Científicas Joint Unit, 500018 Zaragoza, Spain

Edited by Chris Whitfield

Many Gram-negative bacterial pathogens use a syringe-like apparatus called a type III secretion system to inject virulence factors into host cells. Some of these effectors are enzymes that modify host proteins to subvert their normal functions. NleB is a glycosyltransferase that modifies host proteins with N-acetyl-d-glucosamine to inhibit antibacterial and inflammatory host responses. NleB is conserved among the attaching/effacing pathogens enterohemorrhagic Escherichia coli (EHEC), entero-pathogenic E. coli (EPEC), and Citrobacter rodentium. Moreover, Salmonella enterica strains encode up to three NleB orthologs named SseK1, SseK2, and SseK3. However, there are conflicting reports regarding the activities and host protein targets among the NleB/SseK orthologs. Therefore, here we performed in vitro glycosylation assays and cell culture experiments to compare the activities and substrate specificities of these effectors. SseK1, SseK3, EHEC NleB1, EPEC NleB1, and C. rodentium NleB blocked TNF-mediated NF-κB pathway activation, whereas SseK2 and NleB2 did not. C. rodentium NleB, EHEC NleB1, and SseK1 glycosylated host GAPDH. C. rodentium NleB, EHEC NleB1, EPEC NleB1, and SseK2 glycosylated the FADD (Fas-associated death domain protein), SseK3 and NleB2 were not active against either substrate. We also found that EHEC NleB1 glycosylated two GAPDH arginine residues, Arg197 and Arg200, and that these two residues were essential for GAPDH-mediated activation of TNF receptor-associated factor 2 ubiquitination. These results provide evidence that members of this highly conserved family of bacterial virulence effectors target different host protein substrates and exhibit distinct cellular modes of action to suppress host responses.

The type three secretion system is a syringe-like apparatus used by Gram-negative bacteria to deliver virulence proteins (effectors) into infected host cells (1). Enteropathogenic Escherichia coli (EPEC)2 and enterohemorrhagic E. coli (EHEC) inject effectors that target NF-κB pathway components to inhibit host anti-bacterial and inflammatory responses. NleC cleaves the p50 and p65 NF-κB subunits (2). NleD cleaves the host JNK and p38 MAPK (3). NleH1 prevents the nuclear translocation of the NF-κB subunit RPS3 (4).

NleB was previously characterized as a glycosyltransferase that catalyzes the transfer of GlcNAc to protein substrates (5–7). NleB glycosylates arginine residues (6, 7). Several host proteins such as the death domain-containing proteins FADD, TRADD (tumor necrosis factor receptor type 1-associated death domain protein), and RIPK1 (receptor interaction serine/threonine-protein kinase 1) are targets of NleB N-GlcAcylation activity (6). Glycosylation of TRADD Arg235 contributes to inhibiting NF-κB pathway activity, whereas SseK2 and NleB2 did not (5). NleB glycosylates arginine residues (6, 7). Several host proteins such as the death domain-containing proteins FADD, TRADD (tumor necrosis factor receptor type 1-associated death domain protein), and RIPK1 (receptor interaction serine/threonine-protein kinase 1) are targets of NleB N-GlcAcylation activity (6). Glycosylation of TRADD Arg235 contributes to inhibiting NF-κB pathway activity, whereas SseK2 and NleB2 did not (5).

Salmonella enterica strains encode up to three NleB orthologs named SseK1, SseK2, and SseK3 (8–10). Conflicting results of the roles of the SseK proteins in Salmonella virulence have been presented. Deletion of sseK1, sseK2, or sseK3 was reported not to be deleterious to Salmonella persistence (10). Deletion of the sseK genes failed to impact Salmonella replication in RAW264.7 cells but resulted in attenuation in competitive infection models (8). Another study reported that mutation of sseK1 and/or sseK2 did not alter Salmonella virulence during systemic infection of BALB/c mice (9). SseK1 and SseK2 additively inhibit TNF-induced NF-κB activation and cell death
Functional analysis of NleB and SseK effectors

A

| Effector | C. rod NleB | EHEC NleB1(AAA) | EHEC NleB1 | EPEC NleB1 | EHEC SseK1 | EHEC SseK2 | EHEC SseK3 | SseK1 |
|---|---|---|---|---|---|---|---|---|
| TNF (30’) | + | + | + | + | + | + | + | + |
| Cytosol | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 |
| lkB | 40 | 60 | 80 | 100 | 120 | 140 | 160 | 180 |
| HA | 55 | 70 | 85 | 100 | 115 | 130 | 145 | 160 |
| PARP | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 |
| Tubulin | 55 | 70 | 85 | 100 | 115 | 130 | 145 | 160 |

B

| Effector | C. rod NleB | EHEC NleB1(AAA) | EHEC NleB1 | EPEC NleB1 | EHEC SseK1 | EHEC SseK2 | EHEC SseK3 | SseK1 |
|---|---|---|---|---|---|---|---|---|
| TNF (5’) | + | + | + | + | + | + | + | + |
| Cytosol | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 |
| Ub | 20 | 40 | 60 | 80 | 100 | 120 | 140 | 160 |
| HA | 55 | 70 | 85 | 100 | 115 | 130 | 145 | 160 |
| Ub | 55 | 70 | 85 | 100 | 115 | 130 | 145 | 160 |
| TRAF2-FLAG | 55 | 70 | 85 | 100 | 115 | 130 | 145 | 160 |

