Bombesin Regulates Cyclin D1 Expression through the Early Growth Response Protein Egr-1 in Prostate Cancer Cells

Dongmei Xiao,1 Dharmaraj Chinnappan,1 Richard Pestell,2 Christopher Albanese,2 and Horst Christian Weber1

1Section of Gastroenterology, Boston University School of Medicine, Boston, Massachusetts; and 2Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Cancer Center, Washington, District of Columbia

Abstract

Our previous studies indicate that the activation of mitogen-activated protein kinase (MAPK) pathway is involved in bombesin-induced cell proliferation in prostate cancer cells. Cyclin D1 is a critical regulator involved in cell cycle progression through the G1 phase into the S phase, thereby contributing to cell proliferation. Mostly, mitogen-stimulated expression of cyclin D1 is attributed to the extracellular signal-regulated kinase (ERK) activation. Here, we found that bombesin induced human cyclin D1 expression on both mRNA and protein levels in DU-145 prostate cancer cells. Mutational analyses showed that bombesin-enhanced cyclin D1 transcription required the binding of nuclear proteins to the –143 to –105 region of the human cyclin D1 promoter, which contains binding sites for transcription factors Sp-1 and early growth response protein (Egr-1). Do novo protein synthesis was requisite for bombesin-induced cyclin D1 expression. Further studies showed Egr-1 was induced upon bombesin stimulation. The induction of Egr-1 expression and its binding to the cyclin D1 promoter were essential for bombesin-enhanced cyclin D1 transcription. Inhibition of MAPK pathway with either the MEK1 inhibitor PD98059 or a dominant-negative Ras mutant, RasN17, abolished bombesin-induced cyclin D1 activation. Taken together, bombesin-induced cyclin D1 expression in prostate cancer cells is mediated by Egr-1 activation and the interaction of Egr-1 with the Egr-1/Sp1 motif of the cyclin D1 promoter through the activation of MAPK pathway. These findings represent a novel mechanism of bombesin-dependent stimulation of mitogenesis by regulating directly the cell cycle in prostate cancer. (Cancer Res 2005; 65(21): 9934-42)

Introduction

Prostate cancer represents the most frequently diagnosed malignancy in men and the importance of neuroendocrine peptides, such as bombesin, as growth factors involved in the progression of this disease has been widely recognized. The amphibian tetradecapeptide bombesin was originally isolated from the skin of the frog, Bombina bombina. Its mammalian homologue gastrin-releasing peptide (GRP) is a 27-amino-acid peptide. The COOH-terminal decapeptide of GRP is similar to that of bombesin and possesses all the biological activity of bombesin. In humans, both bombesin and GRP bind with high affinities to the GRP receptor (GRPR), a member of the G protein-coupled receptor superfamily. Both bombesin and GRP have been shown to stimulate growth of androgen-independent prostate cancer cells with the expression of functional GRPR, which was ablated by either selective GRPR antagonists or antibodies against GRP (1–5). The selective antagonists of the GRPR also inhibited the growth of a number of prostate carcinoma models, either cancer cells in vitro or xenografts in syngeneic rats or nude mice (6–8). Furthermore, GRPR was found to be abundantly expressed in a variety of human prostatic carcinomas, whereas normal prostatic epithelium lacks GRPR expression (9–11). Markwalder et al. (9) reported that GRPR was detected, often in high density, not only in all invasive prostate carcinomas but also in all the studied cases of prostatic intraepithelial neoplasia lesions, the earliest phase of neoplastic transformation of the prostate. These studies indicate that neuroendocrine bombesin-like peptides and GRPR play an important role in the carcinogenesis and progression of prostate cancer.

Cyclin D1, the regulatory subunit of several cyclin-dependent kinases (CDK), is required for progression of the G1 phase in mammalian cells. It is a critical target for proliferation signals in G1 phase. Cyclin D1 is induced by extracellular signal-regulated kinases through a Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase (ERK) cascade (12). Conversely, inhibition of the Ras pathway inhibited cyclin D1 gene expression (13). In prostate cancer, ERK activation was dramatically increased upon the stimulation with mitogenic stimulation (14–18). Moreover, the level of activated ERK increased with increasing Gleason score and tumor stage in prostate tumors (19), suggesting that the enhanced activation of the MAPK signal pathway correlates with prostate cancer progression to a more advanced and androgen-independent disease. Bombesin has been shown to activate the MAPK pathway in Swiss 3T3 cells and other human cell lines via its Gq-coupled receptor in either a PKC-dependent or a PKC-independent manner (20–22). More recently, our studies showed that bombesin induced ERK activation via epidermal growth factor receptor transactivation, which was required for bombesin-stimulated cell proliferation in prostate cancer cells (5). Therefore, we wondered whether bombesin stimulation might directly regulate cell cycle progression and cyclin D1 expression in prostate cancer cells. Our studies found that bombesin enhanced human cyclin D1 expression through the induction and activation of the early growth response protein-1 (Egr-1) owing to its binding activity at a cis-regulatory element in the human cyclin D1 promoter in a MAPK pathway-dependent manner. These findings represent a novel mechanism of bombesin-dependent stimulation of mitogenesis by directly regulating the cell cycle in prostate cancer.
Materials and Methods

