S179D Prolactin Increases Vitamin D Receptor and p21 through Up-regulation of Short 1b Prolactin Receptor in Human Prostate Cancer Cells

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Abstract

In this study, we further investigated the mechanisms by which pseudophosphorylated prolactin (S179D PRL) inhibits the growth of human prostate cancer cells. When treated with S179D PRL for 3 days, LnCAP cells responded by increasing expression of the vitamin D receptor (VDR) and the cell cycle regulatory molecule, p21, whereas PC3 and DU145 cells did not. After 5 days of treatment, both PC3 and DU145 cells responded. Untreated LnCAP cells express the short 1b form (SF1b) of the human prolactin receptor, but DU145 and PC3 cells express only low amounts of this receptor until elevated by treatment with S179D PRL. DU145 and PC3 cells become sensitive to the negative effects of S179D PRL on cell number after induction of the SF1b. Transfection of either SF1b or SF1a into PC3 or DU145 cells made them sensitive to S179D PRL in the 3-day time frame, a finding that was not duplicated by transfection with the long form of the receptor. Treatment of LnCAP cells with S179D PRL increased long-term activation of extracellular signal-regulated kinase 1/2 (ERK1/2). This did not occur in PC3 and DU145 cells until transfection with SF1a/SF1b. Blockade of ERK signaling eliminated S179D PRL-stimulated expression of the VDR and p21 in LnCAP cells and transfected PC3 and DU145 cells. We conclude that initiation of alternative splicing to produce SF1b, and subsequent altered signaling, contribute to the growth inhibitory mechanisms of S179D PRL. This is the first indication of a role for short prolactin receptors in the regulation of cell proliferation.

Introduction

Although a role for prolactin in prostate physiology has been recognized for some time (1–6), its clinical importance in the development of prostate hyperplasia and prostate cancer may have been underestimated. This is most likely due to a failure of clinical studies to find a clear correlation between increased circulating prolactin and disease (7–9). At the time these clinical studies were conducted, the pituitary was considered the only source of prolactin, and prolactin was considered a classic, unmodified polypeptide. It is now known that many extrapituitary tissues, including the prostate, synthesize prolactin (reviewed in ref. 10) and that prolactin is posttranslationally modified in a variety of ways that can dramatically alter biological activity (reviewed in ref. 11). Thus, even if prolactin plays an important role in prostate disease, it is possible that there would be little correlation between total circulating prolactin and disease severity. Local production of prolactin has the potential to influence prostate physiology without significant effect on circulating levels of prolactin and different forms of prolactin have been shown to be recognized differently in many immunoassays (11, 12).

More recent work from a number of laboratories has shown that prolactin is a growth factor for the normal prostate (13–16); in mice overexpressing rat prolactin, there is massive prostate hyperplasia in all lobes of the gland (14), and knockout of the prolactin or prolactin receptor (PRLR) gene produces a prostate that is smaller than usual and is resistant to SV40 induction of intraepithelial neoplasia (15, 16). Studies on normal human prostate tissue in vitro have shown an autocrine prolactin growth loop (13) and previous studies from this laboratory have shown that this autocrine growth loop is maintained in LnCAP, DU145, and PC3 prostate cancer cells (17). This latter study also showed that the autocrine prolactin produced by the prostate cancer cells was an unmodified form. In addition, it showed that a molecular mimic of the naturally phosphorylated variant of prolactin, S179D PRL, could block cell proliferation in vitro and the initiation and growth of DU145 cell tumors in nude mice (17). Thus, one form of prolactin is associated with growth, whereas another antagonizes growth. S179D PRL has also been shown to promote the expression of prostate-specific proteins in vivo and, despite the accepted importance of androgens in the expression of these proteins, to concurrently reduce the levels of circulating testosterone and dihydrotestosterone (18). At present, it is unknown whether the effects on prostate-specific gene expression and circulating androgens are direct or indirect.

