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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Attaching-and-Effacing Pathogens Exploit Junction Regulatory Activities of N-WASP and SNX9 to Disrupt the Intestinal Barrier

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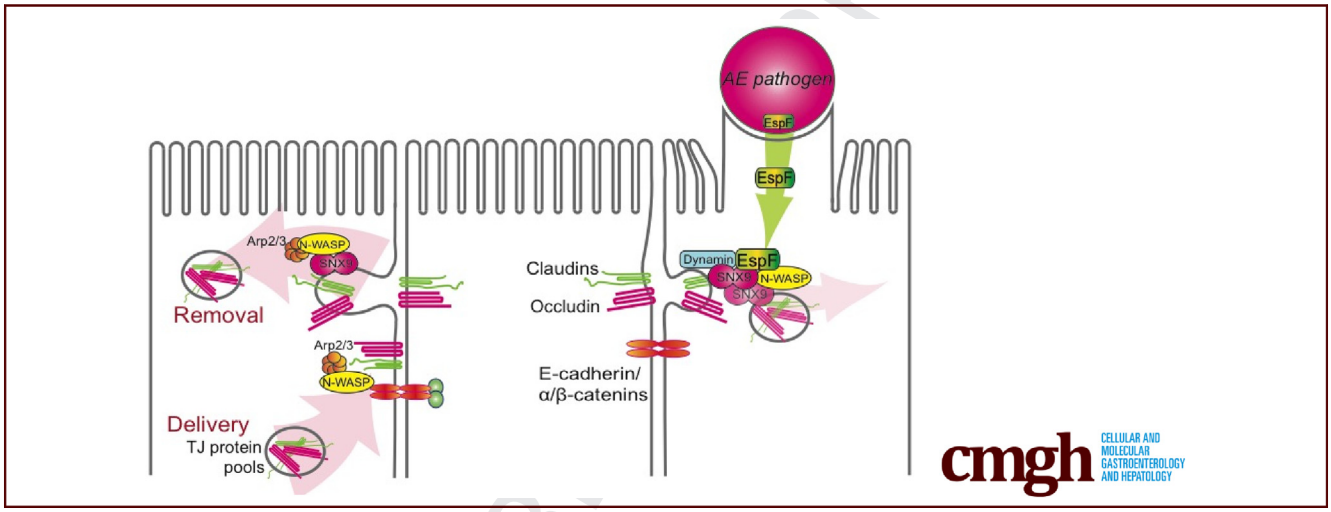

SUMMARY

During enteric infection, the attaching-and-effacing pathogen virulence factor EspF targets host Neural Wiskott-Aldrich Syndrome protein and sorting nexin 9 to promote junctional disruption and intestinal barrier loss. On this basis, we propose a novel role for the actin cytoskeletal regulatory protein Neural Wiskott-Aldrich Syndrome protein in controlling intestinal epithelial apical junction complex stability.

BACKGROUND & AIMS: Neural Wiskott-Aldrich Syndrome protein (N-WASP) is a key regulator of the actin cytoskeleton in epithelial tissues and is poised to mediate cytoskeletal-dependent aspects of apical junction complex (AJC) homeostasis. Attaching-and-effacing (AE) pathogens disrupt this homeostasis through translocation of the effector molecule EspF. Although the mechanisms underlying AJC disruption by EspF are unknown, EspF contains putative binding sites for N-WASP and the endocytic regulator sorting nexin 9 (SNX9). We hypothesized that N-WASP regulates AJC integrity and AE pathogens use EspF to induce junction disassembly through an N-WASP– and SNX9-dependent pathway.

METHODS: We analyzed mice with intestine-specific N-WASP deletion and generated cell lines with N-WASP and SNX9 depletion for dynamic functional assays. We generated EPEC and *Citrobacter rodentium* strains complemented with EspF bearing point mutations abolishing N-WASP and SNX9 binding to investigate the requirement for these interactions.

RESULTS: Mice lacking N-WASP in the intestinal epithelium showed spontaneously increased permeability, abnormal AJC morphology, and mislocalization of occludin. N-WASP depletion in epithelial cell lines led to impaired assembly and disassembly of tight junctions in response to changes in extracellular calcium. Cells lacking N-WASP or SNX9 supported actin pedestals and type III secretion, but were resistant to EPEC-induced AJC disassembly and loss of transepithelial resistance. We found that during in vivo infection with AE pathogens, EspF must bind both N-WASP and SNX9 to disrupt AJCs and induce intestinal barrier dysfunction.

CONCLUSIONS: Overall, these studies show that N-WASP critically regulates AJC homeostasis, and the AE pathogen

effector EspF specifically exploits both N-WASP and SNX9 to disrupt intestinal barrier integrity during infection. *(Cell Mol Gastroenterol Hepatol 2017;■:■–■; https://doi.org/10.1016/j.jcmgh.2017.11.015)*