Materials. Antibodies for Egr-1 and Sp1 were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against cyclin D1 and β-actin were purchased from Sigma (St. Louis, MO). Inhibitors for MEK (PD98059) and PKC (GF19203X) were obtained from Calbiochem (San Diego, CA). The specific, high-affinity GRPR antagonist (D-F5-Phe2,D-Ala1)-bombesin-(6-13)methyl ester (ME) was kindly provided by Drs. R.T. Jensen and T.W. Moody. Human cyclin D1 promoter constructs (−1745 to −163, −144, −66, and −22) have been described previously (13). Series of pTK81 (HSV-TK) firefly luciferase reporter gene constructs were gifts from Dr. A.K. Rustgi (23). Egr-1 expression plasmids (wild-type Egr-1 and mutant Egr-1 with a deletion of DNA-binding domain) were kind gifts from Dr. I. Belle (24).

Cell culture and transient transfection. DU-145 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Eagle's MEM supplemented with 10% FCS and penicillin/streptomycin (50 IU and 50 μg/mL, respectively) at 37°C in 5% CO2. Transient transfection with indicated plasmid DNAs were done while the cells were 70% to 80% confluent by using LipofectAMINE reagent (Invitrogen Corp., Carlsbad, CA). The Escherichia coli β-galactosidase gene in the plasmid pCMVβ-gal (Promega, Madison, WI) was used as reference gene to monitor transfection efficiency. All experiments were done in triplicate and repeated independently at least thrice. Twenty-four hours after transfection, medium was changed and cells were maintained for another 24 hours in serum-free medium before treatment and lysis. Data are reported as luciferase activity normalized to the cotransfected monitoring plasmid β-galactosidase gene activity.

Western blot analysis. Cells were harvested and lysed in ice-cold lysis buffer (0.15 mol/L sodium chloride, 50 mmol/L Tris, 0.5% Triton X-100, and protease inhibitors cocktail from Roche, Indianapolis, IN) for 30 minutes followed by centrifugation at 14,000 rpm for 10 minutes and the pellet was discarded. Protein concentration of the cell lysate was detected by protein assay kit from Bio-Rad ( Hercules, CA) and adjusted so that each sample contained an equal amount of protein. Protein samples were dissolved in loading buffer [60 mmol/L Tris-HCl (pH 6.8), 2% SDS, 100 mmol/L DTT, and 0.01% bromophenol blue], heated to 100°C for 5 minutes, and loaded onto the gel in electrophoresis buffer containing 25 mmol/L Tris-HCl (pH 8.3), 250 mmol/L glycine, and 0.1% SDS. At the completion of electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Science, Piscataway, NJ). Membranes were blocked with 5% nonfat powdered milk in TBS [10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20] and incubated with the primary antibody at 4°C overnight followed by horseradish peroxidase–conjugated second antibody. Immunocomplexes were visualized using the enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Northern blot hybridization. Total cellular RNA was isolated using the RNaseasy kit from Qiagen (Valencia, CA). Aliquots of 20 μg of RNA were denatured in gel-running buffer [0.04 mol/L 3-(N-morpholino) propane-sulfonic acid/10 mmol/L sodium acetate/0.5 mmol/L EDTA (pH 7.5)/50% formamide/6% formaldehyde] and electrophoresed on a 1.3% agarose/6% formaldehyde gel. The integrity of the RNA samples was determined by the visualization of 28S and 18S rRNA bands with ethidium bromide staining (10 μg/mL). After electrophoresis at 10 V/cm, the RNA was transferred from the gel to a nitrocellulose filter by capillary action, as described by the manufacturer (Schleicher & Schuell, Keene, NH). Hybridization was done using radioactively labeled human cyclin D1, Egr-1, Sp1, and β-actin cDNA probes as previously described in detail (4). Autoradiographs were developed after exposure to X-ray film at −70°C, using a Cronex intensifying screen (DuPont, Wilmington, DE).

