Transcriptional Regulation of Human Protease-Activated Receptor 1: A Role for the Early Growth Response-1 Protein in Prostate Cancer

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Abstract

Transcriptional regulation plays a central role in the molecular pathways underlying preferential cancer growth and metastasis. In the present study, we investigated the regulation of human protease-activated receptor 1 (hPar1) gene overexpression in the malignant androgen hormone-resistant phase. We found increased hPar1 RNA chain elongation and no change in message stability in cells with high levels of PAR1 expression, indicating that increased transcription is largely responsible for the overexpression of hPar1 in prostate tumor progression. Enforced expression of early growth response-1 (Egr-1) plasmid markedly enhanced luciferase activity driven by the hPar1 promoter. The neuroendocrine peptide bombesin significantly induced hPar1 expression and increased the ability of the cells to invade Matrigel, an effect abolished by expression of hPar1 small interfering RNA, showing the importance of hPAR1 in invasion. Bombesin also markedly enhanced Egr-1 binding to the hPar1 promoter in vivo and in vitro. These data suggest that bombesin enhances Egr-1 expression leading to increased hPar1 transcription, thereby increasing PAR1 expression and function. Immunohistochemistry of prostate tissue biopsy specimens revealed a direct correlation between the degree of prostate cancer malignancy, PAR1 expression, and EGR-1 expression. Altogether, we show that transcriptional regulation of hPar1 in the aggressive hormone-resistant prostate cancer stage is controlled in part by the transcription factor Egr-1 and may play a central role in malignancy, an important indicator of malignancy. [Cancer Res 2007;67(20):9835–43]

Introduction

It is well established that cancer growth, development, and metastasis involve the alterations in the expression of a gene network. Deciphering the transcriptional pathways that regulate preferential expression of the genes that trigger cancer development may identify the basic determinants of cancer initiation and progression (1, 2). Prostate cancer progresses in distinct stages from prostatic intraepithelial neoplasia to locally invasive carcinoma and metastatic spread (3). Genes that act as transcription factors, cytokines, and differentiation factors. Egr-1 binds to GC-rich elements and is mainly regulated by posttranscriptional mechanisms, such as phosphorylation (8–10). The protein contains a highly conserved DNA binding domain composed of three zinc fingers located in the COOH-terminal domain (11, 12). All human prostate cancer cells tested express high levels of Egr-1. In contrast, there is no Egr-1 expression in prostate normal cells (13, 14). The notion that Egr-1 plays a central role in prostate cancer progression is strongly supported by observations in Egr-1–deficient mice showing impaired prostate tumorigenesis (15). Egr-1 has been shown to bind both nerve growth factor-I A binding protein (NAB) 1 and NAB2, regulatory proteins that repress Egr-1 transcriptional activity. Whereas NAB1 is widely expressed at low levels, NAB2 displays tissue-restricted regulation and is downregulated in primary prostate carcinomas (16). Thus, the loss of Egr-1 repressor NAB2 along with Egr-1 up-regulation play together a critical part in prostate cancer progression. In addition, neuroendocrine differentiation has generated interest as a likely contributor to prostate cancer progression. Accumulating evidence supports the idea that malignant prostate cells can differentiate into neuroendocrine-like cells (17) and probably secrete essential factors that promote prostate cancer progression (17, 18).

Here, we explored the molecular mechanisms underlying increased PAR1 expression in malignant cancer cells, revealing a role for the transcription factor Egr-1 in transcriptional regulation of hPar1 and in the progression of prostate cancer.
Materials and Methods

Cell lines. The prostate adenocarcinoma cell lines LNCaP, CL1, PC3, and DU-145 were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin. 293T cells were cultured in DMEM supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin. All culture medium and supplements were purchased from Beit Haemek.

