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Control of NOD2 and Rip2 dependent innate immune activation by GEF-H1

Yun Zhao^{1,2}, Carmen Alonso Cotoner^{1,2}, Isabel Ballester^{1,2}, Joo Hye Song^{1,2}, Sun Young Chang^{1,2}, Bayasi Guleng^{1,2}, Seiji Arihiro^{1,2}, Peter J. Murray³, Ramnik Xavier^{1,2,4}, Koichi S. Kobayashi⁵, and Hans-Christian Reinecker^{1,2}

¹Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital and Harvard Medical School, Boston, MA USA

²Division of Gastroenterology, Massachusetts General Hospital, Boston. MA, USA

³St. Jude Children's Research Hospital, Memphis, TN, USA

⁴Center for Computational and Integrative Biology Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

⁵Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA

Abstract

Background—Genetic variants of nucleotide-binding oligomerization domain 2 (NOD2) lead to aberrant microbial recognition and can cause chronic inflammatory diseases in patients with Crohn's disease.

Methods—We utilized gene specific siRNA mediated knockdown and expression of GEF-H1 in wildtype, Rip2- and Nod2-deficient macrophages, HCT-116 and HEK 293 cells to determine the role of GEF-H1 in NOD2 and Rip2 mediated NF- κ B dependent induction of proinflammatory cytokine expression. Confocal microscopy was used to determine subcellular distribution of GEF-H1, Rip2 and NOD2.

Results—We identified guanine nucleotide exchange factor H1 (GEF-H1) as an unexpected component of innate immune regulation during microbial pattern recognition by NOD2. Surprisingly, GEF-H1 mediated tyrosine phosphorylation of Rip2, which occurred during signaling by NOD2, but not in the presence of the 3020insC variant of NOD2 associated with Crohn's disease. GEF-H1 functioned downstream of NOD2 as part of Rip2-containing signaling complexes and was responsible for phosphorylation of Rip2 by Src tyrosine kinase. Human Rip2 variants lacking the tyrosine target of GEF-H1-mediated phosphorylation were unable to mediate NF- κ B activation in Rip2-deficient macrophages and failed to transduce NOD2 signaling. GEF-H1 is required downstream of NOD2 as part of Rip2-containing signaling complexes and was responsible for tyrosine phosphorylation of Rip2.

Address correspondence to: Hans-Christian Reinecker, M.D., Massachusetts General, Hospital 55 Fruit Street GRJ 711, Boston, MA, USA 02114 tel 617 724-2172, fax 617-726-3673, hans-christian_reinecker@hms.harvard.edu.

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Conclusion—GEF-H1 connects tyrosine kinase function to NOD-like receptor signaling and is fundamental to the regulation of microbial recognition by ubiquitous innate immune mechanisms mediated by Rip2 kinase.

Keywords

Crohn's disease; NOD-like receptors; Src-kinase; tyrosine phosphorylation; innate immune regulation

Introduction

A diverse array of receptors act together to recognize microbiota via conserved motifs of bacterial compounds or effectors to facilitate signaling that initiates the innate immune response and triggers subsequent regulation of adaptive immunity (1). Conversely, genetic variants in microbial recognition pathways can cause autoimmune or chronic inflammatory diseases.

GEF-H1 is encoded by the *arhgef2* gene and was originally identified as an oncoprotein member of the DBL family, which activates Rho GTPases in hematopoietic cells (2, 3). GEF-H1 is a guanine nucleotide exchange factor with a DH (DBL-homology) domain that promotes loading of GTP onto Rho GTPases, a PH (pleckstrin-homology) domain involved in subcellular targeting, and an N-terminus phorbol-ester/DAG-type zinc finger as recognized functional motifs (2, 3). Recently, GEF-H1 was recognized as a new component of pathogen recognition by the caspase-recruiting domain (CARD)-containing NLR family member NOD1 (4).