Electrophoretic mobility shift assay. Nuclear extracts were prepared by using the nuclear extract kit from Active Motif (Carlsbad, CA) as the manufacturer suggested. Nucleotide sequences of the sense strand of the double-stranded oligonucleotides were as follows: oligoA (cyclin D1 promoter spanning nucleotides −140 to −119), 5'-GGCGCCCCCGCCCCCGCCCTCC-3'; oligoB (cyclin D1 promoter spanning nucleotides −124 to −103), 5'-GGGCCGCCCGCCCCGCCCCCGCCCTCC-3'; oligoB(Mt), 5'-GGGCCGCCGCCATGCCCGCCCCCTCC-3'. Sense and antisense oligodeoxynucleotides were synthesized by Invitrogen and annealed to form a double-stranded DNA. The double stranded DNA probes were 5'-end-labeled using T4 polynucleotide kinase (Promega, Madison, WI) and [γ-32P] ATP (New England Nuclear, Boston, MA). Binding reactions were done mixing 5 μg nuclear extract with 20,000 cpm of 5'-end-labeled DNA probes in 10 μL of electrophoretic mobility shift assay (EMSA) buffer (10 mmol/L Tris-HCl (pH 7.5), 1 mMol/L MgCl2, 0.5 mMol/L EDTA, 0.5 mMol/L DTT, 50 mmol/L NaCl, 5% glycerol, and 0.05 mg/mL poly(dexoyinosinic-deoxyctydilic acid)]. For competition analysis, both 50× and 100× molar excess of unlabeled competitor oligodeoxynucleotides were also added to the mixture. After incubation at room temperature for 20 minutes, the mixture was separated in a 6% nondenaturing polyacrylamide gels in 0.5× Tris-borate EDTA electrophoresis buffer at 4°C and then the gels were dried for autoradiography. For the supershift assays, 2 μg of antibodies (Santa Cruz Biotechnology) were incubated with nuclear extract for 20 minutes at room temperature before adding radiolabeled DNA probes.

Results

Bombesin induces cyclin D1 mRNA and protein expression. First, we tested the effect of bombesin stimulation on cyclin D1 mRNA expression in exponentially growing DU145 cells. Cells were treated with bombesin as indicated, total cellular RNA was isolated at different time points after the treatment and RNA samples were subsequently subjected to Northern blot analysis. As shown in Fig. 1A, the human cyclin D1–specific mRNA of 4.7 kb was found constitutively expressed in DU145 cells. The stimulation of cells with bombesin resulted in a time-dependent increase of cyclin D1 mRNA. Increased cyclin D1 mRNA levels could be detected as early as 4 hours after bombesin treatment, reached a maximum by about 8 hours, and was sustained up to 12 hours after the treatment, before returning to its basal level at 18 hours.

We then determined the relationship of cyclin D1 mRNA expression with its protein expression after bombesin stimulation using Western blot analysis (Fig. 1B). Human cyclin D1 protein levels were shown to increase between 6 to 18 hours after bombesin treatment with a maximum response occurring at about 9 hours.

![Figure 1. Bombesin induces human cyclin D1 mRNA and protein expression in a time-dependent manner. DU145 cells were treated with bombesin (10 nmol/L) for the indicated time after 48 hours of serum starvation followed by isolation of total cellular RNA and protein for either Northern blot (A) or Western blot (B) analysis.](image-url)
Bombesin-induced human cyclin D1 transcriptional activity is mediated through the −143 to −105 region of its promoter. To test whether transcriptional mechanisms account for bombesin-stimulated induction of cyclin D1 mRNA expression in DU145 cells, we did transient transfection experiments with human cyclin D1 promoter mutations with and without bombesin stimulation. These plasmids contained constructs with different 5′ deletions of the human cyclin D1 promoter linked to the luciferase reporter gene (Fig. 2A, top). As shown in Fig. 2A (bottom), we found that human cyclin D1 genomic sequences contained in constructs pA3-1745 to pA3-141 mediated basal promoter activity in DU145 cells, whereas shorter sequences did not. Furthermore, bombesin treatment resulted in at least 2-fold enhancement of cyclin D1 promoter activity for the promoter constructs pA3-1745, pA3-163, and pA3-141. Different concentrations of bombesin tested (1, 10, and 100 nmol/L) yielded similar induction of cyclin D1 promoter activity for the pA3-1745, pA3-163, and pA3-141 promoter constructs (data not shown). However, when the sequence between nucleotides −141 to −66 was deleted, the stimulatory effect was completely abolished. These results indicate that the human cyclin D1 genomic DNA sequence between nucleotides −141 and −66 contains critical sequences required for basal and bombesin-inducible transcriptional activity in DU145 cells.