Small interfering RNA constructs. We used U6 promoter-driven and lentivirus (pLentilox 3.7)-mediated delivery cassette of small interfering RNA (siRNA) specific for hPar1. For this, a sequence of 19 nucleotides of the hPar1 coding region was selected for stem-and-loop oligonucleotide siRNA. The selected sequences were submitted to a BLAST search against the hPar1 coding region, and a terminator sequence poly T. The sticky end of the DNA oligonucleotides: 19 bases of hPar1 quenclinker(9bases), reversecomplementofthe19basesof hPar1 were added to the antisense strand oligos. Both sense and antisense region, and a terminator sequence poly T. The sticky end of the DNA oligonucleotides: 19 bases of hPar1 coding sequence, the loop sequence linker (9 bases), reverse complement of the 19 bases of hPar1 coding region, and a terminator sequence poly T. The sticky end of the Xhol site was added to the antisense strand oligos. Both sense and antisense sequences were phosphorylated at the 5'-ends. The sense sequence oligos were annealed to their respective antisense oligos. siRNA cassette sequences were then ligated into pLentilox 3.7 vector (Van Parij’s Laboratory). We created four such siRNA cassettes from the hPar1 gene.

Preparation of lentivirus. Lentivirus particles were generated by a lentiviral system (VSV-G), and the transfer vector pLentilox 3.7. One day before transfection, 293T cells were plated to 60% confluency. On the next day, cells were fed with the following three vectors: packaging (CMV-D R8.91), envelope (CMV- A) hPar1 expression as determined by real-time PCR analyses. Calculation of hPar1 mRNA isolated from cells treated with DRB for the indicated times.

Real-time PCR. Real-time quantitative PCR analysis was done with an automated rotor gene system RG-3000A (Corbett Research). The PCR mix (20 μL) was composed of 10 μL QPCR SYBR green mix (ABgene), 5 μL of diluted cDNA (each sample in a sixplicate), and a final concentration of 0.3 μmol/L from each primer. PCR conditions were as follows: an initial denaturation step at 95°C for 15 min and 40 cycles of denaturation at 94°C for 15 s, hybridization at 57°C for 30 s, and elongation at 72°C for 30 s. The primers used for this reaction are as follows: upstream hPar1, 5'-GGATGATGTTCTGGAGAGCC-3'; downstream hPar1, 5'-GGATGATGTTCTGGAGAGCC-3'; and downstream human GAPDH, 5'-CTAGACGGCAGGTGTCAGG-3'.

PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

Regulation of hPar1 transcription in prostate cancer cells. A, hPar1 mRNA stability. i. Northern blot analysis of hPar1 and control gene 28S mRNA isolated from cells treated with DRB for the indicated times. ii. histogram of the relative band intensity showing that hPar1 mRNA half-life (t1/2) is ~30 min in cells expressing high or low levels of hPar1 mRNA. Columns, mean (n = 4); bars, SD. iii. histogram of the relative hPar1 mRNA expression as determined by real-time PCR analyses. Calculation of hPar1 mRNA half-life (t1/2) indicated a t1/2 of ~27 min in cells expressing high hPar1 (CL1) and a t1/2 of ~32 min in cells expressing low hPar1 (LNCaP). Columns, mean (n = 6); bars, SD. B, nuclear run-on assay, i. a representative assay shows that hPar1 RNA elongation is markedly increased in cells expressing high hPar1 levels (CL1 and PC3) compared with cells expressing low levels of hPar1 (LNCaP). ii. histogram representing the relative transcription activities showing 6.5- to 8-fold increase in hPar1 transcript in PC3 and CL1 cells compared with LNCaP cells. Columns, mean (n = 6); bars, SD. Statistical significance determined by Student’s t test compared with LNCaP cells: * P < 0.005 (PC3); ** P < 0.005 (CL1).
GAPDH primers were used as an internal control. The expression level of hPar1 without treatment was considered as 100% and the expression at different time points was compared with this 100% value.

Transfection and luciferase expression assay. 293T cells at 60% to 80% confluency, grown for 48 h in steroid-depleted cell culture medium, were transfected with 2 µg of the hPar1 promoter fragment plasmid DNA Puc- HTR/-4.1(F1) (kindly provided by Dr. Marschall S. Runge, University of North Carolina at Chapel Hill, Chapel Hill, NC). Egr-1 plasmid (kindly provided by Dr. Ian de Belle, Laval University Hospital Center Research Center, Quebec City, Quebec, Canada) or empty expression plasmid (2 µg), and CMV/Jgal (0.3 µg) in Fugene 6 transfection reagent (Boehringer Mannheim) according to the manufacturer’s instructions. After 48 h, the cells were lysed in 0.1 mL of lysis buffer (Promega). Cell lysis was transferred to a 1.5-mL microcentrifuge tube and cleared by centrifugation at 12,000 rpm for 2 min at 4°C. Luciferase activity was measured by mixing 20 µL of supernatant in a 96-well microtiter plate with 100 µL of luciferase assay substrates (Promega) and read using a luminometer (Mithras LB 940, Berthold Technologies).

