J. Biochem. 125, 669-675 (1999)

Down-Regulation of Protein Tyrosine Phosphatase Gene Expression in Lactating Mouse Mammary Gland

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Received October 23, 1998; accepted December 14, 1998

Detailed analysis of protein tyrosine phosphatase (PTP) expression in mouse mammary gland and mammary epithelial cells using a set of degenerate primers corresponding to the PTP core domain sequence revealed the presence of 16 different receptor-type and intracellular PTPs. Northern blot and RT-PCR analyses revealed that some PTPs were up-regulated during gestation, suggesting that these enzymes are involved in development of mammary gland. However, expression of most PTPs dramatically decreased during lactation, whereas the β-casein gene expression was increased and remained at a high level. At the involution stage after weaning, most PTPs were up-regulated and their expression returned almost to the virgin level. Such up-regulation was also induced by forced weaning in lactating mother mice. These results suggest the possible contribution of PTPs to the development, involution, and remodeling of mammary gland and their possible inhibitory action on maintaining high expression of milk genes during lactation.

Key words: involution, lactation, mammary gland, prolactin signaling, protein tyrosine phosphatase.

Protein tyrosine phosphorylation has been shown to play critical roles in regulating fundamental cellular processes such as proliferation, differentiation, and development (*1*) and to be controlled by the balance of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) (*2*). PTPs seem to be as diverse as PTKs, but much less is known about the regulation and biological function of PTP activity and specific PTP substrates (*3*).

The PTPs comprise a diverse family of receptor-type and intracellular molecules including various isoforms resulting from alternative splicing (*4*). Receptor-type PTPs have extracellular segments containing immunoglobulin and fibronectin homology domains, and an intracellular part mostly consisting of two PTP domains, of which the N-terminal one is the more active. In contrast, most intracellular PTPs have only one PTP domain but carry various additional subdomains such as GLGF repeats (PTPH1, PTP-BAS), an SH2 domain (SHP-1, SHP-2), or a PEST domain (PTP-PEST, PEP-PTP, HCSF) at their N-terminus or C-teminus. These motifs are thought to mediate their intracellular localization and biological function through substrate specificity (*4, 5*).

The mammary gland undergoes dramatic changes in morphology and function during gestation, lactation, and involution under the control of a variety of peptide and steroid hormones, growth factors, cytokines, and extracellular matrix (*6*). Prolactin has been shown to trigger the signal transduction pathway leading to the transcriptional activation of mammary-specific genes (*7-9*). In this pathway, JAK2 and STAT5, which is also known as mammary gland factor (MGF), play essential roles. Although the importance of PTKs such as JAK2 in mammary development and differentiation processes has been demonstrated, the involvement of PTPs in these processes remains unclear. To begin to address the importance of PTPs in mammary differentiation, we attempted to isolate cDNAs which encode PTPs expressed in mouse mammary gland and mammary epithelial cells. In this paper, we show that 16 different PTPs are expressed in both tissue and cells and that most of them are up-regulated during gestation and involution but highly down-regulated during lactation, where maximal expression of β-casein was observed, suggesting their possible contribution to development, involution, and remodeling of mammary gland and their possible inhibitory action on maintaining high expression of milk genes during lactation.

MATERIALS AND METHODS

Animals and Tissue Preparation—Female mice, mated at 10 weeks of age, were purchased from Japan SLC (Hamamatsu) and fed with standard laboratory chow (Japan SLC) *ad libitum*. The mice were individually housed in a room maintained at 22 to 24°C and about 50% relative humidity, and had free access to food and tap water. At indicated days, mice were sacrificed under anesthesia, and mammary glands and other tissues were excised, immedi-

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Abbreviations: PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; STAT, signal transducers and transactivators of transcription.

ately frozen in liquid nitrogen, and stored at −80°C until use.

RNA Preparation—Total RNA was prepared from the mammary tissues of virgin, pregnant, lactating, and involuting mice or cultured cells with ISOGEN reagent (Nippon Gene, Tokyo) according to the manufacturer's instructions. Poly(A)$^+$ RNA was isolated using Oligotex™-dT30 super (TaKaRa, Kyoto). The RNA samples were stored at −80°C until use.

