Differential expression of the growth hormone receptor and growth hormone-binding protein in epithelia and stroma of the mouse mammary gland at various physiological stages

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Abstract

Increasing evidence suggests that GH is important in normal mammary gland development. To investigate this further, we studied the distribution and levels of growth hormone receptor (GHR) and GH-binding protein (GHBP) in the mouse mammary gland. At three weeks of age, the epithelial component of the right fourth inguinal mammary gland of female mice was removed. These animals were then either maintained as virgins until they were killed or they were mated. One group of the mated mice was killed on day 18 of pregnancy and the remaining mated animals were allowed to carry their pups until term and were killed on day 6 of lactation. At the time of death, both the intact left and the de-epithelialized right mammary glands were collected from all three groups. Some of the intact glands served as a source of epithelial cells, free of stroma. The mRNA levels for GHR and GHBP were measured in intact glands, epithelia-cleared fat pads, and isolated mammary epithelial cells. GHR and GHBP mRNAs were expressed in both the mammary epithelium and stroma. However, the levels of both GHR and GHBP mRNAs were significantly higher in the stroma as compared with the epithelium component. This increase for both mRNAs was from 3- to 12-fold at each physiological state examined. In the intact gland, both GHR and GHBP transcripts were highest in virgins, declined during late pregnancy, and the lowest levels were found in the lactating gland. GHBP and GHR protein concentrations were also assessed in intact glands and epithelia-free fat pads. Similar to the mRNAs, GHR and GHBP protein levels (means ± s.e.m.) in intact glands were highest in virgin mice (0·891 ± 0·15 pmoles/mg protein and 0·136 ± 0·26 pmoles/mg protein respectively), declined during late pregnancy (0·354 ± 0·111 pmoles/mg protein and 0·178 ± 0·039 pmoles/mg protein respectively), and were lowest during lactation (0·096±0·037 pmoles/mg protein and 0·017±0·006 pmoles/mg protein respectively). Immunocytochemistry utilizing specific antisera against mouse (m) GHR and mGHBP revealed that the two proteins are localized to both the stroma and parenchyma of mouse mammary glands, with similar patterns of immunostaining throughout the different physiological stages analyzed. GHR immunolocalized to the plasma membrane and cytosol of mammary epithelial cells and adipocytes, whereas the GHBP immunostaining was nuclear and cytosolic. In conclusion, we report here that GHR and GHBP mRNAs and proteins are expressed in both the epithelium and the stroma of mammary glands of virgin, pregnant, and lactating mice. In intact glands, GHR and GHBP proteins, as well as their transcripts are higher in abundance in virgin relative to lactating mice. At all physiological stages, GHR and GHBP mRNA levels are higher in the stroma compared with the parenchyma. These findings indicate that the actions of GH in the mammary gland are both direct through its binding to the epithelia, and indirect by binding to the stroma and stimulation of IGF-I production which, in turn, affects mammary epithelial development.


Introduction

The two primary components of the mammary gland are the parenchyma or epithelia, a branching ductal system which develops into milk-secreting acini, and the surrounding adipose stroma which serves as a matrix for the proper growth and differentiation of the parenchyma.

In newborn murines, the parenchyma is rudimentary and consists of a small tree of primary ducts ending in small terminal end buds (Imagawa et al. 1994, Medina 1996). Ductal elongation and branching continues at a slow rate until the onset of puberty, when increased mitotic activity in the end buds causes the ducts to penetrate the stroma. Terminal end buds that have reached the edge of the fat pads regress and then differentiate into alveoli. The slow mitotic rate in alveolar cells limits ductal growth and allows the maintenance of duct-free stromal zones until pregnancy. During gestation and early lactation, intense...
epithelial cell proliferation leading to increased ductal branching and lobulo-alveolar development causes the inter-ductal spaces to be filled with secretory lobules (Imagawa et al. 1986). As the alveoli enlarge and differentiate into milk-producing and -secreting structures, the stromal mass decreases (Sakakura 1991) due to lipid depletion from mammary adipocytes (Elias et al. 1973). After lactation, secretory epithelial cells are gradually lost by apoptosis and the mammary gland undergoes involution (Walker et al. 1989, Strange et al. 1992).

