Transcriptional Regulation of Human \textit{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 \textit{human protease-activated receptor 1} (\textit{hPar1}) gene overexpression in the malignant androgen hormone-resistant phase. We found increased \textit{hPar1} RNA chain elongation and no change in message stability in cells with high levels of \textit{PAR1} expression, indicating that increased transcription is largely responsible for the overexpression of \textit{hPar1} in prostate tumor progression. Enforced expression of \textit{early growth response-1} (\textit{Egr-1}) plasmid markedly enhanced luciferase activity driven by the \textit{hPar1} promoter. The neuroendocrine peptide bombesin significantly induced \textit{hPar1} expression and increased the ability of the cells to invade Matrigel, an effect abolished by expression of \textit{hPar1} small interfering RNA, showing the importance of \textit{hPar1} in invasion. Bombesin also markedly enhanced \textit{Egr-1} binding to the \textit{hPar1} promoter \textit{in vivo} and \textit{in vitro}. These data suggest that bombesin enhances \textit{Egr-1} expression leading to increased \textit{hPar1} transcription, thereby increasing \textit{PAR1} expression and function. Immunohistostaining of prostate tissue biopsy specimens revealed a direct correlation between the degree of prostate cancer malignancy, \textit{PAR1} expression, and \textit{EGR-1} expression. Altogether, we show that transcriptional regulation of \textit{hPar1} in the aggressive hormone-resistant prostate cancer stage is controlled in part by the transcription factor \textit{Egr-1} and may play a central role in invasiveness, 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 in the development of promalignant prostate cancer are beginning to emerge. Transcription factors important for the progression from localized carcinoma \textit{in situ} to invasive tumor may provide essential targets for gene therapy.

We have shown previously that \textit{human protease-activated receptor 1} (\textit{hPar1}) plays a central role in epithelial malignancies (4, 5). Functional androgen response elements within the \textit{hPar1} promoter were identified based on transfection studies using \textit{hPar1} promoter-luciferase reporter constructs, electrophoretic mobility shift assay (EMSA), and chromatin immunoprecipitation (ChIP) analysis. Consistent with the hypothesis that androgen regulates \textit{hPar1} expression \textit{in vivo}, prostate cancer biopsy specimens showed high \textit{hPar1} levels in tumor glandular cells, whereas tumor tissues taken from the same individual after hormone ablation treatment exhibited very little to no \textit{hPar1} expression as well as markedly reduced tumor size (6). A major drawback of hormone ablation therapy is that the reduction in tumor size is temporary: the tumor eventually proceeds to the hormone-refractory and drug-resistant stage (7). Although initially we found androgen regulation of the \textit{hPar1} gene (e.g., induced by androgens and markedly reduced after hormone ablation therapy), high \textit{hPar1} expression was found in hormone-resistant prostate malignant carcinomas as well (6). We thus decided to elucidate the molecular basis of \textit{hPar1} overexpression in hormone-independent prostate cancer progression. We hypothesize that \textit{hPar1} overexpression stems at least in part from transcriptional regulation (and not gene amplification) as shown by transcription rates.

Evidence from several independent laboratories using microarray analyses and animal genetic models highlights the central involvement of the transcription factor \textit{early growth response-1} (\textit{Egr-1}) in prostate carcinoma progression. \textit{Egr-1} is a nuclear phosphoprotein that is rapidly (within minutes) induced by growth factors, cytokines, and differentiation factors. \textit{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 \textit{Egr-1}. In contrast, there is no \textit{Egr-1} expression in prostate normal cells (13, 14). The notion that \textit{Egr-1} plays a central role in prostate cancer progression is strongly supported by observations in \textit{Egr-1}–deficient mice showing impaired prostate tumorigenesis (15). \textit{Egr-1} has been shown to bind both nerve growth factor-I A binding protein (NAB) 1 and NAB2, regulatory proteins that repress \textit{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 \textit{Egr-1} repressor NAB2 along with \textit{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 \textit{Egr-1} in transcriptional regulation of \textit{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 humangenome to ensure that targeting was specific to hPAR1. To construct the hairpin, stem-and-loop siRNA expression cassette, we used the following 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 XhoI 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 Parijs’s Laboratory). We created four such siRNA cassettes from the hPar1 gene.

**Preparation of lentivirus.** Lentivirus particles were generated by a packaging system, in which 293T cells were cotransfected with the following three vectors: packaging (CMV-D8.91), envelope (CMV-D8), 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 fresh medium and transfected with the three plasmids using Fugene6 transfection reagent. Medium was changed 24 h after transfection. On days 2 and 3 after transfection, medium was collected to recover viral particles. The collected medium was centrifuged for 1 h at 40,000 rpm to concentrate the viral particles to 1×106.

**RNA extraction and reverse transcription-PCR.** Total RNA was prepared using the TRI reagent (Molecular Research Center, Inc.) as described by the manufacturer. One microgram of RNA was used for cDNA synthesis using Moloney murine leukemia virus reverse transcriptase and oligo(dt) (both from Promega). hPar1 transcripts were amplified using Taq polymerase (Bioline) in a 20 µL total PCR reaction. Initial melting was carried out at 95°C for 3 min followed by 32 cycles of 95°C for 1 min, 56°C for 30 s, and 72°C for 45 s; 5 min at 72°C was used for final extension after cycling. PCR primers were as follows: upstream hPar1, 5’-GCCAGAATTC-CAAAAGCAACAA-3’; downstream hPar1, 5’-GAGATGATGCAGGAGTTGT-3’; upstream human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-CCACCCATGCCAATTCATTGGC-3’; and downstream human GAPDH, 5’-TCTAGACGGCAGTCGTCACC-3’. PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

**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’-GGATGATGGTCTGGAGAGCC-3’; downstream hPar1, 5’-GCTGTCGTTGGGAGTTGTA-3’; upstream human GAPDH, 5’-GGATGATGGTCTGGAGAGCC-3’; and downstream human GAPDH, 5’-AAACGCCCTAGATCATCAAGC-3’.

