Breast Cancer Protein PS2 Synthesis in Mammary Gland of Transgenic Mice and Secretion into Milk

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PS2, a small estrogen-inducible secretory polypeptide with structural analogies to a growth factor, is produced by approximately 50% of human breast tumors. The function of PS2 is, however, unknown. To determine whether PS2 may play an autocrine role in the development of mammary tumors we constructed transgenic mice bearing fusion constructs designed to direct the expression of human PS2 in the lactating mammary gland under the control of the whey acidic protein (WAP) promoter. Mouse lines bearing the genomic PS2 gene under the control of the WAP promoter region (WAP-PS2–2) failed to express the transgene. However, mice harboring the fusion construct WAP-PS2–1, in which the PS2 coding sequence is inserted into the 5′ untranslated region of the complete WAP gene, were observed to express the transgene. Expression was restricted to the secretory epithelium of the mammary gland during lactation, and PS2 protein was secreted into the milk. Nevertheless, no mammary gland dysplasia was observed, and PS2 expression had no discernable effect upon the physiology and/or development of the suckling young or the transgenic mother. (Molecular Endocrinology 3: 1579–1584, 1989)

INTRODUCTION

PS2 was originally identified as an estrogen-induced mRNA species in the breast cancer cell line MCF7 (1–3). The PS2 gene encodes an 84-amino acid polypeptide from which an N-terminal signal sequence is removed to yield a 7-kDa cysteine-rich secretory protein (3–5). In view of these properties a possible role as a growth factor or other intercellular messenger protein has been discussed (3, 6, 7). PS2 is produced by approximately 50% of human breast tumors (8), and PS2 antigen can be detected in the urine of tumor-bearing patients (Rio, M.-C., and P. Chambon, in preparation). Although PS2 was believed to be specific to breast cancer tissue, we recently reported the detection of PS2 protein in gastric fluid of normal individuals of both sexes (9). In support of a role as a signalling protein we (9) and others (10–12) have noted a sequence similarity between PS2 and a porcine protein, PSP (pancreatic spasmolytic polypeptide), that appears to intervene in the regulation of gastric acid secretion and intestinal motility (13, 14). The function of the human PS2 protein is, nevertheless, unknown.

To test the hypothesis that PS2 might play an autocrine or paracrine role in stimulating the growth of breast tumor cells, we have constructed transgenic mouse lines in which the human PS2 gene is transcribed from a mammary gland-specific promoter. We have examined the specificity and effect of human PS2 protein expression in transgenic mice, and the results of these experiments are reported.

RESULTS

Construction of Transgenic Mice Bearing WAP-PS2 Fusion Constructs

The promoter of the mouse whey acidic protein (WAP) gene has previously been used to target expression of foreign proteins to the mammary gland of transgenic mice (15–17). Two constructs were designed in order to direct PS2 expression to the mammary gland. In WAP-PS2–2 the 5′ control elements of the intact PS2 gene were replaced by the promoter region (*Eco*RI-*Kpn*I) of the genomic WAP gene (Fig. 1B). A second construct, WAP-PS2–1, avoids deleting elements from the WAP gene that might be essential for mammary- or lactation-specific expression; the human PS2 cDNA was inserted into the 5′ untranslated region of the intact mouse WAP gene (Fig. 1A). This fusion gene parallels the construct we previously assembled to direct foreign protein production in the mammary gland of transgenic sheep (18).

The hybrid genes were freed of bacterial plasmid sequences and microinjected into fertilized mouse eggs. Three founder mice carried the WAP-PS2–1

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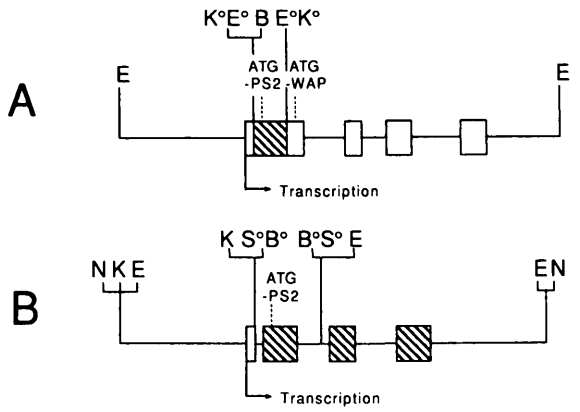

Fig. 1. WAP-PS2 Fusion Genes

A, Construct WAP-PS2–1. B, WAP-PS2–2. Both constructs contain 2.5 kb of 5′ flanking sequences from the mouse WAP gene. Restriction sites are: E, *Eco*RI; K, *Kpn*I; B, *Bam*HI; N, *Not*I; S, *Sma*I. K°, E°, S°, and B°, Sites destroyed during cloning. Exons and coding sequences are *boxed*; PS2 sequences are *cross-hatched*; WAP sequences are shown by *open boxes*. Trancription and translation start sites are indicated.

transgene, of which only one transmitted to progeny (line 33, 20 copies/haploid genome). Seven mouse lines were obtained harboring the WAP-PS2 2 construct. Southern analysis of the integrated transgenes failed to reveal rearrangement.

Tissue Specificity of WAP-PS2 Transgene Expression

To determine the distribution of transcripts from the WAP-PS2 transgenes, tissues from transgenic female mice during lactation were examined for the presence of mRNA species hybridizing to a radiolabeled PS2 cDNA probe. All control mice and tissues from nonlactating animals failed to present transcripts hybridizing to the probe. Further, all seven lines harboring the WAP-PS2–2 construct similarly failed to present PS2-hybridizing transcripts in any tissue examined (analysis as in Fig. 2; not presented).