Two Nucleotide-binding domain, Leucine-Rich repeat containing receptor C (NLRC) family members, NOD1 (NLRC1) and NOD2 (NLRC2) activate innate immunity during microbial infection by recognizing bacterial molecules produced by the synthesis and/or degradation of peptidoglycan (5–7). Recently, the NOD/Rip2 pathway has been shown to have a major role in innate antimicrobial defense activation (8, 9). NOD2 further has a key function in defining adaptive immune regulation for T_H1 driven granulomas in the intestine, in regulating respiratory tolerance in T_H2 driven allergic inflammation and in modulating NF- κ B activation during *Mycobacterium tuberculosis* infection (10–12). A frameshift mutation in the LRR domain of NOD2 (3020insC/L1007fsinsC), and two other single nucleotide polymorphisms within the LRRs (R702W and G908R) are associated with the development of Crohn's disease (13–15). NOD2 Crohn's disease variants have been linked to diminished defensin expression in the ileum (16, 17) and altered TLR signaling, leading to a reduced induction of T_H1 effector cells (18). Genetic variants of NOD2 are associated not only with Crohn's disease but also with Blau syndrome, psoriatic arthritis and asthma (10, 19, 20). Recognition of muropeptides induces oligomerization of the NACHT (named after NAIP, CIITA, HET-E and TP1) domains of NODs and initiates the recruitment of interacting proteins binding to serine/threonine protein kinase Rip2 (RICK/CARDIAK/Ripk2) via CARD-CARD interactions activating I- κ B kinase (IKK) leading to phosphorylation and degradation of I- κ Bs (21). Rip2 is a member of the receptor-interacting protein (RIP) family of kinases, which have emerged as essential sensors of NLR, TLR and TNF- α signaling for the activation of NF- κ B (22–25). Rip2 is a critical downstream mediator of NOD1 and NOD2 signaling, although the molecular details of how the NOD/Rip complex stimulates NF- κ B activation are only partially understood.

In this report, we show that GEF-H1-controlled innate immune responses were dependent on Rip2 for NF- κ B activation. GEF-H1 was part of a signaling complex containing NOD2 and Rip2 and was required for the induction of tyrosine phosphorylation of Rip2. This

activation-dependent post-translational modification of Rip2 was required for ligand-induced NF- κ B activation by NOD2, but was impaired in the presence of the 3020insC NOD2 variant associated with Crohn's disease. We further identified Src as a non-receptor tyrosine kinase able to tyrosine phosphorylate Rip2 in the presence of GEF-H1. Thus, GEF-H1 is a central component of innate immune regulation by Rip2, intersecting Src kinase function with NLRC signaling.

Material and Methods

Cell lines and bone marrow-derived macrophages

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Massachusetts General Hospital (Permit Number: A3596-01). HEK293 cells and HCT116 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA USA) and grown in DMEM containing 10% FBS and a 0.5% penicillin G/streptomycin mixture. Cells were plated for 24 hours before transfection with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA USA) according to the manufacturer's protocols. Bone marrow-derived macrophages were isolated from Rip2-deficient mice and NOD2-deficient mice and cultured in RPMI1640 containing 10% FBS, 0.5% penicillin G/streptomycin mixture and 20 ng/ml MCSF for 7 days. Transfection was carried out using Amaxa® Mouse Macrophage Nucleofector® kits (Amaxa, Lonza Group, Cologne, Germany).

GEFH1 expression in intestinal biopsies from IBD patients

Human colonic biopsies were obtained after informed consent in accordance to the guidelines and protocols established at Massachusetts General Hospital from patients with IBD undergoing colonoscopy. Biopsies were collected in pairs, one belonging to an inflamed area and the other to a non-inflamed area. Data are presented as amount of GEFH1 mRNA relative to GAPDH and the fold increase is normalized to the amount of mRNA present in the non-inflamed tissue for each patient. Tissues were processed for RNA extraction. Total RNA was reverse-transcribed using an iScript™ cDNA synthesis kit (Bio-Rad). Real time quantitative PCR was performed using the iQ™ SYBR® Green super mix (Bio-Rad). Briefly, 100 ng of reverse transcribed cDNA were used for each PCR reaction using 250 nM of forward and reverse primers. The PCR conditions were: 4 min at 95°C, followed by 35 cycles as follows: 94°C for 15 s, 59°C for 40 s. The threshold cycle (CT) values were calculated by subtracting the calibrator gene GAPDH. The GEFH1 mRNA content of the non-inflamed area was given the value 1 in each pair.

