Modulators of Inflammation Use Nuclear Factor-κB and Activator Protein-1 Sites to Induce the Caspase-1 and Granzyme B Inhibitor, Proteinase Inhibitor 9*

Received for publication, January 14, 2002, and in revised form, August 12, 2002
Published, JBC Papers in Press, August 12, 2002, DOI 10.1074/jbc.M200379200

From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801-3602

Padma Kannan-Thulasiram and David J. Shaprio‡

Proteinase inhibitor 9 (PI-9) inhibits caspase-1 (interleukin (IL)-1β-converting enzyme) and granzyme B, thereby regulating production of the pro-inflammatory cytokine IL-1β and susceptibility to granzyme B-induced apoptosis. We show that cellular PI-9 mRNA and protein are induced by IL-1β, lipopolysaccharide, and 12-O-tetradecanoylphorbol-13-acetate. We identified functional imperfect nuclear factor-κB (NF-κB) sites at −135 and −88 and a consensus activator protein-1 (AP-1) site at −308 in the PI-9 promoter region. Using transient transfections in HepG2 cells to assay PI-9 promoter mutations, we find that mutational ablation of the AP-1 site or of either NF-κB site reduces IL-1β-induced expression of PI-9 by ~60%. Mutational ablation of the two NF-κB sites and of the AP-1 site nearly abolishes both basal and IL-1β-induced expression of PI-9. Nuclear extracts from IL-1β-treated HepG2 cells exhibited strong, IL-1β-inducible binding to the NF-κB sites and to the AP-1 site. Electrophoretic mobility shift assays show that after IL-1β treatment c-Jun/c-Fos and JunD bind to the AP-1 site, whereas the p50/p65 heterodimer binds to the two NF-κB sites. Estrogens induce PI-9, but induction of PI-9 by estrogens and IL-1β is not synergistic. In transiently transfected, estrogen receptor-positive HepG2ER7 cells, estrogens do not interfere with IL-1β induction, whereas IL-1β exhibits dose-dependent repression of estrogen-inducible PI-9 expression. Our surprising finding that the pro-inflammatory cytokine IL-1β strongly induces PI-9 suggests a novel mechanism for regulating inflammation and apoptosis through a negative feedback loop controlling expression of the anti-inflammatory and anti-apoptotic protein, PI-9.

The cellular serine proteinase inhibitor (serpin), proteinase inhibitor 9 (PI-9), is unique in its ability to inhibit inflammation and apoptosis (1–5). PI-9 is a potent inhibitor of caspase-1-mediated inflammation (4, 5) and of granzyme-B-induced apoptosis (3, 6). The primary role of caspase-1 (interleukin-1β (IL-1β)-converting enzyme, ICE protease) is to mediate immune and inflammatory reactions by catalyzing the maturation of the pro-inflammatory cytokines, IL-1β and IL-18. IL-1β and IL-18 are synthesized as inactive precursors that are converted to their active forms by proteolytic cleavage by caspase-1 (7). Recent studies implicate IL-1β in the pathogenesis of diseases such as atherosclerosis, hepatitis, and cirrhosis of the liver (5, 8, 9). The expression of PI-9 is dysregulated and inversely related to caspase-1 activity and IL-1β production in atherosclerotic plaques, suggesting that PI-9 may be involved in the regulation of inflammatory and immune responses in vivo (5).

In addition to its anti-inflammatory action, PI-9 acts as an anti-apoptotic protein by inhibiting the serine protease, granzyme B (3). Granzyme B is found in granules produced by cytolytic T lymphocytes (CTLs) and natural killer (NK) cells. CTLs and NK cells use perforin- and granzyme B-containing granules and the FAS mediated pathway to destroy neoplastic cells, or cells infected with intracellular pathogens (10, 11). In target cells, granzyme B induces apoptosis in part by proteolytically cleaving several inactive pro-caspases and thereby converting them into their active forms (12). The expression of high levels of PI-9 in CTLs, immune-privileged cells, and dendritic cells may protect these cells against misdirected granzyme B released during immune responses (3, 13–15). A recent study described a close correlation between the level of expression of PI-9 or its mouse orthologue, SPI-6, and the ability of several tumor cell lines to evade apoptosis mediated by CTL and NK cells. These data led the investigators to suggest a role for elevated expression of PI-9 in the ability of some tumors to evade immune attack (6).

Because PI-9 is likely to play an important role in modulating inflammation and apoptosis, the regulation of PI-9 gene expression is of particular interest. Previously, we showed that PI-9 is an estrogen-inducible gene in human liver and in HepG2 human hepatoblastoma cells (8). PI-9 gene transcription is induced by binding of estrogen-estrogen receptor complex to a unique downstream estrogen-responsive unit (16). In the course of that work we identified a consensus activator protein-1 (AP-1) site at −308 and showed that deletion of the AP-1 site did not alter estrogen induction of PI-9. Here we report that further analysis of the PI-9 promoter region identifies three potential imperfect nuclear factor-κB (NF-κB) sites. AP-1 and NF-κB are ubiquitous transcription factors and pleiotropic regulators of the inducible expression of numerous genes involved in the modulation of processes important in inflammatory and host defense events (17, 18).

The AP-1 transcription factor is a heterodimer composed of members of the Fos, Jun, and Fra families of proto-oncogenes. In different cell and promoter contexts, AP-1 alters gene expression in response to growth factors, cytokines, oxidative stress, and phorbol esters (19–21). NF-κB is composed of ho-
modemeric and heteromeric complexes of the Rel family of proteins, p65 (Rel A), p50/p105, c-Rel, p52/100, and Rel B (21, 22). Activation of NF-xB by extracellular signals such as cytokines, phorbol esters, lipopolysaccharide (LPS), and oxidative stress leads to the phosphorylation and subsequent degradation of its inhibitor, IxB, allowing NF-xB to translocate into the nucleus, bind to specific DNA sites in the promoter regions of target genes, and activate their transcription (23, 24).

