Characterization of Δ7/11, a functional prolactin-binding protein

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Abstract

Prolactin is essential for normal mammary gland development and differentiation, and has been shown to promote tumor cell proliferation and chemotherapeutic resistance. Soluble isoforms of the prolactin receptor (PrlR) have been reported to regulate prolactin bioavailability by functioning as ‘prolactin-binding proteins’. Included in this category is Δ7/11, a product of alternate splicing of the PrlR primary transcript. However, the direct interactions of prolactin with Δ7/11, and the resulting effect on cell behavior, have not been investigated. Herein, we demonstrate the ability of Δ7/11 to bind prolactin using a novel proximity ligation assay and traditional immunoprecipitation techniques. Biochemical analyses demonstrated that Δ7/11 was heavily glycosylated, similar to the extracellular domain of the primary PrlR, and that glycosylation regulated the cellular localization and secretion of Δ7/11. Low levels of Δ7/11 were detected in serum samples of healthy volunteers, but were undetectable in human milk samples. Expression of Δ7/11 was also detected in six of the 62 primary breast tumor biopsies analyzed; however, no correlation was found with Δ7/11 expression and tumor histotype or other patient demographics. Functional analysis demonstrated the ability of Δ7/11 to inhibit prolactin-induced cell proliferation as well as alter prolactin-induced rescue of cell cycle arrest/early senescence events in breast epithelial cells. Collectively, these data demonstrate that Δ7/11 is a novel regulatory mechanism of prolactin bioavailability and signaling.

Key Words

- prolactin
- prolactin receptor
- mammary
- alternative splicing
- prolactin-binding protein

Introduction

Prolactin is a ubiquitous and pleiotropic polypeptide hormone that plays a critical role in breast development, differentiation, and lactation (Das & Vonderhaar 1997, Trott et al. 2008). It is produced by lactotrophic cells within the anterior pituitary, as well as by many extrapituitary tissues including the mammary epithelium, skin, placenta, uterus, brain, and immune cells (Oakes et al. 2008). Through the use of diverse animal models, over 300 functions have been identified for prolactin, including an influence on behavior, electrolyte balance, the regulation of metabolism, and immune response (Bole-Feyssot et al. 1998, Bernichtein et al. 2010). The co-expression of the prolactin receptor (PrlR) in these tissues suggests that an autocrine–paracrine loop of action exists (Hovey et al. 2002, Oakes et al. 2008, Trott et al. 2008). However, due to the inability to distinguish

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between pituitary and extrapituitary prolactin (Bernichtein et al. 2010) and the difficulty in detecting the protein in tissue culture models (Ginsburg & Vonderhaar 1995), our comprehension of the physiological role of extrapituitary prolactin, as well as its regulation, remains poorly understood.

The PrlR is a member of the type-1 cytokine receptor superfamily, which includes the receptors for GH, colony-stimulating factor, and interleukins (Bole-Feyssot et al. 1998). These receptors are single-chain transmembrane proteins that consist of an extracellular, a transmembrane, and an intracellular domain (Vonderhaar et al. 1985, Trott et al. 2004, 2008). Upon ligand binding, receptor homodimerization occurs and cell signaling is induced through the activation of receptor-associated kinases. The canonical prolactin signaling pathways include Jak2/STAT5, c-Src/Fyn, PI3K/akt, Nek3-Rac1, and Grb2-MAPK pathways (Hennighausen et al. 1997, Harris et al. 2004, Nguyen et al. 2008). Adding to the complexity of this multifaceted role of prolactin, the primary transcript of the PrlR has alternative splice products that yield different lengths of the cytoplasmic tails, each of which are reported to have distinctive signaling properties (Clevenger et al. 2009).

Autocrine/paracrine regulation of bioavailable extrapituitary prolactin to its receptor has been proposed to be regulated by soluble forms of the PrlR (Dannies 2001, Kline & Clevenger 2001, Trott et al. 2003). This mechanism of regulation has been demonstrated for many proteins including tumor necrosis factor-α, ciliary neutrophic factor, GH, leptin, interleukins 1, 2, and 6, transferrin, and nerve growth factor (Rose-John & Heinrich 1994, Dannies 2001). These soluble receptor ‘binding proteins’ are generated either by alternate splicing of mRNA for the receptor or through post-translational proteolytic cleavage of the extracellular domain (ECD) of the receptor. To date, two prolactin-binding proteins (PrlBPs) have been described in human serum, milk, and cell lysates (Kline & Clevenger 2001, Trott et al. 2003). Kline & Clevenger (2001) first characterized a PrlBP in human serum and milk by co-precipitating PrlBPs with antibodies generated against human prolactin and the ECD of the human PrlR, as well as by demonstrating the binding affinity of the PrlBPs for prolactin and GH. The structural properties of the PrlBPs were confirmed using limited proteolysis and mass spectrometry, and the ability of PrlBPs to antagonize the growth-promoting function of prolactin was demonstrated in a cell culture model (Kline & Clevenger 2001).

