Paracrine Calcitonin in Prostate Cancer is Linked to CD44 Variant Expression and Invasion

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Abstract. Background: Calcitonin (CT) exerts an autocrine/paracrine influence on prostatic tumor invasion through coupling to transduction protein Gsα. Cell adhesion glycoprotein CD44 variant v7-v10 also facilitates invasion, but its modulation by the CT-Gsα system was unexplored. Materials and Methods: LnCaP, PC-3 and metastasis-derived PC-3M cell lines were studied, including cells modified therefrom: Gsα-QL, expressing mutant constitutively active Gsα protein, and CT+, overexpressing CT. CD44 variant expression was evaluated in vivo after orthotopic implantation into nude mice, and in vitro by real-time RT-PCR and Western blotting. Results: Both mRNA and protein levels of the CD44 variant were minimal in PC-3M tumor implants, but elevated in Gsα-QL. Exogenous CT stimulated invasion into Matrigel strongly in LnCaP and CT+, and less in PC-3 and Gsα-QL. By Western blot analysis, untreated Gsα-QL and CT+ cells overexpressed CD44 variant compared with LnCaP or PC-3. By quantitative RT-PCR, exogenous CT dose-dependently increased CD44 variant mRNA to seven-fold. Pharmacologic agents that stimulated or inhibited Gsα activity or stimulated adenyl cyclase produced proportionate dose-dependent effects on both CD44 variant expression and Matrigel invasion. Conclusion: This paracrine factor, acting through cyclic AMP, regulates the expression of CD44v7-10, which modulates the tumor phenotype.

Normal prostatic epithelium contains receptors for a variety of neuroendocrine factors that exert a mitogenic influence. One of them is the 32-amino acid peptide calcitonin (CT). We have demonstrated, by both in situ hybridization and immunohistochemistry in prostate cancer, that CT expression is increased in proportion to tumor grade (1). Exogenously added CT stimulates the proliferation of normally slow-growing LnCaP prostate cancer cells by increasing cAMP concentration and cytoplasmic Ca2+ transients, and increases their chemotaxis by currently unknown mechanisms (2, 3). Moreover, both primary prostatic tumor and cultured prostate cancer cells express high-affinity CT receptors (2), establishing an autocrine/paracrine role for CT. The receptor site for CT has been demonstrated to be coupled with the membrane-bound stimulatory GTP-binding protein, Gsα (4, 5). Ligand-induced and/or constitutive activation of Gsα in prostate cancer augments tumor growth (6, 7) and invasion (8). We (G.V.S.) have constructed PC-3M cell variants called CT+ and Gsα-QL, which stably express CT or constitutively active Gsα, respectively. Gsα-QL cells show increased cAMP accumulation, increased DNA synthesis (7), faster proliferation, greater invasiveness in vitro, and more metastasis formation in vivo (8).

Cell adhesion molecules such as transmembrane glycoprotein CD44, by forming complexes with extracellular matrix protein, facilitate tumor cell dyshesion and migration through a matrix. The standard isoform of CD44 (CD44s) results from expression of its exons 1-5 and 16-20 with intervening exons spliced out; longer splice variant isoforms (CD44v1-10) result from altered mRNA processing to include sequences from one or more of exons 6-15. CD44s expression is ubiquitous, whereas CD44v are restricted to epithelia. Carcinomas express novel and increased variant isoforms, reflecting dysregulated mRNA processing. In
several studies from this laboratory, we have shown that a variant isoform of CD44 is characteristically present in prostate cancer, but not benign prostate (9-12). In this paper, we show that exogenous or endogenous CT stimulates invasion to varying extents in four prostate cancer cell lines that represent the spectrum of invasiveness. Given that the CD44v7-10 isoform is characteristically overexpressed in primary (9-11) and metastatic (11) prostate cancer and facilitates cell invasion (11), we sought to determine whether its expression was altered by endogenous or exogenous stimulation of CT-Gsα signaling. We verified that this interaction existed and that Gsα and CT+ cells also overexpressed CD44v7-10. Moreover, we discovered that pharmacologic manipulation of Gsα or adenylyl cyclase overexpressed CD44v7-10. Additionally, we reported that this interaction existed and that Gsα and CT+ cells also overexpressed CD44v7-10. Furthermore, we discovered that pharmacologic manipulation of Gsα or adenylyl cyclase significantly modulated CD44v7-10 expression and invasion.

