Chapter

Superior Stimulation of β-Casein mRNA Accumulation by Pseudophosphorylated Prolactin: Enhanced Transcription and Message Stabilization

Wei Wu, Changhui Deng, Jennifer L. Brockman, Linda A. Schuler and Ameae M. Walker

Abstract

A proportion of secreted pituitary prolactin (PRL) is phosphorylated. However, because most commercial sources of PRL are recombinant proteins without post-translational modification, the importance of PRL phosphorylation to the production of milk proteins is an understudied area. Here, we have examined the effect of PRL phosphorylation on expression of the milk protein, β-casein, using a phospho-stable mimic of the phosphorylated form (S179D-PRL) and analyzing promoter activation and mRNA stability over a 7-day treatment period in response to this and unmodified PRL. At equivalent concentrations, the phospho-mimic showed a superior ability to activate a −2300 → +490 region of the promoter, but not an artificial promoter consisting of three Stat5 consensus sites upstream of a minimal promoter. Unlike unmodified PRL, S179D-PRL was also able to stabilize β-casein mRNA. These effects of S179D-PRL were eliminated by incubation in the MAP kinase pathway inhibitor, U0126, bringing promoter activation down to the level seen with unmodified PRL and essentially eliminating the effect on mRNA stability. These results support an important role for the posttranslational phosphorylation of PRL and signaling through the MAP kinase pathway in the production of this milk protein.

Keywords: hormone regulation, posttranslational modification, phosphorylated prolactin, molecular mimicry, S179D prolactin, beta-casein, mRNA stabilization, length of promoter

1. Introduction

PRL phosphorylation in the pituitary is regulated physiologically [1, 2]. Phospho-PRL has been demonstrated in rat [3], mouse [4], sheep, avian [5], bovine [6], and human [7] pituitary extracts. The phosphorylated form is very stable and cleared

from the circulation with similar kinetics to the unmodified hormone [6–8]. Differential function analysis of unmodified and phospho-PRL demonstrated different biological activities [9, 10]. Recent studies have utilized a molecular mimic of phospho-PRL to prevent conversion to the unmodified form during the course of an experiment. This mimic was made by substituting an aspartate residue for the normally phosphorylated serine [11, 12], thereby producing S179D-PRL.

Previous work from our laboratories has demonstrated different activities for unmodified PRL (U-PRL) and S179D-PRL in the control of proliferation in the mammary gland and mammary cells in culture, with U-PRL promoting cell proliferation and S179D-PRL antagonizing this effect [12–16]. Despite these demonstrations, which indicate that mammary cells recognize and respond to the two kinds of PRL differently, there has since been very limited investigation of the importance of PRL phosphorylation to the production of milk proteins. In large part, this is likely due to the lack of availability of purified phosphorylated PRL, another reason we developed the phospho-mimic, S179D-PRL.

Using S179D-PRL, we have previously shown [14] that compared to U-PRL this phospho-mimic had a superior ability to stimulate β-casein expression. This was surprising since S179D-PRL has an inferior ability to activate Stat5 [13], activation of which is crucial to milk protein production [17].

Here, we have investigated the molecular mechanisms resulting in superior β-casein expression in response to S179D-PRL by examining the relative roles of transcriptional and posttranscriptional activities and MAP kinase signaling to the accumulation of β-casein mRNA in response to each PRL.

2. Materials and methods

2.1 Mammary cell culture and differentiation

HC11 cells were a gift from Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland). Cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FBS, 5 μg/ml insulin (Sigma, St. Louis, MO, USA), 10 ng/ml epidermal growth factor (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. Once confluent, they were grown 3 more days with daily medium changes. The medium was then removed, and the cells were washed five times with RPMI 1640. Cells were then treated with priming medium-RPMI 1640 supplemented with 10% charcoal-stripped horse serum (Cocalico Biologicals, Reamstown, PA, USA), antibiotics, 10 μg/ml insulin, 1 μg/ml hydrocortisone (Sigma) for 24 h followed by induction in priming medium plus U-PRL, or S179D-PRL (changed daily). This essentially follows Taverna et al. [18] except that 1 μg/ml, which maximally stimulates the endpoints measured here, instead of 5 μg/ml PRL, was used in this study. Potential differences in the uptake or degradation of U-PRL and S179D-PRL were monitored by ELISA and Western blot of media samples.

