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A Molecular Mimic of Phosphorylated Prolactin (S179D PRL) Secreted by Eukaryotic Cells Has a Conformation with an Increased Positive Surface Charge Compared to That of Unmodified Prolactin†

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ABSTRACT: S179D prolactin (S179D PRL) is a pseudophosphorylated form of human PRL which has potent antitumor and anti-angiogenic activities in vivo. This molecule binds to the same forms of the PRL receptor (PRLR) as unmodified PRL, yet this binding results in different intracellular signaling and biological end points. Since it is now clear that PRLRs are predimerized and therefore that ligand binding must initiate signaling by inducing a conformational change in the receptor dimer, we hypothesized that S179D PRL had an altered conformation compared to unmodified PRL. The conformation of the ligand−receptor ternary complex would therefore also have an altered conformation, and thus, different signaling molecules would be activated. Here we present evidence in support of this hypothesis by demonstrating, in contrast to unmodified PRL, that S179D PRL has reduced nickel and zinc binding capacity and a higher affinity for heparin and DEAE. Conformational changes have occurred since these features are counterintuitive on the basis of the simple substitution of a serine with a negatively charged aspartate residue. To demonstrate that these particular properties of S179D PRL were not due to misfolding of the molecule during production, S179D PRL was expressed in two different mammalian cell lines. Also investigated was the potential for production of S179D PRL as a soluble cytoplasmic, or secreted periplasmic, protein in *Escherichia coli*.

S179D PRL[1] is a pseudophosphorylated form of human PRL in which the serine residue at the 179 position is replaced with aspartate (*1*). This molecule has both in vitro (*2*−*4*) and in vivo inhibitory effects on tumor growth (*4*) and angiogenesis (*5*, *6*) and in general has biological activities different from those of unmodified PRL (reviewed in refs *7* and *8*). There are multiple isoforms of human PRL receptors (*9*), and therefore, one possible way S179D PRL and unmodified PRL might have produced different biological effects was by interaction with different receptors. However, in cells reported to express only a single form of the receptor (Nb2 receptors), unmodified PRL and S179D PRL signal differently (*10*). In addition, when the ability of unmodified PRL and S179D PRL to interact with the long and two short human PRL receptors (PRLR) was compared in transfected HEK 293 cells by bioluminescence resonance energy transfer (BRET) experiments, they were each able to initiate a BRET signal from the same homo- and heteroreceptor pairs (*11*). Nevertheless, several studies analyzing human (*2*, *3*, *6*, *11*) and rodent (*12*, *13*) cell lines have shown that unmodified PRL and S179D PRL signal differently. For example, unmodified PRL activates Jak2 and Stat5, whereas S179D PRL inhibits Stat5a activation in response to unmodified PRL (*2*). Both ligands activate ERK; for unmodified PRL, this is rapid and transient, whereas for S179D PRL, the activation is delayed and prolonged, leading to inhibition of the cell cycle (*3*, *6*, *12*) and apoptosis (*6*). Unlike unmodified PRL, S179D PRL also inhibits rapid ERK 1/2 activation in response to basic fibroblast growth factor (*6*). If unmodified PRL and S179D PRL interact with and pair the same receptor isoforms, we hypothesized that these two forms of PRL must have different conformations. In this way, ligand−receptor conformations would be different, resulting in association of different scaffolding and intracellular signaling molecules. This hypothesis was cartooned in a recent review (*8*).

Previously, we demonstrated that S179D PRL had decreased hydrophobicity compared to that of unmodified PRL. This was expected with the addition of an extra charged amino acid, but

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[1]Abbreviations: PRL, prolactin; S179D PRL, PRL in which serine 179 is mutated to an aspartate; DEAE, diethylaminoethyl cellulose; BRET, bioluminescence resonance energy transfer; Jak2, Janus kinase 2; Stat5, signal transducer and activator of transcription factor 5; ERK, extracellularly regulated kinase; RER, rough endoplasmic reticulum; IPTG, isopropyl β-D-1-thiogalactopyranoside; HEK 293, human embryonic kidney; CHO, Chinese hamster ovary; ATCC, American type Culture Collection; CD, circular dichroism; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass analysis; SDS, sodium dodecyl sulfate; MEIA, microparticle enzyme immunoabsorbent assay; NIDDK, National Institute for Diabetes and Digestive and Kidney Diseases; HuVEC, human umbilical vascular endothelial cells; DPBS, Dulbecco's phosphate-buffered saline; IMAC, metal-affinity chromatography.

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the magnitude of the effect was much larger than expected (*14*), a finding suggestive of a change in conformation. However, the S179D PRL preparations used for most previous experiments were produced by refolding of protein derived from *Escherichia coli* inclusion bodies, so different conformations could possibly have been the result of abnormal folding. If a protein is difficult to fold or there is any question about proper refolding from inclusion bodies, eukaryotic secretion expression systems are the ultimate choice. These systems provide a quality control system in which poorly folded or slow folding protein in the rough endoplasmic reticulum (RER) is recognized, aided in refolding by chaperone proteins, or eliminated by digestion in proteasomes (reviewed in ref *15*).

By analyzing the properties of material produced in eukaryotic expression systems, we conclude that differences between unmodified PRL and S179D PRL in terms of their ability to bind nickel, zinc, heparin, and DEAE are the result of different conformations after appropriate folding. This supports the hypothesis that these two forms of PRL likely interact differently with predimerized receptors and hence that the ternary complex formed in each case has the capacity to signal differently. Large-scale production of proteins by expression in eukaryotic systems is expensive; this study therefore also investigated the possibility of production using either soluble cytoplasmic expression or periplasmic expression in *E. coli.* These studies provided confirmatory evidence that unmodified PRL and S179D PRL had different conformations and established that previous methods based on refolding from inclusion bodies were the optimal methods of production.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Expression Constructs, and DNA Manipulations. The following *E. coli* strains were used as hosts for protein expression: Origami B [F^- ompT hsdSB(rB^-mB^-) gal dcm lacY1 aphC (DE3) gor522::Tn10 trxB(KanR, TetR)], BL21 [F^- ompT hsdSB(rB^-mB^-) gal dcm], W3110 [F^- IN(rrnD-rrnE)1 lambda-], BL21-CodonPlus(DE3)-RIPL [B F^- ompT hsdS(rB^-mB^-) dcm^+ Tetr gal λ(DE3) endA Hte [argU proL CamR] [argU ileY leuW Strep/SpecR]]. So that the names would not be too cumbersome, we have abbreviated them as follows. For the plasmids, the designation PRL stands for unmodified PRL and 179 stands for S179D PRL. All of the current work is concerned with human PRL, and so in this instance, we have just used PRL and not hPRL as in some of our previous publications. The promoter herein designated pL should more completely be designated pλP_L. The expression plasmids used for periplasmic secretion and cytoplasmic (cy) expression consisted of two types: IPTG-inducible [periplasmic expression: pTac-PRL and pTac-179; cytoplasmic expression: pTac-cyPRL, pTac-cy179, pT7SCII-cyPRL, and pT7SCII-cy179 (*1*, *16*)] and temperature-inducible constructs [periplasmic expression: pL-PRL and pL-179; cytoplasmic expression: pL-cyPRL and pL-cy179]. pTac-179 was obtained by directed mutagenesis using pTac-PRL as the template (*16*) employing primers 5′-GATCGATC**GGATCC**-TTATCAGCAGTTGTTGTTGTGGATGATTCGGCACTT-CAGGAGCTTGAGATAATTGTCGATTTTATGGTCATC-CCTGCG-3′ (BamHI restriction site in bold) and 5′-ATCGAT-CGACTACCGTTGTATTCTCTGCGTCCGCCTTCGCTT-TGCCCATCTGTCCCGGCGGG-3′ (NdeI restriction site in bold). Cytoplasmic plasmids pTac-cyPRL and pTac-cy179 were obtained with PCR technology using pTac-PRL as a template and the primers 5′-GGG**CATATG**CCCATCTGTCCCGG-CGGGGCT-3′ (NdeI restriction site in bold) and 5′-GAT-CGATC**GGATCC**TTATCAGCAGTTGTTGTTGTGGATG-ATTCGGCACTTCAGGAGCTTGAGATAATTGTCGATT-TTATGGTCATCCCTGCG-3′ (for S179D PRL, BamHI restriction site in bold) and 5′-GATCGATC**GGATCC**TTAT-CAGCAGTTGTTGTTGTGGATGATTCGGCACTTACTG-AGCTTGAGATAATTGTCGATTTTATGGTCATCCCTG-CG-3′ (for unmodified PRL, BamHI restriction site in bold), and pL-PRL was obtained by inserting the NdeI/BamHI fragment containing the pre-PRL cDNA sequence from pTac-PRL in a pL-GH plasmid digested by the same enzymes (*17*). The same procedures were carried out for pL-179, pL-cyPRL, and pL-cy179, which were constructed from the inserts obtained from pTac-179, pTac-PRL, and pTac-cy179 digested with NdeI and BamHI, respectively. The expression vectors employed for eukaryotic expression were p658-PRL (*18*), p658-179, pcDNA-PRL, and pcDNA-179. The p658-179 vector was obtained by inserting a fragment containing the desired mutation into the p658-PRL vector as a HindIII−BamHI fragment (*18*). The pcDNA-PRL and pcDNA-179 vectors were constructed by inserting the coding sequences of unmodified PRL and S179D PRL as HindIII−BamHI inserts from p658-PRL and p658-179, respectively, into a pcDNA 3.1 expression vector (Invitrogen, Carlsbad, CA). CHO cells (ATCC, Manassas, VA) and HEK 293 cells (ATCC) were used to harbor these plasmids for production of both kinds of PRL. All DNA manipulations were performed according to Sambrook et al. (*19*). The sequences generated by PCR were checked by DNA sequencing. Expression in CHO cells was from stable transfections, while that in HEK 293 cells was from transient transfections.