Figure 1. NleB/SseK orthologs have differential activities toward host NF-κB signaling. A, analysis of lkBα degradation and NF-κB p65 subunit nuclear translocation. HEK 293T cells were transfected with the indicated plasmids and treated 24 h later with 50 ng/ml TNF-α for 30 min. The cells were lysed and immunoblotted using the indicated antibodies. Tubulin and poly-(ADP-ribose) polymerase (PARP) were used to normalize cytosolic and nuclear protein concentrations, respectively. The asterisks used in quantification panels indicate significantly different protein abundance as compared with the TNF-α control (n = 3, ANOVA). B, analysis of TRAF2 polyubiquitination. HEK 293T cells were transfected with the indicated plasmids and treated 24 h later with 50 ng/ml TNF-α for 5 min. Cell lysates were immunoprecipitated (IP) using FLAG antibody and immunoblotted for ubiquitin (Ub). Asterisks used in quantification panels indicate significantly different TRAF2-UB signal intensity as compared with the TNF-α control (n = 3, ANOVA). C. rod, C. rodentium.

Results

Differential activities of NleB orthologs against NF-κB activation

NleB is a glycosyltransferase that glycosylates several host proteins with GlcNAcs (5–7). EPEC NleB1 targets TRADD, FADD, RIPK1, and TNFR1 to disrupt TNF signaling (6, 7). EHEC NleB1 and Citrobacter rodentium NleB glycosylate GAPDH to disrupt TRAF2 activation and downstream NF-κB signaling (5). To clarify potential functional differences among the NleB/SseK orthologs and their activities toward GAPDH. Although EHEC NleB1 glycosylates GAPDH with GlcNAc (5), EPEC NleB1 does not (6), despite 98.6% identity between the two proteins. The present work was undertaken to compare the activities of the different NleB/SseK orthologs and clarify their substrate specificity by combining data from in vitro glycosylation assays and mammalian cell culture assays. Mass spectrometry and site-directed mutagenesis were used to demonstrate that EHEC NleB1 glycosylates GAPDH and NF-κB on Arg197 and Arg200. These residues were essential for GAPDH-mediated activation of TRAF2 ubiquitination.

NleB orthologs differentially glycosylate GAPDH and FADD

We then sought to determine whether the NleB/SseK effectors differ in their ability to glycosylate host substrates with NF-κB signaling. Although EHEC NleB1 glycosylates GAPDH, it does not GlcNAcylate TRIM32 (12). The contribution of each SseK family member to bacterial virulence remains to be clarified.

Another paradoxical result concerns EHEC and EPEC NleB1 and their activities toward GAPDH. Although EHEC NleB1 glycosylates GAPDH with GlcNAc (5), EPEC NleB1 does not (6), despite 98.6% identity between the two proteins. The present work was undertaken to compare the activities of the different NleB/SseK orthologs and clarify their substrate specificity by combining data from in vitro glycosylation assays and mammalian cell culture assays. Mass spectrometry and site-directed mutagenesis were used to demonstrate that EHEC NleB1 glycosylates GAPDH and NF-κB on Arg197 and Arg200. These residues were essential for GAPDH-mediated activation of TRAF2 ubiquitination.

in macrophage infection experiments (11). SseK3 binds TRIM32, an E3 ubiquitin ligase involved in TNF signaling and interferon induction, although SseK3 does not GlcNAcylate TRIM32 (12). The contribution of each SseK family member to bacterial virulence remains to be clarified.

Another paradoxical result concerns EHEC and EPEC NleB1 and their activities toward GAPDH. Although EHEC NleB1 glycosylates GAPDH with GlcNAc (5), EPEC NleB1 does not (6), despite 98.6% identity between the two proteins. The present work was undertaken to compare the activities of the different NleB/SseK orthologs and clarify their substrate specificity by combining data from in vitro glycosylation assays and mammalian cell culture assays. Mass spectrometry and site-directed mutagenesis were used to demonstrate that EHEC NleB1 glycosylates GAPDH and NF-κB on Arg197 and Arg200. These residues were essential for GAPDH-mediated activation of TRAF2 ubiquitination.

Results

Differential activities of NleB orthologs against NF-κB activation

NleB is a glycosyltransferase that glycosylates several host proteins with GlcNAcs (5–7). EPEC NleB1 targets TRADD, FADD, RIPK1, and TNFR1 to disrupt TNF signaling (6, 7). EHEC NleB1 and Citrobacter rodentium NleB glycosylate GAPDH to disrupt TRAF2 activation and downstream NF-κB signaling (5). To clarify potential functional differences among the NleB effectors encoded by A/E pathogens, as well as the three NleB orthologs in S. enterica (SseK1/2/3), we transfected HEK293T cells with plasmids expressing each individual effector and then quantified both lkbα degradation and the nuclear translocation of the NF-κB p65 subunit after stimulating the cells with TNF.

