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Cortactin-Binding Protein 90 (CBP90) Expression in the Mouse Mammary Glands during Prolactin-Induced Lobuloalveolar Development

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ABSTRACT—We have previously performed suppression subtractive hybridization to identify genes that were induced during prolactin (PRL)-driven lobuloalveolar development of the mammary gland. This suggested that cortactin-binding protein 90 (CBP90), which is known to be a brain-specific protein that binds to cortactin, was expressed under the regulation of PRL in the mammary glands (preliminary observation). In this study, the expression of CBP90 was examined in the mammary glands of mice under manipulated hormonal circumstances. PRL treatment by 9 days of pituitary grafting induced CBP90 expression in the normal mammary glands but not in the cleared fat pads, while cortactin was expressed constitutively in both the normal mammary glands and the cleared fat pads. Unlike milk proteins, longer treatment with PRL (36 days of pituitary grafting) did not increase the expression level of CBP90 mRNA, while it slightly increased the cortactin mRNA level. Mammary CBP90 mRNA expression was induced by pituitary grafting but not by progesterone treatment in PRL-deficient mice, while pituitary grafting induced mammary CBP90 expression in ovariectomized PRL-deficient mice only when estrogen and progesterone were appropriately supplemented to permit the formation of alveolar buds. The CBP90 protein was detected by immunohistochemistry in the luminal epithelium of the alveolar buds and more faintly in the ductal epithelium. Thus, from the unique expression pattern, CBP90 may be useful as a molecular marker for the hormone-stimulated development of mammary alveolar buds.

Keywords: mammary gland, cortactin-binding protein 90, lobuloalveolar development; prolactin knockout mouse; molecular marker

INTRODUCTION

The mammary gland originates from a simple ductal-alveolar system that invades the cutaneous fat pad with branching to form an advanced ductal-alveolar system after puberty. The ductal system becomes decorated with alveolar buds to form a lobuloalveolar system during maturation and pregnancy, and finally produces milk during lactation (Horseman, 1999). Studies on gene-disrupted mice have shown that prolactin (PRL) and its paralogues from the placenta are essential for the genesis, development and maintenance of the alveolar buds as well as for lactation

(Horseman, 1997; Brisken *et al.*, 1999; Vomachka *et al.*, 2000). Although the morphological and physiological changes in the mammary glands associated with pregnancy and lactation are fascinating at any steps, studies on lactogenic hormone actions have generally been focused on the biosynthesis of milk proteins and carcinogenesis. How lactogenic hormones direct the development of the alveolar buds and what molecules are involved in the reorganization of mammary physiology have been less explored, although the roles of some molecules such as transforming growth factor- β , parathyroid hormone-related protein (PTHrP) and osteoprotegerin ligand (OPGL) have been successfully reported in the developing mammary glands (Gorska *et al.*, 1998; Dunbar and Wysolmerski, 1999; Fata *et al.*, 2000). Accumulation of information on the expression of many other genes is necessary for the better understanding of

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Table 1. PCR primers and conditions

| Gene Name | | Primer Sequences (5' 3') | Amplified region (nt) | Product Size (bp) | Annealing Temperature (°C) | Number of Cycles |
|-----------|-----------|---------------------------|-----------------------|-------------------|----------------------------|------------------|
| CBP90 | Sense | CCTACGGTGCACAGTTTACATTAC | 1267–2146 | 880 | 62 | 29 |
| | Antisense | TGACATTTCCCTGGGCAGCAGCTTG | | | | |
| Cortactin | Sense | TTGACAAGGACAAGGTGGATAAAAG | 602–1265 | 664 | 64 | 30 |
| | Antisense | TCATAGATGGGGCTGGAGGGTGGTC | | | | |
| GAPDH | Sense | ACCACAGTCCATGCCATCAC | 520– 971 | 452 | – | – |
| | Antisense | TCCACCACCTGTTGCTGTA | | | | |

morphogenesis and physiology of the PRL-driven mammary development.

