Alternative splicing to exon 11 of human prolactin receptor gene results in multiple isoforms including a secreted prolactin-binding protein

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Abstract

Endocrine and autocrine prolactin (PRL) exerts effects on normal breast and breast cancer cells, and high serum PRL is a poor prognostic factor for colorectal cancer. Here we tested the hypothesis that short isoforms of the PRL receptor (PRLR) in human tissue regulate the actions of PRL in cancer. Using 3' RACE we isolated five splice variants of the human PRLR (hPRLR), three of which encode the complete extracellular binding domain. Two of these isoforms, short form 1a (SF1a) and short form 1b (SF1b), possess unique intracellular domains encoded by splicing to exon 11 from exons 10 and 9 respectively. A third novel isoform ($\Delta 7/11$) reflects alternative splicing from exon 7 to exon 11 and encodes a secreted soluble PRL-binding protein. Additional splice variants of SF1b and $\Delta 7/11$ that lacked exon 4 ($\Delta 4$ -SF1b and $\Delta 4$ - $\Delta 7/11$) were also identified. Functional analyses indicated that hPRLR-SF1b is a strong dominant-negative to the differentiative function of the PRLR long form while hPRLR-SF1a is a weaker dominant-negative. Differential abundance of SF1a, SF1b and $\Delta 7/11$ expression was detected in normal breast, colon, placenta, kidney, liver, ovary and pancreas, and breast and colon tumors. Taken together, these data indicate the presence of multiple isoforms of the hPRLR that may function to modulate the endocrine and autocrine effects of PRL in normal human tissue and cancer.

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Introduction

Prolactin (PRL) is a polypeptide hormone that has a clear role during mammary carcinogenesis in rodents (Wennbo *et al.* 1997) and may contribute to the initiation and progression of human breast cancer (Hankinson *et al.* 1999). Serum PRL levels are also a recognized risk factor for recurrence and reduced survival time for both breast and colorectal cancer (Bhatavdekar *et al.* 1992, 2000, Patel *et al.* 1994). The effects of PRL may be realized through either the endocrine synthesis of PRL or its local autocrine/paracrine synthesis in various tissues including breast and colon cancer (Clevenger *et al.* 1995, Ginsburg & Vonderhaar 1995, Touraine *et al.* 1998, Bhatavdekar *et al.* 2000, Llovera *et al.* 2000). A recent report of a soluble PRL-binding

protein present in human serum and milk derived from an as yet unidentified site(s) (Kline & Clevenger 2001) adds further complexity to the mechanism by which PRL probably acts on normal and neoplastic tissues.

PRL acts through the PRL receptor (PRLR), a member of the large class-1 cytokine receptor superfamily (Bole-Feysot *et al.* 1998). The PRLR is composed of an extracellular ligand-binding domain, a transmembrane region and an intracellular domain (Kelly *et al.* 1989). The gene contains ten exons including five alternative exon 1 sequences, where only exons 3 through 10 are coding exons (Hu *et al.* 1999, 2002). Different isoforms (long form (LF), intermediate form (IF) and short form (SF) of the PRLR have been named for the length of their intracellular domain

generated by alternative splicing of the PRLR gene (Ormandy *et al.* 1998, Hu *et al.* 1999). Functional clones have been characterized for the human (hPRLR-LF (exons 1 to 10; Boutin *et al.* 1989) and IF (same as hPRLR-LF but lacks part of exon 10; Kline *et al.* 1999). The most extensively characterized isoform is the hPRLR-LF that transduces both mitogenic and differentiative signals (Lesueur *et al.* 1991, O'Neal & Yu Lee 1994, Jabbour *et al.* 1998, Llovera *et al.* 2000).

SFs of the PRLR have been cloned from the rat (Boutin *et al.* 1988), mouse (Davis & Linzer 1989), sheep, cow and goat (Bignon *et al.* 1997) although their roles are largely unknown. Alternative splicing from exon 9 to an exon unrelated to exon 10 generates the rat and mouse SFs. The ruminant SFs are generated by alternative splicing to include 39 bp located 5' of exon 10 (Bignon *et al.* 1997). The rat short PRLR exerts a dominant-negative effect on differentiative signals transduced by the LF (Berlanga *et al.* 1997), while the SF in sheep does not transduce a differentiative signal, but is capable of phosphorylating Stat5 (Bignon *et al.* 1999). The mouse RS1 isoform facilitates mitosis in mouse fibroblasts (Das & Vonderhaar 1995). During the preparation of this manuscript, Hu *et al.* (2001) described a novel exon 11 of the human PRLR (hPRLR) gene and two SFs of the hPRLR (SF1a and SF1b). These isoforms were shown to be dominant-negatives to the differentiative function of the hPRLR-LF.

In this study we performed 3' RACE to search for SFs of the hPRLR, assuming similar 3' splicing occurred in humans as in rodents (Bignon *et al.* 1997, Ormandy *et al.* 1998). Two SFs of the hPRLR (SF1a and SF1b) were cloned from human placenta that led to subsequent cloning of three additional variants ($\Delta 7/11$, $\Delta 4$ -SF1b and $\Delta 4$ - $\Delta 7/11$) from breast cancer cDNA. Both hPRLR-SF1a and hPRLR-SF1b were shown to function as dominant-negatives to the differentiative effect of the hPRLR-LF, whereas $\Delta 7/11$ encodes a secreted, soluble PRL-binding protein. Expression of these isoforms was detected in normal human tissues and breast and colon cancer.

Materials and Methods

3' RACE

3' RACE was performed on Marathon-Ready human ovary cDNA (Clontech, Palo Alto, CA,

USA). Three 5' gene-specific primers designed from the hPRLR-LF cDNA (GenBank Accession M31661) were used for 3' RACE. The sequences of these primers were hPRLR2D (5'-GGAGTGCA TGGAGTCCAGCGACC-3') in exon 7, and NhPRLR2e (5'-ATGGTGACCTGCATCTTTCC GCCAGTTC-3') and NhPRLR2c (5'-TGGGCC AAAAATAAAAGGATTTGATGC-3'), both in exon 9. These 5' primers were used in three rounds of nested PCR. The first round of amplification used hPRLR2D with adapter primer AP1, the second round used NhPRLR2e with adapter primer AP2 on products from the first reaction, and the third round used NhPRLR2c with AP2 on products from the second reaction. Genuine RACE products from the final two reactions were identified by electrophoresis side by side on 1.2% agarose gels. Products from the third PCR were gel-purified and cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA) prior to sequencing.