2.2 Transfection and β-casein luciferase assays

Primed HC11 cells were transfected with −2300 → +490 of the proximal rat β-casein promoter [19] subcloned into pGL3Basic (Promega, Madison, WI, USA) and a CMV-β-galactosidase construct. Sub-confluent cultures in six-well plates were transfected with β-casein luciferase DNA (2 μg), β-gal DNA (0.5 μg), and 10 μl

lipofectamine/5 ml. After transfection, cells were treated with one or other form of PRL for 24 h in the absence or presence of U0126 (10 μM). For the 7-day experiment, cells were treated with the PRLs for 6 days, with daily medium changes, prior to transfection. For some experiments, cultures were transfected with an artificial promoter consisting of three Stat5 consensus sites upstream of a minimal promoter [20].

2.3 β-Casein mRNA stability

HC11 cells were induced for 1–7 days with daily medium changes and RNA was isolated. To test the mRNA stability, the transcription inhibitor 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB) (Promega) was added (50 μg/ml) in the continued presence of the PRLs and in the absence and presence of the MEK1/2 inhibitor, U0126 (10 μM). Real-time PCR reactions were 12.5 μl SYBR Green PCR master mix (Applied Biosystems), 2.5 μl of 10 μM mouse β-casein forward primer (5′-CCC GTC CCA CAA AAC ATC CAG CC-3), and 2.5 μl reverse primer (5′-ATT AGC AAG ACT GGC AAG GCT G-3′), or 2.5 μl of mouse GAPDH forward primer (5′-CCA TGG AGA AGG CTG GGG-3′), and 2.5 μl reverse primer (5′-CAA AGT TGT CAT GGA TGA CC-3′), 1 μl diluted RT product and 6.5 μl ddH_2O with 10 min 95°C followed by cycles of 95°C, 1 min; 55°C, 30 s; 72°C, 45 s; and 80°C, 10 s. Both annealing temperature and Tm of mouse β-casein and GAPDH primers are similar, allowing co-amplification and a comparative C_T method for quantification of gene expression calculated by $2^{-\Delta\Delta C}{}_T$.

2.4 Statistical analysis

Data were subjected to ANOVA with posttests comparing specific groups and Bonferroni corrections for multiple comparisons. Data are presented as mean ± SEM. The minimal number of experiments and replicates within each experiment was 3. Analysis of the real-time RT-PCR data was as per ABI PRISM 7700 Sequence Detection System User Bulletin #2.

3. Results

Real-time RT-PCR allowed us to determine steady-state transcript levels as a function of time in response to U-PRL and S179D-PRL (**Figure 1**).

β-Casein mRNA increased over the 7-day period in response to each PRL. S179D-PRL was more efficacious than U-PRL, resulting in over twice the level of β-casein transcripts after 3 days, and 3–4 times higher levels after the full 7-day incubation. In order to determine whether these were effects on promoter activity, we utilized a β-casein promoter-luciferase construct. Importantly, this construct included the −2300 to +490 region of the β-casein promoter and not the usually employed, much smaller −344/−1 portion. As can be seen in **Figure 2A**, both PRLs stimulated reporter activity after 1 day of exposure. However, S179D-PRL was twice as effective as U-PRL. This was not the result of differential stability of these PRL forms since examination of the 24-h media from these incubations by ELISA and Western blot using an antibody, which recognizes both forms equally showed no evidence of different uptake or degradation of the PRLs (data not shown). Conduct of this experiment in the presence of U0126 demonstrated that the ERK pathway was important for the superior ability of S179D-PRL to activate the promoter (**Figure 2A**). In contrast, this inhibitor had no significant effect on U-PRL-stimulated activity and did not alter controls.