Recently, suppression subtractive hybridization screening has been performed for the genes induced by PRL in the developing mammary glands in order to determine the genes regulated by PRL in the mammary glands (unpublished data). The result suggested that cortactin-binding protein 90 (CBP90), a 'brain specific' protein that binds to cortactin, was expressed under the regulation of PRL in the mammary glands. The function of CBP90 is currently unknown except that it interacts with cortactin and modifies cortactin functions (Ohoka and Takai, 1998). Cortactin is a perimembrane protein that binds to filamentous actin and mediates downstream signaling of receptor tyrosine kinases and, potentially, G protein-coupled receptors (Huang *et al.*, 1998; Zitzer *et al.*, 1999; Higgs, 2001). The cortactin signal is also associated with the invasion of breast cancers (Kairouz and Daly, 2000). Therefore, CBP90 may play a role in the PRL-induced mammary development through the modification of the cortactin signal. In the present study, we characterized CBP90 as a PRL-dependently regulated molecule in the mouse mammary epithelial cells.

MATERIALS AND METHODS

Animals and hormone treatment

Adult female PRL-deficient (PRL^{-/-}), heterozygous (PRL^{+/-}) and wild type C57Bl/6J mice were kept under the conditions described elsewhere (Hou *et al.*, 2000). Pituitary grafting and ovariectomy were performed in 14 to 16-week-old mice as described (Matsuda and Mori, 1996). Steroid hormone treatment was performed by the subcutaneous grafting of steroid-containing beeswax tablets (Markaverich *et al.*, 1984). The amount of estradiol-17 β and progesterone given to a mouse was 1 μ g and 8 mg, respectively, unless noted otherwise in the text. The steroid tablets were replaced with new ones every 9 days. Mammary cleared fat pads were made by removal of epithelial components from the inguinal (4th) mammary glands in 3-week-old mice (Young, 2000).

RT-PCR

The inguinal (4th) mammary gland was stored at -80°C after careful removal of the lymph node. Total RNA was prepared with TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH) from the frozen tissue, and was treated with DNase I (Promega Corp., Madison, WI). The RNA from 3-5 mice were pooled. The first strand cDNA was made from 2 μ g of total RNA with random hexamers as described (Matsuda and Mori, 1997). The cDNA originat-

ing from 0.1 μ g of RNA was subjected to PCR with Takara Ex Taq Hot Start Version (Takara Shuzo K.K., Tokyo, Japan) in 24- μ l reaction volume. The primer sequence, annealing temperature and number of cycles are listed in Table 1. Each PCR cycle was composed of 95°C for 20 sec, the annealing temperature for 30 sec and 72°C for 1 min.

"Virtual" northern hybridization

"Virtual" northern hybridization is an alternative method to conventional northern hybridization, in which full-length cDNA is used instead of RNA (Franz *et al.*, 1999). Pooled total RNA was prepared as described above. Poly A⁺ mRNA was recovered with Oligotex-dT30 (Qiagen, Inc., Valencia, CA). Full-length cDNA was made with SMARTTM PCR cDNA Synthesis Kit (Clontech, Inc, Palo Alto, CA) and amplified by 14 cycles of PCR according to the manufacturer's instructions. The PCR product (0.5 μ g) was electrophoresed on a 1.5% agarose gel and transferred onto a Hybond N⁺ membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The membrane was hybridized with alkaline phosphatase-labeled DNA probes generated with AlkPhos Direct DNA Labeling Kit (Amersham Pharmacia Biotech). The signal was detected by CPD-StarTM chemiluminescence on an ECL film (Amersham Pharmacia Biotech).

The probe for CBP90, cortactin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was made from a plasmid insert that contains an RT-PCR product of each gene. The primer sequences are shown in Table 1.