A second soluble form of the PrlR was subsequently described (Trott et al. 2003). This PrlR isoform results from alternative splicing of the mRNA from exon 7 to exon 11, thus giving its name Δ7/11. Expression of Δ7/11 has been reported in human colon and breast tumor samples, as well as in tumor-associated histologically normal breast tissue. Fluorescent immunohistochemistry illustrated a vesiculated intracellular distribution, consistent with its fate as a secreted protein. Similar to the previously identified human PrlBP, Δ7/11 was shown to bind to GH. However, its binding affinity for prolactin and the physiological function are yet to be described.

Herein, we demonstrate the ability of Δ7/11 to directly bind prolactin using novel proximity ligation assays and traditional immunoprecipitation techniques. The biochemical properties and relative binding affinities of Δ7/11 for prolactin were investigated. Δ7/11 was found to be glycosylated and was not detectable in human milk, two properties that distinguish it from PrlBP. Furthermore, we demonstrate the ability of Δ7/11 to alter the prolactin-induced cell proliferation and signaling of breast cancer cells.

### Materials and methods

#### Cell culture

T47D, MDA MB231, MCF7, Hs578t, and Chinese hamster ovary K1 (CHO) cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured as recommended. Cells were passaged using trypsinization (0.05% trypsin–EDTA; Invitrogen) and counted on a hemocytometer using trypan blue exclusion.

#### Production of recombinant Δ7/11

Recombinant protein was produced based on published data (Trott et al. 2003), isolated, and purified by ProteinOne (Rockville, MD, USA) in their Escherichia coli expression system. Protein was validated via sequencing and western blot analysis.

#### Generation of human PrlR isoform overexpressing CHO cells and collection of conditioned media

Design of the flag-tagged human PrlR isoform constructs was described previously (Trott et al. 2003). CHO cells were plated in normal growth medium. Twenty-four hour post-plating, cells were transfected with either pEF6C (empty vector) or pEF6C-Δ7/11DNA using Fugene HD Transfection Reagent (Roche Applied Science). After 2 days of growth, stably transfected cells were selected in the

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presence of blasticidin (2 \( \mu \)g/ml; Invitrogen). The presence of the protein was confirmed via dot blot analysis using FLAG (Sigma) and PrlR isoform-specific antibodies (Antibody Production and Purification Unit, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA). For collection of conditioned media, near-confluent stably transfected CHO cells were washed and placed in serum-free media for 24 h. Media were collected, centrifuged to remove cells, and then directly used to treat the cells. When indicated, conditioned media were concentrated using an Amicon Ultra centrifugation filter (MWCO 10K; Millipore, Billerica, MA, USA).

**Generation of human prolactin antibody**

The prolactin antibody was produced by PRIMM (IMGEN Technologies, Cambridge, MA, USA) via immunization of rabbits with a recombinant His-tagged prolactin protein (residues 23–220, GI: 531103) and affinity column purified by the Antibody Production and Purification Unit (APPU; National Cancer Institute, Bethesda, MD, USA). Initial affinity column purification was followed by an additional purification using a GE Superdex 200 2.6/60 on an Akta Purifier (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) in PBS containing 0.1% sodium azide. The resulting antibody was validated by western blot analysis against the immunizing protein.

**Solution-phase proximity ligation assay**

All assay probes and reagents were purchased from Applied Biosystems and used as directed. Two sets of probes were used. The first set of probes was designed to detect \( \Delta 7/11 \) using the SF1a rabbit MAB and the commercially available mouse MAB that detect the ECD of the PrlR (Invitrogen Cat.# 35-9200). The second set of probes was designed to detect human prolactin and used the prolactin antibody (PRIMM) described above. Applied Biosystems linked the short DNA strands to the primary antibodies for detection. Each set of probes (primary antibodies linked to the DNA probe) was used for dual recognition of the target protein complex in situ, i.e. in their physiological context. If the DNA strands are in close proximity, they interact to form a unique DNA amplicon, which is subsequently amplified and detected by quantitative real-time PCR. The procedure is as follows. Briefly, diluted samples and the solution-phase proximity ligation assay (spPLA) probes (62.5 pM) were incubated overnight at 4 °C. The following day, ligation buffer mix was added to each well, incubated for 10 min at 37 °C, and then inactivated by a 5 min incubation at 95 °C. Samples were then mixed with TaqMan Fast PCR Master Mix (Life Technologies) and products measured using the StepOne-Plus Real Time PCR System (Life Technologies). Data were analyzed via the \( \Delta C_t \) method. Controls were reaction mixture alone and reaction mixture containing the probe pairs only (no protein added). For detection of \( \Delta 7/11 \) in human serum, a panel of normal and matched breast cancer serum samples was purchased from SeraCare Life Sciences, Inc. (Oceanside, CA, USA).