The ability of PI-9 to modulate inflammatory and apoptotic events and to function upstream of NF-xB and AP-1 suggests that the PI-9 transcription initiation site led us to examine the ability of agents that modulate inflammation to regulate PI-9 gene expression. The pro-inflammatory cytokine, IL-1, and other pro-inflammatory agents including LPS and TPA induced PI-9 mRNA and protein in liver cells. Using transient transfection of reporter gene constructs containing mutationally inactivated NF-xB and AP-1 sites, we found that the NF-xB sites at -139 and -88 and the AP-1 site at -308 are all necessary for efficient IL-1 induction of PI-9. Binding studies and gel shift assays showed that after treatment of the cells with IL-1, c-Jun, JunD, and c-Fos bind to the AP-1 site. After IL-1 treatment, the p50/p65 NF-xB heterodimer binds to the two NF-xB sites. Using a construct derived from a cellular gene induced by estrogen and pro-inflammatory agents, we show that a pro-inflammatory agent (IL-1) suppresses estrogen induction. Despite PI-9 anti-inflammatory and anti-apoptotic effects, the pro-inflammatory cytokine IL-1x produced as a result of caspase-1 action was a potent inducer of PI-9 in several human cell lines. This study suggests a novel form of end-product regulation. IL-1x strongly induces PI-9, which in turn inhibits caspase-1, blocking the formation of additional active IL-1x.

EXPERIMENTAL PROCEDURES

Generation of AP-1 and NF-xB Mutations in the PI-9 Promoter—The full-length PI-9 promoter cloned upstream of a firefly luciferase gene in the plasmid pGL3-Basic (16) was used to generate mutations in the AP-1 and NF-xB binding sites. Mutations were generated using the Stratagene QuikChange kit (Stratagene, La Jolla, CA) in the presence of 1.5 betaine (Sigma). In each case, the AP-1 or NF-xB sequences of the PI-9 promoter were mutated to a HindIII site. The ERU was mutated at the imperfect estrogen response element with a two-base change and at one of the consensus estrogen response elements creating a HindIII site. The mutations in the ERU primer are underlined: 5'-GGGCGAGCTGGGAGGACCTTACGCTCTGCAGCAGC-3'. All constructs were verified by sequencing using the BIG DYE terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Cell Culture, Transient Transfections, and Luciferase Assays—Twenty-four hours before transfection, HepG2 cells and HepG2ER7 cells, generously provided by Dr. D. Zajchowski (8) were plated in Dulbecco’s modified Eagle’s medium, 10% charcoal dextran-treated fetal bovine serum, and penicillin-streptomycin. Transfections were carried out using calcium phosphate coprecipitation in 12-well plates with 50 ng of PI-9-luciferase construct, 15 ng of pRLSV40 (Promega, Madison, WI) as internal standard, and 1.9 ng of pTZIP18U as carrier. In experiments using mouse xestrol, 25 ng of CMVxER was used. After 14–16 h, the transfected cells were shocked with 20% glycerol and plated in medium with or without mediators as indicated in the figure legends. In transient transfection experiments using pyrrolidine dithiobiscarbamate (PDTC), HepG2 cells were treated with 240 nM PDTC. Cells were harvested and assayed using the dual-luciferase assay kit according to the manufacturer’s protocol (Promega).

HeLa cells were cultured under the same conditions as HepG2 cells. However, transfections were performed using Lipofect Plus reagent (Invitrogen) according to the manufacturer’s instructions with the following DNA concentrations: 50 ng of PI-9 promoter-luciferase construct, 15 ng of pRLSV40, and 0.3 ng of pTZIP18U. Transfected cells were incubated with or without 5 ng/ml IL-1x (R&D Systems, Minneapolis, MN) for 24 h, and dual luciferase assays were performed.

MCF-7 cells were maintained in minimal essential medium with Hank’s salts, 10 mM Hepes, 10% heat-inactivated fetal bovine serum, and penicillin-streptomycin. Transient transfections were performed with LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions with 540 ng of PI-9 promoter-luciferase construct, 10 ng of pRLSV40, and 0.8 ng of pTZIP18U. After the addition of liposomes, the cells were treated with 5 ng/ml IL-1x for 24 h before harvesting the cells.

CHO-S cells were maintained in Dulbecco’s modified Eagle’s/F-12 medium, 10% heat-inactivated fetal bovine serum, l-glutamine, and penicillin/streptomycin. Transient transfections were performed using LipofectAMINE 2000, with 25 ng of PI-9 promoter-luciferase construct, 10 ng of pRLSV40, and 0.8 ng of pTZIP18U.

Quantitative Reverse Transcription-PCR—Total RNA from HepG2ER7 cells and from Daudi cells (maintained in RPMI, 5 mM Hepes, 1 mM sodium pyruvate, 2 mM l-glutamine, 10% heat-inactivated fetal bovine serum, and penicillin-streptomycin) was reverse-transcribed using Superscript II (Invitrogen) according to the manufacturer’s protocol and treated with RNase-free DNase for 15 min at 37 °C followed by phenol-chloroform extraction. Reverse transcription was performed using 1 μg of total RNA, 5 μM random hexamer primers, and Moloney murine leukemia virus (Invitrogen) according to the manufacturer’s directions at 37 °C for 1 h. A 1-μl aliquot of the reverse transcription reaction was used for thermocycling. Each PCR reaction contained 12.5 μl of 2× Taqman master mix (PE Biosystems, Foster City, CA), 300 ng forward and reverse primers, and 200 ng of PI-9 probe (5′-bam-catacaacagaasgact- ggctttaaaataacca-tamra-3′) (fam, 6-carboxyfluorescein; tamra, 6-carboxytetramethylrhodamine) and 25 μl of total volume. Following the initial control, 18 S RNA was used controlling both control primers and probe (PE Biosystems, Foster city, CA). Detection and data analysis were carried out on an ABI PRISM 7700 sequence detection system.