Cell Culture—Mouse mammary epithelial cell line HC11 (7) was generously provided by Dr. B. Groner (Institute of Experimental Cancer Research, Tumor Biology Center, FRG). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 5 μg/ml insulin, and 10 ng/ml epidermal growth factor (EGF) until confluency. Then, hormone induction was performed for 48 h in RPMI 1640 medium containing 10% FCS, 0.1 μM hydrocortisone (Sigma), 5 μg/ml ovine prolactin (Sigma), and 5 μg/ml insulin.

RT-PCR—Degenerate primers were designed according to the consensus sequences for two highly conserved amino acid stretches with the catalytic domains of PTPs: FWX-MXW and HCSAG(S/I/V)G. Random primed cDNA up to 50 ng from lactating mammary gland or HC11 cell RNA was used as a template for PCR reaction. Both sense and antisense primers were added to 100 μl of reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% bovine serum albumin, 200 μM dNTPs, 1 unit of Taq DNA polymerase (TaKaRa), and template cDNA. PCR amplification was carried out on a thermal cycler for 40 cycles of 94°C for 1 min, 42°C for 1 min, and

TABLE I. **Frequency of specific PTP clones obtained by RT-PCR on cDNA from pregnant mammary gland and lactogenic hormone-treated mouse mammary epithelial cells (HC11).**[a]

PTP	Mammary gland	Mammary epithelial cells
Receptor-type PTPs		
PTPα	3.0	4.3
PTPε	3.0	0.3
PTPκ	25.3	45.6
PTPλ		0.3
LAR	12.7	0.9
PTPσ		3.1
DEP-1	18.1	
Intracellular PTPs		
PTP1B	9.1	6.8
MPTP	3.0	3.1
SHP-1	3.0	
PTP-PEST	6.1	
HCSF		0.6
PTP-BAS		0.3
PTP-MEG2	12.3	27.9
PTP-RL10		3.1
PTP36		2.6
Non-PTPs	4.4	1.1

[a]The number of clones of each PTP is expressed as a frequency (%) relative to the total number of plasmid clones: 145 for mammary gland, 248 for mammary epithelial cells.

TABLE II. **Oligonucleotide primers for RT-PCR to amplify specific PTPs.**[a]

PTP	GenBank Acc. No.	Target residues
Receptor-type PTPs		
PTPα	M36033	21-651
PTPε	D83484	643-1285
PTPκ	L10106	1126-1612
PTPλ	U55057	3965-4700
LAR	Z37988	990-1759
PTPσ	X82288	4993-5779
DEP-1	D45212	3281-4086
Intracellular PTPs		
PTP1B	M97590	79-1394
MPTP	M81477	28-1236
SHP-1	M90389	91-729
PTP-PEST	X63440	40-662
HCSF	U49853	12-1390
PTP-BAS	Z32740	1-634
PTP-MEG2	AF013490	1190-1890
PTP-RL10	D37801	301-935
PTP36	D31842	2995-3716
β-Casein	M26940	95-702

[a]For sense and antisense primers, 20 nucleotides each were synthesized and purified by gel filtration chromatography.

