

# MAP kinases contribute to IL-8 secretion by intestinal epithelial cells via a posttranscriptional mechanism

HUMBERTO B. JIJON,<sup>1</sup> WILLIAM J. PANENKA,<sup>1</sup>  
KAREN L. MADSEN,<sup>2</sup> AND HOWARD G. PARSONS<sup>1</sup>

<sup>1</sup>Gastrointestinal Research Group, University of Calgary, Calgary T2N 4N1; and <sup>2</sup>Division of Gastroenterology, University of Alberta, Edmonton, Alberta, Canada T6G 2C2

Received 5 March 2001; accepted in final form 15 January 2002

**Jijon, Humberto B., William J. Panenka, Karen L. Madsen, and Howard G. Parsons.** MAP kinases contribute to IL-8 secretion by intestinal epithelial cells via a posttranscriptional mechanism. *Am J Physiol Cell Physiol* 283: C31–C41, 2002. First published February 6, 2002; 10.1152/ajpcell.00113.2001.—The intracellular pathways that regulate intestinal epithelial gene expression are poorly understood. In this study we examined the roles of extracellular signal-regulated kinase (ERK) and p38 in the expression of interleukin-8 (IL-8) and intercellular adhesion molecule-1 (ICAM-1) using the human intestinal cell line HT-29. HT-29 cells were treated with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the presence or absence of ERK and p38 pathway inhibitors. TNF- $\alpha$  treatment resulted in increased IL-8 and ICAM-1 protein and mRNA synthesis, increased ERK and p38 activity, and activation of the transcription factors activator protein-1 (AP-1) and nuclear factor- $\kappa$ B (NF- $\kappa$ B). Inhibition of the ERK and p38 pathways attenuated IL-8 secretion but did not alter ICAM-1 expression. Furthermore, AP-1 and NF- $\kappa$ B DNA binding was not affected by ERK and p38 inhibition. In contrast, ERK and p38 inhibition resulted in the accelerated degradation of the IL-8 mRNA, suggesting that in HT-29 cells, p38 and ERK contribute to TNF- $\alpha$ -stimulated IL-8 secretion by intestinal epithelial cells via a posttranscriptional mechanism that involves stabilization of the IL-8 transcript.

enterocyte; intestinal inflammation; signal transduction; mRNA stability

INTESTINAL EPITHELIAL CELLS (IECs) are critical to the barrier and absorptive functions of the gastrointestinal tract. In addition, a growing body of evidence suggests that these cells, which line the luminal surface of the gut, act as sentinels of the mucosal immune system. IECs express numerous receptors, adhesion molecules, and proinflammatory mediators that allow IECs to communicate with the immune system. Consequently, IECs undergo functional changes, which contribute to many pathological features of intestinal inflammation (14, 25).

An important regulator of epithelial function during inflammation is the cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Its levels are elevated in both human inflam-

matory bowel diseases and animal models of intestinal inflammation. Furthermore, therapies that target TNF- $\alpha$  may provide promising new alternatives for the treatment of intestinal inflammation (41). Importantly, TNF- $\alpha$  induces profound changes in epithelial function, including alterations in permeability (32), cell cycle progression (9, 26), nutrient absorption (33), and gene expression (45). However, the intracellular events that underlie these changes are poorly understood.

A consequence of TNF- $\alpha$  stimulation in numerous cell types is the activation of mitogen-activated protein kinases (MAPKs). At least three distinct groups of MAPKs have been identified in mammals, including the extracellular signal-regulated kinases (ERKs), the c-Jun NH<sub>2</sub>-terminal kinases (JNKs), and p38. Of these, the most extensively studied are the ERKs, which are acutely stimulated by growth and differentiation factors, heterotrimeric G protein-coupled receptors, and cytokine receptors. JNKs and p38 family members are generally implicated in responses to cellular stress, inflammation, and apoptosis (6, 30). MAPK activation has been shown to result from dual phosphorylation on threonine and tyrosine residues by an upstream kinase termed a MAPK kinase (MKK). ERK, JNK, and p38 family members are activated by MKK1/2, MKK4/7, and MKK 3/6, respectively. MKKs, in turn, are activated by MKK kinases (6, 30). Although the roles the ERKs play during epithelial proliferation and differentiation are well established (1, 9), little is known about the role of these kinases in IECs in the context of inflammation.

In this study, we hypothesized that activation of MAPKs contributes to TNF- $\alpha$ -elicited changes in proinflammatory gene expression in IECs. In particular, we focused on the secretion of interleukin 8 (IL-8) and the expression of intercellular adhesion molecule-1 (ICAM-1), which are known to play important roles in the recruitment of circulating inflammatory cells (35).

TNF- $\alpha$  treatment of HT-29 cells leads to the rapid activation of MAPK pathways as well as ICAM-1 and IL-8 expression. However, in contrast to ICAM-1, TNF- $\alpha$ -stimulated IL-8 secretion was potently suppressed

Address for reprint requests and other correspondence: H. Jijon, Division of Gastroenterology, Dept. of Medicine, Gastrointestinal Research Unit, 519 Newton Bldg., Univ. of Alberta, Edmonton, AB, Canada T6G 2C2 (E-mail: hjijon@ualberta.ca).

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by treatment with ERK and p38 inhibitors. Importantly, we provide evidence that the ERK and p38 pathways contribute to IL-8 secretion via a posttranscriptional mechanism involving the stabilization of the IL-8 transcript.

## MATERIALS AND METHODS

**Materials.** Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO).

**Cell culture.** HT-29 and T84 cells were obtained from American Type Culture Collection (Rockwell, MA) and cultured in RPMI 1640 and F-12/DMEM media, respectively (GIBCO, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal calf serum (FCS; Cansera, Rexdale, ON, Canada), 2 mM glutamine, 1 mM sodium pyruvate, 2% sodium bicarbonate, and 10 mM HEPES. Cells were grown in 12-well tissue culture plates (Falcon) for the determination of IL-8 protein, 6-well plates for measurement of MAPK activity and Western blotting, and 25-cm<sup>2</sup> flasks for the determination of IL-8 mRNA by RT-PCR.

Confluent monolayers (passage 25–45) were incubated with human recombinant TNF- $\alpha$  (10 ng/ml; R&D Systems, Minneapolis, MN) in the presence or absence of various concentrations of the ERK pathway inhibitor PD-98059 (11), the nonspecific MAPK inhibitor apigenin (29), the p38 inhibitor SB-203580 (Calbiochem, San Diego, CA) (7), or the nuclear factor- $\kappa$ B (NF- $\kappa$ B) inhibitor MG132 (Calbiochem) (37). Cells were treated with MAPK inhibitors for 30 min before treatment with TNF- $\alpha$ . TNF- $\alpha$  (10 ng/ml) was chosen from dose-response experiments because this concentration gives a maximal response (data not shown). Control monolayers were treated with an equal volume of vehicle (DMSO). No changes in total de novo protein synthesis were detected by [<sup>35</sup>S]methionine labeling following treatment with TNF- $\alpha$  for 3 h in the presence or absence of various inhibitors (data not shown). Likewise, no cytotoxicity was detected with the lactate dehydrogenase release assay (data not shown). Before experiments designed to measure MAPK activation were performed, cells were incubated in serum-free media overnight to reduce growth factor-mediated MAPK activation. All experiments were conducted in serum-free media.

