We have identified a novel exon 11 of the human prolactin receptor (hPRLR) gene that is distinct from its rodent counterparts and have demonstrated the presence of two novel short forms of the hPRLR (S1a and S1b), which are derived from alternative splicing of exons 10 and 11. S1a encodes 376 amino acids (aa) that contain partial exon 10 and a unique 39-aa C-terminal region encoded by exon 11. S1b encodes 288 aa that lack the entire exon 10 and contains 3 amino acids at the C terminus derived from exon 11 using a shifted reading frame. These short forms, which were found in several normal tissues and in breast cancer cell lines, were expressed as cell surface receptors and possessed binding affinities comparable with the long form. Unlike the long form, neither short form was able to mediate the activation of the β-casein gene promoter induced by prolactin. Instead they acted as dominant negative forms when co-expressed with the long form in transfected cells. Due to a marked difference in the cellular levels between the two short forms in transfected cells, S1b was more effective in inhibiting the prolactin-induced activation of the β-casein gene promoter mediated by the long form of the receptor. The low cellular level of S1b was due to its more rapid turnover than the S1a protein. This is attributable to specific residues within the C-terminal unique 39 amino acids of the S1b form and may represent a new mechanism by which the hPRLR is modulated at the post-translational level. Since both short forms contain abbreviated cytoplasmic domains with unique C termini, they may also exhibit distinct signaling pathways in addition to modulating the signaling from the long form of the receptor. These receptors may therefore play important roles in the diversified actions of prolactin in human tissues.

Prolactin receptors (PRLRs) are members of the cytokine receptor superfamily, which are expressed as single transmembrane proteins in multiple prolactin target tissues (1). PRLRs are present in several forms that are generated from alternative splicing of the receptor gene and mainly differ in their intracellular domains (1–3). The PRLR gene has a complex structure that is amenable to alternative splicing that leads to the generation of multiple PRLR transcripts (2). The genomic region of the long form human PRLR (hPRLR) comprises eight exons (exons 3–10) in which exon 8 codes for the transmembrane domain and exon 10 codes for most of the intracellular domain (4). While exon 2 is a common noncoding exon corresponding to the middle region of the 5′-untranslated region of the hPRLR mRNA, there are multiple alternative first exons, hE1a and hE1N (4) and more recently hE1N2–5 (5), each of which is transcribed into the 5′-end sequence of the hPRLR mRNA under the control of their respective promoters and is alternatively spliced onto the common exon 2. However, the 5′ diversity of the hPRLR gene does not alter the N-terminal coding region of the hPRLR. In humans, an intermediate form of the hPRLR was identified from the breast cancer cells that was derived from partial nucleotide sequence deletion of exon 10 at nt +1010/+1581 of the hPRLR gene (6). While the long form receptor has been linked to several signaling pathways including Jak-Stat and mitogen-activated protein kinase that may account for much of the diverse functions of prolactin, the signal transduction of the intermediate form of the receptor remains unknown.

To date no short forms of the hPRLR that utilize alternative exons downstream of exon 10 have been reported. The aim of this study was to isolate new forms of the human PRLR and to study their functional differences from the long form of the receptor. In the present work, we identified two novel forms of the hPRLR, S1a and S1b, which are derived from alternative splicing between exon 10, well conserved among the human and rodents, and exon 11, distinct from those of the rodents at both nucleotide and amino acid levels. We have demonstrated that both short forms are transmembrane receptors expressed on cell membrane and capable of ligand binding. These forms lack intrinsic activity in Jak-Stat5 activation but can act as dominant negative forms. They are widely expressed as is the long form and may exert distinct functions. Significant differences of expression are observed between the two short forms. These findings are of significance to further understanding of the diverse actions of prolactin in human tissues.

**EXPERIMENTAL PROCEDURES**

3′-Rapid Amplification of cDNA Ends of Human PRLR mRNA—3′ Rapid amplification of cDNA ends was performed as described.

