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Different Biological Effects of Unmodified Prolactin and a Molecular Mimic of Phosphorylated Prolactin Involve Different Signaling Pathways†

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ABSTRACT: Previous work has shown that naturally phosphorylated prolactin antagonizes the growth-promoting activities of unmodified prolactin (U-PRL) and that this effect is duplicated by a molecular mimic, S179D PRL. At the same time, the S179D PRL is a superagonist with regard to expression of some PRL-regulated genes. We have asked whether the different activities of U-PRL and S179D PRL are the result of differential signaling. HC11 cells (a normal mouse mammary cell line) were grown to confluence, primed with hydrocortisone, and then exposed to the PRLs. A 15 min incubation of PRL-naive cells led to substantial tyrosine phosphorylation of Jak 2 and Stat 5a by U-PRL and an essentially equivalent Jak 2 activation by S179D PRL. The latter, however, was accompanied by reduced tyrosine phosphorylation of Stat 5a. EMSA analysis using a Stat 5 binding site showed both PRLs to cause equivalent binding of nuclear proteins and that most of what bound was complexed through Stat 5a. Phosphoamino acid analysis of Stat 5 showed S179D PRL to double the amount of serine phosphorylation versus that seen with U-PRL. Analysis of the MAP kinase pathway showed U-PRL capable of activation of ERKs 1 and 2 but that signaling via ERKs 1 and 2 was greater with S179D PRL. A 7-day incubation in either PRL increased β-casein mRNA levels, but S179D PRL caused a 2-fold increase over that seen with U-PRL. The increase, over that seen with U-PRL, was blocked by the MAP kinase inhibitor, PD98059. After 7 days of treatment with S179D PRL, expression of the short PRL receptor was doubled, and signaling showed a greater dependence on the MAP kinase pathway (2.9-fold increase in ERK 1 and 2 activation). We conclude that although both PRLs use both pathways to some extent, U-PRL signals primarily through Jak 2−Stat 5 whereas S179D PRL signals primarily through the MAP kinase pathway especially after prolonged exposure. This is the first demonstration of differential involvement of signaling pathways by different forms of PRL.

Work from this laboratory has recently described the differential effects of recombinant unmodified prolactin (U-PRL)[1] and a molecular mimic of phosphorylated PRL (S179D PRL) on the development of the adult rat mammary gland (*1*). Thus, U-PRL was shown to promote ductal growth and the growth of alveoli, and S179D PRL was shown to inhibit ductal and alveolar growth and yet to promote β-casein gene expression. The growth and anti-growth effects of these PRLs in the rodent mammary gland have recently been duplicated in another laboratory (C. Ormandy, Garvan Institute, Sydney, Australia, personal communication). Given the differential effects of these two PRLs in the mammary gland, it seemed likely that their interaction with PRL receptors resulted in the use of differential signaling pathways within mammary epithelial cells. Differential intracellular signaling of these two PRLs has previously been demonstrated in the Nb2 lymphoma system (*2*). However, the PRL receptor of Nb2 cells is unique (*3*), and analysis of signaling via this unique receptor may not provide accurate insight into signaling in other tissues. All normal rodent tissues express both a long and a short form(s) of the PRL receptor (*4*). The ratio of long to short receptor varies from tissue to tissue and has been shown to vary with physiological state in some tissues (*4*). The significance of this differential expression is still unclear. Both forms of the normal PRL receptor are identical in their extracellular domains and differ in their cytoplasmic domains (*5*). They have both been shown to activate Jak 2 (*6*), but only the long form of the receptor activates Stat 5 as a result (*6*, *7*). In addition, the long and short forms of the receptor also activate the MAP kinase

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[1] Abbreviations: PRL, prolactin; U-PRL, unmodified prolactin; S179D PRL, mimic of phosphorylated prolactin in which serine 179 is replaced by an aspartate residue; EMSA, electromobility shift assay; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; Jak 2; Janus kinase 2; Stat 5, signal transducer and activator of transcription 5; NIDDK, National Institute for Diabetes, Digestive and Kidney Diseases; NICHD, National Institute for Child Health and Human Development; USDA, United States Department of Agriculture; GIBCO, Grand Island Biological Company; RPMI, Roswell Park Memorial Institute; MALDI, matrix-assisted laser desorption ionization; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; Tris/TRIZMA, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PVDF, polyvinylidene fluoride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; GAS, γ interferon activating sequence; TBE, Tris−borate−EDTA; SSC, sodium citrate/sodium chloride; UV, ultraviolet; PCR, polymerase chain reaction.

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pathway via ras and raf (*8*, *9*). These appear to be the main signaling pathways, although a large number of other signaling molecules have been shown to be activated by PRL in a variety of tissues (reviewed in ref *10*).

In this study, we have used a normal mouse mammary cell line to investigate the relative use of the Jak 2−Stat 5 pathway and the MAP kinase pathway by each form of PRL. Although both PRLs use both pathways to some extent, the results demonstrate that U-PRL predominantly uses Jak 2−Stat 5a, whereas S179D PRL predominantly uses MAP kinase.

EXPERIMENTAL PROCEDURES

Mass Spectrometry. The phosphorylation status of normal human PRL extracted from pituitaries (standard PRL) was assessed by mass spectrometry. The human PRL was in part a gift from Dr. A. Parlow (Harbor-UCLA, Torrance, CA) and in part provided through the auspices of the Hormone and Pituitary Program of the NIDDK, NICHD, and USDA. The PRL (AFP3855A, B3) was dissolved in water and washed to reduce ion contamination by three exchanges of water in a Microcon YM-10 (Millipore Corp., Bedford, MA) with a 10 kDa molecular mass cutoff. The washed material was completely dissolved in water containing 0.1% trifluoroacetic acid, and aliquots were subjected to MALDI (matrix-assisted laser desorption ionization) using sinapinic acid as the matrix. The choice of matrix is crucial to appropriate resolution of the phosphorylated forms. Accuracy in this molecular mass range is ±0.1%.

Recombinant PRLs. The recombinant human PRLs were expressed in *Escherichia coli* to avoid posttranslational modifications and were produced and characterized as previously described (*11*). Since both PRLs were expressed at similar levels and were isolated and folded in tandem, they served as controls for one another for any potential non-PRL contaminants. Serine 177 in rat PRL is the major site of phosphorylation (*12*) and is equivalent to serine 179 in human PRL (Swiss Protein Database). Serine 179 of human PRL has also been shown to be phosphorylated by the intragranular kinase of rat pituitary secretory granules (*13*). Mutation of S179 to an aspartate residue produced a molecular mimic of monophosphorylated PRL. An aspartate residue mimics a phosphoserine by approximate size of the side chain and by carrying a negative charge. This kind of mimicry is now commonplace in enzymology where both functional and structural studies show equivalency between the natural phospho form and the mimic (e.g., ref *14*).