Western Blot Analysis—Cell proteins were separated on a 15% SDS-polyacrylamide gel and transferred using the trans-blot semi-dry transfer cell (Fisher) at 15 V for 15 min. The membrane was blocked overnight with 3% nonfat milk and 0.05% Tween followed by a 1-h incubation with the primary PI-9 polyclonal antibody diluted 1:2,000 in 1% nonfat milk and 0.2% Tween in phosphate-buffered saline. After a 15-min wash (PBS with 0.05% Tween), the membrane was incubated for 1 h with the secondary goat-anti rabbit antibody diluted at 1:10,000. Following a 1-h wash, antigen-antibody complexes were detected using West Chemiluminescent substrate (Pierce).

Preparation of HepG2 Cell Nuclear Extract—Nuclear extract from HepG2 cells was prepared as described previously (25) with modifications. Briefly, cells were washed twice with ice-cold phosphate-buffered saline and collected after centrifugation at 900 × g for 5 min. Cells were resuspended in ice-cold buffer containing 10 mM Hepes, pH 7.8, 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 2 μg/ml pepstatin and kept on ice for 15 min. Cells were lysed in 0.1% Nonidet P-40 and vortexed for 10 s, and the nuclear pellet was recovered after centrifugation at 13, 000 × g for 10 s at 4 °C. The nuclear pellet was resuspended in ice-cold buffer containing 20 mM Hepes, pH 7.8, 0.4 M NaCl, 1 mM EDTA, 1.5 mM MgCl2, 1 mM dithiothreitol, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 2 μg/ml pepstatin and incubated on ice for 20 min with shaking. Nuclear extract was retained after centrifugation at 14,000 rpm for 5 min at 4 °C, and the supernatant was separated into aliquots and stored at -80 °C. Protein concentration was determined by the Bradford method (Bio-Rad).

Transcription Factor Binding Assays—To measure AP-1 or NF-xB activation and their interaction with sites of the PI-9 promoter, competition experiments were performed using the Trans-AM AP-1 and NF-xB p50 kit according to the manufacturer’s protocol (Active Motif, Carlsbad, CA). Briefly, an oligonucleotide containing either a consensus AP-1 or NF-xB site immobilized on a 96-well plate was incubated with 5 μg of untreated or 1.5 μg of IL-1x (5 ng/ml)-treated HepG2 nuclear extract in the presence of double-stranded oligonucleotides containing as competitor either an AP-1 or NF-xB site from the PI-9 promoter or a mutated version of the site. Binding specificity was tested in competition assays using a 60-fold excess of either the AP-1 or the NF-xB site from the PI-9 promoter or in a mutated mutation specific to either c-Jun or p65 was added following the addition of a secondary antibody conjugated to horseradish peroxidase. c-Jun and p65 activation was measured at 450 nm. The AP-1 and NF-xB sites of the PI-9 promoter used as competitors were: AP-1 (t-317 to -297), 5′-ctcggtact-cagctgctct-3′, NF-xB (t-144 to -121), 5′-agcttgagtcaccttcatt-3′, NF-xB (t-97 to -74), 5′-agcttgagtcaccttcatt-3′, and NF-xB sequences used as competitors were 5′-ctcggtagttgcaggagc-3′ and 5′-agcttgagtcaccttcatt-3′, respectively.

AntibodySuper Shift Assays—Double-stranded oligonucleotides of the same AP-1 and NF-xB sites used in the transcription factor binding
**Induction of PI-9 by Pro-inflammatory Agents**

**RESULTS**

**IL-1β and LPS Induce PI-9 mRNA and Protein**—Previously we identified a consensus AP-1 site at −308 of the PI-9 promoter region (16). We examined the PI-9 promoter for other transcription factor binding sites potentially involved in controlling PI-9 gene expression. Three potential NF-κB sites at −1078, −135 and −88 were identified (Fig. 1A). The site at −135 differs from the consensus NF-κB binding sequence by 1 nucleotide, whereas the other 2 sites differ from the consensus NF-κB sequence by 2 nucleotides. Because pro-inflammatory agents regulate the expression of many genes involved in inflammatory processes through AP-1 and NF-κB proteins (26–30), we tested the ability of the pro-inflammatory agents IL-1β and LPS to induce expression of the native cellular PI-9 gene and protein in an estrogen receptor-positive clone (HepG2ER7 (8)) of HepG2 human hepatoblastoma cells and in a well studied B cell line, Daudi cells. Using quantitative real-time PCR to measure PI-9 mRNA levels, IL-1β induced cellular PI-9 mRNA in HepG2ER7 cells by −6-fold (Fig. 1B). We previously used Northern blot analysis to show that estrogen induction of PI-9 mRNA is rapid, and PI-9 mRNA levels remain elevated for at least 1 day (8). Induction of PI-9 mRNA by IL-1β was rapid, with a 4–5-fold induction in 4 h. Induction was maximal at −6-fold at 8 h, and PI-9 mRNA remained fully induced through the 24 h of the experiment (Fig. 1B). Western blot analysis showed that IL-1β induced PI-9 protein within 4 h and that PI-9 levels remained elevated for at least 48 h (Fig. 1B).

