Prolactin induces growth inhibition and promotes differentiation of CHO cells stably transfected with prolactin receptor complementary DNA

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Abstract **We have characterized a stable and functional transfectant of the rabbit prolactin receptor in Chinese hamster ovary cells, and investigated the action of prolactin (PRL) on the growth and differentiation of this transfectant (clone E32). PRL induced a significant inhibition of E32 cell proliferation. Growth inhibition correlated with gene induction of the molecular marker of ovarian differentiation cholesterol side chain cleavage P450 ($P450_{scc}$). Both effects were inversely proportional to cell confluence. The limits and potential development of such transfected cellular systems are discussed.**

Key words: Prolactin receptor; Growth inhibition; Differentiation marker; Signal transduction; Transfection

1. Introduction

Prolactin is a pituitary hormone eliciting numerous effects in vivo [1]. In mammary gland, PRL is responsible, along with insulin and glucocorticoids, for milk protein gene expression [2]. In the gonads, PRL stimulates steroidogenesis and growth of ovarian follicles [1]. The role of PRL on cell proliferation has also been studied using lymphocyte-derived cell lines [3]. Previously, we have shown that mammary gland explants could be used to study the effects of PRL on milk protein gene expression [4]. We have also shown that CHO cells co-transfected with rabbit prolactin receptor (rbPRL-R) cDNA and with a plasmid bearing a β-lactoglobulin promoter upstream from the chloramphenicol acetyl transferase (CAT) gene, respond to PRL by increased CAT activity [5], which is reminiscent of the differentiation action of PRL obtained either in vivo or with mammary explants. In 3T3-F442A pre-adipocytes, growth hormone (GH) exhibits anti-mitogenic activity and induces these cells to differentiate to adipocytes [6,7]. By contrast, CHO cells transfected with growth hormone receptor (GH-R) cDNA proliferate in response to GH [8]. Thus, it seems that, depending on the tissue, GH and PRL, the receptors of which belong to the same superfamily of cytokine receptors [1], can either promote differentiation or induce proliferation of a given cell type. Therefore, in the present work we have selected and characterized a stable rbPRL-R transfectant in CHO cells and investigated the action of PRL on the proliferation/differentiation of this clone, to determine to what extent transfection of rbPRL-R would allow us to observe the same effects as those already seen in mammary cells.

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Abbreviations: oPRL, ovine prolactin; kb, kilobases; CAT, chloramphenicol acetyl transferase; mAb, monoclonal antibody; pAb, polyclonal antibody; Ig, immunoglobulins; GH-R cDNA, growth hormone receptor complementary DNA; LIF, leukemia inhibitory factor; CNTF, cilliary neurotrophic factor, OM, oncostatin M; EPO, erythropoietin; rbPRL-R, rabbit prolactin receptor.

2. Materials and methods

2.1. Materials

Bovine $P450_{scc}$ cDNA was kindly provided by Dr. Evan R. Simpson [9]. oPRL was a gift of the National Pituitary Agency, USA.

2.2. CAT assay

CAT assay has already been described [10].

2.3. Microsome preparation and binding assays

After rinsing with PBS, confluent cells were scrapped off the dish into 1 ml of 25 mM Tris, pH 7.4, 5 mM EDTA, 10 mM $MgCl_2$ and pelleted. Cells were resuspended in 0.5 ml of 25 mM Tris, pH 7.4, 10 mM $MgCl_2$, sonicated and the lysate used directly for binding studies. For assay, 200 μg of microsomes were made up in a final volume of 0.5 ml of 25 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.1% BSA (TMBA) and incubated overnight at room temperature in the presence of 5×10^4 cpm ^{125}I-labeled oPRL (specific activity ≈ 50 μCi/μg) with or without unlabled oPRL standards of appropriate concentration. Binding was determined by adding 3 ml TMBA followed by centrifugation for 15 min at $3000 \times g$. Pellet radioactivity was counted in an LKB scintillation spectrometer. For Scatchard analysis, data were processed with a regression analysis package, and results expressed as fmol/mg proteins.