Materials and Methods

**Cell lines and culture.** LnCaP cells were purchased (American Type Culture Collection (ATCC), Manassas, VA, USA) and incubated in RPMI 1640 with L-glutamine, 10% fetal calf serum, and antibiotics at 37°C in a 5% CO2 incubator. PC-3 cells (also from ATCC) were incubated in Ham’s F12-K medium plus the above supplementary ingredients. The CT+ cell line was prepared by stably transfecting PC-3 metastatic (PC-3M) tumor cells with mammalian expression vector pcDNA3.1, containing cDNA to express the CT peptide constitutively. The second line, Gα-QL, was prepared by transfection with this same vector containing cDNA for Gα-QL, a mutant, constitutively active Gα protein that lacks GTPase activity (6). PC-3M, Gα-QL and CT+ cells were maintained in complete medium (RPMI 1640 supplemented with L-glutamine, 5% fetal calf serum, 12% horse serum, 50 U/mL penicillin, 50 µg/mL streptomycin and 20 µg/mL fungizone).

**Prostatic tumor formation in vivo.** Metastasis-derived PC-3M parental cells, or those stably expressing plasmids for either wild-type Gα or Gα-QL (8), were orthotopically injected into dorsolateral lobes of the prostates of nude mice. The protocol used 1x10⁶ cells in 20 µl Hank’s Balanced Salt Solution, as described previously (13). Each treatment group included five to eight mice. The mice were monitored for tumor growth and distant metastases in lymph nodes by palpation. Necropsy was performed 5 weeks after the implantation and tumor tissue was harvested from the prostates (8). The specimens were formalin-fixed, paraffin-embedded.

**Effect of calcitonin on cell invasion.** Studies were performed in six-well two-tier invasion chambers (Collaborative Biomedical Products, Bedford, MA, USA). 2.5x10⁵ LnCaP, PC-3, Gα-QL, or CT+ cells per well were seeded in the upper insert in 2 mL serum-free basal medium (RPMI 1640 medium containing 0.1% BSA, 150 mg/mL of G418, 4 mM L-glutamine, 100 µg/mL penicillin G and 100 µg/mL streptomycin). The lower chamber contained 2 mL of chemoattractant medium: 70% complete medium, 10% fetal bovine serum, plus 20% conditioned medium obtained from subconfluent cultures.

CT was obtained (Peninsula Laboratories, San Carlos, CA, USA) and added to medium at a 50 nM final concentration, more than sufficient to induce DNA synthesis (7) for test groups of each of these four cell lines. CT was omitted from control groups of each of the four cell lines. The incubations were carried out for 36 h. After this period, upper inserts were removed, and uninvaded cells removed from the bottom Matrigel surface using cotton swabs. The invasive cells could penetrate through the Matrigel layer and were situated on the outside bottom of the upper insert. Gel inserts were fixed, stained using Diff Quik staining (Dade Diagnostics, Aguac, PR, USA), and mounted on glass slides. The total number of cells on the entire gel was counted. Based on three experimental trials, the absolute numbers of treated invaded cells were normalized to those of control cells (11), and the results were reported as mean ± standard error.