**Proliferation assays**

Breast cancer cells, proliferating in log phase, were placed in RPMI-1640 containing 0.01% charcoal-stripped serum (control media) for 24 h followed by 3 days of treatment. For treatment with conditioned media: cells were treated with fresh conditioned media from stably transfected CHO cells expressing either human \( \Delta 7/11 \) or the empty vector control in the presence or absence of 100 ng/ml of recombinant human prolactin. For treatment with recombinant protein, cells were treated with control media, control media with 100 ng/ml of prolactin, control media with 250 ng/ml of recombinant \( \Delta 7/11 \), or the combination. For all proliferation assays, cells were trypsinized and counted on a hemocytometer using trypan blue exclusion. Data represent three independent experiments ± s.d.

**Cell cycle analysis**

For cell cycle analysis, \( 2 \times 10^4 \) cells/cm\(^2\) were plated in growth medium for 24 h and then washed and incubated in media containing 0.01% charcoal-stripped serum overnight. Cells were then treated with prolactin, \( \Delta 7/11 \), or the combination, as indicated, in media containing 0.01% charcoal-stripped serum for 3 days. Following the treatment, cells were washed in DPBS and fixed in 70% ethanol for 24 h to 3 days. On the day of analysis, cells were treated with RNase (100 \( \mu \)g/ml; Invitrogen) for 20 min at 37 °C, followed by incubation with propidium iodide (50 \( \mu \)g/ml in DPBS; Invitrogen) for 60 min at 4 °C. Prior to analysis on the FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA), cells were filtered through a 40 \( \mu \)m nylon mesh filter. Side and forward scatter were used to exclude cell debris/dead cells/clumps, and a second dot plot was employed to gate propidium iodide-positive cells. A minimum of 10,000 cells was acquired. The percentage of cells in the G\( _0/G_1 \), S, and G\( _2/M \) phase, and CV were
Deglycosylation assays

Human milk (a kind gift from Dr Gilbert Smith; National Cancer Institute, Bethesda MD, USA), human serum, recombinant Δ7/11 protein, and conditioned media collected from CHO cells stably expressing Δ7/11 were subjected to deglycosylation using the PNGase kit from New England BioLabs ( Ipswich, MA, USA) as directed. Briefly, samples were denatured for 10 min at 100°C, followed by incubation with the PNGase enzyme cocktail for 1 h at 37°C. Reducing sample buffer (Bio-Rad) was added, samples were incubated for 10 min at 90°C, and then separated by SDS–PAGE. Proteins were transferred to PVDF membranes using the iBlot (Invitrogen) at P3, for 7 min. The membranes were blocked for 1 h in 5% milk in TBS buffer with 0.1% Tween (TBST) at room temperature, and then incubated with primary antibody (5 μg/ml of SF1a rabbit MAB) overnight at 4°C, washed, incubated with the appropriate secondary antibody conjugated to Alexa680 (Invitrogen) in TBST with 5% milk for 1 h at room temperature, and then imaged using the Li-COR Odyssey (Lincoln, NE, USA). The membranes were then stripped with 1 M NaOH for 5 min, washed, probed with a PrLR antibody that recognized the ECD of all PrLR isoforms (5 μg/ml; Invitrogen), and imaged as described above.

For cell culture deglycosylation studies, T47D cells were transiently transfected with a FLAG-tagged human Δ7/11 construct (previously described in Trott et al. (2003)). Three days post-transfection, cells were treated with 500 ng/ml of tunicamycin (Sigma) for 48 h. This concentration was previously determined to affect the glycosylation of proteins, and not protein synthesis (Banerjee et al. 1993). Cells were treated for 2 days; conditioned media were collected and concentrated using the Amicon Ultra-10 filtration system (EMD Millipore). Cell lysates were collected and separated into membrane and cytosol fractions using the BioVision FractionPREP kit (Milpitas, CA, USA) as directed, and then analyzed via western blot as described below. For immunofluorescence detection of Δ7/11 localization, cells were briefly fixed in 100% methanol, washed, and then blocked in 1× PBS containing 5.0% goat serum and 1.0% BSA (blocking buffer) for 30 min at room temperature. Cells were then incubated with a 1:50 dilution of the primary antibody (anti-FLAG; Sigma) in 2% BSA and 2% goat serum overnight at 4°C, washed with 1× PBS, and then incubated with the appropriate secondary antibody (anti-mouse Alexa Fluor 488; Invitrogen) at a 1:1000 dilution in blocking buffer for 30 min at room temperature. Coverslips were mounted using Prolong Gold antifade reagent with DAPI (Invitrogen). Imaging was performed using the Carl Zeiss LSM510 confocal imaging system (Carl Zeiss MicrolImaging) at ×20 magnification.