Plasmids

The β casCAT plasmid contains 344 bp of the rat β -casein promoter upstream of the chloramphenicol acetyl transferase (CAT) reporter gene (both gifts from L Hennighausen, NIDDK, NIH, Bethesda, MD, USA). The pEFlacZ plasmid (Invitrogen) contains the elongation factor- $\alpha 1$ (EF) promoter upstream of the lacZ (β -galactosidase) reporter gene. The hPRLR LF cDNA, a gift from Paul A Kelly (INSERM, Paris, France), was subcloned into pCRScript (Stratagene, La Jolla, CA, USA) and sequence verified prior to being subcloned into the pEF6C expression vector (Invitrogen). pCMV-GFP contains GFP cDNA under control of the CMV promoter (a gift from David Kerr, University of Vermont, VT, USA).

Cloning of hPRLR cDNAs

Primers were designed over the ATG translation initiation site (hPRSF2; 5'-AAGAGAAGGCAGCC AACATGAAG-3') and at the 3' end of the exon 11 sequence (hPRSR2; 5'-TAAGATCCAACCAGGT TGC GAAG-3'). Total RNA (1 μ g) from human placenta was used for the 25 μ l RT reaction using MMLV-RT (Life Technologies, Gaithersburg, MD, USA) primed with both oligo dT and random hexamers. PCR was performed on 5 μ l RT product using the Advantage cDNA PCR kit (Clontech).

Reaction conditions were 94 °C for 1 min then 94 °C for 30 s, 63.5 °C for 1 min, and 72 °C for 3 min for 45 cycles. PCR products were gel purified, cloned into pCR2.1 (Invitrogen) and sequenced in both directions. Fragments with the correct sequence were subcloned into the pEF4C (Invitrogen) expression vector. The $\Delta 7/11$, $\Delta 4$ - $\Delta 7/11$ and $\Delta 4$ -SF1b cDNAs were amplified by PCR using reverse-transcribed breast cancer RNA, gel purified, then cloned into pCR2.1 (Invitrogen) for sequencing to generate a consensus from multiple clones.

Generation of FLAG-tagged PRLR cDNAs

The hPRLR-LF cDNA was C-terminal tagged with the FLAG epitope using the primers LF5P 5'-CACCGGCCACTCTGTTGAATGAAG-3', and LFFLAG3P 5'-CGCTTCGAAT**CATTATCGT CATCGTCTTTGTAGTCGT**GAAAGGAGTG TGTAAC-3' to generate a PCR product encompassing the 3' coding region of the hPRLR-LF sequence 1621 to 2101 bp (M31661) with a FLAG sequence (bold) inserted prior to the stop codon. The cDNA was sequence verified, then excised from pCR2.1 with XbaI and BstBI and ligated into the similarly digested pEF6 hPRLR-LF construct.

The hPRLR-SF1a, hPRLR-SF1b and hPRLR- $\Delta 7/11$ cDNAs were C-terminally tagged with the FLAG epitope using the following primers: SF5P 5'-CGCGGATCCAAGAGAAGGCAGCCAACA TGAAG-3' for all constructs plus either SF1a-FLAG3P (5'-GCCGGAATT**CATTATCGT CATCGTCTTTGTAGTCCT**GGAAGTGTGGT CAATGTTG-3') for both hPRLR-SF1a (amplifies 1–1145 of AF416619 plus FLAG sequence) and hPRL- $\Delta 7/11$ (amplifies 18–821 of AF492470 plus FLAG sequence) or SF1b-FLAG3P (5'-GCCGGAATT**CATTATCGT CATCGTCTTTGTAGTC**AGGGGTCACCTCCAACAGAT-3'; amplifies 1–881 bp of AF416618 plus FLAG coding sequence) for hPRLR-SF1b. These PCR products were digested with BamHI and EcoRI, purified and cloned into pEF4A or pEF6C (digested with BamHI and EcoRI) and sequence verified.

Cell culture and transfection

Chinese hamster ovary cells (CHO-K1) were maintained in α -MEM (Life Technologies) sup-

plemented with 10% fetal bovine serum (Life Technologies), penicillin (100 U/ml) and streptomycin (100 µg/ml; Life Technologies). Transfections were performed using Fugene (Roche Molecular Biochemicals, Indianapolis, IN, USA) at a ratio of 1 µg DNA to 3–4 µl Fugene. Transfection efficiencies were controlled by co-transfection with pEFlacZ or monitored by parallel transfection with pCMV-GFP. To examine PRLR signaling to the β -casein promoter, CHO-K1 cells were plated in six-well dishes at 2.1×10^5 cells/well the day before transfection. Each well was transfected with 1.5 µg PRLR and/or empty vector (pEF6C) DNA, 0.5 µg β casCAT DNA and 0.2 µg pEFlacZ DNA. Cells were transfected for 24 h then the media changed to α -MEM supplemented with ITS+1 (BD Biosciences, Palo Alto, CA, USA), penicillin, streptomycin and dexamethasone (1 µM; Sigma, St Louis, MO, USA), with or without ovine PRL (oPRL; 400 ng/ml; Sigma). After 48 h, the cells were scraped into 1 × Reporter Lysis Buffer (Promega, Madison, WI, USA) and the supernatant stored at –80 °C until assayed. For live cell binding assays, CHO-K1 cells were plated in 12-well dishes at 1×10^5 cells per well the day before transfection, and then transfected for 24 h using 4.5 µl Fugene plus 0.5 or 1.5 µg DNA per well. The cells were then incubated in serum-free medium (α -MEM, ITS+1, penicillin and streptomycin) for a further 16–24 h and were confluent at the time of the binding assay. When hPRLR-LF was transfected with various ratios of either hPRLR-SF1a or hPRLR-SF1b, a total of 1 µg DNA or 1.5 µg DNA was used respectively with 4.5 µl Fugene.

For secreted protein binding assays, CHO-K1 cells were plated in six-well dishes at 2.4×10^5 cells per well the day before transfection, and then transfected for 24 h using 2 µg DNA per well. The cells were then incubated in serum-free medium (α -MEM, ITS+1, penicillin and streptomycin) for a further 24 h.

For immunocytochemistry, CHO-K1 cells were plated onto glass chamber slides (Nalge Nunc International, Rochester, NY, USA) and transfected for 72 h, prior to immunostaining.

Reporter gene assays

Cell lysates were assayed for CAT activity using [14 C]chloramphenicol (0.025 mCi/ml; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the

CAT Enzyme Assay System (Promega) at 37 °C for 2 h. The butyrylated [^{14}C]chloramphenicol was extracted with 300 μl xylene, back extracted once with 100 μl 0.25 M Tris-HCl (pH 8), then 200 μl of the organic phase was counted using Ecoscint A (National Diagnostics, Atlanta, GA, USA) in an LS6000IC scintillation counter (Beckman, Fullerton, CA, USA). β -Galactosidase activity was measured using a 96-well colorimetric assay (β -galactosidase Enzyme Assay System; Promega) and the absorbance determined using an ELx800UV microtiter plate reader (Bio-Tek Instruments Inc, Winooski, VT, USA).