**In situ hybridization for CD44v9.** CD44v9 mRNA was evaluated in tumors grown in nude mice. An antisense DNA probe for CD44v9 was designed (Invitrogen, Carlsbad, CA, USA) as follows: Biotin- AAGGACCCAGTCCTACGTAGGTACACTGGGAA-Biotin. A sense probe was designed in the reverse sequence. Sections from paraffin-embedded tissue were floated in DEPC-treated water baths for slide preparation. In situ hybridization was performed as previously (10). Briefly, after deparaffinization, tissue was digested with Proteinase K (DAKO, Carpenteria, CA, USA). The probe was diluted to a final concentration of 100 nM in CEP Hybridization buffer (Vysis, Downers Grove, IL, USA) delivering 20 ng/slide, diluted to a final concentration of 100 nM in CEP Hybridization buffer (Vysis, Downers Grove, IL, USA) delivering 20 ng/slide, heat to 80°C to prevent secondary structure formation, coveredslipped, and hybridized at 37°C overnight. Two stringency washes were done using 2X SSC with 0.1% Nonidet P-40 at 73°C for 5 min. Slides were transferred to PBS pH=7.4 and peroxide block was performed for 10 min with 3% H₂O₂ in methanol. Detection was accomplished using the tyramide signal amplification method of the GenPoint kit (DAKO).

**Immunohistochemical staining.** CD44v7-10 reactivity was evaluated in tumors grown in nude mice. Five µm sections were deparaffinized in xylene and alcohols and soaked for 30 min in Tris-buffered saline, pH=7.5 with 0.1% Tween-20 (TBST). Slides were subjected to steam heat antigen retrieval in citrate buffer, pH=6 for 2 x 30 min. Slides were quenched in 3% H₂O₂ in methanol for 10 min and rinsed well in distilled water, then in TBST. An “Inhibitor solution” (Ventana) was applied for 4 min followed by blocking antibodies for 10 min (20% normal swine serum in Tris-HCl, pH=7.6). Specific monoclonal anti-CD44v7/8 antibody (SeroTec, Raleigh, NC, USA) was used at 1:100 or anti-CD44v9 antibody from the supernatant of mouse hybridoma cells HB-258 (American Type Culture Collection, Manassas, VA, USA) was used neat. The specificity of these antibodies was confirmed by Western blot using colon cancer as a positive control. Antibody was applied at 4°C, overnight. Biotin block (Ventana) was applied for 3 min. A secondary antibody (Dako LSAB Kit mouse/goat/rabbit) was applied for 25 min. Slides were rinsed in TBST, covered with avidin-biotin complex for 25 min, and rinsed in TBST. Diaminobenzidine served as the chromogen, and slides were counterstained with hematoxylin. Negative controls consisted of application of non-immune whole rabbit serum at 1:300 dilution.

**SDS-polyacrylamide gel electrophoresis and Western blot analysis for CD44v9.** A pellet from treated or untreated cultured cells was lysed in RIPA lysis buffer (Upstate Biologicals, Lake Placid, NY, USA) freshly supplemented with the protease inhibitors: 2 µg/mL Pepstatin A, 1.5 µg/mL Leupeptin and 1 mM PMSF. The cell lysate
was treated with an equal volume of 2% SDS and the protein concentration was estimated by the Lowry method.

One part of 5x sample buffer (formulated as described by Bio-Rad, Hercules, CA, USA) was added to four parts of the solubilized proteins containing 40 μg of sample/lane. The proteins were then denatured for 5 min in 100°C boiling water bath and loaded into wells of a 4% stacking gel (10). Five μL of protein marker (RPN 756V Amersham Pharmacia, Piscataway, NJ, USA) were also loaded on each gel. SDS-PAGE was performed according to the Laemmli method (15) using 12% polyacrylamide gels. After electrophoresis, the protein was blotted onto nitrocellulose (Trans-Blot Transfer Medium, Bio-Rad). The membrane was hybridized overnight to primary antibody, CD44v7-10 expression was assessed using neat HB-258 supernatant, as detailed in the immunostaining section above. Goat anti-mouse IgG labeled with horseradish peroxidase (Pierce, Rockford, IL, USA) was used as a secondary antibody at 1:10,000 dilution. Reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce, Rockford IL, USA), or a colorimetric system (Opti-4CN kit, Bio-Rad). To verify equal protein loading, all membranes were next re-hybridized with mouse monoclonal anti-α-tubulin (clone DM1A, Sigma, St. Louis, MO, USA) at 1:2000 dilution and developed as above. A 50-kD band was expected.