These main stages of mammary growth and differentiation are dependent on endocrine, paracrine, and autocrine control mechanisms which involve complex interactions between steroid hormones, polypeptide hormones, and growth factors (Lyons et al. 1958, Forsyth 1989, Rillema 1994). The major player in mammary gland growth and development, as well as in lactation, is widely considered to be prolactin (PRL) (Lyons et al. 1958, Vonderhaar 1987). Although growth hormone (GH) has long been known to contribute to ductal and lobulo-alveolar growth, this effect was attributed to structural similarities between GH and PRL, enabling GH to bind to and activate PRL receptors (Vonderhaar 1987). Recently, however, GH was shown to be a more potent mammogen than PRL (Kleinberg et al. 1990). This was not limited to the lactogenic human (h) GH, which can activate human PRL receptors (Hughes & Friesen 1985), but was also true for rat and ruminant GHS known to act through the GH receptors (GHR) only. Furthermore, GHR mRNAs have been localized to mammary glands of many different species (Hauser et al. 1990, Jammes et al. 1991, Tiong & Herington 1991), suggesting a direct action for GH in mammary function.

In a recent study, we found GHR and GH-binding protein (GHBP) transcripts in the mouse mammary gland (Ilkbahar et al. 1995), and we also reported a gestation- and lactation-associated down-regulation of the two messages in mouse inguinal glands. Expression levels of both GHR and GHBP mRNAs gradually decreased throughout pregnancy and were further reduced in the lactating gland, reaching an overall 7-fold reduction by day 6 of lactation when compared with mammary tissue of virgin mice.

In the present study, we set out to determine where in the mammary gland (epithelia or stroma) the GHR and GHBP are expressed and to examine what causes the reduction in GHR and GHBP expression during pregnancy and lactation.

Materials and Methods

Animals and experimental design

All protocols for the care and use of animals in this study were approved by the Chancellor’s Animal Research Committee of the University of California, Santa Cruz. Timed pregnant (day plug found=day 0 of pregnancy) and age-matched female virgin Swiss-Webster mice were purchased from Simonsen Laboratories (Gilroy, CA, USA). All mice were kept in a temperature-controlled (18–25 °C) vivarium, with lights on between 0600 h and 2000 h, and food was available ad libitum.

Young virgin mice at three weeks of age were anesthetized by a single intra-peritoneal injection of Nembutal (90 µg/kg body weight). The right inguinal mammary gland (fourth pair) was cleared of epithelia by cauterization, whereas the gland on the left side was left intact. After surgery, the mice were divided into two groups. At 8 weeks of age, one group of mice was mated, and the remaining group was maintained as virgins until killed. Of the mated animals, some were killed on day 18 of gestation, but others were kept until after parturition. The parous mice nursed 8–14 pups each until the sixth day of lactation, when they were killed. Intact mammary tissues (left side) and epithelia-free fat pads (right side) from the fourth pair of glands were collected. The intact glands were either immediately processed to isolate epithelial cells or were frozen for later use to assess the levels of expression of the GHR and GHBP (see Fig. 1).

Figure 1 Schematic representation of the experimental procedure used for the studies. See Materials and Methods for a more detailed description.
**Isolation of epithelial cells from mouse mammary glands**

Intact inguinal mammary glands freshly dissected from 8-week-old nonpregnant (5–6 glands per pool), pregnant (1–4 glands per pool), and lactating (2–3 glands per pool) animals were minced, then incubated at 37 °C with gentle shaking for 1 h in Hanks balanced salt solution (HBSS) containing 0·004% DNAse and 0·1% collagenase (digestion medium). Three pools of epithelial cells for each physiological state (virgin, pregnant, lactating) were disassociated. After the dissociation was complete, the cell suspension was fractionated on a 38% continuous Percoll gradient. The epithelial cells were washed extensively in HBSS and any endothelial contaminants were removed by sedimentation. The purity of the resulting epithelial cell population was verified by microscopic analysis. The cells were frozen on dry ice and stored at −70 °C until RNA extraction.

**RNA analysis**

Total RNA was extracted from intact glands, epithelia-free fat pads and pools of epithelial cells (n=3) obtained from nonpregnant, pregnant, and lactating mice. Each of the tissue samples was processed independently for extraction and analysis of RNA.

RNA was isolated by the single-step guanidinium-isothiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987). The integrity of the extracted RNA was confirmed by gel electrophoresis. RNA concentrations were determined by spectrophotometry.

