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Mechanism of Interferon-γ–Induced Increase in T84 Intestinal Epithelial Tight Junction

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Interferon-γ (IFN-γ) is an important proinflammatory cytokine that plays a central role in the intestinal inflammatory process of inflammatory bowel disease. IFN-γ induced disturbance of the intestinal epithelial tight junction (TJ) barrier has been postulated to be an important mechanism contributing to intestinal inflammation. The intracellular mechanisms that mediate the IFN-γ induced increase in intestinal TJ permeability remain unclear. The aim of this study was to examine the role of the phosphatidylinositol 3-kinase (PI3-K) pathway in the regulation of the IFN-γ induced increase in intestinal TJ permeability using the T84 intestinal epithelial cell line. IFN-γ caused an increase in T84 intestinal epithelial TJ permeability and depletion of TJ protein, occludin. The IFN-γ induced increase in TJ permeability and alteration in occludin protein was associated with rapid activation of PI3-K; and inhibition of PI3-K activation prevented the IFN-γ induced effects. IFN-γ also caused a delayed but more prolonged activation of nuclear factor-κB (NF-κB); inhibition of NF-κB also prevented the increase in T84 TJ permeability and alteration in occludin expression. The IFN-γ induced activation of NF-κB was mediated by a cross-talk with PI3-K pathway. In conclusion, the IFN-γ induced increase in T84 TJ permeability and alteration in occludin protein expression were mediated by the PI3-K pathway. These results show for the first time that the IFN-γ modulation of TJ protein and TJ barrier function is regulated by a cross-talk between PI3-K and NF-κB pathways.

Introduction

Intestinal epithelial cells provide a barrier function against noxious substances in the intestinal lumen (Hollander 1998; Ma and Anderson 2006). Intestinal epithelial tight junctions (TJs) are the apical–most junctional complexes and act as a structural and functional barrier against paracellular permeation of luminal substances (Hollander 1998; Ma and Anderson 2006). The defective intestinal epithelial TJ barrier allows increased paracellular permeation of luminal substances including toxic luminal antigens that promote inflammatory response in the intestine (Hollander 1998). The defective intestinal TJ barrier exists in many inflammatory disorders including in Crohn's disease (CD), NSAID-associated enteropathy, postinfectious irritable bowel syndrome, alcoholic liver disease, and infectious diarrheal syndromes (Hollander 1998; Ma and Anderson 2006). The defective TJ barrier has been implicated as an important pathogenic factor leading to intestinal inflammation (Hollander 1998; Ma and Anderson 2006). Accumulating evidence indicates that an important biological action of proinflammatory cytokines is to induce a functional opening of the intestinal TJ barrier, allowing increased paracellular permeation of toxic luminal substances (Hollander 1998; Madara and Stafford 1989; Ma and others 2004; Bruewer and others 2005; Ma and Anderson 2006). Thus, in addition to their direct effects on immune activation, proinflammatory cytokines also promote intestinal inflammation by disrupting the intestinal TJ barrier (Madara and Stafford 1989; Ma and others 2004; Bruewer and others 2005; Ma and Anderson 2006).

Interferon-γ (IFN-γ), a proinflammatory cytokine produced by cells of the innate immune system, plays an important role in the initiation and propagation of the inflammatory process in CD and other inflammatory disorders of the gut (Reinecker and others 1993). The IFN-γ levels are markedly elevated in intestinal tissue of CD patients and

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lead to production and stimulation of other potent proinflammatory cytokines including interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) (Reinecker and others 1993; Fuss and others 1996). The proinflammatory mechanisms attributed to IFN-γ include stimulation of inflammatory cytokines, activation of acute phase responses, and disturbance in intestinal TJ barrier function (Reinecker and others 1993; Fuss and others 1996; Hommes and others 2006). The importance of IFN-γ in intestinal inflammation of CD has been well-validated by clinical studies demonstrating the efficacy of anti-IFN-γ antibody treatment in patients with severe active CD (Hommes and others 2006). The role of IFN-γ in the alteration of intestinal TJ barrier has been well-established (Madara and Stafford 1989; Adams and others 1993; Youakim and Ahdieh 1999; Bruewer and others 2003; Willemsen and others 2005). The IFN-γ induced increase in TJ permeability has been postulated to play an important pathogenic role in promoting intestinal inflammation (Madara and Stafford 1989; Bruewer and others 2003; Bruewer and others 2005; Willemsen and others 2005). However, the intracellular pathways and mechanisms that mediate the IFN-γ induced increase in intestinal TJ permeability have not been well-delineated. Understanding the intracellular pathways involved in IFN-γ modulation of intestinal permeability could be important in devising potential therapeutic strategies to induce retightening of the TJ barrier during various inflammatory conditions.

The major aim of this study was to investigate the intracellular pathways that mediate the INF-γ induced increase in intestinal TJ permeability, using filter-grown T84 monolayers as an *in vitro* intestinal epithelial model system. Previous studies have suggested that some of the proinflammatory responses related to IFN-γ may be regulated by activation of phosphatidylinositol 3-kinase (PI3-K) or nuclear factor-κB (NF-κB) pathways (Jones and Clague 1995; Haller and others 2002; Sheth and others 2003; Kaur and others 2005). In this study, we examined the role of PI3-K and NF-κB pathways in mediating the IFN-γ induced increase in intestinal TJ permeability. Our results suggest that PI3-K pathway activation is required for the IFN-γ induced increase in T84 TJ permeability. Additionally, our data suggest that a cross-talk between the PI3-K and NF-κB pathways is necessary for the IFN-γ modulation of the TJ barrier.

Materials and Methods

Materials

Trypsin, FBS, and other cell culture reagents were purchased from Life Technologies (Gaithersburg, MD). Penicillin, streptomycin, and PBS were purchased from GIBCO-BRL (Grand Island, NY). Transwell permeable filters were purchased from Corning (Corning, NY). IFN-γ and EGF (epidermal growth factor) was purchased from R & D Systems (Minneapolis, MN). Anti-occludin and anti-claudin antibodies were obtained from Zymed Laboratories (San Francisco, CA), p85 antibody, phosphor-AKT antibody and pan-AKT antibodies were obtained from Cell Signaling Technologies (Danvers, MA). Cell culture media [Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12], LY294002, wortmannin and pyrolidine dithiocarbamate (PDTC) were purchased from Sigma (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated secondary antibodies for Western blot analysis were purchased from Zymed Laboratories and Cell Signaling Technologies. FACE and enzyme-linked immunosorbent assay (ELISA) reagents were obtained from Active Motif (Carlsbad, CA).

