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Nod2 is an intracellular sensor of a specific bacterial cell wall component, muramyl dipeptide, and activation of Nod2 stimulates an inflammatory response. Specific mutations of Nod2 have been associated with two inflammatory diseases, Crohn disease and Blau syndrome, and are thought to contribute to disease susceptibility through altering Nod2 signaling. Association of disease with inappropriate activation of Nod2 highlights the importance of proper regulation of Nod2 activity. However, little is known about specific regulation of the Nod2 pathway. We performed a biochemical screen to discover potential regulators of Nod2 and identified Erbin, a protein involved in cell polarity, receptor localization, and regulation of the mitogen-activated protein kinase pathway, as a novel Nod2-interacting protein. In our studies, we demonstrate specific interaction of Erbin and Nod2 both *in vitro* and *in vivo* and characterize the regions required for interaction in both proteins. We found that Nod2-dependent activation of NF-κB and cytokine secretion is inhibited by Erbin overexpression, whereas Erbin$^{-/-}$ mouse embryo fibroblasts show an increased sensitivity to muramyl dipeptide. These studies identify Erbin as a regulator of Nod2 signaling and demonstrate a novel role for Erbin in inflammatory responses.

The immune system has evolved many mechanisms to identify, neutralize, and destroy pathogens. On the front line of defense against these microbial invaders is the innate immune system, a broad specificity inflammatory response that includes the activation and recruitment of phagocytic cells to sites of infection. Specialized molecules of the innate immune system, called pattern recognition molecules (PRMs),[2] recognize conserved components of microorganisms that are not found in the host, called pathogen-associated molecular patterns, and stimulate the innate immune response. There are several families of PRMs that include scavenger receptors, Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain containing proteins (NODs). These different families of PRMs act at distinct cellular locations, with secreted scavenger receptors in the extracellular space, transmembrane TLRs functioning at both the plasma membrane and intracellular vesicles, and cytosolic NODs sensing the intracellular environment (1, 2). All of these PRMs work in concert to generate an effective innate immune response to counter microbial pathogens.

Nod2 is a member of a family of over 20 cytosolic proteins characterized by the presence of a conserved NOD (1). These proteins contain an amino-terminal effector-binding region that consists of protein-protein interaction domains including caspase recruitment domains (CARDs), pyrin, and baculovirus inhibitor repeat domains, a central NOD that acts to oligomerize these proteins, and carboxyl-terminal leucine-rich repeats (LRRs) that are required to detect ligands. These molecules are thought to play key roles in pathogen defense through sensing bacteria and generating an inflammatory immune response.

Nod2 detects bacteria by recognizing a specific component of the peptidoglycan cell wall, muramyl dipeptide (MDP) (3, 4). Stimulation of Nod2 by MDP is thought to cause the oligomerization of Nod2 and the recruitment of a protein kinase RICK (also called RIP2, RIPK2, or CARDIAK). The recruitment of RICK to Nod2 activates downstream signaling pathways such as the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways (5–7). Activation of these pathways results in stimulation of both the innate and adaptive immune responses through the up-regulation of pro-inflammatory molecules, such as cytokines, chemokines, and cell adhesion molecules.

Specific mutations in the *NOD2* gene have been associated with inflammatory disease (8–11). One of these diseases, Crohn disease (CD), is a chronic inflammatory bowel disease characterized by granulomatous inflammation localized primarily to the ileum. Two missense mutations in *NOD2*, R702W and G908R, as well as a frameshift mutation, L1007fsinsC, are the main *NOD2* mutations associated with CD. *NOD2* mutations of at least one allele are found in ~50% of CD patients in the Western hemisphere. Individuals that carry two of these mutated *NOD2* alleles have a 20–40-fold increased risk in developing CD (8, 11, 12). These mutations are grouped near or within the ligand-sensing region of Nod2, the LRRs. Although there is some controversy over the effects of these mutations on Nod2 signaling, peripheral blood mononuclear cells and macrophages from CD patients homozygous for these mutations show severe defects in both NF-κB activation and cytokine secretion in response to MDP treatment, suggesting that these Nod2 mutations result in a loss-of-function phenotype in humans (13–17). Another major inflammatory disease associated with Nod2 mutations is Blau syndrome (BS). BS is an autosomal dominant disease characterized by granulomatous arthritis, uveitis, and skin rashes (10). The Nod2 mutations associated with BS, R334W/Q and L469F, are missense mutations located within the NOD oligomerization domain. These

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[2] The abbreviations used are: PRM, pattern recognition molecule; TLR, Toll-like receptor; NOD, nucleotide-binding oligomerization domain; CARD, caspase recruitment domain; LRR, leucine-rich repeat; MDP, muramyl dipeptide; MAPK, mitogen-activated protein kinase; CD, Crohn disease; BS, Blau syndrome; MEF, mouse embryo fibroblast; LPS, lipopolysaccharide; TNF, tumor necrosis factor; TAP, tandem affinity purification; MCP-1, monocyte chemoattractant protein-1; HA, hemagglutinin; EGFP, enhanced green fluorescent protein.

mutations result in ligand-independent NF-κB activity, suggesting that these are gain-of-function mutations resulting in constitutive activation of Nod2 signaling (18, 19). The association of both loss-of-function and gain-of-function Nod2 mutations with inflammatory disease highlights the importance of proper regulation of Nod2 signaling.

To date, there is little known about the regulation of the Nod2 signaling pathway. To identify potential protein regulators of Nod2, we performed a biochemical screen to isolate Nod2-interacting proteins. In this screen, we identified Erbin as a novel Nod2-interacting protein. Erbin has been previously shown to play roles in cell polarity, receptor localization, specialized cell-cell contacts, and regulation of MAPK signaling (20). In this report, we demonstrate that Erbin binds specifically to Nod2 and inhibits Nod2-dependent NF-κB signaling in response to MDP stimulation. These results identify a novel regulator of Nod2 signaling and uncover a role for Erbin in inflammatory responses.

MATERIALS AND METHODS

Primary Cells and Cell Lines—The monocyte cell line MonoMac6 (Deutsche Sammlung von Mikroorganismen und Zellkulturen) was maintained in RPMI + 10% fetal bovine serum + penicillin/streptomycin + 1× nonessential amino acids + 2 mM glutamine + 1 mM sodium pyruvate + 10 μg/ml insulin. The Caco2 epithelial cell line was maintained in Dulbecco's modified Eagle's medium + 20% fetal calf serum + 1% nonessential amino acids and polarized as in (21). LoVo, SW480, HEK293T, HEK293-TAP, and HEK293-TAP-Nod2 cell lines were cultured in Dulbecco's modified Eagle's medium + 10% fetal bovine serum + penicillin/streptomycin. The cell lines, HEK293-TAP, and HEK293-TAP-Nod2 were created by infection of HEK293 cells with either TAP retrovirus or TAP-Nod2 retrovirus, followed by selection with puromycin. The generation of Erbin-deficient mice will be described in a separate report.[3] Primary mouse embryo fibroblasts (MEFs) were isolated from 14.5-day-old Erbin$^{+/+}$, Erbin$^{+/-}$, and Erbin$^{-/-}$ 129/C57 mouse embryos following standard protocols and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (22). MEF cell lines stably expressing Nod2 were created by infection of MEFs with HA-Nod2 retrovirus and selection with puromycin.