Real-time RT-PCR for CD44 variant. A pellet was obtained from PC-3M cells that had been plated at 20,000/well in two 6-well plates. At 70% confluency, cells were serum-starved for 8 h, then four wells were treated for 12 h with each of the following: 0, 1.0, 10, or 100 nM CT. Total RNA was extracted in Trizol (Invitrogen), resuspended in 20 μL of diethylpyrocarbonate-treated water, and its concentration was measured. Complementary DNA (cDNA) was synthesized from 4 μg total RNA in a 20 μl reaction mixture, with reverse transcriptase buffer: (Tris 500 mmol/L, pH 8.3, potassium chloride 500 mmol/L, magnesium chloride 80 mmol/L, dithiothreitol 100 mmol/L), 10 units RNAasin ribonuclease inhibitor (Promega, Madison, WI, USA), oligo(dT)15 primer 25 μmol/L (Promega), 2 mmol/L each of dNTP and 150 units avian myeloblastosis reverse transcriptase (Invitrogen). PCR reaction used 4 μg cDNA plus the manufacturer’s master mix (Applied Biosystems, Foster City, CA, USA) in a volume of 25 μL. Samples were run in triplicate with primers and probe for CD44v4; samples were also amplified with 18S ribosomal RNA primers and detected with an 18S probe (all Applied Biosystems). The amplification protocol was as follows: hold 50°C 2 min, 95°C 10 min, then 40 cycles of (95°C for 0:15 and 60°C for 1:00) using the iCycler iQ protocol was as follows: hold 50°C 2 min, 95°C 10 min, then 40 cycles of (95°C for 0:15 and 60°C for 1:00) using the iCycler iQ protocol was as follows: hold 50°C 2 min, 95°C 10 min, then 40 cycles of (95°C for 0:15 and 60°C for 1:00). The amplification protocol was as follows: hold 50°C 2 min, 95°C 10 min, then 40 cycles of (95°C for 0:15 and 60°C for 1:00) using the iCycler iQ protocol was as follows: hold 50°C 2 min, 95°C 10 min, then 40 cycles of (95°C for 0:15 and 60°C for 1:00).

Pharmacologic manipulation of Gα protein and adenyl cyclase. To stimulate the Gα protein in PC-3 cells, the non-hydrolyzable GTP analog Gpp(NH)p was obtained (Sigma). Inhibition of Gα protein activity in Gα-QL cells was accomplished using a non-hydrolyzable GDP analog, GDP-βS (Sigma), for which the effective dose was expected to be up to 200 μM (16). Cultured cells were permeabilized with saponin at a 0.01% concentration for 7 min, then the saponin was removed and G-protein drugs (or for controls, no drug) were added. To compare the effect of stimulating adenyl cyclase in PC-3 and Gα-QL cells, forskolin was obtained (Sigma). Cells were treated with 5-100 μM forskolin dissolved in DMSO and untreated cells received DMSO alone. All stimulatory or inhibitory drugs were added to 4 mL cell culture at graded concentrations, for 36 h, after which cells were harvested for protein preparations and immunoblotting as above. In separate experiments, 250,000 cells/well were treated with these same pharmacologic agents while Matrigel invasion assays were carried out as detailed in the second paragraph of Materials and Methods.

### Results

Gα activity correlates with CD44v9 expression in vivo. In situ hybridization and immunostaining for CD44v9 were performed on sections of tumor grown in nude mice, consisting of PC-3M cells, Gα-wild-type and Gα-QL cells. The PC-3M cells showed virtually no mRNA signal (Figure 1A), the Gα-wild-type cells showed slight cytoplasmic reactivity (Figure 1B), and the Gα-QL cells showed intense reactivity (Figure 1C). Immunostaining demonstrated similar trends for CD44v9 protein (Figures 1D-F) and CD44v7/8 (data not shown).

Effect of exogenous or endogenous CT or constitutively active Gα on invasion. The effect of CT on Matrigel invasion was tested in the normally noninvasive LnCaP cells and invasive PC-3, Gα-QL and CT+ cells (Figure 2). The number of invaded LnCaP cells per insert was the smallest with or without added CT, but the addition of CT more than doubled the number of invaded cells compared to that of controls. An increase of similar magnitude was seen in CT+ cells. Lesser increases in invasion were noted in PC-3 cells. Gα-QL cells exhibited the highest invasiveness among the cell lines tested in this study, and exogenous CT did not significantly increase invasiveness.

