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Aldolase C/Zebrin Gene Regulation by Prolactin During Pregnancy and Lactation

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Prolactin (PRL) is necessary for the genesis of mammary alveolar buds and for lactation. A cDNA library enriched for PRL-dependent genes was made by suppression subtractive hybridization. Aldolase C/zebrin (AldC/zebrin), a "brain-specific" aldolase, was found to be PRL-dependent in the mouse mammary glands. AldC/zebrin was preferentially expressed in the alveolar buds. Expression of the gene in the ovary was also evident. During pregnancy, mammary AldC/zebrin mRNA levels were elevated beginning at midpregnancy (d 10 of pregnancy) in accordance with the genesis of the lobuloalveolar system, and the expression level was gradually increased through the end of pregnancy. Lactating mammary gland contained a very high level of AldC/zebrin mRNA, and the gene expression decreased during involution. By contrast, levels of aldolase A and B mRNA expression in the mammary glands were less affected by pregnancy and lactation. The selective regulation of AldC/zebrin may contribute to a shift in nutrient metabolism during pregnancy and lactation to facilitate epithelial growth and biosynthesis of milk constituents.

Key Words: Mammary gland; glucose metabolism; gene profiling.

Introduction

Pregnancy and lactation require profound physiologic adaptations in many maternal organ systems, which are controlled by the actions of reproductive hormones from the pituitary gland, ovaries, and placenta *(1)*. Although the physiologic changes associated with pregnancy and lactation are essential for mammalian reproductive success, studies of lactogenic hormone actions have generally been focused narrowly on questions regarding the biosynthesis of milk proteins or carcinogenesis. The broader questions of how lactogenic hormones direct the "homeorhetic" reorganization of mammary and maternal physiology, and what molecular events underlie these adaptations have been less well explored. The mammary glands originate from rudimentary accumulations of epidermal cells in the embryo and form a primary tubular system among the skin fibroblasts prenatally. After estrogen levels increase during puberty, the mammary epithelial tubules invade the fat pad with branching to form an advanced tubular system. The epithelial ducts become decorated with subordinate branches and alveolar buds, especially during pregnancy. Finally, the mammary glands develop an extensive lobuloalveolar epithelial network, which produces milk during lactation. After weaning, involution of the mammary glands occurs, and the mammary epithelium stays at a retarded state until the next pregnancy *(1)*. These irreversible events and reversible cycles of the mammary gland are regulated mainly by peptides from the pituitary gland and placenta, and steroids from the ovary and placenta. In mice with targeted disruptions of the prolactin (PRL) or PRL receptor genes, the mammary glands comprise a fully developed tubular epithelial system but lack subordinate branches and alveolar buds *(2–5)*. These results provide genetic proof that direct and/or indirect PRL signaling is essential for the development of the mammary lobuloalveolar system, as well as for milk synthesis.

We are interested in discovering genes that are involved in breast development and the homeorhetic adaptation of the mammary glands during PRL-driven mammogenesis and lactation. Suppression subtractive hybridization (SSH) screening was performed to identify genes regulated by PRL in the mammary glands. Expression of several genes was shown to be PRL dependent, and Aldolase C/zebrin (AldC/zebrin) was among them. AldC/zebrin is one of three isoforms of the glycolytic/gluconeogenic enzyme, fluctose-1,6-bisphosphate aldolase (Ald), which catalyzes the bidirectional conversion of fructose-1,6-bisphosphate with glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The expression of AldC/zebrin is ubiquitous during early development but is highly restricted to neuronal cells in the brain in adulthood *(6,7)*. AldC/zebrin is also known as the antigen identified by the Zebrin II monoclonal antibody, which has long been used for recognition of Purkinje cells in the adult cerebellar cortex *(8)*. Outside of the brain, AldC/zebrin is expressed in actively proliferating cancerous cells *(6,9)*.

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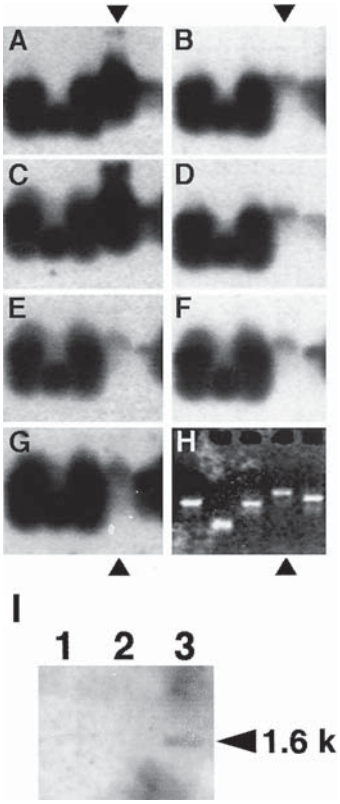

Fig. 1. Screening of mammary cDNA library by polymerase chain reaction–Southern hybridization. For primary screening, the PCR-amplified inserts of plasmid clones were transferred to membranes and hybridized with (**A**) pitgr/P_4 and (**B**) pitgr/cont forward-subtracted probes; (**C**) pitgr/cont forward- and (**D**) reverse-subtracted probes; and nonsubtracted probes made from cont (**E**), P_4 (**F**), and pitgr (**G**) cDNA. (**H**) Image of PCR products in an ethidium biomide–stained agarose gel. The arrowhead indicates a pitgr-dependent band. Secondary screening was done with AldC/zebrin cDNA probe by virtual Northern hybridization (**I**). The probe hybridized with a 1.6-kbp product (large arrowhead) contained in pitgr cDNA (lane 3) but not with cont and P_4 (lanes 1 and 2, respectively).