**Determination of IL-8 protein.** In experiments in which IL-8 secretion was measured, epithelial monolayers were stimulated with 10 ng/ml TNF- $\alpha$  for 3 h. IL-8 protein in supernatants was measured via ELISA as follows: 96-well Maxisorp ELISA plates (Nunc, Rochester, NY) were coated with 4  $\mu$ g/ml capture monoclonal anti-IL-8 antibody (R&D Systems) in PBS (pH 7.4) overnight. Plates were then blocked overnight (5% sucrose, 0.05% sodium azide, and 1% BSA in PBS, pH 7.4). Plates were washed four times between all steps with 0.05% Tween 20 in PBS, pH 7.4. Samples (100  $\mu$ l) and standards (0–4,000 pg/ml human recombinant IL-8; R&D Systems) were incubated in the plates overnight. Biotinylated polyclonal anti-IL-8 antibody (R&D Systems) was added (20 ng/ml in PBS, pH 7.4), and plates were incubated for 2 h. Streptavidin-horseradish peroxidase (HRP, 100  $\mu$ l; Southern Biotechnology Associates, Birmingham, AL) was added for 1 h, followed by development with 100  $\mu$ l of soluble 3,3',5,5'-tetramethylbenzidine (Calbiochem). The reaction was stopped with acid (0.5 M H<sub>2</sub>SO<sub>4</sub>), and plates were read immediately at 450 nm with an ELISA plate reader (UV max; Molecular Devices, Sunnyvale, CA). All steps were carried out at room temperature. ELISA was sensitive to <30 pg/ml.

**Measurement of mRNA.** Total RNA was isolated from HT-29 monolayers grown in 25-cm<sup>2</sup> flasks with the use of Trizol RNA isolation reagent (GIBCO) following manufactur-

er's instructions. mRNA (2  $\mu$ g) was reverse transcribed and amplified with the polymerase chain reaction (PCR) by using the "primer dropping" method as described by Wong et al. (44). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers were included in the reactions as endogenous internal standard to control for variations in product abundance due to differences in individual reverse transcription and PCR reaction efficiencies. PCR products were visualized and quantified following agarose gel electrophoresis and ethidium bromide staining by using a digital camera (Geldoc Imaging System, Bio-Rad, Hercules, CA). Densitometry measurements are expressed as the ratios of ICAM-1 or IL-8 mRNA to GAPDH. Comparisons were made only when samples were isolated, amplified, and run together in the same gel. Multiple exposures were compared and measurements taken at intensities below pixel saturation. For measurement of mRNA decay, HT-29 cells were grown in six-well plates and treated with 10 ng/ml TNF- $\alpha$  for 1 h to allow new mRNA synthesis. Monolayers were then treated with the mRNA synthesis inhibitors actinomycin D (5  $\mu$ g/ml) or  $\alpha$ -amanitin (2  $\mu$ g/ml) in the presence or absence of PD-98059 (25  $\mu$ M) or SB-203580 (10  $\mu$ M). Total RNA was isolated at 30-min intervals following mRNA synthesis inhibition for up to 3 h as described above. Comparisons were drawn only among samples isolated and amplified together and run in the same gel. Actin was used as the internal control in these experiments because subtle changes in GAPDH mRNA decay were observed following treatment with MAPK inhibitors (data not shown).

The human IL-8 and ICAM-1 sequences were obtained from GenBank and used to design the primer pairs. The primers chosen hybridize to genomic regions separated by at least one intron. The primer sequences used for PCR are as shown in Table 1.

**Quantitative measurement of mRNA.** Quantitative measurement of mRNA was conducted by using a commercial mRNA ELISA (R&D Systems) following manufacturer's instructions. Total RNA (10  $\mu$ g) was used for analyses and compared with authentic in vitro-transcribed IL-8 mRNA standards. Concentrations were determined spectrophotometrically with a nucleic acid calculator (GeneQuant, Biochrom, Cambridge, UK).

**SDS-PAGE.** For experiments involving the measurement of phospho-ERK, -JNK, and -p38, confluent HT-29 cells were incubated overnight in serum-free media to reduce background activity. HT-29 monolayers were stimulated with 10 ng/ml TNF- $\alpha$  in the presence or absence of inhibitors and harvested in Mono Q buffer (1.08 g of  $\beta$ -glycerophosphate, 38.04 mg of EGTA, 0.5 ml of Triton X-100, and 200  $\mu$ l of 1 M MgCl<sub>2</sub> per 100 ml) at different times. After sonication for 30 s, samples were centrifuged at 12,000 rpm for 1 min to remove insoluble material and protein concentrations were

Table 1. PCR primer sequences

Primer Name	Sequence
IL-8 forward primer	5' TCAGAGGTCAGACTTGGTGTCTCT 3'
IL-8 reverse primer	5' CACACAGTGAGAATGGTTCTCTCC 3'
ICAM-1 forward primer	5' GGCAAGAACCTTACCCTACG 3'
ICAM-1 reverse primer	5' GAGACCTCTGGCTTCGTACG 3'
GAPDH forward primer	5' AGCCTTCTCCATGGTGGTGAAGAC 3'
GAPDH reverse primer	5' CGGAGTCAACGGATTGGTTCGTAT 3'
Actin forward primer	5' CGTGGGCGCCCTAGGCACCA 3'
Actin reverse primer	5' TTGGCCTTAGGGTTCAGGGGGG 3'

IL-8, interleukin-8; ICAM-1, intercellular adhesion molecule-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



determined with a commercial Lowry assay (Bio-Rad) by using BSA standards in Mono Q buffer. Lysate concentrations were adjusted to ensure even protein loading, mixed with an equal volume of 2× protein sample buffer [130 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 5%  $\beta$ -mercaptoethanol, trace bromphenol blue, 4 mM sodium orthovanadate (Calbiochem), and 2  $\mu$ M microcystin (Calbiochem)], boiled for 2 min, and separated via electrophoresis (10% acrylamide gels, 100  $\mu$ g protein/lane).

**Western blotting.** After electrophoresis, proteins were transferred for 1.5 h at 400 mA in transfer buffer (25 mM Tris-base, 150 mM glycine, and 10% methanol) onto a polyvinylidene difluoride membrane (Millipore). Membranes were blocked for 1 h with 3% skim milk and incubated overnight in primary antibody. The antibodies used were as follows: anti-ERK-1 (1:3,000, rabbit; Upstate Biotech, Lake Placid, NY), anti-phospho-ERK1/2 (1:1,000, rabbit; New England Biolabs, Beverly, MA), anti-p38 (1:1,000, rabbit; New England Biolabs), anti-phospho-p38  $\alpha/\beta$  (1:1,000, rabbit; New England Biolabs), and anti-phospho-JNK1/2 (1:500, mouse monoclonal; New England Biolabs). Secondary staining was conducted with HRP-conjugated goat sera specific for mouse or rabbit Ig as required (1:3,000; Amersham, Baie d'Urfe, QC, Canada), followed by chemiluminescent detection with a commercial reagent following manufacturer's instructions (Lumilight; Roche, Laval, QC, Canada). Comparisons were made only among samples isolated and transferred together onto the same membrane. Multiple exposures were done to ensure that film was not overexposed. To confirm equal loading of protein, all Western blots using phospho-specific antibodies were stripped and reprobed with antibody against the nonphosphorylated kinase or anti-ERK.