GHR and GHBP mRNA levels were quantitated by ribonuclease protection assay (RPA), as previously described (Ilkbahar et al. 1995). A 256-nucleotide (excluding the polylinker) antisense GHR/GHBP (GHR/BP) riboprobe and an 18S ribosomal (r) RNA riboprobe for use as an internal standard were generated according to the manufacturer’s directions, using MAXIScript and MEGAscript (Ambion, Austin, TX, USA) in vitro transcription kits respectively. The GHR/BP riboprobe was complementary to a 150-nucleotide portion of the mouse (m) GHR and mGHBP mRNA encoding the hormone-binding domain, shared by mGHR and mGHBP, as well as to 106 nucleotides of the mGHBP mRNA encoding the hydrophilic tail of the mGHBP. Therefore, in the RPA a 150-nucleotide fragment of this complementary RNA probe was protected by mGHR mRNA, whereas the full length of 256 nucleotides of the probe was protected by mGHBP mRNA, as demonstrated previously (Ilkbahar et al. 1995). Mouse 18S rRNA protects 80 nucleotides of the internal control probe. RPAs were performed on 10 μg total cellular RNA. Signal intensities of bands corresponding to GHR, GHBP and 18S rRNA were quantitated by phosphor-imaging.

After correction for background, the data were analyzed for heterogeneity of variance with Bartlett’s test. To achieve homogeneity of variance (P>0·05), the data were transformed to log scale, followed by one-way analysis of variance with completely randomized design. Additional analysis was carried out with Duncan’s multiple range test, in which P<0·05 was considered significant. Nontransformed data are shown in Fig. 2.

**mGHR and GHBP analysis**

Epithelia-free fat pads dissected from virgin (4 pools of 8–12 animals per pool), 18-day pregnant (3 pools of 6–10 animals per pool), 6-day lactating (5 pools of 9–12 animals per pool) mice, and intact mammary glands from identical virgins (5 pools of 8–12 animals per pool), 18-day pregnant (5 pools of 6–10 animals per pool), and 6-day lactating (5 pools of 2–9 animals per pool) mice were washed in 0·9% saline, immediately frozen after collection and stored at −70 °C.

Microsomal membranes of these tissues were prepared as described previously (Camarillo et al. 1998). Briefly, tissues were homogenized on ice, in 4 × (w/v) homogenization buffer (300 mM sucrose, 50 mM HEPES with protease inhibitors, pH 8·0) using a Polytron homogenizer. The homogenates were centrifuged at 20 000 g for 30 min. The resulting supernatant was centrifuged at 100 000 g for 1 h to pellet microsomal membrane fractions. The pellets were then washed in a buffer consisting of 50 mM HEPES, 10 mM EDTA and protease inhibitors (pH=7·5) and recentrifuged. Membrane proteins were solubilized by strong agitation of the microsomal membrane pellet suspended in RIA buffer (10 mM Na2HPO4, pH 7·5, 10 mM EDTA, 150 mM NaCl, and 0·01% (w/v) Thimerosal) containing 2% Triton X-100. An aliquot of each membrane preparation was assayed by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) to determine total protein concentration.

Mouse GHR (Camarillo et al. 1998) and mGHBP (Cramer et al. 1992) radioimmunoassays developed in our laboratory were used for determination of GHR and GHBP concentrations in the mammary tissue membrane preparations. Samples were diluted in RIA buffer with 2% Triton X-100 and each dilution was assayed in triplicate. The anti-mGHR antiserum was obtained by injection of a 14-amino acid synthetic peptide corresponding to the carboxyl terminus of mGHR. A synthetic peptide corresponding to the hydrophilic ‘tail’ of the mGHBP was used to immunize rabbits for the generation of anti-mGHBP antiserum. Since the sequence of the peptides used for immunization was unique to mGHR or mGHBP respectively, the generated antisera specifically recognize mGHR or mGHBP.

Mouse GHR and GHBP concentrations quantitated by RIA were normalized for total membrane protein concentration in each sample as determined by BCA assay. Differences in mGHR and mGHBP concentrations...
between whole intact glands and cleared fat pads at different physiological states were compared by analysis of variance followed by Fisher's protected least difference test. Differences were considered to be significant at \( P < 0.05 \). Results are expressed as means \( \pm \) S.E.M.