**Figure 1. 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 cells. 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 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).

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**Figure 2. Egr-1 induces hPar1 promoter activity and increased expression following bombesin treatment.** A, 293T cells were transiently cotransfected with hPar1-luciferase (LUC) reporter and either empty expression vector or an Egr-1 expression plasmid. Luciferase activity was normalized to β-gal activity to control for transfection efficiency. Columns, mean (n = 6); bars, SD. Statistical significance determined by Student’s t test compared with vector-transfected cells: *, P < 0.005. B, bombesin induces hPar1 expression in DU-145 cells in a dose- and time-dependent manner. RT-PCR analysis of hPar1 done on RNA isolated from DU-145 cells before and after bombesin treatment. Top, cells were treated with the indicated concentration of bombesin for 2 h; bottom, cells were treated for the indicated time with 10−8 M bombesin. Images shown are representative of three independent experiments.

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 PBS. After washes, blots were incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were detected by the enhanced chemiluminescence reagent (Pierce).
status of epithelia. Color was developed using a DakoCytoVision 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 scrapped 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 hypotonic 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 (Hylocks) for the hPar1 putative Egr-1 sites (5'-ccagtagggcacagggcggca-3') and for a mutated version of the putative hPar1 Egr-1 sites (5'-ccagtagggctcaggtagggcggca-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 RediPrimer 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(dexoyinosinic–deoxyctydylid 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 noncompetitive competition experiments. The samples were loaded on a prerun (100 V for 1 h at room temperature) 6% polyacrylamide gel (acryl/ C0 354 to C2 /C2) and electrophoresed in 0.25/C2 prerun (100 V for 1 h at room temperature) 6% polyacrylamide gel (acryl/C0 354 to C2 /C2) and electrophoresed in 0.25/C2 prerun (100 V for 1 h at room temperature) 6% polyacrylamide gel (acryl/C0 354 to C2 /C2). The samples were loaded on a prerun (100 V for 1 h at room temperature) 6% polyacrylamide gel (acryl/C0 354 to C2 /C2) and electrophoresed in 0.25/C2 prerun (100 V for 1 h at room temperature) 6% polyacrylamide gel (acryl/C0 354 to C2 /C2)

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%) at room temperature for 10 min to cross-link histone proteins to DNA and then glycine (to a final concentration of 0.125 C2) was added to plates to quench formaldehyde. Soluble chromatin was prepared as follows: cells were washed and detached from the dish by scraping 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. Immunocomplexes with the beads were washed with radioimmunoprecipitation assay buffer followed by a wash with Tris-EDTA, and then the immunocomplexes were recovered by adding elution buffer [1% SDS, 1% NaCl, 1% 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 was as follows: 5'-accttagggccgcctggtcgtctggc-3' (forward) and 5'-ggtaagatcaggggtccaagc-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 TITANUM 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 oligo. 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 a control. 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 (GGGCGGGCGGGCGGGCGG). 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 activity. 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/Sp1 binding motifs (21). Bombesin treatment
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 regions 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

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**Figure 5.** Bombesin promotes an increase in PAR1-dependent invasion of DU-145 cells into Matrigel. A, Western blot analysis of hPAR1 protein levels. A noticeably reduced level in protein is seen following hPar1-siRNA infection compared with empty vector–infected cells. B, DU-145 cells were treated with SFFLRN with or without bombesin (10^{-8} mol/L, 2 h) in the presence or absence of a control viral vector or a viral vector encoding hPar1-siRNA. Cells were plated onto Matrigel-coated filters as described in Materials and Methods. After overnight incubation, cells that had migrated through the Matrigel were photographed and counted as described. Top, photomicrographs of representative fields from the indicated conditions; bottom, quantification of invasion using cell counts of representative fields. Columns, mean (n = 6); bars, SD. Statistical significance determined by Student’s t test compared with bombesin-treated cells; *, P < 0.001.
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 SFLLRN, 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 immunohistology 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 epithelia 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 analysis 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 Egr-1 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 (44). It has been reported that gastrin-releasing peptide (GRP), the human homologue of bombesin, acts like bombesin and that both function via high-affinity binding to their receptors. GRP receptors (GRP-R) are members of the GPCR family and absent in normal prostate epithelium but abundantly expressed in a variety of human prostatic carcinomas (45–47). Furthermore, although high GRP-R density was found in invasive prostate carcinomas, there is also GRP-R expression in intraepithelial neoplasia (46). The involvement of GRP-R in prostate carcinoma has been shown by application of either selective GRP-R antagonists or neutralizing antibodies, both resulting in impaired growth of androgen-independent prostate cancer cells in vitro and in vivo in mouse models (44, 48–50). This further highlights the presence and significance of neuroendocrine cells in invasive prostate carcinoma, independent of androgen levels and function. Our finding that bombesin enhances Egr-1–mediated hPar1 induction in prostate cancer is consistent with these reports and indicates a central function of GRPR in mediating Egr-1 expression. The molecular mechanism of bombesin-enhanced Egr-1 expression is unknown.

Our studies show for the first time the presence of a functional Egr-1 binding motif at the 5′ ATG proximal region of hPar1 and provide evidence that Egr-1 plays a central role in the transcriptional regulation of hPar1 in androgen-independent prostate cancer progression.

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