The genomic sequence between nucleotides −141 to −66 contains two putative Egr-binding motifs and two potential Sp1-binding motifs that overlap with the 3′ putative Egr motif (Fig. 2B, top). To examine more precisely the function of these putative binding sites, we tested various reporter constructs encompassing the wild-type sequence of human cyclin D1 promoter region between nucleotides −143 and −105 and corresponding mutants (CC → TA) of both putative Egr motifs (Fig. 2B, top) by transient transfection studies in DU145 cells. This genomic sequence had been subcloned into the heterologous herpes simplex virus thymidine kinase (HSV-TK) minimal promoter fused to the luciferase reporter gene in the pTK81 plasmid (23). As shown in Fig. 2B (bottom), basal transcriptional activity of the wild-type construct (40W) was significantly enhanced in response to bombesin stimulation (1 and 10 nmol/L) and this responsiveness was maintained as long as the 3′ putative Egr-1/Sp1 motif was preserved as shown in the mutant construct 402M. In contradistinction, using the 3′ Egr motif mutant constructs 401M and 403M, basal transcriptional activity was greatly decreased and bombesin-mediated responsiveness was abolished. Taken together, these results indicate strongly that only the 3′ putative Egr-1/Sp1 motif in the −143 to −105 bp promoter region is required for bombesin-inducible human cyclin D1 transcriptional activation.

De novo protein synthesis is requisite for bombesin-induced human cyclin D1 expression. To determine whether bombesin-induced cyclin D1 expression is dependent on de novo protein synthesis, we measured cyclin D1 expression in the present of cycloheximide. Quiescent DU145 cells were pretreated with or without cycloheximide (10 μg/mL for 30 minutes) and then stimulated with bombesin (100 nmol/L) for the indicated time periods. Cell lysates were prepared and used for Western blot to determine cyclin D1 and Egr-1 protein expression. As shown in Fig. 3A, in the absence of cycloheximide, bombesin enhanced cyclin expression. When the cells were pretreated with cycloheximide, bombesin-induced cyclin D1 expression was not detected, indicating that de novo protein synthesis is required for bombesin induction of cyclin D1 expression.

**Figure 2.** The proximal Egr-1 site in the −143-bp to −105-bp region of the human cyclin D1 promoter is requisite for bombesin (Bn)–induced cyclin D1 promoter activity. A, top, schematic depiction of mutant human cyclin D1 promoter constructs. These constructs harbor different 5′ deletions of the human cyclin D1 promoter linked to the luciferase reporter gene. DNA sequences representing putative cis-regulatory elements are shown schematically. Bottom, effect of bombesin on human cyclin D1 5′ promoter deletion activity as determined by transient transfection in DU145 cells. Columns, means from at least three independent experiments, each done in triplicate; bars, ± SE. B, top, human cyclin D1 promoter region −143 to −105 harboring two Egr-1 motifs and their corresponding mutants were cloned into the pTK81 plasmid with the minimal promoter HSV-TK luciferase reporter gene (23). Bottom, DU145 cells, transiently transfected with the indicated constructs, were treated with bombesin (1 and 10 nmol/L) for 8 hours before lysis for luciferase and β-galactosidase activity from three independent experiments, each done in triplicate; bars, ± SE.
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D1 expression in the same time-dependent manner as shown in Fig. 1, whereas cycloheximide pretreatment completely abolished bombesin-inducible cyclin D1 expression. However, bombesin-induced Egr-1 expression was not affected by cycloheximide pretreatment. These data indicate that bombesin-induced cyclin D1 expression requires de novo protein synthesis.

Egr-1 is stimulated by bombesin and required for bombesin-dependent human cyclin D1 promoter activity. Because we determined the genomic sequence with two putative binding sites for Egr-1 and Sp1 required for bombesin-induced cyclin D1 promoter activity and that bombesin-enhanced cyclin D1 expression requires de novo protein synthesis, we next tested whether the Egr-1 and Sp1 expression were induced upon bombesin stimulation in DU145 cells. As shown in Fig. 3B, both Egr-1- and Sp1-specific mRNA were found constitutively expressed in DU145 cells. Bombesin treatment of DU145 cells resulted in a rapid and time-dependent increase of Egr-1 mRNA expression but not that of Sp1. Bombesin-enhanced Egr-1 mRNA expression occurred as early as 10 minutes and was sustained up to 30 minutes with a peak induction at about 15 minutes after bombesin stimulation (Fig. 3B). Consistent with the increase in Egr-1 mRNA, bombesin treatment of DU145 cells also yielded enhanced Egr-1 protein expression in DU145 cells. Egr-1 protein expression was induced by bombesin stimulation as early as 1 hour and lasted up to 16 hours with a peak at 8 hours (Fig. 3C).

Bombesin stimulation of DU145 cells did not affect Sp1 protein expression as detected by Western blot up to 24 hours after peptide stimulation (data not shown). These data suggest that in DU145 prostate cancer cells Egr-1 expression is regulated and induced by bombesin, whereas bombesin stimulation has no effect on Sp1 expression.

