Interferon-γ Augments CARD4/NOD1 Gene and Protein Expression through Interferon Regulatory Factor-1 in Intestinal Epithelial Cells*‡

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Although intestinal epithelial cells appear to be functionally hyporesponsive to normal intestinal flora, human intestinal epithelial cells can respond to enteroinvasive bacteria and induce an inflammatory response. This initial inflammatory response leads to the recruitment of polymorphonuclear leukocytes to the affected site in vitro and in vivo. CARD4/NOD1 is a potential cytosolic receptor for peptidoglycan in mammalian cells that resembles pattern-resistance proteins of plants. In this context, CARD4/NOD1 is the candidate for a recognition protein of intracellular bacteria or peptidoglycan in intestinal epithelial cells. In this study, we demonstrate that CARD4/NOD1 is constitutively expressed in intestinal epithelial cell lines and isolated primary intestinal epithelial cells. Interferon-γ (IFNγ), which is a potent pro-inflammatory cytokine in intestinal mucosal inflammation, activates CARD4/NOD1 mRNA transcription in a time- and dose-dependent manner and results in augmentation of CARD4/NOD1 protein in SW480 cells. Promoter analysis of CARD4/NOD1 indicates that interferon regulatory factor-1 (IRF-1) binding motif (−791 to −782) is essential for the effect of IFNγ. Nuclear extracts from SW480 cells treated with IFNγ show specific binding of oligonucleotides corresponding to this IRF-1-binding motif, which was supershifted by anti-IRF-1 antibody in electrophoretic mobility shift assay. Overexpression of IRF-1 protein activates the CARD4/NOD1 promoter but not the deletion mutant of the IRF-1-binding site in a co-transfection assay of IRF-1 expression plasmid with CARD4/NOD1 promoter. These studies suggest that the Th1 cytokine, IFNγ, activates CARD4/NOD1 transcription and regulate innate immune mechanisms in the condition of intestinal mucosal inflammation.

In the gastrointestinal tract, intestinal epithelial cells form a structural and functional barrier against numerous luminal bacteria, pathogenic and nonpathogenic. Previous studies indicate that IECs are relatively hyporesponsive to extracellular bacterial components such as lipopolysaccharide. However, when either bacterial components or invasive bacteria gain access to intracellular compartments, they may readily induce cellular responses by IECs (1–3).

Plants were first noted to have both cytosolic and membrane pathogen-binding proteins that contribute to resistance to pathogenic microorganisms (4–6). More recently, mammalian cells have also been shown to express binding pattern recognition receptors including membrane toll-like receptors (TLRs) and cytosolic pathogen recognition proteins designated NODs. CARD4/NOD1 and CARD15/NOD2 were first identified as homologues of Apaf-1. CARD4/NOD1 and CARD15/NOD2 have highly conserved domain structures composed of caspase recruitment domains, nucleotide-binding domains, and leucine-rich repeats (7–9). Leucine-rich repeats domain are responsible for recognition of pathogen-associated molecular patterns by TLRs. Similarly, the leucine-rich repeat domain in NOD2 provides the recognition of bacterial peptidoglycan (10, 11). NODs activate NF-kB through a RICK signaling pathway following peptidoglycan stimulation (12–14).

Disorders of mucosal inflammation, such as inflammatory bowel disease, are associated with destruction of the mucosal barrier, which results in increasing susceptibility to luminal pathogen and penetration of normal flora. The interaction between luminal microorganisms and intestinal epithelial cells has been considered a critical point for initiation and perpetuation of mucosal inflammation in murine colitis models and human inflammatory bowel disease (15, 16). Therefore, we investigate the expression of the cytosolic bacterial pathogen recognition molecule, CARD4/NOD1 in IECs, and the regulation by the pro-inflammatory cytokine IFNγ, which has been implicated as a key regulatory cytokine in inflammatory bowel disease.