HC11 Cells. HC11 cells, which are normal mouse mammary epithelial cells, were generously provided by Dr. Nancy Hynes (Friedrich Meischer Institute, Basel, Switzerland) through Dr. Margaret Neville (University of Colorado, Denver, CO). They were routinely cultured in RPMI 1640 with 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY), 5 μg/mL insulin (Sigma Chemical Co., St. Louis, MO), and 10 ng/mL EGF (GIBCO-BRL). When the cells had been confluent for 2 days, the medium was changed to "priming medium" (RPMI 1640, 10% charcoal-stripped horse serum, 10 μg/mL insulin, and 1 μg/mL hydrocortisone) for 1−2 days. In the first experiments, standard PRL, U-PRL, or S179D PRL was then added to give a final concentration of 5 μg/mL, and the cells were lysed after 15 min. In the second set of experiments, 1 μg/mL U-PRL or S179D PRL was used. In the third set, different doses of S179D PRL were titrated against 100 ng/mL U-PRL. The priming protocol was provided by Peggy Neville and was based on one designed by Taverna et al. (*15*). Of special note for this study was analysis of signaling with or without only the PRLs. In other words, unlike studies published by other laboratories (e.g., ref *16*), all three lactogenic hormones were not added simultaneously. Instead, the cells had been exposed to insulin throughout and to hydrocortisone for 1−2 days prior to the addition of PRL.

HC11 cells are the only cell line that responds to PRL by increasing β-casein gene expression. This is therefore the only cell line in which one can analyze natural regulation. Even in this cell line, however, the level of β-casein expression is relatively low, and others have demonstrated that there is an increasing response to PRL treatment for as much as 5 days (*17*). Since our goal was to analyze mRNA levels by Northern blot and to assess differential total stimulation between the two PRLs, we utilized a 7-day stimulation period.

When β-casein gene expression was analyzed, cells were incubated in a 1 μg/mL amount of each PRL for 7 days with a change of medium each day. Signaling in 7-day PRL-incubated cells was assessed by removal of PRL for 2 h prior to reexposure. As for initial exposure, reexposure was for 15 min. In some experiments, the MAP kinase inhibitor, PD98059 (25 μM), was included in the 7-day PRL incubation. This concentration of PD98059 has been shown to decrease PRL-stimulated MAP kinase activity in HC11 cells by more than 90% (*16*).

After PRL exposure, cells were rinsed with 0.01 M phosphate-buffered saline, pH 7.4, and were then scraped from the dish into 20 mM Tris, pH 7.4, containing 150 mM NaCl, 10 mM each of sodium pyrophosphate, sodium fluoride, and sodium vanadate, 10 μg/mL each of aprotinin, leupeptin, and pepstatin, 1 mM PMSF, 0.02% sodium azide, 1 mM EDTA, and 1% Triton X-100. After a 1 h rotation at 4 °C, lysates were cleared by centrifugation at 13000*g* for 10 min, and the supernatants were saved for immunoprecipitation.

Immunoprecipitation. Lysates containing 4 mg of protein were used for immunoprecipitation. Antibodies were as follows: polyclonal anti-Jak 2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-Stat 5b (Zymed Laboratories, Inc., San Francisco, CA), polyclonal anti-pan Stat 5 and anti-Stat 5a (Santa Cruz Biotechnology), and polyclonal anti-MAP kinase (Promega, Madison, WI). Four micrograms of purified antibody was added to 1 mL of lysate and incubated for 2 h overnight (overnight used for anti-MAP kinase, but 2 h or overnight made no difference to the Stat 5 results) at 4 °C. Antigen−antibody complexes were precipitated by addition of 30 μL of washed protein G−Sepharose slurry (Amersham Pharmacia Biotech, Piscataway, NJ) and incubation for 2 h at 4 °C. The pellets were washed three times in lysis buffer and then placed in reducing SDS sample buffer at 95 °C for 10 min before being loaded on a 7.5% polyacrylamide gel. For Figure 6, whole cell lysates were subjected to electrophoresis.

Western Blot. After protein transfer to nitrocellulose membranes in 48 mM Trizma, 30 mM glycine, 0.1% SDS, and 10% methanol (pH 8.3), membranes were blocked with

10% nonfat milk in wash buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% BSA, 0.1% Tween 20). Blocked membranes were probed with anti-phosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY) diluted 1:2000 in wash buffer or with anti-phospho MAP kinase (Promega) diluted 1:5000 for 2 h at room temperature. After being washed three times for 15 min, the blot was further incubated in goat anti-mouse conjugated to horseradish peroxidase (Sigma) at 1:2000 or goat anti-rabbit conjugated to horseradish peroxidase (Sigma), as appropriate, for 1 h at room temperature. After five washes, horseradish peroxidase positive bands were detected with ECL reagent (Amersham Biosciences, Piscataway, NJ) followed by autoradiography. Blots were subsequently stripped for 1 h at 70 °C in 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris, pH 6.8. After further blocking and washing, membranes were reprobed with anti-Jak 2, anti-Stat 5a, anti-Stat 5b, or anti-MAP kinase diluted 1:1000 in wash buffer, and either goat anti-rabbit or goat anti-mouse peroxidase conjugated antibodies were used, as appropriate.

Controls included tests of the second antibodies alone for each of the immunoprecipitating antibodies. Proteins were identified by molecular mass in reference to coelectrophoresed colored standards (Bio-Rad, Hercules, CA) and by overlay of the autoradiograms derived from sequential blots. Each blot analyzing phosphotyrosine included a positive control, which also served as a control for thorough stripping of membranes. For each blot, several exposures were used to ensure that film development was in the linear range.