Plasmids and Reagents—MDP (Bachem) was used at 10–1000 ng/ml, ultra-pure LPS from *Escherichia coli O55:B5* (Sigma) was used at 10–100 ng/ml, and human TNFα (Roche Applied Science) was used at 10 ng/ml. Anti-HA (Covance, monoclonal HA.11 and Santa Cruz, rabbit polyclonal Y-11), anti-Myc (Santa Cruz, monoclonal 9E10, rabbit polyclonal A-14), and anti-FLAG (Sigma, monoclonal M2) antibodies were used at 1:1000 for Western blot analysis. Anti-Nod2 (monoclonal 2D9 (23) and rabbit polyclonal sera (11)) and anti-Erbin (rabbit polyclonal sera (21)) antibodies were used at 1:500 for Western blot analysis. The monoclonal anti-T7 antibody (Novagen) was used at 1:10,000 in Western blot assays.

Many of the plasmids used in these studies have been previously described and include the Nod2 mammalian expression construct pcDNA3-HA-Nod2 (11); Nod2 mutants, pcDNA3-Nod2ΔLRR (W741x), and pcDNA3-Nod2CARDs (Q335x) (19); the retroviral expression plasmids, pMXneo-FLAG-Nod2, pMXp-HA-Nod2, pMXp-HA-Nod2 R702W, pMXp-HA-Nod2 G908R, and pMXp-HA-Nod2 L1007fs (13); the Erbin mammalian expression constructs, Myc-Erbin, Myc-Erbin mutPDZ, Myc-Erbin P315L (21, 24), pRK5-Myc-LRR, and pRK5-Myc-965/1371 (25); the yeast plasmids, pBTM116-Erbin, and pACT2-PKP4 (26); Nod family member expression plasmids, pcDNA3-HA-Apaf-1 (27), pcDNA3-T7-Cryopyrin, pcDNA3-T7-Ipaf (28), and pcDNA3-HA-Nod1 (29); and the luciferase assay plasmids pBVIx-Luc, and pEFBOS-βgal (29). The plasmids EGFP-C1 (Clontech), pACT2 (Clontech), and pcDNA3 (Invitrogen) were purchased from their respective companies.

The plasmids that were generated for these studies and their construction are described briefly below. The yeast two-hybrid Nod2 plasmid, pACT2-Nod2, was generated by amplifying human Nod2 cDNA by PCR and cloning it into the BamHI and XhoI sites of pACT2. The Nod2 deletion constructs of the Nod2 NOD domain (amino acids 265–744) and LRRs (amino acids 744–1040) were amplified by PCR to incorporate SpeI and EcoRI restrictions sites and cloned into the XbaI and EcoRI sites of pcDNA3HA, resulting in the incorporation of a HA tag on the amino terminus of the proteins. The retroviral expression construct pMXp-TAP-Nod2 was constructed by PCR amplifying the tandem affinity purification (TAP) tag consisting of an immunoglobulin-binding domain separated from a calmodulin-binding domain by a TEV protease cleavage site from pNRTIS-33 (30) and cloning into the retroviral vector pMXpuro along with a HA-Nod2 cDNA fragment from pcDNA3-HA-Nod2 in a three-fragment ligation. The retroviral expression constructs pMXp-HA-Nod2 R334W and pMXp-HA-Nod2 L469F were generated by site-directed mutagenesis of pMXp-HA-Nod2 using the QuikChange XL kit (Stratagene) according to the manufacturer's instructions. The Erbin deletion construct, pcDNA3-Myc-Erbin 392/964, was generated by PCR amplifying amino acids 392–964 of Erbin using primers to incorporate XbaI and EcoRI restriction sites into the product and cloning it into the same sites of pcDNA3-Myc to generate a fusion protein with an amino-terminal Myc tag.

Tandem Affinity Protocol Purification—The protocol used was adapted from Ref. 31. Briefly, the cell lysates were made from HEK293-TAP and HEK293-TAP-Nod2 cells using Nonidet P-40 lysis buffer (10 mM Hepes-KOH, pH 7.4, 142 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.2% Nonidet P-40, 1× Roche Applied Science Complete protease inhibitor mixture). The cell lysates were bound to 400 μl of IgG-Sepharose (Amersham Biosciences 6 FastFlow beads) for 2 h at 4 °C, followed by washing with 60 ml of Nonidet P-40 lysis buffer and elution with 0.5%SDS. Eluted proteins were trichloroacetic acid-precipitated, trypsin-digested, and analyzed by liquid chromatography-tandem mass spectral analysis at the University of Victoria BC Proteomics Centre.

Immunoprecipitation—Lysates were made from cells using Nonidet P-40 lysis buffer and precleared by incubation with recombinant protein G-Sepharose 4B (Zymed Laboratories Inc.) for 30 min at 4 °C. Precleared lysates were incubated with immunoprecipitating antibody overnight at 4 °C, followed by collection of antibody-protein complexes with protein G-Sepharose 4B for 1 h at 4 °C. Immunoprecipitates were washed six times in 1 ml of Nonidet P-40 lysis buffer, separated by SDS-PAGE, and analyzed by Western blot.

Yeast Two-hybrid Analysis—L40 yeast were transformed with the LexA bait plasmid pBTM116-Erbin and a GAL4 prey plasmid, pACT2, pACT2-PKP4, or pACT2-Nod2, using the *S.c.* EasyComp Transformation kit (Invitrogen) according to the manufacturer's instructions. Transformants were plated on SD-Leu-Trp or SD-Leu-Trp-His plates for identification of positive interactions. Transformants were also tested for β-galactosidase activity using the PXG assay as described in Ref. 32.

Immunofluorescence—Immunofluorescent staining and confocal microscopy were performed as described in Ref. 21.

Luciferase Reporter Gene Assays—The cells were plated 4×10^4 cells/ml in 24-well plates and transfected with plasmid DNA using Lipofectamine PLUS according to the manufacturer's instructions (Invitrogen). Transfected cells were stimulated for 16–18 h with 100 ng/ml

[3] S. Marchetto, F. Fiore, D. Birnbaum, and J.-P. Borg, manuscript in preparation.