PCR analysis of breast tumor biopsies

Collection of patient samples was performed in accordance with the guidelines of the National Cancer Institute’s Institutional Review Board, protocol OH99-C-NOS7. Total RNA was isolated from tumor biopsies using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. RNA was reverse transcribed using MMLV reverse transcriptase (Invitrogen) and primed with oligo-dT and random hexamers (Invitrogen). cDNA was amplified using gene primers that recognize all three isoforms of the PrLR, as described in Trott et al. (2003), and PCR Master Mix (Roche Applied Science). PCR data were analyzed via gel electrophoresis.

Western blotting

Equal concentrations of protein, as determined by the Bradford assay, were separated by SDS–PAGE under reducing conditions. Membranes were blocked in 5% non-fat milk in TBST for 1 h at room temperature, then incubated with primary antibody (P38, P-P38 (Tyr 182), and PERK, Santa Cruz; FLAG, Sigma; PSTAT5 and PAKT, Cell Signaling Technology) overnight at 4°C in TBST +5% BSA, washed, and incubated with the appropriate secondary antibody conjugated to HRP (GE Healthcare) in TBST with 5% milk for 1 h at room temperature. Peroxidase activity was detected using the enhanced chemiluminescence detection system (ECL Plus, GE Healthcare) as directed. GAPDH was used as a control to show equal loading (Santa Cruz). Western blots were quantified using NIH ImageJ 64.

Immunoprecipitation

Recombinant human prolactin (500 ng) and recombinant human Δ7/11 (1 μg) were incubated alone or in combination with either the SF1a rabbit MAB (5 μg/ml) or a commercially available antibody directed against human prolactin (5 μg/ml, PRIMM) in PBS, overnight at 4°C. The next day, protein A/G PLUS beads (Invitrogen) were added; samples were incubated for 1.5 h at 4°C, and then washed in PBS. The immunobead complexes were calculated using MODFIT-LT software (Verity Software House, Topsham, ME, USA).
pelleted, resuspended in reducing sample buffer (Bio-Rad), and the eluted proteins were subjected to SDS-PAGE and western blot analysis as described above. Primary antibodies were a PrlR ECD antibody (1:500; Invitrogen) or a polyclonal antibody directed against human prolactin (2 μg/ml). Secondary antibodies were goat anti-mouse FITC (1:3000; Invitrogen) or clean-blot secondary anti-rabbit (1:100; Pierce Biotechnology, Rockledge, IL, USA). Controls included beads incubated with the proteins but no antibody and beads incubated with normal rabbit serum. For conditioned media immunoprecipitation, the same procedure was performed with the following modifications: i) 800 ng of recombinant human prolactin, ii) 1.0 ml of concentrated conditioned media from Δ7/11-expressing CHO cells was the source of Δ7/11 and concentrated conditioned media from CHO cells expressing the empty vector were used as the control.

**Statistical analysis**

Data were evaluated for significance via t-tests or one-way ANOVA with the appropriate post hoc analysis (Tukey, Bonferroni) using GraphPad InStat Software version 3.0b (San Diego, CA, USA). Data were considered as significant at P < 0.05.

**Results**

**Evaluation of recombinant Δ7/11 and prolactin binding interactions**

Δ7/11 was identified as an alternative splice variant of the primary human PrlR transcript using RT-PCR of breast tumor tissue RNA (Trott et al. 2003). Figure 1A illustrates the alternate splicing of the exons found within the discussed PrlR isoforms, and Fig. 1B shows the extracellular and intracellular domains present in the membrane bound and soluble PrlR variants (adapted from Trott et al. (2003)). Bioinformatic analysis suggested that Δ7/11 was a secreted binding protein and the ability of Δ7/11 to bind the ligand was examined via immunoprecipitation of FLAG-tagged Δ7/11 with 125I-labeled human GH (hGH). The results confirmed the ability of Δ7/11 to bind the ligand, and the ability of Δ7/11 to bind 125I-hGH was competitively inhibited by the addition of ovine prolactin (Trott et al. 2003). However, the direct interaction of Δ7/11 with prolactin was not investigated. Therefore, to directly assess the ability of Δ7/11 to bind prolactin, a novel in-solution proximity ligation assay (spPLA) was used.

A MAB directed against the short form 1a isoform of the PrlR (SF1a) and a PrlR ECD antibody (ECD) were each linked to DNA strands to form the detection probes. Upon proximal binding of the probe pairs to their respective target proteins, the cDNA strands hybridize and are ligated to form a unique DNA amplicon, which is subsequently detected by quantitative real-time PCR. Based on the structure of the PrlR isoforms (Trott et al. 2004), the SF1a and ECD set of probes will detect only the SF1a and Δ7/11
proteins. Initial optimization experiments demonstrated the detectable range of Δ7/11 protein, 2 pg–4 ng (Fig. 2A). Additional probes to detect human prolactin were developed using a human specific antibody (design/production described in Materials and methods). The specificity of the prolactin probes to human prolactin compared with ovine prolactin was also evaluated, and the results show a preferential detection of human prolactin (Fig. 2B). To further establish the selectivity of this assay, we next examined whether a similarly sized and relevant peptide hormone, hGH, could be detected in the assay. The results confirmed the specificity of the assay as the hGH was below detectable limits of the assay (Fig. 2C).