The PRLR also comes in multiple forms, primarily produced in this instance by alternative splicing (19–24), although the form which constitutes the binding protein in serum may also be produced by proteolytic cleavage (25). In normal human tissues, one long and two short forms of the human PRLR are the most abundant (24, 26). These forms of the receptor have identical amino acid sequences in their extracellular and transmembrane domains but differ in the signal-transducing cytoplasmic domain. The two short forms of the receptor are produced by alternative splicing of exons 10 and 11 (23, 24). The long form of the receptor can use an array of signaling pathways including Janus-activated kinase 2/signal transducers and activators of transcription 5 (JAK2/STAT5), the mitogen-activated protein kinase pathway (MAPK), fyn activation of K channels, etc. (reviewed in refs. 27–30). The recently cloned short forms of the receptor contain a box 1 domain (23, 24), which in the long form is required for the activation of JAK2 (30, 31). Theoretically therefore, each short form has the potential to activate extracellular signal-regulated kinase 1 and 2 (ERK1/2) via the Ras/Raf pathway. They are currently thought to be unable to activate STAT5a (24). Therefore, prolactin-induced signal transduction is
likely to be different in cells predominantly expressing either the long or short forms of the receptor.

Previous work from this laboratory using a mouse mammary cell line has shown that treatment with S179D PRL increased expression of at least one of the mouse versions of short PRLRs (32), which suggests that short PRLRs may be important in the mechanism of action of S179D PRL. Increased expression of short PRLRs was also associated with increased long-term activation of ERK1/2 (32). There are considerable differences between the mouse and human short receptors (compare refs. 21, 33 with refs. 24–26), which preclude significant extrapolation from the mouse to human systems. For example, there are three short forms of the mouse receptor which only differ by a few amino acids in their cytoplasmic domains, but in humans, only two short receptors have been identified; one of which is shorter and one of which is longer than the mouse short receptors. The two human short receptors differ in length by almost 100 amino acids in their cytoplasmic domains, suggesting that they may signal very differently from each other.

A large amount of evidence shows that sustained activation of MAPK stimulates p21 expression (34–37), whereas short-term activation may have the opposite effect (38). p21 inhibits cell cycle progression and promotes apoptosis (reviewed in ref. 39), although there are some recent reports associating increased expression with a more malignant phenotype (39). PC3 cells express lower levels of p21 than the less malignant and more differentiated LnCAP cells (40). Transfection of p21 adenovirus into LnCAP, DU145, and PC3 prostate cancer cell lines has also been shown to arrest cell cycle progression (41). Vitamin D has potent antiproliferative effects in normal prostate cells and human prostate cancer models (42), and progression (41). Vitamin D has potent antiproliferative effects in normal prostate cells and human prostate cancer models (42), and LnCAP cells are more sensitive to growth inhibition by vitamin D than either DU145 or PC3 cells (43). The effects of vitamin D are mediated in large part via interaction with a nuclear receptor, the vitamin D receptor (VDR; reviewed in ref. 44). Increased expression of the VDR, brought about by transfection, increases the sensitivity of DU145 and PC3 cells to growth inhibition by vitamin D (43). Expression of the VDR is regulated by MAPK activation (45) and there is a VDR response element (VDRE) in the p21 promoter (46). One of the ways that the MAPK pathway can influence p21 expression is therefore through elevation of VDR levels.

In the current study, we further investigated the mechanism whereby S179D PRL inhibits the growth of prostate cancer cells. To do this, we analyzed both p21 and VDR expression as measures associated with decreased cell proliferation considered likely to arise from short receptor signaling. We have determined that S179D PRL up-regulates expression of the short 1b form of the human PRLR and that signaling through the short PRLR and MAPK is required for S179D PRL to increase expression of p21 and the VDR. This is the first study to report positive signal transduction by a human short receptor form and the first study to link expression of short PRLRs to cell cycle–regulating proteins.

Materials and Methods

**Production of recombinant prolactins.** S179D PRL is a pseudophosphorylated prolactin made by substituting an aspartic acid for the naturally phosphorylated serine. Recombinant S179D PRL and unmodified prolactin were made and purified in parallel in BL21 bacterial cells as previously described (47).

**Cell culture.** LnCAP, PC3, and DU145 cells were purchased from American Type Culture Collection (Rockville, MD). Cells were routinely grown in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO2 in air.