Cells of the immune system contain high levels of PI-9 (3, 31). To determine whether the cellular PI-9 gene was still inducible in these cells, we looked at the ability of the widely studied pro-inflammatory agent, LPS, to induce PI-9 in a well studied B cell line, Daudi cells. LPS activation of caspase-1 leads to IL-1β secretion in several systems (32, 33). It was therefore of interest to determine whether this important pro-inflammatory agent would elicit induction of the anti-inflammatory protein, PI-9. LPS treatment of Daudi cells elicited a progressive induction of PI-9 mRNA, which increased in level throughout the 24 h of the experiment (Fig. 1C). LPS induced PI-9 mRNA >-7-fold and strongly induced PI-9 protein (Fig. 1C). Thus, a rapid and robust induction of PI-9 mRNA and protein is elicited by the pro-inflammatory agents, IL-1β and LPS.

**TNF-α and TPA Induce PI-9 Gene Expression**—We further analyzed induction of PI-9 by another pro-inflammatory agent that often acts through AP-1 and NF-κB sites, tumor necrosis factor-α (TNF-α). HepG2 cells were transiently transfected with a construct containing the PI-9 promoter region, driving the expression of a luciferase reporter gene (16), and exposed to varying concentrations of TNF-α. TNF-α elicited a concentration-dependent 4–5-fold increase in PI-9 promoter activity, with induction reaching a plateau at 5 ng/ml TNF-α (Fig. 2A).

LPS, IL-1β, and TNF-α are all agents related to inflammatory processes. It was of interest to determine whether a different class of regulatory agents known to work through AP-1 and NF-κB sites could also regulate PI-9 expression. As shown in Fig. 2B, the phorbol ester, TPA, a well known tumor pro-
FIG. 2. TNF-α and TPA induce PI-9 expression. A. HepG2 cells were transfected with our native human PI-9 promoter (~1482 to +314)-luciferase construct (16) with the indicated concentrations of TNF-α for 24 h and then assayed for luciferase activity. Fold induction represents the increase in luciferase activity in response to TNF-α with the vehicle-treated sample (0.1% BSA) set equal to 1. The data represent the mean ± S.E. of three separate transfections. B. HepG2ER7 were treated at the indicated times with 20 ng TPA. RNA was isolated from HepG2ER7 and analyzed by quantitative reverse transcription-PCR as described under “Experimental Procedures.” Proteins from TPA-treated HepG2ER7 cells were fractionated by SDS-PAGE and analyzed by Western blot using polyclonal antibody to recombinant PI-9.

moter, induced PI-9 mRNA ~7-fold and strongly induced PI-9 protein in HepG2ER7 cells (Fig. 2B). These data demonstrate that cellular PI-9 gene expression is regulated by diverse agents known to act through NF-κB and AP-1 sites.

The Two Proximal NF-κB Sites and the AP-1 Site Are Important in IL-1β Induction of PI-9 Gene Expression—To characterize the roles of the three putative NF-κB sites and of the AP-1 site in induction of PI-9 gene expression, we constructed a series of plasmids containing mutations that inactivate the AP-1 and NF-κB sites. We transiently transfected the PI-9 promoter constructs into HepG2 cells and examined the ability of IL-1β (at 5 ng/ml) to induce PI-9 expression (Fig. 3). Because none of the mutant promoters we tested exhibited increased basal expression of PI-9 (data not shown), a reduced fold induction by a transfected mutant promoter is due to less IL-1β-induced expression than is seen with the wild-type promoter. Mutational ablation of the putative NF-κB element at −1078 had a minimal effect on IL-1β induction, indicating that this element does not play a significant role in IL-1β regulation of PI-9. In contrast, mutational ablation of the putative NF-κB sites at either −135 or at −88 reduced IL-1β induction of PI-9 by ~60%. Mutation of both of the proximal NF-κB sites resulted in a modest further reduction of the IL-1β response. The effect of mutational inactivation of the AP-1 site at −308 was similar to the effect of mutating individual NF-κB sites, with PI-9 expression reduced by ~55% relative to the wild-type promoter. These data indicate that the two proximal imperfect NF-κB elements and the consensus AP-1 site all contribute to IL-1β induction of PI-9 gene expression. Mutational inactivation of both NF-κB sites and the AP-1 site completely abolished IL-1β induction and reduced basal PI-9 promoter activity to extremely low levels (Fig. 3). Consistent with a role for both the proximal NF-κB sites and the AP-1 site in IL-1β induction of PI-9, the inhibitor of IκB degradation, pyrrolidine dithiocarbamate, suppressed IL-1β inducibility of PI-9 by ~60% but did not completely abolish induction (data not shown).

IL-1β Induces c-Jun and p65 Proteins That Bind to Their Sites in the PI-9 Promoter—To test whether proteins in nuclear extracts from IL-1β-induced HepG2 cells exhibit enhanced interaction with the AP-1 and NF-κB sites in the 5′-flanking region of the PI-9 promoter, we used a quantitative antibody-based assay to detect and quantify binding by c-Jun and p65, members of the AP-1 and NF-κB families, respectively. Nuclear extract from control and IL-1β-treated HepG2 cells was incubated with an oligonucleotide containing the AP-1 consensus sequence in the PI-9 gene (5′-ACTCAGT-3′), bound protein was reacted with c-Jun antibody, and the extent of c-Jun binding was determined using a second antibody conjugated to horse-radish peroxidase. Extract from IL-1β-treated HepG2 cells showed a 4-fold increase in c-Jun binding to the PI-9 AP-1 site (Fig. 4A). Binding to the immobilized AP-1 site was specifically competed by the PI-9 AP-1 site (Fig. 4A, PI-9 AP-1). The inability of a mutated AP-1 site to compete for binding (Fig. 4A, Mut AP-1) supports the view that the induced protein was c-Jun.