Binding assays

Live cell binding assays were performed in triplicate or quadruplicate on transiently transfected live CHO-K1 cells approximately 40 h after transfection. Cells were washed once with PBS (pH 7.4) then were incubated in 0.5 ml binding medium (α -MEM, 0.1% BSA, 12.5 mM Hepes, pH 7.3) supplemented with ^{125}I -human growth hormone (hGH) (10^5 c.p.m. at 2200 Ci/mmol; Amersham Pharmacia Biotech) in the presence of various concentrations of unlabeled oPRL (0–3000 ng/ml) for 4 h at room temperature. Cells were then washed with PBS and lysed with 3% SDS. Bound ^{125}I -hGH was counted in a Packard Multi-Prias γ -counter (Perkin Elmer). Competitive binding data for the various hPRLR isoforms were analyzed using GraphPad Prism3 (GraphPad Software Inc., San Diego, CA, USA).

Secreted protein binding assays were performed 48 h after transfection. Cells were incubated overnight in serum-free media containing ^{125}I -hGH (200 000 c.p.m./ml) with or without 10 μg oPRL. Media (0.8 ml) was immunoprecipitated in duplicate using α -FLAG M2 mouse monoclonal antibody (mAb) affinity gel (Sigma) and bound ^{125}I -hGH was counted in a EG&G Wallac 1272 Clinigamma γ -counter (Perkin Elmer).

Immunoprecipitation and Western analysis

Transfected CHO-K1 cells were harvested and sonicated in homogenization buffer (25 mM Tris pH 7.5, 2 mM MgCl_2 , 1 mM EDTA, 1 mM dithiothreitol, 1 μM leupeptin, 1 μM pepstatin A, 1 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ trypsin inhibitor, 20 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride, 1% Triton

X-100). Total protein (2 mg) was subjected to immunoprecipitation with 40 μl α -FLAG M2 mouse mAb affinity gel (Sigma).

Medium was harvested from transfected CHO-K1 cells and 0.8–1 ml was immunoprecipitated using 20 μl (10 μl packed gel volume) α -FLAG M2 mouse mAb affinity gel as per the manufacturer's instructions.

From total cell lysate, 80–100 μg protein was subjected to 10% SDS-PAGE. Proteins were transferred to Hybond nitrocellulose EC membrane (Amersham Pharmacia Biotech) and probed with either mouse α -FLAG M2 Monoclonal IgG Antibody (Sigma), followed by horseradish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody, or mouse α -FLAG M2 Monoclonal IgG Antibody-HRP conjugate (Sigma). Immunoreactivity was detected using enhanced chemiluminescence (ECL+plus; Amersham Pharmacia Biotech). Molecular size determinations were made using MagicMark Western Standard (Invitrogen).

Fluorescent immunocytochemistry

Cells were washed five times with PBS, fixed in 1% paraformaldehyde in PBS (pH 7.4) for 10 min at RT and then permeabilized with 0.2% Triton-X 100, 1% horse serum in PBS for 5 min on ice. Cells were then incubated for 30 min with mouse α -FLAG M2 Monoclonal IgG Antibody-HRP conjugate (1:100), followed by 30 min with Cy3-conjugated AffiniPure Rabbit Anti-HRP (1:250; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Slides were mounted with DAPI antifade media (Molecular Probes, Inc., Eugene, OR, USA).

Northern analysis

Total RNA was extracted from transiently transfected CHO-K1 cells using Trizol (Life Technologies). Following DNase treatment, total RNA (10 μg) was subjected to Northern analysis. The blot was probed with a 716 bp fragment from the extracellular domain generated by PCR using 0.2 μM of the forward (PRLR 5'; 5'-AAGGAAACATTTCACCTGCTG-3') and reverse (PRLR 3'; 5'-AATCCTTTTATTTTGGCCC-3') primers and an annealing temperature of 54 °C. The probe was labeled with [α - ^{32}P]dCTP using RediprimeII (Amersham Pharmacia Biotech) and

Table 1 Sequences, annealing temperature and expected amplicon sizes for primer pairs used to detect expression of hPRLR isoforms and housekeeping genes

Gene	Amplicon size (bp)	Annealing temperature (°C)	5' Primer (5'–3') and 3' primer (5'–3')
hPRLR-SF1a	795	60	CCAGCGACCTTCATTCAGATAC
hPRLR-SF1b	641	60	GCCACATCCTTCACAAACACC
Δ7/11	471	60	
hPRLR-LF	1487	62	TGGGAGATCCATTTTGCTGGGC GCTGGAGAAAGAGGCAAGTGG TTCACCATGAATGATACAACCG
hPRLR-LF	398	58	TCAGACAAAAGGGAAGGGC GAGAATCCTGAAACAACCCACACC
hPRLR-LF	398	60	TTGACCTTGTGAATCTCCACATAATCC GATGAGAAGGACCCACGGCGTCTGTTCCG
Ribosomal protein S9	431	68	GAGACAATCCAGCAGCCCAGGAGGGACA TGAAGGTCGGAGTCAACGGATTTGGT
hGAPDH	983	68	CATGTGGGCCATGAGGTCCACCAC TGAAGGTCGGTGTGAACGGATTTGGC
Mouse GAPDH	983	60	CATGTAGGCCATGAGGTCCACCAC ATCTGGCACCCACACCTTCTACAATGAGCTGCG
β-Actin	838	60	CGTCATACTCCTGCTTGCTGATCCACATCTGC

hybridized overnight with the blot at 65 °C in Church buffer (Church & Gilbert 1984). Following hybridization the blot was washed four times with $0.5 \times \text{SSC}$ ($0.15 \text{ M NaCl}/0.015 \text{ M}$ trisodium citrate pH 7.6), 0.1% SDS at 65 °C, and subjected to autoradiography.