2.4. Cell proliferation assay

E32 cells were starved for 5 h in GC3 medium containing 50 ng/ml insulin. This step eliminates traces of PRL-like activity in the culture medium and makes cells more responsive to PRL. In addition, insulin concentration was lowered in this experiment (normally 3 μg/ml) because it enhances cell proliferation (not shown). Cells were then incubated in the absence or presence of 2×10^{-8} M (400 ng/ml) oPRL for 24 h in the same medium, and then labeled for 2 h in RPMI containing 1.5 μCi/ml [^{3}H]thymidine (Amersham, UK). After rinsing with PBS, the incorporated radioactivity was determined by scintillation counting.

2.5. Northern blots

Northern blotting and quantitation of mRNA expression were performed as described [11], except that 20 μg/lane of total RNA were used instead of poly(A). In brief, blots were first hybridized with $P450_{scc}$ cDNA [9], then stripped and re-probed for β-actin transcript. The resulting two autoradiograghs were scanned with a Pharmacia LKB ImageMaster DTS, and results were expressed in arbitrary units as the intensity ratio of the $P450_{scc}$ band to the β-actin band of the same track.

2.6. Western blots

After rinsing with PBS, 8×10^6 confluent cells were scraped with a rubber policeman in 1 ml PBS per 10 cm diam. dish, and pelleted at room temperature in a single microtube for 5 min at 5000 rpm. The cell pellet was either boiled in reducing sample buffer, or lysed for 30 min at 4°C on a rotating wheel in 1 ml of 20 mM Tris, pH 8, 137 mM NaCl, 2.7 mM KCl, 1% NP40, 10% glycerol, 1 mM PMSF, 5 μg/ml each leupeptin, pepstatin, and aprotinin. In the latter case, insoluble material was removed by centrifugation for 10 min at 15,000 rpm in a cold microfuge, and solubilized proteins of the supernatant were immunoprecipitated with the indicated antibodies and protein A-Sepha-

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rose (Sigma). Total or immunoprecipitated proteins were resolved by SDS-PAGE, and electro-transferred onto nitrocellulose. Blots were revealed with anti-rbPRL-R polyclonal antibody 46 [12], alcaline phosphatase-linked second Ab and NBT/BCIP dye. For metabolic labeling, 8×10^6 cells were incubated at 37°C in a damp atmosphere and 5% CO_2, for 20 min in methionine- and cysteine-deprived DMEM, then for 2 h in 5 ml of the same buffer supplemented with 0.5 mCi Tran^{35}S-label (ICN), and processed as above. Immunoprecipitated labeled proteins were separated by SDS-PAGE and revealed by autoradiography.

3. Results

3.1. Characterization of prolactin receptor in stably transfected CHO cells

In a previous paper we described the isolation of E3, a stable CHO cell transfectant of the rbPRL-R [5]. In order to check whether E3 was a pure clone or a heterogenous population, E3 cells were diluted and each independent subclone obtained from a single cell was tested for [^{125}I]oPRL binding, as described in section 2. Among 10 randomly chosen subclones, only 3 displayed significant binding properties over that of parental E3 (not shown). Subclone E32 was retained for its highest binding capacity and used in subsequent experiments.

Scatchard analysis indicated that E32 cells contained about 9000 binding sites per cell, with a $K_a = 10.8$ nM^{-1} for oPRL (Fig. 1I). The capability of the receptor to transduce the PRL signal was investigated by transfecting E32 cells with an eukaryotic expression vector containing 4 kb of the ovine β-lactoglobulin promoter upstream of the CAT gene [5]. As illustrated in Fig. 1II, addition of 400 ng/ml oPRL to the culture medium for 72 h induced a 3-fold increase of CAT activity compared