**Electrophoretic mobility shift assays.** HT-29 cells were grown in six-well plates and stimulated with TNF- $\alpha$  in the presence or absence of MAPK inhibitors for various times. Nuclear extracts were prepared as follows. Media were aspirated and monolayers were harvested on ice by scraping after 15-min incubation in hypotonic lysis buffer [10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.625% Nonidet P-40, 3 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1  $\mu$ g/ml leupeptin, and 20  $\mu$ g/ml aprotinin]. Lysates were centrifuged for 1 min at 15,000 rpm, and the cytosolic fraction was aspirated. The nuclear pellet was resuspended in hypertonic buffer (20 mM HEPES, 0.42 M NaCl, 5 mM EDTA, 10% glycerol, 5 mM DTT, and 1 mM PMSF) and incubated at 4°C for 30 min. Samples were then centrifuged at 15,000 rpm for 10 min, and nuclear extracts were aliquoted and frozen in liquid nitrogen. These were stored at -70°C until assayed. An aliquot was used for protein determination as described in SDS-PAGE using BSA standards solubilized in hypertonic buffer. Nuclear protein (20  $\mu$ g) was preincubated with reaction buffer (1 mM EDTA, 100 mM NaCl, 10 mM Tris·HCl, 1 mM MgCl<sub>2</sub>, 4% glycerol vol/vol, and 1 mM DTT) for 20 min at room temperature before incubation with <sup>32</sup>P-labeled oligonucleotides. Labeling of oligonucleotides [NF- $\kappa$ B, 5'-AGTTGAGGG-GACTTTCAGGC-3'; activator protein-1 (AP-1), 5'-CGCTTGATGATCCAGCCGAA-3' (Santa Cruz Biotechnology, Santa Cruz, CA)] was performed using T4 polynucleotide kinase (GIBCO) following manufacturer's instructions. Nuclear extracts were incubated at room temperature with labeled oligonucleotides and were separated by electrophoresis in a 6% TBE (Tris base, boric acid, EDTA) acrylamide gel. Gels were dried for 1 h in a gel drier (Bio-Rad), and autoradiography was conducted at -70°C with Kodak X-OMAT film. Electrophoretic mobility shift assays (EMSAs) were also scanned with a Storm 850 PhosphorImager (Molecular Dy-

namics, Sunnyvale, CA). The NF- $\kappa$ B:DNA complex was found to contain p65 by supershifting with a p65-specific antibody (Upstate Biotech, Saranac, NY), and the specificity of the oligonucleotide was confirmed by competition with excess unlabeled probe. Likewise, AP-1-containing complexes were supershifted by anti-Fos (Santa Cruz Biotechnology) and, to a lesser extent, by anti-pan Jun (Santa Cruz Biotechnology) and were competed by excess unlabeled oligonucleotide.

**Flow cytometry.** Cells were stimulated with TNF- $\alpha$  (10 ng/ml) for 24 h in the presence or absence of MAPK inhibitors. Although these inhibitors are not likely to be active throughout this time period, ERK and p38 activation are transient events occurring within the first hour, a time frame during which these inhibitors are active (see Fig. 4). Cells were then harvested with trypsin-EDTA (GIBCO) and washed in RPMI supplemented with 0.1% FCS and 5 mM EDTA. Cells were labeled with mouse anti-human ICAM-1 (Becton Dickinson, San Jose, CA) and with goat anti-mouse FITC (Becton Dickinson). Stained suspensions were analyzed by flow cytometry (Becton Dickinson) gating on viable cells (10,000 events). Mean channel fluorescence, which correlates with fluorescence intensity, was determined from the peak of positively stained cells and recorded on a log scale. The number of ICAM-1 surface molecules was determined by calibrating the flow cytometer with the use of identically stained microbeads with different calibrated binding capacities of goat anti-mouse IgG following manufacturer's instructions (Quantum Simply Cellular, Flow Cytometry Standards, San Juan, PR) using commercial software (QuickCal, Flow Cytometry Standards).

**Statistical analysis.** Unless otherwise stated, data provided are from representative experiments with comparable results obtained in additional experiments. Where data are expressed as means  $\pm$  SD, statistical analyses were performed by using statistical software (SigmaStat, Jandel, San Rafael, CA). Differences between means were evaluated by using analysis of variance or paired *t*-tests where appropriate. Specific differences were tested using the Student-Newman-Keuls test. *P* < 0.05 was considered statistically significant.

## RESULTS

**TNF- $\alpha$  stimulates de novo IL-8 and ICAM-1 mRNA and protein synthesis.** Transformed intestinal epithelial cell lines have previously been shown to express IL-8 and ICAM-1 both constitutively and following treatment with TNF- $\alpha$  (10, 12). Because these genes share similar *cis*-acting elements (NF- $\kappa$ B, AP-1, CCAAT/enhancer-binding proteins), it was expected that these genes should be regulated in a similar manner (20, 34). Examination of the kinetics of these events using semiquantitative RT-PCR revealed that the time courses of ICAM-1 and IL-8 mRNA accumulation following treatment with TNF- $\alpha$  are similar (Fig. 1A). However, measurement of ICAM-1 expression on the cell surface by flow cytometry showed that ICAM-1 protein expression is delayed relative to IL-8 secretion (Fig. 1B). A 1.6-fold increase in ICAM-1 expression (representing an increase from  $\sim 5 \times 10^3$  to  $1 \times 10^4$  ICAM-1 molecules/cell) occurred 24 h poststimulus compared with a 28-fold increase (150 pg/ml to 4 ng/ml) in IL-8 secretion observed at 6 h.

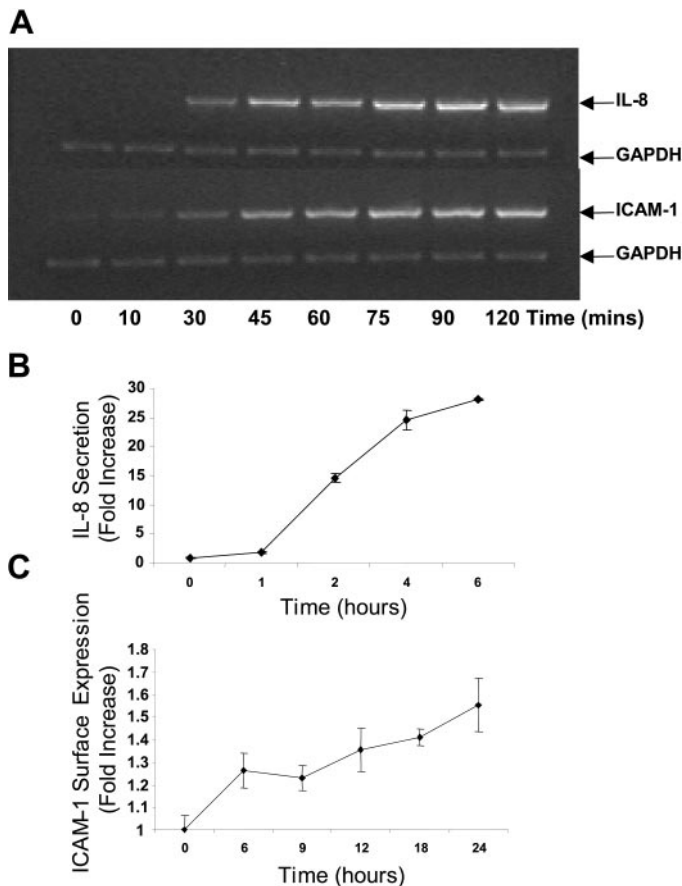


Fig. 1. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) stimulates the de novo accumulation of intercellular adhesion molecule-1 (ICAM-1) and interleukin-8 (IL-8) mRNA. IL-8 and ICAM-1 mRNA accumulation was measured by semiquantitative RT-PCR (A). IL-8 secretion was measured via ELISA (B), and expression of ICAM-1 on the cell surface was measured by flow cytometry (C) as outlined in MATERIALS AND METHODS. Cells were treated with TNF- $\alpha$  (10 ng/ml) for times shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data represent means  $\pm$  SD of 4 independent experiments run in triplicate.

*TNF- $\alpha$  stimulates p38 and ERK1/2 in HT-29 cells.* TNF- $\alpha$  has been shown to activate numerous signaling cascades including the ERKs, JNK, and p38 (31). Activation of these kinases was assayed by using antibodies specific for the phosphorylated, active forms of these kinases by Western blotting. As shown in Fig. 2A, ERK1 and -2 are transiently phosphorylated following treatment with TNF- $\alpha$ , with maximal phosphorylation evident by 15 min and decaying thereafter. The time course of activation is indistinguishable for ERK1 and -2 and consistent with previous observations in IECs (27) (Fig. 2A).

Having observed ERK activation following TNF- $\alpha$  treatment, it was of interest whether other MAPKs were also activated. Figure 2B shows an anti-phospho-p38 blot of TNF- $\alpha$ -treated cells. The phosphorylated form of p38 was evident by 5 min, with signal peaking at 15 min and decaying thereafter. The control Western blot for p38 shows the slow appearance of a doublet corresponding to the active and inactive forms of p38.