E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase.
scribed previously (7) using total RNA isolated from the breast cancer cell T-47D. Forward specific primer (hP23, +970/+992, 5'-ATTACCATCATTGAAATGATAACACC-3') located at exon 8 and oligo(dT) were used for amplification of the 3' species of the hPRLR mRNA. The PCR products were directly subcloned into pCR2.1 by the TA cloning procedure (Invitrogen, Carlsbad, CA) and were further sequenced for the inserts. The full lengths of the hPRLR isoforms were cloned from the T-47D cell by RT-PCR using a 5' common forward primer (hP1, +284/262, 5'-GGAGGCTGAAATCCCCAGACC-3') and the specific reverse primer to the 3' novel sequence (hP40, 5'-TCCACAGCTTTACCTTGG-3') (exon 11).

RT-PCR Analysis of hPRL mRNA in Human Tissues—RT-PCR was performed as described previously (8) using total RNA isolated from human tissues of normal adult subjects (25–40 years old) including mammary gland, ovary, testis, liver (male) (Cooperative Human Tissue Network, Philadelphia, PA), and T-47D cells. First-strand cDNA was synthesized using random primers and SuperScript II reverse transcriptase (Life Technologies, Inc.). The primers used for PCR were the forward hP23 (exon 8) and the reverse hP45, 5'-ATGCTCTTCAGTCTAGTG-3' (exon 11), and the reverse primer hP50, 5'-TGGACATTTGGATCCACCACTGACATT-3' (exon 10).

Generation of the hPRLR Expression Plasmids and Their Mutants and of the hPRLR-Green Fluorescence Protein (GFP) Fusion Protein Expression Plasmids—The hPRLR cDNA forms were subcloned into pDNA-7 (vector) at the EcoRI site. A chimeric receptor that contains the C-terminal 39 aa of the S1a form within the intracellular domain of the long form at aa 442 was achieved by inserting the 39-aa (117-bp) PCR DNA fragment into the XhoI restriction site of the long form at nt 1325. The point mutation or deletion within the unique C-terminal region of the S1a form was performed as described previously (9). The full-length coding regions of the long, S1a, and S1b forms of the hPRLR were subcloned into the pEGFP-N2 vector (CLONTECH) at sites so that the receptors were in-frame with GFP and so that the junction between the receptor and GFP of the three fusion proteins differs minimally. All plasmids were verified by restriction enzyme mapping and sequencing.

Cell Culture and Transfection—Both COS-1 and HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in a CO2 incubator. The hPRLR plasmids were transiently transfected into COS-1 and HEK293 cells using LipofectAMINE Plus reagent (Life Technologies, Inc.).

Confocal Microscopy Study of hPRLR Cellular Localization Using Living Color GFP System—The hPRLR-GFP plasmids (2 µg/ml) or pEGFP2 vector only (2 µg/ml) were transfected into COS-1 cells grown on poly-l-lysine coated cover slips using LipofectAMINE Plus reagent. After 24 h of incubation the cells were examined in an inverted microscope under a 40× oil-immersion objective (Nikon, Inc., Melville, NY) and a Bio-Rad laser confocal microscope system (MRC-1024).

RESULTS

Identification of Novel hPRLR mRNA Species and the Corresponding Genomic Sequence—To isolate potential subtypes of the hPRLR, we performed 3′-rapid amplification of cDNA ends analysis of the hPRLR mRNA from the human breast cancer cell line T-47D using a specific 5′ primer located at the extracellular domain (exon 8) proximal to the transmembrane region, which is common to all known forms of the receptor (Fig. 1, top and bottom). We identified two new hPRLR mRNA species that diverge from the long form at two sites. One deviates at the junction (nt 786) between exon 9 and 10 (aa 285) with omission of exon 10, and the other form results from an internal splicing site within exon 10 at nt 1010 (aa 337) (Fig. 1, top and bottom), both followed by a novel 3′-end sequence of 420 bp (GenBank™ accession number AF214012) that contains a putative poly(A) signal (AGTAA, Fig. 1, middle) different from that of the long form (AATATA). To examine whether this novel 3′ sequence is derived from an alternative exon of the hPRLR gene, we isolated a gene fragment containing this sequence from a human placenta genomic DNA clone and determined that this sequence represents an additional exon (exon 11) of the hPRLR gene (Fig. 1). The intron-exon junction at the 5′-end of exon 11 conforms to the consensussplicing pattern (CT-rich . . . AG), and the junction at the 3′ polyadenylation site conforms to the genomic recognition site (poly(A) signal . . . GT-rich) for the 3′ cleavage and polyadenylation (Fig. 1, middle). This exon contains a polyadenylation
signal, AGTAAA, at 15 bp upstream of the poly(A) tail that is a weaker variant form of the poly(A) signal AATAAA (11).