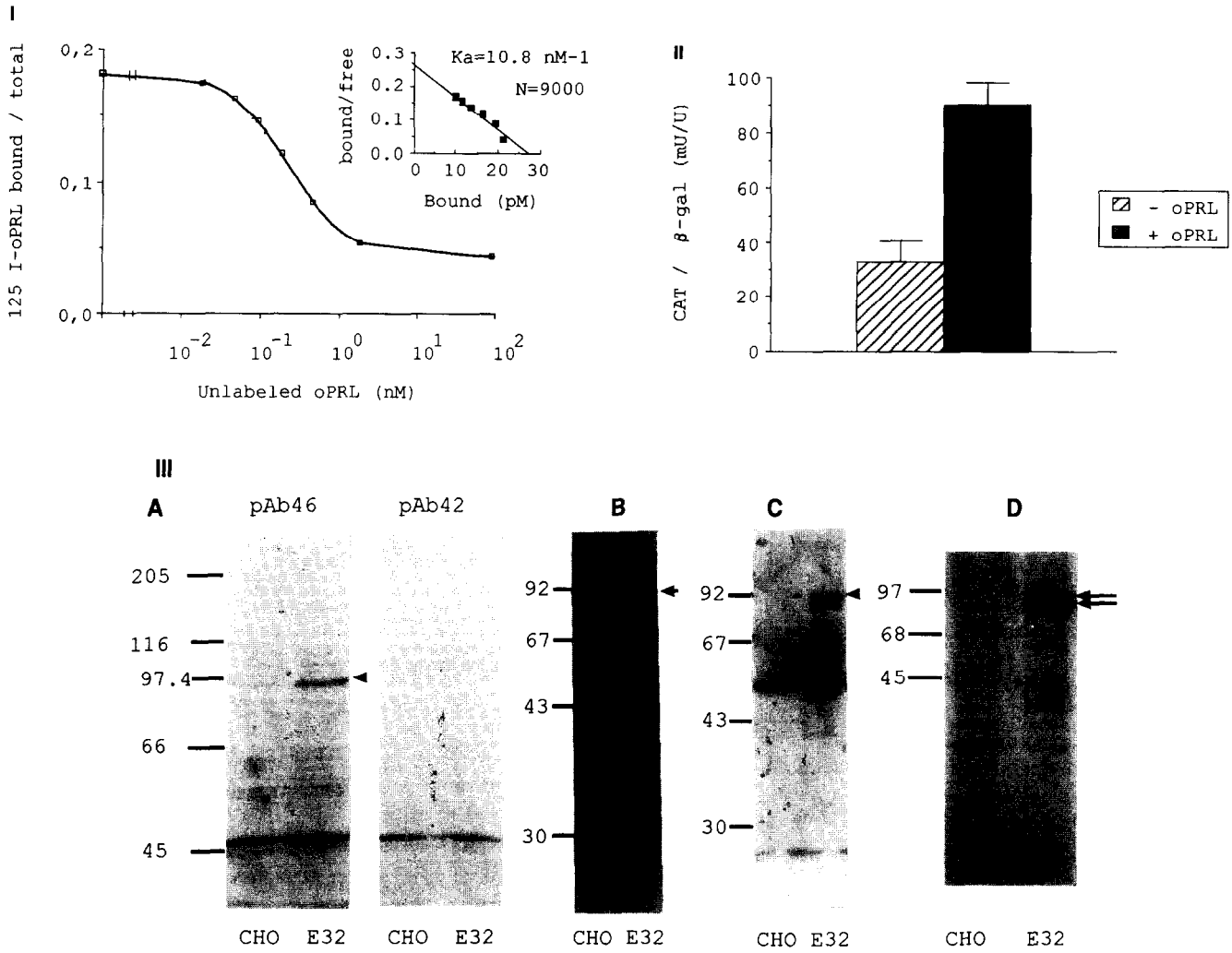

Fig. 1. Characterization of rabbit prolactin receptor in stably transfected CHO cells. (I) Displacement curve by unlabled oPRL of [^{125}I]oPRL binding to E32 cell microsomes. Inset: Scatchard plot of competition assay, 9000 binding sites/cell, $K_a = 10.8$ nM^{-1}. (II) Functional test of the rbPRL-R expressed by E32 cells (see section 3 and [10] for details). CAT activity present in E32 cells before (hatched bar) and after (grey bar) addition of 400 ng/ml oPRL for 72 h. Data are expressed as the ratio of CAT mU to β-galactosidase (β-gal) U to normalize the results for transfection efficiency [10]. (III) Estimated size of rbPRL-R expressed by E32 cells. E32 (or untransfected CHO K1) cells were either boiled in reducing sample buffer (A), or lysed in 1% NP40 in the presence of protease inhibitors (B–D). In the latter case, solubilized proteins were immunoprecipitated by either anti-rbPRL-R polyclonal antibody (pAb) 46 (B,D), or anti-rbPRL-R monoclonal antibody M110 [12] (C). Total (A) or immunoprecipitated (B,C) proteins were resolved by SDS-PAGE and electro-transferred onto nitrocellulose. Blots were revealed as described in section 2. Specificity was demonstrated by use of pre-immune serum 42 (A, pAb42). In D, E32 (or untransfected CHO K1) cells were metabolically labeled and processed as described in section 2. The position of molecular weight markers and of the receptor are indicated by numbers on the left and arrows on the right of each blot, respectively.

to that of non-stimulated cells. Thus, rbPRL-R expressed by E32 cells was able to transduce the prolactin signal to a transfected target gene promoter.

The size of rbPRL-R expressed in E32 cells was estimated by Western blotting with different Ab's (Fig. 1III, A–C). In all cases, a single species was detected at 90/100 kDa that often migated as a doublet. Since rbPRL-R purified from mammary gland was shown to migrate as a 42 kDa species under reducing conditions [14], we wondered whether that species would also be present in E32 cells but be hidden on blots by Ig heavy chains. To clarify this point, we metabolically labeled E32 cells and revealed immunoprecipitated bands by direct autoradiography (Fig. 1III, D). Here again, only the doublet migrating at 100 kDa was visible.