Phosphorylation of MAPKs resulted in a slight electrophoretic mobility shift, which often results in the appearance of a second, slightly retarded band. This is confirmed by the phospho-blot showing only a single band representing the phosphorylated form of p38.

Jobin et al. (22) have previously reported the activation of JNK following TNF- $\alpha$  stimulation of IECs. We confirm these findings as shown in Fig. 2C. The kinetics of JNK activation are similar to those observed for ERK, with active JNK evident 15 and 30 min post-stimulation. Two isoforms of JNK, p54 (JNK1) and p46 (JNK2) are evident and correspond to the specificity of the antibody.

*ERK and p38 are required for IL-8 secretion following TNF- $\alpha$  stimulation.* Having shown that p38 and ERK are activated following TNF- $\alpha$  treatment, we inquired whether these kinases are required for TNF- $\alpha$ -elicited proinflammatory gene expression. IL-8 is a well-characterized chemokine with an important role in inflammation and angiogenesis (41). It was therefore of interest to determine whether ERKs and p38 contribute to TNF- $\alpha$ -stimulated IL-8 secretion. Figure 3A shows that treatment of HT-29 cells with the ERK pathway-specific inhibitor PD-98059 30 min before TNF- $\alpha$  treatment resulted in >40% reduction in IL-8 secretion compared with controls ( $P < 0.001$ ). Likewise, treatment of HT-29 cells with the p38-specific inhibitor SB-203580 resulted in an ~30% reduction in IL-8 secretion ( $P < 0.001$ ) (Fig. 3B).

Figure 3C shows the combined effect of simultaneous treatment of HT-29 cells with both PD-98059 and SB-203580. As shown, the effect is additive in that 70%

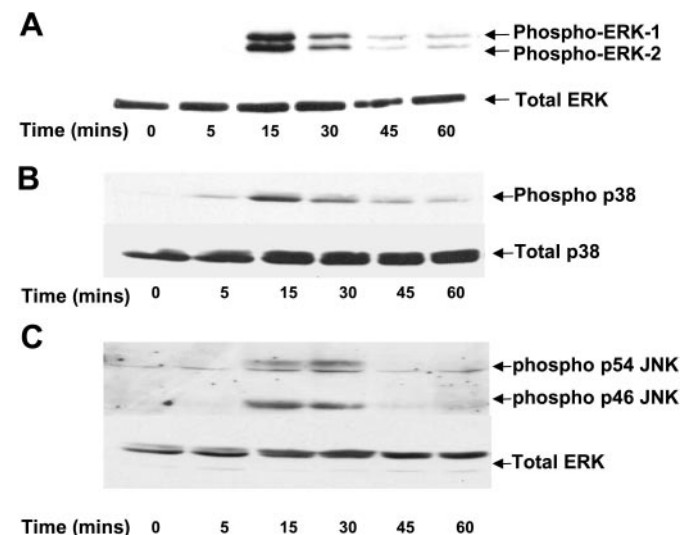


Fig. 2. TNF- $\alpha$  stimulates the extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and c-Jun NH<sub>2</sub>-terminal kinase (JNK) pathways. HT-29 cells were treated with TNF- $\alpha$  (10 ng/ml) for various times, and whole cell extracts were prepared for Western blotting. Extracts were immunoblotted with antibodies specific for the phosphorylated (phospho) forms of ERK (A), p38 (B), and JNK (C). All gels were run at least 3 times, and equal protein loading was confirmed by protein assay and by densitometric analysis of control Western blots by using antibodies specific for the nonphosphorylated (total) forms of these kinases (see MATERIALS AND METHODS).



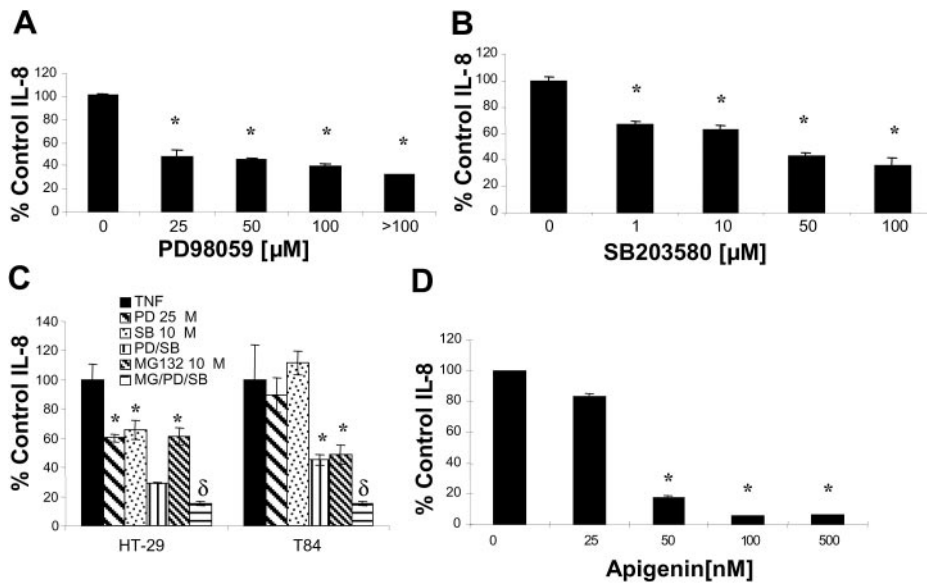


Fig. 3. Inhibition of ERK and p38 attenuate IL-8 secretion in intestinal epithelial cells (IECs). HT-29 cells were treated with various concentrations of the ERK and p38 pathway inhibitors PD-98059 (A;  $*P < 0.001$ ) and SB-203580 (B;  $*P < 0.001$ ) for 30 min before and during TNF- $\alpha$  treatment (10 ng/ml, 3 h). Control monolayers were treated with an equal volume of vehicle (DMSO). C: effects of combined treatments with PD-98059 (25  $\mu$ M) and SB-203580 (10  $\mu$ M) in HT-29 and T84 cells are shown ( $*P < 0.01$ ) as well as the effects of combined treatments with MAPK inhibitors and 10  $\mu$ M MG132 ( $^{\delta}P < 0.001$ ). The effects of a nonspecific MAPK inhibitor apigenin are shown in D ( $*P < 0.01$ ,  $^{\delta}P < 0.001$ ). Data represent means  $\pm$  SE of at least 4 experiments done in triplicate.

inhibition was achieved, highlighting an important role for these pathways in the secretion of IL-8 ( $P < 0.001$ ). To assess the relative contribution of MAPKs to IL-8 secretion relative to the contribution of the NF- $\kappa$ B pathway, we also compared the effects of the NF- $\kappa$ B inhibitor MG132 (37, 38) in the presence or absence of ERK and p38 inhibitors. Complete inhibition of IL-8 secretion was obtained by using 100  $\mu$ M MG132 (data not shown). On the other hand, partial inhibition occurred when 10  $\mu$ M MG132 was used. Interestingly, simultaneous inhibition of ERK and p38 in combination with 10  $\mu$ M MG132 resulted in  $>80\%$  reduction in IL-8 secretion.

We also examined the effects of ERK and p38 inhibitors on the colonic epithelial cell line T84. As shown in Fig. 3C, treatment of T84 monolayers with either 25  $\mu$ M PD-98059 or 10  $\mu$ M SB-203580 did not result in a reduction in IL-8 secretion. However, when used in combination, these drugs attenuated IL-8 secretion by  $\sim 50\%$ . Furthermore, inhibition of ERK and p38, when combined with 10  $\mu$ M MG132, resulted in almost complete ablation of IL-8 secretion (Fig. 3C).