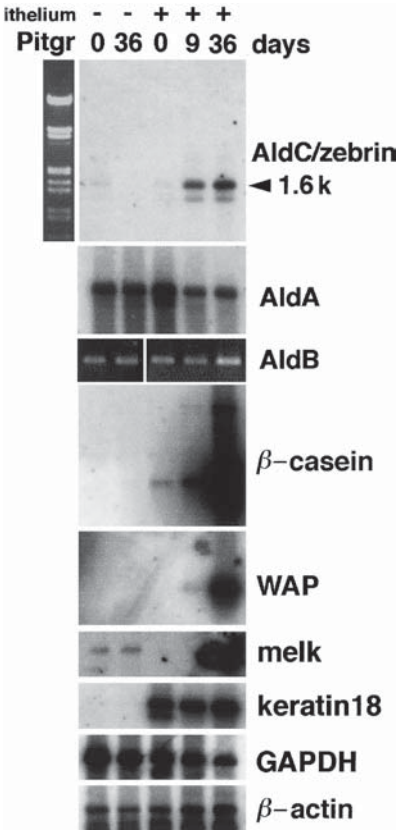

Fig. 2. Expression of mRNA for Ald and other genes in the mammary glands and cleared fat pad in pituitary-grafted PRL-KO mice. Three isoforms of Ald, two milk proteins (β-casein and WAP), a fat pad marker (melk), an epithelical marker (keratin 18), and the housekeeping genes (GAPDH and β-actin) were analyzed. PCR-amplified Smart® cDNA from the cleared fat pad (left two lanes) and mammary glands (right three lanes) were subjected to PCR (for AldB) and virtual Northern analysis (for the other genes). The term of the hormonal stimulation is indicated above the lanes. The upper left panel indicates the molecular size marker, λ-DNA digested with *Eco*RI and *Hind*III.

Here, we introduce a novel relationship between PRL, which is an essential mammotrophic hormone, and mammary expression of AldC/zebrin, which has been considered to be a "brain-specific" enzyme.

Results

Identification of AldC/Zebrin by SSH Cloning

A representative example of the primary screening of cDNAs cloned by SSH is shown in Fig. 1. A clone was positive to cDNA from pituitary graft treatment subtracted by progesterone (P_4) or control (cont) tissue cDNA, but not to the reverse-subtracted or nonsubtracted probe (Fig. 1A–H). Thus, the clone appeared to be induced by PRL, but not secondarily regulated by PRL via its stimulatory effect on P_4 secretion from the ovary. In the secondary screening by virtual Northern hybridization, 17 clones derived from eight different genes were finally determined to be PRL dependent. One of the eight genes was determined to be AldC/zebrin when the cDNA was sequenced, and a 1.6-kb cDNA of AldC/zebrin was observed in reverse-transcribed mRNA from mammary of gland pituitary-grafted (pitgr) mice (Fig. 1I). Because AldC/zebrin had never been reported in the mammary gland and was considered to be brain specific, we chose to follow up this gene with more detailed studies.

Expression of AldC/zebrin mRNA was examined in the mammary glands and cleared mammary fats pads of pitgr wild-type (WT) mice by virtual Northern hybridization (Fig. 2). High-level expression of the 1.6-kb product was observed only in the PRL-stimulated mammary glands, whereas only a trace level was evident in the mammary glands without the hormonal stimulation. Cleared fat pad expressed very low levels of AldC/zebrin mRNA even after pituitary grafting. Two typical milk proteins, β-casein and whey

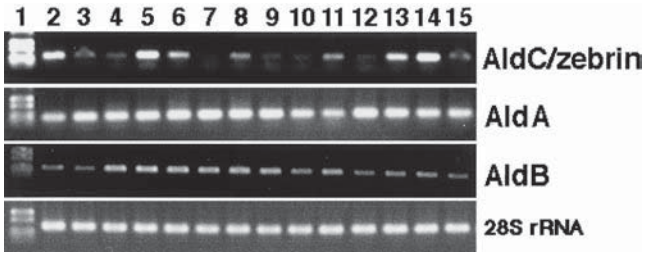

Fig. 3. Distribution of three types of Ald mRNA in virgin female mice. Expression of mRNA of AldA, AldB, and AldC was examined by reverse transcriptase (RT)-PCR. Lane 1, size marker; lane 2, mammary glands after 9 d of pitgr treatment; lanes 3–15, organs from normal intact mice (lane 3, mammary glands without hormone treatment; lane 4, liver; lane 5, brain; lane 6, kidney; lane 7, heart; lane 8, lung; lane 9, spleen; lane 10, thymus; lane 11, salivary gland; lane 12, abdominal fat body; lane 13, adrenal gland; lane 14, ovary; lane 15 uterus).

acidic protein (WAP), were expressed moderately at 9 d and strongly in the mammary tissue at 36 d after the pituitary grafting. Unlike the milk protein genes, mammary AldC/zebrin expression was stimulated by 9 d of pituitary grafting and was not markedly increased by further treatment with PRL (9 d vs 36 d). Semiquantitative PCR analysis using serially diluted cDNA samples suggested that the content of AldC/zebrin transcript (vs glyceraldehydes phosphate dehydrogenase [GAPDH] or β-actin) in the mammary tissue was increased by approx 32-fold by 9 d of pituitary grafting (data not shown). This increase was not caused simply by an increase in epithelial component in the tissue sample because a marker of epithelium, K18, mRNA content relative to either GAPDH or β-actin was not affected by PRL. The melk mRNA, which was expressed in the stroma of cleared mammary fat pads, was not detectable in normal mammary glands, confirming that the majority of cDNA in the whole-mammary-glands cDNA sample was derived from the epithelium. In contrast to AldC/zebrin, mammary AldA mRNA expression was decreased to about 20–30% of the control level by pituitary grafting (quantitative data not shown). AldB was not detected by virtual Northern hybridization but was detectable by PCR, and the expression level did not appear to be altered by PRL.