Both EHEC and EPEC NleB1, as well as C. rodentium NleB and S. enterica SseK1 and SseK3, prevented lkbα degradation and suppressed p65 nuclear translocation (Fig. 1A). Neither NleB2 (the EHEC and EPEC proteins are identical), SseK2, nor an enzymatically inactive form of EHEC NleB1 in which the NleB1 DAD active site is mutated (NleB1(AAA)) (5) were active in this assay.

We also examined the extent to which TRAF2 polyubiquitination was inhibited and found that both EHEC and EPEC NleB1, C. rodentium NleB, and SseK1 inhibited TRAF2 polyubiquitination to a greater extent as compared with the other NleB orthologs (Fig. 1B). However, the extent of inhibition was not as significant for EPEC NleB1, as compared with EHEC NleB1, C. rodentium NleB, and SseK1 (Fig. 1B).

NleB orthologs differentially glycosylate GAPDH and FADD

We then sought to determine whether the NleB/SseK effectors differ in their ability to glycosylate host substrates with NF-κB signaling. Although EHEC NleB1 glycosylates GAPDH, it does not GlcNAcylate TRIM32 (12). The contribution of each SseK family member to bacterial virulence remains to be clarified.
GlcNAcylation assays. Serial dilutions of NleB/SseK enzymes (6.25–200 nM) were incubated with either GAPDH or FADD (1 μM) in 50 mM Tris-HCl, pH 7.4, 10 mM UDP-GlcNAc, 10 mM MnCl₂, and 1 mM DTT. The samples were then subjected to Western blot detection using an anti-R-GlcNAc monoclonal antibody. 

B. Titration of NleB/SseK orthologs in GAPDH and FADD GlcNAcylation assays. Serial dilutions of NleB/SseK enzymes (6.25–200 nM) were incubated with either GAPDH or FADD (1 μM) and then subjected to Western blot detection using an anti-R-GlcNAc monoclonal antibody.

C. Quantification of GAPDH glycosylation. The data shown in B were quantified by normalizing GlcNAc-GAPDH signals to total GAPDH signals. The data shown are representative of three independent experiments.

D. Quantification of FADD glycosylation. We were unable to detect a stable GST pulldown (Fig. 2E). His-GAPDH or His-FADD were individually incubated with each NleB/SseK ortholog then subjected to GST pulldown assays using glutathione-Sepharose beads (GE Healthcare). Protein complexes were eluted with 10 mM reduced glutathione followed by SDS-PAGE analysis. GST was used as negative control. C. rodentium.

Figure 2. NleB/SseK orthologs differentially glycosylate GAPDH and FADD. A, in vitro GlcNAcylation assays. NleB/SseK enzymes (200 nM) were incubated for 4 h at room temperature with either with GAPDH or FADD (1 μM) in 50 mM Tris-HCl, pH 7.4, 10 mM UDP-GlcNAc, 10 mM MnCl₂, and 1 mM DTT. The samples were then subjected to Western blot detection using an anti-R-GlcNAc monoclonal antibody. B, titration of NleB/SseK orthologs in GAPDH and FADD GlcNAcylation assays. Serial dilutions of NleB/SseK enzymes (6.25–200 nM) were incubated with either GAPDH or FADD (1 μM) and then subjected to Western blot detection using an anti-R-GlcNAc monoclonal antibody. C, quantification of GAPDH glycosylation. The data shown in B were quantified by normalizing GlcNAc-GAPDH signals to total GAPDH signals. The data shown are representative of three independent experiments. D, quantification of FADD glycosylation. The data shown in B were quantified by normalizing GlcNAc-FADD signals to total FADD signals. The data shown are representative of three independent experiments. E and F, pulldown assays to detect binding between the NleB/SseK orthologs and GAPDH (E) or FADD (F). His-GAPDH or His-FADD were individually incubated with each NleB/SseK ortholog then subjected to GST pulldown assays using glutathione-Sepharose beads (GE Healthcare). Protein complexes were eluted with 10 mM reduced glutathione followed by SDS-PAGE analysis. GST was used as negative control. C. rodentium.
Figure 3. EHEC NleB1 glycosylates GAPDH on Arg\textsuperscript{197} and Arg\textsuperscript{200}.

A, in vitro GlcNAcylation of WT and GAPDH mutants by EHEC NleB1. The indicated GAPDH proteins (1 μM) were incubated for 4 h at room temperature with EHEC NleB1 (200 nM) in 50 mM Tris-HCl, pH 7.4, 10 mM UDP-GlcNAc, 10 mM MnCl\textsubscript{2}, and 1 mM DTT. Samples were then subjected to Western blot detection using an anti-R-GlcNAc monoclonal antibody. B, mass spectrometric analysis of WT GAPDH tryptic digests. An Arg-GlcNAc modified peptide LWDRDGALQNIIPASTGAAK was identified only when WT GAPDH was incubated with UDP-GlcNAc and NleB (right panels). The top right panel shows extracted ion chromatograms for the GlcNAc-modified LWDRDGALQNIIPASTGAAK peptide (red trace, \(m/z = 600.31^{+}\)) and for the unmodified LWDRDGALQNI-