RT-PCR analysis of PRLR expression

Two collections of pooled tissue cDNA from 14- to 76-year-old humans ($0.2 \text{ ng}/\mu\text{l}$; Clontech) were used as the templates for PCR ($5 \mu\text{l}$ per reaction). All samples were from both males and females except for liver (from males), lung (from females) and sex-specific tissues. Total RNA from a series of cancerous and normal human breast cell lines, and ten matched normal and cancerous breast samples, was extracted using Trizol and $1 \mu\text{g}$ used for RT reactions. Total RNA ($1 \mu\text{g}$) from 26 breast cancer samples, nine matched normal and cancer colon samples, and three additional tumor samples was also used for RT reactions. PCR was performed on $2.5 \mu\text{l}$ RT product using PCR Master Mix (Roche) with 0.2 – $0.4 \mu\text{M}$ of each primer. The sequence of primers used to detect hPRLR-SF1a, hPRLR-SF1b and Δ7/11 after 40 rounds of amplification are presented in Table 1. Primer sequences used to

amplify hPRLR-LF with 35 cycles of PCR are given in Table 1. The sequence of the various short PRLR isoform products was confirmed after they were cloned into pCR2.1 (Invitrogen). The integrity of RNA was determined using primers for either human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH; 24–27 cycles; Clontech; Table 1), ribosomal protein S9 (23 cycles; Clontech; Table 1) or β-actin (25 cycles; Clontech; Table 1). The integrity of RNA from CHO-K1 cells was tested using mouse GAPDH primers (22 cycles; Clontech; Table 1). For all PCR analyses the number of cycles used was selected to provide the full range of amplicon intensity across a linear range for the group of samples tested. The intensity of agarose gel-resolved PCR products was quantified using NIH Image and expressed relative to the level of the corresponding housekeeping gene.

Statistics

Levels of CAT activity following treatment with and without PRL were compared by Student's *t*-test. One-way ANOVA was used to compare total specific binding for the different PRLR isoforms. Specific binding by Δ7/11 in media was analyzed using Student's *t*-test.

A	
GTGACCCCTTGATGTTGGGTGCCTCTCATTACAAAAATCTCAAATCTTACAGACCAAGAA	60
AAATCTCAAGTCAAGGAAGACTTGCGGTGTTACAAAGGCAACATTGACCACAGTCCAGT	120
AGAGCTGAAGAGCATATACCAGAATTACAGGAACCTCAACATTTTCTCCCCTCTGTCCAACC	180
ACAGGACAGACTGTGCACCCCAGGGAGATGCTGTGTGTCCACTGGCCTCACTGACTTGGA	240
TTACTCCTGCAGCACGTGACCAGCCAGCTCATATGATCACTCCCTACTGCCCCCAAAGCA	300
GAATTGACTGGGAGCTGGAAACCCACACAAAAAGGGACAATTATATTGAATGTAACATAC	360
TCCCTCTGCTTACCTAGTGCCTTCACCTCCTATGGGCATTTGGAGTAAAAGGTTGTTATG	420
CAGGTGTTTGTGAAGGATGTGGCCAAGTCAGTTGTCTCCCTTACAGGGTGACCCAAGATT	480
AATGATATCTACCCATCCCCAGTCCCTCGACCCCCTAGTTGCAATCAGCTTTGTCACTGT	540
CCCCCTTAGGCACTGCCATGTAAAGCACGTGGTCCCCCATTCTGAAGCAGTGGGAAACCT	600
AGAAGAGATGTACTCTTCGCAACCTGGTTGGATCTTAAAAACATGGTTCTAAC (A)	653
B	
LF KGYSMVTCLFPPVPGFKIKGFDAHLLKKGKSEELLSALGCQDFPPTS DYEDLLVEYLEVD	318
IF KGYSMVTCLFPPVPGFKIKGFDAHLLKKGKSEELLSALGCQDFPPTS DYEDLLVEYLEVD	318
SF1a KGYSMVTCLFPPVPGFKIKGFDAHLLKKGKSEELLSALGCQDFPPTS DYEDLLVEYLEVD	318
SF1b KGYSMVTCLFPPVPGFKIKGFDAHLLVTP	288
Δ7/11	228
LF DSEDQHLM SVHSKEHPSQGMKPTYLD PTD SGRGSCDSP SLLSEKCEEPQANPSTFYDPE	378
IF DSEDQHLM SVHSKEHPSQEREQROAQEARD	349
SF1a DSEDQHLM SVHSKEHPSQGDPLMLGASHYKNLKSYP RKISSOGR LAVFTKATLTTVO	376
Δ7/11GDPLMLGASHYKNLKSYP RKISSOGR LAVFTKATLTTVO	268
LF VIEKPENPETHTHTWDPQCISM EGKIPYFHAGGSKCSTWPLPQPSQHNP RSSYHNITDVCE	438
LF LAVGPAGAPATLLNEAGKDALKSSQTIKSREEGKATQQREVESFHSETDQDTPWLLPQEK	498
LF TPFGSAKPLDYVEIHKVNKDGALSLLPKQRENSGKPKPGTPENNKEYAKVSGVMDNNIL	558
LF VLVPDPHAKNVACFEESAKEAPP SLEONQAEKALANFTATSSKCRLOLGGLDYLD PACFT	618
LF HSFH	622

Figure 1 Exon 11 sequence and derived amino acid sequences of two short hPRLR isoforms and one soluble isoform. (A) Nucleotide sequence of exon 11. The polyadenylation signal is boxed. (B) Amino acid sequence alignment of the intracellular domains of hPRLR long form (LF), intermediate form (IF), short form 1a (SF1a) and short form 1b (SF1b) with corresponding exon 11-encoded amino acid sequence from the Δ7/11 isoform. The proline-rich box 1 (proximal to the transmembrane domain) and the more distal box 2 are boxed. The intracellular amino acids in SF1a, SF1b and IF that differ compared with LF are underlined.

Results

Cloning of five hPRLR splice variants

An exon 11 of the hPRLR gene was identified by 3' RACE from human ovary cDNA and subsequent comparison with the draft human genome sequence (<http://www.ncbi.nlm.nih.gov/genome/>

guide/human/). The clone contained sequence from exon 9 of the hPRLR-LF that spliced to exon 11 (Fig. 1). Using RT-PCR, we subsequently identified five alternative splice variants containing exon 11 sequence (Fig. 2). For two of these, hPRLR-SF1a (GenBank Accession AF416619) and hPRLR-SF1b (GenBank Accession AF416618),

Figure 2 Exon structure and splice sites of seven known hPRLR isoforms. Four of the seven forms (long (LF), intermediate (IF), short form 1a (SF1a) and short form 1b (SF1b)) share exons 3–9. The LF splices from exon 9 to exon 10. The IF and SF1a share the first 154 bp of exon 10 before splicing to 3' sequence in exons 10 and 11 respectively. SF1b splices from exon 9 to exon 11. The $\Delta 7/11$ isoform splices from exon 7 to exon 11. Two additional isoforms, $\Delta 4$ -SF1b and $\Delta 4$ - $\Delta 7/11$, are exon 4 deletion variants of SF1b and $\Delta 7/11$ respectively. I=box 1. II=box 2.