**Effects of unmodified prolactin or S179D PRL on expression of vitamin D receptor and p21.** For experiments shown in Figs. 1A–F, 2A–D, 4A–D, and 5A–F, 4 × 10³ cells were seeded per well of a 6-well, 35-mm cell culture plate. After the cells were confluent, they were washed with serum-free medium and incubated in serum-free medium (Figs. 1A–F, 2A–D, 4A–D, and 5A–C and F) or medium containing 10% charcoal-stripped horse serum (Figs. 5D–E and 6A–F; Cocalico Biologicals, Reamstown, PA) in the absence or presence of one of the prolactins (1 μg/mL). Serum was used in experiments requiring transfection or incubation in PD98059. Charcoal stripping was used to reduce both vitamin D and prolactin content. Key parts of Figs. 1, 2, 4, and 5 were repeated in medium with charcoal-stripped serum to be sure that inclusion or exclusion of serum did not alter the result. Cells were treated from 0 minute to 5 days and processed for protein or RNA extraction.

** Effects of S179D PRL on cell number.** Cells were seeded at 1,000 per well of a 96-well plate. After allowing the cells to attach overnight, they were washed thrice and treated with different concentrations of S179D PRL or RNA extraction.

** Figure 1.** Western blot of the VDR and p21 response to unmodified prolactin or S179D PRL, after incubation for 3 days. A and B, LnCAP cells; C and D, PC3 cells; E, and F, DU145. Only LnCAP cells responded and only to S179D PRL. Both unmodified prolactin and S179D PRL were used at 1 μg/mL. Columns, means; bars, ±SE. * P < 0.05 versus S179D PRL; †, P < 0.001 versus S179D PRL.
Short PRLR Up-Regulates VDR and p21/waf1

PCR assay for prolactin receptor mRNA. Cells for this purpose were grown in 75-cm² flasks and incubation in S179D PRL was for 3 days in serum-free medium. Total RNA was extracted using TRIzol reagent (Invitrogen) and 1 µg was used for reverse transcription reactions using Moloney murine leukemia virus-RT and oligo dT. PCR was done on 2.5 µL reverse transcription product using PCR Master Mix (Roche, Indianapolis, IN) as described (24). Primer sequences used to amplify SF receptors as follows: forward CCAGCGACCTTCATTAGATAC and reverse GCCCATCTCTCCAACACCC. These primers are designed to recognize both SF1a and SF1b, but the size of the product is different. As a housekeeping gene, hGAPDH primer sequences were used: forward CATGTTGGCCATGAGGTCCACCAC and reverse TGAAGGTCGGTGGAACCGATTGGC. The PCR procedure was standardized for the amount of RNA and number of cycles so that the conditions used were in the linear range.

Transfection of the short receptors. Cells at 50% to 70% confluency were washed with serum-free medium thrice and subjected to the Invitrogen LipofectAMINE protocol with low DNA and low transfection reagent. All plasmid DNA was purified using a Qiagen purification kit (Qiagen, Valencia, CA). A 0.5 µg/mL of an expression construct containing human short or long PRLR cDNA was added to each well of a 6-well plate together with 0.25 µg/l β-galactosidase construct to normalize for transfection efficiency. After allowing transfection to proceed for 12 to 18 hours, the medium was discarded and replaced by medium containing the prolactins with and without PD98059 (25 µmol/L) in 10% charcoal-stripped horse serum for 3 days. After treatment, the cell lysate was prepared for Western blot.

Preparation of cell extracts. Cells were rinsed with Dulbecco's PBS (Invitrogen) and lysed with a buffer containing 20 mmol/L Tris-HCl (pH 7.4), 140 mmol/L NaCl, 0.05 mmol/L EDTA, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 25 µg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L Na3VO4, 10 mmol/L NaF, 1 mmol/L EGTA, and 1% NP40. After centrifugation at 12,000 × g for 10 minutes, the supernatant was collected. This was used for analysis of ERK activation and immunoprecipitation of SF1b receptor. The pellet obtained was resuspended in 3 volumes of nuclear extraction buffer (20% glycerol, 20 mmol/L PMSF, 1 mmol/L DTT, and 0.1 mmol/L Na2VO4) and incubated on ice for 30 minutes. This was combined with the previous supernatant for analysis of VDR and p21. Protein concentration was measured by the Bradford method.

