Modulation of Nod2-dependent NF-κB signaling by the actin cytoskeleton

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Summary

Actin disruption by CytochalasinD (CytD) and LatrunculinB (LatB) induced NF-κB activation in myelomonocytic and intestinal epithelial cells. In an attempt to elucidate the mechanism by which actin disruption induced IKK activation, we studied the human Nod2 protein, which was able to induce NF-κB activation and whose expression was restricted to myelomonocytic and intestinal epithelial cells. Nod2 is thought to play key roles in pathogen defence through sensing bacteria and generating an inflammatory immune response. We showed that actin disruption by CytD significantly and specifically increased Nod2-mediated NF-κB signaling. Nod2 was fully partitioned in the Triton-X-100-insoluble fraction but translocated into the soluble fraction after CytD treatment, demonstrating that the presence of Nod2 in the detergent-insoluble pellet was specific to actin cytoskeleton. Confocal analysis also revealed a Nod2 colocalization with membrane-associated F-actin. Colocalization and co-immunoprecipitation assays with endogenous Rac1 have shown that Nod2 associated with activated Rac1 in membrane ruffles through both its N-terminal caspase recruitment domains (CARD) and C-terminal leucine-rich repeats (LRRs). Membrane ruffle disruption by a Rac1 dominant negative form primed Nod2-dependent NF-κB signaling. The recruitment of Nod2 in Rac-induced dynamic cytoskeletal structures could be a strategy to both repress the Nod2-dependent NF-κB signaling in unstimulated cells and rapidly mobilize Nod2 during bacterial infection.

Key words: Actin cytoskeleton, NF-κB, Nod2, Rac1

Introduction

The cytoskeleton is involved in many aspects of cellular function, such as cell movement (Wittmann and Waterman-Storer, 2001), muscle contraction (Clark et al., 2002), phagocytosis (Friedland et al., 2001) and mitosis (Nanninga, 2001). Several studies suggested a link between cytoskeleton dynamics and alteration of gene expression through direct interactions with members of transduction pathways. Indeed, it has been demonstrated that cytoskeleton-disrupting factors could activate specific protein kinases (Shtil et al., 1999; Christerson et al., 1999) or transcription factors (Sotiropoulos et al., 1999; Mack et al., 2001; Holsinger et al., 1998). Recently, we have shown that actin disruption induced a significant activation of the transcription factor NF-κB in myelomonocytic cells (Kustermans et al., 2005).

The transcription factor NF-κB regulates the transcription of an exceptionally large number of genes, particularly those involved in immune and inflammatory responses, the control of apoptosis and cell proliferation (for a review, see Ghosh et al., 1998). Inappropriate regulation of NF-κB is directly involved in a wide range of human disorders, including a variety of cancers (for a review, see Luque and Gelinas, 1997), neurodegenerative diseases (for a review, see Grilli and Memo, 1999), arthritis (for a review, see Foxwell et al., 1998) and many other inflammatory diseases. NF-κB binds specific DNA sequences as dimers of the Rel/NF-κB family members (for a review, see Ghosh et al., 1998). NF-κB complexes are sequestered in the cytoplasm of most resting cells by inhibitory proteins belonging to the IκB family (for a review, see Ghosh et al., 1998). Upon stimulation with proinflammatory agents such as cytokines (TNFα, IL-1β) or bacterial lipopolysaccharides (LPS), IκBα is phosphorylated, polyubiquitinated, then degraded by the 26S proteasome, allowing NF-κB to translocate to the nucleus and transactivate its target genes (for reviews, see Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002). IκBα is phosphorylated on Ser32 and Ser36 by a specific IκB kinase (IKK). IKK is a complex composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO (for reviews, see Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002).

We have recently demonstrated that the actin disruption by a variety of agents, including cytochalasin D (CytD), latrunculin B (LatB) and jasplakinolide (JP) leads to NF-κB activation in various myelomonocytic cell lines and human monocytes through an IKK-dependent pathway (Kustermans et al., 2005). No significant NF-κB activation in response to actin disruption could be observed in other cell types such as HeLa cells, murine fibroblasts or human T lymphocytes. Simultaneously, it has been shown that treatment of human intestinal epithelial cells with CytD or LatB resulted in

increased NF-κB activation and IL-8 expression (Németh et al., 2004).

In an attempt to elucidate the mechanism by which actin disruption induces IKK activation, we turned to the human Nod2 protein which is able to induce NF-κB activation and whose expression is restricted to myelomonocytic and intestinal epithelial cells (Ogura et al., 2001a; Gutierrez et al., 2002; Berrebi et al., 2003). Nod2 is a member of a family of over 20 cytosolic proteins characterized by the presence of a conserved nucleotide-binding oligomerization domain (NOD) (Inohara et al., 2005). These molecules play key roles in pathogen defence through sensing bacteria and generating an inflammatory immune response. Nod2 detects bacteria by recognizing muramyl dipeptide (MDP), a specific component of the peptidoglycan present in the cell walls of Gram-negative and Gram-positive bacteria (Girardin et al., 2003; Inohara et al., 2003). In addition to a central NOD, Nod2 contains an N-terminal effector binding region that consists of two caspase-recruitment domains (CARD) and C-terminal leucine-rich repeats (LRRs) that are required to detect MDP (Girardin et al., 2003; Inohara et al., 2003). Stimulation of Nod2 by MDP causes Nod2 oligomerization and the recruitment of a protein kinase RICK (also called RIP2) through a CARD-CARD interaction (Ogura et al., 2001a; Inohara et al., 2000). RICK then oligomerizes to transmit the Nod2 signal directly to the IKK complex, leading to activation of the NF-κB signaling pathway.

Three main genetic variants within the coding region of Nod2 have been associated with susceptibility to Crohn's disease (CD), a chronic inflammatory disease of the gastrointestinal tract (Hugot et al., 2001; Ogura et al., 2001b). These mutations (two missense mutations, R702W and G908R, as well as a frameshift mutation, L1007fsinsC) are grouped near or within the ligand-sensing region of Nod2 (LRRs). Although there is some controversy over the effects of these mutations on Nod2 signaling, PBMCs and macrophages from CD patients homozygous for these mutations show severe defects in both NF-κB activation and cytokine secretion in response to MDP, suggesting that these Nod2 mutations result in a loss-of-function phenotype in humans (Inohara et al., 2003; Li et al., 2004; Maeda et al., 2005; Netea et al., 2005; van Heel et al., 2005).

In this paper, we showed that actin disruption increased Nod2-mediated NF-κB activation. To understand the way in which Nod2 senses perturbations of actin dynamics, we performed subcellular localization assays and demonstrated that Nod2 is sequestered in actin cytoskeleton structures such as lamellipodia and membrane ruffles in association with the small RhoGTPase, Rac1.

Results

Actin disruption increases Nod2-mediated NF-κB activation and IL-8 secretion

HEK293 cells were transiently co-transfected with a Nod2 expression plasmid and a NF-κB reporter construct before being treated with actin-disrupting agents. We used two inhibitors of actin polymerization such as cytochalasin D (CytD) (Sampath and Pollard, 1991) and latrunculin B (LatB) (Coue et al., 1987) to induce actin cytoskeleton alterations. No significant induction of NF-κB-mediated transcription was observed when HEK293 cells transfected with a control plasmid were exposed to CytD or LatB (Fig. 1A). Because overexpression of Nod2 induces NF-κB activation (Ogura et al., 2001a), HEK293 cells were transfected with a small amount of the Nod2 expression plasmid. A small increase of LUC activities was detected in Nod2-expressing cells (about

