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Cutting Edge: Bacterial Modulation of Epithelial Signaling via Changes in Neddylation of Cullin-1

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The human enteric flora plays a significant role in intestinal health and disease. Certain enteric bacteria can inhibit the NF-κB pathway by blockade of IkB-α ubiquitination. IkB-α ubiquitination is catalyzed by the E3-SCF\(^{BTrCP}\) ubiquitin ligase, which is itself regulated via covalent modification of the cullin-1 subunit by the ubiquitin-like protein NEDD8. Neddylation is a biochemical event associated with diverse cellular processes related to cell signaling, however, physiological regulation of cullin neddylation has not been described in mammalian systems. We report that interaction of nonpathogenic bacteria with epithelial cells results in a rapid loss of neddylated Cul-1 and consequent repression of the NF-κB pathway. This observation may explain the ability of intestinal bacterial communities to influence diverse eukaryotic processes in general and inflammatory tolerance of the mammalian intestinal epithelia specifically. The Journal of Immunology. 2005, 175: 4194–4198.

Virtually unique among mammalian cell types, the intestinal epithelia coexists in intimate contact with a normal flora of ~10^{12} prokaryotic organisms without activation of proinflammatory responses that would typically be evoked in other tissues. We have observed that colonization of human model epithelium in vitro with certain strains of non-pathogenic bacteria could abrogate the host cell responses to subsequent proinflammatory challenges by blockade of the key proinflammatory/antiapoptotic NF-κB pathway (1). This effect was mediated by the inhibition of IkB-α ubiquitination (but not phosphorylation), thus preventing regulated IkB-α degradation, NF-κB nuclear translocation, and the subsequent transcriptional activation of proinflammatory/antiapoptotic genes. Additionally, we observed that these bacterial interactions inhibited ubiquitination of β-catenin, suggesting a common mechanism of action.

Substrate phospho-IkB-α and phospho-β-catenin are normally ubiquitinated by a specific ubiquitin ligase complex designated E3-SCF\(^{BTrCP}\) (2, 3). This and other E3-SCF complexes are themselves regulated by transient covalent modifications. The ubiquitin homologue NEDD8 must be conjugated to the cullin subunit of the E3-SCF complex on Lys\(^{20}\) for optimal ubiquitin ligase activity (4–7). Furthermore, NEDD8 modification of cullin-1 (Cul-1) has been demonstrated to be necessary for the ubiquitination of IkB-α and p100/p105 and the subsequent activation of NF-κB in mammalian cells (8–12), for ubiquitination of Dorsal, a Drosophila homologue (13), as well as for a myriad of signaling events in organisms as disparate as yeast and plants (Arabidopsis).

NEDD8 is a small protein of 81 aa that shares 80% homology (60% identity) with ubiquitin (6). “Neddylation” of substrate proteins proceeds by a successive series of enzymes structurally related to the ubiquitination enzymes, including an ATP-dependent charging enzyme (E1) and enzymes with transferase/ligase activities (E2/E3) (14). Unlike ubiquitination, however, substrates for neddylation are restricted to proteins of the cullin class (4, 7, 15). Cullins are components of E3-SCF complexes, an observation supportive of the generally accepted hypothesis that Cul-1 neddylation evolved as a regulatory modification specific to the ubiquitination machinery, and thus represents a control point in many signaling pathways and cellular processes governed by regulated protein degradation. However, to date, physiologic regulation of Cul-1 neddylation has not been described in a mammalian system, though it is well described in plant signaling pathways (16).

Materials and Methods

Bacterial strains

Salmonella typhimurium nonpathogenic mutant strains phoP\(^{+}\) and fluC\(^{−}\) fluB\(^{−}\), and Escherichia coli human commensal strains were grown in Luria-Bertani broth in a rotary shaker at 37°C for 6–8 h. This culture was diluted 1/1000 in 100 ml of Luria-Bertani broth and grown 16 h at 37°C under microaerophilic conditions as previously described (1). Lactobacillus rhamnosus GG was obtained from the American Type Culture Collection (ATCC cat. no. 53103) and grown in Lactobacillus selection broth as above for E. coli. Bacteroides thetaiotaomicron was obtained from the American Type Culture Collection (ATCC cat. no. 29148) and was grown in trypticase-yeast extract-glucose medium for 24 h under anaerobic conditions.