Cell cultures

T84 intestinal epithelial cells (*passage 53*) were purchased from the American Type Culture Collection (Rockville, MD) and maintained at 37°C in DMEM/Ham's F-12 medium supplemented with 16 mM $NaHCO_3$, antibiotics, and 5% fetal bovine serum. The cells were kept at 37°C in a 5% CO_2 environment. Culture medium was changed every 2 days. T84 cells were subcultured after partial digestion with 0.25% trypsin and 0.9 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS. Only T84 cells from passage 55 to 61 were used to maintain consistency. Unless stated otherwise, in all IFN-γ was added to the basolateral compartment only.

Determination of epithelial monolayer resistance and paracellular permeability

An epithelial voltohmeter (World Precision Instruments, Sarasota, FL) was used for measurements of the trans-epithelial electrical resistance (TER) of the filter-grown T84 intestinal monolayers as previously reported (Jones and Clague 1995; Boivin and others 2007). To study the dose effects of IFN-γ on TER, T84 monolayers were treated with increasing doses ranging 1–100 ng/mL over a 48-h time period. The effect of IFN-γ on T84 paracellular permeability was determined using an established paracellular marker: inulin (Jones and Clague 1995; Boivin and others 2006). For determination of mucosal-to-serosal flux rates of inulin, T84-plated filters having epithelial resistance of 1500–2000 Ω cm^2 were used. Known concentrations of ^{14}C-Inulin tracer were added to the apical solution. Low concentrations of permeability marker were used to ensure that negligible osmotic or concentration gradient was introduced. ^{14}C-Inulin was measured using liquid scintillation on a Beckmann instrument (Beckman-Coulter, Fullerton, CA).

Assessment of protein expression by Western blot analysis

To study the effect of IFN-γ on PI3-K p85 regulatory subunit and occludin protein expression, T84 monolayers were treated with IFN-γ (10 ng/mL) for varying time periods. At the end of the experimental period, T84 monolayers were immediately rinsed with ice-cold PBS, and cells were lysed with the lysis buffer. For TJ protein western blots, lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 500 μM NaF, 2 mM EDTA, 100 μM vanadate, 100 μM PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 40 mM paranitrophenyl phosphate, 1 μg/mL aprotinin, and 1% Triton X-100 was used. For PI3-K p85 subunit expression, both Triton-X soluble and insoluble fractions were obtained as previously described and p85 subunit translocation from Triton-X

soluble to insoluble fraction was used to assess PI3-K activation (Sheth and others 2003). Cell lysates were centrifuged to yield a clear lysate, supernatants were collected, and protein measurement was performed using Bio-Rad Lowry Protein Assay kit (Bio-Rad Laboratories, Hercules CA). Laemmli gel loading buffer was added to the lysate containing 10 μg of protein and boiled for 7 min, after which proteins were separated on an SDS-PAGE gel. Proteins from the gel were transferred to the membrane (Nitrocellulose Membrane; Bio-Rad Laboratories) overnight. The membrane was incubated for 2 h in blocking solution (5% dry milk in TBS-Tween 20 buffer). The membrane was incubated with appropriate primary antibodies in blocking solution. After being washed in TBS-1% Tween buffer, the membrane was incubated in appropriate secondary antibodies and developed using the Santa Cruz Western Blotting Luminol Reagents (Santa Cruz Biotechnology, Santa Cruz, CA) on the Kodak BioMax MS film (Fisher Scientific, Pittsburgh, PA).

Assessment of PI3-K and Akt activation by FACE assay

Activation of the PI3-K and Akt enzymes were determined by an in-situ ELISA using phospho-specific and total antibodies (FACE assay, Active Motif, Carlsbad, CA). Experiments were carried out according to the manufacturer's protocol. In brief, T84 cells were treated with IFN-γ and/or inhibitors for the appropriate time periods. The cells were then fixed, washed and lysed on the ELISA plate. The cells were incubated with blocking solution followed by incubation in primary antibody (either anti-phospho-Akt or PI3-K or anti-Akt or PI3-K antibody) containing solution. The cells were then labeled with HRP-conjugated secondary antibody. After rinsing, chemiluminescent solution was added and the bound antibody chemiluminescent signal was determined on a plate luminometer. Results are expressed as the ratio of phospho-(PI3 or Akt) antibody to total (PI3 or Akt) antibody (run as separate experiments).

Isolation of nuclear extracts and ELISA for NF-κB p65 DNA binding

Filter-grown T84 cells were treated with 10 ng/mL IFN-γ for increasing time points (2–24 h). Cells were washed with ice-cold PBS, scraped, and centrifuged at 14,000 rpm for 30 s. To isolate nuclear proteins, the cell pellets were resuspended in 200 μL hypotonic buffer (Nuclear Extract kit, Active Motif, Carlsbad CA) and incubated on ice for 15 min to lyse the cells. After centrifugation at 14,000 rpm for 30 s, pelleted nuclei were resuspended in 30 μL complete lysis buffer (also from Active Motif). After incubation on ice for 20 min, the resuspended nuclei were centrifuged at 14,000 rpm for 20 min to collect the nuclear proteins in the supernatant. Nuclear protein concentrations were determined using the Bradford method. The NF-κB DNA-binding activity assay was performed using Trans-AM ELISA-based kits from Active Motif (Carlsbad, CA) according to the manufacturer's protocol. Nuclear extract (5 μg) was added to individual wells on the plate containing the bound oligonucleiotide with consensus NF-κB sequences and incubated for 1 h. Rabbit anti-NF-κB p65 antibody was added to the well to bind NF-κB p65 from the nuclear extract. After incubation for 1 h, anti-rabbit HRP-conjugated IgG were added to the well and incubated for 1 h. Subsequently, developing solution was added, then stop solution was added. The absorbance at 450 nm was determined using the SpectrraMax 190 (Molecular Devices, Sunnyvale, CA).

NF-kB-dependent promoter activation assay

To study the NF-κB activation, serum embryonic alkaline phosphatase (SEAP) reporter assay was performed as per manufacturer's instructions (Clontech, CA). Briefly, T84 cells were transfected with plasmid vector encoding NF-κB p65 consensus sequence responsive promoter and SEAP reporter gene, using the transfection reagent Genejuice (Novagen). The culture media was replaced after 24 h. At 48 h, the transfected cells were treated with IFN-γ. In inhibitor studies, the cells were pre-treated with inhibitor for 1 h prior to treatment with IFN-γ. The SEAP activity in the supernatant was determined using the BD Great EscAPe™ chemiluminescence detection kit (Clontech, CA) as per the manufacturer's protocol. SEAP activity was expressed as relative light unit (RLU).