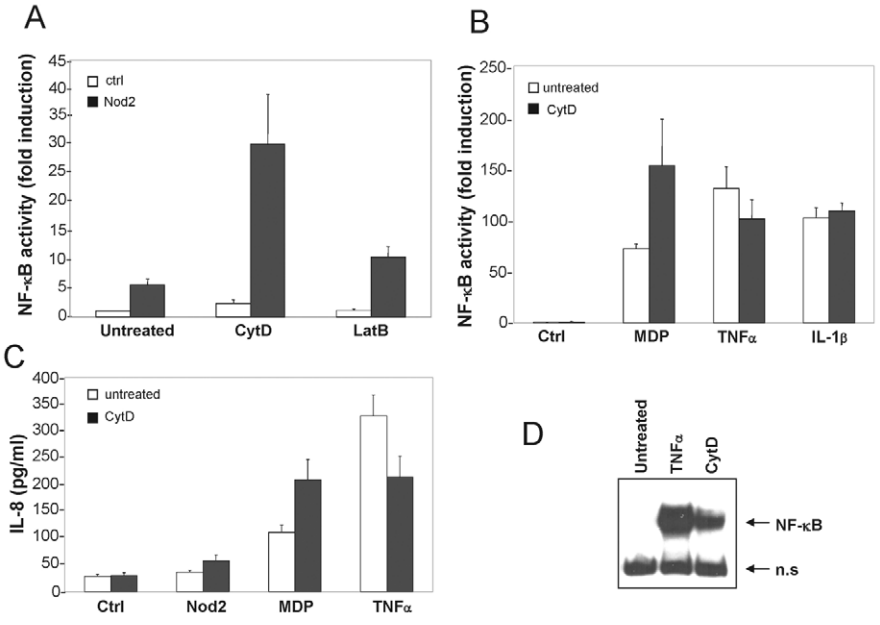

Fig. 1. Actin disruption increases Nod2-mediated signaling. (A) HEK293 cells were transfected with 500 ng of pcDNA3 and 50 ng of $(\kappa B)_5$LUC in the presence (black bars) or not (white bars) of 6 ng pcDNA3-Nod2. Twenty-four hours post transfection, cells were untreated or treated with CytD (50 μM) or LatB (1 μM) for 7 hours before being harvested for LUC assays. Values represent the mean + s.d. of triplicate cultures. (B) HEK293 cells were transfected with 500 ng pcDNA3, 50 ng $(\kappa B)_5$LUC and 10 ng of empty vector (Ctrl, TNFα and IL1β) or plasmid encoding Nod2 (MDP). Twenty-four hours post transfection, cells were treated (black bars) or not (white bars) for 7 hours with CytD (5 μM), alone or in combination with MDP (100 ng/ml), TNFα (10 U/ml) or IL-1β (100 U/ml) before being harvested for LUC assays. Values represent the mean + s.d. of triplicate cultures. (C) HEK293 cells were transfected with 500 ng pcDNA3 and 10 ng of empty vector (Ctrl, TNFα) or plasmid encoding Nod2. Twenty-four hours post transfection, cells were treated (black bars) or not (white bars) with CytD (5 μM), alone or in combination with MDP (100 ng/ml) or TNFα (10 U/ml). After 18 hours, the supernatants were harvested for IL-8 quantification by ELISA. Values represent the mean + s.d. of triplicate cultures. (D) HT-29 cells were treated or not with TNFα (100 U/ml) or CytD (10 μM) for 2 hours before being harvested for nuclear extraction. The DNA-binding activity of nuclear proteins (5 μg) to a ^{32}P-labeled κB probe was estimated by EMSA. n.s., non-specific band.

fivefold) whereas a significant NF-κB activation was observed in Nod2-expressing cells after CytD or LatB treatment (30- or 10-fold, respectively) (Fig. 1A). LatB was less efficient than CytD.

Under physiological conditions, Nod2 is activated by its microbial ligand, MDP (Girardin et al., 2003; Inohara et al., 2003). We tested whether the actin disrupting treatment could also have a synergistic effect on Nod2-dependent NF-κB activation following MDP stimulation. As expected, MDP efficiently induced NF-κB activation in HEK293 cells transfected with a small amount of Nod2 (up to 72-fold, Fig. 1B). Interestingly, actin disruption further increased the MDP-dependent NF-κB signaling (up to 155-fold). In addition, we found that the modulation of NF-κB signaling by actin cytoskeleton was specific to Nod2 and MDP because CytD did not affect TNFα- or IL-1β-mediated NF-κB activation in HEK293 cells (Fig. 1B).

To confirm the modulating effect of actin cytoskeleton in the Nod2-mediated NF-κB signaling, we measured the release of IL-8 in the supernatant of HEK293 cells transfected with Nod2 and stimulated by CytD and MDP, alone or in combination. The Fig. 1C shows that the amount of IL-8 in the supernatant correlated with NF-κB activation. Indeed, the amount of IL-8 found in the supernatant of HEK293 cells that were transfected with Nod2 and stimulated with MDP (111 pg/ml) was significantly increased by CytD (209 pg/ml) whereas the IL-8 secretion in response to TNFα was partially inhibited by actin disrupting treatment.

Finally, we analyzed the NF-κB activation in response to CytD in HT-29 intestinal epithelial cells expressing endogenous levels of Nod2 (Barnich et al., 2005a). Since HT-29 cells are difficult to transfect with DNA plasmids, we estimated NF-κB activation in these cells by determining the DNA-binding activity of nuclear extracts on a probe carrying a κB site (electromobility shift assay: EMSA). As shown in Fig. 1D, actin disruption by CytD in HT-29 cells also leads to a significant NF-κB activation.

Both CARDs of Nod2 are necessary and sufficient to induce NF-κB activation after CytD treatment

Nod2 is characterized by the presence of three motifs: two N-terminal caspase recruitment domains (CARD1 and CARD2), a central nucleotide-binding oligomerization domain (NOD) and C-terminal leucine-rich repeats (LRRs) (Fig. 2A). Both CARDs are involved in a homophilic CARD-CARD interaction with RICK, which in turn interacts with the γ subunit of the IKK complex (Ogura et al., 2001a). NOD is required for Nod2 oligomerization, which promotes the proximity of RICK

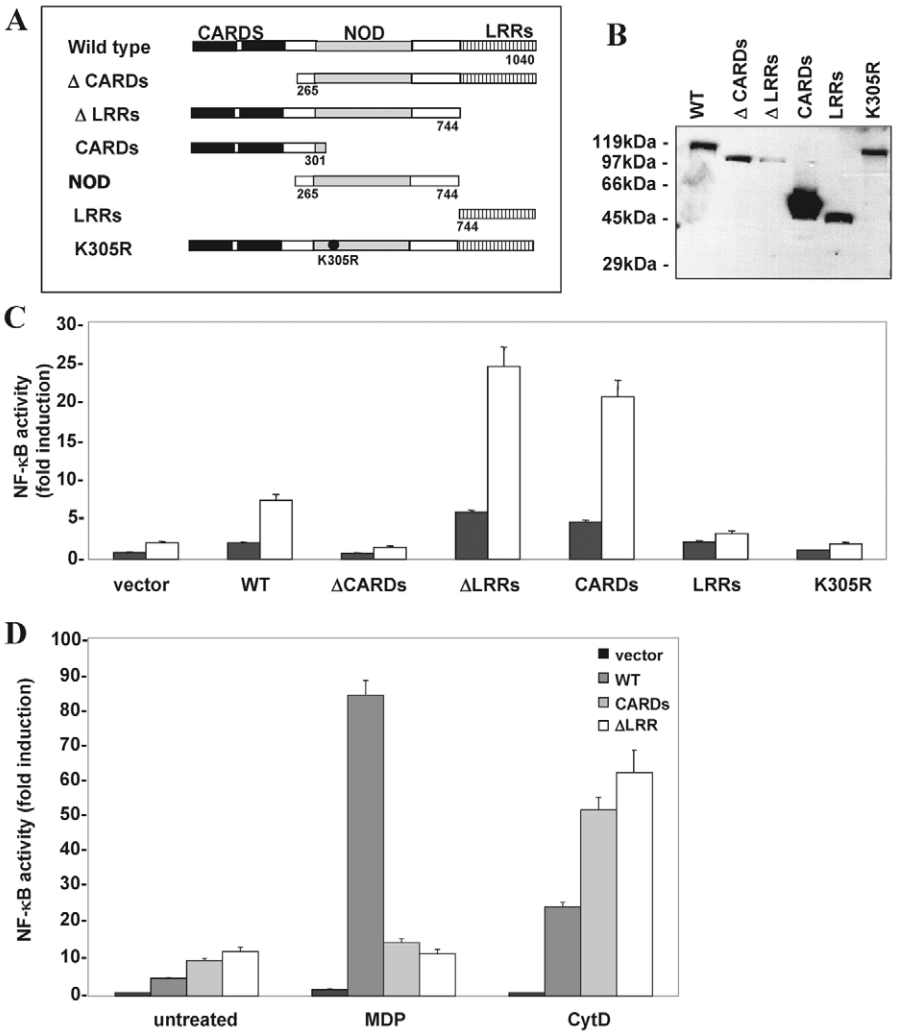

Fig. 2. Behavior of the mutant Nod2 proteins. (A) Wild-type and mutant Nod2 proteins. CARDs, NOD and LRRs are indicated by black, shaded and hatched boxes, respectively. Numbers represent amino acid residues in the Nod2 protein. (B) Expression analysis of wild-type (WT) and mutant Nod2 proteins. HEK293 cells were transfected with 5 μg of plasmids producing the indicated HA-tagged Nod2 proteins. Twenty-four hours post transfection, expression of various Nod2 proteins was analyzed by immunoblot with monoclonal anti-HA antibody. (C) Response of HEK293 cells expressing mutant Nod2 proteins to CytD. HEK293 cells were transfected with 500 ng pcDNA3 and 50 ng $(κB)_5$LUC in the presence of 6 ng of plasmids producing the indicated HA-tagged mutant Nod2 proteins. Twenty-four hours post transfection, cells were treated (white bars) or not (black bars) with CytD (50 μM) for 7 hours before being harvested for LUC assays. Values represent the mean + s.d. of triplicate cultures. (D) Differential responsiveness of the mutant Nod2 proteins to CytD and MDP. HEK293 cells were transfected with 500 ng pcDNA3 and 50 ng $(κB)_5$LUC in the presence of 10 ng of plasmids producing the indicated HA-tagged mutant Nod2 proteins. Twenty-four hours post transfection, cells were untreated or treated with CytD (50 μM) or MDP (100 ng/ml, in the presence of calcium phosphate) for 7 hours before being harvested for LUC assays. Values represent the mean + s.d. of triplicate cultures.