Protein Expression and Collection of PRL and S179D PRL. Periplasmic protein expression and soluble *E. coli* cytoplasmic expression were conducted as described previously, as were inclusion body denaturation and refolding (*1*, *20*). In all cases, unmodified PRL and S179D PRL were prepared and analyzed side by side. The PRLs secreted by CHO cells into serum free medium were collected and then concentrated by tangential flow filtration (Millipore Corp., Bedford, MA) using a membrane with a cutoff range of 10 kDa. For circular dichroism (CD) studies, the proteins were purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) using previously described conditions (*21*) and concentrated by lyophilization. PRLs were lyophilized with a sucrose to an unmodified PRL:S179D PRL ratio of 300:1 to prevent aggregation or misfolding. This results in a 93% mean lyophilization recovery of the PRLs, as judged by HPLC and a radioimmunoassay (data not shown). The results were also cross-checked with material concentrated with filters. The PRLs secreted by HEK 293 cells into serum free medium were collected in conditioned medium and concentrated using a Microcon YM-10 centrifugal filter unit (NMWL 10 kDa). Concentration with filters removes salts and low-molecular weight substances that could potentially interfere with column retention and allows for diluent exchange into a buffer suitable for each chromatography. This process was also applied to lyophilized samples prior to column analyses. All analyses were repeated a minimum of three times for each PRL form and each column type. Standard PRL was either purified recombinant material or material extracted from pituitary glands obtained from A. F. Parlow (Torrance, CA) in conjunction with the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

Analytical Reverse-Phase High-Performance Liquid Chromatography (*RP-HPLC*). To compare the properties of unmodified PRL and S179D PRL in conditioned medium with those of other preparations, the different PRLs were analyzed by RP-HPLC as described previously (*21*).

Mass Spectrometry (*MALDI-TOF*). The purity and molecular mass of the PRL preparations were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Analysis was performed on a Voyager DE (PE Biosystems, Foster City, CA) in positive ion mode using an acceleration voltage of 40 kV. Calibration of the machine was conducted with a mixture of cytochrome *c* (M_r = 12362) and myoglobin (M_r = 16952). The matrix consisted of 100 mM sinapinic acid in an acetonitrile/methanol/water mixture (1:1:1) (*13*, *16*). The accuracy of this instrument is 0.01%.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (*SDS−PAGE*) *and Immunoblotting*. Analysis of the PRLs included electrophoresis on reducing 12% SDS−polyacrylamide gels. The gels were stained with Coomassie Blue R-250, or the proteins were transferred by electrophoresis to a nitrocellulose membrane for immunoblotting with rabbit polyclonal anti-PRL antibody. Bound antibody was usually detected with protein A labeled with ^{125}I followed by autoradiography, as previously described (*16*). In the heparin-affinity chromatography study, binding of the first antibody was detected using horseradish peroxidase-coupled secondary anti-rabbit antibodies and the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Metal-Affinity Chromatography (*IMAC*). Conditioned medium, concentrated and desalted as described and containing the different PRLs at concentrations of up to 1 mg/mL, was applied to a column prepacked with Chelating Sepharose Fast Flow gel [Pharmacia Biotech, Upsala, Sweden, XK16/40; 10 cm × 16 mm (inside diameter)] precharged with Ni(II) following the recommendations of the manufacturer. Equilibration was conducted with 50 mM sodium phosphate (pH 7.2), 0.8 M NaCl, and 0.1% Tween 20 (buffer B). After application of the sample, the column was washed to baseline absorbance, and then the PRLs were eluted with buffer B containing increasing concentrations of imidazole. A flow rate of 100 mL/h was maintained throughout. Fractions were analyzed using reducing SDS−PAGE and immunoblotting with an antibody that recognizes both forms of PRL.

DEAE Anion-Exchange Chromatography. One milliliter HiTrap columns were packed with DEAE-Sepharose Fast Flow ion-exchange resin (Amersham Biosciences) and equilibrated with 25 mM HEPES (pH 8.0). PRLs (25 μg) were diluted to 2 mL in the same buffer and applied to the columns. After the flow through had been collected, the bound fractions were eluted stepwise by washing the column with 0.1, 0.2, and 0.3 M NaCl in the same buffer. Fractions were collected and concentrated with Microcon YM-10 centrifugal filter units (NMWL 10 kDa) (Millipore).

Heparin-Affinity Chromatography (*HAC*). One milliliter prepacked heparin-Sepharose columns (Amersham Biosciences) were equilibrated with 10 mM phosphate buffer (pH 7.0). PRLs (25 μg) were diluted to 2 mL of the same buffer and applied to the columns. After the flow through had been collected, the bound fractions were eluted stepwise with 0.5, 1.0, and 1.5 M NaCl in the same buffer. Fractions were collected and concentrated with Microcon YM-10 centrifugal filter units (NMWL 10 kDa) (Millipore).

Quantification of the PRLs before the Bioassay. Hormone concentrations were determined by a number of methods, including microparticle enzyme immunoabsorbent assay (MEIA) using ABBOTT reagents on an AxSYM system (Abbott diagnostics, Abbott Park, IL) at the University of Sao Paulo Hospital and densitometry of Coomassie blue-stained gels in comparison with NIDDK standard human PRL. Both isoforms are equally recognized by the antibody from the MEIA system, and both variants are also equally detected by immunoblotting and radioimmunoassay using NIDDK anti-human PRL.