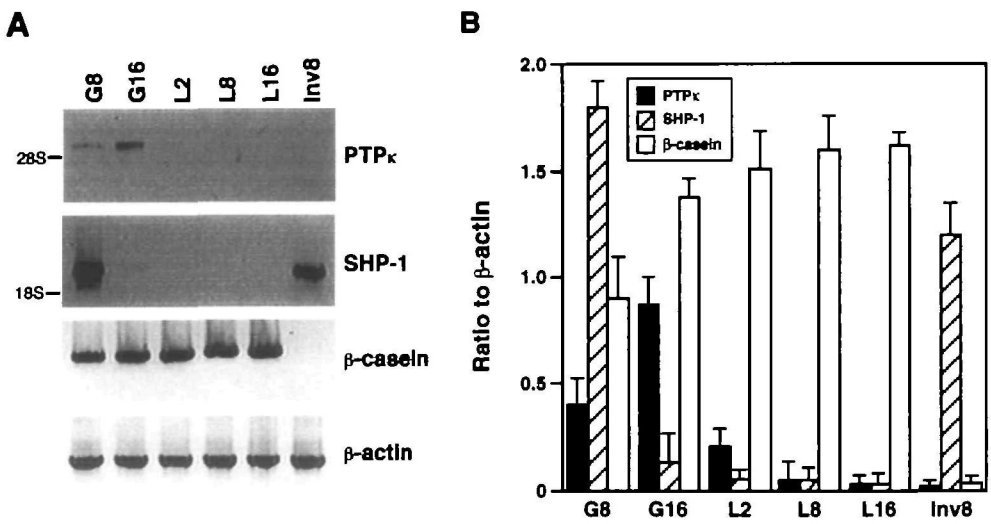
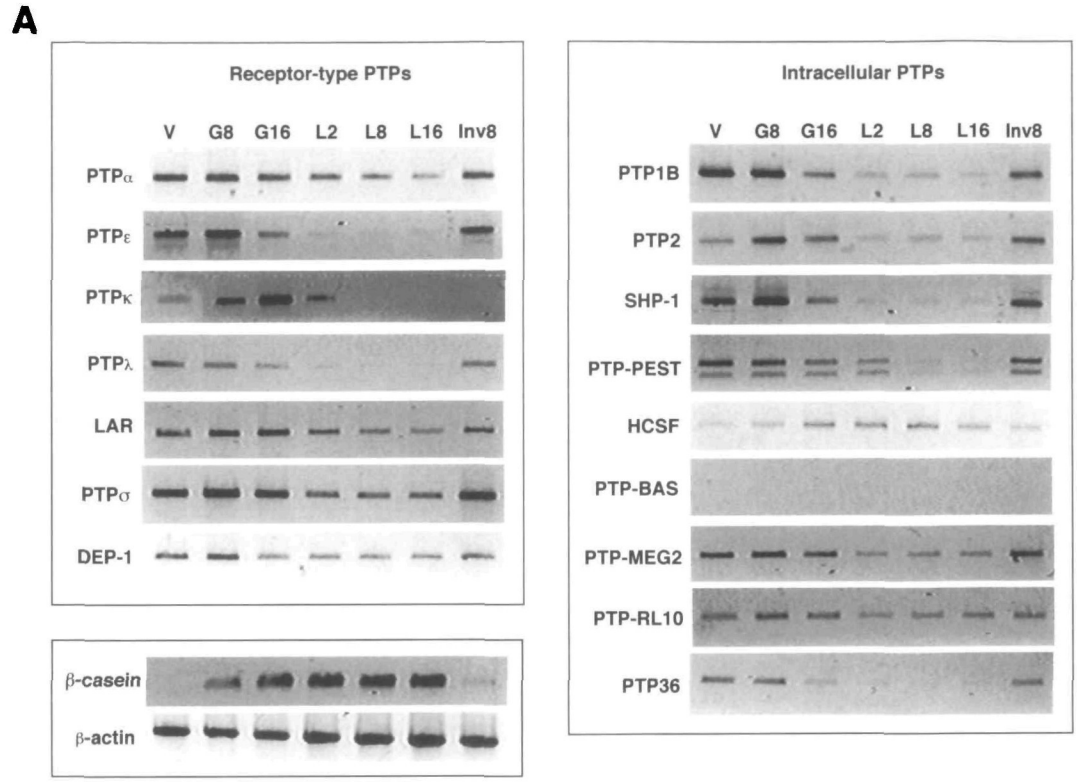


Fig. 1. **Differential expression profiles of mouse PTPκ and SHP-1 mRNAs in mouse mammary gland.** A: Northern blots containing 2 μg of poly(A)$^+$ RNA from day 8 and 16 of gestation (G8 and G16, respectively), day 2, 8, and 16 of lactation (L2, L8, and L16, respectively), and day 8 of the involution period after weaning (Inv8) were probed with ^{32}P-labeled PCR products for mouse PTPκ and SHP-1 sequentially. The same blot was reprobed with ^{32}P-labeled cDNAs for β-casein and β-actin as internal controls. B: the expression levels of PTPκ and SHP-1 mRNAs were quantitated and shown as the ratio of radioactivity of each mRNA band to that of the β-actin mRNA band. The plot shows amounts of transcripts relative to the earliest gestation stage (G8) as 100%. Values are shown as means±SEM of three independent determinations.