Nuclear run-on assay. Nuclei isolated from 5 × 10⁶ cells were centrifuged in a conical tube and washed twice with ice-cold PBS. Cells were then lysed on ice for 5 min in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 3 mmol/L CaCl₂, and 2 mmol/L MgCl₂. After centrifugation (15 min at 500 × g), the supernatant was removed and replaced by 1 mL lysis buffer. Remaining intact cells were lysed by NP40 lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 3 mmol/L CaCl₂, 2 mmol/L MgCl₂, 1% (v/v) NP40]. Then, cells were transferred and homogenized in ice-cold Dounce homogenizer until the nuclei appear free of membrane components by phase-control microscope. Nuclei were then centrifuged and resuspended in 200 µL of glycerol storage buffer [50 mmol/L Tris-HCl (pH 8.3), 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 40% (v/v) glycerol] and stored at −80°C. Transcriptional elongation was carried out as follows: frozen nuclei were thawed and resuspended in 1 volume (200 µL) of reaction buffer (30% glycerol, 2.5 mmol/L DTT, 7.0 mmol/L KCl, 0.5 mmol/L of recombinant ATP, recombinant CTP, and recombinant GTP, 100 µCi [³²P]UTP (1 mg/mL heparin, RNase inhibitor) and reaction was carried out for 20 min at 30°C. Nascent labeled RNA was isolated by TRI reagent as mentioned above. RNA was resuspended in hybridization buffer and heated for 1 min at 65°C and hybridized to CDNA immobilized to prehybridized nitrocelulose membrane strips. After overnight hybridization, membranes were washed with 2x SSC and 0.2% SDS and exposed to X-ray film.

mRNA stability and Northern blot analysis. Cells were treated with the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) for various times. RNA was isolated as mentioned above, and Northern blot analysis was done. Total RNA (10 µg from CL1 cells and 30 µg from LNcaP cells) was electrophoresed on 1% formaldehyde-agarose gels and transferred to Hybond-N membranes (Amersham Pharmacia Biotech UK Limited). The membranes were hybridized (42°C, 18 h) with [α-³²P]dCTP-labeled (RediPrimer II, Amersham Biosciences UK Limited) probe for human Par1. After hybridization, membranes were washed and exposed to X-ray films. We used the 28S RNA as a control for RNA loading.

Matrigel invasion assay. Blind well chemotaxis chambers with 13-mm-diameter filters were used for this assay. Polycylinyprolidized-free polycarbonate filters, 8-mm pore size (Costar Scientific Co.), were coated with basement membrane Matrigel (25 µg/filter) as described previously (19). Briefly, the Matrigel was diluted to the desired final concentration with cold distilled water, applied to the filters, and dried under a hood. Cells (2 × 10³ to 3 × 10⁵), suspended in DMEM containing 0.1% bovine serum albumin (BSA), were added to the upper chamber. Conditioned medium of 3T3 fibroblasts was applied as a chemoattractant and placed in the lower compartment of the Boyden chamber. Assays were carried out at 37°C in 5% CO₂. More than 90% of the cells attached to the filter after incubation overnight. At the end of the incubation, the cells on the upper surface of the filter were removed by wiping with a cotton swab. The filters were fixed and stained with Diff-Quick System (Dade Behring, Inc.). Cells from various areas of the lower surface were counted and each assay was done in triplicate.

Western blotting analysis. Cells were solubilized in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, and protease inhibitor cocktail (Sigma), including 5 µg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate, for 30 min at 4°C. After centrifugation at 12,000 × g for 20 min at 4°C, the supernatants were transferred and the protein content was determined. Lysates (50 µg) were loaded on a 10% SDS-PAGE followed by transfer to Immobilon-P membrane (Millipore). Membranes were blocked and probed with Anti-PAR1 thrombin receptor monoclonal antibody, ATAP2 (Santa Cruz Biotechnology, Inc.), suspended in 3% BSA in 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/mL NaCl, and 0.5% Tween 20. After washes, blots were incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were detected by the enhanced chemiluminescence reagent (Pierce).