Statistical analysis

Results are expressed as means ± SE. Statistical significance of differences between mean values was assessed with Student's *t*-tests for unpaired data. All reported significance levels represent two-tailed p values. A p value of <0.05 was used to indicate statistical significance. All experiments including Western blot analysis, DNA binding assays, promoter activity studies, and functional studies were repeated a minimum of three times to ensure reproducibility.

Results

Effect of IFN-γ on T84 intestinal epithelial TJ permeability

The effect of IFN-γ on T84 intestinal epithelial TJ permeability was determined by measuring TER and paracellular permeability. First, the apical versus basolateral membrane specificity of IFN-γ effect on T84 TJ permeability was examined. IFN-γ (10 ng/mL) was added either to the basolateral, apical or combined apical and basolateral compartments. The addition of IFN-γ to the basolateral compartment produced a progressive drop in T84 TER (Fig. 1A). However, apical treatment of IFN-γ did not have any significant effect on T84 TER (Fig. 1A). The extent of drop in TER was similar whether IFN-γ was added to the basolateral compartment alone or to both basolateral and apical compartments together. These results indicated that IFN-γ action at the basolateral compartment was both required and sufficient for the IFN-γ induced increase in T84 TJ permeability. These results are consistent with the presence of IFN-γ receptor on the basolateral membrane surface, where IFN-γ is secreted into the interstitial fluid by immune cells. The time course of IFN-γ (10 ng/mL) effect on T84 TER is shown in Figure 1B.

IFN-γ caused a time-dependent drop in T84 TER with a maximal drop occurring between 48 and 72 h (Fig. 1B).

As in previous reports (Madara and Stafford 1989; Adams and others 1993; Youakim and Ahdieh 1999), increasing concentration of IFN-γ (0–100 ng/mL) caused a dose-dependent decrease in T84 TER (Fig. 1C). The maximal drop in TER was reached at IFN-γ concentration of 10 ng/mL, and increasing the concentration of IFN-γ above 10 ng/mL did not cause a further drop in T84 TER. In separate studies, the relationship between IFN-γ effect on TER and paracellular permeability was examined, IFN-γ caused a time-dependent drop in TER and a time-dependent increase in trans-epithelial permeability to paracellular marker inulin (Fig. 1D). There was a direct linear relationship between IFN-γ induced drop in TER and increase in paracellular permeability with relative correlation coefficient of $r = 0.96$ (data not shown) indicating that IFN-γ induced decrease in T84 TER directly correlates with an increase in paracellular permeability.