72°C for 1 min. The PCR products were separated on a 1.5% agarose gel. The bands around 350–400 bp were excised, subcloned into pBluescript KS(+) (Stratagene), and sequenced by the chain termination method (*10*).

For the PCR amplification of individual PTPs, both sense and antisense primers were selected based on the reported cDNA sequences. Target nucleotide sequences of the primers are listed in Table II. The primer set for mouse



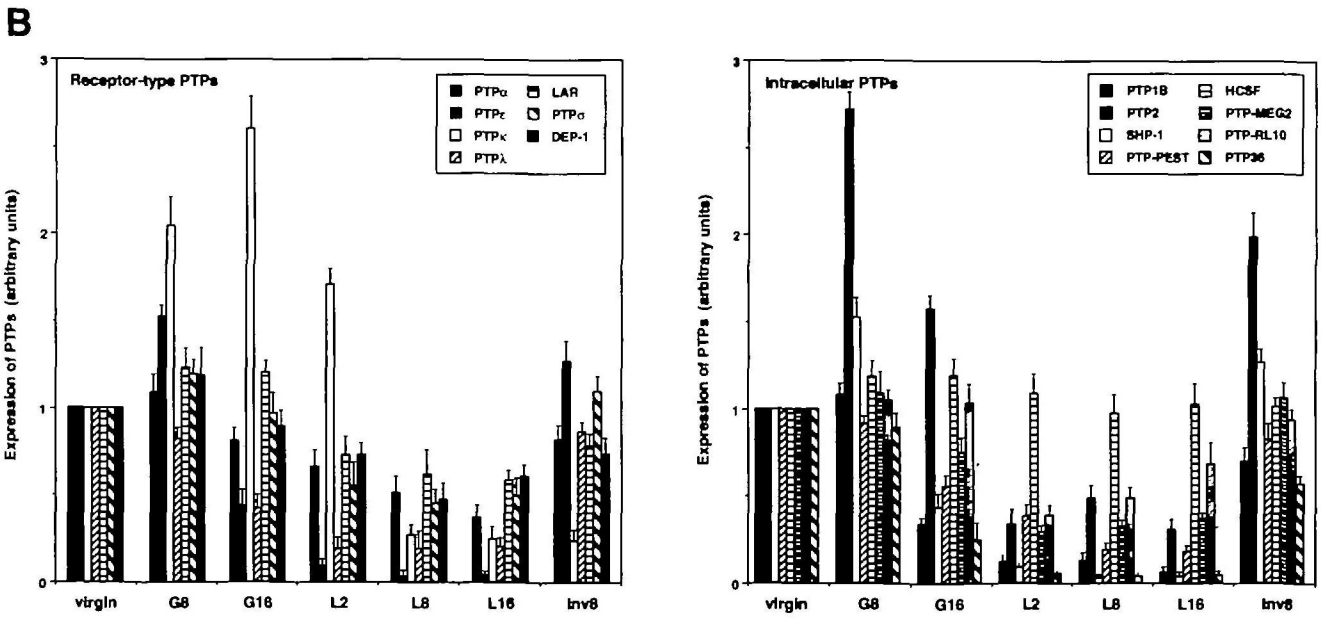


Fig. 2. **Determination of differential expression profiles of the PTPs in mouse mammary gland by RT-PCR analysis.** PCR amplification was carried out as described in "MATERIALS AND METHODS" using specific sets of primer and cDNAs from mammary gland of various stages, including virgin. Aliquots of the PCR product were run on a 1.0% agarose gel, then subjected to Southern analysis (panel A) and densitometric analysis using Densitograph for Macintosh (ATTO, Japan) (panel B). The expression level of each PTP was normalized to that of β-actin, and the plot shows the amounts of the PCR product relative to that at the virgin stage. Graphical data for PTP-BAS are omitted due to the lack of signals on the Southern blot. For PTP-PEST, graphical data for the upper band are presented. β-Casein expression is shown as a positive control. Values are shown as means ± SEM of three independent determinations.

β-actin was obtained from Clontech and the amplified band is 540 kb in size. PCR amplification was done using 50 ng of cDNA prepared from poly(A)$^+$ RNA under the conditions described above except for annealing temperature (50°C) and the number of cycles (20 cycles). Aliquots of the PCR product were separated on a 1.0% agarose gel, blotted to Hybond-N+ membranes (Amersham), then the membranes were probed with the digoxigenin-labeled cDNA for each PTP.