The sequences of coding and 5'-flanking region of mammary AldC mRNA were identical to the cDNA previously reported in the brain.

Expression of Ald/Zebrin mRNAs in Mammary Glands and Other Organs

Expression of mRNA of AldA, AldB, and AldC was examined in the mammary glands and other organs in virgin female PRL-knockout (KO) mice (Fig. 3). High-level expression of AldC mRNA was observed in the brain (lane 5) and ovary (lane 14). AldC/zebrin was highly expressed in hormone-stimulated mammary glands (9 d of pituitary grafting) as well (lane 2). Pituitary grafting did not affect the expression of AldC mRNA in other organs other than mammary gland (data not shown). Compared with AldC/zebrin, AldA and AldB appeared to be more ubiquitously distributed among tissues of the body.

Hormonal Control of Mammary Alveologenesis and Expression of AldC/Zebrin

Effects of estradiol 17-β (E_2) and P_4 on the genesis of mammary alveolar buds and AldC/zebrin expression were examined in pitgr $PRL^{-/-}$ mice. Adult female virgin mice were ovariectomized (OVX), and 2 wk later they were treated with PRL (by pituitary grafting), 1 mg (+) or 20 mg (++) of E_2 and/or 8 mg of P_4 for 9 d, as shown in Fig. 4A. Mammary glands 9 d after pituitary grafting were comparable with those of d 10 of pregnancy. Among OVX pitgr mice, only the group treated with both E_2 and P_4 had alveolar buds and expressed a high level of AldC/zebrin mRNA (Fig. 4B,C). Thus, PRL, E_2, and P_4 were necessary for the development of the alveolar buds and maximal induction of AldC/zebrin expression. In addition, a high dose of E_2 attenuated the AldC/zebrin expression and alveolar bud formation (Fig. 4H). Expression of β-casein was also induced under the conditions in which alveolar buds were formed. WAP was not detected under the conditions of 9 d of hormone exposure in this experiment. Thus, AldC/zebrin expression appeared to be linked to the genesis of alveolar buds, AldC/zebrin and β-casein were similarly regulated by hormones during the early stages of lobuloalveolar development, whereas WAP was not induced.

In another set of experiments all mice were given pituitary grafts. After 18 d, some were OVX and given steroids, and some were surgically removed of the grafted ectopic pituitary. Finally, the mammary tissue was examined after an additional 18 d (Fig. 5). Highly developed mammary glands with clustered alveolar buds were observed after 36 d of pituitary graft, which resembled the tissue in mice of late pregnancy. High levels of AldC/zebrin, WAP, and β-casein mRNAs were expressed in the mammary glands. On the other hand, mammary alveolar buds involuted, and expression of AldC/zebrin, and WAP decreased following removal of the PRL-producing pituitary graft. Interestingly, the experimentally "involuted" mammary glands were still decorated with small alveolar buds and obviously differed from the virgin mammary glands without a history of PRL stimulation, in spite of the fact that circulating PRL was not detectable by radioimmunoassay (RIA) in these mice. The involuted glands retained a somewhat higher level of β-casein mRNA than that observed in nonstimulated mammary glands. Ovariectomy also induced a mammary involution and a decrease in AldC/zebrin mRNA content, and treatment with E_2 and P_4 compensated for the effect of ovariectomy. The mammary glands of OVX and E_2-treated mice looked involuted, with dilated and thickened ducts, but no lobuloalveoli. Those of OVX, P_4-treated mice were still decorated with many alveolar buds. Mammary expression of AldC/zebrin and WAP in OVX mice supplemented with