**Immunocytochemistry**

Paraffin-embedded sections of methacarn-fixed intact mammary glands and epithelia-free fat pads were immunostained for mGHR and mGHBP using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Deparaffinized and rehydrated tissue sections to be stained for mGHBP were immersed in diluted (1% in di-H\(_2\)O) Antigen Unmasking Solution (Vector Laboratories) and microwaved for two cycles of 5 min each at a setting of 700 W. This step was not necessary for GHR immunostaining. After blocking of nonspecific reactive sites by incubation in undiluted SuperBlock Blocking Buffer (Pierce) for 1 h, the following protocol was used. (1) Primary antisera (anti-GHR or anti-GHBP) or normal rabbit serum as control were diluted 1000-fold in Tris-buffered saline (TBS), 0.5% Tween 20 (Sigma, St Louis, MO, USA), 10% SuperBlock, and 0.01% NaN\(_3\), and added to tissue sections for a 1-h incubation at 22 °C. (2) Endogenous peroxidase was blocked by incubation in ImmunoPure Peroxidase Suppressor (Pierce) for 30 min. (3) Biotinylated secondary (goat anti-rabbit) antibody was diluted (0.5%, v/v) in TBS, 0.5% Tween 20, 10% SuperBlock, and 1.5% normal goat serum and was added to tissue sections for 30 min at 22 °C. (4) Conjugated avidin-biotinylated peroxidase-complex diluted 1:25 in TBS, 0.5% Tween 20 was added to the sections and incubated for 30 min at 22 °C. (5) Tissue sections were then stained with a substrate solution consisting of 0.05% diaminobenzidine and 0.02% H\(_2\)O\(_2\) (Vector Laboratories) in 0.1 M Tris–HCl (pH 7.2). Between each step, the sections were washed in TBS with 0.5% Tween 20. After immunostaining, the tissue sections were counterstained in Harris’ Modified Hematoxylin (Sigma).

On each slide, a control section was incubated with normal rabbit serum that replaced the primary antiserum for either GHR or GHBP. Otherwise, these sections were subjected to a protocol identical to that used on sections immunostained for GHR or GHBP. Other control sections were incubated with anti-GHR or anti-GHBP primary antiserum preabsorbed overnight at 4 °C with 20 µg/ml of the specific peptide to which each antiserum had been raised.

**Results**

**mGHR and GHBP mRNA profiles**

As expected, three sets of bands representing riboprobe fragments protected by mGHR mRNA, mGHBP mRNA or 18S rRNA were observed when total RNA extracted from mammary tissues was analyzed by RPA (Fig. 2). The 256-nucleotide band represents the full length of the mGHR/BP cRNA probe when protected by mGHBP mRNA, the doublet of about 150 nucleotides corresponds to the extracellular domain portion of the probe that was protected by mGHR mRNA, and a doublet of about 80 bases of the internal control probe was protected by 18S rRNA. As noted previously (Ilkbahar et al. 1995), we consistently observe the double bands corresponding to mGHR transcripts and to 18S rRNA and quantitate the two bands together to calculate mGHR
mRNA and 18S rRNA abundance. The likely cause for the mGHR mRNA signal being a doublet was hybrid instability at an A-U-rich region at the junction of exon 7, the last exon encoding the hormone-binding domain, and exon 8a, encoding the hydrophilic 'tail'. The double band we observed for the internal standard was most likely due to persistent secondary structures as a result of insufficient denaturation of rRNA.

The levels of expression of GHR and GHBP mRNA (Fig. 3) in intact inguinal mammary glands were decreased by 30% each in 18-day pregnant animals as compared with virgins. A further 3-fold decrease of both transcripts was observed in mammary glands of 6-day lactating mice. This is consistent with the progressive down-regulation of mGHR and GHBP mRNA expression in mammary tissues by gestation and lactation which we reported earlier (Ilkbahar et al. 1995).

GHR and GHBP mRNAs were expressed in both the parenchymal and stromal components of mouse inguinal mammary glands. The abundance of GHR and GHBP transcripts was significantly higher in epithelia-cleared fat pads (4- to 12-fold and 3- to 10-fold respectively) as compared with mammary epithelial cells, regardless of physiological state. There was no statistically significant difference in the levels of the transcripts among virgin, pregnant, or lactating mice, in either the stromal or the epithelial component of the gland.