Immunohistochemistry. Sections (5 µm) from tumors were deparaffinized and rehydrated. Tissues were then denatured for 4 min in a microwave oven in a citrate buffer (0.01 mol/L, pH 6.0) for PAR1 and Egr-1 immunostaining or in pressure cooker in Tris-EDTA (pH 9.0) for α-methyl-CoA-racemase (AMACR) staining. The sections were incubated overnight at 4°C with either our rabbit polyclonal anti-human PAR1 antibody, monoclonal anti-Egr-1 antibody (Santa Cruz Biotechnology), or rabbit monoclonal anti-AMACR antibody (DakoCytomation). AMACR tumor marker stain was used to support pathologic decision about neoplastic
status of epithelia. Color was developed using a DacoCytomation kit according to the manufacturer’s instructions (Dako North America, Inc.) and counterstained with Mayer’s hematoxylin.

Electrophoretic mobility shift assay. Nuclear extracts were prepared as described previously (20). In brief, cells were scraped in PBS, and after centrifugation, the cell pellet was reconstituted in a hypotonic lysis buffer (10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA) supplemented with 1 mmol/L DTT and a broad-spectrum cocktail of protease inhibitors (Sigma-Aldrich). The cells were allowed to swell on ice for 15 min, then NP40 was added, and cells were lysed by vortex. After centrifugation, nuclear extracts were obtained by incubating nuclei in a hypertonic nuclear extraction buffer (20 mmol/L HEPES (pH 7.9), 0.42 mol/L KCl, 1 mmol/L EDTA) supplemented with 1 mmol/L DTT for 15 min at 4°C. The supernatant was collected after centrifugation. Complementary oligonucleotide probes were synthesized (Hylabs) for the hPar1 putative Egr-1 sites (5′-ccagtacctggctcagggcaggccgcaaca-3′) and for a mutated version of the putative hPar1 Egr-1 sites (5′-ccagtacctgcctcagggcaggccgcaaca-3′). Oligonucleotides were annealed by heating them to 95°C in Tris-EDTA buffer and cooling slowly to room temperature. The double-stranded probes were labeled with [α-32P]dCTP using RediPrime II. Labeled probes (0.3 ng) were incubated in a total volume of 20 μL with DU-145 cell nuclear extract, 2 μg of poly(dexosinomycin-dexoyctidylid acid), and 2 μL of 10× binding buffer (binding buffer: 100 mmol/L Tris-HCl (pH 8.0), 200 mmol/L KCl, 10 mmol/L MgCl2, 10 mmol/L EDTA, 10 mmol/L DTT, 40% glycerol) at room temperature for 20 min. For competition experiments, 1- to 100-fold concentration of unlabeled double-stranded oligonucleotide was added 15 min before incubation. An oligonucleotide containing a vitamin E binding site was used in nonspecific competition experiments. The samples were loaded on a prerun (100 V for 1 h at room temperature) 6% polyacrylamide gel (acryl/bisacryl = 29:1) and electrophoresed in 0.25× TBE (0.1 mol/L Tris, 0.083 mol/L boric acid, 1 mmol/L EDTA) at 100 V for 3 h at room temperature. The gel then was dried and exposed to X-ray films.