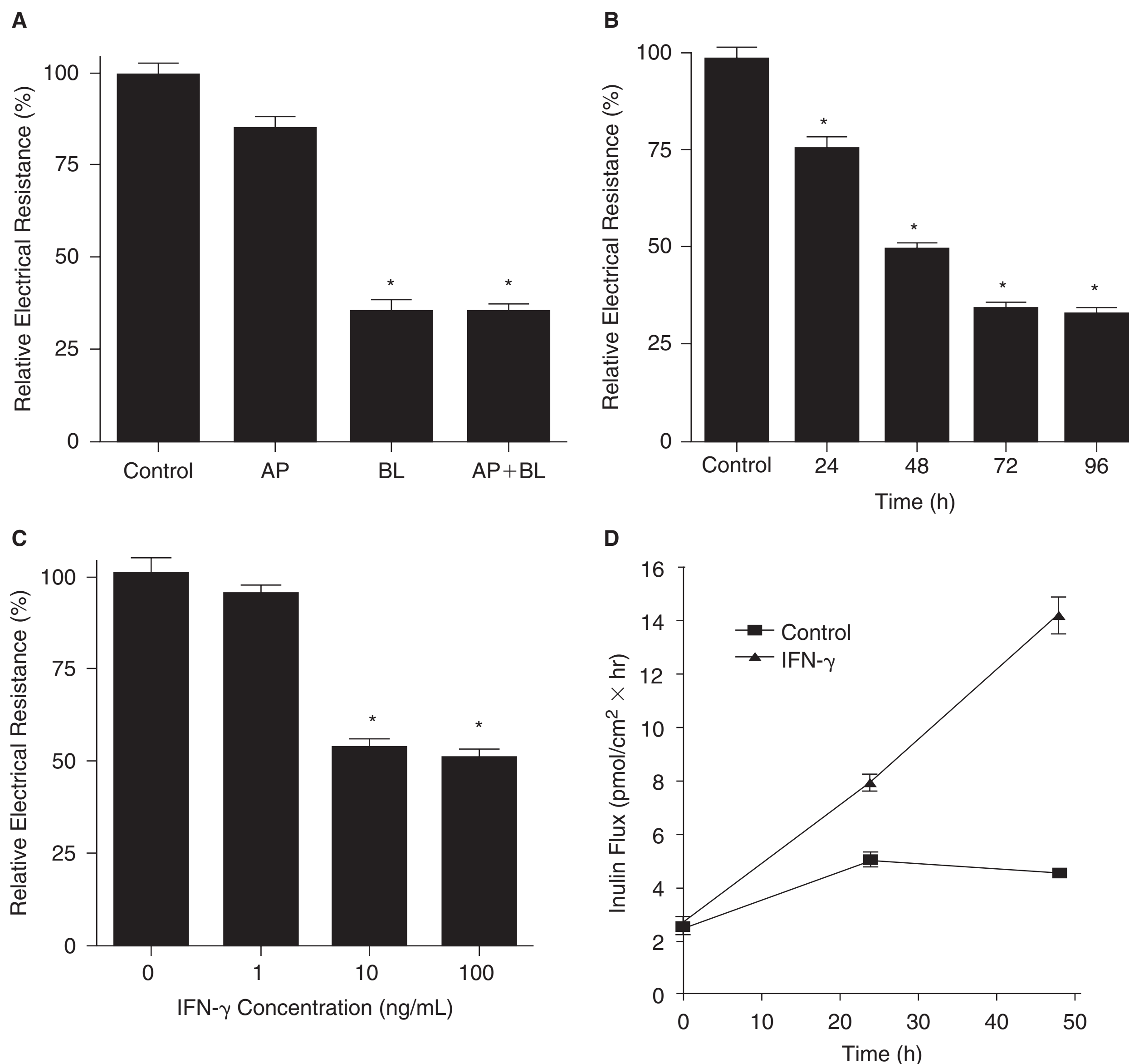

FIG. 1. Effect of IFN-γ on T84 trans-epithelial electrical resistance (TER). (**A**) Membrane specificity of IFN-γ effect on T84 epithelial resistance. IFN-γ (10 ng/mL) was added to either apical (AP), basolateral (BL), or combined apical and basolateral compartments (AP+BL). Addition of IFN-γ to the basolateral or combined basolateral and apical compartments produced a significant drop in T84 epithelial resistance ($n = 3$). $^*p < 0.01$ versus control. (**B**) IFN-γ (10 ng/nl) caused a time-dependent decrease in TER. Data represent means ± SE of epithelial resistance ($n = 4$). (**C**) IFN-γ produced a concentration (0, 1, 10, 100 ng/mL) dependent decrease in T84 TER over the 48-h experimental period. $^*p < 0.0001$ versus control. All experiments were repeated three to six times to ensure reproducibility. (**D**) Time-course effect of IFN-γ (10 ng/mL) on ^{14}C-inulin flux from apical to basolateral compartments ($n = 4$). $p < 0.0001$ IFN-γ versus Control at 48 h.

PI3-K pathway activation is required for the IFN-γ–induced increase in T84 epithelial TJ permeability

In the following studies, the role of PI3-K pathways in IFN-γ induced increase in T84 TJ permeability was examined. PI3-K is made up of two subunits, p85 and p110, both of which are normally present in the cytoplasm in quiescent cells. Upon activation, PI3-K is phosphorylated and translocates to the plasma membrane. The activated PI3-K mediates the recruitment and phophorylation of Akt at Serine 473 (Jones and Clague 1995; Sheth and others 2003; Kaur and others 2005). The IFN-γ (10 ng/mL) effect on PI3-K pathway activation in T84 cells was determined by assessing phosphorylation of PI3-K, translocation of p85 subunit from cytoplasm-to-membrane fraction and by Akt phosphorylation (Jones and Clague 1995; Sheth and others 2003; Kaur and others 2005). IFN-γ treatment resulted in a rapid increase in PI3-K (p85) phosphorylation (Fig. 2A). The increase in P13-K phosphorylation was followed by an increase in Akt phosphorylation (Fig. 2B) (Kaur and others 2005). In previous studies, the translocation of p85 subunit from Triton-x soluble to insoluble fraction has also been used as an indirect measure of cytoplasmic-to-membrane translocation of p85 subunit (Sheth and others 2003). IFN-γ treatment also resulted in a rapid translocation of p85 subunit from Triton-X soluble to the insoluble fraction, suggesting cytoplasmic-to-membrane translocation (Fig. 2C) (Sheth and others 2003). Together, these results indicated that IFN- γ causes an early activation of PI3-K in T84 cells.

Next, the role of PI3-K activation in the IFN-γ induced increase in T84 TJ permeability was examined. In the following studies, PI3-K activation was inhibited by PI3-K inhibitor LY 294002 (20 μM) (Kaur and others 2005). LY 294002 pretreatment significantly inhibited the IFN-γ induced PI3-K and AKT activation (Fig. 2B and C) and the IFN-γ induced drop in T84 TER (Fig. 3). Another PI3-K inhibitor wortmannin also significantly inhibited the IFN-γ induced drop in T84 TER (Fig. 3), indicating that PI3-K activation was required for the IFN-γ induced drop in T84 TER. It should be noted that the PI3-K inhibitors did not completely prevent the IFN-γ induced drop in TER, suggesting the possibility that other signaling pathways may also be involved.

PI3-K/NF-κB cross-talk in IFN-γ modulation of T84 TJ permeability

PI3-K has been shown to activate nuclear transcription factor NF-κB (Haller and others 2002). Previous studies from our laboratory have also suggested that NF-κB pathways may have an important regulatory role in the modulation of TNF-α induced increase in Caco-2 TJ permeability (Ma and others 2004). Therefore, in the following studies, we examined the possibility that a cross-talk between PI3-K and NF-κB pathways may be involved in the IFN-γ modulation of T84 TJ barrier function. In quiescent cells, NF-κB is present mostly in the cytoplasm in an inactive form bound to an inhibitory protein IκB (Ma and others 2004). Upon stimulation, IκB are rapidly degraded and NF-κB becomes activated and translocates to the nucleus. The activated NF-κB binds to the DNA-binding site and induces gene expression (Ma and others 2004). The effect of IFN-γ on NF-κB activation in T84 cells was determined by an ELISA-based DNA-binding assay (Boivin and others 2007) and by promoter assay. Following IFN-γ treatment of filter-grown T84 cells, nuclear fraction was isolated and the binding of activated NF-κB to the DNA probe encoding the κB site was determined. IFN-γ caused a progressive increase in NF-κB binding to the DNA probe (Fig. 4A). The maximal binding of NF-κB occurred at ~8 h. There was no significant increase in NF-κB /DNA binding within the first 2 h of IFN-γ treatment (data not shown). To confirm the specificity of NF-κB binding to the DNA probe, wild-type oligonucleotide (WT) containing the consensus κB binding site was added in excess (100-fold higher concentration) as a competitive inhibitor for NF-κB binding. The addition of excess wild-type κB-oligonucleotide prevented the binding of NF-κB to the DNA probe. The addition of excess oligonucleotide containing a mutated NF-κB-binding motif (MT) did not interfere NF-κB binding. These findings confirmed that IFN-γ activated NF-κB specifically binds to the κB site on the DNA probe. In subsequent studies, the effect of IFN-γ on NF-κB dependent promoter activity was determined by transfecting filter-grown T84 cells with a plasmid vector encoding NF-κB-responsive promoter region (linked to a SEAP reporter gene). IFN-γ (10 mg/mL) produced a time-dependent increase in NF-κB promoter activity as determined by the SEAP assay (Fig. 4B). Consistent with the time-course of the NF-κB/DNA binding studies, the maximal increase in SEAP activity occurred at 12 h (~4 h after maximal DNA binding). Next, the effect of NF-κB inhibitor PDTC on IFN-γ-induced drop in T84 TER was examined (Ma and others 2004). PDTC (100 μM) significantly inhibited the IFN-γ-induced activation of NF-κB (Fig. 4C) and the drop in T84 TER (Fig. 4D), indicating that NF-κB activation was also required for the IFN-γ modulation TJ barrier function. In comparison to PI3-K and Akt activation, which occurs within minutes following IFN-γ treatment (Fig. 2), NF-κB activation occurred later and was more prolonged. In the following studies, a possible cross-talk between activation of PI3-K/Akt pathway and NF-κB activation was examined. To determine whether PI3-K activation was required for NF-κB activation, the effect of PI3-K inhibitor LY294002 on IFN-γ induced NF-κB activation was determined. LY294002 pretreatment inhibited the IFN-γ induced increase (Fig. 5A), suggesting that PI3-K activation was required for the increase in NF-κB activity. In contrast, NF-κB inhibitor PDTC did not effect IFN-γ induced activation of PI3-K activity as assessed by Akt phosphorylation (Fig. 5B). Furthermore, addition of a maximal dose of the strong PI3-K agonist EGF induced almost complete activation of AKT (as assessed by the comparison of phospho-AKT to total-AKT by Western blotting) and similarly demonstrated no inhibition by PDTC. These results suggested that a) IFN-γ induced activation of NF-κB was mediated by a cross-talk between PI3-K/Akt and NF-κB pathways and b) NF-κB inhibition does not inhibit PI3-K activation and c) PDTC does not nonspecifically inhibit the PI3-K pathway.