MATERIALS AND METHODS
Cell Culture—HT-29, Caco2, T84, Colo205, SW480, WiDr, SW48.5, LS174, and SW620 cells were obtained from the American Type Culture Collection (Manassas, VA) and used for experiments after they had reached 90–100% confluence. HT-29 cells were cultured in Dulbecco’s modified Eagle’s medium with 1-glutamine (Cellgro Mediatech Inc., Herndon, VA) supplemented with 10% (v/v) fetal calf serum (Atlanta Biologicals Inc., Norcross, GA) in 6-well tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ). Caco2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum (Atlanta Biologicals Inc., Norcross, GA) in 6-well tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ). T84, SW480, and SW620 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% (v/v) fetal calf serum. T84, SW480, and SW620 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. The cytokines for cell stimulation (IFNγ, tumor necrosis factor α, IL-1β, IL-4, and transforming growth factor-β) were obtained from R & D System, (McKinley, NE).

Reverse Transcription-PCR—Total RNA from each IEC line was isolated by Trizol (Invitrogen) following the manufacturer’s protocols. For reverse transcription, 2 μg of total RNA was transcribed by SuperScript II transcriptase according to protocols of SuperScript first strand synthesis system for RT-PCR (Invitrogen). The PCR mixture contained 1×...
PCR buffer, 1.5 μM MgCl₂, 0.2 μM dNTP mix, each 5’ and 3’ primer at 0.2 μM, 2 units of Taq polymerase (Invitrogen), and 2 μl (10% of total volume) of reverse transcription products from RNA in a total volume of 50 μl. Amplification was performed on a PerkinElmer Life Sciences Thermal Cycler with 40 cycles at adequate conditions. The primers for polymerase chain reaction encoding CARD4/NOD1, CD45, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are: forward, 5’-AAA-GCAAATCGGAACTTCTGG-3’, and reverse, 5’-GGCATAGGACGACGAGA-3’ (CARD4/NOD1, 374bp); forward, 5’-AGCCCGTCTGTTGTTCTCT-3’, and reverse, 5’-ACCTCGATCCTCTGTTTATC-3’ (CD45, 338bp); and forward, 5’-TCATCTCTGGCCCTCCTGCT-3’, and reverse, 5’-CCGACGCTGTTCAACACTT-3’ (GAPDH, 440bp). Annealing temperature was 55 °C for CARD4/NOD1 and 56 °C for GAPDH and CD45. PCR products were subcloned into pcDNA2.1 TOPO vector (Invitrogen) and sequenced using a ABI 3700 PRISM automated sequencer (PerkinElmer Life Sciences). The sequences were confirmed using NCBI BLAST software.

Northern Blot Analysis—For Northern blot analysis, 15 μg/lane of total RNA was loaded on a formaldehyde gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). The cDNA probe for CARD4/NOD1 was obtained by EcoRI and Smal (Promega, Madison, WI) digestion (1.5 kb) of the pCI CARD4-HA plasmid kindly provided by Dr. John Bertin (Millennium Pharmaceuticals Inc., Cambridge, MA). cDNA fragment for CARD4 was obtained by RT-PCR in SW480 treated with IFNγ using forward primer (5’-GACCCTGCTCTGCTCTGT-3’) and reverse primer (5’-GAGCTGCTCTGAGCTGCTG-3’). The PCR product was subcloned into pcDNA2.1 TOPO vector (Invitrogen) and sequenced. cDNA probe for IFNγ was obtained by EcoRI digestion. Labeling of probes with [α-32P]dCTP was performed using Ready To Go DNA Labeling Beads (Amersham Biosciences). Prehybridization and hybridization were performed in QuikHyb Hybridization Solution (Stratagene, La Jolla, CA).

Primary Intestinal Epithelial Cell Isolation—Intestinal epithelial cells were isolated from freshly obtained colonic mucosa obtained from five individual patients (primary IECs 1–5, respectively). Total RNA for each sample was obtained from 10–20 isolated primary intestinal epithelial cells. RT-PCR for CD45 and CD68 was performed to confirm the lack of contamination by nonepithelial cells (data not shown). The sensitivity of RT-PCR of CD45 and CD68 was validated by detection of an appropriate product using total RNA from a single Jurkat cell and THP-1 cell. As an internal control, RT-PCR of GAPDH (440 bp) was performed.