Circular Dichroism (*CD*). CD spectra were recorded with a Jobin Yvon (Edison, NJ) model CD 6 dichrograph. Far-UV CD spectra were acquired over a wavelength range of 190−260 nm by using 1 nm increments. The baseline obtained with solvent alone was subtracted from the sample spectra. The temperature was controlled with a Haake model D8 thermostat and with a thermostated cell holder supplied by Jobin Yvon. The sample concentration was 500 μg/mL in 25 mM NH_4HCO_3 and 100 mM NaCl (pH 8.0). Helicity was calculated as previously described (*22*).

DNA Content Determination. Human umbilical vascular endothelial cells (HuVEC) were cultured for 3 days in SF Medium (Clonetics, San Diego, CA) with reduced bFGF (2.5 ng/mL) containing 5% fetal bovine serum and different S179D PRL preparations at 1 μg/mL. Cells were harvested by trypsinization, washed with cold Dulbecco's phosphate-buffered saline (DPBS), and fixed with 75% ethanol in DPBS at 4 °C for 30 min. Cell pellets were resuspended in 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in DPBS containing 200 μg/mL RNase (Sigma) and 10 μg/mL propidium iodide (Sigma) and incubated at room temperature for 30 min. The fluorescence of individual cells was measured with a FACScan cytofluorimeter equipped with CellQuest (Becton Dickinson, Franklin Lakes, NJ).

Modeling. Models were constructed using the automated homology modeling program, ESyPred3D, by Lambert et al. (*23*) and the solution structure of human prolactin, Protein Data Bank entry 1RW5 by Teilum et al. (*24*), as a template. Analysis was performed with SwissPdbViewer. Energy minimization was performed by 200 steps at steepest descent followed by 200 steps of conjugate gradient and then another 200 steps of steepest descent, with a cutoff value of 10 Å. The models were produced on three different occasions.

RESULTS

To take advantage of the inherent quality control mechanisms present within the RER of eukaryotic cells, we produced a number of CHO clones stably expressing either unmodified PRL or S179D PRL. Figure 1A shows a Western blot of conditioned medium derived from four clones for each type of PRL. Lanes 2−5 contained unmodified PRL, and lanes 6−9 contained S179D PRL. Lanes 1 and 10 contained a standard human recombinant PRL preparation. Expression levels varied among clones, but on average, the clones producing S179D PRL secreted ~50% less into the medium. The upper band is likely glycosylated since it runs at ~25 kDa and is present in the same proportion to the main, monomer/nonglycosylated band, regardless of which PRL is being expressed.

RP-HPLC was utilized to determine the relative hydrophobicity of S179D PRL and unmodified PRL. For both forms of

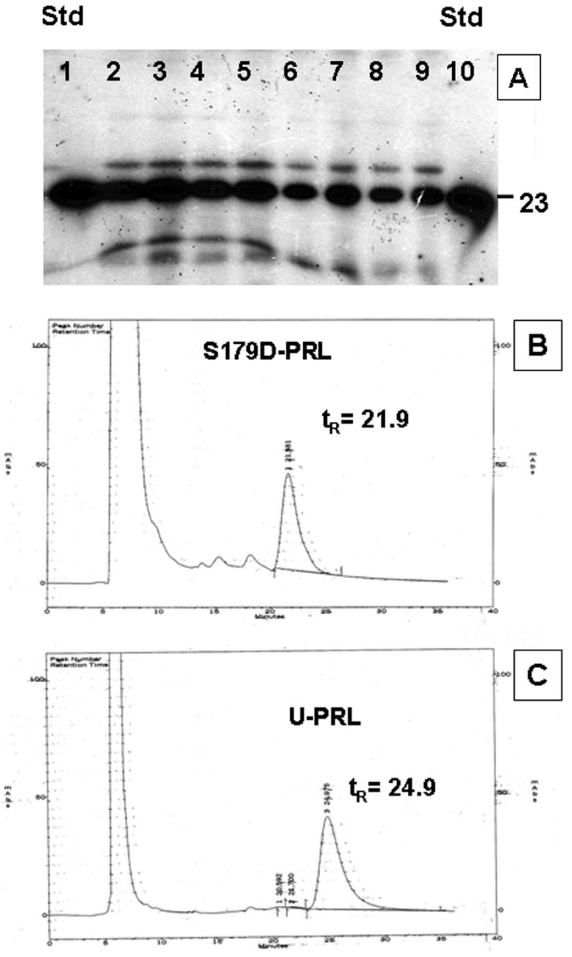

FIGURE 1: Production level and hydrophobicity of material produced by CHO stable cell lines. (A) Immunoblot of conditioned medium: lanes 1 and 10, standard recombinant (Std) PRL (100 ng); lanes 2−5, conditioned medium from different clones expressing unmodified PRL (U-PRL); and lanes 6−9, conditioned medium from clones expressing S179D PRL. (B and C) RP-HPLC analysis of S179D PRL (B) and unmodified PRL (C). Isocratic RP-HPLC on a C Vydac 4 214 TP 54 column [25 cm × 4.6 mm (inside diameter), pore diameter of 300 Å, and particle diameter of 5 mm], connected to a Vydac 214 FSK 54 guard column. The mobile phase consisted of 71% Tris-HCl buffer (50 mM, pH 7.5) and 29% *n*-propanol, with a flow rate of 0.5 mL/min and a column temperature maintained at 45 °C.

PRL, it was necessary to concentrate the conditioned medium for analysis by RP-HPLC. Panels B and C of Figure 1 show the elution profiles for S179D PRL and unmodified PRL, respectively. S179D PRL had a lower retention time (21.9 min) than unmodified PRL (24.9 min) ($p < 0.01$), indicating a reduced hydrophobicity. This reduced hydrophobicity has been previously reported for material expressed in *E. coli* and refolded from inclusion bodies (*14*).

We have previously shown that PRL has sufficient affinity for Ni(II) and other divalent cations for this property to be used in the purification process (*16*). When equivalent preparations of unmodified PRL and S179D PRL concentrated from conditioned medium of mammalian cells were tested, S179D PRL had a much reduced affinity. Panels A and B of Figure 2 show that S179D PRL eluted from an IMAC Ni(II) column with 15 mM imidazole, mostly in fractions 24−29, and unmodified PRL eluted with 60 mM imidazole, mostly in fractions 59−64. A similar result was obtained with Zn(II) (data not shown). All fractions were analyzed, and all fractions positive by Western blot after reducing SDS−PAGE are shown. Since this was conditioned medium and other proteins were present, Western blot demonstration of the hormones in various fractions was necessary. All fractions were tested, although only some are illustrated. Imidazole also absorbs at 280 nm and therefore contributes to OD_{280}, especially at 60 mM. Initial analyses used identical gradients, but to more rigorously test the inability of 15 mM imidazole to elute unmodified PRL, we used a longer time at 15 mM imidazole, as illustrated.

Human PRL has been reported to bind to heparin (*25*). As a third test of surface molecular differences, we analyzed the relative affinity of unmodified PRL and S179D PRL for heparin. Figure 3A shows a Western blot after the stepwise elution of the PRLs from a heparin-Sepharose column. Unmodified PRL eluted at 0.5 M NaCl, but S179D PRL did not. When the NaCl concentration was increased to 1 M, S179D PRL eluted. Thus, S179D PRL has a higher affinity for heparin. Using a more refined stepwise elution with DEAE (Figure 3B), results showed elution of both PRLs starting at 0.1 M NaCl. Nevertheless, the results indicate that S179D PRL had a greater affinity for the DEAE resin since 0.3 M NaCl was required to elute all the S179D PRL, whereas unmodified PRL elution was complete with 0.2 M NaCl.