IFN-γ–induced downregulation of occludin protein expression is mediated by PI3-K activation

Previous studies have shown that IFN-γ causes a significant decrease in occludin protein expression (Youakim and

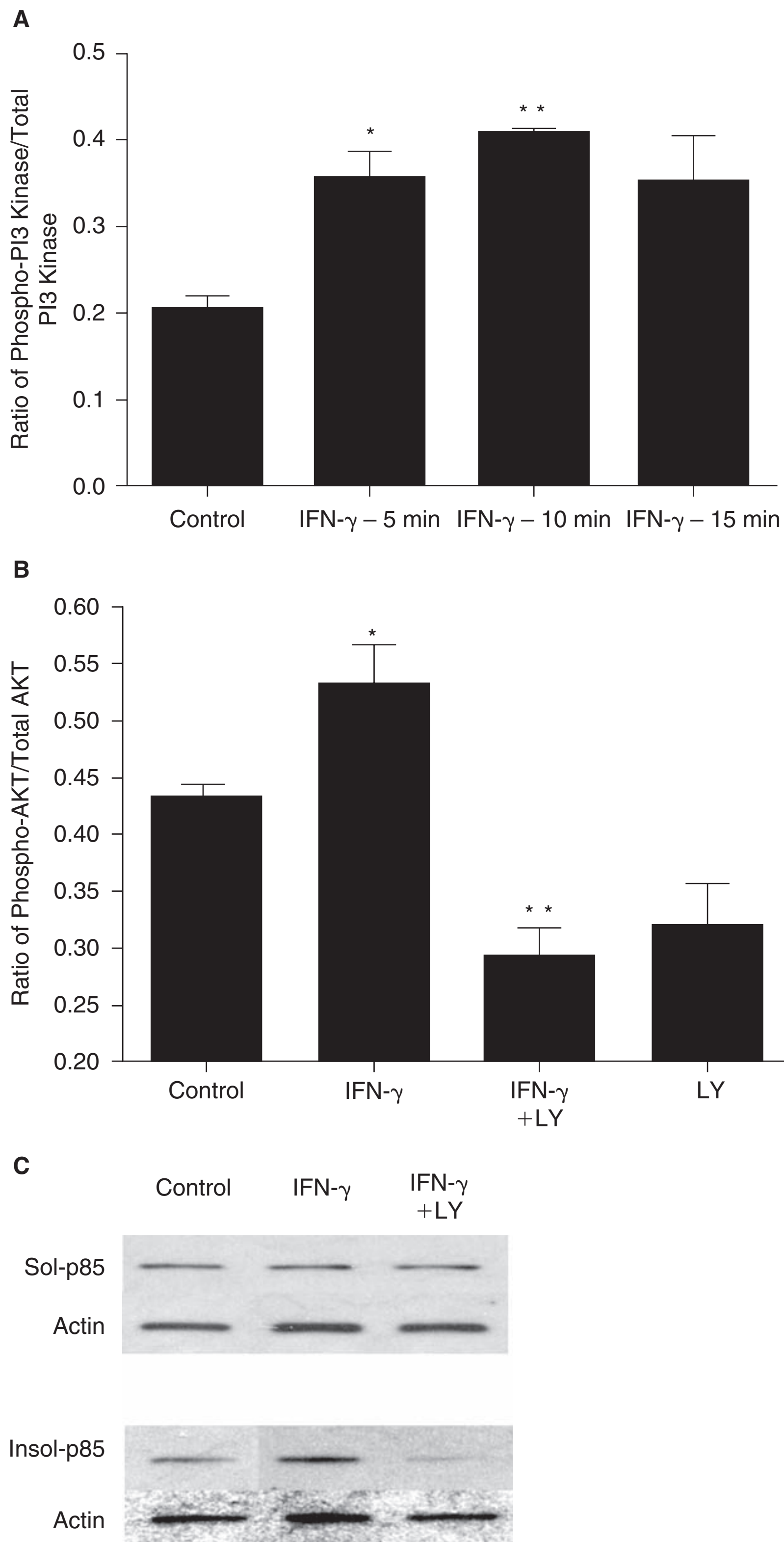

FIG. 2. Effect of IFN-γ on PI3-kinase (PI3-K) and Akt activation. (**A**) IFN-γ (10 ng/mL) caused a significant increase in the ratio of phosphorylated PI3 kinase enzyme to total PI3-K enzyme as measured by FACE at 5 and 10 min (*$p < 0.05$ compared to control). (**B**) IFN-γ (10 ng/mL) caused a significant increase in the ratio of phosphorylated Akt enzyme to total-Akt enzyme as measured by FACE. Addition of LY294002 (20 μM) inhibited Akt phosphorylation to levels below control (*$p < 0.05$ compared to control, **$p < 0.05$ compared to interferon-γ). LY treatment alone also caused a decrease in baseline phosphorylated Akt. (**C**) Filter-grown T84 monolayers were treated with IFN-γ (10 ng/mL) for 5 min with or without LY-294002 (20 μM). PI3-K p85 subunit expression in the soluble and insoluble fractions of T84 cell lysate as determined by Western blot analysis was used as an indirect indication of cytoplasmic-to-membrane translocation. Simultaneously run β-actin blots serve as loading control. IFN-γ treatment (10 μg/mL) caused a rapid increase in PI3-K p85 subunit translocation to the insoluble fraction. The PI3-K inhibitor, LY 294002 (20 μM), prevented the PI3-K translocation to the insoluble fraction.