ChIP and PCR analysis. DU-145 cells were grown for 48 h in serum-free starvation medium and then left untreated or treated with 10−8 mol/L bombesin for 2 h. Following treatment, DU-145 cells were fixed with formaldehyde and added directly to the culture medium (to a final concentration of 1% formaldehyde) at room temperature for 10 min to cross-link histone proteins to DNA and then glycine (to a final concentration of 0.125 mol/L) was added to quench formaldehyde. Soluble chromatin was made as follows: cells were scraped and washed and centrifuged following addition of ice-cold PBS and concentrated following centrifugation for 4 min at 700 × g. The resultant cell pellet was then lysed, centrifuged, and lysed in two consecutive lysis buffers: LB1 [50 mmol/L HEPES-KOH (pH 7.5), 140 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton X-100, protease inhibitor cocktail] and then LB2 [10 mmol/L Tris-HCl (pH 8.0), 200 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 0.1% sodium deoxycholate] and sonicated. Samples were then centrifuged at 13,000 rpm for 10 min and supernatant was collected. For immunoprecipitation, 10 μg of antibodies, prebound protein A, were added to 500 μL of the purified chromatin sample and incubated overnight at 4°C. Immuno-complexes with the beads were washed with radioimmunoprecipitation assay buffer followed by a wash with Tris-EDTA, and then the immuno-complexes were recovered by adding elution buffer [1% SDS, 1% NaCl, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0)] for 10 min at 65°C and then by centrifugation at 14,000 rpm for 10 min. Antibody immunocomplexed DNA was then recovered by phenol/chloroform extraction and ethanol precipitation and resuspended in Tris-EDTA. PCR primer sets were designed to overlap and span the Egr-1 binding sites of the hPar1 promoter. Primer set 1 was as follows: 5′-tccagtagggcagcgggcg-3′ (forward) and 5′-gttagacggtgccgcaaca-3′ (reverse). These primers were first evaluated using the Par1-lucerase construct as DNA template. An additional set of primers was used to show the specificity of Egr-1 binding sites directed to either the 5′-untranslated region (UTR) or 3′-UTR. Quantitative PCR was then done with eluted Egr-1 immunocomplexed DNA using the TITANIUM Taq PCR kit (Clontech Laboratories, Inc.). PCR was also done on nonprecipitated chromatin as a positive control and to correct for input volume. Amplification was carried out for 35 cycles (28 cycles for unprecipitated chromatin input lanes) with denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min. PCR products were separated in 2% agarose gel.

Results

Transcriptional regulation of hPar1: run-on assay. In situ hybridization analyses of hPar1 expression in a range of tissue biopsy specimens and reverse transcription-PCR (RT-PCR) analyses of differentially metastatic prostate cancer cell lines show that hPar1 mRNA is overexpressed in prostate tumor tissues and in metastatic cell lines (6). Fluorescence in situ chromosome

Figure 3. Binding properties of a novel Egr-1 binding site in the hPar1 promoter. Nuclear extract of DU-145 cells and 32P-labeled Egr-1 oligos were used in an EMSA. A, bombesin treatment enhanced binding to the Egr-1 sequence oligos. B, dose-dependent inhibition of binding to 32P-labeled Egr-1 oligos by competition with excess unlabeled Egr-1 consensus sequence oligos. Unlabeled Egr-1 oligos were included in the binding reaction at the indicated fold excess over labeled oligo. C, competition with 25× excess unlabeled mutant Egr-1 consensus (mt) sequence oligos or excess nonspecific oligos had no effect on binding to the hPar1 promoter Egr-1 site. D, anti-Egr-1 antibodies were included in the binding reaction and induced a supershift of the hPar1-Egr-1 binding complex. The presence of an irrelevant control IgG antibody had no effect on mobility of the hPar1-Egr-1 binding complex.
hybridization analyses done on cells of high (e.g., CL1) and low (e.g., LNCaP) metastatic potential showed that hPar1 gene copy number remains the same regardless of hPar1 expression level (data not shown), indicating that hPar1 overexpression does not stem from gene amplification. We therefore analyzed both hPar1 transcription rates and mRNA stability to determine how hPar1 mRNA levels are elevated. To evaluate hPar1 mRNA stability, we treated the cells with the transcription inhibitor DRB for various times and then extracted mRNA and analyzed it by both Northern blotting and real-time PCR to determine levels of hPar1 mRNA. We also determined 28S and GAPDH mRNA levels, respectively, as controls. The ratio of hPar1 mRNA/28S or GAPDH mRNA was calculated for each cell type and time point. Using this ratio, the degradation rates for hPar1 mRNA are very similar regardless of whether the RNA comes from cells with high (e.g., CL1 or PC3; data not shown) or low (e.g., LNCaP) hPar1 expression levels (Fig. 1A).