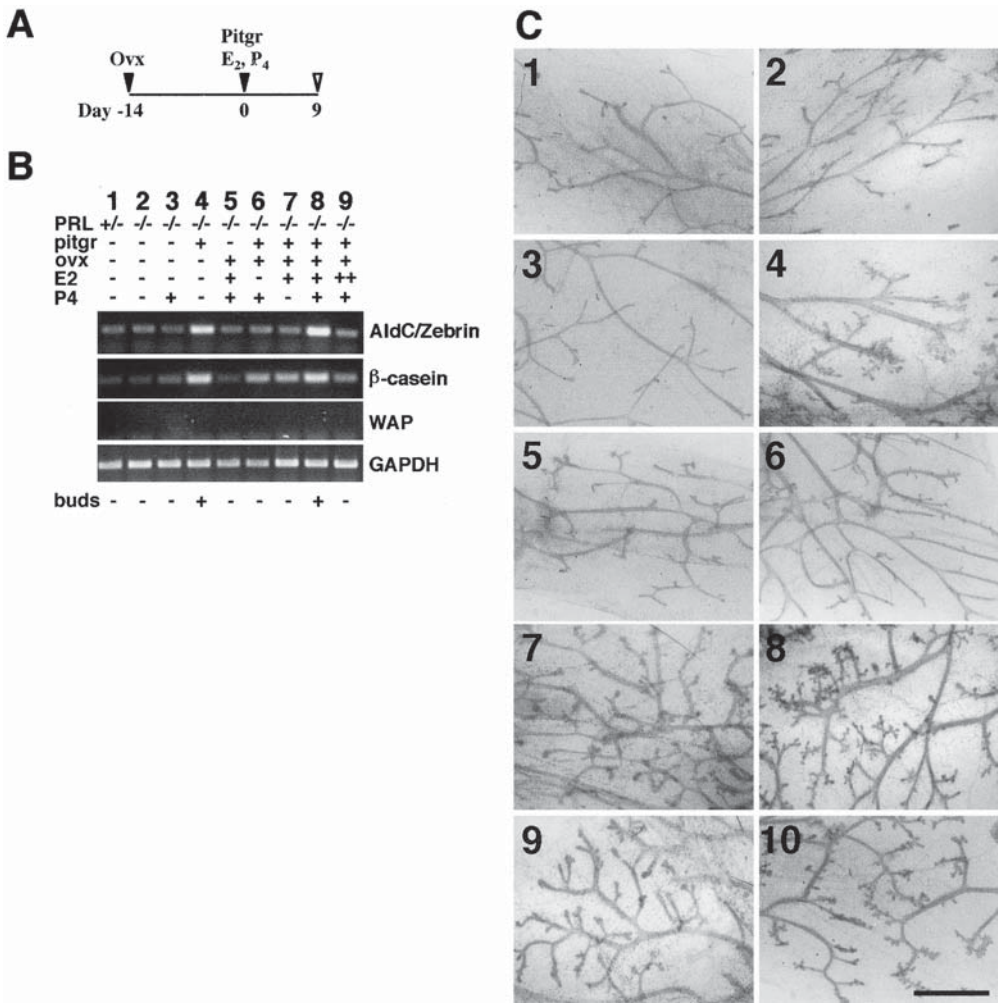

Fig. 4. Effect of PRL, E_2 (+:1 mg; ++: 20 mg) and P_4 (8 mg) on mammary morphology and gene expression in virgin female PRL-KO mice. Mice were treated with various combinations of ovariectomy (ovx), pituitary grafting (pitgr), and treatment with steroid hormones following the schedule indicated in (**A**). Mammary expression of AldC/zebrin, GAPDH, WAP, and β-casein mRNA was analyzed by RT-PCR in hormone-treated mice (**B**). Lanes 1 and 10 are heterozygous ($PRL^{+/-}$) control and pregnant (d 10.5) females, and the others are from PRL-KO mice. Whole-mount pictures 1–9 (**C**) correspond to lanes 1–10 (**B**). All of PRL, E_2, and P_4 were necessary for the high level of expression of AldC/zebrin as well as for the proliferation of alveolar buds (+) and high expression of β-casein. A high dose of E_2 (20 mg, lane 9) alternated development and gene expression.

either E_2 or P_4 was as low as that in virgin mice. Thus, PRL-induced development of the mammary glands seemed to consist of two steps: irreversible formation of alveolar buds and reversible proliferation of the buds. Induction of AldC/zebrin expression was a reversible effect of PRL. In addition, the sum of PRL, E_2, and P_4 was necessary for the maintenance of a high level of AldC/zebrin expression.

AldC/Zebrin Expression in WT Mice During Pregnancy and Lactation

Mammary AldC/zebrin mRNA content was estimated by RT-PCR (Fig. 6) in pregnant and lactating WT mice. AldC/zebrin mRNA began to increase at d 10.5 of pregnancy, in accordance with the genesis of lobuloalveolar structures, and gradually increased until the end of gestation. WAP expression occurred later than that of either β-casein or AldC/zebrin, in the pregnancy/lactation cycle. This result corresponded with the effects of pituitary grafting (Figs. 2 and 4) wherein short-term pituitary grafting (9 d) did not induce WAP, but longer pituitary grafting (36 d) caused a high level of WAP gene expression. From the onset of lactation, mammary glands expressed a very high level of AldC/zebrin, and the high expression was maintained during lactation at least for 10 d. AldC/zebrin expression decreased with involution of the mammary glands caused by weaning of the pups. AldC/zebrin mRNA level in the brain was not altered during pregnancy and lactation (data not shown). By contrast, mammary expression of AldA mRNA appeared to decrease modestly during pregnancy and lactation and to increase again after the onset of involution.

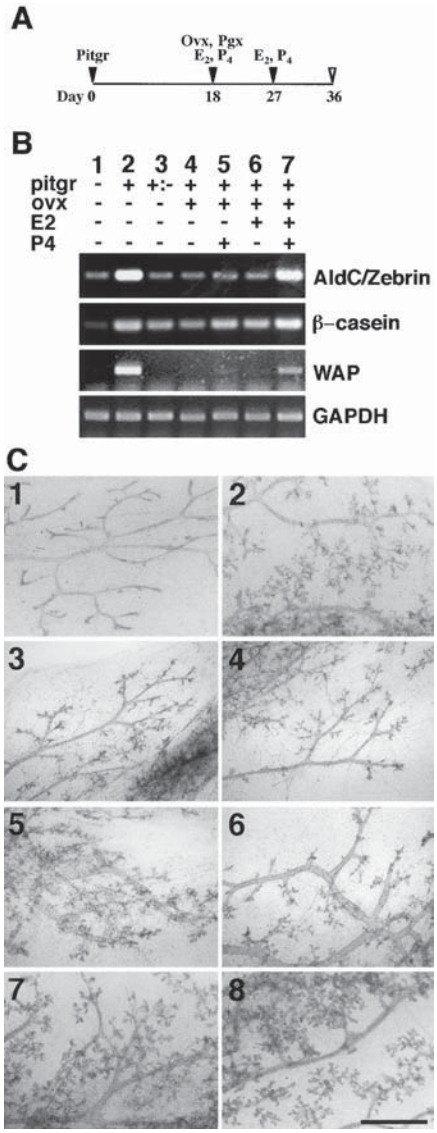

Fig. 5. Effect of PRL, E_2 and P_4 implants on mammary morphology and gene expression in hormone-prestimulated mammary glands of virgin female PRL-KO mice. Mice were given a pituitary graft (pitgr) for 18 d and then were treated with various combinations of ovariectomy (ovx), removal of the grafted pituitary (pgx), and treatment with E_2 (1 μg) and P_4 (8 mg) following the schedule indicated in (**A**). (**B**) Mammary expression of AldC/zebrin milk protein gene and GAPDH mRNA was analyzed by RT-PCR. Lane 1 is the intact control, and the others are from pitgr mice. (**C**) Whole-mount pictures correspond to lanes 1–7 in (**B**) and from d 18.5 pregnant WT mice (panel 8). In our mice, pituitary grafting and P_4 were necessary for maintenance of a high level of AldC/zebrin expression as well as for the proliferation of alveolar buds and high levels of milk protein gene expression. E_2 was not necessary for maintaining alveolar development (panel 5), but was necessary for a high level of AldC/zebrin and milk protein gene expression.