Ahdieh 1999; Sugi and others 2001; Watson and others 2005). In the following studies, we examined the possible involvement of PI3-K and NF-κB pathways in IFN-γ induced decrease in occludin protein expression. Similar to previous reports, IFN-γ caused a decrease in occludin expression (Fig. 6A). Pretreatment with PI3-K inhibitor (LY294002) inhibited the IFN-γ induced decrease in occludin expression (Fig. 6A). Similarly, NF-κB inhibitor PDTC also inhibited the decrease in occludin expression (Fig. 6B). These results suggested that the IFN-γ induced decrease in occludin protein

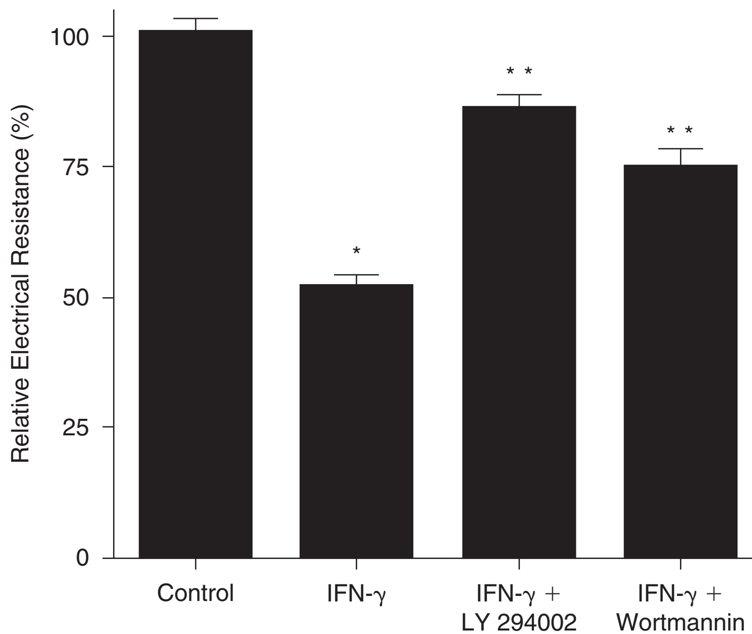

FIG. 3. Effect of PI3-K inhibitors on the IFN-γ induced drop in T84 epithelial resistance. LY294002 (20 μM) and Wortmannin (100 μM) significantly prevented the IFN-γ (10 ng/mL) induced drop in T84 epithelial resistance at 48 h ($n = 4$). *$p < 0.001$ versus controls. **$p < 0.001$ versus IFN-γ-treated monolayers.

was also dependent in-part on PI3-K and NF-κB pathways. It is of interest to note that IFN-γ effect on downregulation of occludin protein expression was associated with an increase in claudin-1 expression and a decrease in claudin-2 expression (Fig. 6C and D).Claudin-3 expression was not affected (data not shown).

Discussion

IFN-γ is a potent proinflammatory cytokine that is markedly elevated in inflammatory bowel disease including Crohn's disease and ulcerative colitis (Breese and others 1993; Reinecker and others 1993; Niessner and Volk 1995; Fuss and others 1996). Recent clinical studies have shown that anti-IFN-γ antibody is effective in the treatment of patients with severe active Crohn's disease (Hommes and others 2006). Previous studies have demonstrated that an important proinflammatory action of IFN-γ is to induce a disturbance in intestinal epithelial TJ barrier function, leading to an increase in paracellular permeation of toxic luminal antigens (Madara and Stafford 1989; Adams and others 1993; Youakim and Ahdieh 1999; Bruewer and others 2003; Willemsen and others 2005). Although the intestinal TJ barrier disruptive action of IFN-γ is well-established (Madara and Stafford 1989; Youakim and Ahdieh 1999; Bruewer and others 2003; Watson and others 2004; Utech and others 2005; Willemsen and others 2005), the intracellular pathways that mediate the IFN-γ effect on intestinal TJ permeability remain unclear.

In the present study, we examined the role of PI3-K in IFN-γ induced increase in intestinal epithelial TJ permeability using filter-grown T84 monolayers (Bruewer and others 2003; Ma and others 2004; Bruewer and others 2005). Our results indicated that IFN-γ causes an early activation of PI3-K (within minutes of IFN-γ treatment) (Fig. 2). The IFN-γ induced PI3-K activation was also associated with activation of Akt, a known target of PI3-K. Subsequently, IFN-γ caused a delayed activation of NF-κB (Fig. 4). The time-course and inhibitor studies demonstrated a cross-talk between PI3-K and NF-κB pathways in the modulation of TJ barrier. Our studies indicated a sequential activation of PI3-K/Akt and NF-κB pathways, and showed that PI3-K/Akt activation was required for the activation of NF-κB pathways. It's important to note that although PI3-K inhibition prevented the NF-κB activation; NF-κB inhibition did not affect the IFN-γ induced activation of PI3-K/Akt pathway (Fig. 5). In combination, our data indicated that IFN-γ activates PI3-K/Akt pathways which, in turn, leads to the activation of NF-κB and NF-κB dependent opening of the TJ barrier. This is the first study to show that IFN-γ modulation of TJ barrier is mediated by a cross-talk between PI3-K and NF-κB pathways. The involvement of PI3-K/NF-κB cross-talk in the regulation of TJ barrier also had not been previously reported in other models of TJ regulation. Of interest is that the IFN-γ induced NF-κB activation demonstrated in this study is delayed (beginning at 4 h) and prolonged (persisting over 24 h) in comparison to the induction by other proinflammatory cytokines such as TNF-α or IL-1β. As we have previously shown TNF-α induced activation of NF-κB occurs almost immediately following TNF-α treatment and is not mediated by PI3-K pathway (Ma and others 2004). In this regard, the mechanism mediating the IFN-γ activation of NF-κB appears to be quite distinct from that involved in TNF-α or IL-1β (Ma and others 2004; Al-Sadi and Ma 2007). It is also interesting to note that the TNF-α effect on intestinal TJ barrier is mediated by NF-κB dependent activation of MLCK gene and protein expression, which leads to the TJ barrier opening (Ma and others 2005). In contrast, IFN-γ induced activation of NF-κB did not affect MLCK gene or protein expression (unpublished data). Thus, it appears that IFN-γ effect on TJ barrier is mediated by a distinct mechanism from that of TNF-α. Further studies are needed to fully delineate the intracellular mechanisms that mediate NF-κB modulation of TJ barrier following IFN-γ stimulation.