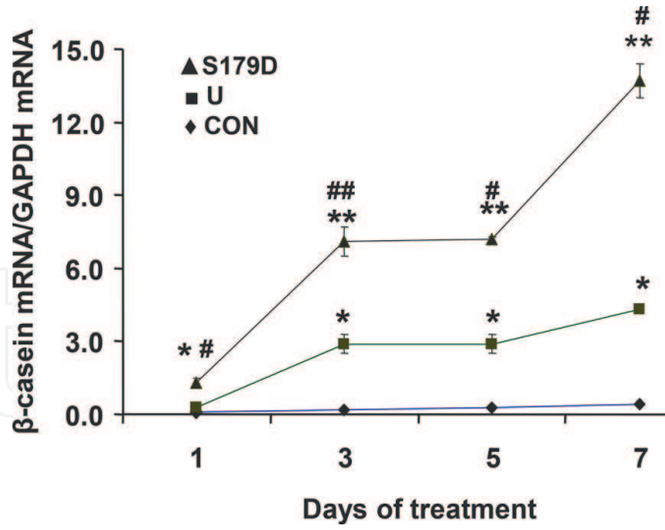

Figure 1.
*Effect of U-PRL and S179D-PRL on β-casein mRNA levels as a function of days of stimulation. *, p < 0.01 versus control (CON); #, p < 0.01 for S179D-PRL (S179D) versus U-PRL (U); ** p < 0.001 versus control; ##, p < 0.05 for S179D-PRL versus U-PRL.*

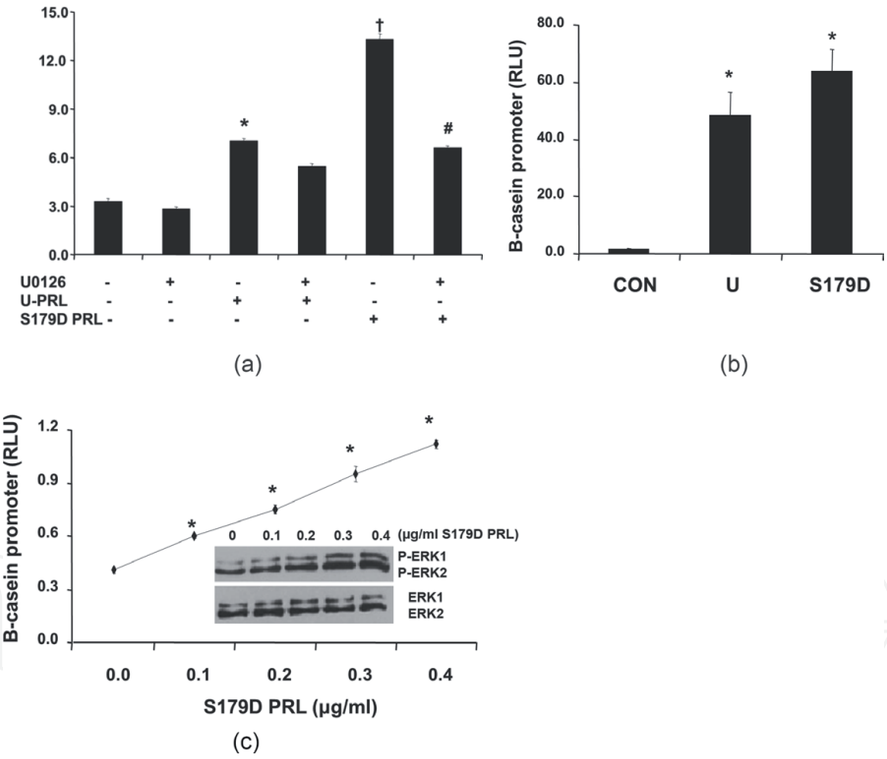

Figure 2.
*Effect of U-PRL and S179D-PRL on the −2300 → +490 β-casein promoter-luciferase. A, transfection at day 0 and assay at day 1 of treatment with and without inhibition by U0126; B, transfection at day 6 and assay at day 7 of treatment; C, ERK activation and β-casein-luciferase activity in response to S179D-PRL. *, p < 0.01 for U-PRL and †, p < 0.001 versus control. #, p < 0.01 versus S179D-PRL.*

The relationship between both ERK activation and promoter activity and dose of S179D-PRL is shown in **Figure 2C**.