In contrast, when we used nuclear run-on assay to determine transcript elongation rates, we found markedly enhanced hPar1 transcription rates in CL1 and PC3 cells, which express high hPar1 levels, compared with LNCaP, which have low levels of hPar1 expression (see Fig. 1B). We conclude that the increase in hPar1 RNA levels in the malignant cells we examined is primarily due to increased hPar1 transcription.

**Egr-1 induces hPar1 promoter activity and mRNA expression.** Analysis of the hPar1 promoter genomic sequence (accession number U63331) reveals a potential Egr-1 motif between −354 and −335 bp (GGGCGGGGCGGGGCGGGGCG). To show the effect of Egr-1 on hPar1 promoter activity, we cotransfected a wild-type Egr-1 expression plasmid and an hPar1 promoter-luciferase reporter (Luc-F1) plasmid into 293T cells. A constitutive reporter h-gal construct was included as an internal control for transfection efficiency and luciferase activity was normalized to h-gal. Expression of Egr-1 resulted in a 4.7 ± 1.2–fold (n = 6) increase in normalized luciferase activity compared with activity in cells cotransfected with an empty expression vector, indicating induction of hPar1 promoter activity by Egr-1 (Fig. 2A). To further investigate the role of Egr-1 on hPar1 expression, we treated DU-145 cells expressing Egr-1 with bombesin, a neuroendocrine peptide known to enhance Egr-1 binding at the putative Egr-1/Specific protein 1 (Sp1) binding motifs (21). Bombesin treatment

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**Figure 4.** ChIP assay shows the in vivo interaction between Egr-1 and the hPar1-Egr-1 consensus site. Top, 5′ flanking sequences of PAR1 promoter and proposed Egr-1 motif; middle, DNA fragments immunoprecipitated with the indicated antibodies (Ab) were purified and the region containing either 3′-UTR or 5′-UTR distal to the Egr-1 consensus site or the Egr-1 proposed site was amplified by PCR. An equal amount (input) of DNA was applied. PCR products generated using hPar1 promoter primers or GAPDH primers to amplify from immunoprecipitated DNA before (lane 1) or after (lane 2) bombesin treatment of DU-145 cells. Nonspecific levels were obtained when either primers directed to 3′-UTR or 5′-UTR (distal from the site of Egr-1) as also GAPDH or a nonspecific control IgG was used to immunoprecipitate the complex. Representative histogram shows quantification of the band intensity for hPAR1 promoter amplification product after normalization to GAPDH. This is a representative figure of at least three independent experiments done.
increased hPar1 mRNA levels in a concentration- and time-dependent manner as shown by RT-PCR analysis (Fig. 2B, i and ii). Maximal hPar1 mRNA induction was achieved by treating the cells with 10\(^{-8}\) mol/L bombesin for 2 h and hPar1 mRNA remained elevated for at least 6 h following treatment.

**hPar1 promoter contains a functional Egr-1 binding motif.**

The enhanced luciferase-promoter activity along with the demonstration that hPar1 mRNA levels are elevated following induction of Egr-1 expression by coexpression of Egr-1 plasmid or by bombesin treatment in cells known to express Egr-1 strongly support the presence of functional Egr-1 motifs in the hPar1 promoter. Bioinformatic analysis predicted the presence of multiple Egr-1 binding motifs located between −354 and −335 bp in the 5'-UTR of hPar1. This region offers five possible combinatorial options to create an Egr-1 site. To investigate Egr-1 binding to the hPar1 promoter in vitro, we did EMSA analyses. Treatment of DU-145 prostate cancer cells with bombesin (10\(^{-8}\) mol/L) markedly enhanced binding of Egr-1 to an oligo of the Egr-1 binding sites from the hPar1 promoter (Fig. 3A). Binding was inhibited in the presence of excess unlabeled oligonucleotides representing a consensus Egr-1 binding sequence. The inhibition was concentration dependent and binding was almost completely depleted using a 25-fold excess of unlabeled consensus Egr-1 site oligos (Fig. 3B). As the band representing the Egr-1 DNA binding site is markedly weakened due to the presence of excess cold consensus Egr-1 motif, a stronger lower band is becoming evident. This may be due to the established similarities between the binding motifs within the Egr-1 family of proteins. The outcome of these similarities is that each member of the family may interact with overlapping sites in a given promoter but with different affinities. Along this line of evidence, induction of EGR-1 often displaces Sp1, Sp3, and Wilms’ tumor 1 (22). These examples indicate that, at a time when a specific binding site is neutralized/abrogated as in our case in the presence of excess cold Egr-1, another binding motif becomes stabilized designated by increased binding properties.