PCR primers sequences were Human GAPDH (product 440 bp): forward primer 5'-TCATCTCTGCCCCCTCTGCT-3' reverse primer 5'-CGACGCCTGCTTCACCACCT-3', human GEFH1 (product 157 bp); forward primer 5'-ATGTCTCGGATCGAATCCCTC-3' reverse primer 5'-ACATGGTCATGCCTGAAACTG-3'.

Plasmids, Antibodies and Reagents

Plasmid encoding Flag-tagged GEF-H1 (Human pCMV6-Entry-GEF-H1) was purchased from OriGene (OriGene Technologies, Inc. Rockville, MD USA), pCMV-ubiquitin was purchased from Open Biosystems (Thermo Scientific, Huntsville, AL USA), Flag-Rip1 was kindly provided by Dr. Zhigang Liu (Institute of Allergy and Immunology School of Medicine Shenzhen University, Shenzhen China), Flag-Rip3 was kindly provided by Dr. Jiahuai Han (The Scripps Research Institute, La Jolla, CA USA) and HA-Rip2 was kindly provided by Dr. Ramnik J. Xavier (Massachusetts General Hospital and Harvard Medical School, Boston, MA USA). HA-Rip2 (Y381A) mutant prepared to generate a Tyr to Ala

mutation was generated by Quickchange[®] Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), Flag-tagged (pCMVtag2c-NOD2) and 3020insC NOD2 mutant mammalian expression vectors were described previously (26). CSK constructs were obtained from Adgene (Cambridge, MA USA).

Immunoprecipitation and immunoblotting

Cells were harvested and proteins homogenated in NP-40 buffer (1% NP-40, 20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 4 mM Na₃VO₄, and 40 mM NaF). HEK293 cells were transfected in 6 well plates using Lipofectamine[™] 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. After 24 hours, proteins were separated as described above. Lysates were incubated with protein G plus agarose (Pierce Thermo Scientific, Rockford, IL) at 4°C for 30 minutes and pre-cleared. Pre-cleared lysates were incubated with anti-HA antibodies at 4°C overnight followed by incubation with agarose beads at 4°C for 4 hours. Precipitated proteins were collected by centrifugation and washed 3 times in washing buffer (0.5% NP-40, 20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 4 mM Na₃VO₄, 40 mM NaF). After washing, proteins were boiled with SDS-PAGE sample buffer at 95°C for 10 minutes for elution and detected by Western blotting. Electrophoresis and transfer were performed as previously described. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) at room temperature for 1 hour and incubated with primary antibodies diluted in blocking solution to a ratio of 1:1000 at 4°C overnight. After washing in TBS with 0.05% Tween-20 (TBS-T), membranes were incubated with appropriate horseradish peroxidase conjugated secondary antibody diluted in blocking buffer for 1 hour at room temperature. Blots were washed 3 times with TBS-T and hybridized bands were detected by Amersham ECL Western blotting detection reagent (GE Healthcare, Piscataway, NJ).

Immunostaining and confocal microscopy

Peritoneal macrophages were harvested at 4 days after Brewer's thioglycollate injection by standard peritoneal lavage technique using cold PBS (4°C), centrifuged, washed, and resuspended in DMEM including 10% FBS. Prior to use, the macrophage subpopulation was isolated by a 1 hour adherence step at 37°C. All remaining non-adherent cells (polymorphs, lymphocytes, and occasional red blood cells) were removed by washing twice with warm PBS. Adherent macrophages were harvested in EDTA/PBS and transfected using Amaxa[®] Mouse Macrophage Nucleofector[®] Kit (Amaxa, Lonza Group). The transfected cells were placed on 4 well Lab-Tek[™] Chamber Slide[™] system slides (Nuncbrand, Thermo Scientific, Rochester, NY USA) 24 hours later, the cells were fixed with -20°C methanol for 2 minutes on ice and permeabilized with 0.5% Triton X-100 (Sigma-Aldridge, St. Louis, MO USA) in TBS for 5 minutes. The permeabilized cells were stained with anti-HA mouse antibody-Alexa Fluor[®] 647 conjugate, 1:100 dilution (Cell Signaling Technology, Danvers, MA USA) or Anti-Flag M2 antibody-Alexa Fluor[®] 555 conjugate, 1:50 dilution (Cell Signaling Technology) for 1 hour. A Bio-Rad Radiance 2000 confocal microscope (Hercules, CA USA) was used for image acquisition and Volocity software (Perkin Elmer, Waltham, MA USA) was used for image analysis.