Boston, MA). Transfection was performed using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer’s instructions. Antibody—Anti-CARD4/NOD1 was raised in rabbits immunized with either the synthetic polypeptide PVSRTQQLRHLHRLGDR-SKFVLC (ND1:1.125–146,22 amino acids (aa)), SLVQRNTSPVETL-HAGRDTLC (ND1:2.440–461,22 aa), or GLNKPEAVKVEDEKRHC (ND1:3.953–992,20 aa) conjugated with keyhole limpet hemocyanin using Imject sulphhydryl reactive antibody production and purification kits (Pierce) (see Fig. 4A). Anti-sera (HM3851, ND1:1; HM3849, ND1:2; and HM3847, ND1:3) were purified using HyTrap protein A HP (Amersham Biosciences). Biotinylation of the anti-CARD4/NOD1 sera was performed using EZ-Link Sulfo-NHS-LC-Biotin (Pierce) according to the manufacturer’s instructions. All sera were affinity-purified by peptides before use. Anti-IRF-1 affinity-purified rabbit polyclonal antibody (C-20) was obtained from Santa Cruz Biotechnology, Inc.

Immunoprecipitation and Immunoblotting—The cells were grown in 6-well plates and stimulated with 10 ng/ml IFNγ for 18 h and washed, and then 300 μl of lysis buffer (1% Triton X-100, phenylmethylsulfonyl fluoride, protease inhibitor, EDTA, Tris/HCl, NaCl, pH 7.4, supplemented with protease-inhibitor mixture, Complete Mini (Roche Applied Science)) was added. After 10 min of incubation, cell-free lysates were obtained and stored at −80 °C. Protein concentration was determined using a Bio-Rad DC protein assay kit. For immunoprecipitation, a total 2 mg of protein was mixed with 2 μg of affinity-purified rabbit anti-CARD4/NOD1 antisem (HM3851) and 10 μl of Hitrap protein A Sepharose beads (Amersham Biosciences). After overnight incubation at 4 °C, the immunocomplexes were washed with lysis buffer and solubilized in 30 μl of SDS sample buffer (1.25% SDS, 2.5% glycerol, 62.5
Fig. 3. Effect of IFNγ on CARD4/NOD1 mRNA transcription. A, Northern blot analysis for CARD4/NOD1 mRNA expression in SW480 cells following addition of 10 ng/ml of IFNγ, tumor necrosis factor α, IL-1β, IL-4, and transforming growth factor-β for 6 h. GAPDH was used as an internal control. B, concentration-dependent effects of IFNγ on CARD4/NOD1 mRNA expression in SW480 cells. The cells were treated with 0.3, 1, 3.3, 10, 33, and 100 ng/ml IFNγ for 6 h. GAPDH was used as an internal control. C, time-dependent effects of IFNγ on CARD4/NOD1 mRNA expression in SW480 cells. The cells were treated with 100 ng/ml IFNγ for 2, 4, 6, 8, 12, 24, and 48 h. GAPDH was used as an internal control.

mm Tris/HCl, pH 6.8, 5% 2-mercaptoethanol). The immunoprecipitated proteins were separated on 4–20% Tris/glycine polyacrylamide gel (Invitrogen), and the proteins were blotted onto polyvinylidene difluoride membranes (PerkinElmer Life Sciences). The membranes were blocked with 5% dry milk, 0.1% Tween 20 in Tris-buffered saline for 2 h at room temperature and then incubated overnight at 4°C with biotinylated rabbit anti-CARD4/NOD1 antisera (HM3847) (100 ng/ml) followed by 1 h of incubation at room temperature with horseradish peroxidase-labeled rabbit anti-biotin antibody (1:1000) (Cell Signaling, Beverly, MA). Staining was detected using the Renaissance chemiluminescence kit (PerkinElmer Life Sciences) and radiographic film. Lysates from COS7 and COS7 cells transfected with pCI CARD4-HA were used as negative and positive controls, respectively.