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The apical junction complex (AJC), consisting of the tight junction (TJ) and adherens junction (AJ), is a multiprotein organelle that is intimately linked to dynamic networks of apical actin,[1–3] which, by simultaneously lending the AJC both stability and plasticity, enable it to control intestinal permeability. Neural Wiskott-Aldrich Syndrome protein (N-WASP) is a ubiquitously expressed protein that interacts with and activates the Arp2/3 complex, leading to robust polymerization of actin filaments. N-WASP has been shown to stabilize newly formed actin filaments and facilitate their incorporation into perijunctional rings critical for E-cadherin–dependent AJ stability.[4] There is also indirect evidence of a role for N-WASP in regulating both the delivery and removal of key junction proteins. A role for N-WASP in delivery of AJC proteins was suggested by studies in which chemical inhibition or depletion of N-WASP in epithelial cell lines was associated with defective delivery of occludin,[5] E-cadherin, and F-actin[6] to nascent AJCs. Evidence for N-WASP–mediated removal of AJC proteins comes from studies of *Drosophila* epithelium lacking Cdc42, N-WASP, or Arp3. In these studies, genetic depletion of any member of the Cdc42/N-WASP/Arp pathway in fly epithelium led to defective internalization of E-cadherin and destabilization of the AJC.[7,8]

As the major regulator of intestinal permeability, the AJC is also the target of a number of bacterial pathogens, including enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E coli* (EHEC), and the closely related mouse pathogen *Citrobacter rodentium*.[9,10] These pathogens use type III secretion to inject effector proteins that modulate a variety of host cellular processes. The first of these translocated proteins, the translocated intimin receptor (Tir), activates N-WASP, which results in localized actin polymerization and the formation of actin-rich pedestals and destruction of adjacent microvilli, lending this class of bacteria their designation as attaching-and-effacing (AE) pathogens.[11]

EPEC, EHEC, and *C rodentium* also disrupt intercellular junctions and induce intestinal barrier dysfunction,[9,10] and bacterial effector protein EspF, common to all 3 pathogens, has been shown to be required for AE pathogen-induced junction disruption.[9] Although the mechanism of EspF-mediated junction disruption remains unknown, EspF contains N-WASP binding sites and is capable of directly stimulating the actin polymerizing activity of N-WASP.[12] In addition to binding N-WASP, EspF also contains a nonoverlapping binding site for sorting nexin 9 (SNX9).[12] Sorting nexins are involved in diverse endocytic vesicle sorting activities, and the specific functions of SNX9 within the epithelium are largely unknown.[13,14] Direct binding of EspF to SNX9 alone appears to be dispensable for mediating EspF-dependent TJ disruption,[12] although a role for EspF binding to N-WASP in mediating TJ disruption has not been reported.

We sought to evaluate the role that N-WASP, as a key regulator of actin dynamics, plays in maintaining AJC homeostasis, and to test whether AE pathogens, through EspF, exploit N-WASP–dependent pathways to disrupt AJC function. We first show in vivo and in vitro that N-WASP regulates AJC homeostasis, and is required for efficient TJ assembly and disassembly. Moreover, we identify a novel pathway whereby the bacterial effector EspF disrupts tight junctions through exploiting an otherwise homeostatic function of both N-WASP and SNX9 in regulating removal of TJ proteins from the AJC.

Materials and Methods

Mice

Mixed strain (FVB/129) mice bearing a conditional *Wasl* knockout (KO) allele ($Wasl^{flox/flox}$) were crossed with villin-Cre transgenic mice[15] to generate $Wasl^{flox/flox}$; $tg^{villin\text{-}Cre}$ mice. Littermate $Wasl^{flox/+}$; $tg^{villin\text{-}Cre}$ mice were used as controls in each experiment. Intestinal N-WASP KO (iNWKO) breeder mice and offspring were housed at either the Center for Comparative Medicine at the Massachusetts General Hospital or the Boston Children's Hospital animal facility. This study protocol was conducted in accordance with the ARRIVE guidelines and was approved by the animal ethics committees of both Massachusetts General Hospital and Boston Children's Hospital.

Western Blot

For Western blot, intestinal epithelial cells were isolated from 8-week-old littermate control or iNWKO mice by EDTA dissociation as previously described,[16] and lysed with ice-cold buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 1% Triton X-100, complete protease inhibitor cocktail). Lysates were centrifuged at 13,000*g* for 15 minutes at 4°C and the supernatant (Triton-soluble cytoplasmic fraction) was collected. The pellet was resuspended in lysis buffer containing 1% sodium dodecyl sulfate and centrifuged, and the supernatant (Triton-insoluble membrane/cytoskeletal fraction) was collected. To asses short hairpin RNA (shRNA)

Abbreviations used in this paper: **ADF, actin depolymerization factor; AE, attaching-and-effacing; AJ, adherens junction; AJC, apical junction complex; Arp, _____; CR, *Citrobacter rodentium*; Crb, Crumbs; DBS100, _____; EcoRI, _____; EHEC, enterohemorrhagic *Escherichia coli*; EM, electron microscopy; EPEC, enteropathogenic *Escherichia coli*; EspF, _____; FITC, fluorescein isothiocyanate; iNWKO, intestine Neural Wiskott-Aldrich Syndrome protein knockout; KO, knockout; N-WASP, Neural Wiskott-Aldrich Syndrome protein; NWKD, Neural Wiskott-Aldrich Syndrome protein knockdown; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; shRNA, short hairpin RNA; SNX9, sorting nexin 9; SNX9KD, sorting nexin 9 knockdown; TER, transepithelial electrical resistance; Tir, translocated intimin receptor; TJ, tight junction; ZO-1, zonula occludens-1.**

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