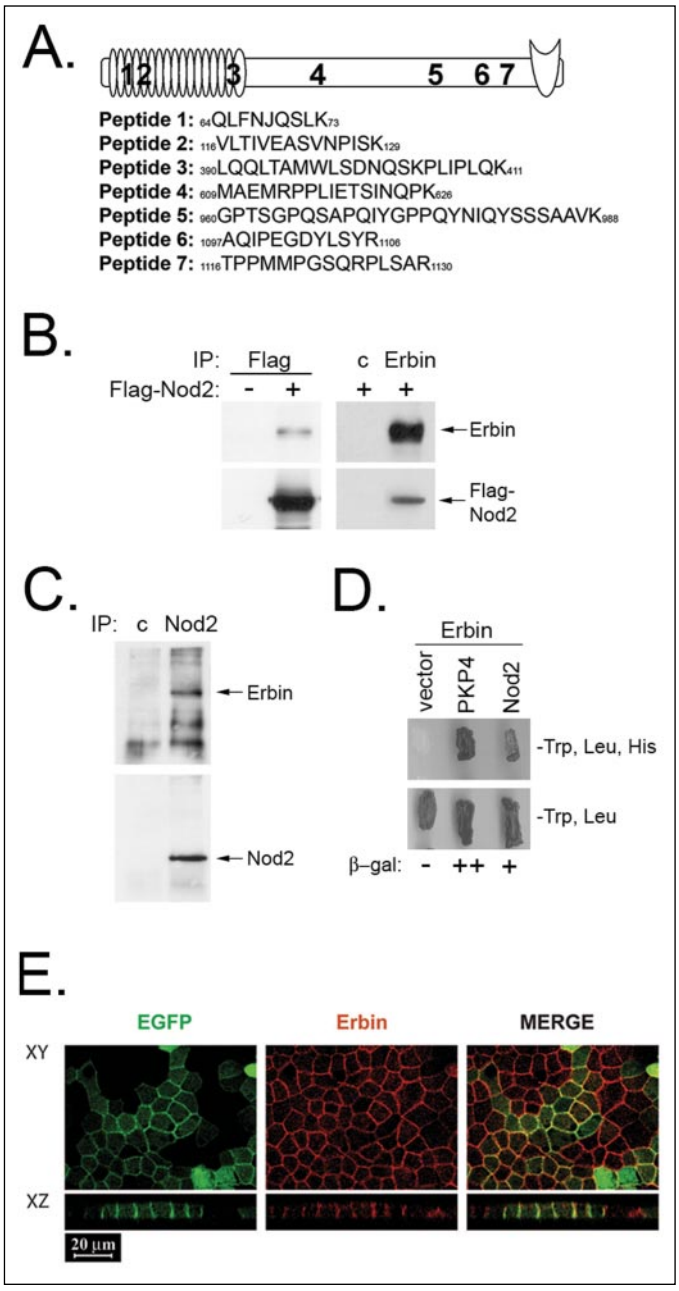

FIGURE 1. **Identification of Erbin as a Nod2-interacting protein.** *A*, Erbin peptides identified by mass spectral analysis in TAP screen. The approximate locations of peptides are shown in the diagram of Erbin protein and amino acid numbers designated by subscript numbers. *B*, co-immunoprecipitation (*IP*) of Erbin and ectopically expressed FLAG-Nod2. Lysates from HEK293T cells transfected with FLAG-Nod2 plasmid (+) or control plasmid (−) were immunoprecipitated with anti-FLAG antibody, anti-Erbin rabbit sera, or normal rabbit sera (*c*), and Western blot analysis was performed with either anti-FLAG (*bottom panels*) or anti-Erbin (*top panels*) antibodies. *C*, endogenous Erbin and Nod2 proteins interact. Lysates from SW480 cells were immunoprecipitated with anti-Nod2 rabbit sera (*Nod2*) or normal rabbit sera (*c*) and immunoprecipitating proteins visualized by Western blot analysis with either anti-Erbin rabbit sera (*top panel*) or monoclonal anti-Nod2 2D9 (*bottom panel*) antibodies. *D*, Erbin and Nod2 interact in a yeast two-hybrid assay. L40 yeast were transformed with Erbin bait plasmid and prey plasmids with plakophilin-4 (PKP4), Nod2, or no insert (*vector*). The ability of these proteins to interact was assessed by growth on restrictive medium (SD-Trp-Leu-His) and activation of a *lacZ* reporter gene (β-gal). *E*, Erbin co-localizes with Nod2 at the basolateral membrane of polarized Caco2 cells. Confocal analysis of polarized Caco2 cells expressing EGFP-Nod2 (*green*) immunostained with an anti-Erbin antibody (*red*) is shown.

MDP and then lysed in 1× reporter lysis buffer (Promega) and assayed for luciferase and β-galactosidase activity (29). The luciferase values were normalized to transfection efficiency by dividing by the raw luciferase values by the β-galactosidase values. The averages and standard deviations were calculated from triplicate wells using Microsoft Excel.

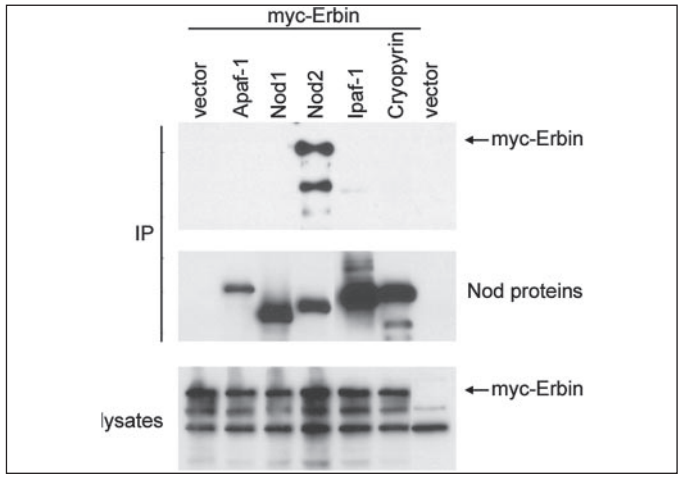

FIGURE 2. **Erbin specifically interacts with Nod2.** HEK293T cells were transiently transfected with plasmids expressing HA-tagged Apaf-1, HA-tagged Nod1, HA-tagged Nod2, T7-tagged Ipaf, T7-tagged Cryopyrin, or empty HA tag or T7 tag vectors, along with an expression plasmid for Myc-tagged Erbin. The cell lysates were immunoprecipitated (*IP*) with either monoclonal anti-HA or anti-T7 antibodies and proteins visualized by Western blot using either anti-Myc (*top* and *bottom panels*), anti-HA, or anti-T7 (*middle panel*) antibodies.

Enzyme-linked Immunoabsorbant Assay —MEFs were plated 10^4 cells/well in 24-well plates, infected with retrovirus for 30 h, and then stimulated with ligand for 16–18 h. MEF-Nod2 stable cell lines were plated at 10^4 cells/well in 24-well plates and transfected with plasmid using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. 16 h post-transfection MEF-Nod2 cells were stimulated with ligand for 8 h. Tissue culture supernatants were collected, and cytokine levels were assayed according to the manufacturer's instructions (R & D Biosystems). The averages, standard deviations, and statistical significance assessed by two-tailed Student's *t* test were calculated using Microsoft Excel software.

RESULTS

Identification of Erbin as a Nod2-interacting Protein—We performed a biochemical screen to identify Nod2-interacting proteins that may function as regulators of Nod2 signaling. In this screen, Nod2-containing complexes were purified from lysates of a HEK293 cell line expressing a tandem affinity tagged Nod2 (HEK293:TAP-Nod2) by affinity chromatography. The Nod2 co-purifying proteins were identified by liquid chromatography tandem mass spectroscopy. Lysates from a HEK293 cell line expressing the affinity tag only (HEK293:TAP) were processed in the same manner as a control for nonspecific binding of proteins during the purification process. Nod2-interacting protein candidates were determined by comparing the peptide profiles from the HEK293:TAP-Nod2 and HEK293:TAP purifications, and proteins that were identified by multiple distinct peptides were considered as candidates.

One Nod2-interacting protein candidate was identified by seven distinct peptides broadly distributed over the protein sequence (Fig. 1*A*). This candidate, Erbin (also called Erbb2ip or densin-180-like protein), was originally identified as a protein that is required for proper localization of the receptor ErbB2 to the basolateral membrane of epithelial cells (21).

The interaction of Erbin and Nod2 was confirmed through both *in vitro* and *in vivo* assays. First, we assessed the interaction of Erbin and FLAG-tagged Nod2 (FLAG-Nod2) from lysates of transiently trans-