Phosphoamino Acid Analysis. After 2 days in priming medium, the medium was changed to phosphate-free DMEM (GIBCO-BRL) containing 1 mCi/mL $H_3{}^{32}PO_4$ (ICN Radiochemicals, Irvine, CA) for 4 h prior to a 15 min incubation in the PRLs. At the end of the 15 min incubation, the cells were lysed, and Stat 5 was immunoprecipitated as above using the anti-Stat 5 antibody. The immunoprecipitate was resolved into its components by reducing SDS gel electrophoresis, and the proteins were transferred to PVDF membranes and then subjected to autoradiography. The Stat 5 band, identified by reference to molecular weight markers, was excised and then hydrolyzed in 6 N HCl (Pierce Chemical Co., Rockford, IL) for 1 h at 110 °C under vacuum. Hydrolysates were dried in a SpeedVac and then redisolved in water. Samples were spotted on thin-layer chromatography films (EM Science, Gibbstown, NJ) and electrophoresed at 450 V for 3 h at 4 °C in 0.5% pyridine containing 0.5 mM EDTA, pH 3.9. After drying and visualization of co-run standards with ninhydrin (0.3% in butanol), the phosphoamino acids were detected by autoradiography and quantified by densitometry.

Electromobility Shift Assay. To obtain a nuclear extract, cells were first scraped off the dish into hypotonic buffer [20 mM Tris, pH 7.4, containing 10 mM NaCl, 1 mM $MgCl_2$, 10 μg/mL each of aprotinin, leupeptin, and pepstatin (Sigma), 1 mM PMSF (Sigma), 10 mM NaF, and 1 mM Na_3VO_4] and allowed to swell on ice. The cells were broken by two passages through a 27 gauge needle, and nuclei were then pelleted by centrifugation. The nuclear pellet was then placed in hypertonic buffer (20 mM Hepes, pH 7.5, containing 420 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 20% glycerol, 10 μg/mL each of aprotinin, leupeptin, and pepstatin, 1 mM PMSF, 10 mM NaF, and 1 mM Na_3VO_4) for 30 min on ice with occasional mixing.

After centrifugation at 12000*g* for 15 min, the supernatants were diluted with equal volumes of the hypotonic buffer to lower the salt concentration. Two micrograms of nuclear extract protein was used in each binding reaction, which was carried out in 10 mM Hepes, pH 7.4, containing 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, and 5 mM DTT. One microgram of antibody or 100× unlabeled oligonucleotide or an equivalent volume was added for 15 min followed by incubation for 45 min at 37 °C with radiolabeled oligonucleotide. The oligonculeotide equivalent to the most proximal GAS site on mouse β-casein (5′-CACGTAGACTTCTTGGAATTGA-3′) was annealed to the complementary sequence and radiolabeled with T4 kinase (GIBCO-BRL, Grand Island, NY) according to the manufacturer's instructions. At the end of the binding reaction, the mixture was analyzed on a 6% polyacrylamide gel (prerun for 30 min at 120 V) in 0.25 × TBE buffer (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, pH 8) for 1.5 h at 200 V. Dried gels were exposed for autoradiographic analysis.

Northern Blot Analysis for the Expression of β-Casein and the Long and Short Forms of the PRL Receptor mRNA. Total RNA, isolated from HC11 cells using the Trizol RNA reagent (GIBCO-BRL), was treated with DNase I (GIBCO-BRL). Equal amounts of RNA (10 μg) from control and test samples were loaded on a 1.0% agarose−formaldehyde gel. The gels were run at 60 V for 3−5 h. The RNA was blotted onto nylon filters (Micron Separations, Inc., Westboro, MA) by capillary transfer with 10 × SSC and fixed by UV crosslinking. The 201 bp mouse β-casein cDNA probe for hybridization was made by PCR. The primers were 5′-CCC GTC CCA CAA AAC ATC C-3′ (forward) and 5′-ATT AGC AAG ACT GGC AAG GCT G-3′ (reverse). A 399 bp cDNA probe which recognizes both the long and short forms of the receptor was made by PCR. The primers were 5′-CCC ACC CAC CAT AAC TGA TG-3′ (forward) and 5′-TCC AGC AGA TGG GTA TCA AAT C-3′ (reverse). Hybridization gave a band at ∼10 kb and a band at ∼8 kb for the long and short receptor message, respectively. A 245 bp specific probe for the short receptor was a gift from Paul Kelly's laboratory (INSERM, Paris, France). Probes were labeled with 50 μCi of [α-^{32}P]dCTP (ICN) using a DECA Prime II DNA labeling kit (Ambion, Austin, TX). The labeled probes were separated by ProbeQuant G-50 microcolumns (Amersham Pharmacia Biotechnology). After 2 h of prehybridization at 65 °C with the hybridization solution (25 M Na_2HPO_4, pH 7.2, 1 mM EDTA, pH 8.0, 7% SDS), hybridizations were carried out at 65 °C for 16−24 h. The filters were then washed in alternating solutions of 20 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, and 5% SDS and of 20 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, and 1% SDS for a total of three times in each. Filters were exposed to Fuji medical X-ray film (Fuji Medical Systems, Inc., Stamford, CT) for 1−7 days at −70 °C. Probe stripping was performed by heating the nylon filter at 95 °C for 10−30 min in a solution of 10 mM Tris, pH 8.0, 1 mM EDTA, and 1% SDS. A mouse 18S rRNA cDNA fragment (DECA template-18S-mouse, 1212 bp) (Ambion, Austin, TX) was used to normalize for errors in RNA loading and transfer. A Kodak 1D image analysis system was used for quantification (Eastman Kodak Co., Rochester, NY). Several exposures were used for each autoradiogram to ensure that film development was in the linear range.