Following optimization of the probes, we tested the ability of recombinant human Δ7/11 to directly interact with prolactin. As shown in Fig. 3A, in vitro incubation of Δ7/11 with prolactin resulted in a dose-dependent increase in the detection of protein interactions using the SF1a and prolactin probe pairs. The maximal detection of protein binding with 10 nM prolactin occurred at 500 nM Δ7/11. When using only prolactin-specific probes, a dose-dependent inhibition of prolactin detection was identified (Fig. 3B). Collectively, these results demonstrate direct protein interactions of Δ7/11 and prolactin.

To confirm the results obtained from the spPLA assays, standard immunoprecipitation experiments were performed. Both recombinant Δ7/11 and Δ7/11-expressing CHO cell-conditioned media were tested for their ability to bind human prolactin. The two sources of Δ7/11 were incubated with prolactin and immunoprecipitated with either the prolactin antibody or the SF1a antibody, followed by western blot analysis using the PrlR ECD or human prolactin antibody. The results confirmed our observations from the spPLA assay, both sources of Δ7/11 bound to prolactin (Fig. 4). Similar to the spPLA assays, we were unable to detect Δ7/11 when incubated with prolactin and immunoprecipitated with the prolactin antibody (Fig. 4, bottom panel). The inability to detect Δ7/11 may be due to variations in the potency and form of Δ7/11 produced in mammals compared with the recombinant Δ7/11 produced by bacteria. The different sources of Δ7/11 may have altered the binding affinity or strength of the protein–protein interactions, which resulted in the loss of the protein complex during the co-immunoprecipitation procedure. Alternatively, additional post-translational modifications of Δ7/11 by mammalian cells might have altered the binding affinity or physically blocked the binding site for the prolactin antibody to bind to its epitope on prolactin. For example, glycosylation (i.e. the addition of a carbohydrate to the Δ7/11 molecule) may cause a steric hindrance in the ability of the prolactin antibody to reach the epitope on prolactin when bound to glycosylated Δ7/11; therefore, only the free prolactin, not bound to Δ7/11, was able to be immunoprecipitated when the prolactin antibody was used to detect the prolactin and Δ7/11 interactions.

Figure 2
Optimization of the solution-phase proximity ligation assays. To generate the detection probes, SF1a and PrlR antibodies were linked to the DNA strands, and upon proximal binding of the antibodies to their respective target proteins, the DNA strands ligate and the PCR amplicon is detected using quantitative real-time PCR. (A) Representative data of Δ7/11 detection using a MAB directed against the extracellular domain and the SF1a isoform of the PrlR. (B) Representative data of ovine and human prolactin (oPRL and hPrl respectively) detection using human prolactin-specific probes. (C) Representative data of GH (hGH) and human prolactin detection using human prolactin-specific probes. Data represent average ΔCt of a minimum of three replicates ± s.d.
Glycosylation of Δ7/11

Previous studies have described a PrlBP that is found in human serum and milk, and is not glycosylated despite the high level of glycosylation on the asparagine residues of the PrlR ECD (Buteau et al. 1998, Kline & Clevenger 2001). Therefore, the glycosylation status of Δ7/11 was investigated. Δ7/11-expressing CHO cell lysates and recombinant Δ7/11 were incubated in the presence or absence of peptide N-glycosidase F, and then evaluated for a shift in electrophoretic mobility by western blot analysis. Δ7/11 was detected using either the SF1a or PrlR ECD MAB, both of which recognize the lower molecular weight Δ7/11 in addition to other PrlR isoforms (SF1a, 57 kDa; Δ7/11, 39.1–40.6 kDa depending on glycosylation; Trott et al. 2004). As shown in Fig. 5, CHO cell lysates repeatedly demonstrated a distinct shift in electrophoretic mobility when treated with the glycosidase, suggesting glycosylation of Δ7/11. The recombinant Δ7/11, produced in bacteria, lacked glycosylation. In addition to the cell lysates and recombinant Δ7/11, human serum and milk were treated with the glycosidase and tested for the presence of Δ7/11. Δ7/11 could not be detected in serum or milk via western blot analysis using either the SF1a or PrlR ECD MAB. However, consistent with previous reports (Kline & Clevenger 2001), the PrlBP was detected in serum and milk and did not exhibit any shift in electrophoretic mobility (Fig. 5B).

