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Blockade of estrogen-stimulated proliferation by a constitutively-active prolactin receptor having lower expression in invasive ductal carcinoma

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ABSTRACT

A comprehensive understanding of prolactin's (PRL's) role in breast cancer is complicated by disparate roles for alternatively-spliced PRL receptors (PRLR) and crosstalk between PRL and estrogen signaling. Among PRLRs, the short form 1b (SF1b) inhibits PRL-stimulated cell proliferation. In addition to ligand-dependent PRLRs, constitutively-active varieties, missing the S2 region of the extracellular domain (ΔS2), naturally occur. Expression analysis of the ΔS2 version of SF1b (ΔS2SF1b) showed higher expression in histologically-normal contiguous tissue versus invasive ductal carcinoma. To determine the function of ΔS2SF1b, a T47D breast cancer line with inducible expression was produced. Induction of ΔS2SF1b blocked estrogen-stimulated cell proliferation. Unlike intact SF1b, induction of ΔS2SF1b had no effect on PRL-mediated activation of Stat5a. However induction inhibited estrogen's stimulatory effects on serine-118 phosphorylation of estrogen receptor α, serine-473 phosphorylation of Akt, serine-9 phosphorylation of GSK3β, and c-myc expression. In addition, induction of ΔS2SF1b increased expression of the cell cycle-inhibiting protein, p21. Thus, increased expression of ΔS2SF1b, such as we demonstrate occurs with the selective PRLR modulator, S179D PRL, would create a physiological state in which estrogen-stimulated proliferation was inhibited, but differentiative responses to PRL were maintained.

Introduction

Both estradiol (E2) and prolactin (PRL) stimulate cell proliferation and differentiation in the mammary gland, and both hormones are required for full development and normal function of the gland. At the same time, increased exposure to either E2 or PRL is associated with an increased risk of breast cancer [1–4]. The functions of these two hormones are intimately linked in multiple ways. For example, E2 increases PRL expression in the pituitary [5] and mammary gland [6], and increases expression of total PRL receptors (PRLR) [7]. E2 stimulation of breast cancer cells results in multiple phosphorylations of the estrogen receptor α (ERα) that are important to ER-mediated transcription, including that leading to stimulated cell proliferation. A particularly important site of phosphorylation is serine-118 [8–11]. PRL also causes serine-118 phosphorylation of ERα [12], but different signaling pathways are used by E2 and PRL to achieve this end [13]. In addition, while PRL stimulation of serine-118 phosphorylation did not significantly increase cell proliferation, it enhanced the response to E2 [13]. In other words, while serine-118 phosphorylation is an important regulator, additional actions of E2 are required for cell proliferation. Some actions of PRL may work through activation of ERα [14], although, at least on the basis of regulated gene expression, other actions of PRL do not [15]. Thus, there are important independent as well as interactive activities of E2 and PRL.

There are multiple isoforms of the human PRLR, primarily produced by alternative splicing of one initial gene transcript [16–19]. PRLRs have a single transmembrane domain (except for secreted versions) and characteristics that class them with a large family of cytokine receptors [20]. The most abundant isoforms in humans,

Abbreviations: BRET, bioluminescence resonance energy transfer; CHTN, cooperative human tissue network; c-myc, cellular proto-oncogene related to myelocytomatosis viral oncogene; Δ, delta – meaning without; DPBS, Dulbecco's phosphate buffered saline; E2, estrogen:17-β estradiol; EDTA, ethylenediaminetetraacetic acid; ER, estrogen receptor with α and β subtypes; ERK, extracellularly regulated kinase; FBS, fetal bovine serum; GAS, gamma activated site; GFP, green fluorescent protein; Gluc, Gaussia luciferase; GSK3β, glycogen synthase kinase 3β; HEK, human embryonic kidney cells; LF, long form of the prolactin receptor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; S179D PRL, a mutant prolactin in which the serine at position 179 has been replaced with an aspartate to mimic naturally-phosphorylated PRL; S2, a region of the extracellular domain of the prolactin receptor proximal to the transmembrane domain; SDS, sodium dodecyl sulfate; SF, short form of the prolactin receptor with subtypes 1a and 1b.