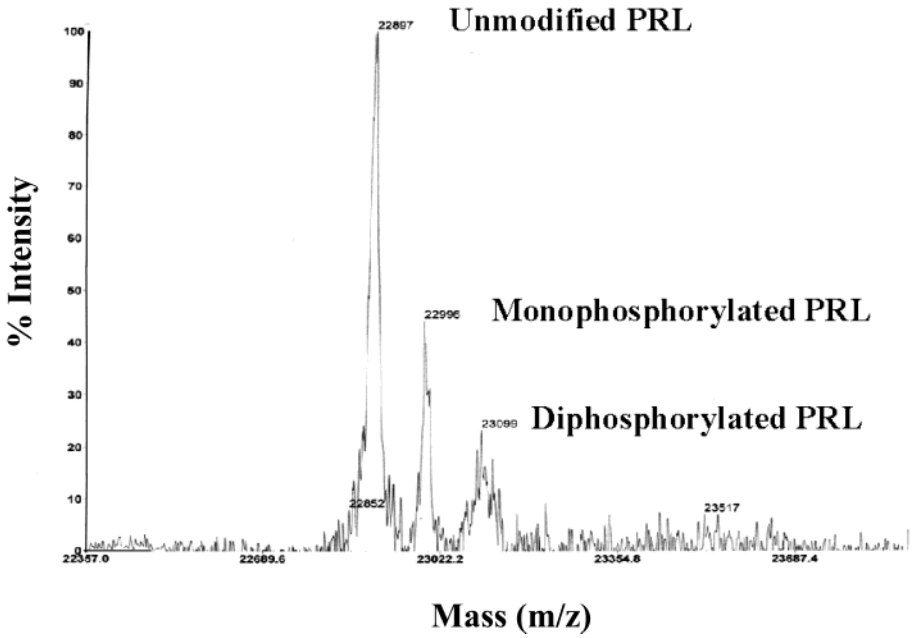

FIGURE 1: Mass spectrometric analysis of NIDDK standard human pituitary PRL. PRL was dissolved in 0.1% trifluoroacetic acid and was subjected to matrix-assisted laser desorption ionization using sinapinic acid as the matrix. The ordinate is in relative units. The numbers next to the peaks are the masses of those peaks in daltons. The accuracy in this molecular mass range is ±0.1%. The main peak at 22897 is unmodified PRL, the peak at 22996 is monophosphorylated PRL, and the peak at 23099 is diphosphorylated PRL.

Statistical Analyses. Data were analyzed by analysis of variance and posttests for comparing specific groups, using Bonferroni corrections for multiple comparisons against a single group.

RESULTS

Mass spectrometry of standard human pituitary PRL demonstrated the presence of mono- and diphosphorylated PRL within the mixture (Figure 1). The first peak at a mass of 22897 Da is U-PRL, which has a calculated mass of 22897.75 Da. It constitutes 62% of the mixture. The second peak at 22996 Da is PRL with one phosphate and one sodium exchanged for a hydrogen. It constitutes 19% of the mixture. The third peak at 23099 Da is PRL with two phosphates and two sodium−hydrogen exchanges. It also constitutes 19% of the mixture. The proportions of each form of PRL were in the range reported previously for rodent PRL (*18*, *19*). Signaling from standard PRL therefore represents a physiological, mixed response to U-PRL and phospho-PRL. This preparation of standard PRL, however, only represents one ratio of U-PRL to phospho-PRL, and the amount phosphorylated varies with physiological status (*18*, *19*) and varies from preparation to preparation distributed as standard.

To clearly differentiate the intracellular signals of U-PRL and P-PRL, we used a recombinant human version of U-PRL and a recombinant molecular mimic of human P-PRL, S179D PRL. In this way we could avoid the potential dephosphorylation of P-PRL to U-PRL. In addition, we compared signaling generated by these two recombinant PRLs to that produced by the standard PRL preparation. In this way, we could compare our results with others in the literature.

As shown in Figure 2, primed HC11 cells show some activation of Jak 2 without the addition of PRL. This occurred even when the cells were allowed to rest prior to and during the 15 min incubation. Addition of standard PRL, recombinant U-PRL, or S179D PRL resulted in a similar degree

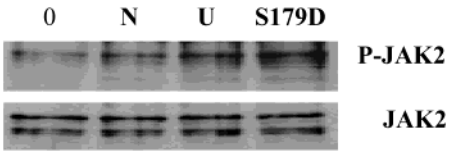

FIGURE 2: Jak 2 activation by U-PRL, S179D PRL, and standard pituitary PRL. HC11 cells were exposed to 5 μg/mL PRLs for 15 min. Immunoprecipitation was with anti-Jak 2. The upper panel was blotted with anti-phosphotyrosine and then stripped and reprobed with anti-Jak 2 to produce the lower panel. Key: 0, no added PRL; N, addition of NIDDK standard human pituitary PRL; U, unmodified recombinant human PRL; S179D, recombinant S179D human PRL. Note that each PRL activates Jak 2 and that the degree of activation is similar, although the background in the S179D PRL-treated lane is higher. This blot is representative of three separate experiments.

of additional Jak 2 activation with, if anything, a larger increase in response to S179D PRL (upper panel blotted with anti-phosphotyrosine). Equal loading of the lanes is demonstrated in the bottom panel blotted with anti-Jak 2. Analysis of Stat 5 tyrosine phosphorylation, however, showed very different responses to the different PRLs. Standard human PRL (which is mostly U-PRL) and U-PRL strongly tyrosine phosphorylated Stat 5a while S179D PRL showed a more modest increase over the control level (Figure 3). By contrast, none of the PRLs was a strong activator of Stat 5b tyrosine phosphorylation in these cells (data not shown).

Following exposure of the cells to each PRL, EMSA analysis using the β-casein GAS site demonstrated that each PRL promoted the nuclear translocation of proteins which formed very similar protein−oligonucleotide complexes, resolving as two to three bands (Figure 4). Competition with anti-Stat 5a and anti-Stat 5b antibodies demonstrated that the majority of the complexes contained Stat 5a, regardless of the PRL type. The absence of the complexes with no PRL and in the presence of competing, unlabeled oligonucleotide shows the specificity of the binding. Excess, radiolabeled

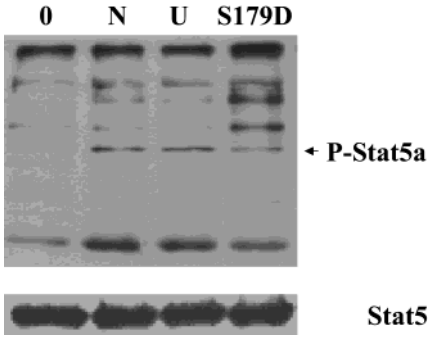

FIGURE 3: Stat 5a activation in response to U-PRL, S179D PRL, and standard pituitary PRL. HC11 cells were exposed to 5 μg/mL PRLs for 15 min. Immunoprecipitation was with anti-Stat 5a. The upper panel was blotted with anti-phosphotyrosine and then stripped and reprobed to produce the lower panel blotted with anti-Stat 5a. Key: 0, no added PRL; N, addition of NIDDK standard human pituitary prolactin; U, addition of unmodified recombinant human PRL; S179D, addition of recombinant human S179D PRL. Note the superior tyrosine phosphorylation by NIDDK PRL and U-PRL and the weaker activation by S179D PRL. This blot is representative of eight separate experiments.