Expression and Distribution of AldC/Zebrin Protein in Mammary Glands in Pregnant and Lactating Mice

The 36-kDa AldC/zebrin protein was detected by immunoblotting in the hormone-stimulated mammary glands and

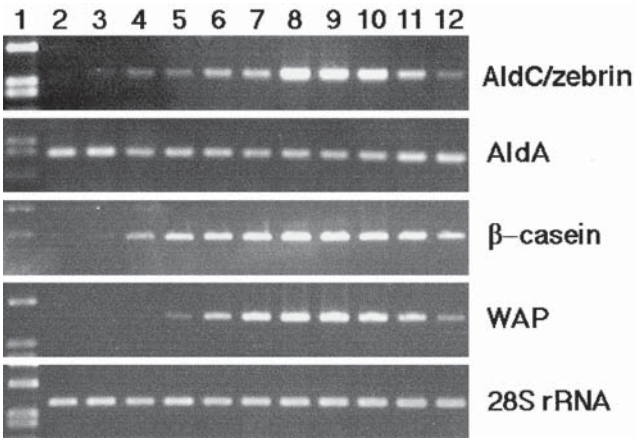

Fig. 6. Expression of AldA and AldC/zebrin examined by RT-PCR during pregnancy and lactation in WT mice. Lane 1: size marker; lane 2: virgin; lanes 3–7: d 6.5, 10.5, 14.5, 17.5, and 19.5 of pregnancy, respectively; lanes 8 and 9: d 2 and 10 of lactation, respectively; lanes 10–12: d 2, 5, and 10 of involution, respectively.

the ovary as well as in the cerebellum (Fig. 7A). The protein was not detectable in the mammary tissue in virgin mice but was detectable in tissue from pregnant (d 14.5) glands and highest in the lactating glands. AldC/zebrin content (per total protein amount) in the mammary glands and ovary was lower than that in the cerebellum. The distribution of AldC/zebrin in the mammary glands was examined by immunohistochemistry (Fig. 7B–D). Specific signal for AldC/zebrin was obtained from the mammary epithelium, especially luminal epithelium of the alveoli, but not from the stroma in the lactating mammary glands (Fig. 7B,C). The signal in the ductal epithelial cells was weak, and primary ducts were similar to the negative control tissue (Fig. 7D). The mammary glands in hormone-nonstimulated virgin mice were also negative for AldC/zebrin (Fig. 7E,F).

Discussion

AldC/zebrin has been considered a brain-specific isoform in adult animals, but here we have observed abundant expression of AldC in the adult mammary glands and ovary. The luminal epithelia of alveolar buds, especially in lactating mice, were responsible for the mammary expression of AldC/zebrin, and E_2, P_4, and PRL were required for high-level expression.

Among the three forms of aldolases, AldA favors glycolysis, i.e., the cleavage of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate in the liver and many other organs. AldB favors hormone-dependent gluconeogenesis by catalyzing the reverse reaction, especially in the liver and kidney. The kinetic properties of AldC/zebrin are intermediate between those of the A and B isoforms *(10)*. The mammary AldC/zebrin is unlikely to play a role in gluconeogenesis, since there is no evidence