Construction of CARD4 Luciferase Reporter Constructs—The genomic sequence adjacent to the human CARD4/NOD1 exon 1 and promoter sequences were found in a GenBank™ working draft sequence of chromosome 7 (see Fig. 5A). The primers were designed to correspond to sequences overlapping the CARD4 exon1 at +21 and also a sequence ~2,128 bp upstream. This putative promoter region was amplified from human genomic DNA (Promega) by PCR using Expand high Fidelity PCR System (Roche Applied Science). The 2,128-bp PCR fragment was cloned into the promoterless luciferase reporter vector pGL3-basic (Promega). This construct was confirmed by sequencing and named pGL-2128. The CARD4 promoter deletions (see Fig. 5B) were generated by restriction enzyme digestion using a blunting kit (Takara Biochemical, Berkeley, CA) or by PCR.

Luciferase Reporter Assay—1 × 10^5/well of SW480 cells were seeded on 12-well plates (Costar, Cambridge, MA). After 24 h, the cells were transiently transfected with reporter plasmids, together with the indicated expression vectors. After 10 h of transfection, the cells were treated with 100 ng/ml of IFNγ for 16 h. The luciferase activity of total cell lysates was measured using the dual luciferase reporter assay system (Promega). The Renilla luciferase reporter gene (10 ng/well) (Promega) was used as an internal control.

Electrophoretic Mobility Shift Assay—Nuclear proteins were prepared as previously described (18). Both double-stranded oligonucleotides corresponding to −807 to −768 bp and mutated oligonucleotides were used as probes or cold competitors to analyze the interaction between IRF-1 protein and DNA (see Fig. 6, A and B). The probes were labeled with T4 polynucleotide kinase (Promega) with [γ-32P]ATP. The binding reaction was performed as previously described (19). For a competition assay, a 100-fold excess of unlabeled oligonucleotide was added to the reaction. To perform supershift assay, the binding mixture was incubated for 10 min at room temperature in the presence of 1 μl of affinity-purified polyclonal anti-IRF-1 antibody (C-20X, Santa Cruz). The samples were fractionated on 4% nondenaturing polyacrylamide gel in 0.5 × TBE buffer (0.045 M Tris-borate, 1 mM Na2 EDTA). The resultant DNA-protein complexes were detected by autoradiography.

RESULTS AND DISCUSSION

Although intestinal epithelial cells are exposed to numerous bacteria and their components, they appear functionally to be hyporesponsive to normal flora or their components in vivo, an adaptation that seems appropriate to protect host from constant immune and inflammatory activation. However, previous studies have indicated that intestinal epithelial cells can respond to enteroinvasive bacteria to stimulate the recruitment of polymorphonuclear neutrophils to an affected site in vivo and in vitro. Enteroinvasive bacteria, but not noninvasive bacteria, activate NF-κB in intestinal epithelial cell lines and induce expression of several genes encoding inflammatory me-
Diators including IL-8, ICAM-1, monocyte chemoattractant protein-1, or inducible nitric-oxide synthase in vitro (1, 2, 20). These results suggest that intracellular presentation of bacteria may lead to initial responses of intestinal epithelial cells that do not occur when bacteria are only encountered in the extracellular environment. Thus, intestinal epithelial cells have mechanisms for the recognition of intracellular bacterial pathogenic components that are presented by invasive bacteria.

