Proteinase Inhibitor 9, an Inhibitor of Granzyme B-mediated Apoptosis, Is a Primary Estrogen-inducible Gene in Human Liver Cells*

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Although liver is an estrogen target tissue, the number of hepatic genes known to be directly induced by estrogen is very small. We identified proteinase inhibitor 9, or PI-9, as being rapidly and strongly induced by estrogen in an estrogen receptor-positive human liver cell line (HepG2-ER7). Since PI-9 mRNA was also induced by estrogen in a human liver biopsy sample, PI-9 is a genuine estrogen-regulated human gene. PI-9 is a potent inhibitor of granzyme B and of granzyme B-mediated apoptosis. Estrogens induced PI-9 mRNA within 2 h, PI-9 mRNA levels reached a plateau of 30–40-fold induction in 4 h, and induction was not blocked by cycloheximide, indicating that induction of PI-9 mRNA is a primary response. The antiestrogen trans-hydroxytamoxifen was a partial agonist for PI-9 mRNA induction, whereas the antiestrogen ICI 182,780 was a pure antagonist. Western blot analysis showed that estrogen strongly increases PI-9 protein levels. Inhibition of transcription with actinomycin D resulted in identical rates of PI-9 mRNA decay in the presence and absence of estrogen. We isolated genomic clones containing the PI-9 promoter region, identified a putative transcriptional start site, and carried out transient transfections of PI-9-luciferase reporter gene constructs. The estrogen, moxestrol, elicited a robust induction from the PI-9-luciferase reporter gene constructs. The estrogen, moxestrol, elicited a robust induction from the PI-9-luciferase reporter gene constructs. The estrogen, moxestrol, elicited a robust induction from the PI-9-luciferase reporter gene constructs.
**Experimental Procedures**

**Differential Display**—Differential display was modified and performed as we recently described (2). Briefly, total cell RNA was prepared using guanidine thiocyanate extraction and centrifugation through cesium chloride (16). Before reverse transcription, the RNAs were DNase-treated. Primer 1 (5'-TTGTTGTAACTTTGTTTA-3') was used for reverse transcription, and the cDNAs were fractionated on a Bio-Rad Gradient Purification column as a calcium phosphate precipitate used for precipitate essentially as described (17), except that the ER cDNA was from the HEGO plasmid (18). Cells were cultured in Eagle's minimal essential medium (Life Technologies, Inc.) supplemented with 1 mM HEPES, 2 mM glutamine, 0.1 mM Eagle's nonessential amino acids, 1.0 mM sodium pyruvate, 50 μg/ml gentamicin, 10% fetal bovine serum, and 10 μM ICI 164,384 during selection in 1000 μg/ml G418 (Life Technologies). Stable ER-expressing clones were identified by immunocytochemical and Western blot analyses (19) using anti-ER antibodies kindly provided by G. Greene (University of Chicago). Using a whole cell binding assay for tritiated 17β-estradiol, we determined that the HepG2-ER7 clonal isolate contains approximately 30,000 ER sites/cell. HepG2-ER7 cells were routinely cultured in Dulbecco's modified Eagle's medium, 10% dextran-coated charcoal-treated fetal bovine serum. Three independent HepG2 cell lines expressing Flag epitope-tagged hERα were isolated using the bicistronic system essentially as we recently described for ER-positive HEla cells (20). The HepG2ER clones were selected in Dulbecco's modified Eagle's medium, 10% dextran-coated charcoal-treated fetal bovine serum containing 800 μg/ml G418 and 50% HepG2-conditioned medium.