To determine the importance of Stat5 in the differential effects of the two PRLs at the promoter, we examined their relative activities at an isolated Stat5 enhancer.

Although both PRLs were able to significantly increase activity, S179D-PRL was slightly less effective (**Figure 3**). This result demonstrated that other regulatory

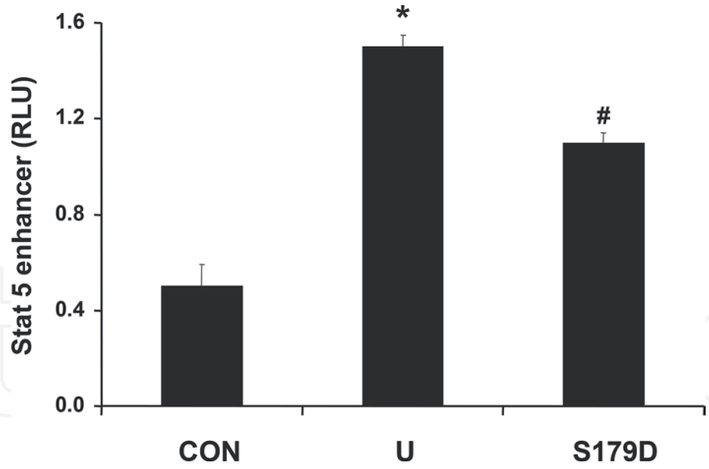

Figure 3.
*Effect of U-PRL and S179D-PRL on day 1 activity of the Stat5 enhancer. *, p < 0.01 and #, p < 0.05 versus control.*

sequences and mediators, in addition to Stat5, were important in the S179D-PRL response in the physiologic context of the β-casein promoter.

By 7 days of incubation, the difference in efficacies of the two PRLs in stimulating β-casein promoter activity was essentially eliminated (**Figure 2B**) and the degree of promoter stimulation in response to both ligands was increased. Based on the results in **Figure 1**, one would not have predicted the loss of the difference between U-PRL