To analyze the functional significance of Δ7/11 glycosylation, enzymatic deglycosylation studies were performed in cultured cells. T47D cells were transiently transfected with FLAG-tagged Δ7/11 and grown in the presence or absence of 500 ng/ml of tunicamycin (Sigma) for 48 h. This concentration was previously determined to affect the glycosylation of proteins, and not protein synthesis (Banerjee et al. 1993). Similar to previous studies showing that glycosylation is required for PrlR long-form membrane localization (Banerjee et al. 1993), we show that the secretion and localization of Δ7/11 is regulated by glycosylation. Both western and immunofluorescence analyses show altered Δ7/11 localization upon treatment. Specifically, cells treated with tunicamycin had increased levels of Δ7/11 in the cytoplasm, and significantly
with control cells, suggesting that non-glycosylated Δ7/11 accumulates in the Golgi area. Collectively, these data suggest that the functional mechanism of Δ7/11 glycosylation involves proper localization and secretion of the protein and has no effect on the ability of Δ7/11 to bind prolactin.

We further examined human serum for the presence of Δ7/11 using the spPLA for enhanced detection. Ten serum samples from breast cancer patients and 13 healthy volunteer serum samples were analyzed. A control sample of serum supplemented with recombinant Δ7/11 was used as a positive control. Three of the healthy volunteer serum samples had low but detectable levels of Δ7/11 (Fig. 6), suggesting that in contrast to the PrlBP, Δ7/11 is not abundantly secreted into the circulation. Δ7/11 has also been identified in both breast tumors and the patient-matched, associated normal breast tissue (Trott et al. 2003). To extend upon this initial observation and investigate Δ7/11 expression with clinical variables, we analyzed an additional 62 primary breast tumor biopsies for Δ7/11 expression. Consistent with previous results, ~10% (6 of 62) of the tumors were positive for Δ7/11 expression (Supplementary Figure 1, see section on supplementary data given at the end of this article). No correlation was found with histotype (lobular vs ductal carcinomas), estrogen receptor positivity of the tumor cells, or with patient age.

**Δ7/11 inhibits prolactin-stimulated proliferation**

To begin to elucidate the functional role of Δ7/11, proliferation assays were performed. T47D breast cancer cells, or with patient age.

**Δ7/11 inhibits prolactin-stimulated proliferation**

To begin to elucidate the functional role of Δ7/11, proliferation assays were performed. T47D breast cancer decreased levels of secreted Δ7/11 (*P*<0.05; Fig. 5C). The shift in the molecular weight of Δ7/11 indicates deglycosylation. Immunocytofluorescence showed an increase in the punctate perinuclear localization of Δ7/11 compared
cells were chosen, as they have been reported to readily respond to prolactin-stimulated proliferation (Das & Vonderhaar 1996, Perks et al. 2004). All cell lines used in the current study have been reported to express levels of prolactin mRNA or secrete low levels of autocrine prolactin (Clevenger et al. 1995, Ginsburg & Vonderhaar 1995, Shaw-Bruha et al. 1997, Vonderhaar 1999, Perks et al. 2004). These studies have shown that in the short time, our cells were treated and the media collected (overnight under minimal media conditions), and that autocrine prolactin secretion is negligible at best. Thus, the finding that the negligible amounts of prolactin are secreted by the cells is not enough to demonstrate significant differences in cell proliferation between the control and Δ7/11-treated cells during the 3-day period observed. To observe maximal effects of the prolactin response, cells were incubated in 0.01% charcoal-stripped serum overnight prior to the treatment for 3 days. As shown in Fig. 7, recombinant Δ7/11 had no effect on cell proliferation alone; however, Δ7/11 was able to completely inhibit prolactin-induced cell proliferation at all concentrations tested (25 and 250 ng/ml, P<0.05). As Δ7/11 was found to be glycosylated (Fig. 5), we investigated whether the glycosylation would alter the observed inhibitory effect of Δ7/11 on prolactin-induced proliferation. Δ7/11-expressing CHO cell-conditioned media were used as the source of Δ7/11, and both T47D and MDA MB231 breast cancer cells were treated with Δ7/11, prolactin, or the combination. Similar to the results obtained with the recombinant protein, Δ7/11 was found to significantly inhibit prolactin-induced cell proliferation in both cell lines (Fig. 7B). The breast cancer cell response to prolactin was reduced overall, potentially due to other cytokines and growth factors secreted into the CHO cell-conditioned media. Lastly, to confirm our findings from the proliferation assays, cell cycle analysis was performed. T47D cells were treated similarly to the proliferation assays; however, the cells were fixed, stained with propidium iodide, and analyzed via flow cytometry 24 h post-treatment to observe initial cell cycle events. In agreement with the proliferation assays, prolactin stimulated an increase in the percentage of cells in the S phase, and Δ7/11 inhibited this response (Fig. 7C).