Relationship between CT and/or Gα and CD44v9 expression in vitro. Since CD44v9 expression facilitates invasion, and CT increased the invasiveness of cell lines, we tested the effect of CT on CD44 variant expression. Triplicate Western blot analyses for CD44v9 were performed in four cell lines spanning the spectrum of invasiveness. The highest CD44v9 expression was seen in unstimulated Gα-QL and CT+ cells, by comparison to the less invasive PC-3 and LnCaP. Addition of CT caused an increased CD44v9 protein expression in all four cell lines tested (Figures 3A-B). The increases in Gα-QL and CT+ cells were not as marked as in LnCaP and PC-3 cells and this probably reflects the high baseline expression of CD44 variant in unstimulated Gα-QL and CT+ cells. At the mRNA level, exogenous CT at doses of 1 or 10 nM doubled expression in PC-3M cells, and 100 nM resulted in 7-fold higher mRNA levels (Figure 4).
Figure 1. CD44v9 messenger RNA and protein in tumor resulting from orthotopic implantation in nude mouse prostates (all 600x). A-C. In situ hybridization. Parental PC-3M cells have no signal (A), but minimal signal is seen in PC-3M cells transfected with a wild-type Gsα plasmid (B), and more abundant signal is noted in Gsα-QL cells, expressing mutant, constitutively active Gsα (C). D-F. Immunohistochemistry. Parental PC-3M cells have no reactivity (D). Cytoplasmic reactivity is seen in PC-3M cells transfected with a wild-type Gsα (E). Gsα-QL cells demonstrate the strongest reactivity (F). The expression is proportional to the differential Gsα protein expression among these cell lines. Therefore, this hormonal signaling system activates CD44v7-10 transcription.

Figure 2. Comparative counts of invasive cells after addition of 50 nM exogenous CT peptide to the upper chamber of Matrigel assays. Invasion was stimulated in four prostate cancer cell lines. Bar graph represents the mean number of invaded cells, normalized to untreated controls, based on triplicate trials ± SD. Increases in invasion by cell line (with unstimulated and stimulated counts in parentheses) were LnCaP, 167% (20-80 and 100-170); PC-3, 30% (626-8604 and 865-8779); Gsα-QL, 38% (969-5820 and 1540-7354); CT+, 119% (829-1425 and 1446-4418). LnCaP results were based on small numbers, precluding estimation of significance. Results may reflect that PC-3 does not express detectable CT receptor mRNA, whereas the PC-3M derived Gsα-QL and CT+ cells and LnCaP do (7).
Pharmacologic activation of Gα or stimulation of adenylyl cyclase stimulates CD44v9 expression. To examine the role of Gαq in the CT-induced increase in CD44v9 expression, we acutely activated Gαq with graded concentrations of stimulatory drug Gpp(NH)p, as described in Materials and Methods. The cell lysates were then processed for Western blot analysis. Dose-dependent increases in CD44v9 were observed. At the highest doses, the PC-3 cells expressed CD44v9 strongly, similar to untreated Gαq-QL cells (Figures 5A-B). Functionally, the result was 50% increased Matrigel invasion. To examine the role of adenylyl cyclase induction of CD44v9 by CT, we tested the effect of forskolin, a direct activator of adenylyl cyclase. Forskolin dose-dependently increased CD44v9 expression in the LnCaP and PC-3 cells (Figures 5C-D). Treatment of all cell lines with the maximally effective forskolin dose caused the greatest increases in Matrigel invasion in LnCaP and PC-3 (Figure 5E). Conversely, the Gαq protein inhibitor GDP-βS reduced CD44v9 expression in Gαq-QL cells to the level of the PC-3 cells, and this effect was dose-dependent up to 200 µM (Figures 5F-G). The drug inhibited Matrigel invasion of Gαq-QL by 32%.