IPASTGAAK peptide (black trace, \(m/z = 549.58^{+}\)). Unlabeled ion chromatogram peaks are not related to the LWDRDGALQNIIPASTGAAK peptide. The corresponding HCD-MS\textsuperscript{2} spectrum of the Arg-GlcNAc-modified LWDRDGALQNIIPASTGAAK peptide shown in the bottom right panel. The blue square denotes the GlcNAc residue. The top left panel shows extracted ion chromatograms for the GlcNAc-modified (red trace, \(m/z = 800.10^{+}\)) and unmodified (black trace, \(m/z = 731.41^{+}\)) LWDRDGALQNIIPASTGAAK peptides, demonstrating that only the unmodified peptide is detected when UDP-GlcNAc and NleB are omitted. The corresponding HCD-MS\textsuperscript{2} spectrum of the unmodified LWDRDGALQNIIPASTGAAK peptide is shown on the bottom left. C, mass spectrometric analysis of arginine-free (R free) GAPDH tryptic digests. The top panels show extracted ion chromatograms for unmodified (black trace, \(m/z = 1013.05^{+}\)) and GlcNAc-modified LWADGAGALQNIIPASTGAAK (red trace, \(m/z = 1114.58^{+}\)) peptides, demonstrating that only the unmodified peptide is detected when GDP-GlcNAc and NleB are included (top right panel) or excluded (top left panel) from the reaction. The corresponding HCD-MS\textsuperscript{2} spectra for the unmodified LWADGAGALQNIIPASTGAAK peptides are shown in the bottom panels. D, mass spectrometric analysis of the R197A/R200A GAPDH tryptic digests. The top panels show extracted ion chromatograms for unmodified (black trace, \(m/z = 1013.05^{+}\)) and GlcNAc-modified LWADGAGALQNIIPASTGAAK (red trace, \(m/z = 1114.58^{+}\)) peptides, demonstrating that only the unmodified peptide is detected when GDP-GlcNAc and NleB are included (top right panel) or excluded (top left panel) from the reaction. The corresponding HCD-MS\textsuperscript{2} spectra for the unmodified LWADGAGALQNIIPASTGAAK peptides are shown in the bottom panels. E, in vitro GlcNAcylation of GAPDH alamine and lysine mutants. The indicated GAPDH proteins (1 μM) were incubated for 4 h at room temperature with EHEC NleB1 (200 nM) in 50 mM Tris-HCl, pH 7.4, 10 mM UDP-GlcNAc, 10 mM MnCl\textsubscript{2}, and 1 mM DTT. Samples were then subjected to Western blot detection using an anti-R-GlcNAc monoclonal antibody. F, mutating GAPDH Arg\textsuperscript{197} and Arg\textsuperscript{200} does not affect NleB binding. Pull-down assays to detect binding between the GAPDH mutants and EHEC NleB1. His-GAPDH mutants were individually incubated with each EHEC NleB1 and then subjected to GST pull-down assays using glutathione-Sepharose beads (GE Healthcare). Protein complexes were eluted with 10 mM reduced glutathione followed by SDS-PAGE analysis. GST was used as negative control.
To determine the functional importance of the GAPDH Arg^{197} and Arg^{200} residues in the context of TRAF2 activity, we mutated Arg^{197} and/or Arg^{200} to either alanine or lysine for use in transfection experiments in a stable GAPDH knockdown cell line (Fig. 4A). We transfected these cells with TRAF2-FLAG and with different Myc-GAPDH mutants. By contrast to WT GAPDH, the GAPDH mutants neither interacted with nor stimulated TRAF2 polyubiquitination (Fig. 4B). Thus, GAPDH Arg^{197} and Arg^{200} are essential both for the GAPDH-TRAF2 interaction and for activating TRAF2 polyubiquitination. Although we had predicted that the GAPDH Arg^{197}/Arg^{200} mutants would thus be resistant to EHEC NleB1-mediated inhibition in these cell culture experiments, our finding that the GAPDH mutants failed to interact with TRAF2 precluded us from testing this hypothesis directly.

**Discussion**

Here we have unequivocally demonstrated that the NleB/SseK orthologs have different acceptor substrate specificities using both *in vitro* glycosylation assays and cell-based assays. We found that EHEC NleB1 and *C. rodentium* NleB glycosylate both GAPDH and FADD, whereas SseK1 only glycosylates GAPDH, and SseK2 and EPEC NleB1 are only active with FADD. Moreover, NleB2 and SseK3 were inactive with these substrates (Fig. 2). The observed glycosylation activities among the NleB/SseK effectors were not tightly linked to their substrate binding, because EPEC NleB1 and NleB2 both bound to GAPDH and SseK1 bound to FADD, despite having no apparent ability to glycosylate these substrates.

Similarly, catalytically inactive mutants such as EPEC NleB1 (active site DAD mutated to AAA) still bind to FADD but do not label it (13). By contrast, we failed to detect stable binding between SseK2 and FADD in pulldown assays, even though SseK2 glycosylated FADD *in vitro*. A similar result was obtained in a previous mutagenesis study of EPEC NleB1 in which several EPEC NleB1 mutants glycosylated FADD yet exhibited weak or no binding in pulldown assays (13). Thus, it is clear that catalytic activity and substrate recognition and binding are distinct aspects of the NleB/SseK effectors.