To further examine the role of Egr-1 in bombesin-dependent cyclin D1 promoter activation, we did transient cotransfection with either the heterologous promoter construct 40W or the native human cyclin D1 promoter construct pA3-141 and a wild-type Egr-1 expression plasmid or its inactivated mutant with the DNA-binding domain being deleted. As shown in Fig. 4, overexpression of wild-type Egr-1 enhanced both the basal and bombesin-inducible human cyclin D1 promoter activity under both experimental conditions. In contrast, when the Egr-1 mutant, lacking the DNA-binding site, was used, cyclin D1 promoter activities in both instances remained unchanged in response to bombesin stimulation (Fig. 4). In summary, these results provide strong evidence that bombesin-induced cyclin D1 promoter activity is mediated by Egr-1.

Bombesin enhanced Egr-1 protein binding activity at the putative Egr-1/Sp1-binding motif of the human cyclin D1 promoter. Our data indicate that the nucleotide sequence the -143 to -105 region of the human cyclin D1 promoter is critical for its bombesin-dependent regulation. To further detail the

Figure 3. Bombesin (BN)–induced cyclin D1 expression requires de novo protein synthesis, and the Egr-1 protein is induced time dependently by bombesin in DU145 cells. A, de novo protein synthesis is required for bombesin-dependent cyclin D1 expression. After being starved in serum-free medium for 48 hours, DU145 cells were treated with 100 nmol/L bombesin at indicated time points in the presence or absence of 10 μg/mL cycloheximide (CHX). Cell lysates were harvested and then used for Western blot using antibodies to cyclin D1 and Egr-1. B–C, bombesin induces Egr-1 expression. DU145 cells were treated with bombesin (10 nmol/L) for indicated time periods after 48 hours of starvation in serum-free medium and harvested for either Northern blot (A) or Western blot (B) analysis.

Figure 4. Egr-1 protein is required for bombesin (BN)–induced human cyclin D1 transcriptional activation. DU145 cells were transiently cotransfected with either the wild-type heterologous promoter construct 40W (A) or the native human cyclin D1 promoter construct pA3-141 (B) with wild-type Egr-1 or the Egr-1 mutant lacking the DNA-binding domain. Luciferase and β-galactosidase activity were measured after 8 hours of treatment with bombesin following 24 hours of serum starvation. Columns, means from three independent experiments, each done in triplicate; bars, ±SE. *, P < 0.05; ** or ***, P < 0.01 and **** or *****, P < 0.001, versus vector alone (one-sided Student’s t test).
sequences involved in this regulation and to identify trans-acting factors binding to this DNA sequence, we did a series of gel shift experiments using the oligonucleotides A and B. OligoA (−140 to −119) spans the 5′ putative Egr motif and oligoB (−124 to −103) spans two potential Sp1 motifs that overlap with the putative 3′ Egr motif (Fig. 2B, top). Both oligoA and oligoB probes were incubated with nuclear extracts from DU145 cells isolated after a time-dependent stimulation with bombesin. EMSA revealed a major and a distinct minor nucleoprotein complex as indicated by arrows A and B (Fig. 5A). When using oligoB, but not oligoA, bombesin stimulation resulted in increased intensity of the nucleoprotein complex indicated by arrow B but not that of arrow A (Fig. 5A). Competition experiments with excess unlabeled wild-type oligoB resulted in the loss of band B but not that of band A, whereas the mutant oligoB did not alter the nucleoprotein complexes (data not shown). Furthermore, whereas excess unlabeled Egr-1 consensus oligonucleotide abolished the nucleoprotein complex band B, band A was diminished by the excess unlabeled Sp1 consensus primer in additional competition experiments. Neither the nucleoprotein complex band A nor band B was affected by excess unlabeled CRE consensus oligonucleotide (Fig. 5B).

In supershift assays using the antibodies against Egr-1, Sp1, and AP2, we showed anti-Egr-1 antibody abolished the nucleoprotein complexes in both bands A and B, whereas anti-Sp1 antibody almost completely eliminated band A, but band B remained unaffected. In contrast, the anti-AP2 antibody did not affect either band A or band B (Fig. 5C). Taken together, our data suggest that bombesin stimulates the enhanced Egr-1 protein recruitment to the putative Egr-1/Sp1-binding motif between nucleotides −124 to −103 of the cyclin D1 promoter.