Circular dichroism (CD) can be used to examine aspects of both secondary and tertiary structure. Figure 4 shows CD spectra for two preparations of S179D PRL (from mammalian cells and inclusion bodies) and compares this with one for standard NIDDK PRL. In the 220−260 nm range, the spectra are almost superimposable (they are offset a little for the sake of display), indicating that there is no significant change in the overall tertiary structure. In the 200−220 nm range, there was a minor alteration in the spectrum for the two preparations of S179D PRL, suggestive of a minor alteration in a region of α helix.

Collectively, these different approaches support a different conformation for S179D PRL versus unmodified PRL when they are expressed in a mammalian system where there is tight control over folding. Major aspects of the structure remain the same, but there are differences in the surface properties of the molecule. Using DeepView, we have modeled the three-dimensional structure of both forms of PRL. Figure 5 shows the entire molecules (A) and an enlarged part of the surface of each (B). The portion shown in panel B was chosen to illustrate one area of surface change and is not at this time proposed as a key to the different affinities for either divalent cations or negatively charged matrices. The internal ribbons show helix 1 colored blue, helix 2 green, helix 3 yellow, and helix 4 red. As one can see, the overall structures are predicted to be very similar, with the four antiparallel helices forming the bulk of the molecule. This is consistent with the CD analyses. Serine 179 is on helix 4 and normally faces the inside of the molecule. Substitution with aspartate at this position alters the orientation of a number of amino acids on the surface, some of which are illustrated in Figure 5B. The models were constructed and subjected to energy minimization on three separate occasions, with the same result.

Most of our previous studies on the biology of S179D PRL used a preparation refolded from *E. coli* inclusion bodies produced in BL21 harboring a pT7SCII construct (*1*). The yield is high, and the cost is low. There is no chance of N-glycosylation and resultant problems arising from batch-to-batch variability in glycosylation that would occur in a mammalian system. Additionally, a purity of ≥98%, as estimated by SDS−PAGE, is obtained after renaturation and refolding. In this study, we have

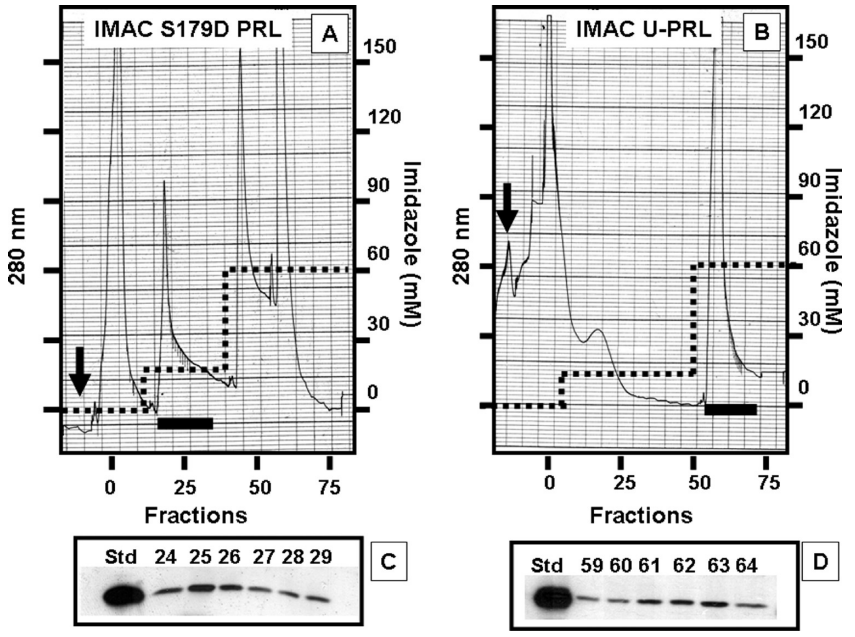

FIGURE 2: Immobilized metal-affinity chromatography (IMAC) of S179D PRL and unmodified PRL from conditioned medium of CHO cells. After application of the sample (~1 mg) prepared from conditioned medium as described in Experimental Procedures, washings were performed with 50 mM sodium phosphate buffer and 0.8 M NaCl (pH 7.2) to remove impurities with a low affinity for the matrix. The hormones were eluted stepwise with buffer containing increasing levels of imidazole: (A) IMAC of S179D PRL and (B) IMAC of PRL. Black bars indicate points at which unmodified PRL or S179D PRL eluted, but all fractions were assayed. Dotted lines indicate points at which the imidazole concentration increased (millimolar). Black arrows show where the samples were applied. (C) Western blot analysis of fractions containing S179D PRL. (D) Western blot analysis of fractions containing unmodified PRL. Numbers above the immunoblots indicate fractions from the respective chromatograms.

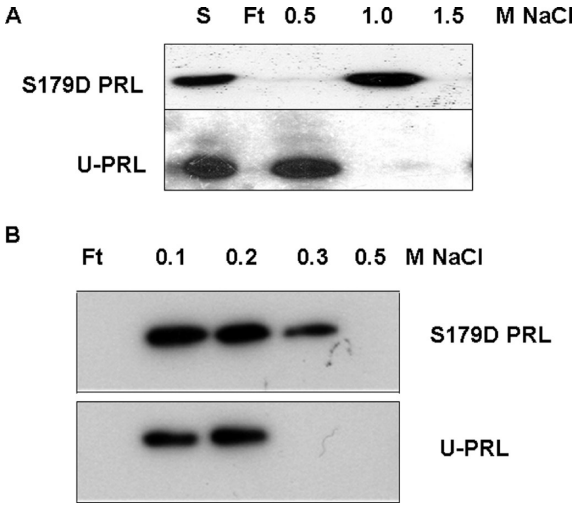

FIGURE 3: Heparin affinity and DEAE anion-exchange chromatography of S179D PRL and unmodified PRL. (A) Heparin affinity. Samples containing 25 μg of unmodified PRL or S179D PRL were applied to the column in phosphate buffer and were eluted stepwise with 0.5, 1, and 1.5 M NaCl. Aliquots were resolved by SDS−PAGE and transferred for immunoblotting: S, applied sample; Ft, flow through. (B) DEAE anion exchange. Samples containing 50 μg of unmodified PRL or S179D PRL were applied to the column in 25 mM HEPES buffer and were eluted stepwise with 0.1, 0.2, 0.3, and 0.5 M NaCl. Aliquots were resolved by SDS−PAGE and transferred for immunoblotting.

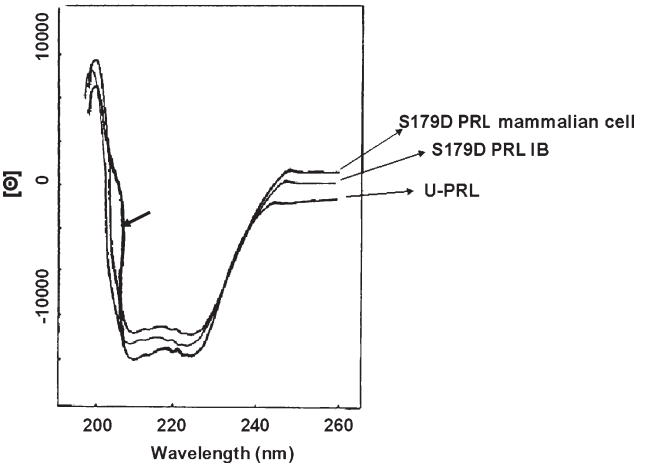

FIGURE 4: Circular dichroism of preparations of S179D PRL and standard NIDDK PRL. Protein samples were diluted in 25 mM NH_4HCO_3 and 100 mM NaCl (pH 8.0), and spectra of S179D PRL or unmodified PRL were measured in the UV range (190−260 nm). Molar ellipticity (θ) is expressed in degrees square centimeters per decimole. S179D PRL IB denotes material refolded from inclusion bodies. An arrow points to a region reproducibly different between S179D PRL and unmodified PRL.

nevertheless examined alternate approaches with theoretical advantages for the production of S179D PRL to determine the most efficient system for production of the properly folded protein. When the yield was sufficient, we have compared the material produced to that derived from mammalian cells (HEK 293 cells). These alternate methods included (1) expression in the periplasmic space using (a) pL and pTac promoters, (b) different signal peptides, and (c) different activation temperatures; (2) expression in cells with reduced protease activities using (a) different promoters and (b) different temperatures; and (3) expression as a soluble cytoplasmic protein using (a) different cell strains, (b) different promoters, and (c) different temperatures.