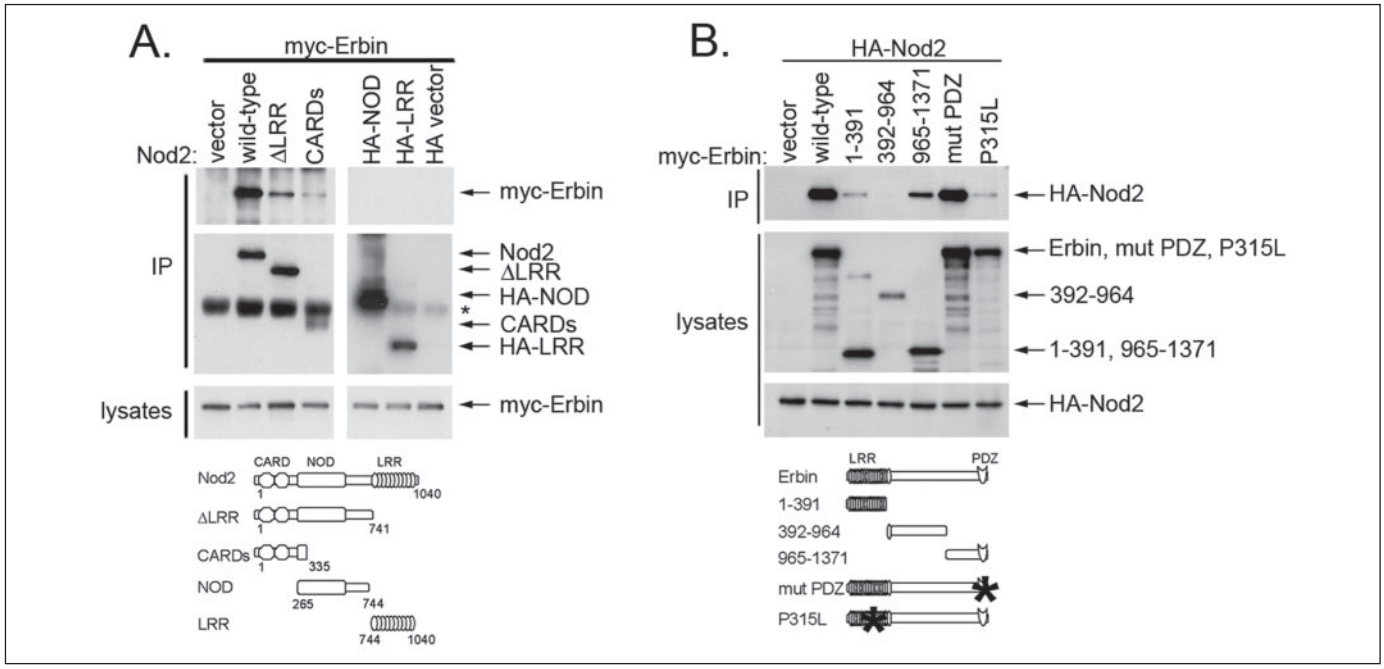

FIGURE 3. **Identification of interacting regions of Nod2 and Erbin.** *A*, the CARDs of Nod2 are necessary and sufficient for interaction with Erbin. Nod2 deletion constructs (diagrammed at *bottom*) were transiently transfected along with Myc-Erbin plasmid into HEK293T cells. The cell lysates were immunoprecipitated (*IP*) with either anti-Nod2 rabbit sera (*left panels*) or anti-HA antibody (*right panels*) and proteins visualized by Western blot with anti-Nod2 rabbit sera (*left middle panel*), anti-HA (*right middle panel*), or anti-Myc (*top* and *bottom panels*) antibodies. Nonspecific bands corresponding to immunoglobulin heavy chain are indicated with *asterisks*. *B*, two regions of Erbin interact with Nod2. Erbin deletion constructs and point mutants (diagrammed at *bottom*) were transiently transfected into HEK293T cells along with HA-Nod2 plasmid. The cell lysates were immunoprecipitated with anti-Myc antibody and proteins visualized by Western blot with anti-Myc (*middle panel*) and anti-HA antibodies (*top* and *bottom panels*).

fected HEK293T cells by co-immunoprecipitation assays. The lysates were immunoprecipitated with either an anti-FLAG or anti-Erbin antibody and analyzed by Western blot. The results of this assay demonstrated that endogenous Erbin protein co-immunoprecipitated with FLAG-Nod2, as well as the converse, where FLAG-Nod2 was pulled down with Erbin in an immunoprecipitation with an anti-Erbin antibody (Fig. 1*B*). Next, we detected interaction of the endogenous Nod2 and Erbin from lysates of a colon epithelial cell line. Immunoprecipitation of endogenous Nod2 from SW480 cell lysates co-immunoprecipitated endogenous Erbin (Fig. 1*C*). In addition, we demonstrated interaction of these proteins in a yeast two-hybrid assay using full-length Erbin as bait. Erbin was found to specifically interact with full-length Nod2, although this interaction was weaker than the interaction with a known binding partner of Erbin, PKP4 (plakophilin-4 or p0071) (Fig. 1*D*). Finally, we assessed the subcellular localization of an enhanced green fluorescent protein (EGFP)-tagged Nod2 and endogenous Erbin in polarized Caco2 epithelial cells and found that Nod2 and Erbin co-localize at the basolateral membrane (Fig. 1*E*). These results confirm that Erbin is a genuine Nod2-interacting protein.

Specificity of Erbin Interaction with Nod2—To determine the specificity of the interaction of Erbin with Nod2, the ability of different NOD family members to co-immunoprecipitate Erbin was assessed by Western blot (Fig. 2). Myc-tagged Erbin and the NOD family members Apaf-1, Nod1, Nod2, Ipaf, and Cryopyrin were expressed in HEK293T cells, and the NOD family members were immunoprecipitated from cell lysates. The association of Erbin with NOD proteins was detected by Western blot analysis. Nod2 was the only NOD family member that interacted with Myc-Erbin in this assay, demonstrating that Erbin binds specifically to Nod2.

Identification of Regions Required for Nod2-Erbin Interaction—The regions of interaction between Nod2 and Erbin were mapped using deletion constructs of the two proteins in co-immunoprecipitation assays. Nod2 has three distinct domain regions, the CARDs, NOD, and LRRs. All of the Nod2 constructs that contained the CARDs were able to co-immunoprecipitate full-length Myc-Erbin, whereas the NOD or LRR domains alone were not sufficient to pull down Myc-Erbin (Fig. 3*A*). Expression of the CARDs construct at high levels is toxic to cells. Therefore, the reduction in the total amount of Erbin pulled down by this construct relative to the other CARD-containing constructs is due to the lower level of expression rather than a decreased affinity of binding to the CARDs alone. These results demonstrate that the CARDs of Nod2 are necessary and sufficient for interaction with Erbin.

The Erbin protein is characterized by 16 amino-terminal LRRs, an LRR-like domain, a large intermediary region, and a carboxyl-terminal PDZ (PSD-90/DLG/ZO-1) domain. Deletion constructs were used that encompassed three different regions of Erbin, the LRRs (amino acids 1–391), the LRR-like domain, and the intermediary region (amino acids 392–964), and the carboxyl terminus, including the PDZ domain (amino acids 965–1371). Two regions of Erbin were able to co-immunoprecipitate full-length HA-Nod2, the regions of amino acids 1–391 and 965–1371, whereas the central region of amino acids 392–964 could not (Fig. 3*B*). The binding of each of these regions was weaker than the full-length Erbin, suggesting that they may cooperate for efficient binding to Nod2.

To further characterize the interaction of Erbin with Nod2, two point mutants of Erbin were tested in a co-immunoprecipitation assay. One point mutation, called mutPDZ (HG1347/8YD), is a mutation in the PDZ domain that abrogates the binding of Erbin to ErbB2 and peptide substrates (21). The other point mutation, P315L, disrupts the function of the LRRs and dramatically affects the ability of Erbin to localize to membranes (24). The mutPDZ construct was able to efficiently co-immunoprecipitate HA-Nod2, whereas the P315L mutation substantially decreased the interaction of these two proteins (Fig. 3*B*). These results suggest that residues within the LRRs and the carboxyl terminus