Because at the time this study was conducted no JNK pathway-specific inhibitors were available, we could not assess the contribution of the JNK pathway. However, we achieved greater inhibition of IL-8 secretion using a naturally occurring, nonspecific MAPK inhibitor, apigenin (29). As shown in Fig. 3D, this compound potently inhibited IL-8 secretion at concentrations as low as 50 nM (80%) ( $P < 0.001$ ). This result suggests the participation of other kinases in the regulation of IL-8 secretion.

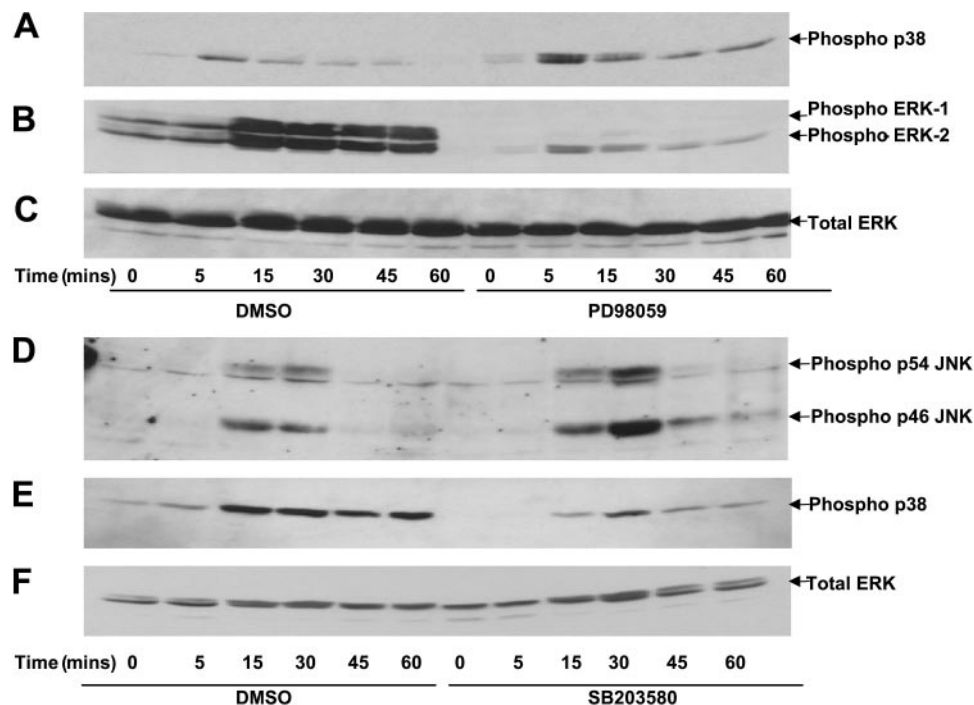
**Evidence for cross talk among the ERK, JNK, and p38 MAPK pathways.** Because simultaneous ERK and p38 inhibition was required in T84 cells to observe a reduction in IL-8 secretion, we asked whether there was a signaling interaction between ERK and p38. For this purpose, cells were treated with either PD-98059 or SB-203580, stimulated for various times with

TNF- $\alpha$ , analyzed for ERK and p38 activation by Western blotting. The results are shown in Fig. 4. Figure 4A shows that treatment with 25  $\mu$ M PD-98059 augments p38 phosphorylation. This is in contrast to the expected reduction in ERK phosphorylation (Fig. 4B). On the other hand, whereas PD-98059 had no effect on JNK phosphorylation, SB-203580 treatment significantly enhanced the phosphorylation of both JNK1 and 2 (Fig. 4D). Figure 4E shows a reduction in p38 phosphorylation in response to SB-203580. ERK phosphorylation was unaffected by this treatment (data not shown). Similar results were obtained using T84 cells (data not shown).

**ICAM-1 surface expression is independent of ERK and p38.** Both IL-8 and ICAM-1 mRNAs are rapidly upregulated in IECs following stimulation with TNF- $\alpha$ . Because of similarities in the kinetics of IL-8 and ICAM-1 mRNA induction and in the structure of the promoters that control these genes, we hypothesized that MAPKs may also regulate the expression of ICAM-1. Figure 5A shows the effect of pretreatment with ERK pathway and p38 inhibitors on ICAM-1 surface expression. Neither PD-98059 nor SB-203580 reduced the number of ICAM-1 molecules expressed on the cell surface compared with vehicle-treated controls. In fact, SB-203580 pretreatment resulted in enhanced ICAM-1 expression ( $P < 0.001$ ). These results are also shown in a conventional flow cytometric histogram on a log scale (Fig. 5, B and C).

**MAPKs are required for IL-8 mRNA accumulation.** We next examined whether ERK and p38 regulated IL-8 expression at the level of transcription. Inhibition of ERK and p38 resulted in decreased IL-8 mRNA accumulation (Fig. 6A;  $P < 0.001$ ). In contrast, ICAM-1 mRNA accumulation was unaffected by either ERK or p38 inhibition (Fig. 6B). IL-8 mRNA was quantified by using a commercial quantitative ELISA, whereas ICAM-1 mRNA was assessed via semiquantitative RT-PCR. At the time this study was conducted, quantita-

Fig. 4. Cross talk between the ERK, JNK, and p38 MAPK pathways. HT-29 cells were treated with TNF- $\alpha$  (10 ng/ml) for times shown in the presence of 25  $\mu$ M PD-98059 or vehicle control (DMSO), and whole cell extracts were prepared for Western blotting. Extracts were immunoblotted with antibodies specific for the phosphorylated forms of p38 (A) and ERK (B). C: a control anti-ERK blot. D–F: effects of an identical experiment where HT-29 cells were instead treated with 10  $\mu$ M SB-203580. Extracts were immunoblotted with antibodies specific for the phosphorylated forms of JNK (D) and p38 (E). F: a control anti-ERK blot. All gels were run at least 3 times, and equal protein loading was confirmed by protein assay.



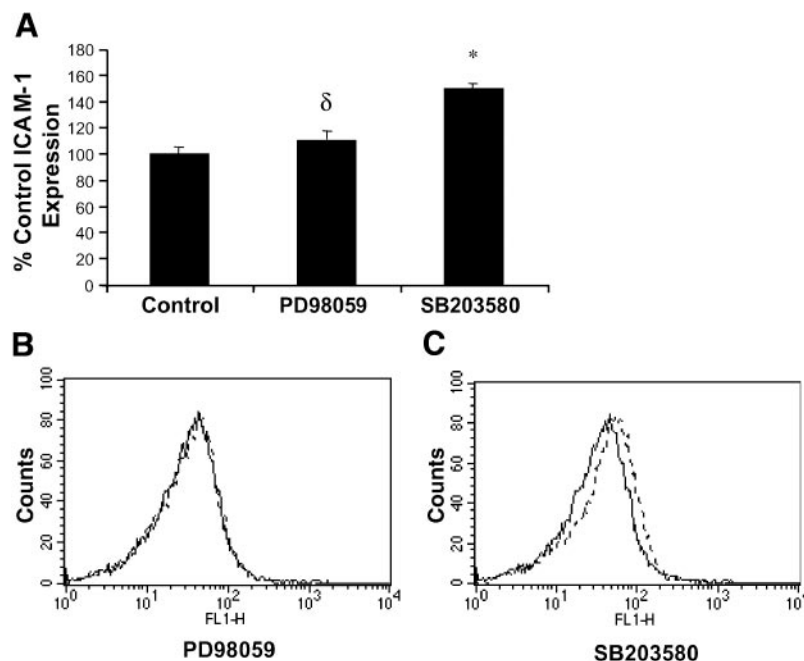
tive ELISA for the measurement of ICAM-1 mRNA was not available. Figure 6B is representative of four separate experiments.

*ERK and p38 contribute to IL-8 secretion independent of NF- $\kappa$ B or AP-1.* Critical to the expression of IL-8 is the activation of the transcription factor NF- $\kappa$ B, a family of ubiquitously expressed homo- and heterodimeric DNA-binding proteins.