Immunohistochemistry

Tissue was fixed in 4% paraformaldehyde-PBS, embedded in paraffin, and cut in 5- μ m sections. After rehydration, the sections were incubated in 10 mM boric acid (pH6.0) at 95°C for 10 min, treated with 0.3% H₂O₂ in methanol for 30 min, and treated with PBS containing 10% normal rabbit serum for 10 min at 25°C. They were reacted with 1:2000-diluted rabbit polyclonal antiserum specific to CBP90 or preimmune serum at 4°C for 16 h. The anti-CBP90 antiserum was kindly donated by Dr. Yoshiharu Ohoka (Osaka University). The specific signal was visualized using Vectastain Elite ABC-PO Kit (Vector Laboratories Inc., Burlingame, CA). Pictures were taken with 'a' Nomarski differential interference contrast microscopy.

RESULTS

CBP90 and cortactin in PRL-stimulated mammary glands and cleared fat pads

Transplantation of an anterior pituitary gland under the kidney capsule is widely used for stimulation of animals with PRL (Matsuda *et al.*, 1994). Expression of CBP90 mRNA was examined in the mammary glands and the cleared fat

pads in pituitary-grafted wild-type mice by "virtual" northern hybridization (Fig. 1). A major 6 kb product and two minor bands at 4.2 and 2.8 kb were observed in the mammary tissue of pituitary-grafted mice, while the mammary glands before pituitary grafting contained a minimal level of CBP90 mRNA. Though the identity of multiple CBP90 transcripts has never been reported, they may be products of alternative splicing or artifacts of PCR amplification of full-length cDNA. The expression level of CBP90 was undetectable in the cleared fat pads, even after pituitary grafting. The signal from CBP90 appeared to be reduced by the longer (36 days) treatment with the pituitary graft. However, GAPDH signal was also weaker in this sample, probably due to an artifact on loading sample or due to the great increase in milk protein cDNA in the sample. β -Casein and whey acid protein gene expression was induced slightly by 9 days, and markedly by 36 days of pituitary grafting (data not shown).

Cortactin mRNA expression was also examined in the mammary glands and cleared fat pads in pituitary-grafted mice by "virtual" northern hybridization and RT-PCR. Unlike

CBP90, cortactin was constitutively expressed both in the cleared fat pads and in the mammary glands during the pituitary graft-induced lobuloalveolar development. The 36-day treatment appeared to increase the gene expression modestly. Examination by RT-PCR revealed that, among 3 types of cortactin, which differ in the number of cortactin repeats, the shortest cortactin C isoform was dominant in the mammary glands.

Hormonal control of the mammary alveolar development and expression of CBP90

The effects of estrogen, progesterone and PRL on mammary CBP90 expression was examined during the lobuloalveolar development in PRL^{-/-} mice. Adult female virgin mice were ovariectomized, and two weeks later they were treated with various combinations of PRL (by pituitary grafting), estradiol-17 β and/or progesterone for 9 days as shown