Full lengths of the two hPRLR species were subsequently cloned from T-47D cells by RT-PCR using the forward primer located at the 5′-untranslated region of the unique sequence (hP44). Two mRNA species of 460 and 306 bp were amplified from all these tissues and cells (Fig. 2, middle) corresponding to the S1a and S1b forms, respectively, as indicated by Southern blot analyses with oligomer probes corresponding to regions common to both short forms (hP34, exon 9) or specific for S1a (hP15, exon 10). This result was further verified by sequencing of the two RT-PCR products (not shown). In addition, a multiple human tissue RNA blot hybridization using a probe corresponding to the exon 11 sequence indicated that exon 11-containing messages (corresponding to both S1a and S1b) are present in a wide range of tissues with relative high abundance in the breast, placenta, uterus, and kidney (data not shown). These results indicated that both short forms of the hPRLR are widely expressed in human tissues as the long form of the receptor. To determine the relative abundance of the long and the two short forms of the hPRLR within a tissue, we estimated the intratissue expression of the three receptor subtypes using a semiquantitative RT-PCR method (Fig. 2, bottom). A 557-bp fragment of the long form was co-amplified with the S1a and S1b forms by including a long form-specific reverse primer (hP50) in the PCR for the two short forms (Fig. 2, middle). Southern blot analyses of the RT-PCR using a probe (hP34) common to all three receptor forms showed that the relative ratios between these forms in all tissues/cells examined were rather consistent. The long form was the most abundant mRNA species (50–60%), the S1a form was second (~30%), and the S1b form was the least abundant (15–20%) (Fig. 2, bottom).

Expression of the S1a and S1b hPRLR in Transfected Cells and Exhibition of Ligand Binding Activities—Both S1a and S1b forms are predicted to be single membrane-spanning proteins. S1a, S1b, and the long form cDNAs were transfected into COS-1 and HEK293 cells, and their expressions were examined by Northern blots, Western blots, and radioligand binding analyses. Using a probe containing extracellular sequences, mRNA transcripts of 2.6, 2.3, and 2.1 kb were revealed corresponding to the S1a and S1b forms, respectively, as predicted from their respective expression constructs (Fig. 3A, lanes 1–4). Transcripts of the 2.0- and 1.8-kb species corresponding to the S1a and S1b short forms, respectively, of the endogenous hPRLRs were observed in Northern blot of poly(A) + RNA from T-47D cells (Fig. 3A, lane 5). The size difference of ~0.2 kb on Northern blots between the S1a and S1b transcripts in both COS-1 and T-47D cells was consistent with the partial exon 10 retention of 153 bp in the S1a form. The slight size difference between the endogenous and transfected mRNA species of S1a and S1b was accounted for by additional sequences flanking the cDNA inserts in the expression vector (pcDNA3.1) used in transfected cells. The apparent molecular masses of the expressed proteins in COS-1 and HEK293 cells were 90, 56, and 42 kDa for the long, S1a, and S1b forms, respectively (Fig. 3, B, lanes 6–8 and C, lanes 9–11). The glycosylation of the extracellular domain of the receptor could account for the differences between the calculated (39 kDa) for S1a and 29 kDa for S1b) and the apparent molecular weights of the short forms (56 kDa for S1a and 42 kDa for S1b).

Confocal microscopy of hPRLR-GFP fusion proteins transiently expressed in COS-1 cells was conducted to visualize subcellular localization of the short forms as well as the long form of the hPRLR. As shown in Fig. 4, the long (L-GFP) and S1b forms (S1b-GFP) were expressed at relatively high levels and were clearly localized on the cell membrane as well as at