EHEC NleB1 glycosylated GAPDH, whereas EPEC NleB1 did not, even though the two proteins are almost identical (98% identity) with only 7 amino acid differences (P35S, K42R, S106N, Y111H, M193I, D302N, and C326S) among the 329 amino acid proteins. Unexpectedly, EPEC NleB1, similar to EHEC NleB1, showed binding affinity for GAPDH despite lacking catalytic activity with this substrate (Fig. 2E), providing evidence that enzyme activity and substrate recognition are distinct aspects that appear not to be coupled in some particular cases. Future site-directed mutagenesis studies may be informative in determining the NleB1 amino acids that govern and dictate substrate specificity and catalysis.

NleB2 did not exhibit detectable glycosylation activity in our assays. Nevertheless, NleB2 bound to GAPDH, but it did not inhibit NF-κB in transfection experiments. NleB2 may glycosylate unknown targets with GlcNAc or another sugar or may lack glycosylation activity. The role of NleB2 is currently unclear, as is why both NleB1 and NleB2 are maintained in EPEC and EHEC genomes.

**Figure 4. Function of GAPDH arginines 197 and 200 in TRAF2 polyubiquitination.** A, GAPDH knockdown efficiency. HEK293T cells were transfected with the shRNA plasmid psi-LVRI6GP targeting the GAPDH 3′-UTR mRNA. Stable cell lines were created using puromycin selection. Tubulin was used to normalize GAPDH abundance. Asterisks indicate protein abundance significantly different from that of the control (n = 3, ANOVA). B, GAPDH R197/Arg^{200} mutants neither interact with nor activate TRAF2 polyubiquitination in *HEK*293T cells. GAPDH stable cell line was co-transfected with FLAG-TRAF2, HA-ubiquitin together with either Myc-GAPDH WT or the indicated GAPDH mutants. After 48 h, cell lysates were immunoprecipitated (IP) with anti-FLAG antibody, followed by immunoblotting with anti-ubiquitin or anti-Myc antibody. The abundance of endogenous GAPDH and transfected forms of GAPDH-Myc is also shown. Asterisks used in quantification panels indicate significantly different TRAF2-Ub signal intensity as compared with the TNF-α control (n = 3, ANOVA). Endo., endogenous; Exo., exogenous; Ub, ubiquitin.

We found that SseK3 has a similar phenotype as compared with EHEC NleB1, EPEC NleB1, and SseK1 in our transfection assays that examined NF-κB pathway inhibition (Fig. 1), despite our *in vitro* assays lacking activity toward FADD or GAPDH.
Functional analysis of NleB and SseK effectors

(Fig. 2). SseK3 binds but does not glycosylate TRIM32, and this interaction is required for inhibiting NF-κB, and this interaction is required for inhibiting NF-κB, suggesting that SseK3 may not be a highly active enzyme. SseK1 and SseK3 have additive effects on inhibiting NF-κB, whereas SseK2 has a moderate impact on this pathway (11). Our in vitro data show that SseK2 glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD, whereas data from transfection experiments (11) indicate that SseK1, rather than SseK2, glycosylates FADD).

It is conceivable that effector activities may differ between in vitro glycosylation assays from their in vivo context as studied in transfection experiments.

NleB2 and SseK3 both contain the conserved DXD motif found in glycosyltransferases, suggesting that they may be enzymatically active, although host substrates for these proteins have not been identified. Additional glycosyltransferase motifs are also found in these two orthologs. NleB2 is 62% identical to EHEC NleB1, whereas SseK3 is 60% identical to SseK1. The absence of their detectable activities toward GAPDH and FADD in vitro does not exclude their potential activities against other substrates that may affect host NF-κB pathway activity. NleB2 and SseK3 might also require different in vitro conditions for optimal enzyme activity.

We showed that EHEC NleB1 glycosylates GAPDH with GlcNAc on two different arginines, Arg197 and Arg200 (Fig. 3), in contrast to the single arginine residue GlcNAcylated in FADD or TRADD (6, 7). Glycosylation of both residues are critical for GAPDH-mediated activation of TRAF2 ubiquitination (Fig. 4B). Our data also suggest that the two Arg197 and Arg200 residues may be essential to maintain the complex in a proper conformation that may be necessary to recruit other proteins to the TRAF2 complex.

The amino acid region surrounding the GAPDH arginine residue targeted by EHEC NleB1 is partly conserved within the other EPEC NleB1 targets TRADD and FADD (WRDGRGAL for GAPDH, WRKVGRSL for TRADD, and WRRLRQRL for FADD) (6, 7), suggesting some commonalities among the recognition sequences. The identification of additional NleB targets, together with site-directed mutagenesis and structural studies, may be helpful in establishing a consensus NleB recognition motif. In summary, we present compelling evidence for distinct substrate specificities of virulence effector enzymes and further demonstrate that substrate binding and catalytic activity of these effectors may be distinct.

Experimental procedures

Cloning

The primers used in this study are listed in Table 1. The plasmids used in this study are listed in Table 2.