Northern Blot Analysis—Northern blot analysis was done as described previously (*11*). Briefly, 2 μg of poly(A)$^+$ RNA was separated on a 1.0% agarose/2.2 M formaldehyde gel and blotted onto Hybond-N+ membranes (Amersham). The membranes were hybridized with the ^{32}P-labeled cDNA fragment of each PTP which had been produced by RT-PCR. The membranes were washed and analyzed by use of an image analyzer model BAS 2500 (Fuji Film, Tokyo). The mRNAs for PTPs were standardized by analyzing β-actin mRNA in each RNA preparation.

RESULTS

To identify PTPs involved in development of mammary gland, we performed reverse transcription-based PCR amplification using poly(A)$^+$ RNA from either pregnant mammary gland or lactogenic hormone-treated mammary epithelial cells (HC11) as a template with degenerate primers that were based on the highly conserved amino acid sequences within the PTP catalytic core domain. This produced specific bands of 350-400 bp. These products were isolated, subcloned, and sequenced. Sequencing analysis identified 10 and 13 different PTP clones in mammary gland and mammary epithelial cells, respectively (Table I). PTPα, PTPε, PTPκ, PTP LAR, PTP1, PTP2, and MEG2 were identified in both tissue and cells. In total, 16 PTP clones were obtained. It is noteworthy that PTPκ which is suspected to play a role in regulating cell contact and adhesion through intracellular association with cadherin-catenin complexes (*12*), was obtained with high frequency. It has been reported that PTP LAR and PTPσ, which are members of the same subfamily, are expressed in mouse mammary gland (*13*). We also obtained the cDNAs for both of them, but not for another member of the family, PTPδ, which is consistent with the previous report (*13*). One of the SH2 domain-containing PTPs, SHP-2 (*14-18*), which is known to be ubiquitously expressed, was not detected by our strategy, whereas SHP-1 (*19-22*) was obtained.

During gestation and lactation, mammary glands develop morphologically and functionally, leading to production and secretion of a large amount of milk protein. To elucidate the differential involvement of the PTPs, poly(A)$^+$ RNAs were prepared from mouse mammary gland during gestation, lactation, and involution and subjected to Northern blot analyses using the PCR products as probes. Figure 1 shows the representative expression profiles of PTPκ (receptor-type) and SHP-1 (intracellular). In the case of PTPκ, the gene expression increased up to day 16 of gestation, then gradually decreased through lactation to the involution period. The same blot was stripped and reprobed with the cDNA for SHP-1. In contrast to the expression profile of PTPκ, SHP-1 was expressed very strongly at day 8 of gestation, but thereafter the expression remained low until it was up-regulated at day 8 of involution. On this day the expression of β-actin was nearly the same as at other stages and only a faint signal was detected for β-casein transcript.

To further elucidate the differential expression of the PTPs identified, specific sets of primers were designed for RT-PCR analysis. Target nucleotide residue numbers are listed in Table II. Poly(A)$^+$ RNAs from mammary glands of virgin mice and mice at the other stages were reverse-transcribed and subjected to PCR amplification as described in "MATERIALS AND METHODS." As shown in Fig. 2, expected bands for all PTP clones except for intracellular PTP-BAS were obtained. All the amplified bands were sequenced and confirmed to be the corresponding PTP sequences by BLAST search, and the amplification was shown to depend on the initial amount of the cDNAs and the number of cycles of PCR reaction (data not shown). PTPα, PTPε, PTPκ, LAR, PTPσ, DEP-1, PTP2, SHP-1, PTP-MEG2, and PTP-RL10 were up-regulated during gestation, suggesting that these PTPs, especially the receptor-type PTPs, are involved in development of mammary glands, because cell-matrix and cell-cell interaction is crucial for the process (*23, 24*). Surprisingly, the expression levels of the PTPs other than HCSF were highly suppressed during the whole lactation period, whereas the β-casein expression remained high in this period (Fig. 2). Also, the expression profiles of selected PTPs (PTPκ, LAR, SHP-1, PTP36, PTP-RL10) in non-mammary tissues, including brain, heart, liver, spleen, and kidney, were found to be expressed almost constantly during gestation, lactation, and post-lactation periods (Fig. 3), implying that the drastic reduc-