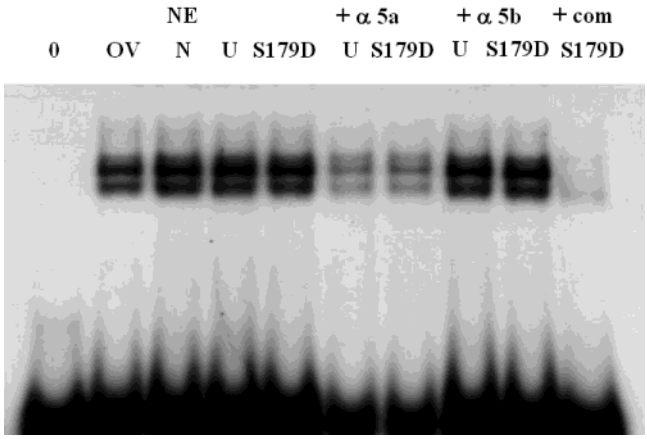

FIGURE 4: EMSA analysis of the β-casein GAS site following stimulation with U-PRL, S179D PRL, and standard pituitary PRL. After stimulation of the cells with each PRL (5 μg/mL, 15 min), nuclear extract proteins (NE) were incubated with radiolabeled GAS site double-stranded oligonucleotide with or without competition by anti-Stat 5a (α 5a), anti-Stat 5b (α 5b) or 100-fold unlabeled oligonucleotide (com). Key: 0, no added PRL; OV, addition of ovine PRL; N, addition of NIDDK standard human pituitary PRL; U, addition of unmodified recombinant human PRL; S179D, addition of recombinant human S179D PRL. Note the very similar complexes produced by NIDDK PRL, U-PRL, and S179D PRL and that formation of complexes in the latter two (only two tested) could be competed for by anti-Stat 5a but not anti-Stat 5b. This autoradiogram is representative of four separate experiments.

oligonucleotide also ensured comparability on a relative quantitative basis.

Phosphoamino acid analysis of immunoprecipitated Stat 5 following phosphate radiolabeling of the cells showed incubation with S179D PRL to result in less tyrosine phosphorylation overall, but in two to three times the level of serine phosphorylation versus that seen with U-PRL (Figure 5). As has been observed by others, there was a constitutive level of serine phosphorylation in cells that had not been exposed to PRL (*16*, *20*), and this level was not increased by U-PRL. Standard PRL gave a result intermediate between the other two PRLs, a result reflective of it being a mixture of U-PRL and P-PRL. The results were normalized to the total radiolabeled phosphate in each lane to correct

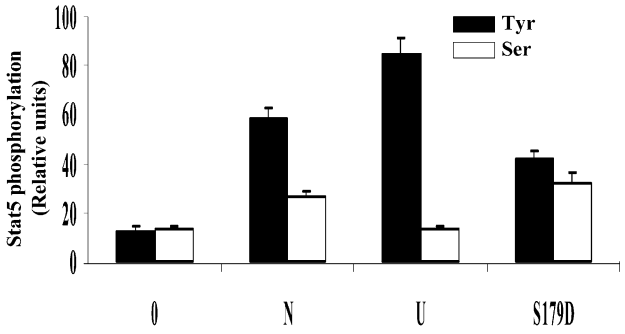

FIGURE 5: Tyrosine and serine phosphorylation of Stat 5 in response to U-PRL, S179D PRL, and standard pituitary PRL. HC11 cells were incubated in 5 μg/mL PRLs for 15 min, lysed, and subjected to immunoprecipitation with anti-Stat 5. Hydrolysis of the gel-purified Stat 5 was followed by phosphoamino acid analysis. The bars show the means ± SE of three separate phosphoamino acid analyses from one radiolabeling experiment and are expressed as relative densitometric units normalized to total phosphate in the hydrolysate to correct for loading. Key: Tyr, phosphotyrosine; Ser, phosphoserine; 0, no added PRL; N, addition of NIDDK standard human PRL; U, addition of recombinant human unmodified PRL; S179D, addition of recombinant human S179D PRL.

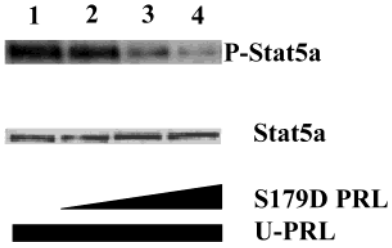

FIGURE 6: Competition between U-PRL and S179D PRL for Stat 5 tyrosine phosphorylation. All cells were incubated in 100 ng/mL U-PRL. Cells in lanes 2, 3, and 4 were additionally incubated in 10, 100, and 1000 ng/mL S179D PRL, respectively. This experiment was conducted without immunoprecipitation of Stat 5a. The upper panel showing phosphotyrosine reactivity was exposed for 5 min. After being stripped and reprobed with anti-Stat 5a, the lower panel was exposed for 20 s. These blots are representative of three separate experiments.

for loading of the film. Both NIDDK PRL and S179D PRL showed a higher amount of free phosphate, suggesting a reduced stability of the phosphoamino acids in these samples. Without this normalization, the differences between the U-PRL and S179D PRL samples would have been larger.

Given the differential effects of U-PRL and S179D PRL on tyrosine phosphorylation of Stat 5a, we compared the titration of one form of PRL against the other. To ensure that the observed differences in tyrosine phosphorylation were not an artifact of preferential immunoprecipitation of serine-phosphorylated Stat 5, this experiment was conducted without immunoprecipitation. Figure 6 illustrates that increasing concentrations of S179D PRL progressively decrease Stat 5a tyrosine phosphorylation in response to U-PRL. Further, this experiment demonstrates that immunoprecipitation was not selecting for subpopulations of Stat 5a.