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referred to as a long form (LF) and short forms (SF) 1a and 1b, have identical extracellular and transmembrane domains, but differ in the length of their intracellular, signaling domains, and therefore in their signaling capabilities [21–24]. An important study in human breast cancer showed an increase in the ratio of LF to total SF receptors to correlate with the presence of disease and disease progression [25]. We extended this observational study by examining the rate of cell growth when breast cancer cells (T47D and MCF7) were transiently transfected with additional LF or SF1b receptors [26]. What we demonstrated was increased cell number with increased LF expression, a result that was counteracted by the co-expression of SF1b.

The short PRLRs have been described as dominant negatives since they inhibit an activity of PRL mediated through the LF and Stat5 activation [18,22,27]. This dominant negative activity of the SFs on Stat5 activation was originally thought to be due simply to the formation of heterodimers between LF and SF receptors and subsequent interference with LF-mediated Stat5 signaling [22]. Indeed, we and others have provided evidence demonstrating such hetero-dimerization [18,22,27,28]. However, we have also shown that expression of SF1b, but not SF1a, down regulates expression of the LF receptor in breast cancer cells by affecting LF mRNA stability [26], thereby adding a new dimension to the dominant negative effect.

For each of the major forms of the human PRLR, a naturally-occurring splice variant missing the S2 region of the extracellular domain (missing approximately half of the extracellular domain normally closest to the membrane) has been described [19]. These were detected using a primer set that recognized the apposition of the S1 and transmembrane regions. Their translation into proteins demonstrated that each was constitutively-active [19,29], confirming the original observation of this property for the ΔS2 LF by Gordou et al. [30].

Here, we have examined the potential role of the constitutively-active variety of SF1b in normal breast health and breast cancer. We show that normal tissue has a higher expression of ΔS2SF1b and that induction of ΔS2SF1b blocks E2-stimulated cell proliferation. However, unlike intact SF1b, ΔS2SF1b does not heterodimerize with the LF PRLR and does not inhibit PRL-stimulated Stat5 activation at the LF PRLR. Instead, induction of ΔS2SF1b increased expression of the cell cycle-inhibiting protein, p21, and interfered with multiple effects of E2, creating a scenario in which proliferation was inhibited, but differentiative responses to PRL could continue.

Materials and methods

Semi-quantitative and real-time RT-PCR

Breast tissue samples were obtained from the Cooperative Human Tissue Network (CHTN) (Midwestern Division, Columbus, OH). The CHTN is funded by the National Cancer Institute (USA) and patient consent for use of samples in research is obtained by them as the distributing entity. Tumors and adjacent normal regions were obtained at surgery and forwarded by CHTN after coding for anonymity and pathological evaluation. Use of anonymous patient samples was approved by the University of California, Riverside Institutional Review Board. Analysis was of samples from nine patients with invasive ductal carcinoma (one male) ranging in age from 47 to 68 years (mean, 60.3). All tumors were ER + PR + HER2–. For RNA extraction, about 0.05 g of the frozen tissue sample was first ground with a mortar and pestle in liquid nitrogen, followed by homogenization in Trizol (Invitrogen, Carlsbad, CA) with a glass homogenizer. Two microgram total RNA was reverse transcribed with M-MLV reverse transcriptase (Invitrogen). For detection of ΔS2 SF1b, cDNA was amplified by PCR using GoTaq DNA polymerase (Promega, Madison, WI). Amplifications were carried out with an initial denaturation at 94 °C for 1 minute, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. A final extension at 72 °C was performed after the last cycle for 5 minutes. PCR products were resolved on a 1% agarose gel and stained with ethidium bromide. The product size for ΔS2SF1b was 188 bp using the primers in Table 1. Real-time RT-PCR was conducted as previously described using SYBR Green PCR Master Mix (e.g. [29]). All primer sequences are listed in Table 1. Relative expression to vehicle controls was analyzed using the $2^{-\Delta\Delta C_T}$ method.