Expression in the periplasmic space of *E. coli* has the potential advantage of producing S179D PRL in bacteria, yielding a recombinant protein without an additional N-terminal

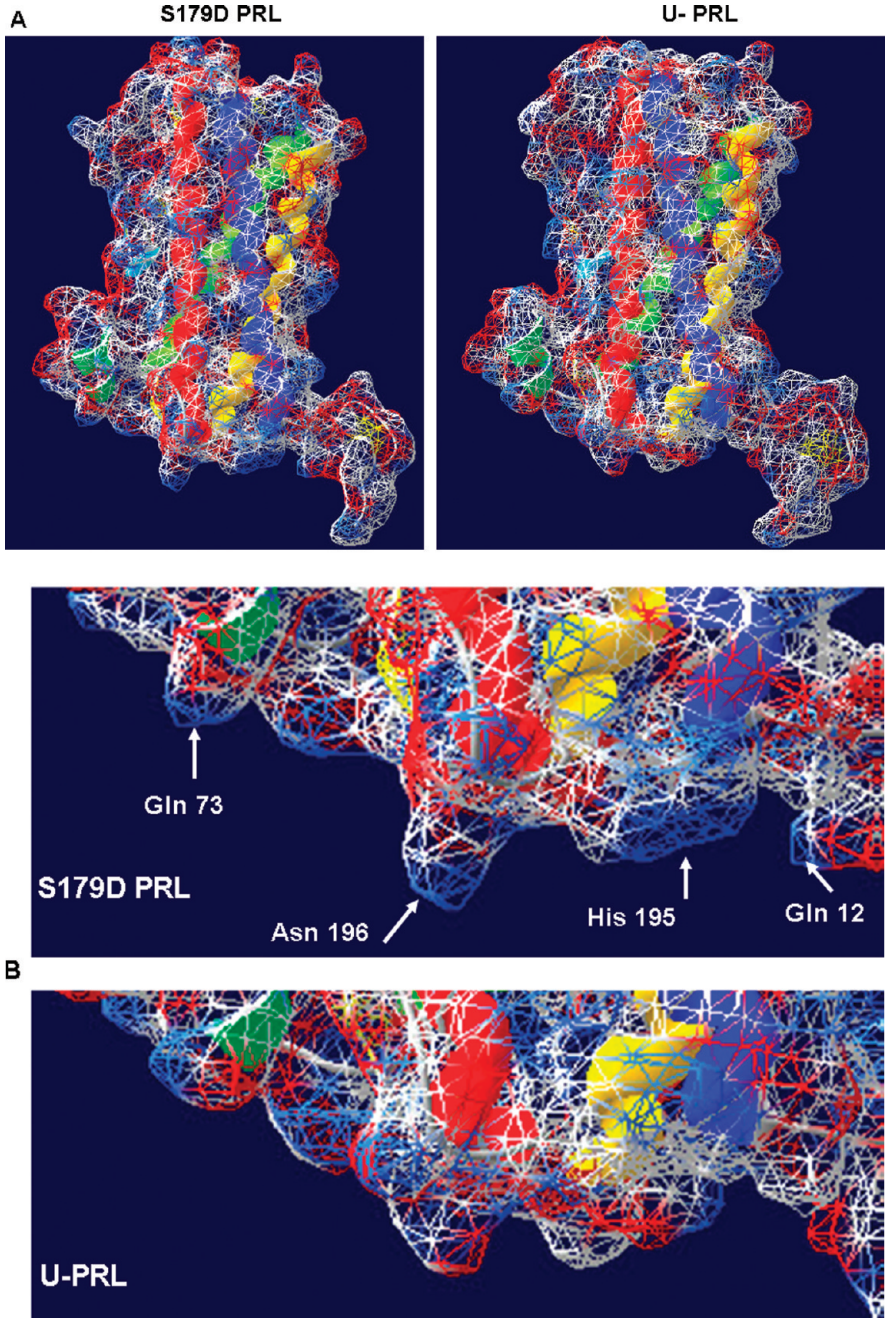

FIGURE 5: Models of S179D PRL and unmodified PRL. The entire molecules are shown in panel A, and a region of the surface is shown in panel B. U-PRL denotes unmodified PRL. The ribbon representations are shown within the model. In the ribbon, helix 1 is colored blue, helix 2 green, helix 3 yellow, and helix 4 red. Arrows indicate amino acids on the surface with altered orientations.

methionine. The periplasmic space also has some chaperone proteins. Although not as efficient as the mammalian versions, these could aid in refolding (*26*). When periplasmic expression of S179D PRL in *E. coli* W3110 was compared to that of unmodified PRL in the same compartment (Figure 6), the yield was poor. This poor yield was not circumvented by using (1) two widely used promoters for large-scale protein production (promoters pL and pTac) (*27*), (2) different signal peptides (with hydrophobicity patterns similar to those of signal peptides from exogenous, strongly expressed periplasmic proteins) (Figure 6 and Table 1), or (3) different activation temperatures for the IPTG or temperature-driven expression systems (Figure 6). The signal peptide with the best protein expression level was p1510, a maltose binding protein signal peptide analogue. This signal sequence was previously described for successful periplasmic secretion of unmodified PRL in a different *E. coli* strain (*16*). The levels of S179D PRL produced were only detectable with immunoblots and were not sufficient for HPLC quantification and characterization. We therefore used MALDI-TOF spectrometry, which has the capacity of identifying small amounts of proteins in complex mixtures. Figure 7A shows the S179D PRL MALDI-TOF spectrum from periplasmic extracts obtained by osmotic shock. The estimated molecular mass of S179D PRL using this technique was 22939 Da, in good agreement with the calculated molecular mass of 22927 Da. MALDI-TOF analysis was able to discriminate S179D PRL produced in the periplasmic compartment from that refolded from cytoplasmic inclusion bodies, which has the extra methionine residue and a mass of 23065 Da (Figure 7B). By comparison, material expressed in HEK 293 cells after transient transfection

gave a mass of 22936 Da (Figure 7C). The 3 Da difference between periplasmic and mammalian expression was within experimental error for this technique (0.01%).

An *E. coli* strain with reduced protease activity (BL21) was used to determine whether the diminished level of S179D PRL expression was due to protease cleavage of this form (Figure 8A). No improvements in S179D PRL yields were achieved. A significant increase in the level of protein expression (a 46% increase) was observed for unmodified PRL, but not S179D PRL (Table 2). The temperature-inducible pL promoter was not efficient at either 37 °C (Figure 8) or 40 °C (data not shown) for the S179D PRL construct. Only pTac-PRL and not pTac-179 was effective in this particular *E. coli* strain.