**Δ7/11 alters breast epithelial cell signaling**

To investigate the mechanisms by which Δ7/11 influenced the prolactin-stimulated cell behavior, the pathways reported to be activated by prolactin were examined. T47D cells were incubated in media containing

![Figure 7](http://www.jme.endocrinology-journals.org)  
**Figure 7**  
Δ7/11 inhibits prolactin-induced breast epithelial cell proliferation.  
(A) T47D breast cancer cells were plated at 2×10^4 cells/cm^2 in growth media. Twenty-four hours post-plating, cells were washed, incubated overnight in media containing 0.01% charcoal-stripped serum, and then treated for 3 days as indicated. Cell number was determined via trypsinization and counting on a hemocytometer using trypan blue exclusion. Data represent mean ± S.D. of three independent experiments. *Indicates significance between Prl and all the other treatments: P<0.05; **indicates significance between C and C + Prl: P<0.05; ***indicates significance between C + Prl and Δ7/11 or Δ7/11 + Prl: P<0.05. Prl=100 ng/ml of prolactin. (B) 24 h conditioned media were collected from CHO cells expressing either the empty vector (C), or the Δ7/11 expression vector (Δ7/11) and used to treat the cells. T47D and MDA MB231 breast cancer cells were plated at 2×10^4 cells/cm^2 in growth media for 24 h, washed, incubated in media containing 0.01% charcoal-stripped serum overnight, and then treated for 3 days as indicated. Cell number was determined as described in (A). Data represent mean ± S.D. of three independent experiments. *Indicates significance between C and C + Prl: P<0.05; **indicates significance between C + Prl and Δ7/11 or Δ7/11 + Prl: P<0.05. (C) The indicated breast cancer cell lines were cultured as described in (A) and treated as indicated for 24 h (C, serum-free media; Prl, 100 ng/ml of prolactin; Δ7/11, 250 ng/ml of recombinant Δ7/11). Following the treatment, cells were fixed, stained with propidium iodide, and analyzed via flow cytometry. Percentage of cells in the S phase was calculated using MODFIT-LS software. Data represent mean ± S.D. of three independent experiments. *Indicates significance between Prl and all the other treatments: P<0.05.
0.01% charcoal-stripped serum for 24 h prior to the treatment for 5 and 60 min with prolactin, Δ7/11, or the combination. As shown in Fig. 8, 24 h culture in low serum-containing media induced early-onset senescence events in the cells, as indicated by the phosphorylation of P38 in the control treatment groups. Treatment with prolactin was able to rescue a significant portion of the cells from P38-induced early senescence/cell cycle arrest as indicated by the significant reduction of P38 phosphorylation in the cell lysates. The addition of Δ7/11 had no significant effect on the cells compared with the controls. The combined treatment of Δ7/11 and prolactin resulted in levels of P38 phosphorylation similar to controls or cells treated with Δ7/11 alone, suggesting that the addition of Δ7/11 was able to inhibit the prolactin-induced rescue of early-onset senescence/cell cycle arrest events. This observation was the most prominent and consistent signaling mechanism observed across all cell lines tested. The basal activation/phosphorylation and prolactin-stimulated activation/phosphorylation of other common signaling pathways varied among the breast cell lines tested (AKT, STAT5, STAT3, and ERK1/2; Supplementary Figure 2, see section on supplementary data given at the end of this article). This is potentially due to the autocrine secretion of prolactin as well as the expression levels of the various PrlR isoforms (Vonderhaar 1999, Trott et al. 2004).

**Discussion**

The data presented in this study show direct interactions between prolactin and Δ7/11, a soluble isoform of the PrlR. We also demonstrate the glycosylation of Δ7/11 and its ability to inhibit prolactin-stimulated cell proliferation. Collectively, these data demonstrate a novel regulatory mechanism of prolactin bioavailability and signaling.

Similar to the PrlBP, the precise source of Δ7/11 generation in breast and colon tissues is unknown. In addition to the nine cell lines analyzed for Δ7/11 message in the initial report (Trott et al. 2003), we screened an additional 12 breast cancer cell lines and two primary normal breast epithelial cell preparations from reduction mammary tissue. Similar to the original report, we were unable to detect Δ7/11 via RT-PCR in these cells grown in vitro on tissue culture dishes (data not shown). This presents the possibility that the source of Δ7/11 in breast tissue originates from stromal or immune cells. Alternatively, as the polarity and 3D organization of epithelial cells is known to directly regulate gene expression (Rooney & Streuli 2011, Schedin & Keely 2011), the possibility remains that breast epithelial cells in a 3D organization or the appropriate extracellular matrix will produce Δ7/11. It is noteworthy that Δ7/11 has only been detected in organized tissues and not in monolayers of cells in culture. It is well recognized that prolactin signaling is altered when cells are cultured in 3D (Prince et al. 2002, Katz & Streuli 2007, Rooney & Streuli 2011); therefore, future studies exploring the role of Δ7/11 on prolactin regulation in polarized cells or on different extracellular matrices would be of interest to the field.