To determine whether IL-1β induced p65 binding activity, we examined the ability of proteins in extracts from control and IL-1β-treated HepG2 cells to bind to an immobilized consensus NF-κB site. Binding of p65 to the consensus NF-κB site was quantitated by incubation with anti-p65 followed by detection with horseradish peroxidase-conjugated secondary antibody. IL-1β treatment induced a 4–5-fold increase in binding of p65 to the consensus NF-κB site (Fig. 4B). Both basal and IL-1β-induced binding to the consensus NF-κB site were effectively competed by the imperfect PI-9 NF-κB sites (Fig. 4B, PI-9 NF-κB −144/−121 and −97/−74). A mutated NF-κB oligonucleotide was unable to compete for binding (Fig. 4B, Mut NF-κB).

These data indicate that IL-1β treatment of HepG2 cells induce c-Jun and active p65 by 4–5-fold. The consensus PI-9 AP-1 site binds c-Jun, and binding of c-Jun is specifically competed by the PI-9 AP-1 site. Oligonucleotides containing the imperfect NF-κB sites at −135 and −88 were effective competitors for binding of p65 to the immobilized consensus NF-κB site.

c-Jun, JunD, and c-Fos Bind to the AP-1 Site, whereas p50 and p65 Bind to the NF-κB Site—To identify the specific Fos/Jun/Fra family members that bind to the AP-1 site and the members of the Rel family that bind to the NF-κB sites in the PI-9 promoter, we performed electrophoretic mobility shift assays with labeled PI-9 AP-1 and NF-κB sites and identified the bound proteins using antibody supershifts. As shown in Figs. 5,
FIG. 3. Both the proximal NF-κB sites and the AP-1 site are important for regulation of PI-9 gene expression. The putative NF-κB sites and the AP-1 site in the PI-9 promoter region were mutagenically inactivated (see “Experimental Procedures”), individually or in combination. The PI-9 promoter-luciferase constructs were transfected into HepG2 cells and incubated with or without 5 ng/ml IL-1β for 24 h. The cells were harvested, cell extracts were prepared, and relative luciferase activity was determined. Fold induction represents the increase in luciferase activity for the wild-type promoter and for the mutated construct in the presence of IL-1β, with the vehicle-treated sample (0.1% BSA) set equal to 1. The data represent the mean ± S.E. for three separate transfections.

A.

B.

FIG. 4. IL-1β induces AP-1 and NF-κB binding activity. HepG2 cells were maintained for 1.5 h in medium containing 5 ng/ml IL-1β (IL-1β, +) or lacking the mediator (IL-1β, −). Nuclear extract was prepared (see “Experimental Procedures”), and 5 μg of nuclear extract were used to show binding to an immobilized consensus AP-1 site (identical to the PI-9 AP-1 site) (panel A) or to an immobilized consensus NF-κB site (panel B) (Active Motif, Carlsbad, CA). A, binding specificity for the AP-1 site was demonstrated by using the consensus AP-1 sequence from the PI-9 promoter (PI-9 AP-1; −317cgcttgacagtgtctcct−297) as competitor or an inactive mutated AP-1 site (Mut AP-1; 5′-cgcttgagagtgcggcggga-3′). B, binding to the immobilized consensus NF-κB site (first bar in each set) and competition with the PI-9 NF-κB oligonucleotides (PI-9 NF-κB −144/−121; 5′-ttggctcaaggtgattctccatat−122 or PI-9 NF-κB −97/−74; 5′-agccagcggacttcctactgg-3′) or with a mutated NF-κB site (Mut NF-κB; 5′-agcttagggactttccagc-3′). The data represent the average ± S.E. for three separate binding assays. OD, optical density.
Induction of PI-9 by Pro-inflammatory Agents

Fig. 5. c-Jun, JunD, c-Fos, and p50/p65 bind to the AP-1 and NF-κB sites of the PI-9 promoter. 20 μg of nuclear extract from control HepG2 cells or HepG2 cells maintained for 1.5 h in IL-1β was incubated with a 32P-labeled double-stranded oligonucleotide containing the AP-1 site from the PI-9 promoter (panels A and B) or with labeled oligonucleotides containing the NF-κB sites located at the −135 or −88 of the PI-9 promoter (see the legend to Fig. 4) in panels C and D, respectively. After the protein-DNA binding reaction, there was an additional incubation with antibodies to individual members of the Jun/Fos/Fra family (panels A and B) or with antibodies to proteins in the Rel/NF-κB family (panels C and D).

A and B, with a labeled oligonucleotide containing the AP-1 site in the PI-9 promoter, specific antibodies against c-Jun, JunD, and c-Fos produced marked supershifts, and a weaker supershift was observed with a JunB antibody. Control PI-9 antibody did not supershift the complex formed by the IL-1β-treated HepG2 nuclear extract. The gel shift experiments also demonstrate strong IL-1β induction of each of the Jun/Fos family members that bind to the PI-9 AP-1 site (Figs. 5, A and B).

Different combinations of the Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA (34). As shown in Figs. 5, C and D, labeled oligonucleotides containing the NF-κB regions at −135 and −88 were supershifted by antibodies against p65 (RelA) and p50. These data are compatible with a p65/p50 heterodimer binding to the NF-κB sites.

**IL-1β Induces PI-9 in Diverse Cell Lines**—Although our data showed that IL-1β induces PI-9 in HepG2 cells, PI-9 is expressed in diverse vertebrate cells (3, 6, 13–15). It was therefore of interest to determine whether other cells had the potential for IL-1β activation of the PI-9 promoter. Because a recent study suggested that the endogenous PI-9 gene may be constitutively overexpressed in many tumor cell lines (6), we carried out transient transfections of three widely used cell lines with the full-length PI-9 promoter-luciferase reporter construct and with the construct in which the two NF-κB sites and the AP-1 site were mutagenically inactivated. IL-1β (at 5 ng/ml) induced luciferase activity from the transfected PI-9 promoter in HepG2 cells, MCF-7, breast cancer cells, CHO-S cells, and HeLa cells by 6-, 4-, 5.5-, and 2.5-fold, respectively (Fig. 6). These data indicate that diverse cells exhibit a capacity for IL-1β induction of PI-9 through its NF-κB and AP-1 sites. The different levels of IL-1β induction in the four cell lines may be due to different levels of IL-1β receptor in the cells. Because IL-1β did not induce PI-9 expression in any of the cells transfected with the triple mutant containing nonfunctional NF-κB and AP-1 sites, the NF-κB and AP-1 sites we identified are essential for IL-1β induction in diverse cells.