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3 Abbreviations used in this paper: Cul-1, cullin-1; siRNA, small inhibiting RNA; MOI, multiplicity of infection; HA, hemagglutinin.
**Transient transfections and luciferase assays**

HeLa or 293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen Life Technologies) or FuGENE 6 (Roche) according to the manufacturer’s instructions. For luciferase reporter assays, cells were transfected with pNF-κB-Luc (Stratagene) and activity was determined using the Dual Luciferase Reporter Assay System (Promega). All transfections were balanced with empty vector to contain 200 ng of DNA.

**Small-inhibiting RNA (siRNA)**

HeLa cells were transfected at 100 nM with the following siRNA duplexes (Dharmacon): Lamin A/C, UBC12 SMARTpool, NEDD8 SMARTpool, or nontargeting siRNA. Forty-eight or 72 h after transfection, cells were stimulated with TNF-α for 30 min, lysed in denaturing SDS-PAGE buffer, and subjected to Western blot analysis. For reporter assays, 20 ng of pNF-κB-Luc was cotransfected with 10–50 nM siRNA. A total of 42–72 h after transfection, cells were treated with TNF-α for 6 h and activity was determined as described previously.

**Immunoprecipitation**

After transfection and expression of epitope-tagged proteins (Flag, hemagglutinin (HA)), cells were washed in HBSS and scraped from wells or plates in a small volume (<1 ml) of nondenaturing lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Nonider P40 or SDS, 1 mM EGTA, pH 8.0, 250 mM MgCl2, Sigma protease inhibitor mixture diluted 1/200, 10 mM NaF, 0.4 mM Na3VO4). The lysates were vortexed for 10 min at 4°C and centrifuged at 13,000 × g for 15 min. The clarified lysates were precleared with IgG Sepharose (Sigma-Aldrich) for 2 h, and then immunoprecipitated for 4 h at 4°C with epitope-tag-Ab-conjugated agarose (anti-HA, Covance; anti-Flag, Sigma-Aldrich). The bound agarose was washed six times in nondenaturing buffer and the immunopurified proteins were eluted with a minimum volume of denaturing SDS-PAGE buffer. For immunoprecipitation of endogenous Cul-1, HeLa cell lysates were prepared as above, incubated with anti-Cul-1 Ab for 1 h, then with protein A-conjugated Sepharose (Sigma-Aldrich) for 1 h, and washed and eluted as described previously.

**Western blot analysis**

Immunoreactive proteins immobilized on nitrocellulose were detected with Abs to 1×BS-α (Santa Cruz Biotechnology), Cul-1 (Zymed Laboratories), Cul-2 (Zymed Laboratories), NEDD8 (Zymed Laboratories), UBC12 (Rockland), HA epitope tag (Covance), or RH epitope tag (RGS-His; Qiagen) using the ECL protocol (Amersham) and a HRP-conjugated donkey anti-rabbit secondary Ab.

**Results and Discussion**

A tempting explanation for the observation of bacterially mediated inhibition of 1×BS-α ubiquitination is that these bacteria attenuate activity of the E3-SCFβ-TrCP ubiquitin ligase. Given the established role of Cul-1 neddylation on SCF activity, we sought to determine whether prokaryotic-eukaryotic interactions could influence this particular post-translational modification. We colonized polarized T84 epithelial cells with various commensal and nonpathogenic bacteria over a time course, prepared lysates, and immunoblotted for Cul-1 and NEDD8. Characteristically, Cul-1 appears as a doublet of 85 and 90 kDa, with the higher molecular mass species presumably representing neddylated Cul-1 (Ref. 17 and Fig. 1, A and B). NEDD8 appears as a free monomer at 8 kDa and associated with cullins around 90 kDa. Both high molecular mass Cul-1 and NEDD8 were virtually abolished in colonized cells (Fig. 1A). High throughput analysis of proteins associated with active (phospho-) 1×BS has demonstrated that SCF complexes contain Cul-1 or the related family member Cul-2 (18). Consistently, loss of high molecular mass Cul-2 was observed under the same conditions as that for Cul-1 (Fig. 1C). Expression of other cullin family members was not observed in our epithelial cell lines (data not shown).