Figure 7A shows the time course of NF- $\kappa$ B activation following TNF- $\alpha$  treatment assayed by EMSA. This NF- $\kappa$ B complex was found to contain p65 by using

supershift assays (Fig. 7C). NF- $\kappa$ B activity peaked at 30 min and waned thereafter in a time frame consistent with the activation of ERK and p38 (Fig. 7A). As shown in Fig. 7C, these inhibitors did not affect the NF- $\kappa$ B DNA binding, suggesting that ERK and p38 act independently of NF- $\kappa$ B. The IL-8 promoter also contains a binding site for the transcription factor AP-1 (34). This transcription factor is also subject to regulation by MAPKs (28); therefore, we examined the activation of AP-1 following TNF- $\alpha$  treatment. As shown in Fig. 7B, there is significant constitutive AP-1 DNA

Fig. 5. ERK and p38 inhibition do not abrogate ICAM-1 protein expression following TNF- $\alpha$  treatment. HT-29 cells were treated for 24 h with 10 ng/ml TNF- $\alpha$  in the presence of either 25  $\mu$ M PD-98059 or 10  $\mu$ M SB-203580, and the number of ICAM-1 molecules per cell was determined by flow cytometry. Results are expressed as percent control (vehicle treated) (A; \* $P$  < 0.001,  $^{\circ}P$  < 0.115) and represent the average of 3 separate experiments. B and C illustrate these data in conventional flow cytometry histograms on a log scale. Results represent 1 of 3 separate experiments.



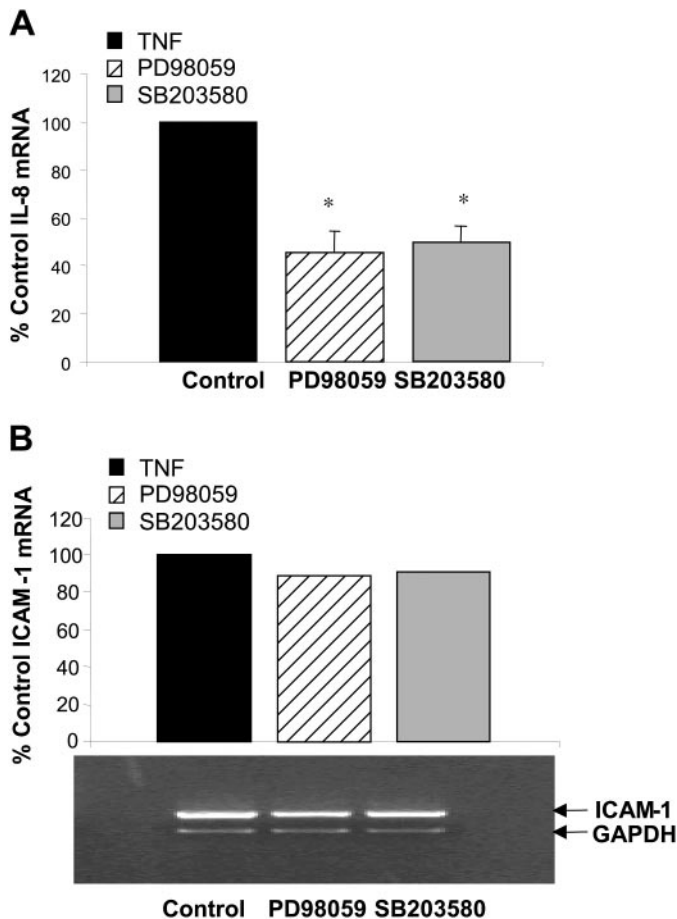


Fig. 6. ERK and p38 inhibition result in reduced IL-8 but not ICAM-1 mRNA accumulation following TNF- $\alpha$  treatment. HT-29 cells were pretreated with 25  $\mu$ M PD-98059 or 10  $\mu$ M SB-203580 for 30 min, followed by 1-h treatment with 10 ng/ml TNF- $\alpha$ . A: effect of ERK and p38 inhibition on IL-8 mRNA accumulation measured via quantitative ELISA. Data represent the average of 3 separate experiments (\* $P < 0.001$ ). B: effect of these treatments on ICAM-1 expression in the same experiment as measured by semiquantitative RT-PCR (see MATERIALS AND METHODS). Results are the percentage of vehicle-treated control determined from the densitometry ratios of ICAM-1 to GAPDH internal control (see MATERIALS AND METHODS) and are representative of 4 separate experiments. Bottom: a representative agarose gel of ICAM-1 and GAPDH RT-PCR products.

binding in resting cells. This is further augmented by TNF- $\alpha$  treatment, with increased AP-1 DNA binding evident by 30 min and sustained for at least 2 h. However, treatment with either ERK or p38 inhibitors did not appreciably affect AP-1 DNA binding (Fig. 7D).

*ERKs and p38 contribute to IL-8 secretion but not ICAM-1 expression via the stabilization of IL-8 mRNA.* mRNA accumulation is the result of the balance between mRNA synthesis and degradation (21). Our data using MAPK inhibitors suggested that MAPKs affect neither IL-8 nor ICAM-1 mRNA transcription. We thus examined an alternative mechanism, the regulation of IL-8 mRNA decay. HT-29 cells were treated with TNF- $\alpha$  for 1 h to allow IL-8 and ICAM-1 mRNA synthesis, followed by treatment with the mRNA synthesis inhibitors actinomycin D or  $\alpha$ -amanitin in the presence or absence of MAPK inhibitors. Actinomycin D

has previously been found to inhibit the degradation of a subset of mRNAs due to inhibition of an actinomycin D-sensitive factor (39). Furthermore, actinomycin D may also cause DNA damage (2). For these reasons we have also employed  $\alpha$ -amanitin, a mechanistically distinct inhibitor of transcription (47). Total mRNA was isolated at various times, and remaining IL-8 and ICAM-1 mRNA was measured by quantitative ELISA (IL-8 only) and semiquantitative RT-PCR (both IL-8 and ICAM-1). Figure 8A shows the effect of ERK and p38 inhibition on IL-8 mRNA decay as measured by RT-PCR. ERK and p38 inhibition resulted in the rapid disappearance of IL-8 mRNA relative to actinomycin D-treated control cells. In contrast, these treatments did not have an appreciable effect on the ICAM-1 transcript (Fig. 8B). Figure 8C graphically presents the rate of IL-8 mRNA decay as measured by quantitative ELISA. After ERK or p38 inhibition, IL-8 mRNA levels fell more rapidly compared with cells treated only with either  $\alpha$ -amanitin or actinomycin D. There was an initial increase in mRNA accumulation, presumably due to incomplete inhibition of mRNA synthesis within the first 15 min at this dose (2  $\mu$ g/ml). Similar results were obtained when using actinomycin D (data not shown). The IL-8 mRNA half-life in TNF- $\alpha$ -treated cells was calculated to be  $250 \pm 17$  min. This time was reduced approximately fivefold following ERK and p38 inhibition ( $P < 0.005$ ).