**Northern Blotting**—cDNA coding for PI-9 was cloned by RT-PCR using 2 μg of LA PCR kit from Promega, Madison, WI according to the manufacturer's instructions. Using total RNA from cells treated with 17β-estradiol, RT-PCR was carried out using PI-9 5'-5'-GGAGGGGTCCATGCTA-3' and 3' (5'-CACCTTTATGGCGATGA-3') primers. The amplified PCR product (nucleotides 87-1240 of PI-9 cDNA, GenBank accession number L40378) was subcloned into the pGEM-T vector (Promega, Madison, WI), and its identity was confirmed by sequencing. The plasmid was then digested with ScaI and used as a template for in vitro transcription using SP6 RNA polymerase to generate an RNA probe containing 421 nucleotides of the PI-9 coding sequence (Fig. 1B). Total cell RNA was prepared using Trizol reagent (Life Technologies) in combination with DNase treatment. 10 μg of total RNA was glyoxal-treated, run on a 1% agarose gel, transferred to a BIODYNE PLUS membrane (Pall, BioSupport Division, Port Washington, NY), blocked with 3% nonfat milk. Incubations with antibodies were for 1 h with a 30-min wash between incubations followed by a final wash for 11 min. 0.1% SDS wash at 65 °C. The probe used for initial screening corresponded to nucleotides 53 to 1205 of the PI-9 cDNA, and the probe used for plaque purification of candidate-positive phage corresponded to nucleotides 53 to 297 (PI-9 cDNA sequence, GenBank accession number L40378). The probes were labeled by random hexamer priming with [α-32P]dCTP (Amersham, Arlington Heights, IL) with [α-32P]dCTP (Amersham, Arlington Heights, IL). After plaque purification, the PI-9 promoter was isolated from the phage by PCR and cloned into the pGEM-T vector (Promega). Sequencing of the promoter was carried out using the Big DYF terminator cycle sequencing kit (Perkin-Elmer). The promoter sequence was deposited in GenBank (accession number AF200208).

**Generation of Estrogen Response Element (ERE) Mutations in the PI-9 Promoter**—Mutations were generated using the Stratagene Quick-Change kit in each of the potential ERE sequences (Fig. 7 legend) by PCR. The ERE was mutated to a HindIII site in the context of the full-length promoter region of the pGL3 promoter plasmid. Because the promoter contains regions with high GC content, to increase the generation of full-length mutant DNAs, GC melt (CLONTECH, Palo Alto, CA) was added to 1x. Thermocycling parameters were altered from the manufacturer's protocol by using an annealing temperature of 48 °C and an extension time of 12.5 min. The presence of the desired mutation was confirmed by DNA sequencing.

**Transfections**—Transfections were performed using HepG2 cells maintained in Dulbecco's modified Eagle's medium (Life Technologies), 10% charcoal dextran-treated fetal bovine serum, and penicillin-streptomycin. Transfections were done in 6-well plates using calcium phosphate precipitation (21) with 3.8 μg of PTZ181 as carrier DNA, 25 ng of pBLSV40 as an internal standard (Promega), 15 ng of CMVhER, and 100 ng of the indicated PI-9 promoter-luciferase reporter plasmid. The PI-9 promoter region was cloned into the NheI and BglII sites of the PGL3 promoter plasmid (Promega). The promoter fragment was generated by PCR using Pfu turbo (Stratagene, La Jolla, CA) and primers containing either NheI or BglII restriction sites. After shocking the HepG2 cells with 20% glycerol, moselast was added to 10% 35S. Cells were harvested 48 h after glycerol shock, and lysates were assayed using the dual luciferase assay kit according to the manufacturers' protocol (Promega).

**Results**

Identification of PI-9 as an Estrrogen-inducible Gene In Human Liver Cells—HepG2-ER7 cells were maintained in 10-7 M 17β-estradiol (E2) or ethanol vehicle for 48 h, the cells were harvested, and the RNAs were analyzed by differential display. The intensity of one band was markedly increased in the sample from E2-treated cells (Fig. 1A, arrow). The band was isolated from the gel, reamplified, sub cloned into a plasmid vector,
Cells were treated with 10⁻²⁸⁻⁴⁴⁰ and the RNA fragment used as a probe in the Northern blots the coding sequence of PI-9 cDNA. A box denotes the coding sequence of PI-9 cDNA. Arrows under the sequence denote the fragment isolated by differential display (DD fragment, nucleotides 286–440) and the RNA fragment used as a probe in the Northern blots (Probe, nucleotides 820–1240). UTR, untranslated region. C, dose-response curve for moxestrol induction of PI-9 mRNAs. The HepG2-ER7 cells were maintained for 48 h in the indicated concentrations of medium containing either MOX or the ethanol vehicle (−) and RNAs were isolated and analyzed by Northern blotting as described in “Experimental Procedures.” The 4.5- and 2.5-kilobase PI-9 transcripts are indicated by arrows (upper panel). β-actin was used as an internal standard.

and sequenced. Data base analysis revealed that the clone was identical to nucleotides 286–440 of PI-9 mRNA (Fig. 1B).