Recently, Philpott et al. (3) reported that bacterial lipopolysaccharide can induce NF-κB activation in microinjected HeLa or Caco2 cells. Girardin et al. (21) demonstrated that invasive Shigella flexneri, but not noninvasive S. flexneri, lead to activation of c-Jun in HeLa cells. Activation of NF-κB and JNK by invasive S. flexneri is independent of the IL-1/TLR pathway in HEK293 cells. After infection with invasive S. flexneri, JNK activation is significantly increased in CARD4/NOD1 overexpressing HEK293 cells. CARD4/NOD1 oligomerization and RICK-IKKε complex are found in CARD4/NOD1-overexpressing HEK293 cells following invasive S. flexneri infection, and these effects are inhibited by a dominant nega-

tive CARD4/NOD1 (21). Collectively, these data suggest that CARD4/NOD1 is a candidate to serve as a pivotal receptor for intracellular bacteria. However, CARD4/NOD1 expression and its regulation have not been previously demonstrated in IEC lines. Therefore, we investigated CARD4/NOD1 mRNA expression in IEC lines.

mRNA Expression of CARD4/NOD1 in IEC Lines and Primary Intestinal Epithelial Cells—Expression of CARD4/NOD1 was assessed by RT-PCR in several independent derived IEC lines: HT-29, T84, Caco2, SW480, SW620, Colo205, WiDr, SW48.5, and LS174. As demonstrated in Fig. 1, CARD4/NOD1 mRNA (product size, 374 bp, confirmed by sequencing) was constitutively expressed in all IEC lines examined. Therefore, we investigated CARD4/NOD1 mRNA expression in IEC lines.

mRNA Expression of CARD4/NOD1 in IEC Lines and Primary Intestinal Epithelial Cells—Expression of CARD4/NOD1 was assessed by RT-PCR in several independent derived IEC lines: HT-29, T84, Caco2, SW480, SW620, Colo205, WiDr, SW48.5, and LS174. As demonstrated in Fig. 1, CARD4/NOD1 mRNA (product size, 374 bp, confirmed by sequencing) was constitutively expressed in all IEC lines examined. Therefore, we investigated CARD4/NOD1 mRNA expression in IEC lines.

Fig. 5. IFNγ activated the human CARD4 promoter. A, schema of CARD4/NOD1 gene. B, schematic structures of pGL CARD4-luciferase deletion mutant. Three IRF-1 binding motifs (IRF-1A, IRF-1B, and IRF-1C) are indicated as black boxes. C, SW480 cells were transiently transfected with 500 ng/well of indicated expression plasmid. 10 h after transfection, the cells were cultured for a further 16 h with (closed bars) or without (open bars) 100 ng/ml of IFNγ, and then luciferase activity was measured. The experiments were performed in triplicate. The data are presented as the relative fold (means ± S.D.) compared with SW480 cells transfected with pGL3 basic empty vector. The results were confirmed in three independent experiments. D, relative induction of luciferase activity by IFNγ. The data are presented as relative fold (means ± S.D.) of luciferase activity of SW480 with IFNγ compared that with medium in three independent experiments. Each experiment was performed in triplicate.
epithelial cells were picked by micropipetting. Markers for lymphocytes and monocytic cells (CD45 and CD68) were not detected in these isolated intestinal epithelial cells by highly sensitive RT-PCR (data not shown). Total RNA from a single Jurkat cell and THP-1 cell served as positive controls for CD45 and CD68, respectively. As shown in Fig. 2B, CARD4/NOD1 expression was detected in all isolated primary intestinal epithelial cells examined.

**Effect of IFNγ on the Expression of CARD4/NOD1—**Regulation of CARD4/NOD1 expression has not been described previously. As shown in Fig. 3A, IFNγ augmented CARD4/NOD1 mRNA expression in SW480 cells. Other cytokines examined (tumor necrosis factor α, IL-1β, IL-4, and transforming growth factor-β) did not affect CARD4/NOD1 mRNA expression. The