Dual-luciferase assay for NF- κ B-dependent promoter activation

Luciferase assays were performed 24 hours after transfection of different vectors using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Renilla luciferase activity was used as an internal control. HEK293 cells were transfected with 10 ng of pNF- κ B firefly luciferase (Clontech, Mountain View, CA USA) and 1 ng of pRL-Renilla vector (Promega) and different vectors with Lipofectamine[™] 2000 transfection reagent as described above. For control experiments,

empty vectors of indicated expression vectors were utilized. All experiments were carried out at least three times.

Small interfering RNA (siRNA)-mediated inhibition of gene expression

NOD2 siRNA, GEF-H1 siRNA and Rip2 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA USA) and 0.5×10^5 HCT116 or HEK293 cells were plated in 24 well plates for 24 hours before transfection. After 48 hours, NF- κ B reporter and pRL-Renilla constructs were transfected into cells and then luciferase detected after 24 hours.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was carried out by Two-way ANOVA followed by Bonferroni post-tests and Student's *t*-test. A *p* value of <0.05 was considered statistically significant.

Results

GEF-H1 is required for Rip2 kinase-dependent innate immune activation by NOD2

To investigate whether GEF-H1-mediated innate immune activation depends on Rip2, we determined NF- κ B activation and cytokine secretion in wildtype and Rip2-deficient macrophages in response to GEF-H1 expression. GEF-H1 expression induced a 12 ± 2 fold increase in the activation of an NF- κ B-dependent luciferase reporter in C57BL/6, but not in Rip2-deficient macrophages (Figure 1A). GEF-H1 expression further served as a signal for the induction of TNF- α and IL-6 secretion in macrophages. The induction of both proinflammatory cytokines by GEF-H1 was dependent on Rip2 (Figure 1A). Furthermore, siRNA-mediated depletion of GEF-H1 prevented NF- κ B activation by muramyl dipeptide (MDP), similar to the depletion of either NOD2 or Rip2 in HCT116 cells, which express NOD2 constitutively (Figure 1B). We also depleted GEF-H1 expression with specific siRNAs in HEK293 cells before transfecting the cells with NOD2 and NF- κ B luciferase reporter constructs. Depletion of GEF-H1 in HEK293 cells significantly reduced baseline NOD2-induced and MDP-induced NF- κ B activation, while control and NOD1 siRNAs did not impair NF- κ B activation in response to MDP (Figure 1B).

To determine whether GEF-H1 regulates NOD2-dependent NF- κ B activation, we expressed GEF-H1 alone or in combination with NOD2 or the NOD2 variant 3020insC in bone marrow (BM)-derived macrophages from NOD2-deficient mice. GEF-H1 significantly enhanced baseline and MDP-induced NF- κ B activation by NOD2 (Figure 1C). GEF-H1 was able to activate NF- κ B independently of NOD2; however, the recognition of MDP required NOD2 in macrophages, as expected (Figure 1C). In the presence of the Crohn's disease-associated NOD2 variant, macrophages failed to respond to NOD2-mediated MDP stimulation, but not to GEF-H1 expression (Figure 1C). We next determined whether GEF-H1 was itself regulated as part of inflammatory signaling cascades. GEF-H1 mRNA was induced in response to MDP stimulation in HCT116 cells (Figure 1D). Furthermore, biopsies from inflamed mucosal areas of Crohn's disease patients demonstrated enhanced GEF-H1 expression compared to non-inflamed areas of the same intestines (Figure 1E). To determine the subcellular localization of the interaction of GEF-H1 and NOD2 signaling, we transfected BM-derived macrophages with constructs encoding for green fluorescent protein (GFP)-tagged-GEF-H1, Flag-tagged NOD2 and HA-tagged Rip2 (Figure 1E). GEF-H1 demonstrated cytoplasmic and cytoskeletal association, but co-localized in vesicular compartments with NOD2 (Figure 1F). Furthermore, these subcellular compartments also contained Rip2, suggesting a close interaction with GEF-H1 and NOD2 in signaling complexes (Figure 1E).