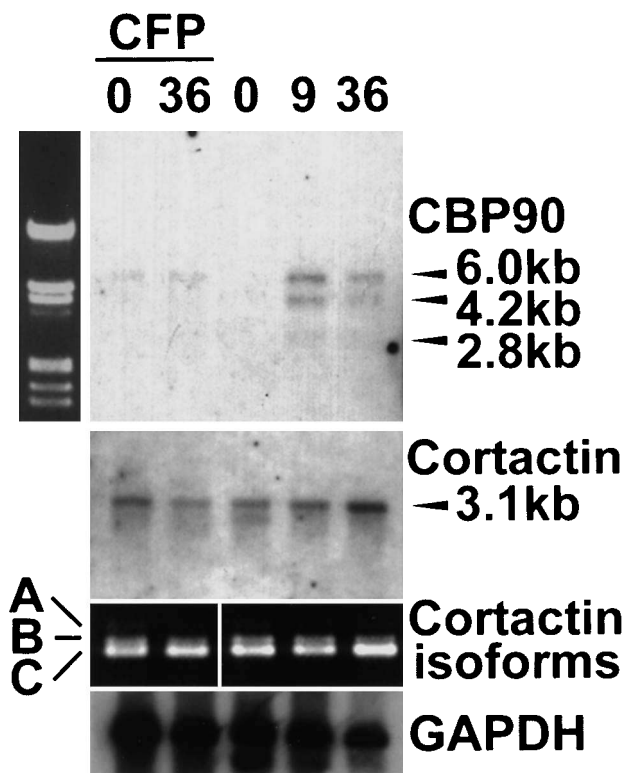


Fig. 1. Expression of mRNA for CBP90 and cortactin in the mammary glands and cleared fat pads in pituitary-grafted wild-type mice. PCR-amplified full-length cDNA from the cleared fat pads (left two lanes) and mammary glands (right three lanes) from 14 to 16-week-old wild-type mice after pituitary grafting was subjected to PCR (for determination of cortactin isoforms) or "virtual" northern analysis (for CBP90, cortactin and GAPDH). The numbers at the top show the days after pituitary grafting. The upper left lane indicates molecular size markers (λ -DNA digested with *EcoR* I and *Hind* III). The lower middle panel shows the ethidium bromide staining of PCR products for cortactin isoforms A (664 bp), B (550 bp) and C (452 bp). CFP, cleared fat pad.

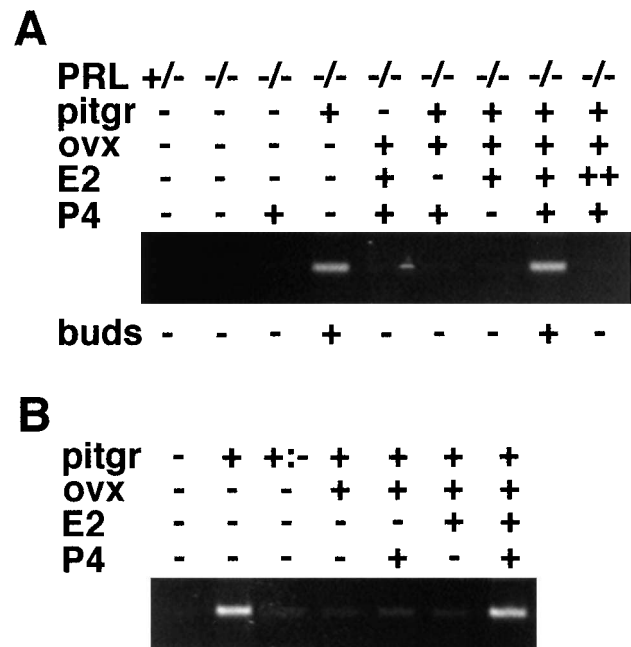


Fig. 2. Effects of pituitary grafting, estrogen and progesterone on the mammary CBP90 gene expression in PRL-deficient mice. Mammary expression of CBP90 mRNA was examined by RT-PCR in 14 to 16-week-old female virgin mice treated with various combination of hormones. (A) Effects of hormones on mammary CBP90 expression during the lobuloalveolar development. Two weeks after ovariectomy (ovx), mice were treated with combinations of pituitary grafting (pitgr), estradiol-17 β (E2) and progesterone (P4) as indicated above the lanes. Nine days later, mammary expression of CBP90 mRNA was examined. Lane 1 is the heterozygous (PRL^{+/-}) control, and the others are from PRL^{-/-} mice. Occurrence of alveolar bud development is indicated at the bottom (buds). (B) Effects of hormones on the maintenance of mammary CBP90 expression. Mice were pretreated with pituitary grafting for 18 days to induce mammary alveolar bud formation and CBP90 expression. Then they were treated with combinations of ovariectomy, estradiol-17 β and progesterone as indicated above the lanes. Alternatively, in a group of mice (lane 3), the elevated PRL level was restored by excising the ectopically grafted pituitary gland (pitgr +/-). Eighteen days later, mammary expression of CBP90 mRNA was examined.

in Fig. 2A. Since progesterone is secreted from the ovary upon PRL stimulation, it has a potential to mediate the systemic action of PRL. As a result, progesterone treatment alone or a normal level of PRL in the wild-type mice failed to induce the mammary CBP90 expression, which confirms the importance of a high level of circulating PRL. In ovariectomized

mice, only the group treated with pituitary grafting, estrogen and progesterone expressed a high level of CBP90 mRNA and had alveolar buds. In addition, a high dose (20 μg) of estrogen prevented the gene expression, and also suppressed alveolar bud formation. Thus, PRL, estrogen and progesterone must be supplied appropriately for the mammary glands to express CBP90 mRNA. Correspondingly, the hormonal conditions that allow mammary CBP90 expression were identical to the conditions appropriate for the development of alveolar buds.