Estrogen Induces PI-9 mRNA—To determine whether PI-9 mRNA was truly estrogen-inducible, we treated the HepG2-ER7 cells with increasing concentrations of the estrogen, moxestrol, which liver cells metabolize more slowly than 17β-estradiol (22). RNA was isolated and analyzed by Northern blotting using an RNA probe corresponding to the 3'-end of the PI-9 protein-coding region (Fig. 1B). In agreement with earlier reports (6, 23), two PI-9 mRNAs approximately 2.5 and 4.5 kilobases in length were detected (Fig. 1C). PI-9 mRNA levels were quite low in the absence of moxestrol. Induction of both PI-9 mRNAs was readily detected at 1 nM moxestrol, and induction was maximal at 10 nM moxestrol. Although the low basal level of PI-9 mRNA makes precise quantitation difficult, quantitation of the RNA bands indicates that moxestrol induces PI-9 mRNA 30–40-fold.

To determine if the moxestrol induction of PI-9 mRNA was a general property of HepG2 human hepatoma cells, we examined the ability of three additional lines of ER-positive HepG2 cells produced using a different protocol based on the production of a bicistronic ER mRNA (20) and prepared in a different laboratory than the one that isolated HepG2-ER7 cells. Moxestrol strongly induced PI-9 mRNA in all three ER positive HepG2 cell lines (data not shown).

PI-9 Is an Estrogen-inducible Gene in Human Liver—These studies employed a transformed, established human liver cell line. Although HepG2 cells are a widely used model for human liver, we wished to more directly examine the ability of moxestrol to induce PI-9 in human liver. A portion of a biopsy sample was obtained from a 61-year old female patient with a diagnosis of autoimmune hepatitis. Tissue samples were incubated with and without moxestrol, and RNA was isolated and analyzed by RT-PCR (Fig. 2B). For comparison, RT-PCR was also carried out using the same protocol on an RNA sample from control and moxestrol-treated HepG2-ER7 cells (Fig. 2A). Denstometric quantitation of the band from the actin internal standard indicated that the actin mRNA level was 1.4-fold higher in the moxestrol-treated biopsy sample than in the untreated control biopsy sample. PI-9 mRNA was virtually undetectable in the control minus moxestrol sample, and precise quantitation of its level by densitometry was therefore difficult. Moxestrol treatment increased the level of PI-9 mRNA by >5-fold. After correcting for the slight increase in the level of expression of the actin control, the fold induction of PI-9 by moxestrol in the human biopsy sample is at least 4-fold and may actually be much higher. These data demonstrate that estrogen induces PI-9 gene expression in human liver.

Antiestrogens Interfere with Moxestrol Induction of PI-9 mRNA—To investigate the effects of antiestrogens on PI-9 gene expression, trans-hydroxytamoxifen or ICI 182,780 was added to the culture medium with and without moxestrol. Trans-hydroxytamoxifen acted as a partial agonist, inducing low levels of PI-9 mRNA and only partially blocking moxestrol-mediated induction of PI-9 mRNAs (Fig. 3A). These data are consistent with several reports that tamoxifen can be a partial agonist in human liver (22, 24, 25). In contrast, a 100-fold excess of the pure antiestrogen ICI 182,780 completely blocked induction of PI-9 mRNAs by moxestrol (Fig. 3B). These data demonstrate that an estrogen-ER complex is required for induction of PI-9 mRNAs. Dexamethasone did not induce PI-9 mRNA and had little or no effect on moxestrol induction of PI-9 RNA (Fig. 3C).

Induction of PI-9 mRNA Is a Direct or Primary Effect of Estrogen—Steroid hormone-regulated genes are often classified by whether their expression is directly controlled by the hormone-receptor complex or whether their regulation is an indirect or secondary effect requiring prior expression of other hormone-regulated genes (reviewed in Ref. 26). To analyze the nature of the estrogen induction of PI-9 mRNA, we determined the time course of induction and the effect of blocking protein synthesis. The induction of PI-9 mRNA in HepG2-ER7 cells was detected as early as 2 h after the addition of moxestrol to the medium, and PI-9 mRNA levels reached a plateau within 4 h (Fig. 4A). When moxestrol was present, the fully induced level of PI-9 mRNA was maintained for at least 48 h (Fig. 4A). The rapid metabolism of E₂ by liver cells enabled us to examine...
with phosphate-buffered saline to remove E₂ and maintained in estrogen-free medium for the indicated times (0, 4, 8, 12, and 24 h), and RNA was extracted, and the samples were analyzed by Northern blot hybridization as described under “Experimental Procedures”.