Immunoprecipitation of prolactin receptor. Cells were plated in 75-cm² flasks and treated with S179D PRL for 1 to 5 days in serum-free medium. Four milligrams of cell extract protein were incubated with 3 µg of an antibody directed against the extracellular domain of the human receptor (Zymed, San Francisco, CA) at 4°C overnight. The immunocomplexes were captured with 100 µL of protein A/G-agarose bead slurry (Upstate Biotechnology, Inc., Lake Placid, NY), followed by image analysis with a Kodak Edas 290 system. Stripping for reprobing occurred in wash buffer with 0.1 mol/L glycine, 1% SDS, and 20% methanol (pH 8.3). The membrane was blocked with 5% nonfat milk in a wash buffer consisting of Dulbecco's PBS containing 0.1% Tween 20. Filters were incubated with primary antibodies, rabbit polyclonal for VDR (1:1,000), p21 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), active MAPK (1:500), total ERK1/2 (1:1,000; Promega), anti-PRLR (1:1,000; Zymed), or monoclonal for goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO) at a dilution of 1:10,000, as appropriate, for 45 minutes at room temperature. After three washes, filters were exposed to enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ) followed by image analysis with a Kodak Edas 290 system. Stripping for reprobing occurred in wash buffer with 0.1 mol/L β-mercaptoethanol and 2% SDS for 30 minutes at room temperature with agitation. Blots were normalized to β-actin, a nonspecific band for predominantly nuclear proteins, the amount of heavy chain for the immunoprecipitates or total ERK, as appropriate. SF1b was identified by molecular weight.

Statistical analysis. Data were analyzed by ANOVA with posttests for comparing specific groups and Bonferroni corrections for multiple comparisons against a single group. The minimal number for each group was 3. All experiments were repeated a minimum of three times except for PCR analysis of receptor expression, which occurred twice.

Results

Both the VDR and cell cycle regulatory protein, p21, have been shown to be important players in the regulation of prostate cancer cell proliferation (39–43). Western blot analysis showed that treatment of LnCAP cells with the form of prolactin that inhibits growth, S179D PRL, but not with the form that promotes growth,
unmodified prolactin, for 3 days resulted in up-regulation of the VDR and p21 (Fig. 1). PC3 cells and DU145 cells, by contrast, showed no effect in this time frame. After a 5-day incubation, however, both PC3 and DU145 cells responded to S179D PRL but not unmodified prolactin, with a similar up-regulation of the VDR and p21 (Fig. 2). This latter result suggests the response to S179D PRL in PC3 and DU145 cells requires the prior production of another factor.

Reverse transcription-PCR analysis of the forms of PRLR expressed in each cell line both in the unstimulated state and in response to S179D PRL for 3 days showed all three cell lines to express low levels of the long form of the PRLR. This expression was not significantly altered by incubation in S179D PRL. Under the conditions used, only the LnCAP cells expressed easily measured quantities of a short form, SF1b, in the unstimulated state. Incubation in S179D PRL had no effect on expression of SF1b in LnCAP cells and yet induced expression in DU145 cells and PC3 cells (Fig. 3A). No SF1a was detected under any conditions in all three cell lines. Analysis of induction of the SF1b receptor in DU145 and PC3 cells by Western blot showed low levels on days 1 to 3 and significant increases by days 4 and 5 of treatment with S179D PRL (Fig. 3B). When the effects of S179D PRL on DU145 and PC3 cell number were analyzed at 3 and 5 days, S179D PRL was found to have no effect at 3 days, but there was a dose-related decrease in cell number after 5 days (Fig. 3C). One would have expected a lesser effect at 3 days because of the reduced number of cell divisions possible in 3 versus 5 days, but no effect was observed.

Although incubation in S179D PRL did not generate a significantly elevated expression of the VDR and p21 in 3 days in PC3 and DU145 cells, transfection of both PC3 cells and DU145 cells with either SF1a or SF1b resulted in a response to S179D PRL within the time frame. Figure 4 shows the p21 response for each cell line and each short receptor, but the same was true for the VDR (data not shown). This result was not simply a question of increased PRLR expression because transfection with the long form of the receptor did not generate a VDR and p21 response to S179D PRL (data not shown).