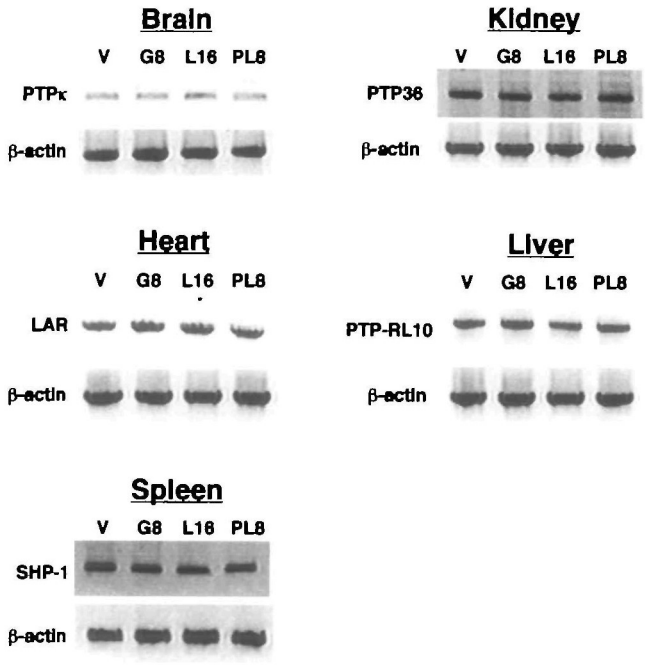


Fig. 3. **Expression of several PTPs in various tissues of virgin, pregnant, lactating, and post-lactating mice.** Indicated tissues were excised from the same mice used in Fig. 2 and subjected to poly(A)$^+$ RNA preparation and cDNA synthesis. PCR amplification was carried out as described in "MATERIALS AND METHODS" using specific primers as indicated. Aliquots of the PCR product were run on a 1.0% agarose gel, then subjected to Southern analysis. As an internal control, PCR amplification for β-actin was done. Values are shown as means ± SEM of two independent determinations.