**Discussion**

Our study produced three related findings: (i) Calcitonin (CT), like CD44 variant v7-10 (11), has functional significance in prostate cancer since it stimulates invasion. Invasion *in vitro* was increased by endogenous or exogenous CT in four cell lines tested. (ii) Endogenous or exogenous stimulation of the CT-Gαq protein system in prostate cancer cells correlated with expression *in vitro* and *in vivo* (in xenografts) of the CD44v7-10 sequence that is characteristically overexpressed in prostate cancer and facilitates invasion (10, 11). (iii) The influence of CT-Gαq on CD44v7-10 expression and invasion could be modulated by applying drugs that stimulate or inhibit Gαq activity or that stimulate adenylyl cyclase.

CT is a 32-amino acid peptide described in the prostate (17) that is secreted by primary prostatic epithelium and at several-fold higher levels in cancer than benign epithelium (18). CT can bind in a saturable fashion to plasma membrane fractions of prostate cancer cells (2). Furthermore, its relevance to *in vitro* growth was studied in explant-derived primary prostate cancer; exogenous CT induced a two-fold increase in DNA synthesis, and anti-CT serum caused a 56% decline (7). CT is also of special interest because its messenger RNA (1, 7) and protein (1) localize to neoplastic secretory cells, whereas expression of other neuroendocrine markers is restricted to the smaller population of neuroendocrine cells that accompany prostatic neoplasia (17). Indeed, we have shown that CT is increased in proportion to tumor Gleason grade and tumor stage (1). CT, thus, may be a determinant of prostate cancer aggressiveness, helping to discriminate the approximate 10% of cases that show the poor prognostic findings of extraprostatic spread or
metastasis. Prostatic neuroendocrine cells lack androgen receptors and thus it has been suggested that tumors with greater neuroendocrine cell populations display faster and more autonomous growth (17).

The receptor for CT is coupled to the G\(_{\alpha}\) protein (2, 4), suggesting a role for its signal transduction cascade (on the inner cytoplasmic membrane) in modulating tumor development. A local increase in secretion of CT in...
aggressive prostate cancer may cause persistent stimulation of G₁α-coupled receptors. To study this phenomenon, G₁α-QL transfectants were established (by G.V.S.) from PC-3M cells. Cells were transfected to express the gsp mutant of G₁α protein that lacks GTPase activity and thus is constitutively active. Prostate cancer cells transfected with this G₁α show increased cAMP accumulation, increased DNA synthesis (7), and greater anchorage-independent growth, basement matrix invasiveness, and metastasis formation (8). Increased G₁α protein activity in prostate cancer growth and invasion has been well-documented (7, 8, 11). The PC-3M-derived cell line CT+ was produced by stably transfecting the cloned cDNA for CT (7).

In our experiments, CT stimulated cancer cell invasion more than two-fold in the LnCaP and CT+ cells and marginally in PC-3. The stimulation in LnCaP cells is in agreement with the finding that, although this cell line does not make endogenous CT, it is positive for CT receptor (7). The weaker stimulation of invasion in PC-3 cells agrees with their having the highest baseline invasion as well as the finding that baseline cAMP is elevated 85-fold after transfection with G₁α-QL cDNA (7).

Our most notable finding was the dose-dependent relationship of CT to CD44v7-10 expression, with maximal 7-fold mRNA response at 100 nM and increased protein levels at 50 nM. CT+ and G₁α-QL cells have the strongest endogenous expression of CD44v9 protein. Further, the addition of exogenous CT increased CD44v9 protein in four cell lines tested, though not necessarily in proportion to the increase in Matrigel invasion which was greatest for LnCaP and CT+. This is, to our knowledge, the first description of CD44v9 expression through signal transduction pathways (2). Forskolin promoted a dose-dependent increase in CD44v9 expression in LnCaP and PC-3.