![Figure 6](image_url)  
**Interaction of IRF-1 with the IRF-1 binding motifs of the human CARD4/NOD1 promoter.** A, IRF-1-binding motifs (IRF-1 A, B, and C in bold type) in CARD4/NOD1 promoter. B, oligonucleotides sequences of wild type and mutated IRF-1 consensus motifs in CARD4/NOD1 promoter (wild type (WT) and mutated sequences (Mut)) for electrophoretic mobility shift assay. IRF-1 consensus sequences are underlined, and mutations are in bold type. C, specific binding of IRF-1 to the IRF-1 consensus sequences in CARD4/NOD1 promoter. Electrophoretic mobility shift assay was performed using 9 μg of nuclear protein from SW480 cells treated with 100 ng/ml of IFNγ for the indicated time periods. The competition assay was performed with a 100-fold excess of unlabeled oligonucleotides. The supershift assays were done by the addition of 1 μg of anti-IRF-1 antibody.

![Figure 7](image_url)  
**IFNγ activates IRF-1 expression in SW480 cells.** A, Northern blot analysis assessing time-dependent IRF-1 mRNA expression in SW480 cells treated with 100 ng/ml of IFNγ for 2, 4, 6, 8, 12, 24, and 48 h. GAPDH was used as an internal control. B, western blot analysis assessing time-dependent nuclear IRF-1 protein expression in SW480 cells treated with 100 ng/ml of IFNγ for 1, 2, and 6 h, respectively. 30 μg/lane of nuclear protein was used.

![Figure 8](image_url)  
**IRF-1 activates CARD4/NOD1 transcription in SW480 cells.** Luciferase activity in SW480 cells co-transfected with 500 ng/well of pGL3-2128 or pGL3-837–546 and the indicated amount of IRF-1 expression plasmid. Luciferase activity was measured after 48 h of transfection. The experiments were performed in triplicate. The data are presented as the relative fold (means ± S.D.) compared with SW480 cells transfected with pGL3 basic empty. The results were confirmed in three independent experiments. The experiments were performed in triplicate.
effect of IFNγ on CARD4/NOD1 mRNA expression is time- and concentration-dependent as assessed by Northern blot analysis (Fig. 3, B and C).

Although CARD4/NOD1 is considered important for recognition for intracellular pathogens, previous studies have not demonstrated active CARD4/NOD1 protein in vitro or in vivo. To investigate the expression of CARD4/NOD1 protein, we generated anti-CARD4/NOD1 sera. The specificity and the sensitivity were confirmed using lysates from COS7 cells transiently transfected with the HA-tagged CARD4 expression plasmid, pCI CARD4-HA (Fig. 4A). Consistent with the regulation of mRNA expression, CARD4/NOD1 protein was also augmented in SW480 cells by IFNγ stimulation (Fig. 4B).

**IRF-1 Is Essential for Up-regulation of CARD4/NOD1 Transcription by IFNγ**—To identify the transcriptional regulation of CARD4/NOD1, we constructed a series of luciferase reporter vectors containing up to 2,128 bp corresponding to the DNA sequence upstream of base 1 and extending 21 bp into the first exon of CARD4/NOD1 (Fig. 5, A and B). Luciferase activity in SW480 cells transfected with a vector containing the entire 2,128-bp upstream DNA (pGL-2128) was 125 ± 16-fold higher than that obtained with the empty pGL3 basic vector. This promoter lacks the TATA box but has several GC-rich motifs in its promoter region. The most distal IRF-1-binding motif (IRF-1A, 550 up to 754) and interferon-stimulated response element were not present in the promoter region of CARD4/NOD1 (Fig. 3, B). The transcriptional activity of IFNγ increased 80% luciferase activity in SW480 cells transfected with pGL-2128.

Luciferase activity of cells transfected with deletion constructs pGL-837–546, pGL-546, and pGL-367 demonstrated that sequences within −837 to −546 promoter are essential for activation by IFNγ. Three IRF-1-binding motifs (IRF-1A, −791 to −782; IRF-1B, −787 to −778; and IRF-1B, −694 to −689) (Figs. 5B and 6A) are clustered in this region. Luciferase activity of cells transfected with pGL-546, pGL-773, and pGL-729 suggested that the most distal IRF-1-binding motif (IRF-1A, −791 to −782) is essential for the IFNγ effect (Fig. 5, C and D).