Mitogen-activated protein kinase pathway is involved in bombesin-dependent cyclin D1 expression. We have previously shown that bombesin activated MAPK pathway and subsequently induced cell proliferation in DU145 cells (5). Cyclin D1, which is required for progression of the G1 phase and is therefore a critical target for proliferation signals in G1 phase, is one of the targets of MAPK pathway. To determine whether the MAPK pathway is involved in bombesin-induced cyclin D1 expression in DU145 cells, we used the compound PD98059, an inhibitor of MEK, the dual specific kinase that activates p44/p42MAPK/ERK by phosphorylation, in two independent experimental approaches. First, we did immunoblot experiments to show cyclin D1 expression and, second, we used transient transfection assays of both promoter constructs as described earlier. As shown in Fig. 6A, PD98059 (20 μmol/L) completely abolished bombesin-enhanced cyclin D1 expression as examined by Western blot, whereas the PKC inhibitor GF109203X (2 μmol/L) had no effect on bombesin-induced cyclin D1 expression. Meanwhile, bombesin-induced cyclin D1 expression

![Figure 5](image-url)
Figure 5. Bombesin (BN) enhances Egr-1 protein–binding activity at the putative Egr/Sp1-binding motif of the human cyclin D1 promoter. A, nuclear extracts from DU145 cells treated or not with bombesin (100 nmol/L) or 10% FCS for the indicated time were incubated with [32P]-end-labeled double-stranded oligonucleotide probes oligoA and oligoB, respectively. Both probes revealed a major and a minor distinct nucleoprotein complex indicated by the arrows A and B. B, in competition experiments, nuclear extracts from DU145 cells treated with 100 nmol/L bombesin for 4 hours were incubated or not with 50- and 100-fold molar excess of the indicated unlabeled oligonucleotides in the presence of [32P]-end-labeled oligoB. Sequences of the double-stranded oligonucleotides of Egr<sup>cons</sup>, Sp1<sup>cons</sup>, and CRE<sup>cons</sup> are 5′-GGATCCA GGCGGGCGAGCGGGGGCGA-3′, 5′-ATTCGATCGGGGGCGGGGCGAGC-3′, and 5′-AGAGATTGCC TGACGTCAGAGAGCTAG-3′, respectively. The arrows indicate the position of two nucleoprotein complexes (Sp1 and Egr-1). C, in supershift assays, 2 μg of the indicated antibodies were preincubated or not with nuclear extracts from DU145 cells treated with 100 nmol/L bombesin for 6 hours before incubation with [32P]-end-labeled oligoB.
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was completely blocked by the pretreatment with the selective GRPR antagonist, ME (Fig. 6A), confirming bombesin induced cyclin D1 expression via specific activation of its high-affinity GRPR. Similarly, our transfection experiments showed that the cyclin D1 promoter responsiveness to bombesin stimulation as shown in Fig. 2, was abolished after pretreatment with the MEK inhibitor PD98059 and the selective GRPR antagonist ME, respectively (Fig. 6B). Corresponding to findings in immunoblot experiments, inhibition of PKC with GFX did not alter the ability of bombesin to stimulate cyclin D1 promoter activity (Fig. 6B).

Ras, rather than Rap1, has been shown to be involved in bombesin-induced MAPK pathway activation in DU145 cells (5). To test whether bombesin-dependent cyclin D1 expression occurs through Ras-dependent or Rap1-dependent MAPK activation, we cotransfected either the heterologous promoter construct 40W or the native cyclin D1 promoter construct pA3-141 along with RasN17 or Rap1N17 or Rap1GAP. As shown in Fig. 6C, over-expression of RasN17 reduced bombesin-inducible promoter activity of both cyclin D1 promoter constructs. Consistent with previous results, Rap1N17 and Rap1GAP did not affect bombesin-inducible cyclin D1 promoter activity (Fig. 6C). Taken together, these data suggest that bombesin-induced cyclin D1 expression is mediated via its high-affinity GRPR and through Ras-dependent MAPK activation.

Discussion

Prostate carcinoma represents the most frequently diagnosed malignancy in men; yet control of advanced disease, usually characterized by androgen-independent tumor progression and frequent neuroendocrine differentiation, is seldom achieved, because the underlying cancer biology is poorly understood. On the other hand, accumulating evidence strongly suggest that neuroendocrine peptides and their receptors might mediate important biological effects in both normal and malignant prostate tissue (25). For instance, owing to virtually ubiquitous ectopic expression of the GRPR in frank prostate carcinoma and, notably, also in prostatic intraepithelial neoplasia, the precursor lesion of prostate malignancy, paracrine and neurocrine interaction of GRP with its specific GRPR might manifest in cell proliferation and migration (2, 7, 8, 10, 26). In the present study, we provide now evidence for a novel mechanism of prostate cancer cell proliferation by agonist-dependent GRPR stimulation (i.e., the transcriptional regulation of cyclin D1).