full-length cDNA possessing exons 3–11 was cloned from placenta. A full-length cDNA for a third, soluble form ($\Delta 7/11$; Genbank Accession AF492470) was cloned from breast cancer RNA. The $\Delta 7/11$ isoform did not contain a transmembrane domain as determined by TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) and was predicted to be a binding protein. Subsequent analysis of breast cancer RNA revealed another two splice variants that lacked exon 4 in the hPRLRSF1b ($\Delta 4$ -SF1b; GenBank Accession AF512796) and $\Delta 7/11$ ($\Delta 4$ - $\Delta 7/11$; GenBank Accession AF493068) sequences. These five variants were alternatively spliced from a single

PRLR gene (Fig. 2) located on chromosome 5. Examination of the human genome map using NCBI Blast (<http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html>) revealed that exon 11 is separated from exon 10 by an intron of 15.1 kb, and is 18.8 kb downstream of exon 9 and 20.7 kb downstream of exon 7. The full-length coding sequence and 3'-UTR of hPRLR-SF1a comprised exons 3 to 9, 154 bp of exon 10 (as present in both the hPRLR-LF and the IF; Kline *et al.* 1999), then 653 bp of exon 11 (Figs 1 and 2). The full-length hPRLR-SF1b coding sequence and 3'-UTR comprised exons 3–9 followed by 653 bp of exon 11 (Figs 1 and 2). The full-length $\Delta 7/11$ isoform comprised exons 3–7 then exon 11 in the same reading frame as hPRLR-SF1a. The splicing sites follow the AG/GT rule of Mount (1982) and there was a non-consensus polyadenylation site of TAAAAA in exon 11, 13 bp upstream of the poly (A)18 tail. The sequences of hPRLR-SF1a, hPRLR-SF1b and $\Delta 7/11$ encode 376 aa, 288 aa and 268 aa precursor proteins. These proteins share homology with both hPRLR-LF and IF for the first 336 aa, 285 aa and 228 aa respectively (Fig. 1). The intracellular domain of hPRLR-SF1a contains both box 1 and box 2 motifs characteristic of the cytokine receptor superfamily (Bole-Feysot *et al.* 1998) whereas hPRLR-SF1b contains only box 1, and $\Delta 7/11$ lacks both. The $\Delta 4$ - $\Delta 7/11$ and $\Delta 4$ -SF1b splice variants both encode either a truncated N-terminal 34 aa peptide (including the signal peptide), or C-terminal proteins that are identical to $\Delta 7/11$ and SF1b respectively, but lack the signal peptide (24 aa) and first 47 aa of the extracellular domain, which would probably abolish their ability to bind PRL.

Differential lactogen binding by hPRLR isoforms

To determine the binding characteristics of the two transmembrane-containing PRLR isoforms (hPRLR-SF1a and hPRLR-SF1b), we transiently transfected CHO-K1 cells with these PRLR expression constructs prior to incubation with 125 I-hGH. CHO-K1 cells transfected with pEF6C alone did not specifically bind 125 I-hGH (data not shown). Likewise they did not express PRLR mRNA as determined by RT-PCR (data not shown). Transfection of CHO-K1 cells

with expression vectors encoding hPRLR-SF1a, hPRLR-SF1b or hPRLR-LF resulted in similar mRNA and protein expression for all three isoforms as determined by Northern analysis and Western analysis (Fig. 3). The size of mRNA transcripts for hPRLR-SF1a, hPRLR-SF1b and hPRLR-LF concurred with the predicted sizes of 2, 1.9 and 3.5 kb respectively. Two PRLR mRNA transcripts of approximately 9 and 3.5 kb were detected in T47D cells, similar to previous results using Northern analysis of hPRLR-LF expression (Boutin *et al.* 1989). Western analysis of FLAG-tagged protein products indicated that SF1a-FLAG, SF1b-FLAG and LF-FLAG encoded translation products of approximately 57, 38 and 93 kDa respectively (Fig. 3B). The predicted sizes of mature SF1a-FLAG and SF1b-FLAG are 41 and 31 kDa respectively (http://www.expasy.ch/tools/pi_tool.html). The hPRLR-LF specifically bound hGH that was competitively displaced by oPRL ($K_m = 3.3$ nM; 95% confidence interval (CI) = 2.0–5.4 nM; Fig. 4A). The hPRLR-SF1a bound a small but specific amount of hGH when 1.5 µg, but not 0.5 µg, of receptor was used in transfection studies (Fig. 4B). The binding affinity for hPRLR-SF1a could not be accurately determined due to the low specific binding (Fig. 4A) although our data suggest it had an affinity for hGH similar to that for hPRLR-SF1b or hPRLR-LF. The hPRLR-SF1b had 3-fold higher total specific binding of hGH than did the hPRLR-LF (Fig. 4B) while binding the ligand with a similar affinity ($K_m = 3.4$ nM; 95% CI = 2.3–5.2 nM; Fig. 4A). The total specific binding capacity of hPRLR-SF1b was 2.4-fold greater than for hPRLR-LF while the specific binding capacity of hPRLR-SF1a was 8-fold lower than for hPRLR-LF. When both hPRLR-LF and hPRLR-SF1b were transfected into CHO-K1 cells at a 1:1 mass ratio, the affinity for hGH was decreased compared with either hPRLR-LF or hPRLR-SF1b alone ($K_m = 9$ nM; 95% CI = 4.4–18 nM; Fig. 4A). When different ratios of hPRLR-LF to hPRLR-SF1b were used, addition of increasing amounts of hPRLR-SF1b to a constant level of hPRLR-LF produced a non-additive increase in specific binding (Fig. 4C). When different ratios of hPRLR-LF to hPRLR-SF1a were used, addition of increasing amounts of hPRLR-SF1a to a constant level of hPRLR-LF produced a decrease in specific binding (Fig. 4D). Similarly, the addition of increasing levels of hPRLR-SF1a to a

Figure 3 Expression of hPRLR long form (LF), short form 1a (SF1a) and short form 1b (SF1b) mRNA in transiently transfected CHO-K1 cells. Cells were transfected for 24 h, the media replaced with serum-free media and the cells harvested for RNA or protein extraction 40 h later. The negative control (Neg) is cells transfected with pEF6C. (A) Expression of hPRLR mRNAs as determined by Northern analysis of total RNA (10 µg). Cells transfected with SF1a, SF1b or LF expression constructs expressed mRNA transcripts of the expected sizes. As a positive control, RNA from T47D cells was analyzed for PRLR mRNA. Equivalence of loading is demonstrated by the intensity of 18S and 28S ribosomal RNA bands. Migration of the RNA ladder (M) is indicated. (B) Western blot analysis of SF1a-FLAG, SF1b-FLAG and LF-FLAG proteins in transfected CHO-K1 cells probed with anti-FLAG antibody. Total cell lysates (80 µg protein) were loaded on the gel. The positive control (+ve) was 100 ng of FLAG bacterial alkaline phosphatase protein.