GEF-H1 interacts with signaling complexes containing Rip kinases and mediates tyrosine phosphorylation of Rip2

We next assembled GEF-H1 and Rip kinase-containing signaling complexes in HEK293 cells. Immunocomplexes forming in the presence of GEF-H1 contained Rip1, Rip2 and Rip3 (Figure 2A). Surprisingly, the interaction with GEF-H1 resulted in the tyrosine phosphorylation of Rip2, but not Rip1 or Rip3 (Figure 2A). A GEF-H1 mutant with a deleted DH domain failed to induce the phosphorylation of Rip2, suggesting that this modification requires the activation of GTPases. We next determined whether tyrosine phosphorylation of Rip2 occurs as part of NOD2-mediated signal transduction (Figure 2B). Rip2 was tyrosine phosphorylated in protein complexes forming in response to the expression of NOD2 but not the 3020insC variant (Figure 2B).

Thus far, only potential serine/threonine phosphorylation of Rip kinases had been implicated in the regulation of their function. We initially utilized phosphorylation prediction algorithms, which indicated putative tyrosine phosphorylation at positions 23, 113, 257 and 474 of human Rip2. In addition, a large-scale proteomics analysis of the human kinome revealed a potential modification of Rip2 by tyrosine phosphorylation at tyrosine 381 (27). Protein sequence alignments revealed that this tyrosine is present only in Rip2 and Rip4, but not in Rip1 or Rip3 (Figure 2C). We therefore created Rip2 variant Y381A in which the tyrosine was replaced with alanine. GEF-H1 was unable to induce tyrosine phosphorylation of Rip2 without Y381 present in the peptide sequence (Figure 2D). Additionally, Rip2 Y381A was unable to induce the serine/threonine phosphorylation of GEF-H1, indicating that phosphorylation of Y381 is an important post-translational modification for the function of Rip2.

Tyrosine phosphorylation of Rip2 is required for GEF-H1 and NOD2-dependent innate immune activation

Y381 phosphorylation of Rip2 is required to initiate proinflammatory cytokine secretion by NOD2 and GEF-H1. TNF- α and IL-6 secretion significantly increased in NOD2-deficient macrophages when the NOD2-GEF-H1 signaling complex was assembled in the presence of Rip2, but not the Rip2 Y381A variant (Figure 3A).

In contrast to wildtype Rip2, the Rip2 Y381A variant also failed to enhance MDP responses in NOD2-deficient macrophages in which either NOD2 or the 3020insC variant were expressed (Figure 3B). Furthermore, GEF-H1 induced NF- κ B activation in the presence of NOD2 was enhanced by Rip2, but not Rip Y381A (Figure 3C).

We also assembled the GEF-H1-NOD2-Rip2 signaling complex in HEK293 cells. NF- κ B activation in response to MDP increased in the presence of NOD2 and GEF-H1. The expression of Rip2, but not the Rip2 Y381A mutant together with GEF-H1 and NOD2 further significantly enhanced NF- κ B activation in response to MDP stimulation. However, in HEK293 cells, the Rip2 Y381A variant failed to completely inhibit MDP-mediated responses, probably due to the expression of wildtype Rip2 expression (Figure 3D). Finally, expression of Rip2 Y381A was unable to restore NOD2 signaling in Rip2-deficient macrophages (Figure 3E). As expected, wildtype Rip2 reestablished responsiveness to MDP in these macrophages, which was further enhanced by NOD2 expression. In contrast, macrophages expressing Rip2 Y381A failed to respond to MDP (Figure 3E). Furthermore, GEF-H1 significantly enhanced MDP-mediated NF- κ B activation only in the presence of the wildtype Rip2, but not the Y381 variant (Figure 3E).