Similar experiments were conducted using the BL21 codon plus expression/secretion system. BL21 codon plus has extra copies of genes that encode the tRNAs that most frequently limit translation of heterologous protein in *E. coli*. Use of BL21 codon plus did not rescue the expression of S179D PRL, nor did it improve the secretion of unmodified PRL (Figure 8).

We studied cytoplasmic expression driven by pTac at lower temperatures (20 and 30 °C) since this encourages better folding in the cytoplasm (*28*). We also conducted cytoplasmic expression with an *E. coli* strain derived from K-12 (Origami B; a *gor*/*trxB* mutant). Here, cytoplasmic folding of heterologous proteins is greatly facilitated due to the oxidizing cytoplasm, making disulfide bond formation possible (*29*). Unlike unmodified PRL, when S179D PRL was produced in its soluble form in the cytoplasm, it was apparently partially degraded into lower-molecular mass forms (Figure 9A,B), and in the BL21 strain, we observed not only a low-molecular mass form but also a higher-molecular mass form (Figure 9A). The pL constructs had very

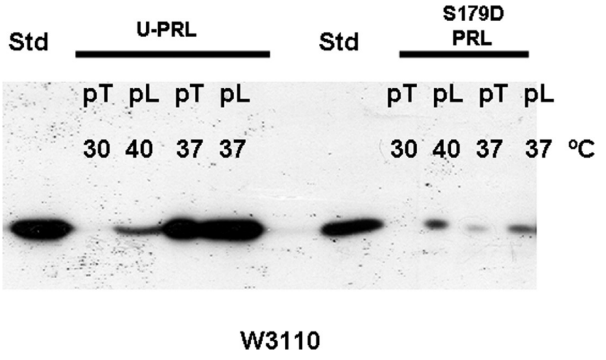

FIGURE 6: Immunoblots of periplasmic extracts from *E. coli* W3110. pT denotes pTac induced with 1 mM IPTG at the temperatures indicated for either pTac PRL or pTac S179D PRL. pL, pL PRL, or pL S179D PRL was induced at the indicated temperatures.

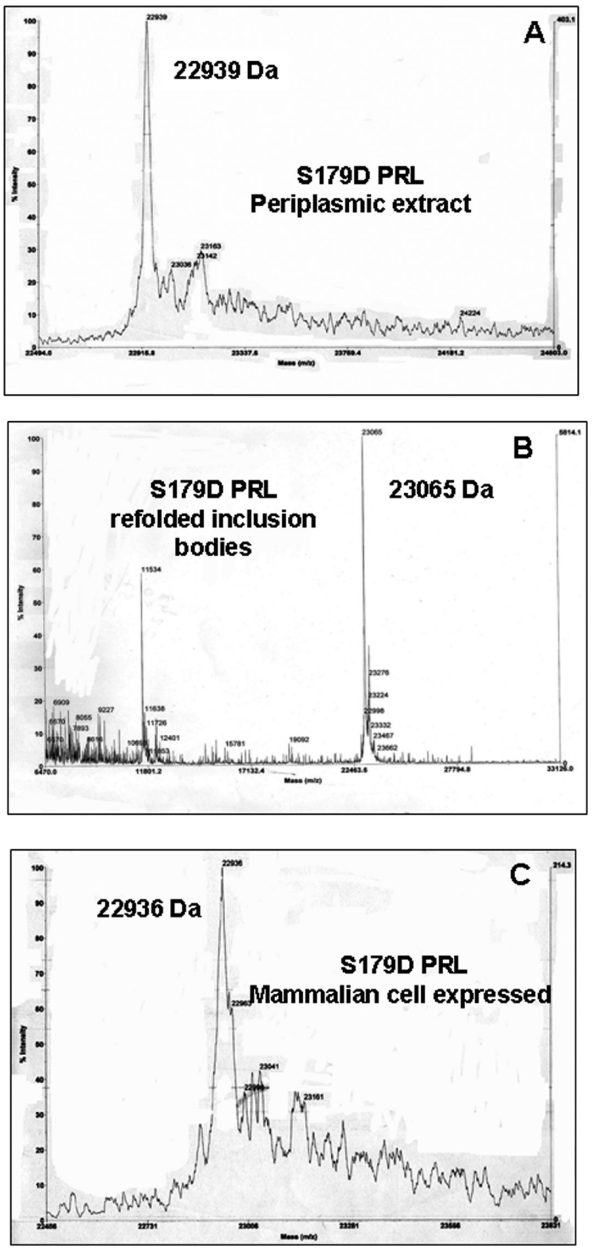

FIGURE 7: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry of S179D PRL preparations. Analysis was performed on a Voyager DE instrument in positive ion mode using 40 kV: (A) periplasmic extract from BL21 transformed with the pTac-179 plasmid, (B) refolded inclusion bodies from BL21 transformed with pT7SCII-179, and (C) conditioned medium from HEK 293 cells transiently transfected with the pcDNA-179 construct.

Table 1: Signal Peptide Sequences and Protein Expression Yields in *E. coli* W3110 with pL and pTac Promoters (micrograms per milliliter per OD unit)[a]

leader sequence	PRL W3110 (pL promoter)	PRL W3110 (pTac promoter)	S179D PRL W3110 (pL promoter)	S179D PRL W3110 (pTac promoter)
1506 MKKIWLALAGLVAFSASA	0.6 ± 0.04	0.55 ± 0.1	ND[b]	ND[b]
1507 MAPSGKSTLLLLFLLLCLPSWNAGA	ND[b]	ND[b]	ND[b]	ND[b]
1508 MKKIWLALAGLVLAFSASA	ND[b]	ND[b]	ND[b]	ND[b]
1510 MKKILALAALTTVVFSASAFA	1.3 ± 0.2	1.1 ± 0.3	0.34 ± 0.03	0.3 ± 0.02
1511 MNHSGKSWLLLLFLLLCLPSWNAGA	ND[b]	ND[b]	ND[b]	ND[b]

[a] All the signal sequences had similar hydrophobic patterns displayed by signal peptides from naturally occurring periplasmic proteins as estimated by MacMolly TetraTM 1997 software for MacOS (Soft Gene GmbH) (data not shown). Signal peptide 1510 is 99% homologous with maltose binding protein, a common periplasmic protein in *E. coli*. [b] Not detectable.

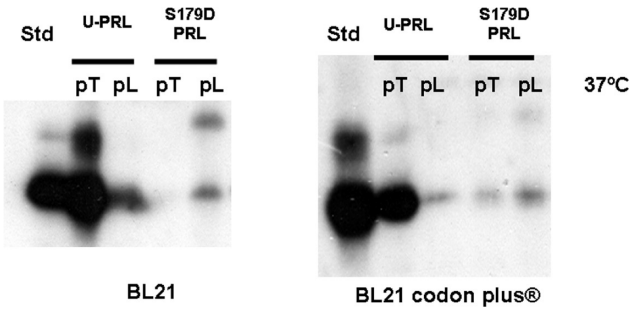

FIGURE 8: Immunoblots of periplasmic extracts from *E. coli*, BL21 and BL21 codon plus. pT denotes pTac unmodified PRL or S179D PRL induced with 1 mM IPTG; pL denotes pL-induced PRL or S179D PRL. All inductions were conducted at 37 °C. Proteins were separated on reducing SDS 12.5% gels prior to immunoblotting. The standard PRL used for comparison was NIDDK-extracted human pituitary PRL.