molecules, followed by the proximity and activation of IKK subunits (Inohara et al., 2000). LRRs are required for the response to MDP (Girardin et al., 2003; Inohara et al., 2003). We wanted to determine the domains of Nod2 that are involved in its modulation by the actin cytoskeleton by using a panel of HA-tagged Nod2 mutants (Fig. 2A). Immunoblot analysis revealed that these mutants were all correctly expressed in transiently transfected HEK293 cells (Fig. 2B). Then, we examined the response of HEK293 cells expressing Nod2 mutants to CytD (Fig. 2C). The behavior of HA-tagged wild-type Nod2 was slightly different compared with the non-tagged wild-type protein. Indeed, the HA-tagged form was less efficient in inducing NF-κB activation, alone or in combination with CytD treatment (Fig. 1A and Fig. 2C). The HA tag at the C-terminal end probably affects the efficiency of oligomerization. Expression of a Nod2 mutant form lacking the two CARDs resulted in total loss of NF-κB activity in the absence or presence of CytD (Fig. 2C). Deletion of the LRRs of Nod2 resulted in enhanced NF-κB activation (Fig. 2C), which could not be explained by increased expression of the mutant (Fig. 2B). This previously reported result (Ogura et al., 2001a), might be explained by the enhanced propensity of this mutant to self-associate. Moreover, this Nod2 mutant lacking the LRRs could still be modulated by CytD as efficiently as the wild-type protein (Fig. 2C). As previously described (Ogura et al., 2001a), expression of both CARDs alone was sufficient for NF-κB activation which was higher than with wild-type Nod2. This observation could partly be attributed to the more important expression of this mutant form (Fig. 2B). Activation of NF-κB mediated by both CARDs could be very efficiently increased by CytD treatment (Fig. 2C). Expression of one CARD alone (CARD1 or CARD2) did not allow NF-κB activation in untreated or CytD-treated cells (data not shown). Indeed, both CARDs are required for the interaction with RICK and NF-κB activation (Ogura et al., 2001a). As expected, NF-κB activity in Nod2-expressing cells, stimulated or not with CytD, was significantly decreased by a dominant negative form of RICK (data not shown). Mutants expressing the LRRs alone or carrying a point mutation of a conserved lysine residue (K305R) involved in nucleotide binding were inactive (Fig. 2C). We compared Nod2 response to CytD and MDP. As previously reported (Girardin et al., 2003; Inohara et al., 2003), Nod2 very efficiently stimulated NF-κB activation (up to 85-fold) in response to MDP whereas the Nod2 mutant forms lacking the LRRs or expressing both CARDs alone failed to induce NF-κB activation in the same conditions (Fig. 2D). On the other hand, the LRRs were absolutely not required for the response to CytD (Fig. 2D). Taken together, these results suggest that both CARDs of Nod2 are necessary and sufficient to induce NF-κB activation after CytD treatment.

Nod2 associates with specific structures of actin cytoskeleton

Since Nod2-mediated NF-κB activation is modulated by the actin cytoskeleton, we postulated that Nod2 could be associated with the actin cytoskeleton. To test this hypothesis and because cytoskeleton-associated proteins constitute a major part of the detergent-insoluble pellets, we detected Nod2 in both Triton-X-100 (TX-100)-insoluble and -soluble fractions. Fig. 3A shows that Nod2 was fully partitioned in the TX-100-insoluble fraction of HEK293 cells transfected with small amounts of HA-tagged Nod2-expressing plasmid. The behavior of non-tagged Nod2 was identical (data not shown). When plasmid concentrations were increased, Nod2 started to accumulate in TX-100-soluble fractions, probably because cytoskeletal structures that interact with Nod2 became saturated (data not shown). The same extracts were immunoblotted with an antibody against β-actin (Fig. 3A). In untreated cells, the major part of cellular actin was detected in the detergent-insoluble fraction, reflecting its association with the cytoskeleton. As expected, treatment of HEK293 cells with CytD induced an almost complete F-actin depolymerization as illustrated by actin translocation from the TX-100-insoluble to the TX-100-soluble fraction. Simultaneously, Nod2, which was partitioned in the detergent-insoluble fraction, was released into the soluble fraction. These results demonstrate that the presence of Nod2 in the TX-100-insoluble pellet is specific to the actin cytoskeleton.

To further investigate whether Nod2 associates with the actin cytoskeleton, Nod2-overexpressing HEK293 cells were labeled with a rabbit anti-Nod2 serum and with rhodamine-conjugated phalloidin, which detects F-actin and analyzed by confocal immunofluorescence. Owing to the detection limits of confocal microscopy, greater amounts of Nod2-expressing plasmid were transfected into HEK293 cells. Consequently, the major part of Nod2 was detected in the cytosol. Nod2 colocalized with F-actin on both sides of long cellular extensions and in lamellipodia-like structures at the tip of these protrusions (Fig. 3B). The subcellular localization of Nod2 was examined in two other cell types with a more specialized actin cytoskeleton. For that purpose, mouse macrophages (Mf4/4) showing numerous F-actin-rich dots corresponding to podosomal adhesion structures and Vero cells (a monkey kidney cell line) rich in stress fibers were used (Fig. 3B). Confocal analysis did not reveal the presence of Nod2 either in stress fibers or in podosomes but showed its colocalization with F-actin at the cell cortex (Fig. 3B, arrowhead).

To support the physiological relevance of these results, we wanted to confirm that endogenous Nod2 could colocalize with F-actin in some cytoskeletal structures. Nod2 is specifically expressed in myelomonocytic and intestinal epithelial cells (Ogura et al., 2001a; Gutierrez et al., 2002; Berrebi et al., 2003). Nod2 is not easily detected by immunofluorescence microscopy because of a low endogenous level. In two recent reports, Nod2 appeared enriched close to the membrane of intestinal epithelial HT-29 cells (Barnich et al., 2005a; Kufer et al., 2006). To determine any eventual recruitment of endogenous Nod2 in the actin cytoskeleton, HT-29 cells were stained for F-actin and for Nod2 with a mix of both 7E11 and 6F6 rat monoclonal antibodies raised against two distinct epitopes. The staining of F-actin in HT-29 cells did not show many actin structures except for the cortical cytoskeleton (Fig. 3C). Interestingly, in a few cells where F-actin accumulated at the cortex in lamellipodia-like structures, Nod2 was recruited to the same location. One example of this colocalization is illustrated in Fig. 3C.

Both CARDs or LRRs are sufficient to target Nod2 in membrane ruffle-like structures

Each Nod2 mutant form was examined for its colocalization with F-actin by confocal microscopy. These experiments were carried out in COS-7 cells because these cells easily