HepG2-ER7 cells were maintained in 10⁻⁴ M cycloheximide (CHX) for 30 min, then 10⁻⁶ M E₂ (CHX + E₂) or ethanol vehicle (CHX) was added, and the cells were maintained for 6 h. RNAs were extracted and analyzed by Northern blotting. C, cycloheximide does not block moxestrol induction of PI-9 mRNA. The cells were maintained in medium either containing or lacking 50 μg/ml cycloheximide (CHX) for 30 min, then 10⁻⁶ M E₂ (CHX + E₂) or ethanol vehicle (CHX) was added, and the cells were maintained for 6 h. RNAs were extracted and analyzed by Northern blot analysis. (−), ethanol vehicle only.

the decline in PI-9 mRNA on removal of E₂ from the culture medium. After removal of E₂ from the culture medium, PI-9 mRNA levels declined rapidly, returning to near basal levels in 24 h (Fig. 4B), indicating that continuous exposure to estrogen is required for maintaining the induced level of PI-9 mRNAs. Inhibition of protein synthesis with cycloheximide resulted in superinduction of PI-9 mRNA, a phenomenon observed with numerous mRNAs (27, 28). Cycloheximide did not block the estrogen-mediated induction of PI-9 mRNA. When E₂ and cycloheximide were both present, PI-9 mRNA levels were high and were approximately the levels expected if E₂ and cycloheximide exhibited additive effects (Fig. 4C). The unusually rapid induction of PI-9 mRNA and the inability of cycloheximide administration to block induction indicate that the induction of PI-9 mRNA is a direct or primary effect of estrogen.

Estrogen Does Not Stabilize PI-9 mRNA—In addition to its widely studied ability to increase rates of gene transcription, estrogen can act posttranscriptionally to alter mRNA stability (29). To examine the decay of PI-9 mRNA in the presence and absence of estrogen, we employed the most widely used method for analyzing mRNA degradation, inhibition of transcription with actinomycin D. After actinomycin D treatment, the rate of PI-9 mRNA decay was the same when the cells were maintained in the presence and absence of estrogen (Fig. 5). Since actinomycin D can sometimes induce artifacts in the measurement of mRNA decay rates (30, 31), it was important to confirm these data using another inhibitor of RNA synthesis. Estrogen also failed to alter the half-life of PI-9 mRNA when 100 μM 5,6-dichlorobenzimidazole riboside was used to inhibit RNA synthesis (data not shown). We therefore conclude that estrogen does not stabilize PI-9 mRNA.

Estrogen Induces Transcription of PI-9—Although cellular PI-9 mRNA is strongly estrogen-inducible and the data of Fig. 5 indicates that estrogen does not increase the stability of PI-9 mRNA, it was important to directly test the idea that estrogen induces PI-9 gene transcription. We therefore screened a human phage library, isolated genomic PI-9 clones, and sequenced the promoter region. To determine the transcription initiation site, we carried out an initial primer extension analysis, which suggested that PI-9 mRNA contained a long −480-nucleotide 5'-untranslated region. The unusually high GC content of the proposed 5'-untranslated region made it impossible to carry out primer extension using primers closer to the putative start site (data not shown). We therefore identified a candidate transcription start site by comparison to consensus transcription initiation elements and confirmed its identity by RT-PCR using 5' primers just upstream and just downstream of the putative start site. The putative transcription initiation
region contained all three elements known to specify a eukaryotic transcription start site, a TATA box, an initiator element, and a downstream promoter element (DPE) (boxed sequences, Fig. 6). The TATA box and the initiator sequence differ from their respective consensus sequences by one nucleotide, and the DPE contains the consensus GACG sequence. In addition, the spacing of these three elements strongly suggests that they are functional. The TATA box and initiator are 27 nucleotides apart, and the DPE is 32 nucleotides downstream of the initiator. Since functional vertebrate initiator elements are usually 25–30 nucleotides downstream of the TATA box (32) and functional DPEs are 28–34 nucleotides downstream of the initiator (32), the TATA box, initiator, and DPE are correctly spaced to form a functional PI-9 initiation site.