Table 2: Periplasmic Protein Expression Yields and Final Optical Densities (OD_{600}) of Different Strains with the pL Promoter

	E. coli strain	protein yield [μg mL^{-1} (OD unit)$^{-1}$]	final OD_{600}
PRL	W3110	1.3 ± 0.2	4.0 ± 0.3
	BL21	1.9 ± 0.4	1.3 ± 0.2
	BL21 codon plus	1.4 ± 0.3	1.0 ± 0.2
S179D PRL	W3110	0.34 ± 0.03	3.8 ± 0.6
	BL21	0.35 ± 0.5	1.3 ± 0.5
	BL21 codon plus	0.40 ± 0.3	1.2 ± 0.1

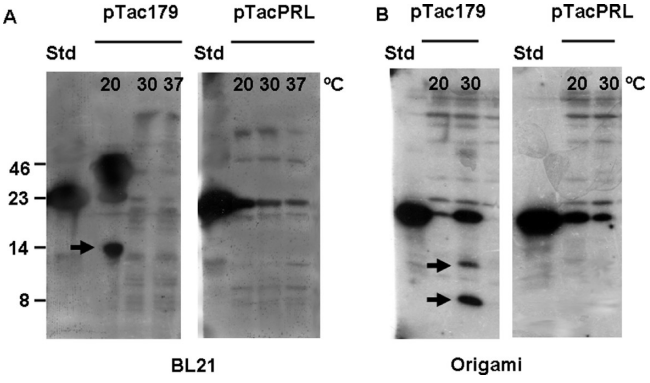

FIGURE 9: Immunoblots of soluble fractions of *E. coli* BL21 or Origami lysates after induction at the indicated temperatures. Arrows indicate possible cleaved products from S179D PRL; numbers to the left of panel A indicate approximate molecular masses.

low yields, with proteins that could not even be detected by immunoblotting. This was true for both PRLs at temperatures lower than 37 °C (data not shown), even though we conducted these activations in strains lacking the λcIts 857 repressor. This is unlike the expression of unmodified PRL in the periplasmic space (*30*). The IPTG induction of pTac proved to be more effective at lower temperatures (Figure 9B).

We also compared the bioactivities of various preparations of S179D PRL. We examined the relative abilities of these preparations to induce apoptosis in human umbilical vein endothelial cells (HuVEC). Shown in Figure 10 are representative flow cytometric histograms and quantification of the subG_0/G_1 area, which is marked as A on the graphs. In addition to showing results with S179D PRL from refolded inclusion bodies, periplasmic expression, and conditioned medium from HEK 293 cells, the graphs also include controls for each of these. Conditioned medium used as the control for S179D PRL expressed in HEK 293 cells (conditioned medium from empty vector-transfected cells) had some apoptotic activity, which is not unexpected since most cells express and secrete some level of pro- and anti-angiogenic factors (*6*, *31*). When the control results were subtracted from the result with the different S179D PRL preparations, it was clear that the activities of all three preparations were very similar, causing apoptosis in 23−27% of cells.

DISCUSSION

The objective of this work was to determine whether S179D PRL and unmodified PRL had different conformations that in turn could explain the initiation of different signals by the two ligands when in a ternary complex with receptors. Clearly, before analysis of different potential conformations, one has to be sure that the material analyzed is properly folded. We also wanted to determine the best method of production of fully functional and properly folded S179D PRL with the intent of eventual therapeutic production. To be sure that folding was correct in various preparations, we took advantage of the quality control mechanisms present in eukaryotic cells, which ensure that only properly folded molecules move from the RER to the Golgi and hence to the exocytotic machinery. This quality control mechanism relies on several sensing enzymes in the lumen of the RER which include a variety of chaperone proteins/oxidoreductases, various protein disulfide isomerases, and rejection by transporter molecules which would normally ferry the properly folded molecule to the Golgi. Retention in the RER allows time for more attempts at proper folding and in the absence of proper folding leads to retrotranslocation of the protein out of the RER and into the cytosol for degradation in proteasomes (reviewed in ref *15*). For example, the presence of a free cysteine in the correct context (i.e., in a protein that does not normally have free cysteines such as PRL) can be sufficient to cause retention and degradation of a protein (reviewed in ref *32*). Cells adapt to changing demands on this quality control mechanism in such a way that the level of output can be increased. If this adaptation is insufficient, cells will undergo apoptosis. In our study, we produced a number of different stable cell clones which by their survival at a certain output means they had adapted to the amount of unmodified or S179D PRL expressed. On average, these clones produced approximately half the amount of S179D PRL as compared to unmodified PRL. Thus, S179D PRL seems inherently more difficult to successfully fold than unmodified PRL. S179D PRL is a molecular mimic of phosphorylated human PRL, and phosphorylation normally occurs in the secretory granule just prior to exocytosis (*33*, *34*) and not cotranslationally. Thus, the RER is not the normal environment for folding of the phosphorylated version of PRL and may therefore be missing some important element necessary for folding the pseudophosphorylated hormone. It is therefore not surprising that folding is more difficult and the resultant yield lower.

Reduced hydrophobicity is expected when a charged aspartate is substituted for a noncharged serine, but the degree of change in hydrophobicity observed suggests that more than this is happening. In other words, there is a change in the surface of the molecule. Further evidence of a changed surface is the reduced affinity for Ni(II) and Zn(II). On the basis of the simple amino acid substitution, one would expect the increased negative charge of S179D PRL versus unmodified PRL to increase rather than

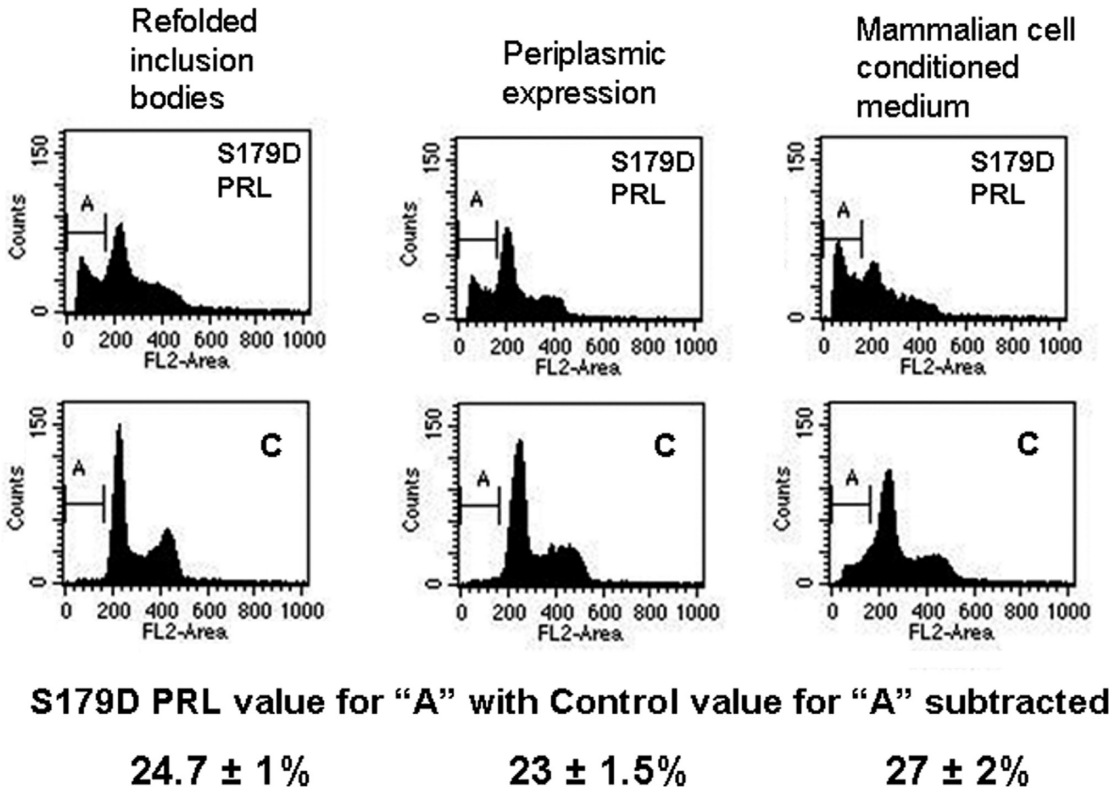

FIGURE 10: Effects of differently expressed preparations of S179D PRL on HuVEC DNA content. Representative flow cytometric analysis histograms following propidium iodide staining of control cells (C) and cells treated with different preparations of S179D PRL. The controls were diluent for the refolded S179D PRL, processed osmotic shock fluid from bacteria not expressing S179D PRL, and conditioned media from HEK 293 cells not expressing S179D PRL. On the graphs, the region marked A contains cells with sub-G_0/G_1 amounts of DNA, shown previously to correspond to cells undergoing apoptosis (*6*). Below the graphs are the mean values of *A* from triplicate determinations with S179D PRL minus *A* from the controls ± the standard error of the mean.