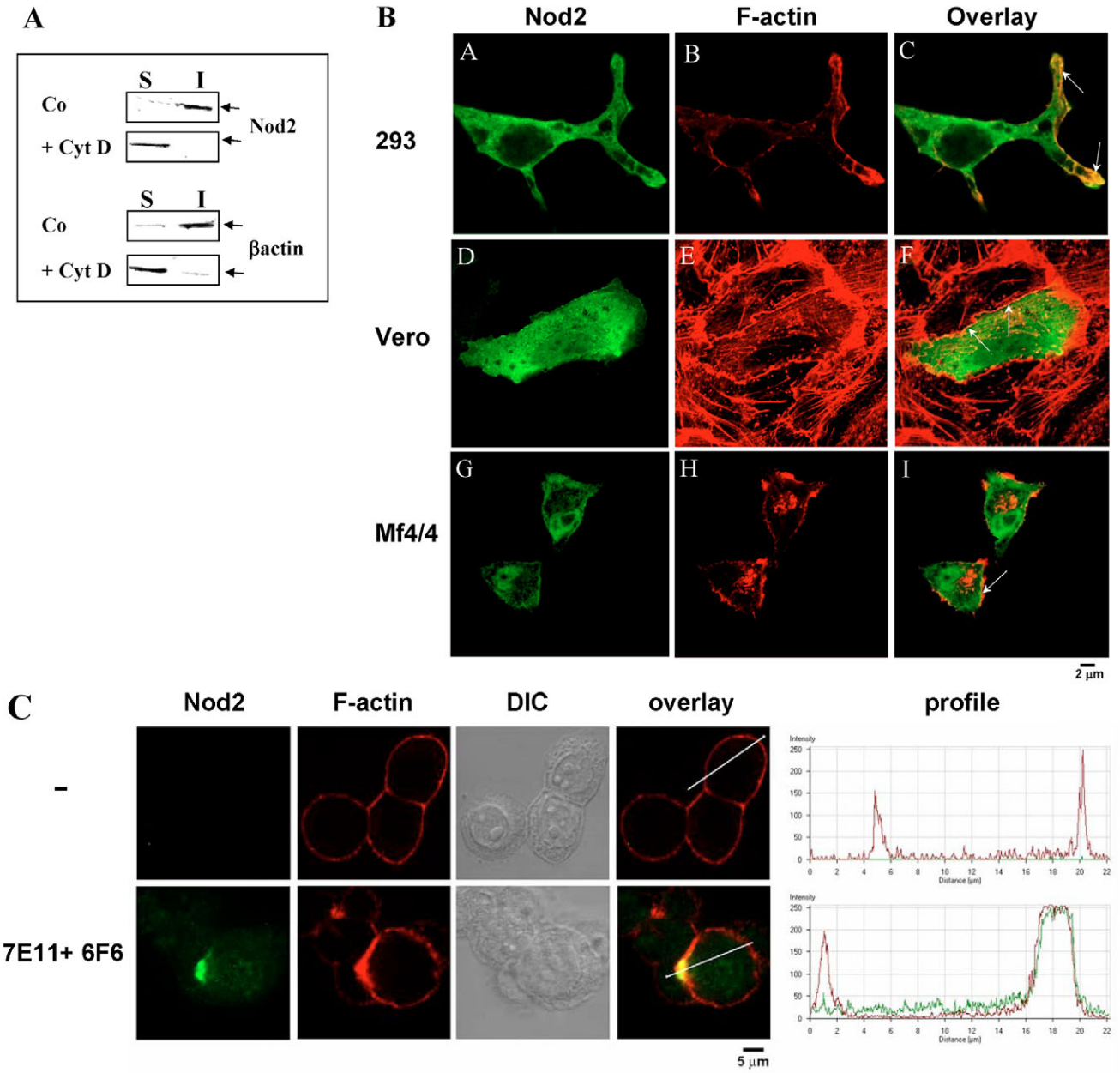

Fig. 3. Nod2 association with actin cytoskeleton. (A) Distribution of the Nod2 protein between Triton-X-100-soluble and -insoluble fractions. HEK293 cells were transfected with 500 ng pcDNA3 and 50 ng of plasmid expressing the HA-tagged Nod2 protein. Twenty-four hours post transfection, cells were treated or not with CytD 50 μM for 1 hour before being harvested for the preparation of Triton-X-100-soluble (S) or -insoluble (I) fractions. Each extract (S, I) was taken from equal number of cells and was separated by SDS-PAGE (10%) followed by western blotting with a rat monoclonal anti-HA or a mouse monoclonal anti-β-actin antibody. (B) Colocalization of ectopically expressed Nod2 with some specific structures of actin cytoskeleton. HEK293, Vero, Mf4/4 cells were transfected with Nod2-expressing plasmid (1 μg). Twenty-four hours post transfection, cells were stained for Nod2 with the rabbit serum (A,D,G) and for F-actin with TRITC-phalloidin (B,E,H). Images were obtained by confocal microscopy. (C,F,I) Merged F-actin and Nod2 images from panels A and B, D and E, G and H, respectively; areas of colocalization are shown in yellow. (C) Colocalization of endogenous Nod2 with some specific structures of the actin cytoskeleton. HT-29 cells were stained for Nod2 with a mixture of both rat anti-Nod2 monoclonal antibodies 7E11 (1:50) and 6F6 (1:50) and for F-actin with TRITC-phalloidin. A negative control (–) without primary antibodies was performed to test for secondary antibody cross-reaction. DIC, differential inference contrast. Diagrams depict the intensity of the fluorescence for each staining along the lines drawn on the overlay images. Arrows indicate areas of colocalization.

form membrane ruffle-like structures when they are cultivated at low confluence in the presence of serum. HA-tagged wild-type or mutant Nod2-expressing COS-7 cells were labeled with the anti-HA mAb and TRITC-phalloidin. Nod2 was located in the cytosol and appeared more concentrated close to the nucleus (Fig. 4A). Moreover, Nod2 was recruited to membrane ruffles each time a transfected cell formed this kind of structure (overlay, Fig. 4A). These results

confirmed colocalization experiments in HEK293 cells (Fig. 3B). Surprisingly, the deletion of both CARDs or LRRs did not modify the cellular distribution of Nod2, which was still able to colocalize with F-actin in membrane ruffles (Fig. 4A). The mutant containing only both CARDs could also associate with the actin cytoskeleton (Fig. 4A). The mutant expressing the LRRs alone showed nuclear staining as well as colocalization with F-actin in membrane ruffles (Fig. 4A). It is worth mentioning that colocalizations were seen less frequently with the mutants containing only both CARDs or LRRs than with wild-type Nod2 or mutants deleted for CARDs or LRRs (data not shown). The mutant containing the NOD alone showed a dashed cytoplasmic staining but failed to colocalize with F-actin (Fig. 4A). Since this domain oligomerized, this dashed staining could result from protein precipitation. Such staining could also be related to a vesicular distribution.

To further investigate the Nod2 domain(s) mediating its association with the actin cytoskeleton, the distribution of the mutant proteins between TX-100-insoluble and -soluble fractions was analyzed. Since the expression levels of the various mutants were very different (see Fig. 2B) and the cytoskeletal structures interacting with Nod2 appeared to be rapidly saturated, HEK293 cells were transfected with

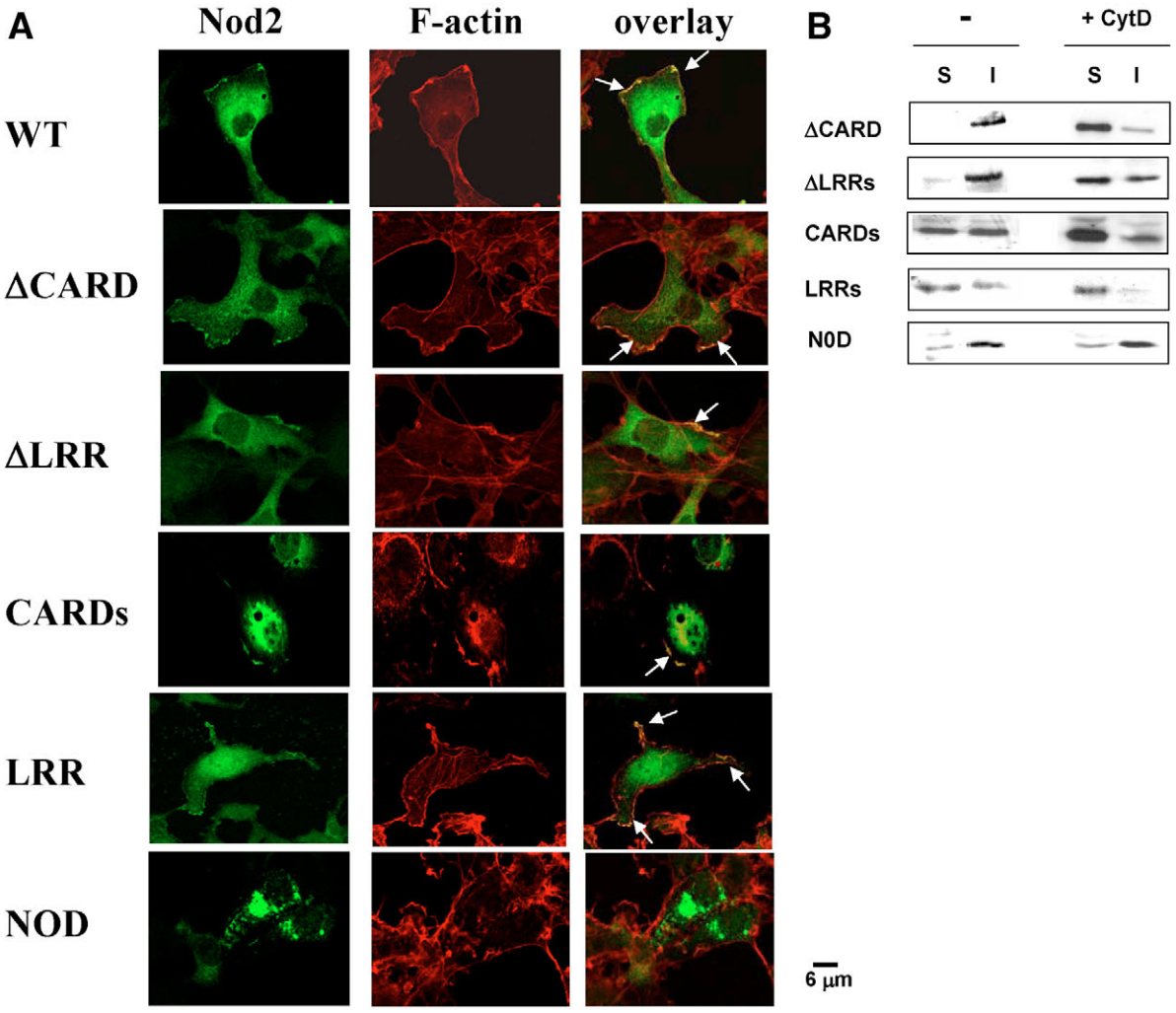

Fig. 4. Both CARDs or LRRs are sufficient to target Nod2 in membrane ruffle-like structures. (A) Colocalization of the wt and mutant Nod2 proteins with F-actin in COS-7 cells. COS-7 cells were transfected with the following amounts of plasmid encoding the HA-tagged or FLAG-tagged wt or mutant Nod2 proteins: $pcDNA_3$-HA-wt Nod2 and $pcDNA_3$-HA-ΔCARDs (1 μg each), $pcDNA_3$-HA-ΔLRRs (4 μg), $pcDNA_3$-HA-CARDs (0.5 μg), pcDNA3-HA-LRRs (1 μg), $pcDNA_3$-FLAG-NOD (2 μg). In each case, the total DNA amount was adjusted to 4 μg with pcDNA3. Twenty-four hours post transfection, cells were stained for the wt or mutant Nod2 proteins with anti-HA mAb or anti-FLAG rabbit serum, and for F-actin with TRITC-phalloidin. Images were obtained by confocal microscopy (Leica TCS NT). Arrows indicate areas of colocalization. (B) Distribution of the mutated Nod2 proteins between Triton-X-100-soluble and -insoluble fractions. HEK293 cells were transfected with the following amounts of plasmid encoding the HA-tagged or FLAG-tagged mutant Nod2 proteins: $pcDNA_3$-HA-ΔCARDs (25 ng), $pcDNA_3$-HA-CARDs (10 ng), pcDNA3-HA-LRRs (100 ng), $pcDNA_3$-FLAG-NOD and $pcDNA_3$-HA-ΔLRRs (2500 ng each). In each case, the DNA total amount was adjusted to at least 2500 ng with pcDNA3. Twenty-four hours post transfection, cells were treated or not with CytD 50 μM for 1 hour before being harvested for the preparation of Triton-X-100-soluble (S) or -insoluble (I) fractions. Each kind of extract (S, I) from equal number of cells was separated by SDS-PAGE (10%) followed by western blotting with anti-HA or anti-FLAG mAbs.