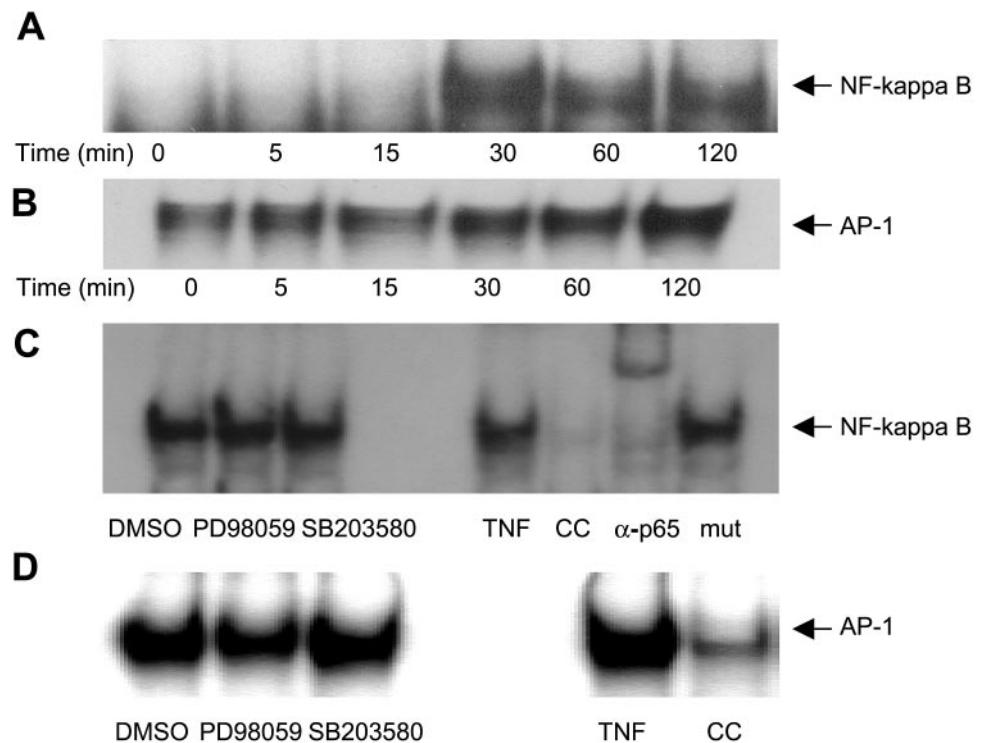
## DISCUSSION

Recent evidence suggests that enterocytes possess unique inflammatory responses (4, 9, 22, 42); however, little is known about how these functions are regulated at the intracellular level. In this study we have examined the role of MAPKs in proinflammatory gene expression in IECs. We report that ERK1/2 and p38 are required for IL-8 secretion by IECs and provide evidence that these kinases act posttranscriptionally via a pathway involving the stabilization of the IL-8 transcript.

Previous studies have examined the signaling cascades triggered by proinflammatory stimuli (e.g., IL-1 $\beta$ , TNF- $\alpha$ ) in intestinal epithelial cells (3, 15, 24). A common effector of these pathways is the transcription factor NF- $\kappa$ B, a pleiotropic transcription factor that controls the expression of multiple genes involved in the inflammatory response, including IL-8 (24), ICAM-1 (23), and inducible nitric oxide synthase (37). We selected IL-8 as a marker of NF- $\kappa$ B activation and enterocyte proinflammatory secretion because of its important role in neutrophil recruitment and inflammation (41). Intestinal epithelial cells have been shown to secrete IL-8 in response to bacterial (13) and viral invasion (48), lipopolysaccharide (12), cytokines (12), and phorbol esters (15). Furthermore, IL-8 is elevated in the context of various intestinal pathologies including Crohn's disease, ulcerative colitis, and possibly intestinal neoplasia (12, 41). Therefore, the examination of signals that trigger IL-8 secretion is relevant to multiple cellular processes.



Fig. 7. MAPKs contribute to IL-8 secretion independently of nuclear factor- $\kappa$ B (NF- $\kappa$ B) or activator protein-1 (AP-1). HT-29 cells were treated with 10 ng/ml TNF- $\alpha$  for times indicated in A and B or for either 30 min (C) or 1 h (D). A–D: nuclear extracts were prepared and analyzed via electrophoretic mobility shift assay (see MATERIALS AND METHODS). A and B depict the time course of NF- $\kappa$ B and AP-1 activation, respectively. In C and D, HT-29 cells were pretreated with 25  $\mu$ M PD-98059 or 10  $\mu$ M SB-203580 for 30 min before treatment with 10 ng/ml TNF- $\alpha$ . Specificity of probes was confirmed by competition with unlabeled probe (indicated as CC in C and D). In C,  $\alpha$ -p65 denotes a sample treated with sera against the p65 subunit of NF- $\kappa$ B (supershift), and mut denotes competition with excess unlabeled oligonucleotide harboring a mutated NF- $\kappa$ B DNA-binding sequence.



Similarly, MAPKs are also activated in response to multiple stimuli, including exposure to cytokines (9, 24, 27), growth factors (16, 27), and bacteria (4, 18). In particular, this study provides evidence that the ERK and p38 MAPK pathways regulate IL-8 secretion in IECs by affecting the rate of mRNA decay. However, whether this occurs independently of NF- $\kappa$ B is still unclear. Vanden Berghe et al. (40) have recently shown that both p38 and ERK contribute to NF- $\kappa$ B p65 transactivation in the context of TNF-stimulated IL-6 secretion in L929 mouse fibrosarcoma cells. Their data show that ERK and p38 inhibition (using PD-98059 and SB-203580, respectively) results in decreased IL-6 production in the absence of changes in NF- $\kappa$ B DNA-binding activity. On the other hand, they observed that PD-98059 and SB-203580 reduced the *trans*-activating activity of the p65 subunit of NF- $\kappa$ B (40). This observation parallels ours in that we also observed a reduction in protein expression (IL-8 in this case) in the absence of changes in NF- $\kappa$ B or AP-1 DNA binding. Furthermore, we observed that the expression of ICAM-1, which is also under the control of NF- $\kappa$ B, is unaffected by ERK inhibition and enhanced by p38 inhibition. This last observation is interesting in that ICAM-1 mRNA synthesis is unaffected by SB-203580 treatment, suggesting that SB-203580 may interfere posttranscriptionally with ICAM-1 surface expression. However, in light of the interaction among ERK, p38, and JNK, it is unclear which of these pathways is involved. However, De Cesaris et al. (8) have recently described a role for the JNK pathway in the expression of ICAM-1 in Sertoli cells. Thus it is possible to speculate that ICAM-1 expression by HT-29 cells following

SB-203580 treatment results from an increase in JNK activity due to relaxation of JNK repression by p38.

We also examined the effect of MAPK inhibitors on the activation of AP-1. Like NF- $\kappa$ B, AP-1 is often activated in response to cellular stress, cytokines, and growth factors (28). However, our data suggest that AP-1 does not play a significant role in IL-8 mRNA synthesis in response to TNF- $\alpha$ , at least not within the first hour. However, MAPK inhibition would be expected to suppress AP-1 activity in that AP-1 expression is known to require both ERK and p38 signaling (17, 28). Figure 7D illustrates that although there is a small increase in AP-1 DNA binding by 30 min, significant DNA binding is only evident after 2 h of stimulation, consistent with a requirement for new protein synthesis. Furthermore, whereas the ICAM-1 promoter also includes an AP-1 binding site, inhibition of the ERK pathway did not affect ICAM-1 mRNA levels.

Recently, Yu and Chadee (46) have shown that cAMP-dependent prostaglandin E<sub>2</sub> stimulation of IL-8 secretion in IECs occurs via a posttranscriptional mechanism. This effect was mapped to the 3' untranslated region (UTR) of the IL-8 gene in the region spanning bases 1902 to 3152. It is unknown, however, whether MAPKs play a role in this response. However, two recent reports (19, 43) suggest that p38 contributes to IL-8 secretion via the stabilization of the IL-8 transcript. These reports suggest that p38 activation in response to IL-1 $\beta$  occurs downstream from MEKK-1 and MKK6. However, although these studies also attribute these effects to the 3' UTR of the IL-8 gene, instability was conferred by sequences between nucleotides 972 and 1310 (43), suggesting the existence of at