To verify that the high molecular mass (90 kDa) Cul-1 detected in immunoblots represented the NEDD8-modified form, NEDD8 immunoreactivity was shown in immunoprecipitations of endogenous Cul-1 in uninfected epithelial cells (Fig. 1B). Furthermore, an epitope-tagged HA-Cul-1 plasmid was transfected into epithelial cells that were subsequently colonized with bacteria. Lysates were immunoprecipitated with anti-HA agarose and immunoblotted with anti-HA and anti-NEDD8 Abs. Immunoprecipitated HA-Cul-1 showed total loss of high molecular mass Cul-1 in the bacterially colonized cells. Interestingly, increased high molecular mass Cul-1 was detected in TNF-treated cells relative to control, and in both cases NEDD8 immunoreactivity could be detected in the HA-Cul-1 precipitated extracts (Fig. 1D). A wide variety of bacteria were able to mediate loss of Cul-1 neddylation with similar kinetics and multiplicity of infection (MOI) requirements. These bacteria include human intestinal commensal bacteria *E. coli*, *L. rhamnosus* GG, and *B. thetaiotaomicron*, as well as mutant strains of *S. typhimurium* rendered nonpathogenic, such as the regulatory mutant *phoP* and the flagellin-deficient mutant *flIC′/flIB′* (Fig. 1B). Collectively, these experiments demonstrate that colonization of epithelial cells by various bacterial strains causes a rapid loss of neddylated Cul-1.

![FIGURE 1](http://www.jimmunol.org)  Bacterial coculture with epithelial cells results in rapid loss of neddylated Cul-1. Epithelial cells were colonized with nonpathogenic and commensal bacteria at a MOI of 10 for 1 h unless otherwise stated. A, Immunoblots with anti-Cul-1 and anti-NEDD8 Abs. Whole-cell extracts from model T84 epithelia colonized by nonpathogenic *S. typhimurium* for 30 min and 1 h at a MOI of 1 and 10 showing 85-kDa Cul-1 and 90-kDa NEDD8-conjugated Cul-1. B, Immunoprecipitation of endogenous Cul-1 showing the high molecular mass (90 kDa) band represents neddylated Cul-1. C, Immunoblot of whole-cell Cul-2 levels in HeLa cells untreated or colonized by commensal *B. thetaiotaomicron*. D, Immunoprecipitation of HA-Cul-1 with anti-HA agarose and immunoblots with anti-HA and anti-NEDD8 Abs of lysates from transfected HeLa cells untreated, challenged with TNF-α for 30 min, or colonized by nonpathogenic *S. typhimurium* for 1 h. E, Immunoblot of whole-cell Cul-1 levels in HeLa or T84 cells colonized by the indicated bacteria for 1 h.
To test whether the bacterially mediated loss of Cul-1 neddylation resulted in the inhibition of the NF-κB pathway, control and bacterially colonized epithelial cells were challenged by a proinflammatory stimulus (TNF-α) and the effects on IkB-α processing were evaluated. First, epithelial cells were treated with MG-262, an inhibitor of the 26s proteasome—a condition that allows visualization of labile phosphorylated and ubiquitinated IkB-α adducts. In cells pretreated with MG-262 and challenged with TNF-α, phosphorylated and ubiquitinated IkB-α species and neddylated Cul-1 were observed as expected (Fig. 2A). Also as expected, Cul-1 neddylation was lost in cells pretreated with MG-262 and colonized with nonpathogenic bacteria in the absence of TNF-α. However, in colonized cells challenged with TNF-α, IkB-α was phosphorylated but no ubiquitinated IkB-α was detected. Interestingly, Cul-1 showed rapid reneddylation (within 15 min) in the presence of TNF-α.

Second, a similar experiment was performed in the absence of MG-262 to evaluate TNF-induced IkB-α degradation. Without bacterial colonization, IkB-α is rapidly degraded in the presence of TNF-α (Fig. 2B). However, when colonized epithelia were challenged with TNF-α, even though Cul-1 returned to a nedded state within 15 min, IkB-α was not degraded and thus, the NF-κB pathway remained blocked. This is consistent with the bacterially mediated inhibition of NF-κB previously described (1). Together, these data show that loss of Cul-1 neddylation correlates with blockade of IkB-α ubiquitination and degradation.

UBC12 is the NEDD8 transferase/ligase enzyme (E2) and, as is typical for enzymes that mediate this function for all ubiquitin-like proteins, uses the Cys111 residue in the catalytic site to form a thioester bond with activated NEDD8 before transfer to a cullin substrate and catalysis of an isopeptide bond (14). Wada et al. (17) described a dominant-negative UBC12 (Cys111Ser) mutant that acts by allowing formation of an irreversible peptide bond between activated NEDD8 and the hydroxyl group on the substituted Ser11 residue, thus sequestering and reducing the free pool of NEDD8. When dominant-negative UBC12 (C111S) and Cul-1 were cotransfected into epithelial cells, loss of neddylated Cul-1 was observed in Cul-1 immunoblots while wild-type UBC12 had no effect (Fig. 3A). Presumably, the sequestration of free NEDD8 irreversibly bound on an “active site trap” results in a blockade of new neddylation and allows de neddyllase activities such as that by the COP9 signalosome, a multisubunit assembly that bears homology to the 16s “lid” regulatory subunit of the proteasome that is physically associated with SCF complexes, to deconjugate NEDD8 from Cul-1 (19, 20). Consistently, dominant-negative UBC12 (C111S) but not wild-type UBC12 overexpression blocked ubiquitination of overexpressed IkB-α (Fig. 3B) and repressed activation of a TNF-α-induced NF-κB-dependent reporter construct (Fig. 3C and Ref. 21), mechanistically demonstrating that loss of neddylated Cul-1 can inhibit IkB-α ubiquitination and NF-κB transactivation in epithelial cells.