decreasing amounts of Nod2 mutant expression plasmids. Fig. 4B shows the results obtained with the lowest concentration of each plasmid, which still allowed a reproducible detection. The mutants lacking either the two CARDs (ΔCARDs) or the leucine-rich repeats (ΔLRRs) preferentially associated with the detergent-insoluble fraction similarly to the wild-type protein (Fig. 3A and Fig. 4B). The mutants composed of the two CARD domains (CARDs) as well as the C-terminal LRR domain (LRRs) were distributed equally between the TX-100-soluble and -insoluble fractions. Unexpectedly, the mutant containing the NOD domain alone was almost completely associated with the TX-100-insoluble pellet. The levels of ΔCARDs, ΔLRRs, CARDs and LRRs mutants in the TX-100-soluble fraction increased after CytD treatment, demonstrating that their presence in the TX-100-insoluble fraction was specific to the actin cytoskeleton. However, the distribution of the NOD domain was not altered by CytD treatment, indicating that its presence in the insoluble fraction does not reflect its association with the actin cytoskeleton (data not shown). This might explain the lack of colocalization between the NOD domain and F-actin in confocal microscopy.

Altogether, these data obtained by molecular approaches and confocal microscopy suggest that the presence of CARDs or LRRs is sufficient to target Nod2 in membrane ruffle-like actin structures. The association of the mutants ΔLRRs and CARDs with the actin cytoskeleton reinforces the results previously reported (Fig. 2C), demonstrating that these mutants could still sense cytoskeleton modifications.

Nod2 interacts with Rac1 in membrane ruffles through CARDs and LRRs

To identify the protein(s) that target Nod2 in membrane ruffles, we looked at Rac1. Rac1 is a member of the Rho family of small GTP-binding proteins that functions as a molecular switch to regulate a variety of biological processes including the regulation of the actin cytoskeleton through formation of lamellipodia and membrane ruffles (Ridley et al., 1992). Fig. 5A shows that in COS-7 cells cultivated in the presence of serum, a small fraction of endogenous Rac1 is activated and colocalizes with F-actin in membrane ruffles.

Concerning the colocalizations between Nod2 and Rac1, the same conclusions as for experiments with F-actin could be drawn (Fig. 5B). Nod2 colocalized with Rac1 in membrane ruffles. The deletion from its N-terminal domain (CARDs) or its C-terminal domain (LRRs) did not prevent Nod2 being recruited in ruffles with Rac1. The mutants containing CARDs or LRRs alone could be also detected in membrane ruffles with Rac1 but to a lesser extent. Again, the nucleotide-binding oligomerization domain (NOD) failed to colocalize with Rac1.

In addition, HA-Nod2 co-precipitated with endogenous Rac1 (Fig. 6A). Nod2 lacking both CARDs or LRRs could still very efficiently interact with Rac1 (Fig. 6A). Co-precipitation of the mutant containing both CARDs alone was less important as compared with the wt or ΔCARDs and ΔLRRs forms (Fig. 6A). The recovery of the mutant containing LRRs alone was very weak (Fig. 6A). As expected, no interaction between FLAG-NOD and Rac1 was detected when lysates were immunoprecipitated with either anti-FLAG or anti-Rac1 antibody and analyzed by western blot (Fig. 6B).

To evaluate the physiological relevance of the interaction between Nod2 and Rac1, HT-29 cells were used because they endogenously express these two proteins at a reasonable level. As shown in Fig. 6C, endogenous Nod2 was co-immunoprecipitated with Rac1, demonstrating that this

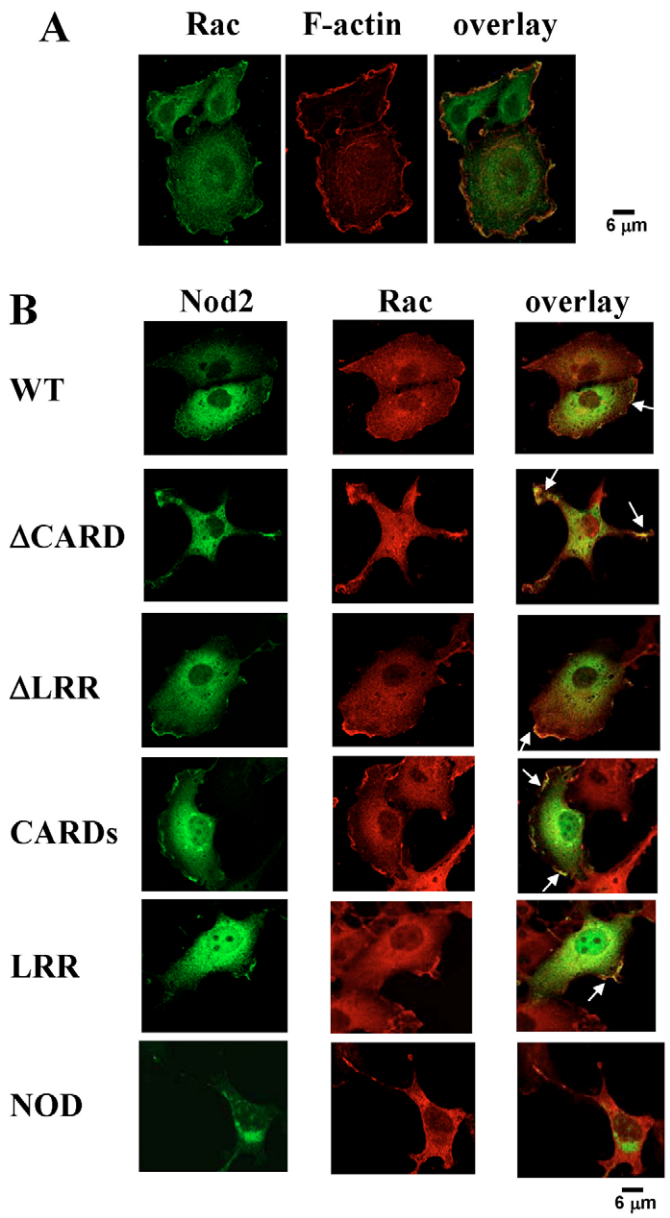

Fig. 5. Colocalization of wt and mutant Nod2 proteins with endogenous Rac1. (A) Rac1 colocalization with F-actin in membrane ruffles. COS-7 cells were stained for Rac1 with the anti-Rac1 mAb and for F-actin with TRITC-phalloidin. Images were obtained by confocal microscopy (Leica TCS NT). (B) COS-7 cells were transfected with the following amounts of plasmid encoding the HA-tagged or FLAG-tagged wt or mutant Nod2 proteins: $pcDNA_3$-HA-wt Nod2 and $pcDNA_3$-HA-ΔCARDs (1 μg each), $pcDNA_3$-HA-ΔLRRs (4 μg), $pcDNA_3$-HA-CARDs (0.5 μg), pcDNA3-HA-LRRs (2 μg), $pcDNA_3$-FLAG-NOD (2 μg). In each case, the total DNA amount was adjusted to 4 μg with pcDNA3. Twenty-four hours post transfection, cells were stained for the wt or mutant Nod2 proteins with monoclonal anti-HA or rabbit anti-FLAG antibodies and for endogenous Rac1 with a monoclonal antibody. Images were obtained by confocal microscopy (Leica TCS NT). Arrows indicate areas of colocalization.