Figure 4 Ligand-binding competition curves for ^{125}I -hGH using CHO-K1 cells transiently transfected with various hPRLR isoforms. (A) Competition binding curves for hPRLR long form (LF), short form 1a (SF1a), short form 1b (SF1b) and a 1:1 ratio of LF: SF1b showing the binding site competition of ^{125}I -hGH using increasing amounts of unlabeled oPRL. The LF, SF1a and SF1b curves are representative of three independent experiments. (B) Total specific binding of ^{125}I -hGH to CHO-K1 cells transiently transfected with LF, SF1a or SF1b. Results are representative of three independent experiments. Means with different letters (a, b, c) are significantly different ($P < 0.0001$). (C) Specific binding of ^{125}I -hGH to CHO-K1 cells transiently transfected with a constant amount of LF (0.5 μg) and various amounts of SF1b. (D) Specific binding of ^{125}I -hGH to CHO-K1 cells transiently transfected with a constant amount of LF (0.3 μg) and various amounts of SF1a. (E) Specific binding of ^{125}I -hGH to CHO-K1 cells transiently transfected with a constant amount of SF1b (0.3 μg) and various amounts of SF1a. All data are means $\pm$ s.e. for triplicate or quadruplicate determinations.

Figure 5 $\Delta 7/11$ is a secreted isoform of the PRLR and specifically binds ^{125}I -hGH. (A) Media or cell lysates from CHO-K1 cells transfected with SF1a-FLAG or $\Delta 7/11$ -FLAG were immunoprecipitated with α -FLAG mAb. Immunoprecipitates were immunoblotted with α -FLAG mAb conjugated to HRP. The equivalent of 250 μl medium or 200 μg cell proteins was loaded in each lane. (B) Medium from $\Delta 7/11$ -FLAG- or LF-FLAG-transfected CHO-K1 cells incubated with ^{125}I -hGH was immunoprecipitated with α -FLAG mAb. No specific binding was obtained with media from cells transfected with LF-FLAG (data not shown). Radioactivity bound by $\Delta 7/11$ -FLAG was normalized for non-specific binding of LF-FLAG media. * $P < 0.005$. Data are means $\pm$ S.E. for quadruplicate determinations. (C, D) Fluorescent immunocytochemistry of (C) $\Delta 7/11$ -FLAG- and (D) LF-FLAG-transfected CHO-K1 cells. Red is Cy3 fluorescence. Blue is DAPI fluorescence. Magnification is $\times 400$.

constant level of hPRLR-SF1b also decreased specific binding (Fig. 4E).

$\Delta 7/11$ is a secreted PRL-binding protein

Since the sequence of $\Delta 7/11$ lacked a transmembrane domain but contained a complete extracellular binding domain, we hypothesized that the protein would be soluble, secreted extracellularly and would bind PRL. Western blot analysis

demonstrated the presence of the $\Delta 7/11$ -FLAG protein in both the media and cell lysates of $\Delta 7/11$ -FLAG-transfected CHO-K1 cells (Fig. 5A). Conversely, the membrane-bound SF1a-FLAG was not present in media (Fig. 5A), although it was clearly present in cell lysates (Fig. 3B). Interestingly, the $\Delta 7/11$ -FLAG protein present in media was slightly larger than that in cells (40.6 compared with 39.2 kDa). The predicted size of the FLAG-tagged mature protein is 29.1 kDa ([http://](http://www.endocrinology.org)

www.expasy.ch/tools/pi_tool.html). We examined the ability of both secreted and intracellular $\Delta 7/11$ to bind ^{125}I -hGH and found that cells containing $\Delta 7/11$ -FLAG were unable to specifically bind ^{125}I -hGH in live cell binding assays (data not shown). However, when the cells were cultured in the presence of ^{125}I -hGH, specifically bound hGH could be co-immunoprecipitated with $\Delta 7/11$ -

FLAG from the media, indicating that secreted $\Delta 7/11$ can bind ligand. Fluorescent immunocytochemistry of permeabilized CHO-K1 cells transfected with $\Delta 7/11$ -FLAG revealed a punctate distribution within the cytoplasm, presumed to be vesicular (Fig. 5C). By comparison, LF-FLAG was cytoplasmic with perinuclear concentration (Fig. 5D), similar to previous observations (Genty *et al.* 1994, Perrot-Applanat *et al.* 1997).

Transmembrane hPRLR isoforms are dominant-negatives for differentiation

The ability of the two transmembrane-containing PRLR isoforms (SF1a and SF1b) to transduce PRL-induced differentiation was tested using CHO-K1 cells co-transfected with a β casCAT reporter and different PRLR constructs. Addition of PRL to CHO-K1 cells transfected with hPRLR-LF stimulated transcription from the β -casein promoter in the presence of insulin and dexamethasone ($P \leq 0.005$; Fig. 6A). In contrast, neither hPRLR-SF1b nor hPRLR-SF1a transduced PRL-induced transcription from the β -casein promoter ($P > 0.05$; Fig. 6A). We also tested whether these SFs could impart a dominant-negative effect on the differentiative signal transduced by the hPRLR-LF. Co-transfection of CHO-K1 cells with a constant amount of

Figure 6 Ability of short hPRLR isoforms to either transduce a differentiative signal or have dominant-negative effects on the hPRLR-LF. CHO-K1 cells were transiently transfected with β casCAT, pEFLacZ and combinations of short form 1a (SF1a), short form 1b (SF1b), long form (LF) or pEF6C (Negative). The levels of CAT activity were normalized for β -galactosidase activity. (A) Transcription from the β -casein promoter in cells transfected with the various PRLR isoform constructs after treatment with either dexamethasone (D) alone or D plus PRL (DPrL). Results are representative of five independent experiments. (B, C) Various amounts of SF1a and SF1b were co-transfected with a constant amount of LF DNA. The total amount of DNA was held constant by adjusting the amount of pEF6C (empty vector) DNA. Ratios are mass ratios of transfected DNA. Each set of results was combined from two experiments and are representative of five independent experiments. (B) SF1b has a strong dominant-negative effect against LF-transduced PRL-stimulation of the β -casein promoter. (C) SF1a has a weak dominant-negative function against the LF-transduced PRL-stimulation of the β -casein promoter. Data are means $\pm$ S.E.