Because previous work had demonstrated that S179D PRL had a superior ability to stimulate β-casein gene expression (*1*) and this was clearly not due to superior tyrosine phosphorylation of Stat 5a or Stat 5b, we investigated the effect of each PRL on the MAP kinase signaling pathway. In these experiments, the two recombinant PRLs were tested for their ability to activate ERKs 1 and 2, and this was concurrently analyzed with activation of Stat 5a. As shown

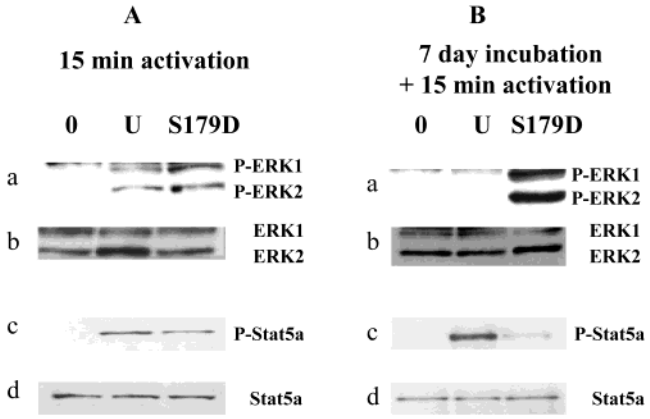

FIGURE 7: Activation of ERK 1, ERK 2, and Stat 5a in response to U-PRL and S179D PRL, both before (panel A) and after (panel B) a 7-day incubation in those PRLs. The cells were incubated for 15 min for panel A in 5 μg/mL PRLs. For panel B, the cells were incubated in the PRLs at 1 μg/mL for 7 days; the PRLs were withdrawn for 2 h and then reapplied for 15 min (5 μg/mL). The upper panels (a) show the result of immunoprecipitation with anti-total ERK and blotting with anti-active ERK. Below them is the same blot stripped and reprobed with anti-total ERK (b). The third panel down shows the result of immunoprecipitation with anti-Stat 5a and blotting with anti-phosphotyrosine (c), and the lowest panel shows the result of stripping and reprobing with anti-Stat 5a (d). In each case, the A panels and B panels were exposed to the same film so that direct comparisons could be made before and after the 7-day incubation. Note the greater activation of ERKs 1 and 2 by S179D PRL and the greater activation of Stat 5a by U-PRL. Also note the upregulation of ERK signaling after the 7-day incubation in S179D PRL and downregulation of Stat 5a signaling. These blots are representative of five separate experiments.

in Figure 7Aa, S179D PRL caused a substantial activation of both ERKs, while the response to U-PRL was weaker. This weaker response to U-PRL is emphasized when one notes the heavier loading of total ERKs shown in Figure 7Ab. At the same time in these experiments, the initial differential responses in terms of Stat 5a were reproduced. In other words, S179D PRL was less effective than U-PRL in causing Stat 5a tyrosine phosphorylation.

To determine whether activation of ERKs 1 and 2 was related to superior β-casein gene expression, we utilized the inhibitor PD98059. As before (*1*), incubation of HC11 cells in either PRL for 7 days resulted in upregulation of endogenous β-casein gene expression (Figure 8). U-PRL doubled the control level (compare bars 1 and 3), while S179D PRL quadrupled the control level (compare bars 1 and 5). Addition of the MAP kinase inhibitor PD98059 inhibited the S179D PRL response (compare bars 5 and 6) while it had no effect on the basal level of expression (compare bars 1 and 2). Importantly, PD98059 also had no effect on β-casein gene expression stimulated by U-PRL (compare bars 3 and 4). Thus MAP kinase activation is linked to the superior β-casein expression seen in response to S179D PRL but is not linked to U-PRL-stimulated β-casein gene expression.

Because one cannot extrapolate signaling after a 15 min exposure to what has happened during the 7-day incubation period required to effectively analyze endogenous gene expression by Northern blot, we reanalyzed signaling at the end of the 7-day period in the same set of cells. Reanalysis of signaling after the 7-day exposure to each of the two PRLs showed a 2.9-fold upregulation of ERK 1 and 2 activation

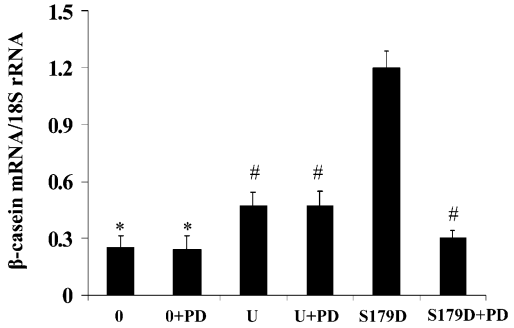

FIGURE 8: Expression of β-casein mRNA in response to U-PRL and S179D PRL in the presence and absence of the MAP kinase inhibitor, PD98059. HC11 cells were incubated in the PRLs for 7 days in the absence or presence of PD98059 (PD). The PRLs were given at 5 μg/mL, and the PD98509 was given at 25 μM. Key: 0, no addition of PRL; 0 + PD, PD alone; U, addition of unmodified recombinant human PRL; U + PD, addition of unmodifed recombinant human PRL plus PD; S179D, addition of recombinant human S179D PRL; S179D + PD, addition of recombinant S179D human PRL plus PD. The data are derived from Northern blots, normalized to 18S rRNA, and are expressed as the mean ± SE of three separate experiments. * = $p < 0.01$; # = $p < 0.05$ versus S179D PRL. Note that PD had no effect on U-PRL-stimulated expression while it inhibited the additional stimulation brought about by S179D PRL.

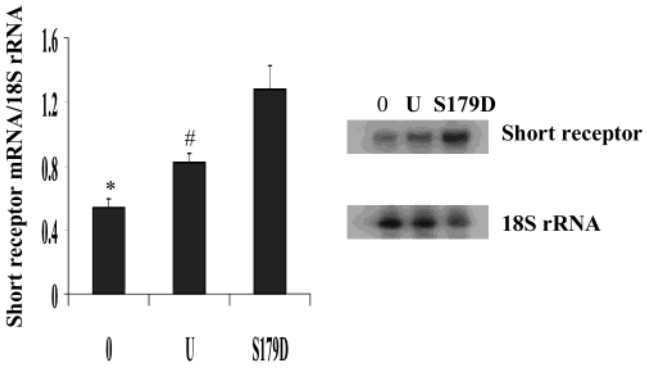

FIGURE 9: Expression of the short PRL receptor in response to a 7-day incubation in 1 μg/mL U-PRL or S179D PRL. Key: 0, no added PRL; U, addition of recombinant unmodified human PRL; S179D, addition of recombinant human S179D PRL. The data are derived from Northern blots using a probe specific for the short receptor, normalized to 18S rRNA, and are presented as the mean ± SE from five separate experiments. This probe does not distinguish among the three short forms of the receptor. * = $p < 0.01$; # = $p < 0.05$ versus S179D PRL. Note the doubling in the expression of the short receptor in response to S179D PRL.

in the S179D PRL-treated cells (compare Figure 7Aa with Figure 7Ba) with concomitant downregulation of Stat 5a activation (compare Figure 7Ac with Figure 7Bc). For each antibody, panels A and B were produced on the same autoradiogram and hence can be directly compared. U-PRL, by contrast, upregulated signaling through Stat 5a (compare Figure 7Ac with Figure 7Bc). Concomitant analysis of long and short PRL receptor expression after the 7-day incubation showed a doubling of the expression of the short receptor as a result of S179D PRL treatment and a smaller increase with U-PRL (Figure 9). Expression of the long receptor changed very little (data not shown).