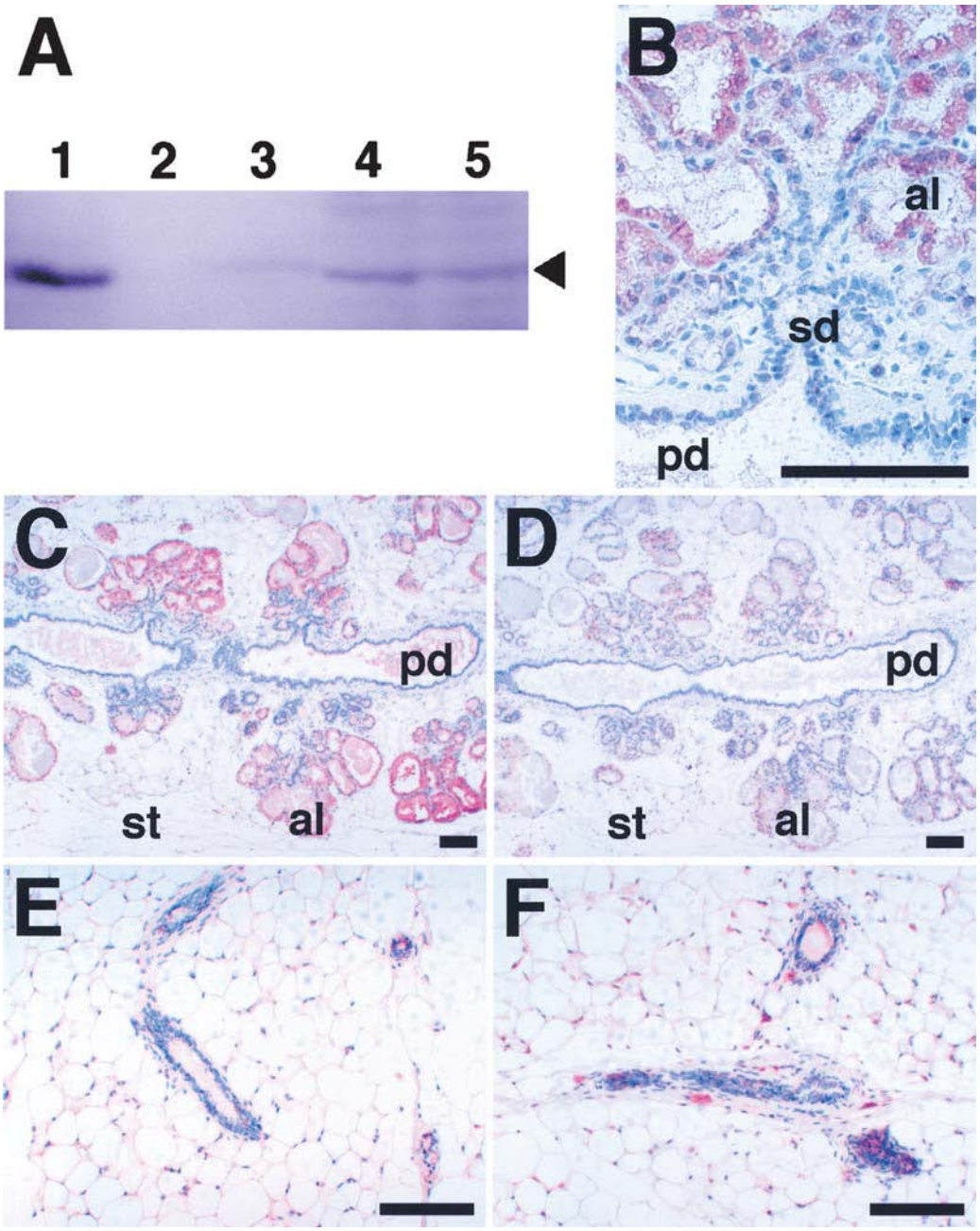

Fig. 7. Expression of AldC/zebrin protein in the mammary glands of female WT mice. (**A**) Detection of AldC/zebrin protein by immunoblotting in the cerebellum (lane 1), mammary glands of virgin (lane 2), d 14.5 pregnant (lane 3) and d 2 lactation (lane 4), and ovary (lane 5). Arrowhead indicates 36-kDa band. Ten milligrams of pooled total protein was loaded in each well. (**B–F**) Images of the mammary tissue section immunostained for AldC/zebrin. Mammary tissues from d 2 of lactation (B–D) and virgin (E,F) mice were reacted with a polyclonal anti-AldC/zebrin antibody (B,C,E) or the antigen peptide–preabsorbed antibody (D,F). Panels C and D are low magnification (×4 objective) and panel B is high magnification ×20 objective) to show the transitions from AldC/zebrin-positive cells and neighboring unstained cells. Note that cytoplasm of alveolar bud epithelium (al) in the PRL-stimulated mammary glands is immunostained intensely with the antibody while the stroma (st), primary (pd) and secondary (sd) appears negative for AldC/zebrin. Bars = 100 μm.

of gluconeogenesis in either the mammary glands or brain. Glucose and its derivatives, including fructose-1,6-bis-phosphate, are not only used as a metabolic fuel but also play other important roles such as provision of NADPH via the pentose phosphate pathway *(11)*. Glucose is also a source material for the synthesis of other key carbohydrates, such as synthesis of ribose and deoxyribose for nucleic acid synthesis; and substrates for the synthesis of glycoproteins, glycolipids, and glycosaminoglycans *(12)*. Expression of AldC/zebrin in developing embryonic tissues, hepatocarcinoma, and stimulated mammary glands implies that this type of Ald takes part in the glucose metabolism in biosynthetically

active tissues that are supplied with nutrients on a priority basis. Levels of AldC/zebrin increase during pregnancy, are highest in lactation, and fall during involution. This pattern appears to coincide with the metabolic demands associated with growth and lactation. During early involution, AldC/zebrin remains high, possibly reflecting the demands associated with tissue remodeling. Abundance of AldC in the lactating mammary epithelial cells observed in the present study implies the indirect contribution of AldC to the synthesis of lactose, a major osmotic regulatory component in the milk *(13)*, by allowing a relatively higher concentration of the substrate vs glyceraldehyde-3-phosphate and dihydroxyacetone phosphate in the cells.

To synthesize large amounts of carbohydrates, such as lactose and oligosaccharides, the lactating mammary gland has a high demand for precursor molecules (i.e., glucose). Increased glucose requirements of the lactating mammary glands necessitate major adjustments in glucose production and utilization in the whole body. Dietary uptake greatly increases during pregnancy and lactation, and hepatic glucose synthesis is increased to accommodate mammary or fetal demands. At the same time, glucose utilization by adipose tissue and muscle is reduced. This response is mediated by reduced tissue sensitivity to insulin, associated with decreased tissue expression of the insulin-responsive glucose transporter, GLUT4 *(14)*. AldA is not only an enzyme but also a linker protein that recruits GLUT4 to the cell surface along the actin cytoskelton, and the substrate for Ald (fructose-1,6-bisphosphate) competitively inhibits Ald-actin binding *(15,16)*. In contrast to other tissues, developing mammary epithelium expresses GLUT1 *(17)*, which does not link with AldA *(15)*, and GLUT 1 is the major form of glucose transporter in the lactating mammary glands. This contributes to a preferential supply of glucose to the tissue.