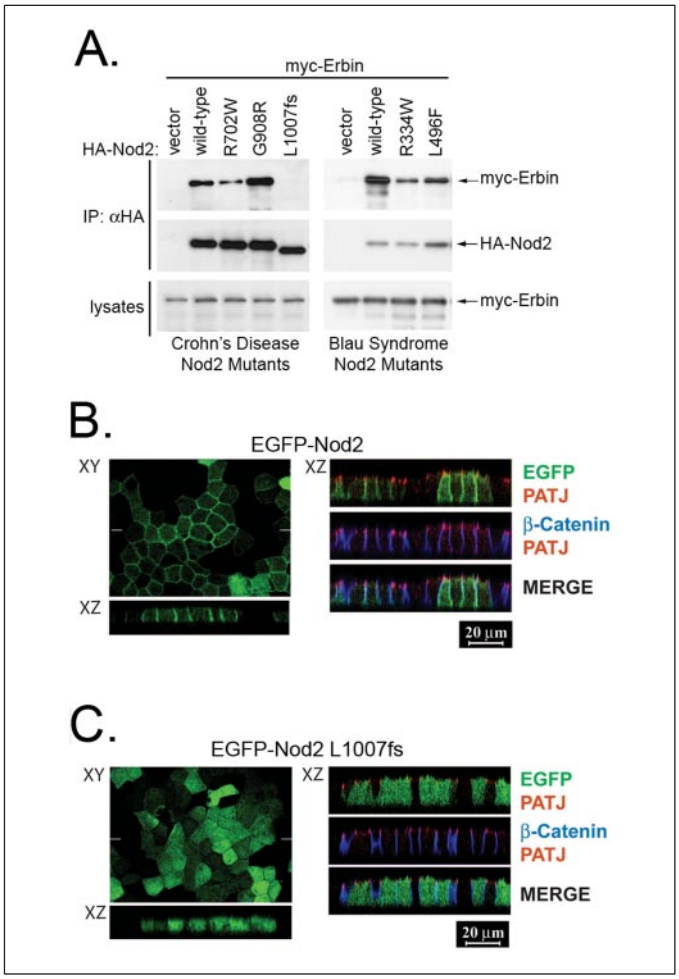

FIGURE 4. **Interaction of Erbin with disease-associated Nod2 mutants.** *A*, HEK293T cells were transiently transfected with HA-Nod2 expression constructs with the indicated mutations and Myc-Erbin plasmid. The cell lysates were immunoprecipitated (*IP*) with anti-HA antibody, and the proteins were visualized by Western blot with anti-Myc (*top* and *bottom panels*) and anti-HA (*middle panel*) antibodies. *B*, wild-type Nod2 is localized at the basolateral membrane of polarized epithelial Caco2 cells. Confocal analysis of Caco2 cells stably expressing EGFP-Nod2 (*green*) following immunostaining with antibodies directed against PATJ (*red*), a subapical marker, and β-Catenin (*blue*), a lateral marker, is shown. *C*, the CD-associated Nod2 mutant, L1007fs, localizes to the cytoplasm of polarized epithelial Caco2 cells. Confocal analysis of Caco2 cells stably expressing EGFP-Nod2 L1007fs (*green*) as performed in *B* is shown.

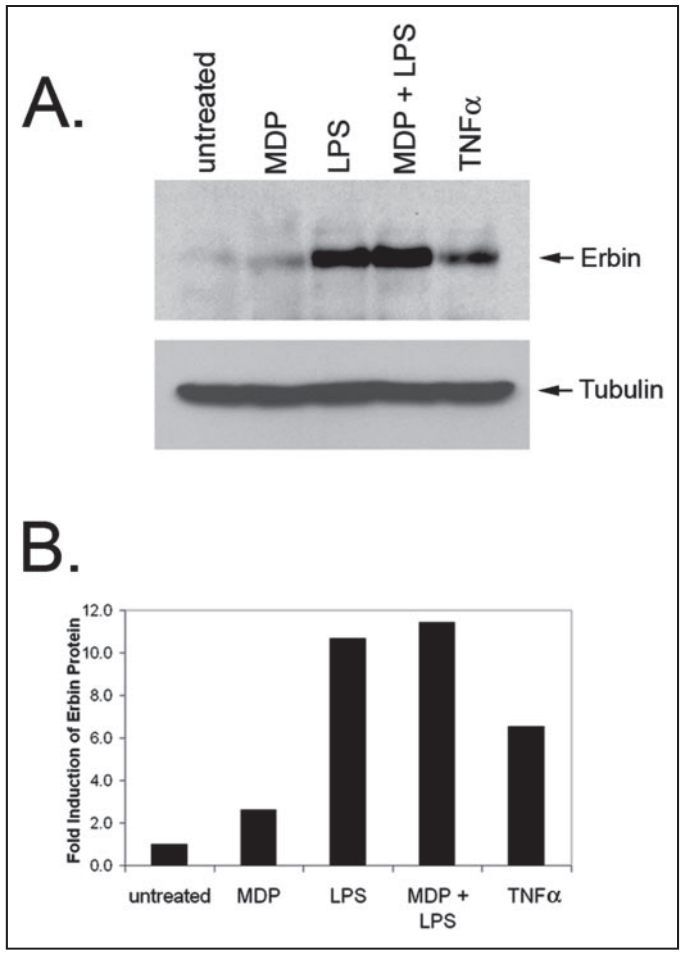

FIGURE 5. *A*, Erbin protein expression is induced by inflammatory stimuli. MonoMac6 cells were stimulated with medium (*untreated*), 1 μg/ml MDP, 100 ng/ml LPS, a combination of MDP and LPS, or 10 ng/ml TNFα for 24 h. Erbin expression in cell lysates determined by Western blot analysis with anti-Erbin rabbit sera (*top panel*) and equal loading confirmed by Western blot with anti-tubulin antibody (*bottom panel*). *B*, quantitation of Erbin protein expression by densitometry. Pixel intensity of untreated sample set at 1 and fold increase of treated samples were calculated.

of Erbin are sufficient for interaction with Nod2, but a functional PDZ domain is not required.

Analysis of the Interaction of Erbin with Nod2 Disease-associated Mutants—Mutations in the *NOD2* gene are associated with two inflammatory diseases, CD and BS. These mutations in humans are associated with either a loss-of-function (CD) or gain-of-function (BS) Nod2 phenotype. The change in activity of these disease-associated Nod2 mutants may be due to altered interactions with protein-binding partners. Therefore, the ability of Erbin to interact with disease-associated Nod2 mutants was tested in co-immunoprecipitation assays. The CD-associated mutations R702W and G908R, as well as the BS-associated mutations R334W and L469F, bound similar amounts of Erbin as compared with the amount bound to wild-type Nod2 (Fig. 4*A*). Another CD-associated Nod2 mutant, L1007fs, lost the ability to interact with Erbin completely. These results suggest that altered Nod2 activity caused by these disease-associated mutations is not correlated with changes in interaction with Erbin.

The loss of interaction between the L1007fs Nod2 mutant with Erbin was surprising, because this *NOD2* mutation results in a carboxyl-terminally truncated protein and leaves the Erbin-interaction domain of Nod2 (the CARDs) intact. This loss of interaction could be explained by either alterations in the protein conformation of this mutant making the CARDs inaccessible for interaction or by an inability of this mutant to localize to the basolateral membrane where Erbin is found. We compared the subcellular localization of the L1007fs Nod2 mutant to the localization of wild-type Nod2 in polarized Caco2 epithelial cell lines expressing EGFP-tagged Nod2 proteins. Wild-type EGFP-Nod2 localized to the basolateral membrane, as detected by co-localization with β-Catenin, a lateral membrane marker (Fig. 4*B*). In contrast, the EGFP-Nod2 L1007fs mutant did not localize to the basolateral membrane but was found in the cytoplasm of these cells (Fig. 4*C*). These results demonstrate that the absence of interaction between Erbin and the L1007fs Nod2 mutant is most likely due to the localization of these proteins to different subcellular compartments.