Protein levels of mGHR and mGHBP

Throughout differentiation of the mammary tissue, concentrations of mGHR and mGHBP as assessed by RIA in both intact glands and the cleared fat pads, paralleled the changes in the expression of mGHR and mGHBP mRNA respectively (Fig. 4). Levels of GHR and GHBP proteins in intact glands were highest in nonpregnant mice (0·891 ± 0·15 pmoles/mg protein and 0·136 ± 0·026 pmoles/mg protein respectively), decreased during late pregnancy (0·354 ± 0·111 pmoles/mg protein and 0·178 ± 0·039 pmoles/mg protein respectively), and were lowest on day 6 of lactation (0·096 ± 0·037 pmoles/mg protein and 0·017 ± 0·006 pmoles/mg protein respectively). In the stroma, mGHR and mGHBP concentrations did not differ significantly among virgin (0·399 ± 0·155 pmoles/mg protein and 0·095 ± 0·012 pmoles/mg protein respectively), late-pregnant (0·552 ± 0·240 pmoles/mg protein and 0·171 ± 0·040 pmoles/mg protein respectively), and lactating states of the mammary tissue (0·278 ± 0·106 pmoles/mg protein and 0·040 ± 0·019 pmoles/mg protein respectively).

Immunocytochemical localization of GHR and GHBP in the mouse mammary gland

Specific staining for mGHR and mGHBP was observed in both the mammary stroma and parenchyma at all developmental stages analyzed. Regardless of physiological stage, the pattern of mGHR or mGHBP immunostaining remained similar among sections obtained from mammary tissues. Staining with anti-GHR revealed the strong localization of GHR to the plasma membrane and cytosol during each developmental state (Fig. 5, A-D). Nuclear GHR immunostaining was rare or absent at any given stage. In contrast, staining with anti-GHBP antiserum was present in the cytosol but most prominent in the nucleus throughout differentiation of the gland (Fig. 5, E-H). This shows the bulk of GHBP is localized internally, with minimal quantities at the plasma membrane. Replacement of primary antiserum with normal rabbit serum completely blocked specific GHR and GHBP immunostaining in virgin, late pregnant, and lactating glands, along with epithelia-free fat pads (Fig. 5, I-L). Preincubation of primary antiserum with the corresponding synthetic peptides also blocked specific immunostaining (not shown).

Discussion

We have previously shown that GHR and GHBP mRNA content decreases progressively during gestation in maternal inguinal mouse mammary glands and that there is a further decrease after parturition (Ilkbahar et al. 1995). In this report, we continued to study the expression of the GHR and GHBP in mouse mammary tissue. We have determined the levels of GHR and GHBP expression in both the epithelial and the stromal component of the mammary gland. In order to do that, we took advantage of the very slow growth rate of the mouse mammary epithelia before puberty, which leaves most of the mammary fat pad in prepubertal animals free of epithelium (Medina 1996). Using the method of DeOme et al. (1959), we surgically removed the rudimentary parenchyma of the right fourth mammary gland, leaving a large portion of the fat pad intact. The contralateral glands which we left intact were either used directly for protein and RNA assessment or served as a source for epithelial cells. This experimental approach allowed us to compare directly the expression levels of mGHR and mGHBP and their transcripts between the parenchyma, stroma, and the whole gland at three stages of development. We found that GHR and GHBP mRNAs and GHBP and GHR proteins were expressed in both epithelia-free fat pads and in isolated epithelial cells in virgin, 18-day pregnant, and 6-day lactating mice. At all three physiological stages, levels of the two transcripts were significantly higher in the fat pads than in the epithelium. Consistent with our previous observation, levels of both messages were highest in virgins, were down-regulated during pregnancy and were lowest in lactating animals. Expression levels of the GHBP and GHR proteins followed similar trends to the profile of mRNA content. GHR and GHBP mRNA, as well as GHBP and GHR protein levels remained comparable in
Figure 3 Mouse GHR (A) and mGHBP (B) mRNA abundance in intact inguinal mammary glands, and stromal and parenchymal components of the gland obtained from nonpregnant, 18-day pregnant and 6-day lactating animals. Each bar represents the means ± S.E.M. (n=3) of phosphor-imaging data. Bars with dissimilar superscripts differ significantly (P<0.05) as assessed by Duncan’s multiple range test. int, intact gland; adp, adipocytes; ept, epithelial cells.
Concentrations of GHR (A) and GHBP (B) in intact mammary glands or cleared fat pads of virgin, 18-day pregnant and 6-day lactating mice. Mouse GHR or mGHBP concentrations were determined by specific radioimmunoassays as described in Materials and Methods. Each bar represents means ± S.E.M. (n=3–5). Bars with dissimilar superscripts differ significantly (P<0.05) as assessed by Fisher's protected least difference test. int, intact gland; adp, cleared fat pad.