Human Tissues—To determine whether the two short forms of the hPRLR are expressed in normal human tissues, we performed RT-PCR analysis of hPRLR mRNAs in human ovary, testis, breast, liver, and peripheral lymphocytes (Fig. 2) using 5′ primer in exon 8 (hP23) and 3′ primer specific for exon 11 (hP44). Two mRNA species of 460 and 306 bp were amplified from all these tissues and cells (Fig. 2, middle) corresponding to the S1a and S1b forms, respectively, as indicated by Southern blot analyses with oligomer probes corresponding to regions common to both short forms (hP34, exon 9) or specific for S1a (hP15, exon 10). This result was further verified by sequencing of the two RT-PCR products (not shown). In addition, a multiple human tissue RNA blot hybridization using a probe corresponding to the exon 11 sequence indicated that exon 11-containing messages (corresponding to both S1a and S1b) are present in a wide range of tissues with relative high abundance in the breast, placenta, uterus, and kidney (data not shown). These results indicated that both short forms of the hPRLR are widely expressed in human tissues as the long form of the receptor. To determine the relative abundance of the long and the two short forms of the hPRLR within a tissue, we estimated the intratissue expression of the three receptor subtypes using a semiquantitative RT-PCR method (Fig. 2, bottom). A 557-bp fragment of the long form was co-amplified with the S1a and S1b forms by including a long form-specific reverse primer (hP50) in the PCR for the two short forms (Fig. 2, middle). Southern blot analyses of the RT-PCR using a probe (hP34) common to all three receptor forms showed that the relative ratios between these forms in all tissues/cells examined were rather consistent. The long form was the most abundant mRNA species (50–60%), the S1a form was second (~30%), and the S1b form was the least abundant (15–20%) (Fig. 2, bottom).

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in intracellular sites. This is consistent with our previous studies that demonstrated in ovarian tissue that a major pool of receptors is hindered even in membrane preparations and only becomes bindable upon detergent solubilization (12). Also other studies demonstrated that a considerable pool of receptors resides at intracellular sites of several tissues (13, 14). The S1a form (Fig. 4, S1a-GFP) was localized for the most part at the cell membrane but expressed at a much lower level than the S1a form (Fig. 4, S1a-GFP) and an exon 11 fragment of 246 bp (nt 68–313 of exon 11) that is specific for the short forms of the receptor (lane 5). B, Southern blot analyses of hPRLR expressed in COS-1 (lanes 6–8) and HEK293 (lanes 9–11) cells using a monoclonal antibody (U5) that recognizes sequences of the extracellular domain of the hPRLR. Vt, vector only. DNA (kb) and protein (kDa) size standards are labeled on the side of gels; arrowheads indicate the sizes of expressed mRNA (kb) and protein (kDa) bands.

Effects of S1a and S1b on Prolactin-induced and Long Form-mediated β-Casein Promoter—The β-casein promoter is a well known prolactin target that can be activated through the long form receptor-mediated Jak-Stat5 signaling pathway (15). To examine whether the S1a and S1b forms can mediate this gene activation, we cotransfected individual receptors with β-casein promoter reporter gene in both COS-1 and HEK293 cells. Stimulation of transfected cells with 100 ng/ml human prolactin for 24 h induced the luciferase activity by 5- (COS-1) or 16-fold (HEK293) in cells transfected with the long form but not with either short form (Fig. 6). This result demonstrated that both S1a and S1b lack intrinsic activity required for the Jak-Stat5 signaling pathway leading to the β-casein promoter activation. To examine whether the two short forms could modulate the signaling of the long form, we cotransfected the long form with the region of the long and short form transcripts amplified by RT-PCR. E100' indicates the 5' part of exon 10. E8–E11, exons 8–11. A, the RT-PCR products were revealed on a 1.5% agarose gel (left) and were analyzed by Southern hybridization (right) with a common probe (hP34, 5'-CTATACATGCTGACCTGATTTCC-3', +780/+806, upper) and S1-specific probe (hP15, 5'-TGAAGACTGTGACTGCTTGAGT-GCCCTGGAATG-3', +867/+883, lower). Std, DNA size standard. B, Southern hybridization (left) of co-amplified long form (557 bp) and short forms (S1a, 460 bp; S1b, 306 bp) of the hPRLR by RT-PCR and the relative intratissue expression among the three forms (right) as measured from PhosphorImager analysis of the autoradiography of the blot. Results are expressed as percentage of total PCR products. The common probe hP34 was used for the hybridization. Data were collected from triplicate measurements of two independent RT-PCR experiments.