Induction of Erbin Protein Expression by Inflammatory Stimuli—Many signaling pathways up-regulate components of their signal transduction cascade, both positive and negative factors, in response to activation. To determine whether Erbin expression increases in response to Nod2 activation, the levels of Erbin protein expression were determined in a monocytic cell line, MonoMac6 (Fig. 5). MonoMac6 cells express Nod2, respond to MDP transcriptionally, and are able to secrete pro-inflammatory cytokines synergistically with co-treatment of MDP and

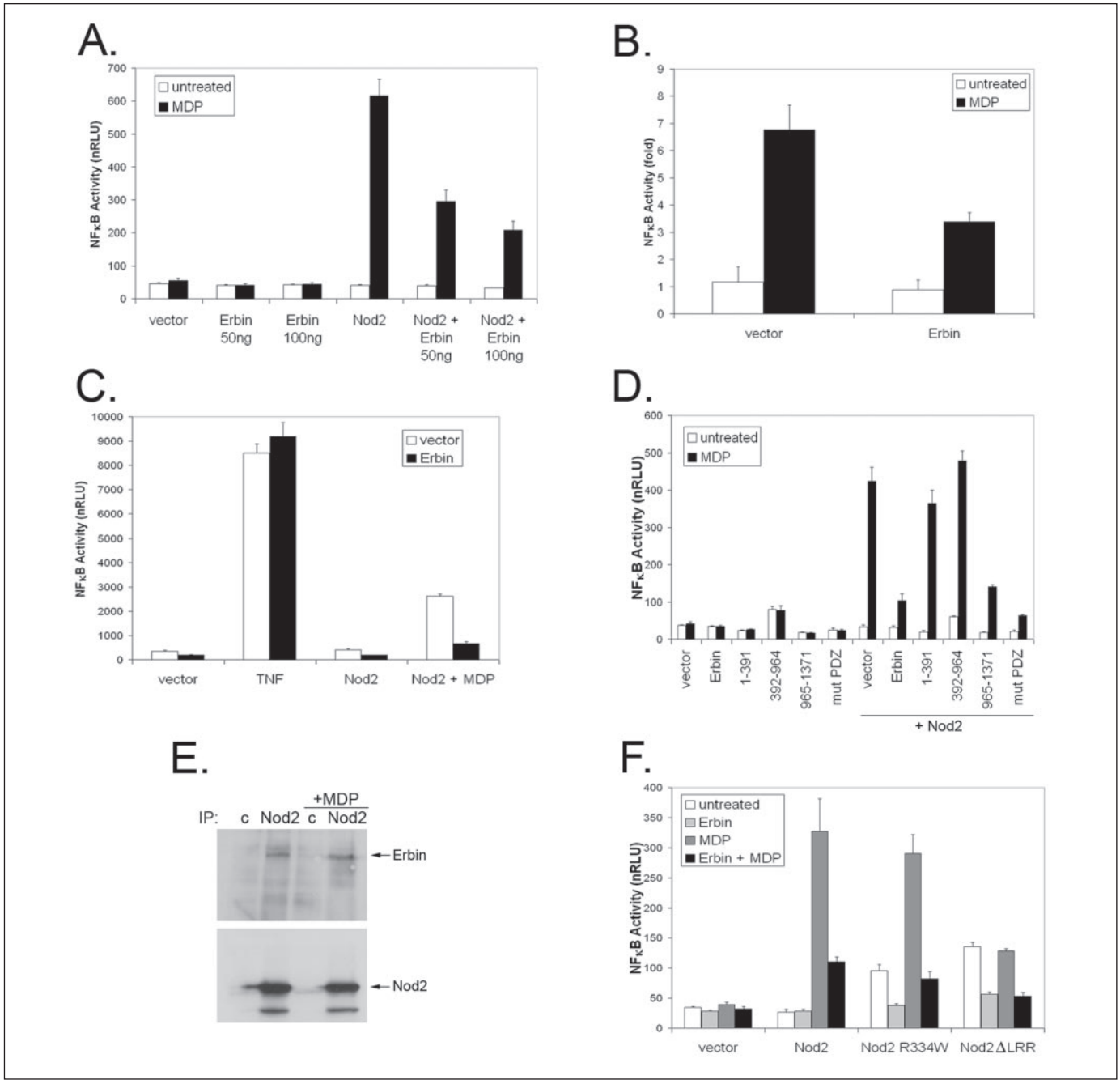

FIGURE 6. **Erbin inhibits Nod2-dependent NF-κB signaling.** *A*, Erbin inhibits Nod2-dependent activation of NF-κB in a dose-dependent manner. Luciferase reporter gene assays were performed in HEK293T cells transiently transfected in triplicate with a NF-κB luciferase reporter plasmid, expression plasmids with Nod2, Erbin, or no insert (vector), along with a β-galactosidase transfection control plasmid. The cells were treated with 100 ng/ml MDP for 16 h followed by measurement of luciferase and β-galactosidase activity in cell lysates. Raw luciferase values were normalized to transfection efficiency (nRLU), and the average and standard deviations were calculated. *B*, Erbin inhibits MDP-induced NF-κB activity in LoVo colon epithelial cells. LoVo cells were transfected in triplicate with a NF-κB luciferase reporter plasmid, empty vector or Erbin expression plasmid, and β-galactosidase transfection control plasmid. The cells were treated and analyzed as in *A*. NF-κB activity is shown as fold increase over untreated samples. *C*, activation of NF-κB by TNFα is unaffected by Erbin. Luciferase reporter gene assays were performed as in *A*, with cells treated with either medium (*untreated*), 10 ng/ml TNFα, or 100 ng/ml MDP for 16 h. *D*, the carboxyl terminus of Erbin is required for the inhibition of Nod2-dependent activation of NF-κB. Luciferase reporter gene assays were performed as in *A*, with cells transfected with deletion constructs and point mutants of Erbin. *E*, association of endogenous Erbin and Nod2 proteins is not affected by stimulation with MDP. LoVo cells were stimulated with 1 μg/ml MDP for 1 h, and the lysates were immunoprecipitated with anti-Nod2 rabbit sera (*Nod2*) or normal rabbit sera (*c*) followed by Western blot analysis with anti-Erbin (*top panel*) or anti-Nod2 2D9 (*bottom panel*) antibodies. *F*, Erbin inhibits both ligand-dependent and ligand-independent Nod2-dependent activation of NF-κB. Luciferase assays were performed as in *A*, with cells transfected with either vector, wild-type Nod2 (Nod2), a constitutively active Nod2 mutant whose activity is enhanced by MDP treatment (Nod2 R334W), or a constitutively active Nod2 mutant that lacks the ligand sensing LRR region (Nod2ΔLRR).

LPS (Ref. 33 and data not shown). In unstimulated cells, Erbin protein was barely detectable by anti-Erbin Western blot analysis of lysates (Fig. 5). With MDP treatment of cells, Erbin protein expression was increased modestly (~2.3-fold), suggesting that Erbin may be a target of Nod2 signaling. In addition, Erbin protein expression was strongly induced in response to LPS (10.7-fold), a combination of MDP and LPS (11.4-fold), and TNFα (6.5-fold). These results demonstrate that Erbin protein expression is regulated by inflammatory stimuli.