To determine whether IRF-1 binds to this IRF-1-binding motif in the CARD4/NOD1 promoter sequences, we performed electrophoretic mobility shift assay using nuclear extracts from SW480 cells with IFNγ treatment. Consistent with the promoter analysis, oligonucleotides corresponding to IRF-1-binding sequences (IRF-1A) in CARD4/NOD1 promoter bound nuclear IRF-1 protein in SW480 cells treated with 100 ng/ml of IFNγ-time-dependently (Fig. 6C, lanes 1–4). The binding of IRF-1 was inhibited by unlabeled oligonucleotides competitor but not by oligonucleotides with mutations at IRF-1-binding motifs (lanes 5 and 6). The band reflecting the complex with IRF-1 oligonucleotides was supershifted by anti-IRF-1 antibody (lanes 4 and 7). Signal transducers and activators of transcription (STAT)-1-binding motifs, IFNγ activation site, and interferon-stimulated response element were not present as far as 5 kb upstream of the promoter region of CARD4/NOD1, as determined by computer analysis using TRANSFAC data base of University of Pennsylvania. We hypothesized that the effect of IFNγ on CARD4/NOD1 expression depends on IRF-1, which has an interferon-γ activation site motif in the promoter region. Transcription of IRF-1 is regulated by IFNγ through Janus tyrosine kinase/STAT pathway (22). To test this hypothesis, we first demonstrated that IRF-1 mRNA and nuclear protein expression were rapidly up-regulated by IFNγ treatment in SW480 cells (Fig. 7). To confirm that over-expressed IRF-1 can activate CARD4/NOD1 promoter, we next performed promoter analysis using SW480 cells co-transfected with IRF-1 expression plasmid. Promoter activity of pGL-2128 was activated 2.0-fold (71.8 ± 0.6 versus 35.9 ± 0.9) in SW480 cells co-transfected with pCDNA IRF-1 expression vector.

The effect of IRF-1 was completely abrogated in pGL-837–546, which lacks the IRF-1 cluster region (Fig. 8). Collectively, these results suggest that rapid augmentation of nuclear IRF-1 protein by IFNγ treatment results in activation of CARD4/NOD1 transcription.

IFNγ is a potent pro-inflammatory cytokine in acute and chronic mucosal inflammation in the intestine (23). In Crohn’s disease and ulcerative colitis as well as murine models of colitis, the Janus tyrosine kinase/STAT pathway is activated in inflamed mucosa (24, 25). As shown in recent studies, the membrane pathogen recognition molecules TLR4 and MD2 are regulated by IFNγ in IECs (26, 27). Augmentation of TLR4 and MD2 by IFNγ priming resulted in acquisition of responsiveness to lipopolysaccharide in HT29 cells, which are relatively hyporesponsive to lipopolysaccharide without IFNγ priming. Secondly, CARD15/MD2, another cytosolic receptor for peptidoglycan, is augmented by tumor necrosis factor α in IECs (28–30). These findings provide evidence that pathogen recognition molecules are modulated by pro-inflammatory cytokines and suggest that the latter promote host defenses to pathogens in inflammatory conditions. In acute and chronic intestinal inflammation, luminal pathogens or their components can invade more easily, because intestinal mucosal barrier function is impaired. In patients with inflammatory bowel disease, it has been suggested that luminal bacteria contribute to the perpetuation of chronic inflammation (31, 32). In this context, the pro-inflammatory cytokine, IFNγ, may readily lead to CARD4/NOD1 augmentation in IECs.

Here, we first demonstrate that IECs express CARD4/NOD1 mRNA and protein and that IFNγ activates transcription of CARD4/NOD1 mRNA through IRF-1 in IECs. These data suggest that intestinal epithelial cells provide a functional as well as structural barrier to the entry of luminal bacteria or their components into the underlying lamina propria via the cytosolic protein CARD4/NOD1.

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