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increasing amount of either the S1a or S1b form along with the /H9252/H -casein reporter gene. HEK293 cells were chosen for this experiment due to the greater -fold induction of reporter activity by prolactin in this cell versus COS-1 cells (Fig. 6). As shown in Fig. 7, S1a inhibited over 50% of the prolactin-induced reporter activity at an equal DNA ratio between the long form and S1b (L/S1b) in transfection, while a DNA ratio of more than 1:8 between the long form and S1a (L/S1a) was required to inhibit 50% of the reporter activity (Fig. 7, A and B). In contrast, an L/S1b DNA ratio of 1:8 nearly abolished the induction of the reporter activity by prolactin (Fig. 7 C), while over 20% of the induced reporter activity still remained at an L/S1a ratio of 1:64 (Fig. 7 B). To examine whether the inhibitory effect of the short forms resulted from ligand competition between the long and short receptor forms, the ligand (hPRL) concentration was increased from 0.1 to 1.0 /H9262/g/ml in transfection studies (Fig. 7 D). The results showed that the inhibition of the long form-mediated activation of the /H9252/H -casein gene by the short forms was independent of the ligand concentration since a 10-fold increase in hPRL concentration did not alter the extent of inhibition (Fig. 7D). These results indicated that S1a and S1b could act as dominant negative forms to inhibit prolactin-induced transcriptional activation of the /H9252/H -casein promoter mediated by the long form of the receptor. However, the S1b form was much more effective than S1a in suppressing the reporter activation mediated by the long form of the receptor. This result appears to be consistent with the low cellular level of S1a protein (Fig. 3) as well as low binding activity on the cell surface (Fig. 5) compared with the S1b protein. As a result, the S1a form requires a much higher DNA ratio over the long form during transfection to achieve a significant inhibitory effect. A post-translational mechanism may account for the differential expression of the two short forms in transfected cells (see below).

**Differential Expression Levels of the Two Short Forms in Cultured Cells**—Western blot analysis showed that the cellular level of S1a is significantly lower than that of S1b or the long form in both COS-1 and HEK293 cells (Fig. 3). This was independent of the expression vector since when these hPRLR forms were cloned into a GFP fusion vector, the cellular level of the S1a-GFP fusion protein was also greatly reduced compared with the long and S1b forms (data not shown). Considering the close structural similarity between S1a and S1b, similar expression level of these two proteins would have been expected when both cDNAs were expressed in transfected cells. However, there was consistent and marked difference of cellular protein levels between the two forms of the receptor (Fig. 8, right). In contrast, the steady-state level of mRNAs from transfected cDNAs of the two forms was similar (Fig. 8, left), indicating that these mRNA species were not significantly different in their transcriptional and post-transcriptional regulation. Thus, the differences in the mRNA nucleotide sequences between the two short forms of the receptor had no effect on the level of protein expression. Studies on the translatability of S1a and S1b mRNA transcripts using both in vitro translation and in vivo translation methods showed (Fig. 9) that S1a protein was as efficiently de novo synthesized as S1b. This finding indicated that the low cellular level of S1a protein is not attributable to the differences in the translatability of the messages.

**Differential Turnover of the S1a and S1b Forms in Transfected HEK293 Cells**—To determine whether the post-transla-
scripts from the long and the chimeric LS1a forms in transfected cells were incubated with 125I-hGH and increasing concentrations of unlabeled hGH. Nonspecific binding was determined in samples containing an excess of unlabeled hGH (1 μg).

FIG. 5. 125I-hGH binding-inhibition curves of the surface hPRLRs on transfected intact COS-1 and HEK293 cells. Intact COS-1 and HEK293 cells were incubated with 125I-hGH and increasing concentrations of unlabeled hGH. Nonspecific binding was determined in samples containing an excess of unlabeled hGH (1 μg).