Erbin Inhibits Nod2-dependent NF-κB Signaling—Stimulation of Nod2 by MDP induces the activation of the NF-κB signaling pathway

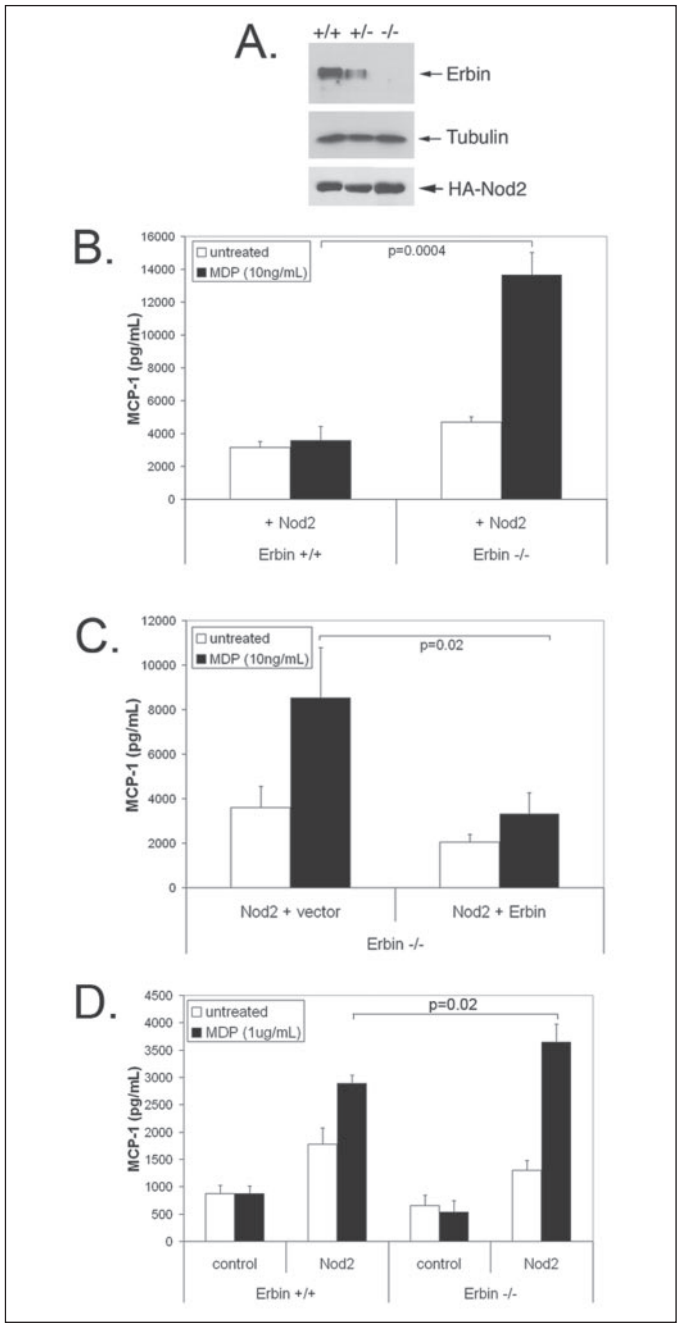

FIGURE 7. **Erbin expression affects Nod2-dependent cytokine secretion.** *A*, Erbin and HA-Nod2 expression in mouse embryo fibroblasts used in these studies. The lysates were analyzed by Western blot using anti-Erbin rabbit sera (*top panel*), anti-tubulin (*middle panel*), and anti-HA (*bottom panel*) antibodies. Genotype is indicated as wild-type (+/+), heterozygous (+/−), or homozygous (−/−) deletion of *ERBIN*. *B*, loss of Erbin expression increases the sensitivity of mouse embryo fibroblasts to MDP. Wild-type or Erbin$^{-/-}$ mouse embryo fibroblast cell lines expressing Nod2 were treated with either medium (*untreated*) or low levels of MDP (10 ng/ml) for 8 h and the secretion of MCP-1 into tissue culture medium was assessed by enzyme-linked immunoabsorbant assay. *B*, the increased sensitivity of Erbin$^{-/-}$ cells to MDP can be reversed by Erbin expression. The Erbin$^{-/-}$ mouse embryo fibroblast cell line expressing Nod2 was transiently transfected with either empty vector or Erbin expression vector for 16 h prior to analysis as in A. *C*, MDP-induced secretion of MCP-1 is dependent on Nod2. Wild-type and Erbin$^{-/-}$ mouse embryo fibroblasts were infected with either control retrovirus or Nod2 expressing retrovirus and then treated with either medium (*untreated*) or 1 μg/ml MDP for 18 h. MCP-1 secretion was assessed as in *A*.

and the production of pro-inflammatory cytokines. The effect of Erbin on Nod2-dependent NF-κB signaling was tested in luciferase reporter gene assays (Fig. 6). First, we assessed the effect of increased Erbin expression on Nod2-dependent NF-κB activity in HEK293T cells. HEK293T cells do not express detectable amounts of endogenous Nod2 and therefore only become responsive to MDP when Nod2 is ectopically expressed. Transfection of increasing amounts of Erbin expression plasmid decreased the amount of Nod2-dependent NF-κB reporter activity in response to MDP treatment in a dose-dependent manner (Fig. 6*A*). We saw similar effects on the MDP-induced NF-κB activity in LoVo cells, a colon epithelial cell line that expresses Nod2 endogenously (Fig. 6*B*). In addition, we found that Erbin inhibition of NF-κB reporter activity was specific to Nod2, because overexpression of Erbin did not affect TNFα-stimulated activation of NF-κB in HEK293T cells (Fig. 6*C*). These results suggest that Erbin functions as an inhibitor of Nod2-dependent NF-κB signaling.

Characterization of Nod2 Inhibition by Erbin—The LRR region of Erbin has been previously shown to inhibit the activation of the Erk signaling pathway (25, 34). To characterize the requirement of the LRRs of Erbin in the inhibition of Nod2 signaling, deletion mutants of Erbin were tested for their ability to inhibit Nod2 signaling in NF-κB reporter assays (Fig. 6*D*). Surprisingly, even though the LRRs (amino acids 1–391) of Erbin interact with Nod2, they did not have an inhibitory effect on Nod2-dependent NF-κB signaling. Instead, another region of Erbin that interacts with Nod2, the carboxyl terminus of Erbin (amino acids 965–1371), was able to inhibit Nod2 activity to a similar extent as full-length Erbin. Mutation of the PDZ domain found in this region of Erbin (mutPDZ) had no effect on its inhibitory activity, suggesting that residues in the adjacent linker region are responsible for Nod2 inhibition.

We examined the molecular mechanisms of Erbin inhibition of Nod2 activity to determine whether Erbin interferes with the activation of Nod2 by MDP or transmission of a signal to downstream effectors of Nod2. We first examined whether the association of endogenous Nod2 and Erbin was affected by MDP stimulation in LoVo cells by a co-immunoprecipitation assay. This assay showed that the interaction of these two proteins was not detectably altered by MDP stimulation (Fig. 6*E*). Next, the effect of Erbin on two constitutively active Nod2 mutants was tested in NF-κB reporter assays. One of the Nod2 mutants, Nod2 R334W, is a mutation associated with Blau syndrome and has a point mutation in the NOD domain that results in constitutive activity that can be enhanced by MDP stimulation (10, 19). The other Nod2 mutant, Nod2ΔLRR, is a truncated Nod2 that is also constitutively active but is unresponsive to MDP because it lacks the ligand-sensing LRRs (19). Both the basal activity and the MDP enhanced activity of the Nod2 R334W mutant was inhibited by Erbin expression (Fig. 6*F*). In addition, Erbin also inhibited the activity of Nod2ΔLRR, indicating that Erbin affects the ability of Nod2 to signal through downstream effectors.