When the short to long PRL receptor ratio is plotted against β-casein gene expression, one can appreciate that an increasing ratio correlates with increasing β-casein gene expression (Figure 10), suggesting a link between increased

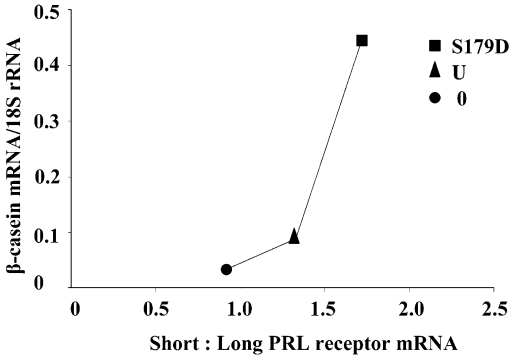

FIGURE 10: Relationship between β-casein gene expression and the ratio of short to long PRL receptor expressed. Cells were incubated in the PRLs at 1 μg/mL for 7 days. The data are derived from five separate experiments using the probe which recognizes both long and short receptor mRNA. Quantification of the long and short receptor was therefore on the same autoradiogram. Note the increasing β-casein expression with increasing short to long receptor ratio.

short receptor expression, increased use of the MAP kinase pathway, and increased β-casein expression.

DISCUSSION

Previous experiments have shown very different biological activities of U-PRL and S179D PRL in the adult rodent mammary gland (*1*). Thus U-PRL promotes growth, while S179D PRL inhibits growth and yet promotes β-casein gene expression. It seemed likely therefore that these two PRLs initiated different signaling pathways in mammary epithelial cells.

S179D PRL is a molecular mimic of human P-PRL. P-PRL has been shown to be a proportion of pituitary PRL in rats, cows, sheep, turkeys, and chickens (*21*−*23*). Previous work had indirectly demonstrated that human PRL was phosphorylated by showing an increase in Nb2 proliferative activity of standard human pituitary PRL after treatment with acid phosphatase (*11*). The current paper shows more directly by mass spectrometry that a proportion of human pituitary PRL is phosphorylated. In experimental animals where this can be analyzed, studies have shown physiological regulation of the proportion phosphorylated (*18*, *19*). Thus, in the second two-thirds of rodent pregnancy, there is a high proportion of U-PRL, but P-PRL increases prior to parturition (*19*; unpublished data). In addition, mammary epithelial cells are exposed via apically oriented receptors (*24*) to high levels of P-PRL in the milk during lactation (*25*, *26*). Analysis of the involvement of different signaling pathways initiated by each of the two recombinant PRLs therefore may help to shed light on how PRL exerts different effects on the mammary gland in pregnancy versus lactation. Although, in this regard, it is clear that steroid hormones inhibit β-casein expression during pregnancy (*27*, *28*), previous work from this laboratory has shown that the form of PRL also plays a role. This differential effect of the two PRLs is not via an effect on estrogen, progesterone, or corticosterone (*1*).

Substitution of a normally phosphorylated serine by an aspartate produces a residue similar in charge and size to a phosphorylated serine; the mimic of the phosphorylated molecule avoids the disadvantage of potential dephosphorylation. This approach is used widely in the study of enzymes turned constitutively on or off by phosphorylation (e.g., ref *14*). Here, we have used the molecular mimic of P-PRL, S179D PRL, to prevent possible interconversion of P-PRL and U-PRL during experimental procedures. Only with this approach can we definitively study the true differences in signaling between the two PRLs.

With U-PRL and the standard human PRL, one can observe signaling via Jak 2 and Stat 5 tyrosine phosphorylation as has now been described by many authors (reviewed in ref *10*). Dimers of tyrosine-phosphorylated Stat 5 are capable of entering the nucleus and binding to GAS sites on the β-casein gene promoter (reviewed in ref *29*). Evidence of similar signaling was present upon stimulation of the cells with S179D PRL but to a reduced degree. When an oligonucleotide equivalent to the β-casein GAS site was used for EMSA analysis, however, complex formation appeared qualitatively and quantitatively similar, regardless of the PRL used. Competition with antibodies showed that the majority of the complex formed in cells stimulated with the PRLs contained Stat 5a.

There is therefore an apparent contradiction in our results: The form of PRL which results in the highest level of β-casein mRNA, S179D PRL, is the one that least efficiently causes tyrosine phosphorylation of Stat 5a, and yet equivalent amounts of Stat 5a complexes are formed with the β-casein GAS site. Phosphoamino acid analysis of equivalent amounts of Stat 5 after stimulation with each PRL showed a doubling to tripling of phosphoserine content with S179D PRL. This suggests that serine phosphorylation of Stat 5 promotes Stat 5−β-casein promoter complex formation and/or stability. Serine phosphorylation of other Stats has been shown to produce more stable Stat−DNA complexes and hence to improve transcriptional efficiency (e.g., ref *30*), but some others have not found serine phosphorylation of Stat 5 in response to PRL (*16*). When comparing the current result with these others in the literature, however, it is important to note that U-PRL had no apparent effect on Stat 5 serine phosphorylation. Most other investigators use either recombinant PRL or various preparations of standard pituitary PRL from a variety of species. The proportion of pituitary PRL that is phosphorylated will vary from preparation to preparation, but the majority of what is present will always be U-PRL. The increase in serine phosphorylation with S179D PRL, which would be equivalent to 100% P-PRL, is large enough that it can be clearly observed over the inherent errors in this kind of experiment. It is entirely reasonable therefore that others have failed to find an effect of PRL on Stat 5 serine phosphorylation (*16*). In agreement with the current results showing PRL stimulation of Stat 5 serine phosphorylation are those of Yamashita et al., which show human PRL-stimulated serine phosphorylation of Stat 5 in both COS-7 and Nb2 cells and that this was accomplished using a proline-juxtaposed serine kinase (*31*). Thus S179D PRL, as a molecular mimic of P-PRL, results in stimulation of both tyrosine and serine phosphorylation of Stat 5, a maneuver which may be responsible for similar β-casein GAS site binding when compared to the more highly tyrosine phosphorylated Stat 5 produced by U-PRL. Is this sufficient, however, to result in superior β-casein gene expression? Work from the Waxman laboratory would suggest, if anything, that serine phosphorylation of Stat 5a is inhibitory to β-casein expression since mutation of serines

to alanines in Stat 5 enhanced expression of an intact β-casein promoter−luciferase reporter (*32*) in response to PRL. This is consistent with the idea that phosphorylation of the serines in Stat 5 is inhibitory to transcription. This same result was found in a second study by Yamashita et al. (*33*). On this latter occasion, however, the investigators additionally determined that the inhibition was removed by incubation in glucocorticoids, and all of our experiments were conducted in cells preincubated in, and in the continual presence of, glucocorticoids.