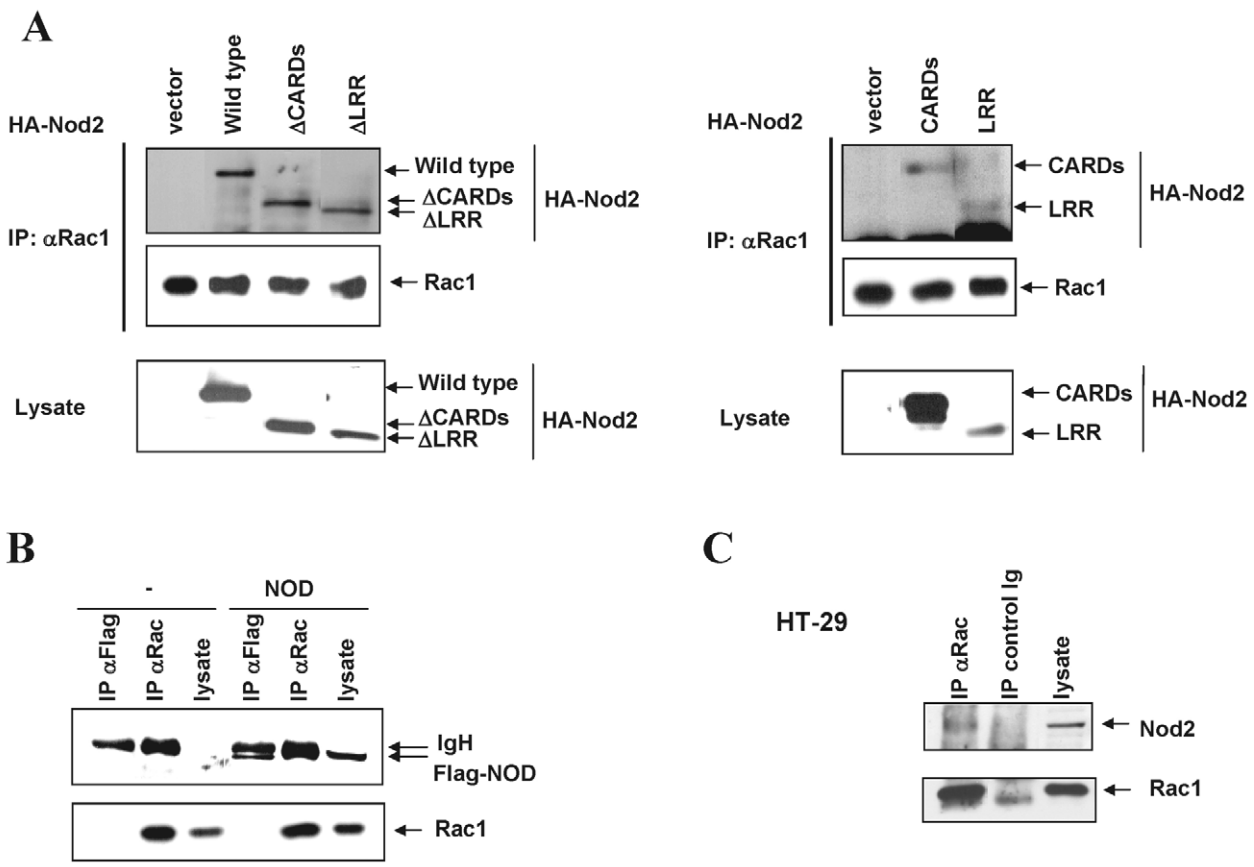

Fig. 6. Co-immunoprecipitation of wt and mutant Nod2 proteins with endogenous Rac1. (A) COS-7 cells were transfected with the following amounts of plasmid encoding the wt or mutant Nod2 proteins: pcDNA$_3$-HA-wt Nod2 and pcDNA$_3$-HA-ΔCARDs (1 μg each), pcDNA$_3$-HA-ΔLRRs (4 μg), pcDNA$_3$-HA-CARDs (0.5 μg), pcDNA3-HA-LRRs (2 μg). In each case, the DNA total amount was adjusted to 4 μg with pcDNA3. Twenty-four hours post transfection, cells were harvested and cell lysates were immunoprecipitated (IP) with anti-Rac1 monoclonal antibody, and proteins were visualized by western blotting with anti-HA (top and bottom panels) or anti-Rac1 monoclonal antibodies (middle panels). (B) COS-7 cells were transfected with 2 μg of pcDNA$_3$-FLAG-NOD. Twenty-four hours post transfection, cells were harvested and cell lysates were immunoprecipitated (IP) with anti-Rac1 or anti-FLAG monoclonal antibodies and proteins were visualized by western blotting with anti-Rac1 (lower panel) or anti-FLAG (upper panel) monoclonal antibodies. Non-specific bands corresponding to immunoglobulin heavy chain (IgH) are indicated. (C) Endogenous Nod2 and Rac1 proteins interact. HT-29 cell lysates (1 mg) were immunoprecipitated (IP) with anti-Rac1 or control mouse monoclonal antibodies and proteins were visualized by western blotting with anti-Rac1 (lower panel) or anti-Nod2 (7E11, 1:100, upper panel) monoclonal antibodies.

interaction can take place in cells where they are expressed (Fig. 6C).

Altogether, these data suggest that Nod2 interacts with Rac1 in membrane ruffles. Co-immunoprecipitation assays allowed us to identify the Nod2 domain(s) that are required for its recruitment in membrane ruffles. When the three domains (CARDs, NOD, LRRs) were analyzed separately, the N-terminal CARDs and, to a lesser extent, the C-terminal LRRs were able to co-immunoprecipitate with Rac1, whereas the central region NOD could not. The binding of each of these regions (CARDs or LRRs) was stronger when they were associated with the central region NOD in the ΔLRR and ΔCARD mutants, respectively, and further increased with the wild-type protein, suggesting that these domains may cooperate for efficient interaction with Rac1.

To confirm that Nod2 associates with membrane ruffles induced by Rac1, a constitutively activated Rac1 mutant (Rac QL) (Perona et al., 1997), which could induce membrane ruffling in the absence of stimulation, was overexpressed in COS-7 cells. The Rac1QL mutant produced an extensive membrane ruffling characterized by a ribbon-like organization of the actin cytoskeleton along the cell periphery, with very sharp colocalization between Rac1 and F-actin (Fig. 7A). A small fraction of Nod2 colocalized with RacQL in these ribbon-like membrane ruffles (Fig. 7B).

COS7 cells expressing a dominant negative Rac1 mutant (Rac N17) (Perona et al., 1997) failed to form typical membrane ruffles and did not show any colocalization between Rac1 and Nod2 (Fig. 8A). As shown in Fig. 8B, membrane ruffle disruption induced by RacN17 primed Nod2-dependent NF-κB activation, suggesting that membrane ruffles could act as a retention receptor for Nod2.

Discussion

The traditional view of the actin cytoskeleton as a passive entity destined to maintain the shape of the cell and promote its motility has changed significantly during recent years. It is now clear that, in addition to these structural functions, the

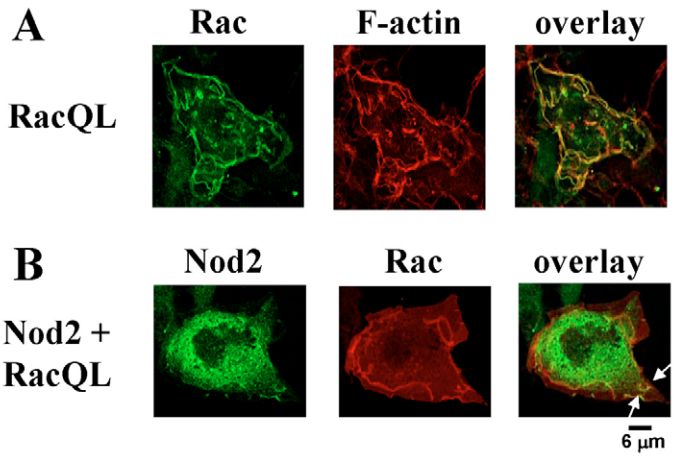

Fig. 7. Nod2 localization in ribbon-like membrane ruffles induced by RacQL. (A,B) COS-7 cells were transfected with 1 μg of plasmid expressing the HA-tagged wt Nod2 protein and 2 μg of plasmid encoding the constitutively activated Rac1 mutant (Rac QL). Twenty-four hours post transfection, cells were stained for Rac1 and F-actin (A) or for Rac1 and Nod2 (B). Images were obtained by confocal microscopy (Leica TCS NT). Arrows indicate areas of colocalization.

actin cytoskeleton plays active roles in a number of processes affecting the biological status of the cell. These include, among others, the formation of membrane domains that establish cell polarity (Bretscher et al., 2002), the creation of synaptic clusters contributing to the signaling output of stimulated lymphocytes (Dustin and Cooper, 2000) and the induction of specific, F-actin-linked transcriptional responses (Sotiropoulos et al., 1999). We have recently demonstrated that actin disruption by CytD and LatB induces a significant NF-κB activation in myelomonocytic cell lines and in human monocytes, through an IKK-dependent pathway (Kustermans et al., 2005). No significant NF-κB activation in response to CytD could be observed in other cell lines such as HeLa cells, murine fibroblasts or human T lymphocytes. Simultaneously, it was shown that the treatment of human intestinal epithelial cells by CytD or LatB resulted in increased NF-κB activation and IL-8 expression (Németh et al., 2004).