**Induction of PI-9 by IL-1β and Moxestrol Is Not Synergistic**—Our studies suggest two regulatory contexts for PI-9. Estrogen-receptor complex induces PI-9 transcription by binding to an ERU downstream of the transcription initiation site (8, 16). Second, IL-1β acts through the AP-1 and NF-κB elements in the 5′-flanking region of the PI-9 gene. To determine whether these regulatory pathways act additively or synergistically to control PI-9 gene expression, we examined the effect of the potent and poorly metabolized estrogen, moxestrol, in the presence or absence of IL-1β on PI-9 expression in transiently transfected HepG2 cells. When saturating concentrations of moxestrol (10 nM) and IL-1β (5 ng/ml) were added together to the culture medium, the induction of luciferase activity from the PI-9 promoter was only slightly greater than the induction seen with moxestrol alone (Fig. 7). At sub-maximal concentrations of moxestrol and IL-1β, induction of PI-9 by moxestrol and IL-1β was clearly greater than with either agent alone but was not additive (Fig. 7, 2 ng/ml IL-1β, 1 nM moxestrol). These data suggest that moxestrol alone can elicit a near-maximal level of PI-9 gene expression. Our previous finding that deletion of the AP-1 site did not reduce the fold induction by moxestrol (16) is also consistent with the view that these two regulatory regions do not act synergistically.

**IL-1β Represses ER Transcriptional Activity but ER Does Not Inhibit IL-1β-induced AP-1 and NF-κB Activity**—Because moxestrol and IL-1β do not act synergistically or additively to induce PI-9 gene expression, we examined whether the pre-
ence of moxestrol or IL-1β interferes with induction by the other regulator. To assess the influence of moxestrol-ER (Mox-ER) on PI-9 induction by IL-1β without the complicating factor of Mox-ER induction through the ERU, we constructed a mutant in which the PI-9 ERU was inactivated. Similarly, we evaluated the influence of IL-1β on induction by Mox-ER using mutants in which the AP-1 and NF-κB sites were mutated (see Fig. 3). HepG2ER7 cells, maintained in IL-1β and/or moxestrol, were transiently transfected with PI-9 promoter-luciferase reporter constructs containing mutations at various combinations of the ERU, the AP-1 site, or the two NF-κB sites. Mutation of the ERU abolished moxestrol induction of the PI-9 promoter but maintained IL-1β induction of PI-9 gene transcription (Fig. 8A, mERU). IL-1β inhibited induction by Mox-ER in transiently transfected HepG2ER7 cells. When the AP-1 site or either of the NF-κB sites was mutated, Mox-ER induction was always lower when IL-1β was present (Fig. 8A). To analyze the effect of IL-1β action on Mox-ER induction independent of the ability of IL-1β to induce PI-9 expression, we used the mutant in which both the AP-1 site and the two NF-κB sites were mutationally inactivated. This mutant has completely lost IL-1β induction (Figs. 3 and 6). IL-1β elicited a dose-dependent reduction in Mox-ER induction of PI-9 promoter activity (Fig. 8B). These results suggest that IL-1β-induced proteins such as NF-κB and/or Fos/Fra/Jun family members inhibit ER-mediated transcriptional activation of the PI-9 promoter. Of course, in the context of the native promoter IL-1β induces PI-9 through the AP-1 and NF-κB sites, masking its negative effect on estrogen induction through the ERU.

**DISCUSSION**

Although its properties and biological roles are only beginning to be identified, PI-9 is emerging as an important modulator of inflammatory and apoptotic processes (3–6, 15). PI-9 inhibits caspase-1 and thereby reduces the production of pro-inflammatory cytokines important in maturation and migration of cells of the immune system. PI-9 also inhibits granzyme B and thereby interferes with granzyme B-mediated apoptosis when a cell is targeted by the immune system. Together these effects of PI-9 represent a powerful multi-level system for modulating the interrelated inflammatory and apoptotic functions of caspase-1 and granzyme B (3–6, 15). Despite its importance, little was known about the regulation of PI-9 gene expression. A novel downstream estrogen responsive unit and an upstream
AP-1 site we recently described (16) were the only reported regulatory elements in the human PI-9 gene. It was therefore of unusual interest to examine the PI-9 promoter region for functional regulatory elements susceptible to control by agents important in inflammatory and apoptotic processes.

Because we identified a consensus AP-1 site and three potential imperfect NF-κB sites in the 5′-flanking region of the human PI-9 promoter, we tested the ability of pro-inflammatory agents known to activate gene expression through NF-κB and AP-1 sites to induce expression of PI-9. IL-1β, LPS, TNF-α, and TPA effectively induced PI-9. The rapid induction of PI-9 mRNA was accompanied by induction of PI-9 protein. Consistent with our findings, after completion of this work, two very recent papers on granzyme B-mediated apoptosis suggested that LPS induces PI-9 in some tumors and in dendritic cells (Refs. 6 and 15; for review, see Ref. 35). However, these studies were limited to qualitative demonstrations of increases in PI-9 mRNA or protein and did not involve analysis of the PI-9 promoter, protein-DNA interaction studies, or investigation of the effects of IL-1β or other regulators. These workers suggested that LPS induction of PI-9 might protect dendritic cells against granzyme B released by CTLs or host cells. PI-9 may therefore exert its anti-inflammatory and anti-apoptotic effects both by reducing the production of pro-inflammatory cytokines that help attract CTLs and NK cells to target cells and by inhibiting the ability of their granzyme B to induce apoptosis of target cells.