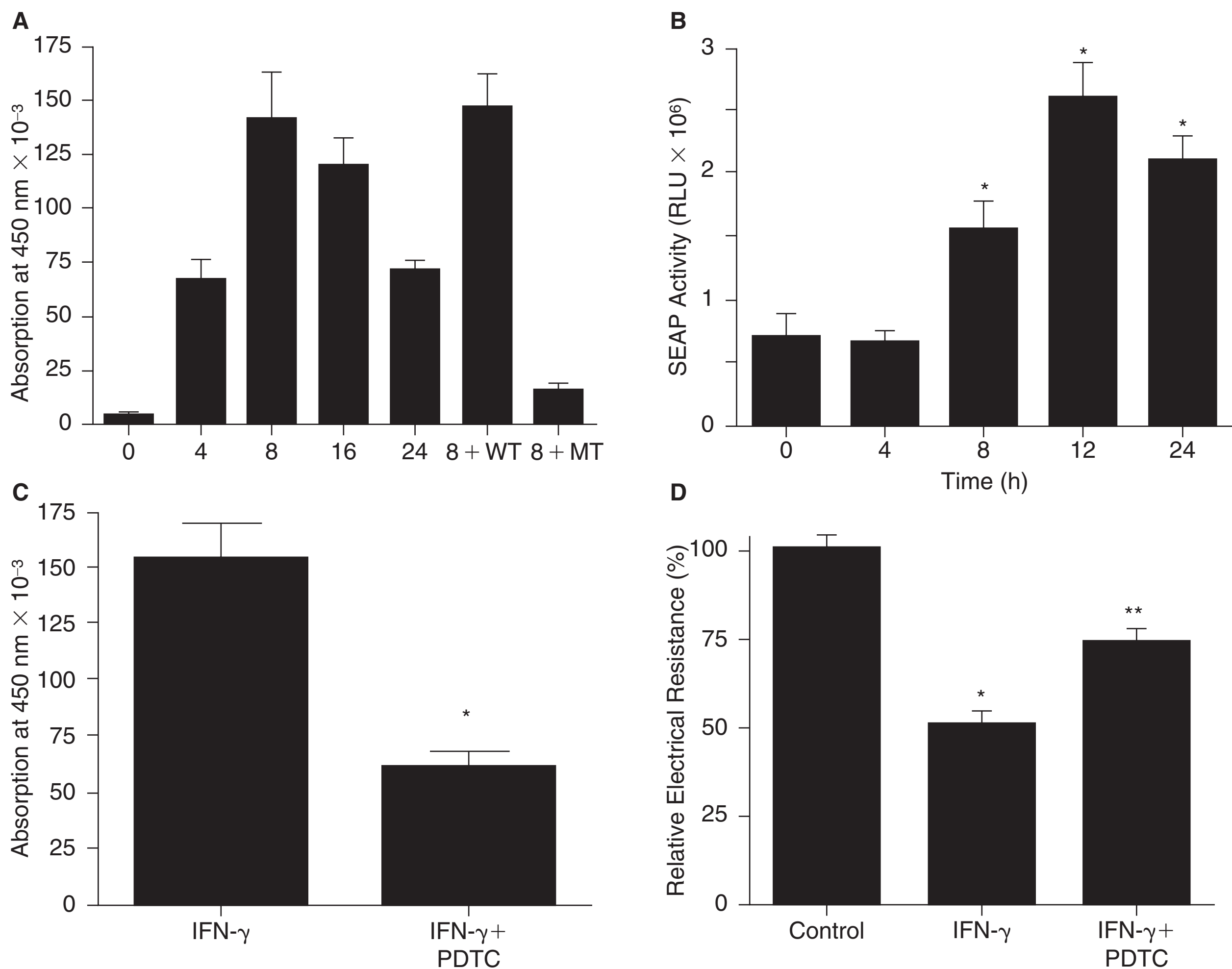

FIG. 4. (**A**) Effect of IFN-γ on NF-κB activation as assessed by NF-κB p65 binding the κB DNA-binding site using ELISA-based DNA-binding assay. IFN-γ (10 μg/mL) caused a time-dependent increase in NF-κB p65 DNA binding, with the maximal binding at 8 h. The increase in IFN-γ induced DNA-binding was inhibited by the addition of excess free "cold" κB-binding site containting oligonucleiotide (WT) to IFN-γ treated samples for 8 h. The addition of excess oligonucleiotide containing a mutated κB-binding site had no effect on DNA binding (MT). (**B**) The effect of IFN-γ on NF-κB-responsive promoter activation. IFN-γ treatment (10 ng/mL) of transfected T84 monolayers resulted in an increase in NF-κB responsive SEAP reporter activity after 8 h, and the increase in activity persisted for more than 24 h. (**C**) NF-κB inhibitors prevent the IFN-γ induced increase in NF-κB DNA binding. IFN-γ treatment induces increased p65 DNA-binding that is inhibited by the addition of PDTC (100 μM). (**D**) Effect of the NF-κB inhibitor, PDTC, on IFN-γ-induced drop in T84 epithelial resistance. PDTC (100 μM) significantly inhibited the IFN-γ (10 ng/mL)-induced drop in T84 epithelial resistance ($n = 4$). *$p < 0.01$ versus controls. **$p < 0.01$ versus IFN-γ-treated monolayers.

Based on the time-course studies, it is highly unlikely that PI3-K/Akt directly activates NF-κB, but requires involvement of other protein kinases or mediators that eventually lead to NF-κB activation. Another possibility is that IFN-γ may lead to secretion of other cytokines that in turn lead to the activation of NF-κB.

In support of PI3-K pathway involvement in the regulation of the intestinal TJ barrier function, previous studies have suggested that IL-4 and IL-13 induced increase in T84 TJ permeability was also mediated by an activation of PI3-K pathways (Ceponis and others 2000; McKay and others 2000). In the T84 monolayers, the IL-4 and IL-13 induced increase in TJ permeability was preceded by an activation of PI3-K pathway and inhibition of PI3-K activation with PI3-K inhibitors prevented the increase in T84 TJ permeability (Ceponis and others 2000). The role of NF-κB pathways in the modulation of intestinal TJ barrier function has also been well-demonstrated in Caco-2 intestinal cells (Ma and others 2004, 2005). Previous studies from several laboratories have indicated that the TNF-α, TNF-α/IFN-γ combination, or IL-1β induced increase in Caco-2 TJ permeability was mediated by NF-κB activation (Ma and others 2004, 2005). The depletion of NF-κB in Caco-2 cells via siRNA induced mRNA silencing completely inhibited the TNF-α induced increase in TJ permeability (Ye and others 2006).