Because previous work in other systems had shown that S179D PRL stimulated ERK1/2 signaling, we determined whether S179D PRL signaled via ERK in the three prostate cancer cell lines. Figure 5 shows that S179D PRL caused an increase in ERK phosphorylation in LnCAP cells which was still apparent at the end of the 3-day incubation (Fig. 5A). This was not true for PC3 or DU145 cells (Fig. 5B-C) until they were transfected with either SF1a or SF1b (only DU145 result shown; Fig. 5D-E). Elevated activated ERK at the end of the 3-day incubation in S179D PRL was therefore the result of the presence (LnCAP), or transfection of (DU145 and PC3), the short receptor. No ERK activation was seen in response to unmodified prolactin under these conditions. Because such prolonged activation of ERK has not been previously reported, we analyzed the effect of S179D PRL on LnCAP cells from 15 minutes through to 5 days of incubation (Fig. 5F). The absence of activated ERK at the 0 time point and the relatively low level at 15 minutes attest to our ability to add...
S179D PRL and extract the cells without artifactual activation of ERK. A graded increase in activated ERK from 15 minutes to 1 hour was observed. The activation levels at 1 hour were then maintained until 3 days followed by a second increase evident at 4 and 5 days.

To test whether ERK signaling resulted in elevated expression of the VDR and p21, replicate 3-day experiments were conducted using the MAPK pathway inhibitor, PD98059. Figure 6 shows that PD98059 eliminated the VDR and p21 response to S179D PRL in LnCAP cells (Fig. 6A-B) and DU145 cells transfected with the short receptor (Fig. 6C-F) and had no effect on the unstimulated expression of these two molecules. The same was true for PC3 cells (data not shown).

Taken together, these results show that S179D PRL signals through either short form of the receptor to achieve prolonged activation of ERK1/2, leading to increased expression of both the VDR and p21. In prostate cancer cells with low levels of SF1b and no SF1a receptors, S179D PRL induced expression of the SF1b isoform and this correlated with the ability of S179D PRL to have a negative effect on cell number.

**Discussion**

In the present studies, we further investigated the mechanisms whereby a molecular mimic of phosphorylated prolactin inhibits the proliferation of human prostate cancer cells. We aimed to further define the physiologic role of phosphorylated prolactin and are interested in the further evaluation of the mimic, S179D PRL, as a potential prostate cancer therapeutic.

A proportion of human pituitary prolactin (49) and that from other species (e.g., ref. 50) is phosphorylated. Phosphorylated prolactin is very stable (50) despite the presence of phosphatases in

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**Figure 4.** Western blot of the p21 response to unmodified prolactin and S179D PRL in PC3 and DU145 cells transfected with short receptors and incubated for 3 days in the prolactins (1 μg/mL). A and C, SF1a-transfected PC3 and DU145 cells, respectively; B and D, SF1b-transfected PC3 and DU145 cells. Columns, means; bars, ±SE. *, P < 0.05; †, P < 0.001 versus S179D PRL.

**Figure 5.** Western blot of ERK activation. A-E, degree of ERK activation at the end of a 3-day incubation in response to either unmodified prolactin or S179D PRL (1 μg/mL). A-C, from nontransfected cells; D and E, from DU145 cells transfected with SF1a (S1a) or SF1b (S1b), respectively. F, extended time course of ERK activation by S179D PRL in LnCAP cells. This time course ran from the more usual 15 and 30 minutes (m) through 1 and 2 hours (h) to 1 to 5 days (d). Representative of at least three separate experiments (A-E).
The proportion of unmodified to phosphorylated prolactin has been shown to be physiologically regulated in rodent systems where this can be analyzed (51, 52). Using human prostate cancer cell lines, we previously showed that the predominant form of prolactin produced by these cell lines is unmodified prolactin because it is able to stimulate Nb2 cell proliferation (17), whereas phosphorylated prolactin is not (50). The proliferation-promoting activity of this autocrine unmodified prolactin can be antagonized because it is able to stimulate Nb2 cell proliferation (17), whereas another preferentially activated MAPK, and yet another was inactive (55). Thus, it is reasonable to propose that the unmodified prolactin up-regulation of the long PRLR (53). In a mouse mammary cell line, this results in the up-regulation of molecules previously shown in many labs to result in decreased cell proliferation and/or increased cell apoptosis (45). ERK activation is necessary for the induction of a differentiation response to vitamin D (45). ERK mediation of the elevation of p21 and the VDR in response to 17ß-D PRL is evident in the experiments using the MAPK pathway inhibitor, PD98059.

Taken together, these results show that part of the mechanism by which 17ß-D PRL regulates cell function is the induction of differential splicing of the PRLR and subsequent increased signaling through a short form of the receptor. For three prostate cancer cell lines, this results in the up-regulation of molecules previously shown in many labs to result in decreased cell proliferation and/or increased differentiation or apoptosis. Up-regulation of these molecules correlates with decreased prostate cancer cell number.

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