Figure 4
Figure 5 Immunocytochemical localization of GHR and GHBP (brown colour) by means of the avidin–biotin system in intact mammary glands of virgin, 18-day pregnant and 6-day lactating mice and in de-epithelialized mammary fat pads. Immunostaining with anti-GHR antiserum at a 1:1000 dilution is shown in virgin (A), late pregnant (B), and lactating (C) glands, and in an epithelia-free fat pad (D). Immunolocalization of GHBP using anti-GHBP antiserum at a dilution of 1:1000 is shown in virgin (E), late pregnant (F), lactating (G) mammary glands, and a cleared fat pad (H). Substitution of the primary antisera with nonimmune rabbit serum diluted 1:1000 (omission of primary antibodies) is shown in control sections of virgin (I), late pregnant (J), lactating (K) glands, and of an epithelia-free fat pad (L). Scale bars represent 10 µm.
fat pads and in epithelial cells among virgin, pregnant, and lactating mice.

This is the first study to provide a direct comparison between stromal and parenchymal contribution of overall GHR and GHBP expression in mammary tissue at both the message and the protein levels. Our results suggest that the gradual decrease in mGHR and mGHBP transcripts during pregnancy and postpartum in the intact maternal inguinal gland is due to the change in the ratio of epithelial to stromal tissues that takes place during pregnancy and continues after parturition, but is not caused by mammary gland-specific down-regulation of GHR/GHBP expression. Due to enormous proliferation of epithelial cells and the reduction in mass of the stroma, the parenchyma is responsible for a proportionally higher fraction of the overall gene expression in the whole mammary gland during gestation and lactation. Therefore, since mGHR and mGHBP are expressed at lower levels in the epithelium than in the stroma, the overall expression in the intact gland is reduced. This hypothesis is also supported by the fact that no difference was found in GHR/GHBP expression in either the epithelial-free stroma or the isolated mammary epithelial cells from virgin, pregnant and lactating mice, demonstrating that the GHR/GHBP levels remained unchanged in each tissue component (epithelia, stroma) of the mammary gland.

The localization of GHRs to epithelial cells and adipocytes of rat mammary glands both immunohistochemically (Lincoln et al. 1990, 1995) and by binding assays (Feldman et al. 1993b) is in accordance with our results. In the present study, GHBP was frequently localized to the cell nucleus in the mammary gland. Nuclear localization of the GHBP has been reported before in various rat tissues and several cell lines (for a review see Lobie et al. 1995). This nuclear localization of the GHBP could be of substantial physiological significance, as it has recently been demonstrated that GHBP enhances signal transducer and activator of transcription (STAT) 5-mediated transcription in response to GH and other cytokines (Graichen et al. 1998).

Growing evidence suggests a more central role for GH in mammary development and lactation, than was recognized before. It has long been known that pubertal mammary differentiation is triggered by estrogen in concert with pituitary hormones (Lyons 1958, Lyons et al. 1958). More recent studies indicate that GH is the pituitary factor which is essential for mammary development in rats (Kleinberg et al. 1990, Feldman et al. 1993a). Feldman and colleagues investigated the ability of native and mutant forms of GHS and PRLs to bind to rat liver GHRs and to stimulate the development of terminal end buds and alveolar structures in estrogen-primed mammary glands from immature, hypophysectomized, castrated, male rats. These investigators found that only GHS capable of binding with high affinity to GHRs induced mammary growth, whereas hPRL, rat PRL, recombinant human PRL, ovine PRL, although able to bind and activate lactogenic receptors, did not stimulate growth (Feldman et al. 1993a). End bud formation in mice (Silberstein & Daniel 1987) and in rats (Ruan et al. 1992) was also observed when GH was administered in slow-release pellets implanted locally, suggesting that the effects of GH on mammary development are local rather than systemic. A comparative study, again utilizing local implants, revealed that both hGH and rGH were significantly more potent mammary mitogens than hPRL and rat PRL (Kleinberg et al. 1990). In addition to its action as a mammogen, GH has also been shown to synergize with PRL to induce and maintain lactation and to regulate milk composition in murines. Studies in our laboratory (Markoff & Talamantes 1980, Thordarson et al. 1986) showed that mGH causes a significant increase in a-lactalbumin and casein secretion from mouse mammary epithelial cells in culture, in addition to enhancing the stimulatory effects of lactogens (mPRL and mouse placental lactogen) on the secretion of a-lactalbumin and insulin-like growth factor (IGF) binding proteins (English et al. 1991, Fielder et al. 1992). In lactating rats, treatment with an antiserum against rGH reduced milk yield by 50% (Madon et al. 1986) and bovine GH was able to maintain lactation in rats receiving bromocriptine as well as anti-rGH injections, although at reduced levels (Flint et al. 1992). In PRL-deficient lactating rats, GH was found to maintain the levels and activity of maternal acetyl-CoA carboxylase, the key enzyme involved in fatty acid synthesis, and of lipoprotein lipase, which is responsible for fatty acid uptake, hence suggesting that GH regulates the fat content of the milk (Barber et al. 1992, Flint et al. 1992).