The C-terminal Unique Region of S1a Confers the Low Cellular Level to the S1a Form—The C-terminal 39-aa region is a unique region among the various forms of the hPRLR. To investigate whether this region may be responsible for the low expression of the S1a form as shown above, we used a pulse-chase experiment using L-[35S]methionine in HEK293 cells transfected with either S1a or S1b cDNA. A clear differential post-labeling protein turnover was observed between the S1a and S1b forms (Fig. 10). While S1a protein began to rapidly decrease from 4 h, S1b displayed only minor changes within 8 h of postlabeling, and it was only minimally but significantly decreased at 13 h. This result was also verified with a pulse-chase experiment in HEK293 cells cotransfected with both short forms (not shown). This result indicated that the S1a protein had a much higher turnover rate than S1b, and thus could contribute to the difference observed in steady-state cellular levels of these proteins given that both forms are equally transcribed and translated (see above).

The C-terminal Unique Region of S1a Confers the Low Cellular Level to the S1a Form—The C-terminal 39-aa region is a unique region among the various forms of the hPRLR. To investigate whether this region may be responsible for the low expression of the S1a form as shown above, we first examined the effects of the unique region on the expression level of a heterologous protein. The entire unique 39 aa region among the various forms of the hPRLR was inserted into the intracellular domain at aa 442 of the long form (named L1S1a) of the receptor. While the level of mRNA transcripts from the long and the chimeric L1S1a forms in transfected cells remained similar (Fig. 11B, lanes 5–8), the insertion caused a decrease in the chimeric protein level by ~85% (Fig. 11B, lanes 1–4). We next examined the effects of deletion or point mutation within this region on the expression level of S1a protein. A deletion of the entire 39 aa from the S1a protein (truncated protein, “trunc”) increased the expression of the protein to a level comparable with the S1b protein (Fig. 11D, lane 3) increased the level of both mutants by ~2-fold compared with that of S1a wild type. These results, which confirmed the findings obtained with the U5 antibody, demonstrated that the amino acid residues within the YRPRK sequence are critical in determining the post-translational regulation of this protein. We further analyzed the effects of deletion or point mutation within the 39-aa region on expression levels of the S1a form. This region contains a basic hydrophilic central domain flanked by more hydrophobic regions. Deletion of aa 353–357 (YRPRK) (Fig. 11C, lane 3) resulted in an increase by 1-fold, and this deletion along with a single mutation of K348N (Fig. 11C, lane 4) resulted in a further increase by ~110%. Due to the low level of S1a protein as detected on Western blot with the U5 monoclonal antibody against an epitope located at the extracellular domain of the hPRLR, we generated a rabbit polyclonal antibody against the C-terminal residues of the S1a form aiming at obtaining a specific reagent with improved sensitivity of detection. As shown in Fig. 11D, this anti-S1a antibody was found to be not only highly specific (Fig. 11D, lower panel, lane 2) but also of enhanced sensitivity (bands shown in the lower panel were obtained with 10 times shorter exposure than those in the upper panel with U5 antibody). Using this anti-S1a antibody, we showed that point mutation Lys-348 to Ala or Asn (Fig. 11C, lane 3) increased the level of both mutants by ~2-fold compared with that of S1a wild type. These results, which confirmed the findings obtained with the U5 antibody, demonstrated that the amino acid residues within the YRPRK sequence and Lys-348 in the 39-aa region play significant roles in regulation of the post-translational process.

FIG. 6. Activities of the β-casein promoter induced by prolactin in hPRLR-transfected cells. The individual hPRLR forms were transfected along with β-casein reporter into COS-1 (top) and HEK293 (bottom) cells. Transfected cells were stimulated with hPRL (100 ng/ml) for 24 h before termination. The activities of the β-casein reporter are shown as percentage of the activity from cells transfected with the long form in the presence of prolactin. The experiments were performed in six-well plates in triplicates. A β-galactosidase plasmid was included in each well of transfection, and the β-galactosidase activities were measured for normalization of the reporter activity. Data were collected from five independent transfections and were expressed as mean ± S.E.

DISCUSSION

In this study, we have identified two short forms, S1a and S1b, of the human PRLR that are generated from alternative splicing of exons 10 and 11 of the hPRLR gene. Both the S1a and S1b short forms were expressed as cell membrane receptors with much abbreviated intracellular domains. These human...
PRLR short forms were capable of ligand binding with binding affinities comparable with that of the long form. However, the two human short forms were unable to mediate the transcriptional activation of the β-casein promoter. This was likely due to the lack in the short forms of the C-terminal region of the long form of the receptor that is required for hormone-induced association and activation of Stat5 (16) and activation of the β-casein promoter (17). Several subtypes of PRLR besides the long form (18) were previously identified in rodents including the short and intermediate forms (19–22). Compared with exons 11 and 12 of rodents, the human exon 11 is unique at both nucleotide and amino acid levels; thus the human S1α and S1β are distinct forms from the rodent short forms. S1α is also distinct from the human intermediate form, which is a variant generated from an internal deletion within exon 10 with no participation of exon 11 (4, 6).