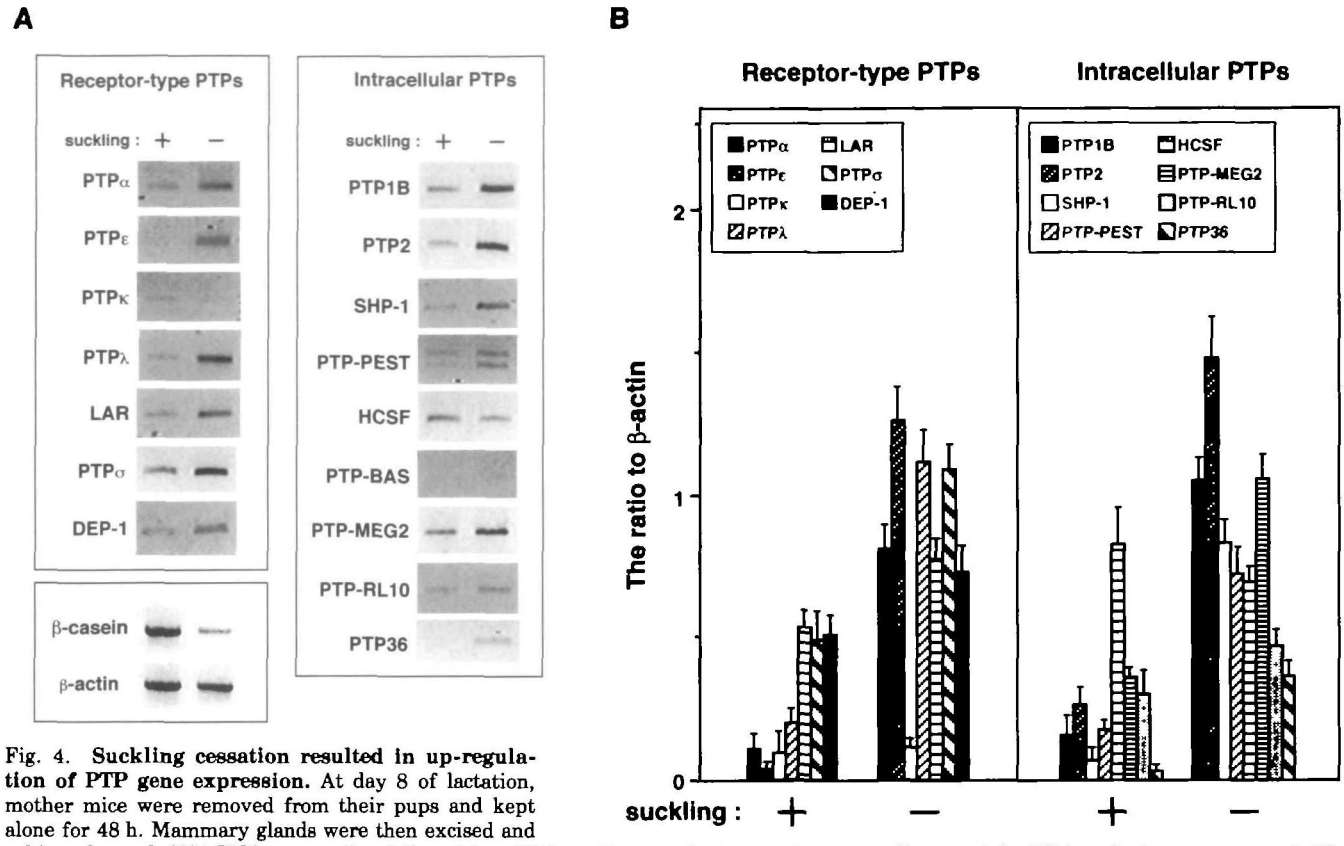


Fig. 4. **Suckling cessation resulted in up-regulation of PTP gene expression.** At day 8 of lactation, mother mice were removed from their pups and kept alone for 48 h. Mammary glands were then excised and subjected to poly(A)$^+$ RNA preparation followed by cDNA synthesis and PCR amplification. Aliquots of the PCR product were run on a 1.0% agarose gel, then subjected to Southern analysis (panel A) and densitometric analysis using Densitograph for Macintosh (Atto, Tokyo) (panel B). Graphical data for PTP-BAS are omitted due to the lack of signals on the Southern blot. For PTP-PEST, graphical data for the upper band are presented. β-Casein expression is shown as a positive control. Values are shown as means ± SEM of two independent determinations.

tion in gene expression of the PTPs is specific event in mammary gland. The additional lower band for PTP-PEST (in Fig. 2A) was shown to be an isoform of PTP-PEST and will be discussed elsewhere (unpublished data).

After weaning, the mammary gland is functionally and morphologically changed, as demonstrated by drastic reduction in milk gene expression. However, unexpected up-regulation of SHP-1 was also found during involution (Fig. 1). Like SHP-1, most of the PTPs except for PTP$\varkappa$ and HCSF were increased to nearly the same level as the virgin and early gestation period (Fig. 2). To confirm that the up-regulated gene expression of the PTPs is associated with involution of the mammary gland or weaning of the pups, artificial involution was induced by forced weaning of the pups from mother mice at mid-lactating stage. At day 8 of lactation, pups were removed from the mother mice for 48 h, then the gene expression profile of individual PTPs in the mammary tissue was determined by RT-PCR as above. As shown in Fig. 4, suckling cessation for 48 h resulted in up-regulation of the PTPs that were shown to be up-regulated in the natural involution period (Fig. 2), whereas a marked reduction in β-casein gene expression was observed. Nearly the same results were observed when the pups were removed from mother mice at day 16 of lactation (data not shown). These results clearly suggest that the PTPs are involved in involution of the mammary gland and possibly remodeling of the tissue for the next reproduction.