The effects of estrogen, progesterone and PRL on the maintenance of mammary CBP90 expression were further examined in previously pituitary-grafted PRL^{-/-} mice. After 18 days of pituitary grafting, some were ovariectomized and given steroids, and in others the engrafted ectopic pituitary was surgically excised. Finally, the mammary CBP90 expression was examined after 18 additional days (Fig. 2B). Here, expression of mammary CBP90 mRNA was maintained only in the mice with a pituitary graft, estrogen and progesterone. Interestingly, mammary glands of mice treated with both pituitary grafting and progesterone after ovariectomy did not contain detectable CBP90 mRNA, although they were still decorated with alveolar buds. In contrast, sustained treatment with pituitary grafting and estrogen without progesterone after ovariectomy did not maintain alveolar buds or CBP90 expression in the mammary gland.

Distribution of CBP90 protein in the mammary gland

The distribution of CBP90 in the mammary glands of wild-type mice was examined by immunohistochemistry (Fig. 3). Specific signals for CBP90 were obtained from the mammary epithelium after 36 days of pituitary grafting, especially in luminal epithelium of both the alveolar buds and mammary ducts (Fig. 3B). The ductal signals were weaker than the signals of alveolar buds. The duct epithelium of the mammary glands untreated with pituitary grafting were negative for CBP90 (Fig. 3A), as well as the section stained with preimmune serum (Fig. 3C).

DISCUSSION

PRL-dependent expression of mammary CBP90 was evident in this study, since progesterone, whose secretion is stimulated by PRL, did not induce mammary CBP90 by itself. Estrogen and progesterone were also required for mammary CBP90 expression, as supplement of both estrogen and progesterone was required for the induction of CBP90 expression in the ovariectomized, pituitary-grafted mice, and as PRL-induced CBP90 expression was maintained after ovariectomy only if both estrogen and progesterone were supplemented. Furthermore, CBP90 was expressed especially in the mammary epithelium of alveolar buds. Even though CBP90 expression was not maintained in alveolar buds after estrogen removal from the general circulation, CBP90 expression was achieved only in the presence of ovarian-steroid and PRL levels that were appro-