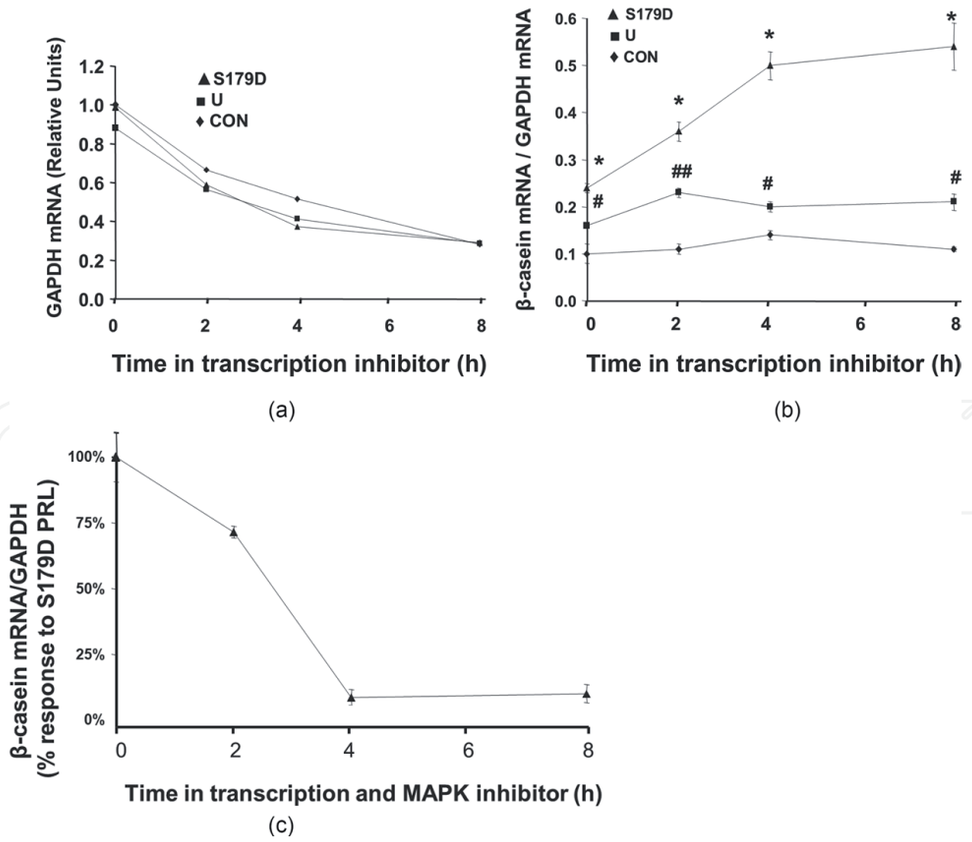

Figure 4.
*Effect of U-PRL and S179D-PRL on mRNA half-life. Following a 6-day incubation in each PRL, the transcription inhibitor, DRB, was added. A, GAPDH mRNA; B and C, β-casein mRNA levels normalized to GAPDH as a function of time after addition of DRB in the absence (B) and presence (C) of U0126 (added at time 0). The ratio at time 0 in the control cells was set at 0.1 in B. *, p < 0.001 for S179D-PRL versus control or U-PRL; #, p < 0.05 for U-PRL versus control; ##, p < 0.001 versus control.*

and S179D-PRL at day 7 if transcription was the most important regulator of steady-state mRNA levels. We therefore analyzed β-casein mRNA stability in response to both PRLs. Cells were incubated without or with the different PRLs for 6 days, and then, the transcription inhibitor, DRB, was added. **Figure 4A**, which plots GADPH mRNA as a function of time after inhibition of transcription, shows the expected decline in mRNA levels, with a half-life of about 4 h. This was not appreciably altered by either PRL, allowing us to use GAPDH to normalize the data for RT-PCR efficiency.

As seen in **Figure 4B**, β-casein transcripts had a half-life of about 4 h since when normalized to GAPDH, the ratio was unaltered by incubation in DRB in the controls or cells incubated in U-PRL. In contrast, incubation in DRB revealed a dramatic effect of S179D-PRL on β-casein mRNA stability. Since time 0 on this graph is after 6 days of U-PRL or S179D-PRL treatment, the effects of the two PRLs on overall mRNA levels are evident prior to DRB treatment. When U0126 was added along with DRB at 0 h, the elevated levels of β-casein mRNA in response to S179D-PRL were reduced by 2 and 4 h, indicating an important role for ERKs in S179D-PRL-induced mRNA stability (**Figure 4C**).

4. Discussion

The results demonstrate that both U-PRL and S179D-PRL increase steady-state β-casein mRNA levels. However, S179D-PRL was more effective than U-PRL after shorter exposures and also elicited a later rise not found with U-PRL. This biphasic response to S179D-PRL suggested the possibility of different mechanisms of mRNA accumulation. A large body of literature has demonstrated rapid effects of PRL treatment on the β-casein promoter [21–23]. However, a second, much smaller number of papers, which have largely been forgotten in recent years, demonstrate that PRL increases β-casein mRNA stability and that this is quantitatively much more important in terms of steady-state mRNA levels than promotion of transcription [24–27]. However, these studies used pituitary extract preparations of PRL, which contained a mixture of both unmodified and phosphorylated PRL, and so the importance of PRL phosphorylation to these activities was unknown.