3.2. *PRL partially inhibits E32 cell growth and promotes differentiation*

In a former paper, we showed that oPRL markedly stimulated β-casein synthesis, but had only a slight positive effect on [^{3}H]thymidine incorporation by rabbit mammary gland explants [4]. Since E32 cells were able to transduce the prolactin signal to a transfected target gene promoter (Fig. 1II), we wondered whether their proliferation would also be stimulated by PRL.

The effect of oPRL on [^{3}H]thymidine incorporation by E32 cells was investigated as described in section 2. Unexpectedly, oPRL partially inhibited E32 cell proliferation, and this effect was inversely proportional to cell confluence (36% inhibition at 15% confluence, 27% inhibition at 30% confluence) (Fig. 2A). At 100% confluence, no effect of oPRL was detected, probably because of spontaneous cell growth arrest by contact inhibition (not shown).

This result prompted us to speculate that oPRL could potentially promote differentiation of E32 cells. Our first attempt to detect morphological changes by electron microscopy examination induced by oPRL was not conclusive (not illustrated). In a second trial, we looked for molecular markers of differentiation. Parental CHO K1 cells originate from an ovary biopsy. The differentiation process of granulosa cells is marked by the onset of steroidogenic enzyme expression, the first of which is cholesterol side chain cleavage P450 ($P450_{scc}$) [15]. Therefore, we followed the expression of $P450_{scc}$ mRNA in E32 cells after stimulation by oPRL. Fig. 2B shows the induction of the endogenous $P450_{scc}$ gene after addition of 400 ng/ml oPRL. As was the case for growth inhibition, this induction was inversely proportional to cell confluence, but some stimulation still existed at 100% confluence (not shown). Finally, unlike CHO cells stably transfected with GH-R cDNA [16], E32 cells did not undergo variation in total protein synthesis under the action of oPRL (not shown).