Previous studies have suggested that IFN-γ induced increase in T84 TJ permeability was related in-part by a decrease in occludin protein expression (Youakim and Ahdieh

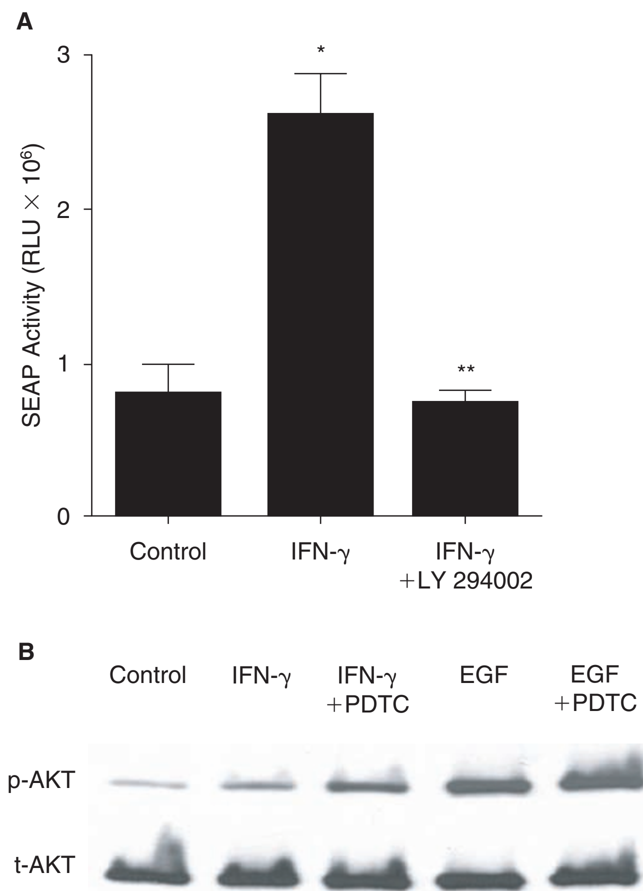

FIG. 5. Cross-talk between PI3-kinase and NF-κB pathways (**A**) The PI3-K inhibitor LY294002 (20 μM) inhibited the IFN-γ-induced increase in NF-κB-responsive promoter activity. $^{*}p < 0.01$ versus controls. $^{**}p < 0.01$ versus IFN-γ-treated monolayers. (**B**) Western blotting of phospho and total-AKT (60 kDA). IFN-γ (10 ng/mL) and EGF treatment (400 ng/mL) caused significant increases in p-AKT levels as compared to controls. Cotreatment with PDTC (100 μM) did not inhibit the increased p-AKT levels in either case. Reprobing for Total-AKT levels are presented as a loading control.

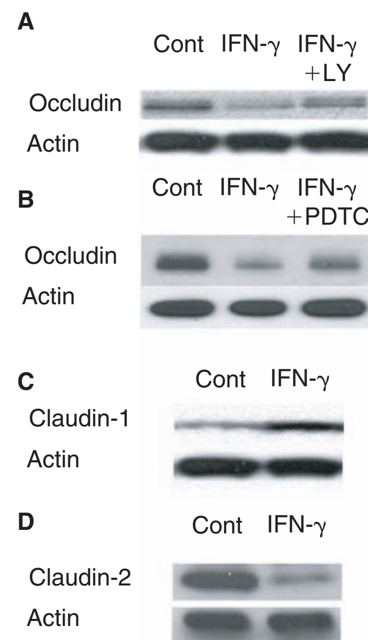

FIG. 6. Effect of IFN-γ on occludin protein expression in T84 monolayers. Filter-grown T84 monolayers were treated with vehicle, IFN-γ (10 ng/mL) or IFN-γ and inhibitors for a 48-h experimental period. Protein expression was determined by Western blot analysis. IFN-γ caused a downregulation of occludin protein expression. IFN-γ induced a downregulation of occluding (68 kDa) protein expression was inhibited by (**A**) the PI3-K inhibitor LY294002 (20 μM) and (**B**) NF-κB inhibitor PDTC (100 μM) . (**C**) IFN-γ treatment (10 ng/mL) induced a significant increase in the expression of Claudin-1 (22 kDa). (**D**) IFN-γ treatment (10 ng/mL) induced a significant decrease in Claudin-2 expression (22 kDa). Simultaneously run β-actin (45 kDa) blots are presented as a loading control for each experiment.

1999; Sugi and others 2001; Watson and others 2005). The IFN-γ induced alteration in TJ protein expression has been implicated as a mechanisms contributing to the disturbance in TJ barrier function (Sugi and others 2001; Kaur and others 2005; Watson and others 2005). However, the intracellular mechanisms involved in TNF-α induced downregulation of occludin protein remain unclear. Our data suggested that the IFN-γ induced decrease in occludin protein expression also required activation of PI3-K and NF-κB pathways. Consistent with our data, a direct binding of PI3-K and occludin protein has been previously demonstrated by capture of PI3-K using a coiled domain of occludin peptide (Nusrat and others 2000). In these elegant studies, using a 27 amino acid occludin sequence as a bait peptide, Nusrat and others (2000) showed that PI3-K binds to the C-terminal region of occludin. Rao and coworkers, using glutathione S-transferase pull-down assay and occludin co-immunoprecipitation also showed direct binding of PI3-K to the C-terminal end of occludin (Sheth and others 2003). They reported that the oxidant induced increase in Caco-2 TJ permeability correlated with an increase in PI3-K activation and increase in PI3-K/occludin binding, and suggested a direct role for PI3-K in the regulation of the TJ barrier function and interaction with occludin protein (Sheth and others 2003). The NF-κB downregulation of occludin expression has also been suggested in astrocytes, where the TNF-α induced decrease in occludin expression was inhibited by NF-κB inhibitors (Ye and others 2006).

In conclusion, our data show for the first time that the IFN-γ induced increase in T84 intestinal epithelial TJ permeability is regulated by a cross-talk between PI3-K and NF-κB pathways. Our data suggest that IFN-γ induced activation of PI3-K leads to a sequential activation of Akt and NF-κB pathways and NF-κB-dependent increase in T84 TJ permeability. The activation of PI3-K and NF-κB pathways was also required for the IFN-γ induced depletion of occludin protein in T84 monolayers. These studies provide new insight into the intracellular pathways that mediate the IFN-γ induced disturbance of intestinal TJ barrier function.

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