Multiple isoforms of prolactin have been identified in the circulation, with the 23 kDa isoform identified as the most prominent in humans (Fonseca et al. 1991). Previous studies have shown that the 23 kDa protein is involved in classic prolactin functions, including exhibiting proliferative and pro-angiogenic properties. Conversely, the 16 kDa form of prolactin was demonstrated as a potent anti-proliferative and anti-angiogenic factor in various cell types both in vitro and in vivo (Bentzien et al. 2001, Kim et al. 2003, Clapp et al. 2006, Nguyen et al. 2007). It would be of interest to investigate the binding affinity for Δ7/11 on these two functionally opposing prolactin isoforms. Our data suggest that Δ7/11 may have a higher affinity for the 23 kDa prolactin as we observed an inhibition of prolactin-stimulated proliferation in all cell types investigated. Furthermore, the 16 kDa prolactin is a cleavage product of the 23 kDa protein, proposed to be formed by the action of the protease cathepsin D (Piwnica et al. 2004). Whether the binding of Δ7/11 to prolactin alters the enzymatic action of cathepsin D is yet to be investigated. These studies may help to elucidate the production and function of these two functionally opposing prolactins.

It is of note that Δ7/11 is glycosylated, though the function of this biochemical modification remains
unknown. The PrlBP is not glycosylated and has the ability to bind both prolactin and GH, as well as inhibit prolactin-stimulated proliferation in a cell culture model (Kline et al. 2002). In the present study, both the glycosylated and non-glycosylated forms of Δ7/11 were able to bind and inhibit prolactin. In an attempt to understand the functional role of Δ7/11 glycosylation, we observed the cellular localization of Δ7/11 in the presence of tunicamycin, an inhibitor of N-glycosylation. Previous studies showed that glycosylation of the asparagyl residues of the ECD of the PrlR is crucial for its cell surface localization, showed that glycosylation of the asparagyl residues of the ECD of the PrlR is crucial for its cell surface localization, but has no effect on PrlR signaling (Cahoreau et al. 1994, Buteau et al. 1998). Similarly, we found that treatment of cells overexpressing Δ7/11 with tunicamycin resulted in the accumulation of protein in the Golgi area. This suggests that the levels of Δ7/11 secreted into the extracellular space can be controlled cell-autonomously by post-translational modifications. In addition to Δ7/11 glycosylation regulating cellular localization, one can hypothesize that the glycosylation may influence the binding affinities for the different forms of prolactin (16 or 23 kDa) or bind the ECDs of the different PrlR isoforms. Given the sequence similarity of Δ7/11 to the PrlR, and the ability of the various PrlR isoforms to homo- and hetero-dimerize, the direct interaction of Δ7/11 and the PrlR is yet another potential mechanism for enhancement or inhibition of prolactin signaling.

Prolactin induces differentiation in mammary epithelial cells through the activation of the JAK/STAT5 signaling pathway (Vonderhaar & Ziska 1989), and has been shown to activate other common proliferative signal transduction pathways in breast epithelial cells (AKT, ERK1/2) (Brisken & O’Malley 2010, LaPensee & Ben-Jonathan 2010). In our studies, we found that alterations in STAT signaling pathways varied across the cell lines (both basal activation and activation by prolactin). We hypothesize that that this is due to the varying amounts of endogenous prolactin secreted by each cell type, as well as the different levels of the PrlR isoforms produced in each cell (Vonderhaar 1999, Trott et al. 2004). Nevertheless, one conclusive pattern was consistently observed in all cell lines tested: we found that the underlying mechanism of Δ7/11-induced inhibition of proliferation involved the regulation of P38 activation. Whether prolactin rescued the cells from cell cycle arrest or early senescence was not determined. However, our data suggest that prolactin was consistently able to rescue the cells from either cell cycle arrest/early senescence events while simultaneously inactivating P38 (i.e. dephosphorylation), thus leading to the observed increase in cell proliferation compared with control cells or cells treated with Δ7/11 alone. Conversely, Δ7/11 blocked the ability of prolactin to rescue the cells in all cell lines tested. The role of prolactin as an inducible survival factor in mammary cells is well known; however, our observation that prolactin may rescue cells from early senescent events via inactivation of P38 presents a unique and understudied mechanisms of prolactin signaling. More studies investigating this novel signaling mechanism are warranted.

In conclusion, we present data detailing the ability of Δ7/11, a soluble secreted form of the PrlR, to directly bind to prolactin and inhibit prolactin-induced cell proliferation. Unlike the previously reported PrlBP, Δ7/11 was found to be glycosylated. Lastly, the inability to detect Δ7/11 in human serum samples suggests that Δ7/11 is a tissue-specific factor responsible for the local regulation of prolactin function. These data highlight the role of Δ7/11 as a novel regulatory factor of prolactin bioavailability.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-12-0201.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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of gene transcription but is crucial for its cell surface targeting.

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