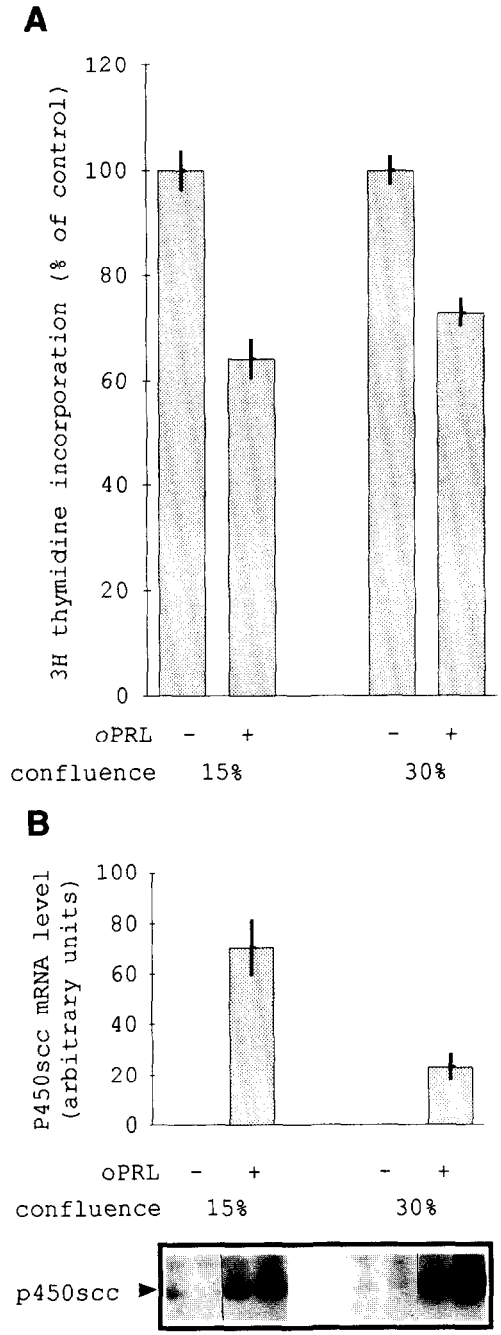

Fig. 2. (A) Effect of oPRL on [^{3}H]thymidine uptake by E32 cells. The experiments were carried out as described in section 2. Cells were seeded so as to reach the indicated confluences at the time of oPRL addition. Results are expressed as the mean ± S.E.M. of two triplicate experiments. (B) $P450_{scc}$ mRNA induction by oPRL in E32 cells. Total RNA of unstimulated, or oPRL-stimulated E32 cells at the indicated confluences was processed as described in section 2. Results of a representative experiment are displayed and expressed as mean ± S.D. of a triplicate assay. Corresponding autoradiography of $P450_{scc}$ mRNA is shown below. The two loadings in each point correspond to two independant culture plates.

4. Discussion

Biochemical and functional characterization of rabbit prolactin receptor was undertaken in the present study by transfecting CHO cells with full length rbPRL-R cDNA. Although histologically unrelated to rabbit mammary gland from which the transfected receptor was cloned, that cellular system proved functional in terms of oPRL stimulation of transfected milk protein target gene, and thus reproduced the result that we had obtained with rabbit mammary gland explants [4]. The difference between explants and CHO cells can be reduced to the action of oPRL on cell growth.

The existence of subclones with binding capabilities very different from that of the first isolated stable transfectant E3