The question of whether these mammogenic and lactogenic effects of GH are direct, mediated by IGF-I, or in concert with IGF-I is currently being investigated. Human GH induction of IGF-I mRNA expression in the rat mammary gland was first reported by Kleinberg and colleagues (1990). Previously, Imagawa et al. (1986) had shown that IGF-I is a mitogen for mouse mammary epithelial cells in culture and that IGF-I can mimic the proliferative effect of insulin. Deeks et al. (1988) demonstrated that optimal growth of rat mammary epithelial cells in culture requires supraphysiological concentrations of insulin, which can be substituted by low levels of IGF-I. These findings suggested that the mitogenic effect of insulin might be mediated through the high affinity type I receptors for IGFs. Indeed, IGF receptors have been found in bovine (Dehoff et al. 1988), ovine (Forsyth 1996), and rat (Lavandero et al. 1991) mammary tissue.

IGF-I is capable of mimicking the effect of GH in stimulating the development of mammary end buds and alveoli in hypophysectomized, castrated, estrogen-treated male rats, when administered locally in implanted pellets (Ruan et al. 1992). These studies indicate that IGF-I can replace GH as a mammogenic hormone in vivo. Several investigators have reported, however, that neither IGF-I
nor IGF-II is capable of substituting for GH to facilitate lactation in murines (Flint et al. 1992, Flint & Gardner 1994). We also showed that the stimulatory effects of GH on protein synthesis in cultured mouse mammary epithelial cells were not mediated through stimulation of IGF-I production, as no IGF-I was detected in the conditioned medium from these cultures (Fielder et al. 1992).

Kleinberg (1997) and colleagues have hypothesized that GH, by interacting with GHRs on stromal cells, induces the production of IGF-I, which then in a paracrine fashion acts synergistically with estrogen to stimulate the formation of end buds and alveoli. Several lines of evidence support this model. For example, IGF-I mRNA in human breast tissue localizes to stromal but not epithelial cells by in situ hybridization (Yee et al. 1989). Also, bovine mammary epithelium does not express IGF-I in culture (Campbell et al. 1991), although the bovine mammary gland expresses IGF receptors (Dehoff et al. 1988).

Recently, Marcotty and coworkers (1994) reported that IGF-I mRNA expression in whole inguinal and abdominal rat mammary glands decreases from the onset of pregnancy until parturition and drops sharply in lactating rats. This profile of IGF-I transcription is similar to the mGHR/BP expression profile in mouse mammary glands which we report here and, similarly, indicates that IGF-I is expressed mostly in the stromal component of the mammary gland. Taken together these studies strongly suggest that GH regulates the expression of IGF-I in the mammary stroma.

In conclusion, we have demonstrated that GHR and GHBP are expressed both in the epithelia and the stroma of the mouse mammary gland and that the levels of both proteins and their messages were higher in the stroma than in the epithelia. Immunocytochemical analysis also revealed that the GHBP is frequently found in the nucleus of the mouse mammary cells. We propose that GH may exert at least some of its effects in the mammary gland through stimulation of IGF-I in the stromal cells. The IGF-I then interacts with IGF-I receptors on the epithelial cells in a paracrine fashion to stimulate epithelial growth and differentiation. However, GH does not exert all its effects on the mammary gland through IGF-I, as it has been shown that GH stimulates protein synthesis in cultured mouse mammary epithelial cells where no IGF-I production was detectable, and IGF-I does not fully compensate for the lack of GH in stimulating milk production in rats.

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