DISCUSSION

Many peptide and steroid hormones such as prolactin, estrogen, and hydrocortisone have been shown to be involved in development of the mammary gland (*6*). Prolactin signaling leading to milk gene expression is particularly well documented, in which the signaling is activated through JAK2 and STAT5 upon prolactin binding to the cognate receptor on the cell surface. But limited information is available about PTPs involved in prolactin signaling and mammary gland development. It has been found by reconstitution experiments that an SH2 domain-containing PTP, SHP-2, plays an essential role in JAK2 activity, STAT5 phosphorylation, and transcriptional induction in prolactin signaling leading to milk gene expression (*25, 26*), whereas another SH2 domain-containing PTP, SHP-1, apparently has no roles in the signaling (*26*). In this study, SHP-2 was not detected by the degenerate primers, possibly because their sequences are unsuitable for SHP-2 amplification. However, RT-PCR using specific primers for SHP-2 produced the expected band, and the differential pattern was indistinguishable from that of SHP-1 (data not shown). Although both SH2-containing PTPs were shown to be expressed in mammary gland during gestation and post-lactation periods, their expression was under the detectable level in mammary epithelial cells cultured under conditions where β-casein gene expression was remarkably

induced (unpublished data). This suggests that other PTPs are also involved in the prolactin signaling in a positive manner. Further experiments will be required to determine endogenous PTPs involved in the mammary-specific expression of genes such as the β-casein gene.

Schaapveld *et al.* reported that gene targeting of PTP LAR resulted in impaired mammary gland development and rapid involution of mammary gland postpartum, and concluded that PTP is an essential mediator in mammary gland development and function (*13*). In this study, RT-PCR analysis showed that LAR is differentially expressed, and the expression profile until parturition was consistent with that reported by the group. The expression profile of LAR-like PTPσ was comparable to that of LAR (Figs. 2 and 3), suggesting that PTPσ also regulates development and function of the mammary gland in a different way, since PTPσ could not substitute for PTP LAR in its biological roles (*13*).

Complex molecular events are involved in the regulation of lactation (*6*). The reduced effect of sex hormones seems to be the most crucial for the initiation of lactation postpartum (*6, 27*). Most of the PTPs were suppressed in the course of lactation, despite of the high expression of milk protein β-casein (Fig. 2). Since limited protein analysis using commercially available antibodies revealed reduced expression of SHP-1 and SHP-2 during lactation (data not shown), reduced gene expression of the PTPs might indicate reduction in protein expression. It is simply assumed that only minimal expression of PTPs might be required for the high expression of the milk protein during lactation or that they might play inhibitory roles in maintaining the process due to their dephosphorylation action on the signaling cascade triggered by lactogenic hormones. HCSF (*28*), which is also known as PTP-K1 (*29*), FLP-1 (*30*), PTP20 (*11*), BDP1 (*31*), was shown to be expressed at relatively high level during lactation, suggesting that this enzyme is involved in milk gene expression at this stage. The expression of this PTP, however, was under detectable level, as were those of SHP-1 and SHP-2 in mammary epithelial cells induced by lactogenic hormone treatment (unpublished data). Alternatively, other undetected PTPs might function in the process.

It is apparent that PTPs are involved in involution to the resting stage as well as development of the mammary gland. Involution of mammary tissue is associated with apoptosis of mammary epithelial cells (*32, 33*), suggesting that up-regulation of PTPs during involution is involved in apoptosis of the cells in the mammary gland. Sato *et al.* reported that an intracellular PTP, FAP-1/PTP-BAS, contributes to the Fas-mediated signal transduction pathway leading to cell death in a negative manner (*34*). Our present data suggest possible involvement of PTPs in apoptosis of mammary epithelial cells in a positive fashion in the mammary gland. However, we cannot exclude the possibility that up-regulation of most PTPs during involution simply reflects the return to the resting level in the mammary gland, where minimal expression of the PTPs is required to maintain the basal function of the tissue.

One might speculate that the drastic up-regulation and down-regulation of most PTPs in mammary gland is associated with cellular tyrosine phosphorylation. This was investigated using cellular extracts of mammary gland at various stages and anti-phosphotyrosine antibodies (PY20), but, unexpectedly, no obvious difference was observed (data not shown). This might simply imply that changes in gene expression of the PTPs other than SHP-1 and SHP-2 do not reflect changes in protein expression, or that the expression of protein tyrosine kinases changes in a similar way to PTPs, resulting in an apparently constant phosphorylation-dephosphorylation status.

We believe that the present study has provided a fundamental framework for further elucidation of PTPs involved in mammary gland development and involution, and in signal transduction pathways triggered by lactogenic hormones such as prolactin.

We thank Berund Groner (Freiburg) for provision of the HC11 cell line.

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