[5] is indicative of either an originally heterogenous population, or a progressive shift undergone by E3, with receptor-producing cells progressively losing their capacity to express this protein. The selected clone E32 encodes about 9000 receptors per cell with a $K_a = 10.8\ nM^{-1}$, which is higher than the value found for E3 ($2.8\ nM^{-1}$ [17]) and for rabbit mammary gland (2.5–3.2 nM^{-1} [18]). This amount of receptor probably explains the clear band seen on Western blots at 100 kDa (Fig. 1III). By contrast, this was not possible in transient transfection of CHO cells, detection of a band requiring the use of Cos cells to obtain sufficient expression [17]. In this respect, receptor size in CHO cells is in agreement with the 88 kDa form expressed by Cos cells [17], the 103 kDa expressed by BAF-3 cells [19], and the 94 kDa produced in a baculovirus expression system [20], all of them using the same cDNA. Interestingly, we have found the same single size receptor in rabbit mammary gland [21]. The above data suggest that (i) at least part of the 42 kDa form previously described in mammary gland [14] might result from artefactual proteolytic cleavage of the 100 kDa species, (ii) the same 100 kDa protein is the only one encoded both by mammary gland and transfected cell lines, although a single cDNA species was used in the latter case whereas four transcripts are present in the former. This confirms our previous observation that all rbPRL-R mRNAs contain the same open reading frame, and differ only by untranslated regions [22].

Although oPRL had the same effect on milk protein gene stimulation in mammary explants [4] and CHO cells expressing rbPRL-R (this study), it had the opposite action on cell proliferation. However, this difference must be considered with caution. Quantitatively, the principal action of oPRL on explants was on β-casein synthesis, stimulation of [^{3}H]thymidine incorporation being a side effect which could be explained (at least in part) by the fact that explants are multicellular structures made of different cell types, and hence are much less amenable to synchronization by serum starvation than are monolayers of homogenous populations of cells such as E32.

During pregnancy, PRL is part of the endocrine complex positively controling the growth of mammary gland [2]. In NB2 lymphoma cells, which express an internally truncated form of PRL-R, oPRL has a proliferative effect [3]. The same is true for BAF-3 cells transfected with full-length rbPRL-R [19], and for murine FDC-P1 cells transfected with the long form of human PRL-R [23]. Thus, as far as we know, the stably transfected CHO cell system described here is the first example in which PRL is growth-inhibitory. In addition, this growth inhibition paralleled the induction of a molecular marker of follicle differentiation, making E32 cells as a potential tool for studying ovarian steroidogenesis, as $P450_{scc}$ is the first enzyme of the steroidogenesis cascade.

At present, we do not know if the association of growth inhibition, differentiation and PRL signaling to milk protein target promoter, concerns CHO cells only or also applies to mammary gland. It is, however, striking that the same receptor (rbPRL-R) is able to sustain proliferation of a pro-B lymphoid BAF-3 cell stable transfectant [19], and to inhibit proliferation of a CHO cell stable transfectant. This suggests a decisive role of the cellular environment, defined by the tissue type in which a given receptor is expressed, on how this receptor will act on cell growth after stimulation by its cognate ligand.

A large amount of work published on signal transduction through receptors of the super-family of cytokine receptors, which encompasses interleukins [24], interferons [25], growth hormone [26], prolactin [27], erythropoietin [28], and LIF/CNTF/OM [29], has provided evidence that non-receptor tyrosine kinases of the Jak family (Jak1, Jak2, or Tyk2) are always involved in the very first steps of this process. In the case of stable transfectants of PRL-R, all these studies have been performed in blood cell-derived cell lines. For instance, Jak2 association with the long or intermediate form of PRL-R, and activation by oPRL have been demonstrated in pro-B lymphoid BAF-3 cells [19], and in NB2 cells [27], respectively. Since the data presented herein suggest a crucial role of the host cell, we are currently investigating the role of Jak kinases in PRL signal transduction to the β-lactoglobulin gene promoter in E32 cells. The next step will be to set up a stable rbPRL-R transfectant of the HC11 mouse mammary cell line [30] so as to work on endogenous milk protein genes, because it is more closely related to mammary tissue than are BAF-3 or NB2 cell lines. In HC11 cellular system, which is even more 'tissue-specific' than CHO cells with respect to the transfected receptor and target gene, will be investigated for the action of oPRL on cell proliferation and differentiation, to test whether the effects described here were restricted to an ovarian-derived cell line or also apply to a mammary-derived cell line.

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