GEF-H1 mediates tyrosine phosphorylation of Rip2 through Src kinase

Non-receptor Src family tyrosine kinases (SFKs) have been implicated in the regulation of pattern recognition receptor signaling transduction following the observation that specific Pan Src kinase inhibitors such as PP2 can protect against LPS induced TNF- α secretion in murine and human macrophages (28–30). We utilized PP2 to determine whether Src kinase was involved in GEF-H1-dependent tyrosine phosphorylation of Rip2. GEF-H1 was unable to induce tyrosine phosphorylation of Rip2 in the presence of PP2 (Figure 4A). Consistent with a role for Src kinases in GEF-H1 and Rip2-dependent signaling, the presence of C-terminal Src kinase (CSK) prevented NF- κ B reporter activation induced by GEF-H1 alone or in the presence of Rip2 (Figure 4B). Furthermore, depletion of Src in HEK293 cells prevented the activation of NF- κ B in response to Rip2 as well as GEF-H1 expression (Figure 4C). We also utilized the constitutively active Src variant Y527F and the dominant negative Src variant K295R to determine the dependency of Rip2 tyrosine phosphorylation by GEF-H1 on this Src family member. The dominant negative Src variant K295R prevented Rip2 tyrosine phosphorylation in immune complexes containing GEF-H1 in contrast to the Src variant Y527F (Figure 4D). While tyrosine phosphorylation of Rip2 was dependent on the presence of GEF-H1 and functional Src kinase, tyrosine phosphorylation of GEF-H1 occurred in the absence or presence of Src Y527F and K295R, indicating that additional tyrosine kinases are involved in the signaling complexes mediating GEF-H1-dependent NF- κ B activation (Figure 4D).

Discussion

We have identified GEF-H1 as a signaling intermediate of an NLRC-dependent signal activation pathway that controls the regulation of innate immune processes mediated by Rip2. We show that GEF-H1 functions downstream of NOD2 in the activation of Rip2 and is required for NF- κ B activation by MDP. Tyrosine phosphorylation at Y381 represents a new activation-dependent post-translational modification of Rip2 that is required for innate immune signaling. While phosphorylation of this tyrosine residue was required for Rip2 activation by GEF-H1, our data do not exclude additional subsequent tyrosine phosphorylation events in Rip2, which may occur during the interaction with NOD2 as it has been proposed that Rip2 functions as a tyrosine kinase autophosphorylating itself during NOD2 signaling (31). We focused on Y381 since the protein motif containing Y381 in Rip2 was present also in human Rip4 but not Rip1 and Rip3, which were not tyrosine phosphorylated by GEF-H1. Activation events leading to human Rip2 and Rip4 activation may therefore differ from those observed for Rip1 and Rip3 which have been described as relying on serine/threonine phosphorylation events for activation and degradation during NF- κ B activation.

We found that GEF-H1 expression was increased in the lamina propria of Crohn's disease patients with active disease. These data indicate that the majority of Crohn's patients, who are not NOD2 deficient have an elevated GEF-H1 expression, which may lead to Rip2 activation. This is consistent with recent evidence suggesting that Crohn's disease patients without NOD2 mutations have enhanced inflammatory responses to mucopeptide signaling (32, 33).

Our findings have implications for devising approaches to target the wide array of innate and adaptive immune mechanisms in which Rip2 is a critical component. Foremost, our data may provide further insights into the mechanisms that prevent the functioning of NOD2-dependent innate immune activation in Crohn's disease patients. The 3020insC mutation in NOD2 prevented Rip2 tyrosine phosphorylation in agreement with the inability of this NOD2 variant to activate NF- κ B signaling and innate immune responses. Reduced Rip2 activation may lead to impaired microbial recognition and defense, which has been proposed

as a common disease cause in Crohn's disease (34). It needs to be determined whether NOD2 variants resulting in enhanced immune signaling associated with Blau syndrome, asthma and psoriatic arthritis lead to distinct interactions with GEF-H1 and regulation of Rip2 phosphorylation.

GEF-H1 may also regulate the role of Rip2 in adaptive immune function, as it is required for successful immunoglobulin isotype switching after immunization with ovalbumin and for the production of IFN- γ in T_H1 cells and NK cells (21, 35). Furthermore, anti-CD3 stimulation of T cells results in an impaired proliferation of Rip2-deficient T cells, correlating with diminished NF- κ B activation and IL-2 production (16, 21, 35).