Cyclin D1, the regulatory subunit of several CDKs, is required for and is capable of shortening the G1-phase progression of the cell cycle (27). The induction of cyclin D1 activates CDKs, which then phosphorylates and inactivates the substrate pRB (27, 28). The inactivation of pRB is an essential prerequisite for cell cycle progression through the G1 phase into the S phase (28). Therefore, the induction of cyclin D1 is thought to play a significant role in cell proliferation by controlling cell cycle progression through the G1 phase. A number of studies have shown that the induction of cyclin D1 expression by various mitogens requires activation of the Ras/MAPK pathway and is temporally preceded by the activation of a class of genes known as immediate-early genes, such as c-jun, c-fos, and Egr-1 (23, 29–32). Along this line, we previously showed that bombesin induced MAPK activation and the expression of immediate-early gene c-fos in androgen-independent prostate cancer cells, which is a requisite for bombesin-stimulated DNA synthesis in prostate cancer cells (4, 5). Therefore we postulated that bombesin might regulate proliferation through the induction of cyclin D1 gene expression in androgen-independent prostate cancer cells. Correspondingly, our experiments for the first time showed that bombesin induced cyclin D1 expression on both mRNA and protein levels in DU145 cells (Fig. 1). Cyclin D1 mRNA was increased from 4 to 12 hours after bombesin treatment in a time-dependent course as previously reported for other mitogens (23, 33, 34). The induction of cyclin D1 mRNA by bombesin seems to correlate with cyclin D1 protein up-regulation, which occurred between 6 and 18 hours after the stimulation (Fig. 1) and required de novo protein synthesis (Fig. 3A). Although the regulation of cyclin D1 may involve transcriptional, post-transcriptional and posttranslational mechanisms, the most important mechanism of cyclin D1 gene activation occurs on the
transcriptional level (35). Multiple cis-regulatory elements such as CRE/activating transcription factor (ATF), SP1/EGR, E2F, and AP1 have been identified in the cyclin D1 promoter (Fig. 2A, top) regulating cyclin D1 transcriptional activation in response to different mitogens in different cell context (29, 31–33, 36–38). Shiozawa et al. (31) showed estrogen-induced activation of the cyclin D1 gene was mediated by binding of c-Jun to the AP1 sequence of the cyclin D1 promoter in normal endometrial glandular cells. Up-regulation of cyclin D1 by growth factors in hepatocytes was mediated by binding cyclic AMP-responsive element binding protein (CREB) and ATF-3 to the CRE/ATF site of the promoter (36, 37). Overexpression of pp60-src in MCF-7 breast cancer cells also activated cyclin D1 through the binding of CREB and ATF-2 to the CRE/ATF motif (38). In chondrocytes, the CRE and activator protein sites were identified as the major determinants in the transcriptional response to transforming growth factor-β and PTHrP, whereby induction of cyclin D1 was reported mediated by transcription factors ATF-2 and CREB (33).

In the present study, we identified by promoter analysis and DNA protein binding studies a region between nucleotides −143 to −105 as critical for bombesin-induced human cyclin D1 regulation. This DNA sequence contains two putative Egr motifs and two potential Sp1 motifs that flank and overlap the 3′ putative Egr motif (Fig. 2B, top). Whereas our data from mutational analyses and EMSA experiments showed the presence of protein complexes binding to both Egr motifs, we further showed that only the Egr/Sp1 site (3′ of the two Egr motifs) was essential for bombesin-induced transcriptional cyclin D1 activation (Fig. 2B, bottom). First, bombesin stimulation resulted in an increased Egr-1 binding only to the Egr/Sp1 site but not to the 5′ Egr site (Fig. 5). Second, in agreement with these EMSA results, we determined that mutation of the Egr/Sp1 motif abrogated bombesin-induced reporter gene activity, whereas mutation of the 5′ Egr site did not abolish this effect (Fig. 2B).

A dual interplay between transcription factors Egr-1 and Sp1 has been described in the regulation of some human genes. Depending on the cellular context, the binding of Egr-1 to the Egr/Sp1 motif can result in either stimulation or inhibition of gene transcription (39–41). In human epithelial cells, both Egr-1 and Sp1 sites are required for maximal induction of the TF promoter by phorbol 12-myristate 13-acetate (PMA) or serum (40). The binding of both Egr-1 and Sp1 to the chromogranin A (CgA) promoter was also required for full gastrin- and PMA-dependent transactivation of the CgA gene (41). In contrast to this stimulatory effect, Egr-1 suppressed the transcription of the β(1)AR gene by competing with Sp1 for binding to their overlapping sites (39). More recently, Zhang et al. (42) showed that Egr-1 physically interacted with Sp1 and sequestered Sp1 as a transcriptional activator of c-met proto-oncogene.