Excess mutant Egr-1 consensus site oligos and nonspecific oligos had no effect on binding, showing that the binding is sequence specific (Fig. 3C). To further substantiate the specificity of the binding, we did a supershift assay using specific anti-Egr-1 antibodies. When antibodies against Egr-1 were added to the EMSA reaction, a supershift in the Egr-1 binding band was observed, whereas no band shift was seen in the presence of a nonspecific IgG, indicating that the band represents Egr-1 binding to the hPar1 promoter Egr-1 site (Fig. 3D).

**In vivo interactions between Egr-1 and hPar1: ChIP analysis.**

To confirm that association between Egr-1 protein and Egr-1 motifs in the hPar1 promoter occurs in vivo, we did PCR on chromatin DNA fragments specifically immunocomplexed with Egr-1. Chromatin fragments were immunoprecipitated from cultured DU-145 cells before and after bombesin treatment using either anti-Egr-1 antibodies or a control rabbit IgG. DNA from the immunoprecipitated complexes was isolated and amplified by PCR using a set of primers synthesized to cover the region between −402 and −131 bp in the hPar1 promoter, which includes the Egr-1 sites between −354 and −335. The PCR signal obtained in the noncomplexed chromatin (input) was used as a control for DNA loading. When specific Egr-1 antibodies were used for precipitation, there was an ∼4-fold increase in PCR product from cells treated with bombesin compared with untreated cells. When control rabbit IgG was used to immunoprecipitate chromatin, only
minimal amounts of PCR product were observed, most likely representing nonspecific, residual immunoprecipitation products. With control IgG, there was no difference in the amount of PCR product generated from immunoprecipitated chromatin from untreated cells versus cells treated with bombesin (Fig. 4). To show the specificity of Egr-1 binding motif within the promoter, we have done PCR using primers directed to either the 3'-UTR or 5'-UTR located distal to the Egr-1 motif. Although equal amounts of input were applied, no specific enrichment of the immunoprecipitated complex was observed following treatment with bombesin (Fig. 4). These data show that bombesin treatment leads to a specific increase in Egr-1 protein-hPar1 DNA complexes, detected by specific primers directed to the designated site in the hPar1 promoter and not by applying primers directed to other regions, such as 3'-UTR or 5'-UTR (distal to the proposed Egr-1 motif).

**Egr-1 induces functional PAR1 expression.** To show the functional relevance of Egr-1–induced hPar1 expression, we used the Boyden chamber Matrigel invasion assay. For this, we have also applied hPar1-siRNA–infected cells before and after siRNA silencing of PAR1. Indeed, DU-145 cells following viral infection of the hPar1-siRNA construct show that, although empty vector construct show distinct levels of PAR1 on hPar1-siRNA infection, a marked reduction in the PAR1 levels is seen (Fig. 5A). DU-145 cells incubated with SFLIRN, a PAR1-activating ligand, show invasion capabilities that are markedly enhanced following bombesin treatment (Fig. 5B, top). To confirm that the bombesin-induced increase in Matrigel invasion capability is due to increased PAR1 expression, we used hPar1-siRNA to block PAR1 expression. We infected DU-145 cells with an hPar1-siRNA viral construct and analyzed their ability to invade Matrigel-coated filters. The presence of hPar1-siRNA counteracted the effect of bombesin, decreasing invasiveness to slightly below the level seen in untreated DU-145 cells (Fig. 5B). Quantification shows that treatment with bombesin induces the invasiveness of DU-145 cells ~3-fold and hPar1-siRNA abrogates invasion capabilities (Fig. 5B), indicating that the increased invasiveness of DU-145 cells following bombesin treatment is dependent on increased human PAR1 levels.