Protein purification

E. coli BL21 (DE3) was transformed with pET42a-nleB, pET42a-tra2, pET28a-gpdh, or pET15b-fadd and grown in LB to an A590 of 0.4. Isopropyl β-d-thiogalactopyranoside (0.5 mM) was added for 4 h, bacteria were pelleted using centrifugation, and the pellet was suspended in 50 mM sodium phosphate, pH 8.0, 0.5 mg/ml lysozyme). The suspension was incubated on ice for 30 min with occasional shaking. An equal volume of 50 mM sodium phosphate, pH 8.0, 2 mM NaCl, 8 mM imidazole, 20% glycerol, 1% Triton X-100 was added, and after 30 min on ice, the bacterial lysate was sonicated and then clarified by centrifugation. The supernatant was incubated with nickel-nitrotriacetic acid beads (Qiagen) with gentle rotation for 2 h at 4 °C, then loaded on a Poly-Prep Chromatography Column (Bio-Rad), and washed with 5 bead volumes of 50 mM sodium phosphate, pH 8.0, 600 mM NaCl, 10% glycerol, 60 mM imidazole. Proteins were eluted in 50 mM sodium phosphate, pH 8.0, 600 mM NaCl, 10% glycerol, 250 mM imidazole.

GlcNAcylation assays

NleB/SseK effector proteins (200 nM) were incubated with either GAPDH or FADD (1 µM) for 4 h at room temperature in 50 mM Tris, pH 7.4, 10 mM MnCl2, 1 mM UDP-GlcNAc, 1 mM DTT. The reactions were terminated by adding 250 mM Tris-HCl, pH 6.8, 0.5 M sodium dodecyl sulfate, 30% glycerol, 5% β-mercaptoethanol, 0.02% bromphenol blue and then immunoblotted using an anti-R-GlcNAc monoclonal antibody (Abcam).

Pulldown assays

Pulldown experiments were performed as described (14). GST-tagged proteins (10 µM) were immobilized on glutathione-Sepharose beads (GE Healthcare) and then mixed with His-tagged prey proteins (10 µM) in 20 mM Tris-HCl, pH 7.9, 20% glycerol, 1 mM EDTA, 5 mM MgCl2, 0.1% Nonidet P-40, 1 mM DTT, 0.2 mM PMSF, 100 mM NaCl, supplemented with 0.33 unit/µl of RNase A and DNase I. After overnight incubation at 4 °C, the beads were washed four times with 20 mM Tris-HCl, pH 7.9, 20% glycerol, 1 mM EDTA, 5 mM MgCl2, 0.1% Nonidet P-40, 1 mM DTT, 0.2 mM PMSF, 1 mM NaCl. Proteins were eluted with 10 mM reduced glutathione and analyzed using SDS-PAGE.

Mass spectrometry

Proteins (10 µg) were precipitated and washed in 90% ice-cold acetone, air-dried briefly, and then resolubilized in 50 mM ammonium bicarbonate containing 0.1% Rapigest (Waters). The samples were reduced in 5 mM DTT at 56 °C for 30 min and subsequently alkylated at room temperature for 30 min by the addition of 10 mM iodoacetamide. Each sample was digested overnight with 0.1 µg of LysC protease (Roche) at 37 °C. Peptides were purified and concentrated using reversed-phased C18 chromatography with in-house packed StageTips (Empore disk–C18, 3M).

LysC digests were separately analyzed using a setup composed of an EASY-nLC 1000 UHPLC (Thermo Scientific) interfaced via a nanoSpray Flex ion source to an LTQ-Orbitrap Velos Pro hybrid mass spectrometer. The EASY-nLC 1000 was operated using a single analytical column setup (PicoFrit Emitter, New Objectives, 75-µm inner diameter) packed in-house with Reprosil-Pure-AQ C18 phase (Dr. Maisch, 1.9-µm particle size). Peptides were separated using a 90-min LC gradient operated using a single analytical column setup (PicoFrit Emitter, New Objectives, 75-µm inner diameter) packed in-house with Reprosil-Pure-AQ C18 phase (Dr.Maisch, 1.9-μm particle size).
acquired in the Orbitrap mass analyzer using a resolution setting of 15,000.

Data processing and analysis were performed using Proteome Discoverer 1.4 (Thermo Fisher Scientific). Enzyme specificity was set to LysC allowing for one missed cleavage site. Full specificity and semispecific cleavages were considered. Peptide identifications with HexNAc modifications were used as variable modifications for cysteine residues. Methionine oxidation and HexNAc modification of arginine were used as variable modifications. Carbamidomethyl was set as a fixed modification. Peptide specificity was set to LysC allowing for one missed cleavage site. Full specificity was set to LysC allowing for one missed cleavage site. Peptide mass tolerance was 10 ppm; fragment ion mass tolerance was 0.02 atomic mass unit; carbamidomethyl was set as a fixed modification. Peptide identifications with HexNAc modifications were used as variable modifications for cysteine residues. Methionine oxidation and HexNAc modification of arginine were used as variable modifications.