Since the expression of Nod2 was restricted to myelomonocytic and intestinal epithelial cells (Ogura et al., 2001a; Gutierrez et al., 2002; Berrebi et al., 2003), we hypothesized that Nod2 could sense actin modulations and convert them into IKK complex activation. We demonstrated that actin disruption by CytD specifically increased Nod2-mediated NF-κB signaling in untreated or MDP-stimulated HEK293 cells. Both CARDs were required to sense actin modulations and convert them in NF-κB activation. Although the C-terminal leucine-rich repeats (LRRs) were necessary to detect MDP, this domain was not required for the response to CytD. Since Nod2 sensed actin perturbations, we examined its association with the actin cytoskeleton. Nod2, at a concentration ensuring the best response to cytD, was partitioned in the detergent-insoluble fraction containing various cytoskeleton-associated proteins. By confocal analysis, we showed that ectopically expressed or endogenous Nod2 colocalized with F-actin in membrane ruffles and/or lamellipodia-like structures. Both N-terminal CARDS and C-terminal LRRs were involved. To identify the protein(s) that target Nod2 to membrane ruffles, we carried out colocalization and co-immunoprecipitation assays with endogenous Rac1. These experiments showed that ectopically expressed Nod2 associated with activated Rac1 in membrane ruffles and/or lamellipodia through its N-terminal CARDS and C-terminal LRRs. These domains seemed to cooperate in the wild-type protein for efficient interaction with Rac1. The physiological relevance of the interaction between Nod2 and Rac1 was supported by the co-immunoprecipitation of endogenous Nod2 with Rac1 in HT-29 cells. Since Nod2 lacks a functional Rac1-binding domain (Rudolph et al., 1998), the interaction between both proteins is probably indirect. The membrane ruffle formation involves the Rac-dependent

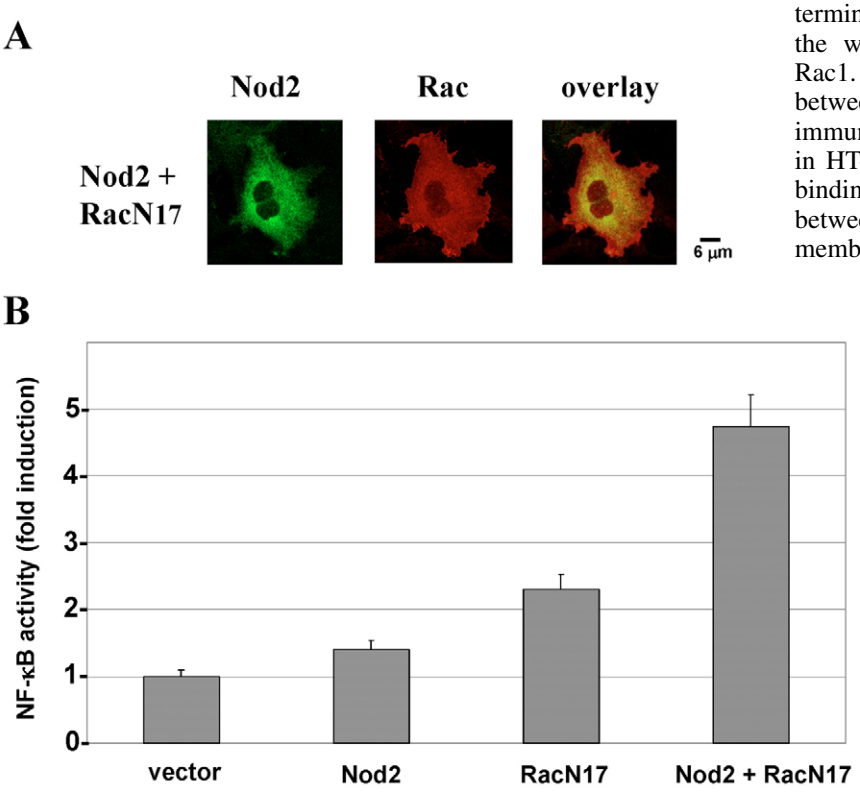

Fig. 8. Effect of a dominant negative Rac1 mutant. (A) COS-7 cells were transfected with 1 μg of plasmid expressing the HA-tagged wt Nod2 protein and 2 μg of plasmid encoding the dominant negative Rac1 mutant (Rac N17). Twenty-four hours post transfection, cells were stained for Nod2 and Rac1 with anti-HA and anti-Rac1 mAbs, respectively. Images were obtained by confocal microscopy (Leica TCS NT). (B) COS-7 cells were transfected with 50 ng of $(\kappa B)_5$LUC in the presence or not of plasmid expressing Nod2 (5 ng) or Rac N17 (100 ng). The DNA total amount was adjusted to 500 ng with pcDNA3. Twenty-four hours post transfection, cells were harvested for LUC assays. Values represent the mean + s.d. of triplicate cultures.

re-localization of WAVE-based complexes to the leading edge to elicit the generation of new actin filaments necessary to drive cell protrusion and motility (Stradal et al., 2004). Nod2 could probably bind one protein from these complexes such as WAVE-1, WAVE-2, Sra-1, Nap-1 or Abi-1 (Stradal et al., 2004).

Nod2, initially partitioned in the TX-100-insoluble fraction, translocated to TX-100-soluble fraction after CytD treatment. The release of Nod2 from cytoskeleton structures allowed it to induce NF-κB activation more efficiently. Moreover, the dislocation of membrane ruffles in RacN17-expressing COS7 cells primed the Nod2-dependent NF-κB activation. This modulating effect of the actin cytoskeleton on Nod2-dependent NF-κB signaling could be physiologically relevant during monocyte activation and/or recruitment into injured tissues, where cellular attachment, migration and phagocytosis result in cyclic shifts in cytoskeletal organization and disorganization. The Nod2 mutant form lacking the C-terminal leucine-rich repeats could still colocalize with Rac in membrane ruffles and could still sense actin modulations and convert them into NF-κB activation. The three main Nod2 mutations associated with CD are located within or near this LRR domain. Although these mutants are deficient in the recognition of MDP, they could still associate with the actin cytoskeleton and activate NF-κB in response to actin cytoskeleton remodelling.

A recent study examined the subcellular localization of wild type Nod2 and CD-associated Nod2 mutants in intestinal epithelial cells (Barnich et al., 2005a). They showed that endogenous Nod2 as well as the expressed protein FLAG-Nod2 is located in the cytosol and appears enriched close to the plasma membrane. The most common Nod2 mutant associated with CD (3020 insC) deleted from the final 33 C-terminal amino acid residues is not recruited to the membrane, indicating that this C-terminal region is responsible for membrane targeting. They also demonstrated that the membrane targeting of Nod2 in intestinal epithelial cells is required for NF-κB activation upon the recognition of MDP. The work from Kufer et al. (Kufer et al., 2006) supported the data of Barnich et al. (Barnich et al., 2005a) with regard the membrane recruitment of Nod2 in HT-29 cells. We did not observe any systematic membrane localization of Nod2 in HT-29 cells. These discrepancies with regard the subcellular localization of Nod2 might be partially explained by the use of different culture conditions, cell fixation methods or antibodies. However, we demonstrated, for the first time, that ectopically expressed and endogenous Nod2 colocalized with F-actin in membrane ruffles and/or lamellipodia-like structures through both CARD and LRR domains.

To date, there is little known about the regulation of the Nod2 signaling pathway. TAK1 (Chen et al., 2004) and GRIM-19 (Barnich et al., 2005b) have been reported to enhance Nod2 signaling. Recently, Erbin was identified as a new Nod2-binding protein and reported to negatively regulate Nod2-mediated NF-κB activation (Kufer et al., 2006; McDonald et al., 2005). Nod2 colocalizes with Erbin at the cell membrane of intestinal epithelial cells (Kufer et al., 2006; McDonald et al., 2005). Erbin was previously shown to play roles in cell polarity, receptor localization (Borg et al., 2000) and regulation of MAPK signaling (Huang et al., 2003). Erbin is also linked to cytoskeleton-associated protein complexes in desmosomes and participates in cell adhesion processes (Izawa et al., 2002). It has been hypothesized that Erbin suppresses the activation of membrane-associated Nod2 until a threshold level of MDP stimulation was reached to prevent inappropriate signaling. In this manner, Erbin might modulate the sensitivity of intestinal epithelial cells to bacterial products.

This work emphasizes the role of actin cytoskeleton in the regulation of Nod2-dependent NF-κB signaling. The reason for the interaction of Nod2 with Rac in membrane ruffles remains to be fully explored. The recruitment of Nod2 in Rac-induced dynamic cytoskeletal structures could be a strategy to both repress the Nod2-dependent NF-κB signaling in unstimulated cells and rapidly mobilize Nod2 during bacterial infection. Invasive pathogens induce their internalization through secreted proteins interfering with cytoskeleton machinery (for a review, see Pizarro-Cerda and Cossart, 2006). One example is the invasion of polarized intestinal epithelial cells by *Salmonella typhimurium* (Shi et al., 2005). Activation of Rac1 by *Salmonella* effector proteins recruited the Abi1-Nap1-PIR121-HSPC300-WAVE2 complex to sites of *Salmonella* invasion at the apical plasma membrane, where WAVE2 stimulated the formation of a highly branched actin network, followed by the appearance of large membrane protrusions surrounding the bacteria, which engulf them. Thus, the complexes driving the polymerization of branched actin filaments at bacterial invasion sites are similar to those recruited in membrane ruffles (Stradal et al., 2004) or in phagocytic pseudopodia (Massol et al., 1998). Since such cytoskeletal structures could harbor Nod2, they might play the role of a scaffold ensuring a close proximity between Nod2 and MDP at apical bacterial invasion sites. This mechanism would be particularly interesting in the intestinal epithelia where Nod2 appears to be restricted to basolateral membranes (McDonald et al., 2005). This hypothesis is reinforced by very recent data showing a recruitment of Nod2 at the entry foci of *Salmonella flexneri* in HeLa cells (Kufer et al., 2006).