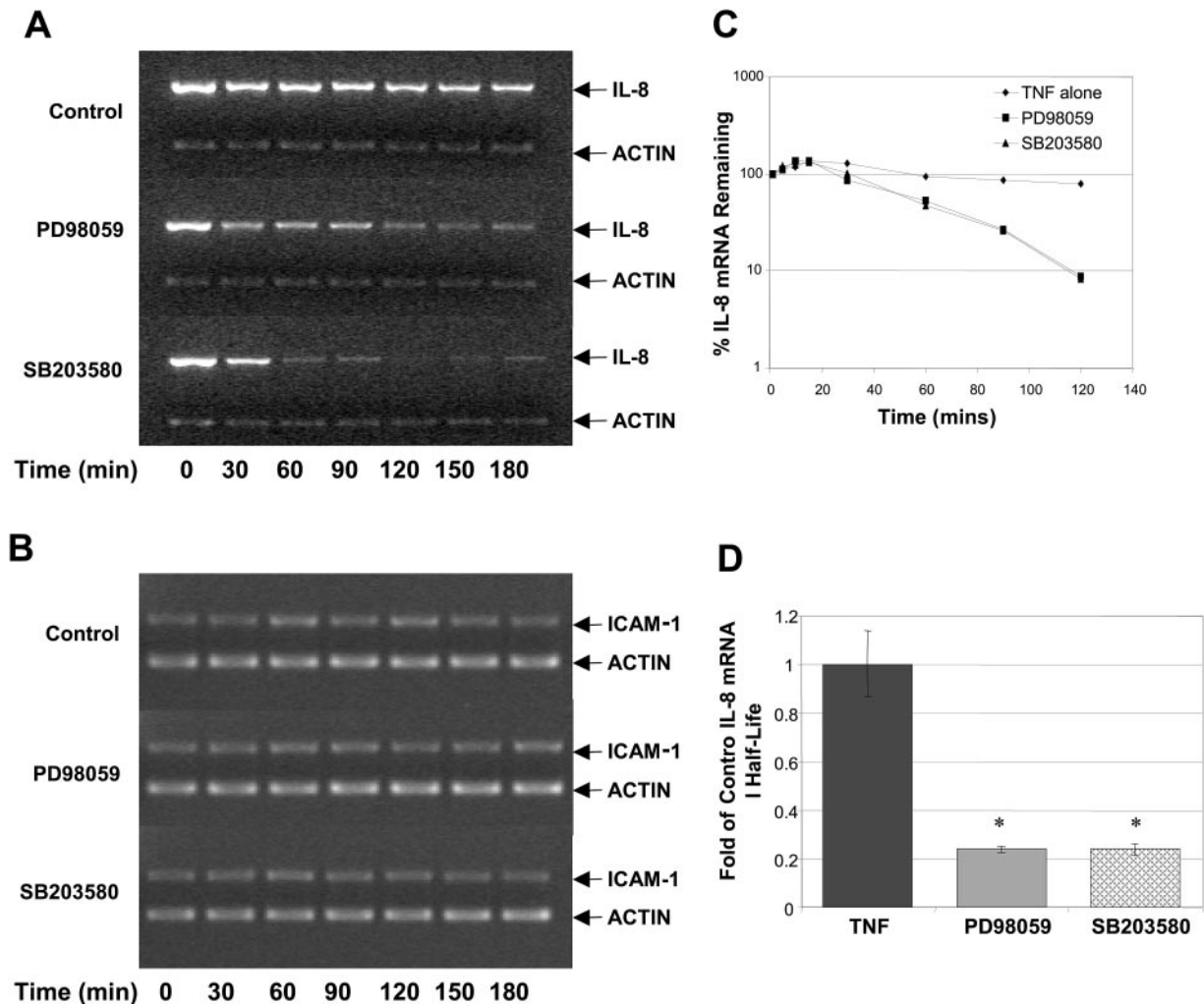


Fig. 8. Effect of ERK and p38 inhibition on IL-8 and ICAM-1 mRNA decay. HT-29 cells were treated with TNF- $\alpha$  (10 ng/ml) for 1 h to allow accumulation of IL-8 and ICAM-1 mRNA. Cells were then treated with the mRNA synthesis inhibitor actinomycin D (5  $\mu$ g/ml) and either vehicle (DMSO), ERK pathway inhibitor (PD-98059), or p38 inhibitor (SB-203580). Total mRNA was then isolated at various times indicated. Effects on IL-8 (A) and ICAM-1 (B) mRNA decay were measured by semiquantitative RT-PCR. Actin mRNA was used as an endogenous control message. Results in A and B are representative of 4 separate experiments. C: effects of ERK and p38 pathway inhibitors on IL-8 mRNA decay as measured by quantitative ELISA. Cells were treated with TNF- $\alpha$  (10 ng/ml) for 1 h followed by treatment with the RNA synthesis inhibitor  $\alpha$ -amanitin (2  $\mu$ g/ml) with or without MAPK pathway inhibitors. D: effect of ERK and p38 pathway inhibitors on the half-life of the IL-8 transcript determined from the curves in C (\* $P$  < 0.005). Values in C and D are means  $\pm$  SD from 3 separate experiments.

least two destabilizing elements within the 3' UTR of the IL-8 gene. The present study supports a role for the p38 pathway in IL-8 mRNA stability in the context of TNF- $\alpha$  signaling and suggests a similar role for the ERK pathway, suggesting that these pathways represent conserved mechanisms for IL-8 regulation.

We have employed two mechanistically distinct inhibitors of transcription in this study because there is evidence indicating that mRNA decay can be disrupted by inhibitors of transcription (actinomycin D) (5, 39). However, the existence of RNA-destabilizing elements insensitive to inhibitors of transcription has also been described (36). Although we cannot rule out such effects on decay, our data would be consistent with the participation of actinomycin D-insensitive RNA-destabilizing element(s). Furthermore, it is possible that

under our experimental conditions the mRNA levels measured during decay may reflect some ongoing RNA synthesis due to incomplete inhibition of transcription. However, in the event that p38 and/or ERK affect p65 transactivation, the transcription inhibitors would affect synthesis to the same extent in all groups. Thus, although the levels of RNA measured may reflect some degree of synthesis, the rates of decay should remain concordant with the degree of RNA degradation. The IL-8 mRNA half-lives were calculated to be 250 min in untreated cells compared with 50 and 49 min for SB-203580- and PD-98059-treated samples, respectively; however, these values are likely elevated. Nonetheless, the slopes of decay are proportional to the rate of decay; hence, we are confident that the fivefold difference observed is accurate (Fig. 8D). In this respect, the

relative contributions of MAPKs to mRNA stabilization and p65 transactivation as well as the specificity of these pathways for different genes in cells of different origin are questions that should lead to interesting future research.

Importantly, we observed differences in the manner in which MAPK inhibitors affect IL-8 secretion between HT-29 and T84 IEC cell lines. Inhibition of p38 or ERK with SB-203580 or PD-98059 did not alter IL-8 secretion in T84 cells. However, when used in combination, simultaneous treatment of T84 cells with PD-98059 and SB-203580 resulted in a 50% reduction in IL-8 production (Fig. 3C). This result suggests that MAPKs are required for maximal IL-8 secretion in T84 cells; however, it also suggests a possible feedback mechanism between these pathways. As shown in Fig. 4, there indeed seems to be interaction between these pathways in that inhibition of ERK augments p38 phosphorylation (A) and p38 inhibition results in enhanced JNK phosphorylation (D). This interaction has previously been described and highlights the importance of examining the effects of these inhibitors on several pathways when ascribing function (6, 30).

In conclusion, this study suggests that MAPKs may play an important role in the regulation of IL-8 secretion in response to TNF- $\alpha$  via a mechanism that involves the stabilization of IL-8 mRNA. We propose that NF- $\kappa$ B activation, although essential in the synthesis of IL-8, must occur in concert with the activation of at least two MAPK pathways for maximal expression to occur. Thus stimuli that activate both MAPKs and NF- $\kappa$ B (e.g., TNF- $\alpha$ , bacteria, and viruses) coordinate maximal IL-8 secretion by IECs.

We thank J. B. McIntyre for expert technical assistance.

This work was supported by research grants from the Crohn's and Colitis Foundation of Canada and the Alberta Children's Hospital Foundation to H. G. Parsons.

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