Peptides and modifications were inspected manually to verify the accuracy of the identifications. Peptide identifications with HexNAc modifications were used as variable modifications for cysteine residues. Methionine oxidation and HexNAc modification of arginine were used as variable modifications. Carbamidomethyl was set as a fixed modification. Peptide specificity was set to LysC allowing for one missed cleavage site. Full specificity was set to LysC allowing for one missed cleavage site. Peptide mass tolerance was 10 ppm; fragment ion mass tolerance was 0.02 atomic mass unit; carbamidomethyl was set as a fixed modification.

Functional analysis of NleB and SseK effectors

HEK293T cells were transfected and treated with TNF-α (50 ng/ml, 5 min). The cells were washed with cold PBS, and cell pellets were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 on ice for 30 min and then mixed with anti-FLAG M2 affinity resin and rotated at 4 °C overnight. The resins were centrifuged at 8,000 × g for 30 min at 4 °C and then washed three times with cold PBS. The resins were resuspended in SDS sample loading buffer, boiled for 10 min, and immunoblotted with appropriate antibodies.

Cell fractionation

Cytosolic and nuclear protein extracts were prepared from HEK293T cells using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo). TNF-α stimulation (30 min, 50 ng/ml) was used to monitor IκBα degradation and phosphorylated p65 nuclear translocation. Poly(ADP-ribose) polymerase and β-tubulin were used to normalize the protein concentrations of nuclear and cytoplasmic fractions, respectively.

Ubiquitination assays

HEK293T cells were transfected and treated with TNF-α (50 ng/ml, 5 min). The cells were washed with cold PBS, and cell pellets were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 on ice for 30 min and then mixed with anti-FLAG M2 affinity resin and rotated at 4 °C overnight. The resins were centrifuged at 8,000 × g for 30 min at 4 °C and then washed three times with cold PBS. The resins were resuspended in SDS sample loading buffer, boiled for 10 min, and immunoblotted with appropriate antibodies.

GAPDH stable cell line construction

shRNAs targeting the GAPDH 3′-UTR mRNA were synthesized and cloned into the psi-LVRU6GP shRNA mammalian expression vector (GeneCopoeia). CSHTCR001-LVRU6GP (GeneCopoeia) was used as a scrambled sequence control. HEK
Table 2

Plasmids used in this study

| Description               | Source                  |
|---------------------------|-------------------------|
| HA-C. rodentium_NleB       | This study              |
| HA-EHEC_NleB1(AAA)         | Ref. 5                  |
| HA-EPEC_NleB1             | This study              |
| HA-NleB2                  | This study              |
| HA-Ssek1                  | This study              |
| HA-Ssek2                  | This study              |
| HA-Ssek3                  | This study              |
| GST-C. rodentium_NleB     | This study              |
| GST-EHEC_NleB1            | This study              |
| GST-EPEC_NleB1            | This study              |
| GST-NleB2                 | This study              |
| GST-Ssek1                 | This study              |
| GST-Ssek2                 | This study              |
| GST-Ssek3                 | This study              |
| His-GAPDH                 | This study              |
| His-GAPDH(R197A)          | This study              |
| His-GAPDH(R200A)          | This study              |
| His-GAPDH(R197A,R200A)    | This study              |
| His-GAPDH(R197K)          | This study              |
| His-GAPDH(R200K)          | This study              |
| His-GAPDH(R197K,R200K)    | This study              |
| GAPDH shRNA in psi-LVRU6GP | GeneCopoeia             |
| CSCHCTR001-LVRU6GP         | scrambled shRNA control |
| Myc-GAPDH                 | Ref. 15                 |
| Myc-GAPDH(R197A)          | This study              |
| Myc-GAPDH(R197A,R200A)    | This study              |
| Myc-GAPDH(R197K)          | This study              |
| Myc-GAPDH(R200K)          | This study              |
| Myc-GAPDH(R197K,R200K)    | This study              |
| Ubiquitin-HA              | Ref. 16                 |
| FLAG-TRAF2                | Ref. 5                  |
| GST-TRAF2                 | This study              |
| His-FADD                  | This study              |

293T cells were seeded onto 100-mm dishes 24 h before transfection and then transfected with 5 μg of plasmids using PolyJet transfection reagent (SignaGen Laboratories). After 48 h of incubation at 37 °C, the cells were passaged in medium containing puromycin (2 μg/ml; InvivoGen) for selection. Puromycin-resistant colonies were isolated by serial dilution in 96-well plates, after which individual clones were expanded in selection medium.

Statistical analyses

Protein abundance was quantified using Li-COR Image Studio software. 1×β degradation, p65 nuclear translocation, and TRAF2 polyubiquitination were analyzed statistically using one-way analysis of variance (ANOVA), p values < 0.05 were considered significant.