PI-9 inhibits caspase-1, and IL-1β is produced by caspase-1 cleavage of the inactive IL-1β precursor. We therefore elected to focus on IL-1β induction of PI-9 gene expression as an unusual example of end-product regulation of gene expression. Our identification of functional imperfect NF-κB sites at −135 and −88 and a consensus AP-1 site at −308 of the PI-9 5′-flanking region (Fig. 1A) was of interest since inducible expression of several AP-1 regulated genes, including α1-antichymotrypsin (28), glutathione transferase (29), and collagenase (36), involves juxtaposed regulatory motifs. Mutational inactivation of the NF-κB sites at −135 and −88 and the AP-1 site at −308, both individually and in combination, indicates that all three sites are important in regulating PI-9 gene expression in most and perhaps all cells.

Using oligonucleotides with sequences corresponding to the AP-1 site of the PI-9 promoter and antibodies specific for members of the Fos/Jun/Fra families, c-Jun, JunD, and c-Fos were identified as the components of DNA-protein complexes in HepG2 cells. Because Jun family members bind to AP-1 sites with a much higher affinity when associated with members of the Fos family (37), in HepG2 cells, a Jun/Fos heterodimer likely mediates regulation at the AP-1 site in the PI-9 promoter.
The IL-1β-signaling pathway activates both AP-1 proteins and NF-κB transcription factor. NF-κB is of central importance in immune and inflammatory responses (23, 24). Our findings suggest that IL-1β-induced expression of PI-9 is mediated by activation of NF-κB and binding of activated NF-κB to imperfect NF-κB sites in the 5′-flanking region of the PI-9 promoter. The NF-κB elements at −135 and −88 could be recognized and bound by the NF-κB p50/p65 heterodimer complex. Both the individual AP-1 family members and the NF-κB p50/p65 heterodimer complex were strongly induced by IL-1β. It is becoming increasingly evident that NF-κB not only exerts a pro-apoptotic function but also exercises an anti-apoptotic role through induction of anti-apoptotic genes such as c-IAP, XIAP, Bcl-2, and c-FLIP (38). Thus, it is not surprising that the anti-apoptotic PI-9 gene is induced in part by NF-κB.

Ligated ERα or glucocorticoid receptor are reported to antagonize cytokine-induced NF-κB or AP-1 activity. The steroid receptors are thought to act as antagonists through physical interaction between NF-κB and the steroid hormone receptor (39–42). Because induction of PI-9 by moxestrol and IL-1β was less than additive, it seemed possible that the estrogen-ER complex might antagonize induction by IL-1β and that IL-1β might antagonize induction by ER. With both an estrogen responsive unit downstream of the transcription start site (8, 16) and two functional NF-κB elements and an AP-1 site upstream of the transcription start site, the PI-9 promoter is an excellent system in which to test the effect of estrogen-NF-kB complex on induction mediated by both NF-κB and AP-1 sites. Using a PI-9 promoter construct with a mutagenically inactivated estrogen responsive unit, we find that Mox-ER is unable to repress IL-1β induction of PI-9 gene expression. Although there have been no previous studies of estrogen down-regulation of a cellular gene responsive to both estrogens and NF-κB, synergistic activation of transcription by an NF-κB site and an AP-1 site may reflect a need to prevent cells from producing levels of PI-9 so high that they completely block IL-1β production and make the cells fully resistant to granulocyte-macrophage colony-stimulating factor (GM-CSF) mediated apoptosis.

By inducing production of IL-1β through caspase-1 cleavage of its inactive precursor, pro-inflammatory agents such as LPS and TNF-α can cause a wide variety of inflammatory conditions (43). The importance of maintaining IL-1β homeostasis to prevent systemic inflammatory reactions is illustrated by the existence of multiple regulatory mechanisms for attenuating IL-1β production. These regulators include pro-caspase-1 and caspase-1 inhibitory proteins like PI-9 (3, 4), ICEBERG (44) and COP (caspase recruitment domain only protein) (45) as well as the IL-1β receptor antagonist (46).

This work leads us to suggest the existence of a novel negative feedback loop in which IL-1β produced by activated caspase-1 negatively regulates its own production by inducing expression of the caspase-1 inhibitor, PI-9. The kinetics of induction of PI-9 protein, whose level begins to increase 4–8 h after the addition of LPS or IL-1β to the culture medium, is consistent with this idea. LPS usually induces rapid activation of caspase-1 and a burst of IL-1β production and secretion (32, 33). We speculate that one way to limit the ability of this system to produce potentially toxic levels of IL-1β for extended periods of time is for IL-1β to inhibit an inducer of its own production, PI-9.

Our intriguing finding that pro-inflammatory agents include IL-1β, LPS, and TNF-α induce expression of the gene coding for the anti-inflammatory protein PI-9 suggests that the PI-9 system may serve as a brake to restrain and attenuate uncontrolled and potentially deleterious inflammatory processes, such as septic shock, and apoptotic processes. The diverse agents that induce PI-9, including, estrogens, TPA, IL-1β, LPS, and TNF-α, suggest that regulated production of PI-9 may have a central role in modulating inflammatory processes and apoptosis mediated by cells of the immune system.

Acknowledgment—We are grateful to Dr. R. Dodson for many helpful comments on the manuscript.