It is not known whether mammary glands and brain share the same promoter for the expression of AldC/zebrin. AldC/zebrin has a typical "housekeeping" type of promoter without canonical TATA and CAAT boxes, but with GC-rich elements. The proximal region (<115 bp) can direct "brain-specific" expression of the gene, but the regions farther upstream are required for a high level of expression *(7,18)*. The involvement of several DNA motifs to which POU domain proteins and HNF-3β, a winged helix factor, competitively bind has been reported *(7,19)*. The tight regulation of AldC/zebrin within a subpopulation of hormone-dependent epithelial cells implies an unexpected subtlety in the control of this enzyme. Interestingly, some other 'brain-specific' genes are expressed in the mammary glands *(20, 21)*, suggesting that mammary glands have employed parts of the gene-regulatory system of the brain during the evolution of the organ as well as that of the bone *(22–24)*. Because numerous animal and cell-based model systems are available, and the mammary gland is a much simpler tissue than the brain, the mammary epithelium will be a good model for studying the transcriptional regulation of AldC gene.

In conclusion, the developing and lactating mammary epithelium of alveolar buds expresses the brain type AldC/zebrin, which may play a role in the homeorhetic regulation of mammary metabolism. The unique expression pattern of AldC/zebrin may be useful as a molecular marker of the physiologic state of the mammary glands.

Materials and Methods

Animals and Hormone Treatment

Sixteen-week-old female PRL-deficient ($PRL^{-/-}$) and WT C57Bl/6J mice were used. $PRL^{-/-}$ mice were made congenic with C57Bl/6J by backcrossing for eight generations, and these mice were kept under the conditions described elsewhere *(25)*. All animal-housing conditions and experimental procedures were conducted under Institutional Animal Care and Use Committee approved protocols.

Pituitary grafting was performed by inserting an anterior pituitary gland that was obtained from heterozygous ($PRL^{+/-}$) mice under the left kidney capsule (pitgr). In some cases, the grafted pituitary gland was surgically removed carefully from the kidney. Grafting of the anterior pituitary under the kidney capsule has been extensively studied as a valid model of hypersecretion of PRL. Lactotrophs, released from dopaminergic inhibition, synthesize and secrete high levels of PRL. Somatrophs are also detected in the grafts, but not other endocrine cells, and growth hormone is not hypersecreted *(26)*. The ratios of biochemical isoforms of PRL may be different in the grafts compared to normal pituitaries, and secretion of unknown factors cannot be excluded. Ovariectomy was done via bilateral incisions in the flanks. Steroid hormone treatment was performed by the sc implantation of steroid-containing beeswax tablets *(27)*. The E_2 and P_4 tablets were made at the concentration of 1 and 500 μg/mg, respectively. The steroid tablets were replaced with new ones every 9 d, if necessary. These surgeries were performed under anesthesia with methoxyflurane. Histologic examinations were done to confirm the success of surgeries.

cDNA Library Construction and Gene Screening

A cDNA library that was rich in PRL-dependent mammary gland genes was made by SSH for comparison of pitgr $PRL^{-/-}$ mice with sham-operated cont and 25 mg of P_4 $PRL^{-/-}$ mice. After 18 d of the hormone treatments, total RNA was prepared with TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH) from the abdominal (fourth) mammary gland tissue after removal of the lymph node and debris. Poly A+ mRNA was recovered with Oligotex-dT30 (Qiagen, Valencia, CA) from pooled total RNA derived from three to five mice, and the first-strand cDNA was generated with a Smart cDNA Synthesis Kit (Clontech, Palo Alto, CA). The cDNA of the genes that were differentially expressed among the three groups were cloned by SSH cloning *(28)* with a PCR-Select® cDNA Subtraction Kit (Clon-tech) according to the manufacturer's protocol. The subtracted *Afa*I-

Table 1
PCR Primers and Conditions

Gene name	Primer sequences (5' → 3')	Annealing temperature (ºC)	No. of cycles
M13 plasmid	CGCCAGGGTTTTCCCAGTCACGAC AGCGGATAACAATTTCACACAGGAAAC	65	30
AldC/zebrin	GTTTATCAAGCGGGCAGAGATGAAC TTCAAACATGGGAGGGCATCTCTAC	62	30
AldA	GAGAACCTGAAGGCAGCCCAGGAGG CCCCTCTTCAATCGCAAGTGGGTAG	62	27
AldB	AGGCATTTTAATAAACATAGTTACC CCAAAAGAACTCTTATTTAATCTGG	56	36
β-casein	TGTGCTCCAGGCTAAAGTTCACTCC AGGTACTGCAGAAGGTCTTGGACAG	62	27
K18	AGAACTAGCCATGCGCCAGTCTGTG AGAGGGCTTCATATTCCTGGGCCTG	62	27
melk	TACCTTCTGCTTCTAGCCAAGAAGG CTAATCTCTTGTAAACCCAGGCATC	62	30
RANKL	GTGAAGACACACTACCTGACTCCTG CACCAGCTCGGAGCTTGAAAAATCC	62	30
WAP	GTCTTGCTGTATAGACTTGGGCTGG ACAAGTCTTCAACTCAGTTCAGTCC	62	30
β-Actin	TCTAGACTTCGAGCAGGAGATGGCC CTAGAAGCACTTGCGGTGCACGATG	62	27
GAPDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	62	27
28S rRNA	CAAACGGTAACGCAGGTGTCCTAAG TAACCTGTCTCACGACGGTCTAAAC	62	24
AldC/zebrin (5'-RACE)	AGTGGTAACAACGCAGAGTACGC CACTTTTTCACACGGTCATCAGCAC	66	30

digested cDNA fragments were subcloned into pPCR-Script® Amp vector (Stratagene Cloning Systems, La Jolla, CA). Thus, subtracted cDNA libraries were constructed.