Regulatory sequences within the β-casein promoter are found over a fairly large region, but most investigators have limited their examination to the activity of the most proximal 344 nucleotides in reporter gene assays. However, this promoter may not detect all the responses to S179D-PRL. CREB, ATF1, and YY1 sites outside of this region can potentially be activated by ERK1/2 (reviewed in [28]). This signaling pathway is a major mediator of S179D-PRL, although U-PRL can also activate these kinases in HC11 cells to some extent [13]. In addition, increased β-casein gene expression can be achieved by removal of YY1 from the promoter [28]. We therefore utilized a −2300 → +490 fragment of the promoter, which includes the CREB, ATF1, and YY1 sites (in addition to the STAT5 site in the −344/−1 region) in an attempt to make the reporter assay more physiologically relevant. It should be noted, however, that even this larger piece does not constitute the whole promoter.

While both PRLs increased activity of this promoter, S179D-PRL was about twice as effective as U-PRL during the first-phase response. This was unexpected, since S179D-PRL is weaker than U-PRL in stimulating STAT5 tyrosine phosphorylation [13, 15], generally thought to be the most important regulator of β-casein expression. The MEK1/2 inhibitor, U0126, eliminated the difference between U-PRL and S179D-PRL on promoter activity, indicating that ERKs 1/2 are important for the superior stimulation with S179D-PRL, but not for the activity of U-PRL. With the construct

containing only the Stat5 enhancer, there was no superior effect of S179D-PRL. Together, these results demonstrate the importance of other transcription factors in S179D-PRL signals to the β-casein promoter.

Increasing β-casein transcripts with duration of S179D-PRL exposure shown in **Figure 1** suggested increased promoter activation over time in culture. However, we have shown that the difference in β-casein promoter activation between S179D-PRL and U-PRL was reduced after 7 days, consistent with a mechanism other than promoter activity for the second phase of the response to S179D-PRL. Our data indicate that S179D-PRL markedly increased β-casein mRNA stability at this time, while U-PRL was without effect. Other investigators have used pituitary-derived PRL, which is a mixture of U-PRL and phosphorylated PRL [27] and hence have not been able to make this distinction.

There are a variety of mechanisms by which β-casein mRNA stability may be regulated, including the length of the poly A tail [29] and effects at the 5′ untranslated region (5′UTR) and other areas of the 3′UTR [26, 27]. ERK1/2 activation has previously been shown to be important for some proportion of the β-casein response to mixed PRL [23]. This is the first demonstration of effects on both transcriptional and posttranscriptional regulation.

Combining these results with previous work on the role of these two PRLs in cell proliferation, we suggest that optimal alveolar development and subsequent lactation are the result of well-orchestrated exposures to combinations of U-PRL, phospho-PRL, and other physiological stimuli including milk removal. U-PRL (or a lactogen acting like U-PRL) stimulates alveolar development [14] and some β-casein expression. Phospho-PRL, which has the capacity to inhibit alveolar development [14, 16], may slow alveolar development after parturition but robustly stimulate β-casein expression by virtue of its ability to increase both promoter activity and mRNA stabilization.

5. Conclusions

These results support an important role for the posttranslational phosphorylation of PRL and signaling through the MAP kinase pathway in the production of the milk protein, β-casein. Furthermore, the results are an important reminder that use of bacterially derived recombinant prolactin that does not have normal secretory pathway posttranslational modifications may not fully represent normal physiology.

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Conflict of interest

The authors declare no conflict of interest that would by any measure affect their impartiality in the presentation of results.

Author details

Wei Wu[1], Changhui Deng[2], Jennifer L. Brockman[3], Linda A. Schuler[3] and Ameae M. Walker[1*]

1 Division of Biomedical Sciences, University of California, Riverside, CA, USA

2 Department of Biochemistry, University of California, Riverside, CA, USA

3 Department of Comparative Biosciences, University of Wisconsin, Madison, WI, USA

*Address all correspondence to: ameae.walker@ucr.edu

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