Tyrosine phosphorylation of Rip2 by GEF-H1 was partially dependent on its GEF function, which has been linked to the activation of RhoA and RhoB (36). Rho GTPases are essential regulators of signaling networks emanating from many receptors involved in innate and adaptive immunity. Host cell activation is triggered by TLRs in response to soluble and particulate microbial structures, including rapid stimulation of RhoA activity. Another Rho-GEF, AKAP13, has been demonstrated to be involved in TLR2-mediated responses (37). Inducible TLR tyrosine phosphorylation and RhoA activity have been linked to activation of Src family kinases, which can be an integral part of TLR signaling complexes (38, 39). Our data linked GEF-H1 and SFK function to the activation of Rip2 and established a role for non-receptor kinases in the regulation of NF- κ B activation by NLRC signal transduction. The involvement of SFKs in microbial pattern recognition has been recognized in experiments in which PP2 has been demonstrated to be effective in reducing cytokine expression and tissue damage in *in vivo* models of LPS-induced lung injury (40). Our data suggests that the ability of PP2 to inhibit IL-6 and TNF- α expression may be linked to the inhibition of GEF-H1-mediated Src-dependent Rip2 activation and may provide an explanation for the observation that PP2 can act as a Rip2 inhibitor (41). A number of small molecule kinase inhibitors are available such as gefitinib and erlotinib which are reported inhibitors of Rip2 phosphorylation with an IC₅₀ between 40 and 50nM (42, 43). Therefore inhibitors maybe available to target enhanced Rip2 activation in inflammatory diseases although the contribution of Rip2 mediated responses to mucosal defense function needs to be elucidated.

We propose a model in which GEF-H1 is required for NF- κ B activation by NOD2 by initiating tyrosine phosphorylation of Rip2 by Src kinase leading to innate immune activation (Figure 5). We were able to show that this is a critical signaling step for the activation of NF- κ B and proinflammatory cytokine expression in Rip2-dependent innate immune responses. This activation pathway for the recognition of intracellular microbial compounds is disabled in the presence of a NOD2 variant found in patients with Crohn's disease. Although NOD2-dependent NF- κ B activation requires GEF-H1, it could also function independently of NLRCs as part of other signaling cascades leading to Rip2 activation. Furthermore, through its interaction with other Rip kinases and activation of SFKs, GEF-H1 function may extend into the coordination of NLRC and TLR signal transduction, defining the outcome of innate immune signaling. The identification of specific tyrosine phosphorylation events required for human Rip2 function initiated by GEF-H1 will help to devise strategies to specifically regulate Rip2 and NLRC-mediated innate immune functions.

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Abbreviations

CARD	caspase-recruiting domain
CSK	C-terminal Src kinase
DAG	diacylglycerol
DH	Dbl homology domain
GEF-H1	guanine nucleotide exchange factor H1
GTP	guanosine triphosphate
IKK	I- κ B kinase
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MDP	muramyl dipeptide
NACHT	neuronal apoptosis inhibitory protein (NAIP), MHC class II transcription activator (CIITA), incompatibility locus protein from <i>Podospora anserina</i> (HET-E), and telomerase-associated protein (TP1)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	nucleotide-binding oligomerization domain (NOD)-like receptor
NLRC	Nucleotide-binding domain, Leucine-Rich repeat containing receptor C
NOD	nucleotide-binding oligomerization domain
PP2	Pan Src kinase inhibitor 2
RICK/CARDIAK/ RIPk2	serine/threonine protein kinase
RIP	receptor-interacting protein
SFK	Src family kinases
Src	sarcoma proto-oncogenic tyrosine kinase
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha

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Figure 1. GEF-H1 mediates NF- κ B activation by NOD2 through Rip2 kinase

(A) NF- κ B activation in bone marrow-derived macrophages from wildtype C57BL/6 or Rip2-deficient mice in response to GEF-H1 expression or γ TriDAP stimulation and subsequent proinflammatory cytokine expression (* indicates $p < 0.05$). (B) NF- κ B activation in response to MDP stimulation in HCT116 or HEK293 cells after siRNA-dependent depletion of Rip2, NOD2, NOD1 or GEF-H1 (* indicates $p < 0.05$, compared to control siRNA). (C) GEF-H1-induced NF- κ B activation in NOD2-deficient macrophages after reconstitution of NOD2 or 3020insC NOD2 variant expression (* indicates $p < 0.05$, compared to NOD2 transfection in the presence of control siRNAs). (D) Analysis of GEF-