We have shown in cotransfection studies that both S1α and S1β forms could act as dominant negative forms to inhibit the prolactin-induced β-casein promoter activation by the long form of the receptor. However, due to its low surface expression resulting from a higher rate of turnover in transfected cells, the S1α form showed significantly weaker inhibitory effects than S1β on the reporter activation mediated through the long form. This was manifested by a marked difference in required long/short form DNA ratios (L/S1α versus L/S1β) for similar inhibitory effects.

**FIG. 7.** Cotransfection of S1α or S1β with the long form in HEK293 cells. S1α (A) or S1β (C) was cotransfected in increasing DNA doses (0.25–8× of the long form) with a constant amount of the long form (0.4 μg) along with the β-casein reporter (0.1 μg) in HEK293 cells. Cells were treated with hPRL (100 ng/ml) for 24 h before termination. The activities of the β-casein promoter are shown as percentage of the activity from cells transfected with the long form of the receptor and stimulated with prolactin (% of long form).

**FIG. 8.** Northern blot and Western blot analyses of hPRLR subtypes in HEK293 cells. Left, Northern blot analysis of hPRLR mRNA from transfected HEK293 cells using probe corresponding to the extracellular domain of the hPRLR. β-Actin mRNA was probed for sample normalization. Right, immunoblot analysis of the hPRLR forms in HEK293 cells transfected with individual receptor cDNAs using U5 monoclonal antibody, which recognizes an epitope in the extracellular domain of the hPRLR. The faint S1α band is immediately below the nonspecific band (N) (arrow). Vt, vector.

**FIG. 9.** *In vitro and in vivo* translation of the short hPRLR forms. Left, *in vitro* labeled proteins corresponding to the long, S1α, and S1β forms are shown in lanes 1–3. Right, *in vivo* labeling with L-[35S]methionine (20 min) of HEK293 cells transfected with S1α and S1β, followed by immunoprecipitation using anti-PRLR antibody (U5). The de novo synthesized S1α and S1β proteins are shown in lanes 5 and 6, respectively.
tory effects during cotransfection. Of the two human short forms, S1b, which contains the shortest intracellular domain of all reported short forms, shares more overall structural similarity with the rat short form since they lack any cytoplasmic region encoded by exon 10 despite their different C-terminal exon 11-encoded regions (20, 21). It is therefore conceivable that S1b may heterodimerize with the long form upon ligand binding and inhibit the activation of Jak2-Stat5 and of the subsequent β-casein gene mediated by homodimerized long form receptors (23, 24). In contrast to the S1b form, S1a has the longest intracellular domain of all short forms, not only containing the C-terminal 39 unique amino acids but also retaining the 52-aa region (aa 285–337) from exon 10, which contains box 2, loosely defined as a 10-aa domain with successive hydrophobic, negatively, and then positively charged residues (1). Therefore the S1a form may retain more functions of the long form than S1b, while its C-terminal unique region may render it functionally distinct from other forms of the hPRLR.

At the nucleotide level, S1a mRNA contains an identical exon 11 sequence as S1b, suggesting that both mRNA transcripts would be comparable in their degradation and translatability. Indeed we showed that the steady-state levels and the translatability of the two short receptor forms were similar in transfected cells, therefore these parameters could not account for the marked difference observed in the protein levels between S1a and S1b. A differential post-translational regulation of the two forms appears to be the major factor in determining the low cellular level of the S1a form since the turnover rate of S1a was much faster than S1b in transfected HEK293 cells. These results indicated that the C-terminal 39-aa region contains the signal(s)/structure that leads the S1a form to a faster degradation pathway than S1b and subsequently to low cellular protein level. The C-terminal 39-aa region of the S1a form is a unique sequence having no significant similarity to any known protein. It is characterized by a middle section rich in basic residues flanked by hydrophobic regions on both sides. This middle section could be involved in a preferential recognition by the protein degradation pathway since deletion of YRPRK or mutation of Lys-348 significantly increased the protein levels.