To confirm the location of the initiation site, we used a method we described previously (33) in which PCR is carried out with primers just upstream and just downstream of the candidate site. If the transcription start site has been correctly identified, the sequence corresponding to the upstream primer will not be reverse-transcribed into cDNA and will not yield a PCR product. PCR using the downstream primer, which should hybridize to a transcribed sequence, should result in a PCR product. As predicted from the sequence data, a strong band of the correct size PCR product was obtained with a primer corresponding to nucleotides +2 to +22 (the numbering is derived from the position of the initiator element), and there was no detectable PCR product using a 5’ primer corresponding to nucleotides −19 to +2 (Fig. 6). Taken together, the sequence and spacing of the initiation site elements and the PCR data localize the transcription start site to this region.

To determine whether moxestrol-ligated ER induced transcription from the PI-9 promoter, we prepared a construct in which a 1.8-kilobase DNA containing the PI-9 promoter region was induced 12-fold by moxestrol. 17β-estradiol (22), in most experiments we used the more slowly metabolized estrogen, moxestrol. 17β-Estradiol and moxestrol elicited similar inductions of PI-9 mRNA (see Fig. 4B with 10 nM MOX in Fig. 1C; and data not shown). Since moxestrol induced PI-9 mRNA in a human liver biopsy specimen and in three additional independently isolated lines of HepG2 cells stably transfected to express ER, PI-9 is an estrogen-inducible gene in human liver. The 30–40-fold estrogen induction of PI-9 mRNA in HepG2-ER7 human liver cells is unusually large. Although several estrogen-inducible human mRNAs have been identi-
levels of PI-9 mRNA in human placenta (23) are due to induction of PI-9 gene expression by the high levels of estrogen to which the placenta is exposed.

CTLs and granzyme B are important mediators of graft versus host disease (34). However, there is as yet no information on the role of estrogen in liver transplant rejection. A more direct relationship between estrogen and apoptosis induced by CTLs exists for chronic aggressive hepatitis. Apoptosis, induced when CTLs recognize viral antigens displayed on the surface of infected cells, appears to be the major factor in the liver damage observed in chronic aggressive hepatitis (35). Iwamura (36) describes the importance of estrogen in protecting liver cells from immunologically mediated attack. A high level of serum estrogen was associated with a less severe level of hepatic injury caused by hepatitis. In a large clinical study, interferon therapy for hepatitis was far more effective in premenopausal women than in postmenopausal women (37). Higher estrogen levels were proposed as the major factor enhancing interferon therapy in premenopausal women (37). Estrogen depletion after menopause or bilateral ovariectomy tends to aggravate chronic hepatitis, even though the disease has been inactive (38). In addition, estrogen was used therapeutically to successfully treat women with chronic aggressive hepatitis, resulting in regression to the inactive state (38). Although estrogen metabolism by liver cells makes it difficult to determine the minimum concentration of estrogen in the cell culture medium that effectively induces hepatic PI-9 mRNA (Fig. 1), the levels of circulating estrogen in women during at least part of the menstrual cycle and pregnancy (39) are likely to be sufficient to induce substantial expression of hepatic PI-9.

Recently Bird and co-workers (6, 7) concluded that it was unlikely PI-9 evolved to protect against a direct hit by a CTL. Instead, they propose that PI-9 neutralizes lower levels of misdirected granzyme B that may inadvertently threaten a bystander cell or CTL. They conclude that it will be difficult to develop a test or assay for this type of indirect protection (7). If such an assay could be developed, future studies relating PI-9 regulation by estrogen to the sensitivity of liver cells to granzyme B-mediated apoptosis would shed light on the role and importance of ER in PI-9 expression and on the function of the immune system.

These studies extend the area of estrogen action to a new class of proteins important in immune system function and in apoptosis. Estrogen induction of PI-9 in human liver cells may have significant biological roles in modulating the extent of hepatic injury caused by hepatitis (viral and autoimmune) and by graft versus host reaction in liver transplants. Our work describes a potential point of intersection between the action of sex steroid hormones at the gene level and a key function of the immune system, induction of apoptosis.

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