We previously investigated the significance of CD44v7-10 in prostate cancer. CD44 standard as well as most other cell adhesion molecules are down-regulated or unchanged in prostate cancer, the sole exceptions being increased expression of MUC18 (22, 23), N-cadherin and cadherin-11 (24). Increased and disorganized expression of CD44 variants characterizes various cancer tissues but not benign tissue. This includes breast, lung, and pancreatic carcinomas, colon cancer (25) and bladder cancer (26, 27), for which it is a serum, urinary, and tissue marker. We have observed, using immunohistochemical staining, Western blotting, in situ hybridization and reverse transcriptase-PCR, that the CD44v7-v10 portion of the variant sequence is overexpressed in prostate cancer, while expression of the CD44v2-v6 isoforms is down-regulated (9-11). Furthermore, this CD44v7-10 overexpression has functional significance; by targeting RNA interference against CD44v9, thus abrogating CD44v7-10 expression, we greatly reduced invasiveness into a Matrigel artificial basement membrane. RNA interference against MUC18, another overexpressed molecule, showed it to be of minor importance in invasion (11).

A possible linkage between G₁α activity and expression of any cell adhesion molecule, including CD44, had remained largely unexplored. CD44v7-10 overexpression in prostate cancer (11) plays a role in invasion analogous to CD44 standard in lung cancer (28). One recent piece of evidence for a link between an autocrine/paracrine factor, its G₁α-coupled receptor, intracellular cAMP, and CD44 standard expression was described in non-small cell lung cancer. The COX-2 product prostaglandin E2 (PGE2) in these cells initiates signaling through the EP4 receptor, alters MMP-2 expression, and increases invasion. CD44 expression was assessed using flow cytometry with a fluorescent antibody. EP4 signaling raised intracellular cAMP, and treatment with PGE2 or agents capable of increasing cAMP was noted to increase CD44 standard protein markedly; mRNA was not studied (29). Our findings with real-time RT-PCR and in situ hybridization provide the first evidence that CD44 is a target gene of CT.

Autocrine factors such as osteopontin can also influence CD44 expression through signal transduction pathways other than G₁α: RET/PTC kinase in transformed thyrocytes (30). Thus, in order to establish that the effects of CT on CD44v9 expression were mediated by G₁α, we performed experiments in which cell lines were subjected to a G-protein stimulatory or inhibitory agent. The normally low expression of CD44v9 in PC-3 cells was elevated to the level of concomitantly grown G₁α-QL by the stimulatory drug Gpp(NH)p, and the high expression in G₁α-QL cells was lowered to that comparable to PC-3 cells with the inhibitory drug GDP-βS. Thus, the effects of CT were largely reproducible by modulating G₁α.

We also investigated the intracellular effectors of CT, since its receptor is capable of independently stimulating both the G₁α-mediated 3′,5′-cAMP and the inositol phosphate/Ca²⁺ signaling pathways (2, 31). Forskolin promoted a dose-dependent increase in CD44v9 expression in LnCaP and PC-3.
cell lines that normally have low expression of this molecule, and increased invasion the most in these two cell lines. Others have studied the role of cAMP in prostate cancer using dibutyryl cAMP and found an increase in DNA synthesis, in vitro cell migration, and invasiveness of LnCaP cells (32). In Gsα-QL and CT+ cells, CD44v9 expression was strong and relatively refractory to forskolin. This result may reflect the aforementioned 85-fold elevation in baseline cAMP after transfection for Gsα-QL cDNA (7). The CT+ cell line is also likely to have high cAMP, making it refractory to forskolin. This suggests that Gsα-mediated cAMP elevation is the predominant intracellular effector mediating CT-stimulated CD44v9 expression. Likewise, Chien and Shah found that forskolin could not mimic the growth-promoting actions of Gsα-QL transfectants, but the calcium channel antagonist nifedipine did potently inhibit DNA synthesis in these cells (6). Taken together with our results, it appears that Gsα increases CD44 variant expression by a cAMP-mediated mechanism but promotes growth by regulating voltage-gated Ca2+ channels (Figure 6). In conclusion, CT peptide stimulation, Gsα activity, and adenylyl cyclase activity correlate positively with prostate cancer’s overexpression of CD44v7-10, in vivo and in vitro. This provides novel evidence that this signal transduction system can interact with a cell adhesion molecule to alter its expression. Since CD44 variant expression determines growth and invasiveness, its regulation by this hormonal transduction system has functional significance.

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