Table 1
Primers used for traditional and real-time PCR.

mRNA	Forward primer 5′–3′	Reverse primer 5′–3′
c-myc	TCGGATTCTCTGCTCTCCTC	CCTGCCTCTTTTCCACAGAA
β-actin	AAAGACCTGTACGCCAACAC	GTCATACTCCTGCTTGCTGAT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
ΔS2 SF1b	ACATAGTTCAGCCAACCATGAATGATAC	AGGGGTCACCTCCAACAGAT
SF1b	TAAATGGTCTCCACCTACCCTGAT	AGGGGTCACCTCCAACAGAT

Cell culture

T47D human breast cancer cells and 293 human embryonic kidney (HEK) cells were purchased from American Type Culture Collection (Manassas, VA). The T47D Tet-On cell line was purchased from Clontech (Mountain View, CA). T47D cells were routinely cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO_2. The specific and control T47D Tet-On cell lines were grown under the same conditions except for the addition of 200 μg/ml Geneticin (G418 sulfate, Invitrogen).

Establishment of T47D tet-on cell lines

The Tet-responsive plasmid was originally developed by Gossen and Bujard [31]. The coding sequence for ΔS2SF1b was excised from a pcDNA3.1(+) expression plasmid described previously [29] with EcoRI and XbaI, and subcloned to the Tet-responsive plasmid. The resulting DNA was then inserted at a XhoI site with a hygromycin resistance gene (including the SV40 promoter and polyadenylation signal) amplified from pcDNA3.1(+)/Hygro (Invitrogen). For the generation of stable cell lines, 10 μg of Tet plasmid DNA containing the ΔS2 SF1b cDNA was transfected into subconfluent T47D Tet-On cells in 10-cm dishes using Fugene HD transfection reagent (Roche Diagnostics, Indianapolis, MN), according to manufacturer's protocol. Stable transfectants were selected with Geneticin (200 μg/ml) and Hygromycin B (200 μg/ml, Invitrogen). Isolated colonies were screened for inducible ΔS2 SF1b expression in response to doxycycline (Clontech) according to manufacturer's guidelines. Stable transfection of an empty vector was performed in parallel to produce control cells.

Immunoprecipitation and western blotting

Whole cell extracts were first prepared by solubilizing cells in cell lysis buffer (0.14 M NaCl, 20 mM Tris base, 10 mM $Na_2P_2O_7$, 10 nM NaF, 1 mM Na_3VO_4, 1% NP-40, 0.02% NaN_3 and 0.5 mM EDTA) supplemented with 1× Complete Mini protease inhibitor cocktail (Roche Diagnostics), followed by two freeze-thaw cycles and clarification. For immunoprecipitation, 500 μg whole cell lysate soluble proteins were incubated with 0.5 μg antibody at 4 °C overnight. Immuno-complexes were captured with protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and the beads were washed three times with cell lysis buffer before solubilization in SDS gel loading buffer.

For Western detection, immunoprecipitates or equal quantities of whole cell lysates (30 μg) were resolved on an 8% or 10% (depending on the molecular weight of the target protein) SDS reducing polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking in 3% bovine serum albumin or 5% non-fat milk for 1 h, membranes were probed with a primary antibody overnight at 4 °C. Antigen–antibody interactions were detected by using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Bar Harbor, ME) and Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA). To ensure equal loading, membranes were stripped with Restore Plus Western blot stripping buffer (Thermo Scientific, Rockford, IL) and re-probed with an internal control antibody.

Anti-phosphorylated ERα (serine-118) was purchased from Upstate Biotechnology (Charlottesville, VA) and was used at 1:1000. Anti-phosphorylated GSK3β (serine-9), anti-phosphorylated Akt (serine 473) and anti-total Akt were purchased from Cell Signaling (Danvers, MA) and used at 1:2000. Anti-PRLR ECD (1A2B1) was purchased from Zymed (Carlsbad, CA) and was used at 1:1000. Antibodies against phosphorylated ERK, ERK, actin, phosphorylated tyrosine (PY20), Stat5, p21, vitamin D receptor, ERα, and ERβ were purchased from Santa Cruz Biotechnology and were used at 1:1000.

Quantification of Westerns used Adobe Photoshop software and normalization to actin and experimental control to allow combination of data obtained on several occasions.

Assay of viable cell number

Cells were seeded at a density of 5×10^3 per well in quadruplicate in a 96-well plate. After 24 h, cells were washed with Dulbecco's phosphate buffered saline (DPBS) and then incubated in phenol red-free RPMI 1640 supplemented with 5% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT) in the presence of 17β-estradiol (1 nM) and/or doxycycline (1 μg/ml). After 3 days, medium was changed to serum-free. Cell number was determined by the MTS formazan conversion assay. The assay

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