REFERENCES

1. Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C., Coughlin, P. B., Gettings, P. G. W., Irvine, J. A., Lomas, D. A., Luke, C. J., Moyer, M. W., Pemberton, P. A., Remold-O’Donnell, E., Salvensen, G. S., Travis, J., and Whistock, J. C. (2001) J. Biol. Chem. 276, 33293–33298
2. Pompeia, J., Koretz, E., and Travis, J. (1994) J. Biol. Chem. 269, 15557–15560
3. Bird, C. H., Sutton, V. R., Sun, J., Hirst, C. E., Novak, A., Kumar, S., Trapani, J. A., and Bird, P. I. (1998) Mol. Cell. Biol. 18, 6387–6398
4. Anderson, R. R., Dahlen, J. R., Strocher, C. A., De Dreu, P., Foster, D. C., Mankovich, J. A., Talania, R. V., Kisel, W., and Giegle, D. A. (1999) Biochem. J. 342, 655–665
5. Young, J. L., Sukhova, G. K., Foster, D., Kisel, W., Libby, P., and Schobert, U. (2000) J. Exp. Med. 191, 1535–1544
6. Medema, J. P., de Jong, J., Potempa, L. T. C., Verdegaal, E. M. E. G., Gaffney, E. P., Limjuco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T., Lee, T. D., Shively, J. E., MacCoss, M., Umoff, R. A., Schmidt, J. A., and Tooci, M. J. (1999) Nature 356, 766–774
7. Kanamori, H., Krieg, S., Rothe, M., Pemberton, P. A., Remold-O’Donnell, E., Salvensen, G. S., Travis, J., and Whistock, J. C. (2001) J. Biol. Chem. 276, 28808–28815
8. Miyamoto, S., and Verma, I. M. (1995) Mol. Cell. Biol. 15, 365–376
9. Koj, A. (1996) Biochim. Biophys. Acta 1317, 84–94
10. Abate, C., Patel, L., Rauscher, F. J., III, and Curran, T. (1990) J. Biol. Chem. 265, 15940–15946
11. Koj, A. (1996) Biochim. Biophys. Acta 1317, 84–94
12. Abate, C., Patel, L., Rauscher, F. J., III, and Curran, T. (1990) J. Biol. Chem. 265, 15940–15946
13. Koj, A. (1996) Biochim. Biophys. Acta 1317, 84–94
14. Bladergroen, B. A., Strik, M. C., Bovenschen, N., van Berkum, O., Scheffer, G. L., Meijer, C. J., Hack, C. E., and Kummer, J. A. (2001) J. Immunol. 166, 3218–3225
15. Medema, J. P., Schuurhuis, D. H., Rea, D., van Tongeren, J., de Jong, J., Bros, S. A., Laban, S., Toes, R. E. M., Toes, M., Schumacher, T. N. M., Bladergroen, B. A., Ossendorp, F., Kummer, J. A., Melief, C. J. M., and Offringa, R. (2001) J. Exp. Med. 194, 657–667
16. Krieg, S. A., Krieg, A. J., and Shapiro, D. J. (2001) Mol. Endocrinol. 15, 1971–1982
17. Zen, K., Karsan, A., Stempien-Otero, A., Yee, E., Tupper, J., Li, X., Eunson, T., Kay, M. A., Wilson, C. E., Winn, R. K., and Harlan, J. M. (1999) J. Biol. Chem. 274, 28808–28815
18. Koj, A. (1996) Biochim. Biophys. Acta 1317, 84–94
19. Abate, C., Patel, L., Rauscher, F. J., III, and Curran, T. (1990) Science 249, 1157–1161
20. Karin, M. (1995) J. Biol. Chem. 270, 16483–16486
21. Johnson, P. F., and McNight, S. L. (1989) Annu. Rev. Biochem. 58, 799–839
22. Miyamoto, S., and Verma, I. M. (1995) Adv. Cancer Res. 66, 255–292
23. Baeuerle, P. A., and Baltimore, D. (1996) Cell 87, 13–20
24. Bégüm, C. H., Song, H. Y., Gao, X., Goedder, D. V., Cao, Z., and Rothe, M. (1997) Cell 89, 373–383
25. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Research 19, 2499
26. Stein, B., and Yang, M. X. (1995) Mol. Cell. Biol. 15, 4971–4979
27. Ni, A., Chau, L., and Chao, J. (1998) J. Biol. Chem. 273, 2784–2781
28. Kerdula, T., Bagno, M., Rydel, R. E., and Travis, J. (2000) J. Neurosci. 20, 7510–7516
29. Suzuki, T., Morimura, S., Diieno, M. B., Yamada, R., Hochi, S.,
Induction of PI-9 by Pro-inflammatory Agents

39. Harnish, D. C., Scicchitano, M. S., Adelman, S. J., Lyttle, C. R., and Karathanasis, S. K. (2000) Endocrinology 141, 3403–3411
40. Tsai, M., and O’Malley, B. (1994) Annu. Rev. Biochem. 63, 451–486
41. Wissink, S., van Heerde, E. C., van der Burg, B., and van der Saag, P. T. (1998) Mol. Endocrinol. 12, 355–363
42. Scheinman, R. I., Gualberto, A., Jewell, C. M., Cidlowski, J. A., and Baldwin, A. S., Jr. (1995) Mol. Cell. Biol. 15, 943–953
43. Dinarello, C. A., and Wolff, S. M. N. (1993) N. Engl. J. Med. 328, 106–113
44. Humke, E. W., Shriver, S. K., Stanovnik, M. A., Fairbrother, W. J., and Dik, V. M. (2000) Cell 103, 59–111
45. Lee, S. H., Stehlik, C., and Reed, J. C. (2001) J. Biol. Chem. 276, 34495–34500
46. Burger, D., and Dayer, J. (2001) in Cytokine Reference: A Compendium of Cytokine and Other Mediators of Host Defense, (Oppenheim, J. J., and Feldmann, M., eds) Vol. 1, pp. 319–336, Academic Press, Inc., San Diego, CA