Ubiquitin-proteasome-mediated proteolysis is a major pathway for protein degradation at the endoplasmic reticulum site (25, 26). The 26 S proteasome contains a multienzyme system (E1, E2, and E3) that recognizes specific structure element(s) of a protein called “degron” and could lead to ubiquitination on lysine residues (27, 28). It was suggested in some proteins such as the yeast hydroxymethylglutaryl-CoA reductase isozyme.

**Fig. 10.** Pulse-chase labeling of the S1a and S1b forms with L-[35S]Met in transfected HEK293 cells. Cells at 70% confluence were transfected with S1a or S1b for 24 h and pulse-labeled with L-[35S]methionine. After the addition of unlabeled methionine to the culture medium with unlabeled methionine. Lysed cellular proteins were immunoprecipitated with U5 antibody and resolved by 8% SDS-polyacrylamide gel electrophoresis. The relative amount of 35S-labeled S1a or S1b was expressed as percentage of that of the control cells (C). The experiment is a representative of three independent experiments with similar results.

**Fig. 11.** Western blot analysis of hPRLR mutants in HEK293 cells. A, schematic representation of the long and S1a hPRLR forms and with insertion, deletion, and/or mutations. L511a, the long form with the unique region of the S1a form; trunc, truncation of the C-terminal 39-aa region of S1a; S1a, wild type; ΔS1a, deletion (353YRPRK357); x and A, point mutations; Ab, specific anti-S1a antibody raised against a peptide of this region. The wild type and mutants of the hPRLR cDNA were transfected in HEK293 cells, and total cell proteins were analyzed by Western blot using anti-hPRLR antibody U5 (B–D) or using anti-S1a antibody (D and E). B, lanes 1–4, chimera protein L511a, is expressed at a reduced level compared with the long form; lanes 5–8, Northern blot hybridized concurrently with hPRLR extracellular domain and the β-actin probe showed that the level of the chimeric L511a mRNA transcript had no reduction in transfected cells compared with the long form. C, deletion of 5 aa (aa 353–357) within the C-terminal 39-aa region of S1a increased the protein expression level. D, specificity of the S1a polyclonal antibody. hPRLR wild type and mutant forms were probed with U5 antibody, which recognizes all forms (upper) and with the anti-S1a specific antibody (lower). No bands in the identical blot were detected with preimmune IgG (not shown). E, Western blot detected with anti-S1a antibody. Point mutation of lysine (Lys-348) significantly increased the S1a level.
(Hmg2P, a membrane protein) that dispersed structural features rather than a primary sequence motif may determine the substrate recognition by the endoplasmic reticulum-associated protein degradation pathway (29). Since the entire deletion of the 39 amino acids of the S1a form increased the protein expression over that observed by deletion and/or mutations, additional sequences within this region might also contribute to the increased degradation of the S1a form (Fig. 11D). There are no known protein-protein interaction domains or motifs identifiable in this region except for the consensus protein kinase A and protein kinase C phosphorylation sites. A 50-aa membrane proximal cytoplasmic domain of erythropoietin receptor was demonstrated to be the determinant for its rapid cellular turnover resulting from proteosome-mediated proteolysis (30). C-terminal domains are often involved in subcellular distribution, trafficking, and degradation of proteins such as glucose transporter 4, p53, cystic fibrosis transmembrane conductance regulator, and aggrecan (31–34). It is reasonable to foresee a mechanism(s) that would control the level of S1a protein expression in certain physiological or pathological conditions. Since the two human short forms are widely expressed in human tissues, they may have important roles in modulating the prolactin signaling through the long form receptor. In addition, the unique cytoplasmic sequence of these forms may confer distinct signaling pathway(s) that may contribute to the functional diversity of prolactin.

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Isolation and Characterization of Two Novel Forms of the Human Prolactin Receptor Generated by Alternative Splicing of a Newly Identified Exon 11
Zhang-Zhi Hu, Jianping Meng and Maria L. Dufau

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