Erbin Inhibits Nod2-dependent Cytokine Secretion—Activation of Nod2 by MDP stimulates the production of pro-inflammatory cytokines. The role of Erbin in Nod2-dependent cytokine secretion was assessed using cells that lack Erbin expression. MEFs were harvested from wild-type and Erbin$^{-/-}$ mice and stable cell lines produced after infection with a retrovirus that expressed Nod2 (Fig. 7*A*). These cells were stimulated with MDP, and the secretion of MCP-1, an NF-κB dependent cytokine, was determined by enzyme-linked immunoabsorbant assay. Erbin expression was found to affect the sensitivity of these cells to MDP treatment, because treatment with low levels of MDP that did not induce MCP-1 secretion in wild-type cells stimulated secretion of MCP-1 in Erbin-deficient cells ($p = 0.0004$) (Fig. 7*B*). This increase in MDP sensitivity was confirmed to be dependent on Erbin through re-expression of Erbin by transient transfection. The secretion of MCP-1 in response to MDP was abrogated with the transfection of Erbin plasmid

($p = 0.02$) (Fig. 7*C*). To demonstrate the requirement of Nod2 in the MDP-induced secretion of MCP-1, wild-type and Erbin$^{-/-}$ MEFs were infected with either a control retrovirus or a retrovirus that expressed Nod2, and the MDP-induced secretion of MCP-1 was assessed by enzyme-linked immunoabsorbant assay. MDP stimulation of MEFs infected with the control virus did not cause secretion of MCP-1, even at high concentrations of MDP (Fig. 7*D*). In contrast, MEFs infected with Nod2 virus secreted high levels of MCP-1 in response to MDP treatment, with Erbin$^{-/-}$ MEFs secreting more MCP-1 than wild-type MEFs ($p = 0.02$) (Fig. 7*D*). These results demonstrate that the level of Erbin expression in cells affects the sensitivity of cells to MDP in a Nod2-dependent manner. This study identifies a novel role for Erbin in inflammation as a regulator of Nod2-dependent NF-κB signaling and pro-inflammatory cytokine secretion.

DISCUSSION

Alterations in Nod2 activity have been correlated with inflammatory disease, highlighting the importance of regulation of Nod2 signaling. To date, identification of specific components of the Nod2 signaling pathway has been limited. The protein kinase RICK has been demonstrated to be an essential component of the Nod2 signaling pathway, because Nod2-dependent activation of NF-κB is blocked in RICK-deficient cells (5). RICK acts to recruit downstream signaling molecules to Nod2, induce the ubiquitinylation of IKKγ (a component of the IKK complex), and promote the activation of NF-κB signaling (7, 29, 35, 36). Recent papers suggest that Nod2 may also interact with GRIM-19 (a protein with homology to the NADPH dehydrogenase complex) and the kinase TAK1 (transforming growth factor-β-activated kinase 1) to activate NF-κB signaling (37–39). In addition, there is one report that the NOD protein, Ipaf (also called CLAN), may heterodimerize with Nod2 and negatively affect Nod2 signaling (40). In this study, we identify a new regulator of the Nod2 signaling pathway, a protein called Erbin. We show that Erbin binds specifically to Nod2 and inhibits the activation of NF-κB and subsequent inflammatory cytokine secretion by Nod2. This is a new function for Erbin, because this protein is best characterized by its scaffolding functions in polarized cells and for its role in regulating another signaling pathway, Erk, involved in cellular growth (20). These results expand the cellular roles of Erbin to include the regulation of inflammatory processes.

We demonstrate that Erbin binds to the CARDs of Nod2 through two distinct regions, one region in the amino terminus of Erbin that includes the LRRs and a second region in the carboxyl terminus that includes a PDZ domain. However, it was found that the PDZ domain, which is required for interaction with many Erbin targets such as ErbB2, PKP4, the p120-like Catenins, δ-Catenin, and ARVCF (Armadillo protein deleted in velo-cardiofacial syndrome), was not required for interaction with Nod2 (21, 26, 41–43). This is similar to what has been reported for interaction of Erbin with PSD-90 and EBP50, where residues between amino acids 965–1240 were required for interaction (25, 34). This region of Erbin has been reported to be alternatively spliced, although the functional significance of this has not been determined. Further analysis will determine whether these Erbin splice variants have altered interactions with Nod2 and contribute to cell type-specific regulation of Nod2 activity (44).

Specific mutations in the *NOD2* gene result in altered activity of Nod2 and are associated with two inflammatory diseases in humans. We examined whether the change in activity of these disease-associated Nod2 mutants could be due to an altered interaction with Erbin. We found no correlation between the ability of these mutants to bind to Erbin and their altered activity, suggesting that there may be several mechanisms that regulate Nod2 activity in addition to interaction with Erbin. Further study of the molecular mechanisms of Nod2 activation and the identification of other regulatory proteins will increase our understanding of Nod2 activity and how alterations of Nod2 signaling contribute to disease pathogenesis.

Interestingly, the L1007fs CD-associated Nod2 mutant was unable to bind to Erbin, even though it contains intact CARDs. This loss of interaction is most likely due to the mislocalization of the L1007fs mutant from the basolateral membrane, where Erbin is found, to the cytoplasm of polarized Caco2 epithelial cells. These findings are in agreement with a recent study that examined the subcellular localization of CD-associated Nod2 mutants in nonpolarized epithelial cells (45). In this study, the authors propose that membrane localization of Nod2 is required for MDP-dependent activation of NF-κB. In light of these observations, we would hypothesize that Erbin suppresses the activation of membrane associated Nod2 until a threshold level of MDP stimulation is reached to prevent inappropriate signaling. In this manner, Erbin may function to modulate the sensitivity of intestinal epithelial cells to bacterial products.

The factors that regulate Erbin protein expression are not well known. Previous studies have demonstrated that Erbin is a target of the tumor suppressor BRCA-1 and that loss of BRCA-1 and Erbin mRNA expression are correlated with a poor prognosis in early primary breast cancer (46, 47). Erbin expression is also increased at the neuromuscular junction after denervation, suggesting that the protein levels of Erbin are regulated by neuronal activity (25). We found that Erbin protein expression is regulated by the pro-inflammatory mediators MDP, LPS, and TNFα. This is similar to the expression of a TLR4 inhibitor, ST2, in response to activation of the TLR4-signaling pathways by LPS (48). Increased expression of ST2 blocks additional stimulation of cells by LPS, and therefore ST2 is thought to be a key regulator of endotoxin tolerance. Our findings suggest that Erbin may play a similar role in down-modulating inflammatory responses.

Erbin has been previously shown to act as a negative regulator of a signaling pathway involved in cellular proliferation, the Erk pathway. Erbin blocks the activation of Erk by ErbB2 in polarized epithelial cells and by the neurofibromatosis 2 protein, merlin, in Schwann cells (25, 34). In our studies, we found that Erbin acts as a negative regulator of another signaling pathway, the Nod2-dependent NF-κB signaling pathway. However, the region of Erbin required for inhibition of Nod2 signaling is distinct from the region of Erbin required for inhibition of Erk signaling. The inhibition of Erk is mediated through disruption of the interaction of upstream activators, Ras and Raf by the LRRs of Erbin (25). We found that a region in the carboxyl terminus of Erbin blocks the transmission of MDP recognition to the downstream effectors of Nod2. Interestingly, the interaction of Erbin with Nod2 was not detectably altered by stimulation of colon epithelial cells with the Nod2 ligand, MDP. This suggests that there may be a conformational change of Nod2 upon MDP stimulation that releases the inhibitory region of Erbin from the CARDs of Nod2 but does not result in complete dissociation of Erbin from Nod2. More studies need to be performed to determine the exact molecular mechanisms involved in Erbin inhibition of Nod2 signaling. Our findings show that Erbin uses two distinct regions to regulate different cellular signaling pathways and that the inhibitory effects of Erbin are downstream of ligand recognition by Nod2.

In conclusion, this study has identified Erbin as a negative regulator of the Nod2 signaling pathway and describes a new role for Erbin in inflammatory signaling. Future studies examining the immune responses of Erbin$^{-/-}$ mice to bacterial infection, as well as evaluation

of tissues from inflammatory disease models, will provide additional information about the role of Erbin in inflammation and disease.

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A Role for Erbin in the Regulation of Nod2-dependent NF-κB Signaling

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