To confirm that repression of UBC12 results in loss of Cul-1 neddylation and NF-κB blockade, siRNA to UBC12 were used and compared against results from NEDD8 siRNA.
UBC12 siRNA was transfected into HeLa cells for 48–72 h, a marked reduction in the ratio of endogenous neddylated Cul-1 and of high molecular mass NEDD8 was observed, consistent with the effects of transfected dominant-negative UBC12 on transfected Cul-1 (Fig. 3D). As expected, NEDD8 siRNA also reduced the whole cell pool of neddylated Cul-1 and high molecular mass NEDD8. Finally, cotransfection of the TNF-α-inducible NF-κB-responsive reporter showed that UBC12 siRNA repressed inducible NF-κB activity to a similar degree as the dominant-negative protein or NEDD8 siRNA (Fig. 3E).

Currently, the precise role that neddylation of cullins plays in the activation of SCF-mediated ubiquitination is not fully understood at a biochemical level. Most models, which are largely derived from in vitro cell-free experimental systems, suggest that the modification of Cul-1 mediated by the NEDD8 E1 (APP-BP1/UBA3) and E2 (UBC12) results in physical recruitment (22) and/or functional activation of the ubiquitin E2 subunit (15, 23). Alternatively, neddylation may serve in the assembly of a substrate binding subcomplex (β-TrCP/Skp1) with an enzymatically active subcomplex (Cul/E2) (24, 25). We are currently pursuing experiments to explore these possibilities.

Mutations leading to loss of function of Cul-1 neddylation status can have profound functional consequences in a variety of experimental models including yeast (20), Arabidopsis (26), Caenorhabditis elegans (27), Drosophila (13), mice (28), and human cells in vitro (19). However, only limited evidence is available implicating environmental signals in the physiologic regulation of Cul-1 neddylation. For example, yeast Cul was shown to be hyperneddylated in response to UV exposure (29). In Arabidopsis studies, wild-type plants showed marked Cul-1 deneddylation when reared in darkness, while mutant plants that fail to respond to light were shown to have an abnormal accumulation of neddylated Cul-1 (16). In this case, deneddylation was achieved by a presumed up-regulation or recruitment of the deneddylating COP9 signalosome. Interestingly, the COP9 signalosome has been shown to be involved in the assembly of a substrate binding subcomplex (β-TrCP/Skp1) with an enzymatically active subcomplex (Cul/E2) (24, 25). We are currently pursuing experiments to explore these possibilities.

How could bacterial colonization of epithelial (or other eukaryotic) cells mediate loss of Cul-1 neddylation? The environment of the intestinal lumen in immediate contact with the epithelium is largely anaerobic and is suffused with not just a complex ecosystem of living bacteria, but also a miasma of small, scarcely produced compounds, and a spectrum of fermentative by-products. Exposure to these bacterial physiochemical stressors may result in compensatory cellular metabolic changes in the epithelium that affect the neddylation machinery, specifically, by limiting activity of UBC12, or augmenting activity of the COP9 signalosome or other deneddylases. A specific example of a metabolic change affecting Cul-1 neddylation was shown in an in vitro system using cytosolic extracts, where ATP depletion with apyrase was shown to efficiently deneddylate Cul-1, while supplementation of the reaction with ATP augmented neddylation (24).

Bacterially derived compounds themselves may directly affect cellular processes as well. Examples of bacterial byproducts with inhibitory activity on proinflammatory signaling are short chain fatty acids such as butyrate, propionate, and acetate produced from carbohydrate fermentation. These compounds have been used therapeutically (by intralumenal instillation) to dampen intestinal inflammation in inflammatory bowel disease, and have been shown to block NF-κB activation in vitro (32). Metabolic products/small molecules produced at the eukaryotic/prokaryotic interface may account for the widely known effects of the bacterial flora on normal intestinal function (33), and may influence a range of eukaryotic regulatory processes.

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Disclosures

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References