Figure 7 Expression of short form 1a (SF1a), short form 1b (SF1b), and long form (LF) mRNA in various normal human tissues as determined by RT-PCR. (A) Human ovary cDNA used for 3' RACE was subjected to PCR using primers that amplify both SF1a and SF1b. The PCR result was consistent over three independent amplifications. (B) Amplification of cDNA (5 μ l; 1 ng) by PCR in a 25 μ l reaction gave consistent results in three separate PCR reactions each performed on two panels of cDNA. The human breast tissue sample contained $\sim$ 2.5 ng cDNA generated from poly A+RNA. hGAPDH was amplified by RT-PCR as an internal control. Sk. Musc.=skeletal muscle; Int.=intestine; M. Gland=mammary gland; Neg=negative control.

hPRLR-LF and increasing amounts of either hPRLR-SF1a or hPRLR-SF1b revealed that hPRLR-SF1b is a strong dominant-negative to the differentiation signal transduced by the hPRLR-LF (Fig. 6B). While hPRLR-SF1a only bound minimal amounts of lactogen (Fig. 4B), it did function as a dominant-negative to the differentiative function of the hPRLR-LF, albeit less effectively than hPRLR-SF1b (Fig. 6C).

Differential expression of PRLR isoforms in tissues and cell lines

Expression of mRNA for the novel PRLR isoforms was examined in various human tissues by RT-PCR using primers in exons 7 and 11 that gave sequence-verified products of 795, 641 and 471 bp for hPRLR-SF1a, hPRLR-SF1b (and Δ 4-SF1b) and Δ 7/11 (and Δ 4- Δ 7/11) respectively. The exon 4 deletion variants that lack both a signal peptide and a complete extracellular domain were only expressed at low levels in certain samples of breast tumor tissue, as detected using primers in exons 3 and 11. The significance of these splice variants is currently under investigation. The hPRLR-SF1b 3' RACE product was originally cloned from human ovary cDNA that expressed hPRLR-SF1b but not

-SF1a (Fig. 7A). Expression of both hPRLR-SF1a and hPRLR-SF1b mRNAs was detected in samples of human breast, placenta, kidney, liver and pancreas. These tissues also expressed hPRLR-LF whereas no expression of Δ 7/11 was evident. By contrast, none of the exon 11-containing hPRLR isoforms was expressed in thymus, skeletal muscle, testis, small intestine, leukocytes, heart, colon, lung or brain (Fig. 7B). The absence of the SFs in two other samples of human ovary cDNA is noteworthy.

We also determined the expression of these three PRLR isoforms in normal breast and cancer cell lines. Both membrane-spanning isoforms (SF1a and SF1b) were expressed in T47D, SKBR3, MDA-MB453 and MDA-MB231 breast cancer cells (Fig. 8). In T47D cells the expression of mRNA for hPRLR-SF1a was notably higher than for hPRLR-SF1b. HS578 breast cancer cells only expressed mRNA for hPRLR-SF1a whereas BT20 breast cancer cells only expressed mRNA for hPRLR-SF1b. No expression of the short PRLR isoforms was detected in MDA-MD435 breast cancer cells or the immortalized normal breast cell lines, A1N4 and MCF-10A. By contrast, hPRLR-LF mRNA was expressed by some cell lines that did not express mRNAs for the short PRLR isoforms.

Figure 8 Expression of hPRLR short form 1a (SF1a), short form 1b (SF1b) and long form (LF) mRNA in various human breast cell lines as determined by RT-PCR. Amplification of RT product gave consistent results in two separate RT-PCR reactions. Ribosomal protein S9 (ribosomal S9) was amplified by RT-PCR as an internal control. MB=MAD-MB; Neg=negative control.

Interestingly, no expression of the secreted $\Delta 7/11$ -binding protein was detected in these various cell lines, perhaps reflective of the *in vitro* environment.

Expression of hPRLR isoforms in breast and colon tumors

We examined the expression of hPRLR-SF1a, hPRLR-SF1b, $\Delta 7/11$ and hPRLR-LF mRNAs in breast cancer tissue from 26 patients (Fig. 9A). Expression of hPRLR-LF was detected in all but one sample of normal breast or breast tumor while expression of one or more SFs was detected in all but three breast tumors and three samples of normal breast. In matched tissues, expression of $\Delta 7/11$ mRNA was evident in three normal and three cancerous breast samples (Fig. 9B).

Given these findings and the candidate role for PRL in colon cancer, we examined PRLR isoform expression in matched normal and tumor colon samples (Fig. 9C). Variable expression of hPRLR-LF, hPRLR-SF1a and hPRLR-SF1b was detected in matched normal and tumor samples. Expression of $\Delta 7/11$ mRNA was found in three normal samples and two colon tumor samples.

Discussion

The present study describes the cloning of five hPRLR isoforms generated by alternative splicing of a single PRLR gene on human chromosome 5. These isoforms contain 3' sequence from exon 11 that exists at a significant distance (15 kb) downstream of exon 10. Both hPRLR-SF1a and

hPRLR-SF1b have a dominant-negative effect on the differentiation signal transduced by the hPRLR-LF, where hPRLR-SF1b is more effective than hPRLR-SF1a. By contrast, the $\Delta 7/11$ isoform encodes a soluble PRL-binding protein. Furthermore, these SFs are differentially expressed in both normal and tumorous breast and colon tissue.

Autocrine PRL is involved in the growth of breast cancer cells (Clevenger *et al.* 1995, Ginsburg & Vonderhaar 1995) and several studies have linked a function of autocrine PRL to breast tumorigenesis (Touraine *et al.* 1998, Vonderhaar 1999, Bhatavdekar *et al.* 2000, Llovera *et al.* 2000). High levels of serum PRL are associated with increased risk of breast cancer (Hankinson *et al.* 1999), recurrence of metastatic breast cancer and reduced survival time (Bhatavdekar *et al.* 2000). The hPRLR-SF1b is a strong dominant-negative to the differentiative function of the hPRLR-LF, leading us to hypothesize that it may regulate hPRLR-LF-mediated differentiation of breast epithelium. Heterozygous PRLR knockout mice with only one copy of the PRLR gene are unable to lactate following their first parturition, while homozygous knockouts never lactate (Ormandy *et al.* 1997). This demonstrates a unique sensitivity of the mammary gland to PRLR levels. Semi-quantitative analysis of amplicon intensities in individual breast tumor samples suggested that 29 of 36 samples had higher levels of hPRLR-SF1a than hPRLR-SF1b, although fully quantitative analysis remains to be performed. Preliminary semi-quantitative analysis also suggested that in the majority of breast tumor samples, the level of hPRLR-SF1a mRNA was higher than in the matched normal tissue. Further confirmation of these results taking into account epithelial cell content remains to be performed. Further studies to elucidate the effects of hPRLR-SF1a and hPRLR-SF1b on PRL-induced mitosis will also help to define whether these isoforms play a role in endocrine or autocrine PRL-stimulated breast cancer growth.