The inserts of cloned plasmids were amplified by PCR using an M13 general primer set (Table 1), subjected to a 1.5% agarose gel electrophoresis, and transferred onto a Hybond N^+ membrane (Amersham, Piscataway, NJ). The membrane was hybridized with alkaline phosphatase–labeled DNA probes generated from the subtracted or nonsubtracted cDNA by AlkPhos Direct DNA Labeling (Amersham). The signal was detected by CPD-Star® chemiluminescence on an ECL film (Amersham).

Identification of mRNA

Virtual Northern hybridization is an alternative method to conventional Northern hybridization, in which full-length cDNA is used instead of RNA *(29)*. Mammary Smart cDNA was prepared from pitgr, P_4, and cont mice as described earlier. The full-length cDNA was amplified by hot-start PCR using Ex-Taq (Takara Shuzo K. K., Tokyo, Japan), and the reaction was stopped at 14 cycles; that is, 3 cycles before it began to plateau. The cDNA concentrations were standardized to the amount of GAPDH or β-actin cDNA that was estimated by Southern hybridization analysis of serially diluted samples. Approximately 0.5 μg of the PCR product per well was subjected to 1.5% agarose gel electrophoresis, followed by capillary blotting to Hybond N^+, and Southern hybridization with AlkPhos®-labeled DNA probes. The DNA probes were derived from glass bead–purified PCR products amplified from the cloned plasmids as described earlier.

Probes for β-casein, WAP, cytokeratin 18 (K18), and a member of the Snf1/AMPK kinase family (melk) *(30)* were also prepared from the PCR product using the specific primers listed in Table 1.

RT-PCR and 5'-RACE

Total RNA was prepared from tissues with TRI-Reagent and treated with DNase I (Promega, Madison, WI). Total RNA (0.1 μg) was reverse transcribed with Superscript® II RT (Invitrogen, Carlsbad, CA) using oligo-$dT_{12–18}$ (Amersham) or random hexamer (Takara) primers. The cDNA was

subjected to 18–35 cycles of PCR amplification with ExTaq for aldolases, β-casein, WAP, and 28S ribosomal RNA. The PCR was manually hot-started, and each cycle comprised 95ºC for 20 s, 30 s at annealing temperature, and 72ºC for 1 min. The primer sets, annealing temperatures, and number of PCR cycles are listed in Table 1.

The cDNA for the 5'-end of AldC/zebrin mRNA was amplified by PCR from the mammary Smart cDNA using a Smart oligo sequence-specific primer and an AldC/zebrin-specific reverse primer (Table 1). The DNA sequence of the PCR product was determined by automated sequencing.

Immunohistochemistry and Immunoblotting

Tissue was fixed in 4% paraformaldehyde–phosphate buffered saline (PBS), embedded in paraffin, and cut in 5-μm sections. After rehydration, the sections were incubated in 10 m*M* boric acid (pH 6.0) at 95ºC for 10 min, 0.3% H_2O_2 in methanol for 30 min, and PBS containing 10% normal rabbit serum for 10 min at 25ºC. They were incubated in 1 ng/mL of goat polyclonal anti-AldC/zebrin antibody (sc-12065; Santa Cruz Biotechnology, Santa Cruz, CA) at 4ºC for 16 h, and the antibody was visualized by the ABC method using a Vectastain Elite ABC-PO Kit (Vector, Burlingame, CA). Tissue sections were counterstained with hematoxylin.

Tissue homogenate samples for sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis were made, and proteins were separated on a 12% gel, transferred onto an Immobilon® polyvinyl difluoride membrane (Millipore, Bedford, MA) by semidry electrophoresis at 2 mA/cm^2 for 1 h, and stained with Ponceau S. After blocking with 5% nonfat dry milk dissolved in PBS (pH 7.5) for 2 h at room temperature, the membrane was incubated with 0.5 ng/mL of anti-AldC/zebrin antibody at 4ºC for 16 h. The antibody was visualized by chromagen staining of alkaline phosphatase–conjugated rabbit polyclonal anti–goat IgG (H+L) (Worthington, Lakewood, NJ).

RIA for PRL

At autopsy mice were killed by CO_2 inhalation for 3 min, and blood samples were collected from the heart just before excising mammary gland tissue. Blood samples were clotted at room temperature for 1 h and at 4ºC for 16 h. They were centrifuged at 3000*g* for 20 min, and the supernatant was stored at –70ºC until used as serum sample for the assay. Serum PRL level was determined by RIA with the use of a mouse PRL assay kit donated by Dr. A. F. Parlow, National Institutes of Health, as described elsewhere *(31)*. The detection limit of the assay was 0.5 ng/mL, and intraassay coefficient of variation was <5% over the whole range of the standard (0.5–64 ng/mL). For high PRL serum (>64 ng/mL), sample was diluted at 1:10 with 1% bovine serum albumin–PBS prior to determination of PRL level by RIA. Assay was done with duplicate samples. Serum PRL was less than the detection limit in the PRL$^{-/-}$ mice, 50–200 ng/mL in pitgr mice, and 8–25 ng/mL in PRL$^{+/-}$ mice.

Whole-Mount Preparation of Mammary Gland Tissue

Whole-mounted mammary glands were stained with safranin O as described previously *(2)*. Briefly, tissue was spread on a glass slide and fixed with Carnoy's fixative for 24 h, defatted with acetone for 24 h, rehydrated with a series of ethanol solution, stained with safranin O solution for 1 h, destained with acid-ethanol solution, dehydrated with a series of ethanol solutions, and mounted with Permount® reagent. The whole-mount tissues were examined with an SZX-10 (Olympus, Tokyo, Japan), and pictures were taken at ×90 optical magnification.

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