Evidence in the current paper supports some substrates of MAP kinase as key regulators of increased β-casein gene expression in response to S179D PRL. Thus S179D PRL, which promotes superior β-casein gene expression (versus U-PRL), is the better activator of MAP kinase. Further, incubation of cells with PD98059 blocked S179D PRL-stimulated β-casein expression. Activation of the MAP kinase pathway can result in activation of CREB and ATF_1, both of which have consensus sequences/binding sites within the β-casein promoter (*29*). In addition, superior activation of β-casein gene expression can be achieved by removal of suppression by the transcription factor YY1 (*29*). Increased β-casein gene expression may therefore be elicited by interactions among all of these transcription factors. Once again, it is important to note that inhibition of MAP kinase had no effect on U-PRL-stimulated β-casein gene expression and hence that our results are not in conflict with previous reports that MAP kinase signaling has no role in PRL activation of β-casein gene expression (*16*). Instead, it is mostly the advantage afforded us by the use of S179D PRL that allows us to see this effect. When comparing to some literature reports, however, it is also important to note the use of a reporter construct assay system by some other investigators, even when using HC11 cells. The usual construct contains only the −344/−1 portion of the β-casein promoter (e.g., ref *16*), and there are CREB, ATF1, and YY1 sites outside of this region. By contrast, we have used the endogenous gene for our analysis. In addition, it is also important to note that we have looked at steady-state mRNA levels after a long incubation in hormone. Effects on mRNA stabilization therefore may also be of consequence since several investigators have reported effects of various agents, including PRL, on the stabilization of β-casein transcripts (*34*−*36*). Ongoing work in the laboratory is designed to determine whether S179D PRL affects mRNA stability or transcription or both.

Long-term incubation in S179D PRL upregulated MAP kinase signaling in the cells and upregulated β-casein gene expression. At the same time, long-term incubation in S179D PRL also upregulated the short PRL receptor. This result suggests, but does not prove, that signaling from the short receptor is responsible for the increased MAP kinase activation seen with S179D PRL. Signaling from the short receptor, however, cannot be responsible for reduced activation of Stat 5a since long-term incubation in U-PRL also upregulated the short receptor to some degree while concurrently upregulating signaling through tyrosine phosphorylation of Stat 5a. Thus, we must conclude that the effect of S179D PRL on Stat 5a activation is a consequence of altered signaling at the long receptor: altered signaling due to the different conformation of the receptor after binding S179D PRL. This dual effect on the long and short receptor may be the mechanism whereby S179D PRL can both inhibit growth in the mammary gland and also stimulate β-casein gene expression (*1*). Since we know that the short PRL receptor alone cannot activate β-casein gene expression (*6*), it is clear that a certain degree of Stat 5a tyrosine phosphorylation must be crucial. Apparently, this does not have to be very large since the activation shown after a 7-day incubation in S179D PRL is much weaker than that seen with U-PRL. Presumably, this reflects a large excess of Stat 5a phosphorylation by U-PRL and reminds us that signaling experiments usually use supraphysiological concentrations of ligand in order to see a robust effect. Titration of S179D PRL against a fixed dose of U-PRL demonstrated competition for tyrosine phosphorylation of Stat 5a. Thus, when used alone, S179D PRL is a partial agonist for Stat 5a tyrosine phosphorylation. However, when used together with the more potent tyrosine phosphorylator of Stat 5a, U-PRL, it acts as an antagonist. At physiological ratios of U-PRL to P-PRL (equivalent to lanes 2 and 3 on Figure 6), there is clear modulation of Stat 5 tyrosine phosphorylation.

The results presented here do not agree with previous reports by other investigators that the short receptor acts as a dominant negative for signaling to β-casein gene expression (*37*). The dominant negative effect was obtained in multiply transfected overexpression systems using the single long and short forms of the rat PRL receptor. Using essentially the same approach, we have duplicated the dominant negative result in CHO cells but with the same reporter construct find S179D PRL to result in greater luciferase activity in HC11 cells (data not presented). There is therefore a significant difference between these systems and natural regulation of β-casein expression in HC11 cells. This may involve the presence of specific signaling molecules or molecules that act as transcription cofactors, an important possibility in light of the fact that HC11 cells are the only mammary cell line capable of expressing endogenous β-casein. Also, if expression of the short receptor exceeds physiological ratios with the long receptor or total receptor expression is very high (as could be produced in the overexpression systems), heterodimer formation between long and short receptors may well artifactitiously result in an inhibition of active long and short receptor dimers and therefore would inhibit β-casein gene expression. In keeping with our contention that upregulation of the short receptor is beneficial to milk protein gene expression is a previous report of a 2-fold upregulation of the short receptor between pregnancy and lactation in rats (*4*). In addition, recent work from the Kelly group has shown replacement of one of the three short forms of the mouse PRL receptor to rescue lactation in the PRL receptor knockout heterozygous animal, whereas the other forms acted as dominant negatives (*38*). Perhaps, therefore, transfection of the rat short form of the receptor into CHO cells cannot duplicate the appropriate short form in mouse HC11 cells. Moreover, in the ovary, it is clear that the short PRL receptor has a distinct function and that it does not act simply as a dominant negative form (*39*).

In conclusion, these results underline the limitations of transfection and reporter construct analyses. More importantly, they demonstrate that U-PRL and S179D PRL signal differently in HC11 cells and that the differences in their signaling can be amplified by long-term exposure. U-PRL primarily uses the Jak−Stat pathway, while S179D PRL

primarily uses the MAP kinase pathway, although both PRLs use both pathways to some extent. In addition, the results suggest a novel and important role for natural expression levels of the short PRL receptor in the enhancement of β-casein expression, a finding currently under further investigation.

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