**PAR1 expression directly correlates with Egr-1 expression and the neoplastic status of prostate tissues.** To show a correlation between expression of hPar1 and Egr-1 in prostate cancer tissues, we immunostained sections of prostate cancer biopsy specimens for both PAR1 and EGR-1. Strong positive staining was observed in neoplastic tissues following immunohistochemistry with both antibodies (Fig. 6, arrowheads). In contrast, very faint or no staining was observed in normal-appearing tissue structures (Fig. 6, arrows). Neoplastic changes were assessed by immunostaining with antibody against AMACR, which showed strong and specific staining in the neoplastic epithelium but no staining of cells with normal appearance. In addition, we observed colocalization of PAR1, EGR-1, and AMACR in neoplastic tissue, indicating that hPar1 expression correlates with Egr-1 expression and the neoplastic potential of human prostate tissue.

**Discussion**

Although a direct correlation has been shown between PAR1 overexpression and tumor malignancy (4–6, 23–29), the molecular mechanism underlying hPar1 overexpression is largely unknown. Recently, Booden et al. (30) proposed that aberrant trafficking of PAR1 (manifested by slowed receptor internalization and/or recycling as well as lack of lysosomal degradation) takes place in breast carcinoma cell lines leading to persistent PAR1 signaling and function. Although persistent signaling is an important facet of tumor malignancy, it provides only one molecular explanation for the pronounced PAR1 overexpression profile observed in cancerous cells. In addition to the increased expression of PAR1 protein, induced hPar1 RNA levels are found in aggressive melanoma (31) and also in a spectrum of tumor biopsy specimens of different origin, including breast (4), ovary (26), endometrium (25), and prostate (6) tissue specimens. We therefore examined transcriptional regulation and mRNA stability of hPar1 in the most aggressive form of prostate cancer, which is hormone resistant.

Previous studies reported an inverse correlation between the expression of activator protein-2α (AP-2α) and PAR1 as well as a direct correlation between Sp1 and PAR1 expression in human melanoma cells (31). It has been proposed that loss of AP-2 in metastatic cells alters the AP-2/Sp1 ratio leading to gain of PAR1.
expression. This hypothesis was verified by microarray assay of melanocyte tissue (32). Although loss of AP-2 was shown in prostate cancer progression as well (33), there was no data about the transcription factors involved in overexpression of hPar1 in the androgen-resistant malignant stage of prostate cancer.

EGR-1 expression results in either promotion (21, 34) or regression (35, 36) of cell proliferation, depending on the cellular context. Accumulating evidence indicates that Egr-1 plays a significant part in the development and progression of prostate cancer (15, 37–40). There is a direct correlation between the level of Egr-1 expression and the prostate tumor Gleason grade (13). In addition, loss of the Egr-1 corepressor NAB2 (41, 42) enhances the Egr-1 levels in prostate cancer. In the present study, we have shown increased binding of Egr-1 to the hPar1 promoter after treatment with bombesin, as shown in vitro by EMSA. We confirmed that this interaction occurs in vivo by ChIP. In addition, we show here that treatment with the neuroendocrine peptide bombesin increases Egr-1 function as manifested by an increase in PAR1-induced invasion in the Matrigel invasion assay. Our studies are consistent with previous studies showing that bombesin induces Egr-1 expression and markedly elevates the expression of cyclin D1 in prostate cancer cells (21) and identify hPar1 as another target of Egr-1 transcriptional activity. Although we show direct binding of Egr-1 to hPar1, we do not exclude the possibility that Egr-1 interacts with Sp1 forming an Sp1/Egr-1 complex, as has been shown to occur in the regulation of hepatocyte growth factor expression (43). In addition, we have not addressed the relative participation of individual Sp1 and Egr1 motifs in the regulation of hPar1. Thus, the mode by which Egr-1 regulates hPar1 expression needs further clarification; however, this study clearly shows the functional involvement of Egr-1 in hPar1 overexpression during prostate carcinoma progression.

Bombesin binds to cells and triggers a signaling cascade via G protein-coupled receptors (GPCR) localized at the cell surface.

References

28. Nierodzi ML, Karpukin S. Thrombin induces tumor growth, metastasis, and angiogenesis: evidence for a
Transcriptional Regulation of Human *Protease-Activated Receptor 1*: A Role for the Early Growth Response-1 Protein in Prostate Cancer

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