decrease its affinity for these divalent cations. Likewise, the increased affinity for heparin and DEAE is the opposite of what one would predict from the amino acid substitution alone. Thus, using the well-folded product of mammalian cells, we have four measures which speak to an altered surface for S179D PRL. The Ni, Zn, heparin, and DEAE results suggest a substantial increase in the positive surface charge which would contribute to reduced hydrophobicity.

We theorize that a difference in conformation between unmodified and S179D PRL results in an altered conformation of the ternary complex between these ligands and paired PRL receptors. Although on one hand there can be surprising functional flexibility in the ligand binding regions of the PRL receptor, as evidenced by the ability of dimeric G129R PRL to initiate a signal (*35*), quite subtle changes in the ligand binding region of the receptor can lead to constitutive activation. For example, a single and conservative amino acid substitution in the human PRL receptor (I146L) leads to constitutive activation (*36*). Single amino acid substitutions in the transmembrane domain of the growth hormone receptor can change the ratio of Stat5 to ERK signaling in response to ligand (*37*), as can a single amino acid twist in the transmembrane domain of the erythropoietin receptor (*38*). Thus, minor changes in receptor conformation, which could result from interaction with ligands with slightly different conformations, could explain the different signaling which results from unmodified versus S179D PRL binding. Unmodified PRL strongly activates Jak2 and Stat5, while there is a minimal response to S179D PRL. S179D PRL also antagonizes Jak2/Stat5 activation in response to unmodified PRL. Both ligands activate ERK; however, for unmodified PRL, this is rapid and transient, whereas for S179D PRL, the activation is delayed and prolonged (*2*, *3*, *6*, *8*, *12*, *13*).

Exactly how substitution of an aspartate for a serine residue affects ligand structure, and as a result the architecture of the ternary complex, awaits specific examination. Meanwhile, it has been proposed from crystallographic evidence that a change in the conformation of S179D PRL could be due to a loss of binding of the serine 179 carbonyl oxygen to a water molecule. The water molecule in unmodified PRL is thought to stabilize the interactions between helices 1 and 4 (*39*).

The increased affinity of S179D PRL toward heparin was an interesting finding. It is noteworthy that many compounds that affect angiogenesis (both positively and negatively) also bind heparin and/or require heparin for their biological activity (e.g., bFGF and endostatin) (reviewed in refs *40* and *41*). S179D PRL is potently anti-angiogenic, whereas unmodified PRL is not (*5*). Although we have previously shown that adding heparin to S179D PRL did not change its anti-angiogenic activity (*6*), it remains possible that there was already sufficient heparin (or similar sulfated proteoglycans) made by the endothelial cells to bind S179D PRL. The possible local accumulation of S179D PRL resulting from binding to sulfated proteoglycans, on cell surfaces or in tissue ground-substance, may account for the greater efficacy of S179D PRL in vivo versus in vitro (*4*, *41*, *42*).

Given the anti-proliferative, anti-angiogenic, anti-inflammatory, and pro-differentiative effects of S179D PRL (reviewed in refs *7* and *8*), this molecule has promise as a cancer therapeutic. It is therefore important to determine the expression system that combines high yield and low cost with a reproducibly and accurately folded product. Many factors (e.g., pH, ionic strength,

and redox conditions) can influence the formation of folding intermediates or "molten globules" of proteins (*43*), and similar molten globules can also occur in the kinetic folding pathway (*44*). The intermediates can be either marginally stable, highly disordered conformations or stable states with nearly native structures (reviewed in ref *45*). While these latter may have the same properties and a significant amount of secondary structure, fluctuating tertiary structure can lead in many instances to unstable active intermediates and therefore unstable bioactivities. Thus, refolding conditions that vary from lab to lab may contribute to different findings, a phenomenon illustrated well by controversies over the bioactivity of endostatin (reviewed in ref *46*).

In this study, we aimed to evaluate a number of potential ways to produce S179D PRL that could be compared with the material expressed in mammalian cells, as well as with the preparation refolded from inclusion bodies already described (*1*). The first approach employed periplasmic expression and secretion of S179D PRL in W3110 *E. coli*, a method already successfully utilized for unmodified PRL in our laboratory (*16*). Unexpectedly, the single amino acid substitution producing S179D PRL impaired protein production. The yield of S179D PRL was lower than that of unmodified PRL in all bacterial expression systems examined. It was not improved by (1) the use of the BL21 codon plus strain (which harbors eukaryotic tRNAs), (2) periplasmic and cytoplasmic production in BL21 cells (a strain with reduced protease activity) with either pTac chemical stimulation at low temperatures or cold shock stimulation of pL (*47*), or (3) expression of S179D PRL as a soluble, cytoplasmic protein at lower temperatures (20 °C). In the latter condition, we observed a low-molecular mass form (~14 kDa) of S179D PRL. Additionally, use of the mutant strain Origami (which promotes the formation of disulfide bonds because of an oxidizing cytosol) also caused apparent partial degradation to two lower-molecular mass (~14 and 8 kDa) derivatives of S179D PRL not present when unmodified PRL was produced. These findings are consistent with a conformation of S179D PRL that is more susceptible to some proteolysis than unmodified PRL. We therefore conclude that the best method for production of S179D PRL utilizes inclusion bodies in which material is protected against proteolysis (*27*). Indeed, many PRL mutants, including S179D PRL, have been successfully expressed in inclusion bodies at the same levels as unmodified PRL (*1*, *48*).

Our method of producing S179D PRL in inclusion bodies appears to result in a refolded protein with properties that are the same as the properties of that expressed in mammalian systems. We have previously shown that this material has a decreased hydrophobicity compared to unmodified PRL (*14*), a result that is duplicated in the current study with material produced in mammalian cells. The CD spectrum of S179D PRL from inclusion bodies is identical to that from mammalian cells, and both are slightly different from that of unmodified PRL. The affinity for heparin is the same (data not shown), and the pro-apoptotic activity of S179D PRL produced from inclusion bodies, periplasmic expression, and mammalian cells is indistinguishable. Thus, we conclude that the method utilized for production, isolation, and refolding from inclusion bodies results in correctly folded S179D PRL.

In summary, we have provided evidence of an altered conformation for S179D PRL compared to unmodified PRL, manifest by a reduced hydrophobicity and increased positive surface charge compared to those of unmodified PRL. This change in ligand conformation is proposed to affect the conformation of the ternary complex with paired receptors in such a way that it may account for the demonstrated ability of the two ligands to initiate different intracellular signals. Analysis of different expression systems for S179D PRL also demonstrates that expression in inclusion bodies is the most efficient, and comparisons with the material expressed in mammalian systems demonstrates that proper folding is achievable.

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