Materials and Methods

Materials

Cytochalasin D (CytD) and MDP (muramyl dipeptide) were purchased from Sigma (St Louis, MO). Latrunculin B (LatB) was obtained from Calbiochem (La Jolla, CA). The protease inhibitors set (complete) was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Tumor necrosis factor (TNFα) and interleukin-1 (IL-1β) were purchased from Peprotech. The reporter construct $(\kappa B)_5$Luc was purchased from Stratagene. Expression plasmids encoding wild-type Nod2 and HA- or FLAG-tagged Nod2 forms (WT, ΔCARDs, ΔLRRs, CARDs, NOD, LRRs, K305R) and the rabbit serum anti-Nod2 were obtained from G. Nunez (University of Michigan Medical School & Comprehensive Cancer Center, Ann Harbor, MI) and have been described (Ogura et al., 2001a). The rat monoclonal anti-Nod2 antibody 7E11 has been previously described (Kufer et al., 2006). The plasmids encoding constitutively active (Rac1QL) and dominant negative (Rac1N17) Rac1 mutants have been described (Perona et al., 1997). Antibodies used in this study were monoclonal anti-β–actin antibody (Sigma), monoclonal and rabbit polyclonal anti-FLAG antibody (Sigma), rat monoclonal anti-HA antibody (clone 3F10, Roche Molecular Biochemicals, Mannheim, Germany) and monoclonal anti-Rac1 antibody (Upstate Biotechnology).

Generation of the anti-Nod2 monoclonal antibody 6F6

An internal peptide of Nod2 (387RFTDRERHCSPTDPTS402) was synthesized and coupled to key-hole limpet hemocyanin or ovalbumin (PSL, Heidelberg, Germany). Rats were immunized with 50 μg peptide-keyhole limpet hemocyanin using CPG 2006 and incomplete Freund adjuvant as an adjuvant. Supernatants were tested in a differential ELISA and analyzed by western blotting using extracts from HEK 293T cells and transiently transfected HEK 293T cells expressing FLAG-nod2. 6F6 specifically recognizes ectopically expressed Nod2 in western blots.

Cell culture and transfection

Mouse macrophages Mf4/4, a gift of R. Beyaert (University of Ghent, Belgium) (Desmedt et al., 1998), were cultivated in RPMI 1640 supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS) and β-mercaptoethanol (50 μM). The human embryonic kidney HEK293 and intestinal epithelial HT-29 cells were cultivated in MEM or EMEM, respectively, supplemented with 2 mM glutamine, non-essential amino acids and 10% FBS. Vero (a monkey kidney cell line, ATCC CCL-81) and COS-7 cells were grown in M199 medium and DMEM supplemented with 2 mM glutamine and 10% FBS, respectively. The cell lines were transfected using the fugene liposome technique according to the protocol of the supplier (Roche Molecular Biochemicals, Germany).

Gene reporter assays

Twenty-four hours after transfection with a reporter plasmid such as $(\kappa B)_5$LUC, cells were treated or not with CytD, LatB or MDP for 7 hours. Then, cells were lysed for the determination of LUC activities in cellular extracts (luciferase reporter gene assay; Roche Molecular Biochemicals, Germany). The luciferase activity of the samples was normalized with the protein concentration measured by the Bradford method (Bio-Rad, Hercules, CA).

IL-8 ELISA

IL-8 concentrations in HEK293 cell supernatants were assessed by ELISA as described by the supplier (ImmunoSource).

Nuclear protein extraction

Cells were washed with cold phosphate-buffered saline and resuspended in lysis buffer containing 10 mM HEPES-KOH pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 0.5% IGEPAL, 1 mM PMSF, 1 mM DTT and protease inhibitors (Complete, Roche). After incubation on ice for 10 minutes and centrifugation at 20,000 ***g*** for 30 seconds, the pellet was resuspended in saline buffer (50 mM HEPES-KOH, pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 50 mM KCl, 400 mM NaCl, 10% (v/v) glycerol, 1 mM PMSF, 1 mM DTT and protease inhibitors) and incubated for 30 minutes on ice. After centrifugation at 20,000 ***g*** for 15 minutes, the supernatant containing the nuclear proteins was harvested and stored at –80°C. The protein content was measured by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich).

Electrophoretic Mobility Shift Assay (EMSA)

In brief, 5 μg of nuclear proteins were incubated for 30 minutes at room temperature in a volume of 10 μl with 0.2 ng ^{32}P-labeled oligonucleotide probe in binding buffer [20 mM HEPES-KOH (pH 7.9), 75 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM $MgCl_2$, 1 mM DTT] containing 2 μg BSA and 1.25 μg poly(dI-dC)-poly(dI-dC) (Amersham Pharmacia Biotech, Roosendael, The Netherlands). DNA-protein complexes were then resolved by electrophoresis on a non-denaturing 6% (w/v) polyacrylamide gel for 2 hours at 300 V in 0.25× TBE (2.5 mM Tris, 2.5 mM H_3BO_3, 2 mM EDTA). The gels were then dried and autoradiographed on a Fuji X-ray film. The sequence of the double strand κB probe was as follows: 5′-GGTTACAAGGGACTTTCCGCTG-3′ and 5′-TTGGCAGCGGAAAGTCCCT-TGT-3′. The oligonucleotide probes were labeled by infilling with the Klenow DNA polymerase (Roche).

Preparation of detergent-soluble and -insoluble cell extracts

Lysis buffer contains 50 mM PIPES-KOH (pH 6.9), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5% (v/v) glycerol, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, 1 mM ATP and protease inhibitors (Roche). Cells were lysed with approximately 50 volumes of pre-warmed buffer. After incubation for 10 minutes at 37°C, samples were centrifuged at 100,000 ***g*** for 60 minutes at room temperature. Supernatants containing detergent-soluble cell proteins are harvested and put on ice. Pellets comprising detergent-insoluble proteins are resuspended in the same volume as supernatants with ice-cold lysis buffer.

Immunoprecipitation

Cells were lysed on ice for 10 minutes in modified RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Igepal, 0.25% sodium deoxycholate, 1 mM PMSF, protease inhibitors). Lysates (1 ml containing 500 μg to 1 mg of proteins) were precleared with 40 μl of protein G-Agarose slurry (Santa Cruz Biotechnology) for 1 hour at 4°C. Following centrifugation for 10 minutes at 4000 rpm, the lysates were collected and incubated overnight with 4 μg anti-Rac1 or anti-FLAG mAb or a control IgG mAb. Immune complexes were collected with 40 μl protein G-Sepharose slurry (Santa Cruz Biotechnology) for 2 hours at 4°C and washed three times with modified RIPA buffer before being analyzed by immunoblot.

Immunoblot

One volume of loading buffer (60 mM Tris-HCl pH 6.8, 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.03% bromophenol blue) was added to protein samples that, after boiling for 5 minutes, were electrophoresed on polyacrylamide-SDS gel and electro-transferred to PVDF membranes (Roche, Mannheim, Germany). After probing with primary and secondary antibodies, membranes were finally analyzed with an ECL system (Amersham, UK).

Immunofluorescence microscopy

The detection of ectopically expressed Nod2 by immunofluorescence was performed as follows. Cells grown on coverslips were rinsed with warmed PBS and fixed with 4% (w/v) paraformaldehyde/PBS for 30 minutes at 37°C. After washing with PBS, the cells were incubated in PBS containing 1% (v/v) FBS and 0.1 % Triton X-100 for 30 minutes at 37°C. In colocalization assays between Nod2 proteins and F-actin, coverslips were incubated with either a rabbit serum anti-Nod2 or a rat anti-HA mAb or a rabbit serum anti-FLAG for 60 minutes at 37°C. After washing with PBS + 1% FBS, coverslips were incubated with 20 μg/ml FITC-conjugated anti-rabbit or anti-rat IgG secondary antibodies (DAKO A/S, Denmark) and 0.1 μg/ml TRITC-phalloïdin (Sigma) for 45 minutes at 37°C. Following a PBS rinse, coverslips were mounted with SlowFade Light Antifade reagent (Molecular Probes). Samples were analyzed by confocal microscopy (Leica TCS NT, Leica). In colocalization assays between Rac1 and Nod2, coverslips were incubated with the following primary antibodies: an anti-Rac1 mAb and, to detect Nod2, either an anti-HA rat mAb or anti-FLAG rabbit serum. Secondary antibodies were a highly cross-adsorbed anti-mouse IgG-Alexa Fluor 546 (Molecular Probes) and a highly cross-adsorbed anti-rat IgG Alexa Fluor 488 (Molecular Probes) or an anti-rabbit IgG-FITC (DAKO A/S, Denmark). When colocalization between Rac and F-actin was tested, the anti-Rac1 mAb was used followed by the FITC-conjugated anti-mouse IgG secondary antibody (DAKO A/S, Denmark) and TRITC-phalloidin (Sigma).

The detection of endogenous Nod2 by immunofluorescence was carried out according to a protocol previously described (Lamsoul et al., 2005). Briefly, HT-29 cells cultivated on glass coverslips were fixed with Immunohistofix (A Phase, Belgium) for 10 minutes at room temperature, followed by incubation in 100% methanol at –20°C for 6 minutes. The cells were washed with PBS, blocked in PBS containing 0.5% gelatin (Bio-Rad) and 0.25% BSA and incubated with a mix of both rat anti-Nod2 monoclonal antibodies 7E11 and 6F6 (1:50) diluted in the blocking solution. The preparations were then washed with PBS containing 0.2% gelatine and incubated with TRITC-phalloidin (Sigma) and a highly cross-adsorbed anti-rat IgG Alexa Fluor 488 (Molecular Probes). After washing, samples were mounted in DABCO-based medium (ICN Biomedicals) and analyzed with a laser-scanning confocal microscope (LSM 510; Zeiss).

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