References

1. Galán, J. E., Lara-Tejero, M., Marlovits, T. C., and Wagner, S. (2014) Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. Annu. Rev. Microbiol. 68, 415–438

2. Yen, H., Ooka, T., Iguchi, A., Hayashi, T., Sugimoto, N., and Tobe, T. (2010) NleC, a type III secretion protease, compromises NF-κB activation by targeting GFP in vivo. PLoS Pathog. 6, e1000231

3. Baruch, K., Guir-Arie, L., Nadler, C., Koby, S., Yerushalmi, G., Ben-Neriah, Y., Yoge, O., Shaulian, E., and Tsaban, I. (2011) Metalloprotease type III effectors that specifically cleave JNK and NF-κB. EMBO J. 30, 221–231

4. Gao, X., Wang, F., Mateo, K., Callegeri, E., Wang, D., Peng, W., Puentes, I., Li, F., Chaussee, M. S., Finlay, B. B., Lenardo, M. J., and Hardwidge, P. R. (2009) Bacterial effector binding to ribosomal protein s3 subverts NF-κB function. PLoS Pathog. 5, e1000708

5. Gao, X., Wang, X., Pham, T. H., Feuerbacher, L. A., Lubos, M. L., Huang, M., Olsen, R., Mushegian, A., Slawson, C., and Hardwidge, P. R. (2013) NleB, a bacterial effector with glycosyltransferase activity, targets GAPDH function to inhibit NF-κB activation. Cell Host Microbe 13, 87–99

6. Li, S., Zhang, L., Yao, Q., Li, L., Dong, N., Rong, J., Gao, W., Ding, X., Sun, L., Chen, X., Chen, S., and Shao, F. (2013) Pathogen blocks host death receptor signalling by arginine GlcNAcylation of death domains. Nature 501, 242–246

7. Pearson, J. S., Giogha, C., Ong, S. Y., Kennedy, C. L., Kelly, M., Robinson, K. S., Lung, T. W., Mansell, A., Riedmaier, P., Oates, C. V., Zaid, A., Mühlen, S., Crepin, V. F., Marches, O., Ang, C. S., et al. (2013) A type III effector antagonizes death receptor signalling during bacterial gut infection. Nature 501, 247–251

8. Brown, N. F., Coombes, B. K., Bishop, J. L., Wickham, M. E., Lowden, M. J., Gal-Mor, O., Goode, D. L., Boyle, E. C., Sanderson, K. L., and Finlay, B. B. (2011) Salmonella phage ST64B encodes a member of the SseK/NleB effector family. PLoS One 6, e17824

9. Kujat Choy, S. L., Boyle, E. C., Gal-Mor, O., Goode, D. L., Valdez, Y., Vallance, B. A., and Finlay, B. B. (2004) SseK1 and SseK2 are novel translocated proteins of Salmonella enterica serovar typhimurium. Infect. Immun. 72, 5115–5125

10. Kidwai, A. S., Mushamiri, I., Niemann, G. S., Brown, R. N., Adkins, J. N., and Heffron, F. (2013) Diverse secreted effectors are required for Sal- monella persistence in a mouse infection model. PLoS One 8, e70753

11. Günster, R. A., Matthews, S. A., Holden, D. W., and Thurston, T. L. (2017) SseK1 and SseK3 type III secretion system effectors inhibit NF-κB signalling and necroptotic cell death in Salmonella-infected macrophages. Infect. Immun. 85, e00100–17

12. Yang, Z., Soderholm, A., Lung, T. W., Giogha, C., Hill, M. M., Brown, N. F., Hartland, E., and Teasdale, R. D. (2015) SseK3 is a Salmonella effector that binds TRIM32 and modulates the host’s NF-κB signalling activity. PLoS One 10, e0138529

13. Wong Fok Lung, T., Giogha, C., Creuzburg, K., Ong, S. Y., Pollock, G. L., Zhang, Y., Fung, K. Y., Pearson, J. S., and Hartland, E. L. (2016) Mutagenesis and functional analysis of the bacterial arginine glycosyltransferase effector NleB1 from enteropathogenic Escherichia coli. Infect. Immun. 84, 1346–1360

14. Nguyen, T. N., and Goodrich, J. A. (2006) Protein-protein interaction assays: eliminating false positive interactions. Nat. Methods 3, 135–139

15. Sen, N., Hara, M. R., Ahmad, A. S., Cascio, M. B., Kamiya, A., Ehmns, J. T., Agrawal, N., Hester, L., Doré, S., Snyder, S. H., and Sawa, A. (2009) GOSPEL: a neuroprotective protein that binds to GAPDH upon S-nitrosylation. Neuron 63, 81–91

16. Lim, K. L., Chew, K. C., Tan, J. M., Wang, C., Chung, K. K., Zhang, Y., Tanaka, Y., Smith, W., Engelender, S., Ross, C. A., Dawson, V. L., and Dawson, T. M. (2005) Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation. J. Neurosci. 25, 2002–2009
NleB/SseK effectors from *Citrobacter rodentium*, *Escherichia coli*, and *Salmonella enterica* display distinct differences in host substrate specificity

Samir El Qaidi, Kangming Chen, Adnan Halim, Lina Siukstaite, Christian Rueter, Ramon Hurtado-Guerrero, Henrik Clausen and Philip R. Hardwidge

*J. Biol. Chem.* 2017, 292:11423-11430.
doi: 10.1074/jbc.M117.790675 originally published online May 18, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M117.790675

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 16 references, 4 of which can be accessed free at

http://www.jbc.org/content/292/27/11423.full.html#ref-list-1