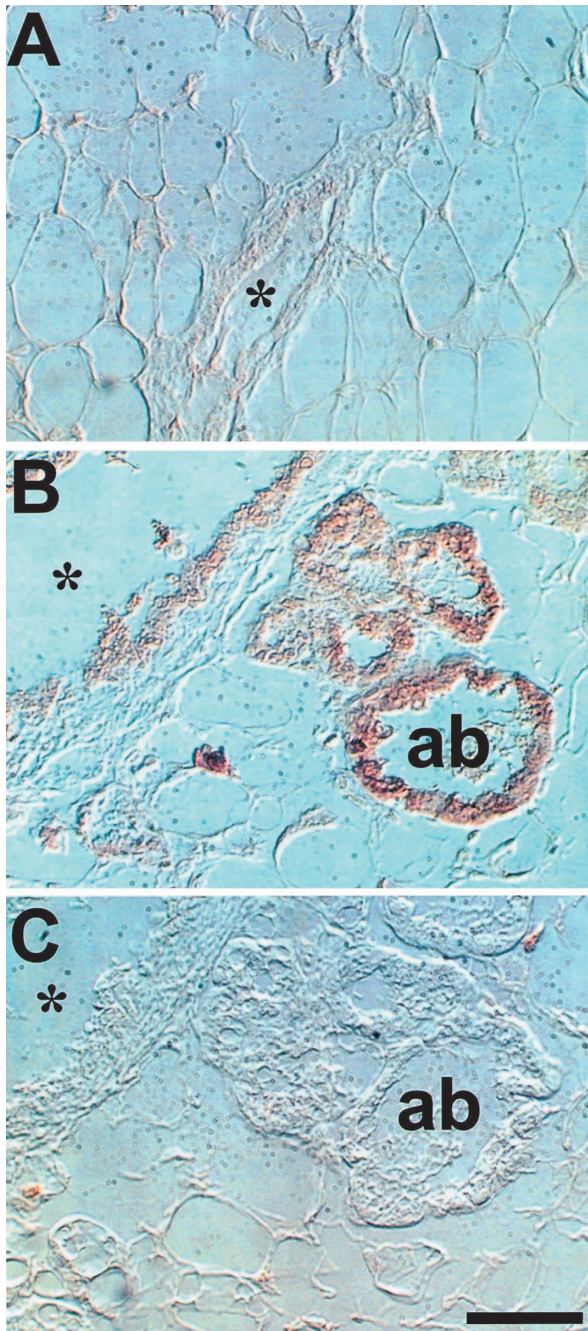


Fig. 3. Expression of CBP90 protein in the mammary glands. Fourteen to 16-week-old virgin female wild-type mice were transplanted with pituitary glands and killed after 36 days. Distribution of CBP90 was examined by immunostaining of the mammary gland of non-treated (A) and pituitary-grafted (B) mice. Pictures were taken with Nomarski differential interference contrast microscopy. Negative control for panel B using preimmune serum instead of the primary antiserum is shown (C). ab, alveolar bud; *, duct. Bar = 50 μm .

prate for the development of mammary alveolar buds. Thus, CBP90 may be a useful marker for the development of alveolar buds.

CBP90 was first characterized by Ohoka and Takai (1998) as a cortactin-binding protein that binds to the src homology 3 (SH3) domain of cortactin through a proline-rich domain in the C-terminal region. So far, the function of the protein is unknown except that it interacts with cortactin and may modify cortactin functions. Cortactin, the product of the EMS1 gene, is a perimembrane signaling protein that binds to filamentous actin and mediates downstream signaling of receptor tyrosine kinases (Huang *et al.*, 1998; Higgs, 2001). Cortactin is seen in cell types with actin-based contractile capacity, including myoepithelium (Wu and Montone, 1998) and many other cells including mammary cancer cells (for review, see Weed and Parsons, 2001). The expression of cortactin is increased in some breast carcinomas, and overexpression of the gene modifies the invasive properties of the mammary cells (Kairouz and Daly, 2000). Cortactin also interacts with somatostatin receptor subtype 2 (SSTR2) through cortactin-binding protein 1 (Zitzer *et al.*, 1999), while SSTR2 mediates growth inhibitory signal of somatostatin in the mammary cells (Hatzoglou *et al.*, 2000). Though it is unknown which cortactin isoform is major in other tissues, the isoform C, which was the major isoform in the mammary gland, is reported to bind to CBP90 (Ohoka and Takai, 1998). These findings suggest that cortactin is involved in the regulation of morphogenesis and growth of the mammary epithelial cells, and imply that CBP90 plays a role in the PRL-induced mammary development through modifying the cortactin function.

CBP90 has been introduced as a brain-specific molecule that is exclusively expressed in the brain. Interestingly, information of mammary expression of traditional 'brain-specific' or 'neuron-specific' marker proteins is being accumulated (*e.g.*, Schumacher *et al.*, 1994). In fact, a brain-specific type of aldolase was also found in the PRL-stimulated mammary epithelium in our study (unpublished data). Molecular similarities in hormone-stimulated mammary epithelial cells and the brain, together with a similarity on signaling molecules between cells of the mammary tissue and those of the bone during pregnancy and lactation (*i.e.*, PTHrP, OPGL, etc.; Dunbar and Wysolmerski, 1999; Fata *et al.*, 2000), may be useful to understand the evolutionary history of the mammary glands. On the other hand, hormone-stimulated mammary glands have a much simpler organization than the brain and may provide a good experimental model on studying the function of some 'brain-specific' molecules.

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