Compared with the well-documented effects of the hPRLR-LF, neither hPRLR-SF1a nor hPRLR-SF1b transduced a differentiation signal to the β -casein promoter. Both box 1 and box 2 in the intracellular domain of the PRLR are necessary for activation of the Jak2/Stat5 pathway during differentiation (DaSilva *et al.* 1994, Pezet *et al.*

Figure 9 Expression of hPRLR short form 1a (SF1a), short form 1b (SF1b), $\Delta 7/11$ and long form (LF) mRNAs in normal and tumor samples of breast and colon as determined by RT-PCR. Amplification of cDNA gave consistent results in two independent RT-PCR reactions. Neg=negative control. (A) Human breast cancer samples. hGAPDH was amplified by RT-PCR as an internal control. (B) Matched pairs of normal and cancerous human breast tissue. β -Actin was amplified by RT-PCR as an internal control. (C) Matched pairs of normal and cancerous human colon tissue. hGAPDH was amplified by RT-PCR as an internal control. T, tumour; N, normal.

1997). The intracellular domains of the rat Nb2 IF, human IF and hPRLR-SF1a all contain box 1 and box 2 and are all approximately the same length. However, neither the human IF nor hPRLR-SF1a has a tyrosine residue at or near position 382. This Y382 in the rat IF is absolutely necessary for PRL-responsive gene transcription (Lebrun *et al.* 1995). The human IF can activate Jak2 but cannot stimulate cell proliferation (Kline *et al.* 1999). hPRLR-SF1a contains two tyrosine residues downstream from box 2 at positions Y347 and Y353 that are not present in the IF, and it remains to be tested whether hPRLR-SF1a can initiate cell proliferation. It is conceivable that hPRLR-SF1a, like the IF, can activate Jak2. PRL activation of

Jak2 leads to constitutive activation of ErbB2 (Yamauchi *et al.* 2000), a contributing factor in the growth of 25–40% of breast cancers (Maguire & Greene 1989), so this is an important question to be answered.

The novel splice variant $\Delta 7/11$ differs in size (40 kDa) from the PRL-binding protein (32 kDa) identified and more recently characterized in human serum and milk (Clandinin *et al.* 1986, Kline & Clevenger 2001). $\Delta 7/11$ is expressed in normal and cancerous breast tissue, and therefore likely to be secreted in milk, and may be involved in regulating the function of PRL during breast tumorigenesis. Further determination of the tissue distribution of $\Delta 7/11$ is currently under

investigation. It is noteworthy that the size of $\Delta 7/11$ differs between the secreted and intracellular forms and may reflect different post-translational processing of the secreted protein. Differences in glycosylation have been observed previously between intracellular/membrane proteins and secreted proteins in CHO-K1 cells (Hooker *et al.* 1999, Morrison *et al.* 2000). Along these lines, the vesiculated intracellular distribution of $\Delta 7/11$ is of interest. Recently, Hu *et al.* (2001) identified that SF1a, but not SF1b, is similarly distributed, raising the possibility that the 3' sequence shared by $\Delta 7/11$ and SF1a directs this localization.

It would appear that multiple poly A signals are present in exon 11 given that the poly (A)18 tail on our 3' RACE product was a further 233 bp downstream of that reported by Hu *et al.* (2001). This phenomenon has been reported for numerous genes (Edwards-Gilbert *et al.* 1997). Our analysis also indicates that 68 bp of 3' flanking sequence proposed by Hu *et al.* (2001) is actually transcribed 3'-UTR. It is intriguing that there was no consensus AATAAA polyadenylation site in the cDNA. This is not unprecedented because a small number of other mammalian mRNAs have been found with non-consensus polyadenylation sites (Sheets *et al.* 1990, Hirma *et al.* 1999), although these are less efficient in directing cleavage and polyadenylation (Sheets *et al.* 1990). The strength of a polyadenylation signal can directly influence the amount of mRNA transcribed (Edwards-Gilbert *et al.* 1997), which may partially explain why the expression level of $\Delta 7/11$, hPRLR-SF1a and hPRLR-SF1b mRNAs was lower than that for hPRLR-LF.

In the course of this study, we also identified two potentially non-functional PRLR isoforms that omit exon 4 but splice to exon 11. Previous works have described splice variants that lack exons 4 and 5 of the extracellular domain (Kline *et al.* 1999), exon 6 of the extracellular domain (Laud *et al.* 2000) and either exon 4, exons 5 and 6, or midway of exon 4 through midway of exon 6 (Nagano *et al.* 1995). Although these latter three variants were assumed to splice to exon 10, we have found isoforms with deletion of exon 4 that spliced in the same manner as either SF1b or $\Delta 7/11$. In addition, a splice variant that lacks part of exon 4 through to part of exon 6 and splices the same as SF1a at the 3' end (data not shown), that we have found, has a

5 bp repeat at the two splice sites, and the AG/GT splice site consensus does not apply. This rare 'splicing' could be a result of recombination (or template-switching) caused by RT (S Mount, personal communication).

We found that the addition of either hPRLR-LF or hPRLR-SF1a to a constant amount of hPRLR-SF1b decreased the total number of binding sites. Moreover, heterodimers of hPRLR-LF:SF1b (1:1) had a slightly lower affinity for hGH than either hPRLR-SF1b or hPRLR-LF alone. The effect of heterodimerization on the affinity of receptors has been documented for a number of transmembrane cytokine receptors, where receptors of low and intermediate affinity can interact to form a high-affinity receptor (Hayashida *et al.* 1990, Kitamura *et al.* 1991, Goldstein *et al.* 1992). At this stage the mechanism behind the decrease in affinity for PRL by heterodimers of hPRLR-LF and hPRLR-SF1b and the effect on the total number of binding sites is not clear.

In conclusion, we have identified five isoforms of the hPRLR, two of which act as dominant-negatives to the differentiative function of the hPRLR-LF, while a third encodes a secreted PRL-binding protein. These isoforms are expressed independently and in different ratios by various human tissues and cell lines. The ratio of $\Delta 7/11$, hPRLR-SF1a and hPRLR-SF1b mRNA expression in colon and breast cancer changes between normal and cancer states, suggesting that these isoforms may play a role in regulating the effects of endocrine and/or autocrine PRL – in normal and cancer cells.

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