H1 mRNA expression in NOD2-expressing HCT116 cells in response to MDP and in non-inflamed and inflamed intestinal tissues of patients with Crohn's disease (* indicates $p < 0.05$, compared to baseline). **(E)** GEF-H1 mRNA expression in colon biopsies from Crohn's disease patients. **(F)** 3D reconstruction of confocal microscopic image series of a macrophage demonstrating co-localization of GEF-H1, NOD2 and Rip2. The combined image includes X-Z sections of the 3D reconstruction with white color indicating the presence of signals representing GEF-H1, Rip2 and NOD2. The white bar represents 5 μm .

Figure 2. GEF-H1 interacts with Rip kinases and specifically phosphorylates Rip2
(A) Analysis of immune complexes containing GEF-H1, which form in the presence of Rip kinases. GEF-H1 was immunoprecipitated from HEK293 cells after transfection with GEF-H1, Rip1, Rip2 and Rip3 expression constructs and analyzed by Western blotting with indicated antibodies. **(B)** Analysis of Rip2-containing immunocomplexes in the presence of NOD2 or the 3020insC NOD2 variant in HEK293 cells. **(C)** Alignment of Rip kinase protein sequences containing the new tyrosine phosphorylation sites in Rip2 responsible for GEF-H1-mediated NF- κ B activation. **(D)** Analysis of immune complexes forming in the presence of GEF-H1, Rip2 or the Rip2 Y381A mutant in HEK293 cells.

Figure 3. Tyrosine phosphorylation of Rip2 is required for NF-κB activation

(A) Proinflammatory cytokine production in NOD2-deficient macrophages after reconstituting NOD2 expression in the absence or presence of GEF-H1, Rip2 or the Rip2 Y381A variant (* indicates $p < 0.05$). (B) NF-κB activation in NOD2-deficient macrophages transfected to express NOD2 or the Crohn's disease associated NOD2 variant 3020insC in the absence or presence of Rip2 or its signaling-deficient Y381A variant (* indicates $p < 0.05$). (C) NF-κB activation in NOD2-deficient macrophages in response to MDP in which NOD2 was reconstituted in the presence of additional GEF-H1 and either Rip2 or Rip2 Y381A (*indicates $p < 0.05$, compared to control siRNA). (D) NF-κB activation in response

to the assembly of the GEF-H1-NOD2-Rip2 signaling complex in the absence or presence of the Rip2 Y381A variant in HEK293 cells (* indicates $p < 0.05$). **(E)** NF- κ B activation in Rip2-deficient macrophages after Rip2 and Rip2 Y381A variant reconstitution in the absence or presence of GEF-H1 and NOD2 (*, indicates $p < 0.05$).

Figure 4. GEF-H1-mediated phosphorylation of Rip2 is dependent on Src kinases
(A) Analysis of Rip2-containing immune complexes in the presence of GEF-H1 and various amounts the SFK inhibitor PP2. **(B)** NF- κ B activation in HEK293 cells by GEF-H1 in the absence or presence of Rip2 and CSK (* indicates $p < 0.05$). **(C)** NF- κ B activation in HEK293 cells with or without siRNA-mediated depletion of Src. NF- κ B activation in response to Rip2 or GEF-H1 overexpression in HEK293 cells before and after depletion of Src kinase expression by siRNA. **(D)** Analysis of Rip2-containing immune complexes forming in HEK293 cells in the absence or presence of GEF-H1 of constitutively active or dominant negative Src variants.

Figure 5. Model of GEF-H1 function in the regulation of Rip2 activation during microbial recognition by NLRC family members

We propose a model in which GEF-H1 is required for NF- κ B activation by NOD2. GEF-H1 is part of Rip2- and NOD2-containing immune complexes and initiates tyrosine phosphorylation of Rip2 by Src kinase, which is required for its activation. In the presence of the Crohn's disease-associated NOD2 variant 3020insC or GEF-deficient GEF-H1 mutants or the Rip2 Y381A variant, Rip2 tyrosine phosphorylation and NF- κ B activation are impaired.