However, in this study, we did not observe any evidence for the presence of dual interplay between Egr-1 and Sp1 in bombesin-induced cyclin D1 transcriptional activation. EMSA supershift assays and the competition experiments clearly showed the specific Egr-1 binding to the Egr-1/Sp1 motif within the human cyclin D1 promoter and Egr-1 recruitment occurred in response to bombesin stimulation only at the Egr-1/Sp-1 motif. Furthermore, in agreement with these findings, we found that bombesin rapidly induced expression of Egr-1-specific mRNA and protein in a time-dependent manner but not that of Sp1 (Fig. 3B and C). We further investigated the role of Egr-1 in bombesin-dependent transcriptional regulation of the human cyclin D1 promoter using two different reporter systems, one in the heterologous HSV-TK promoter and the other in the native human promoter context. In both instances, overexpression of wild-type Egr-1 resulted in enhanced basal and bombesin-inducible cyclin D1 promoter activity. In contrast, when the Egr-1 mutant lacking the DNA-binding domain was used, bombesin-induced cyclin D1 promoter activity was almost completely abrogated (Fig. 4). More interestingly, although Mora et al. (43) recently reported that androgen-independent prostate cancer PC3 cells maintained a long-lasting, heavily phosphorylated state of Egr-1, we observed Egr-1 was not expressed, or expressed in a very low level in these cells, in which cyclin D1 expression could not be induced by bombesin stimulation (data not shown). Therefore, our data strongly suggest that bombesin-mediated cyclin D1 activation is regulated through the expression and activation of Egr-1 in DU145 cells. Although Sp1 did not regulate bombesin-induced cyclin D1 promoter activity, it might still be responsible for the basal expression of cyclin D1. Occupancy of the site may prevent the formation of a repressive chromatin structure (44). It is thus conceivable that the mutation of the site might result in decreased promoter activity of cyclin D1 gene as we observed in transfection experiments with the mutant constructs (Fig. 2B).

Egr-1 is an immediate-early response gene that encodes a zinc finger nuclear transcription factor whose expression is induced by mitogens, stress, and differentiation factors. Expression of Egr-1 results in either promotion or regression of cell proliferation depending on cell context. Recently, evidence has accumulated indicating Egr-1 plays a crucial role in the development and progression of prostate cancer (24, 43, 45–48). Egr-1 expression levels were reported elevated in human prostate carcinomas correlating with grade and stage (47). Inhibition of Egr-1 expression in prostate cancer cells decreased cell proliferation, whereas stable expression of Egr-1 in normal human prostate epithelial cells promoted transformation (46). Moreover, inhibition of Egr-1 expression reverses transformation of prostate cancer cells in vitro and in vivo (45), and the mouse models suggest that Egr-1 is required for tumor progression (45, 48). Our findings in this study further indicate the biological importance of Egr-1 in prostate cancer. Here, we showed for the first time that the neuroendocrine peptide bombesin enhanced Egr-1 expression in prostate cancer cells, which subsequently resulted in increased transcriptional regulation of the human cyclin D1 gene through recruitment of Egr-1 to a distinct cyclin D1 promoter site. Thus, the enhanced Egr-1 expression may represent an alternative pathway mediating bombesin-induced mitogenesis in prostate cancer cells.

Several studies have shown that the ERK activation is an essential regulator of mitogen-stimulated expression of cyclin D1 (12). In most cases, activation of the Ras/Raf/MEK/ERK cascade plays a pivotal role in cyclin D1 gene expression (49, 50). Conversely, inhibition of either Ras or MEK inhibits cyclin D1 gene expression. In agreement with these findings, we found ERK activity was essential for bombesin-induced cyclin D1 expression. Inhibition of ERK activity with the MEK inhibitor PD98059 resulted in a concomitant loss of bombesin-induced cyclin D1 expression (Fig. 6A). Furthermore, overexpression of RasN17, a dominant-negative form of Ras, reduced bombesin-induced cyclin D1 transcriptional activity. ERK activation has been shown to be dramatically increased in prostate cancer upon the stimulation with various mitogenic stimulators (15–18, 25). The activation of ERK in prostate cancer cells can either reduce apoptosis or, more commonly, increase cell proliferation (15–18, 25). We have
previously shown bombesin induces cell proliferation in prostate cancer cells through ERK activation, whereas inhibition of ERK activation hinders bombesin-induced cell proliferation (5). Therefore, this study now provides novel evidence that ERK activation may play a crucial role in bombesin-stimulated mitogenesis in prostate cancer by directly affecting the cell cycle machinery through transcriptional up-regulation of cyclin D1 expression. Accordingly, inhibition of GRPR activation and receptor-dependent downstream intracellular signaling might constitute an attractive target for prostate cancer treatment.

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References

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Dongmei Xiao, Dharmaraj Chinnappan, Richard Pestell, et al.


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