The mammary glands of lactating WAP-PS2–1 transgenic animals contained a new transcript at approximately 1100 nucleotides that was absent from other tissues (Fig. 2). The size of the PS2-hybridizing transcript was as expected for a correctly spliced and polyadenylated hybrid PS2-WAP transcript. To confirm that expression of the WAP-PS2 transgene is directed to the epithelium of the lactating mammary gland, tissue sections were analyzed by *in situ* hybridization to radiolabeled WAP- and PS2-specific probes. In Fig. 3 the WAP and PS2 probes produce an identical hybridization pattern.

To estimate transcript level, duplicate Northern blots of mammary gland RNA were hybridized with PS2 and WAP cDNA probes (Fig. 2). Scanning densitometry of the autoradiogram allowed us to calculate that the hybrid transcript level is approximately 2.5% of the level observed for endogenous WAP transcript. It is not known whether this discrepancy reflects differences in transcription rate or transcript processing and/or stability.

Expression of Human PS2 Protein

The WAP promoter specifically directs expression to secretory epithelial cells of the mammary gland (see above) and (15). To determine whether the hybrid WAP-PS2 transcript is correctly translated into a secreted protein product we used Western blotting to analyze milk from lactating WAP-PS2–1 transgenic females for the presence of PS2 protein. In Fig. 4 the transgenic milk contains a band reactive with PS2 antiserum that comigrates with PS2 protein purified from MCF-7 culture supernatant. We conclude that the PS2-coding sequence is translated *in vivo* and that some or all of the protein product passes through the normal secretion pathway. Western analysis of milk from WAP-PS2–2 transgenic mice failed to reveal the presence of PS2 protein (not shown). Because patients bearing PS2-positive mammary tumors secrete PS2 in their urine (Rio, M.-C., and P. Chambon, in preparation), body fluids from lactating WAP-PS2–1 transgenic animals were analyzed for the presence of PS2 protein. However, we failed to detect cross-reactive material in either blood or urine (Fig. 4). Scanning densitometry of Western blot autoradiograms revealed that the level of PS2 protein in transgenic mouse milk was approximately 1.5 μg/ml, whereas WAP was present in both normal and transgenic animals at approximately 2 mg/ml (not presented). Even allowing for the low PS2 transcript level in these animals (2.5% of WAP, above), the appearance of PS2 protein in milk is approximately 10-fold reduced from the expected value, suggesting a defect in the translation, stability, or secretion of the PS2 protein. However, the concentration of PS2 protein in transgenic mouse milk (1.5 μg/ml) is approximately 15-fold higher than that obtained in supernatants of MCF-7 human breast cancer cells (0.1 μg/ml) (5).

Absence of Pathology

Because PS2 expression is associated with human breast tumors but not with normal mammary tissue, a potential role as an autocrine growth factor has been discussed. However, histological examination of transgenic females expressing PS2 in their mammary epithelial cells failed to exhibit any dysplasia of the mammary gland (Fig. 5; see also Fig. 3), in distinct contrast to transgenic females expressing known oncogenes, such as v-Ha-*ras* and c-*myc*, under the control the same mammary-specific promoter (15, 17, 19). Despite a structural similarity to the porcine protein PSP that can regulate digestive function in both pigs and rodents (see *Introduction*), no evident morphological or physiological abnormalities of digestive function were observed in suckling mice receiving PS2 protein in milk from lactating transgenic females. Further, no statisti-

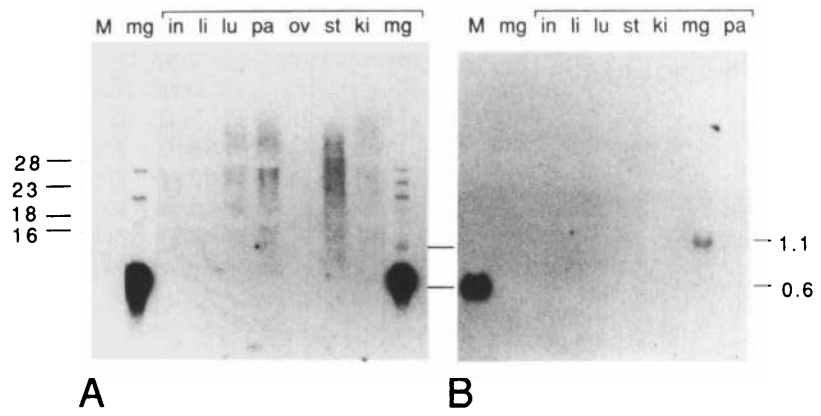

Fig. 2. Northern Analysis of Tissues from WAP-PS2 Transgenic Mice

RNA samples (10 μg) from different tissues of lactating WAP-PS2–1 transgenic female 33 were analyzed for hybridization to probes specific to WAP (A) and PS2 (B). *Overlined* transgenic tissues were: in, intestine; li, liver; lu, lung; pa, pancreas; ov, ovary; st, stomach; ki, kidney; mg, lactating mammary gland. Control samples were: M, RNA from MCF-7 breast cancer cells; mg, mammary gland from a lactating nontransgenic female mouse. rRNA size markers (S values) are indicated (*left*), and the calculated sizes of the transcripts (kilobases) are given (*right*).

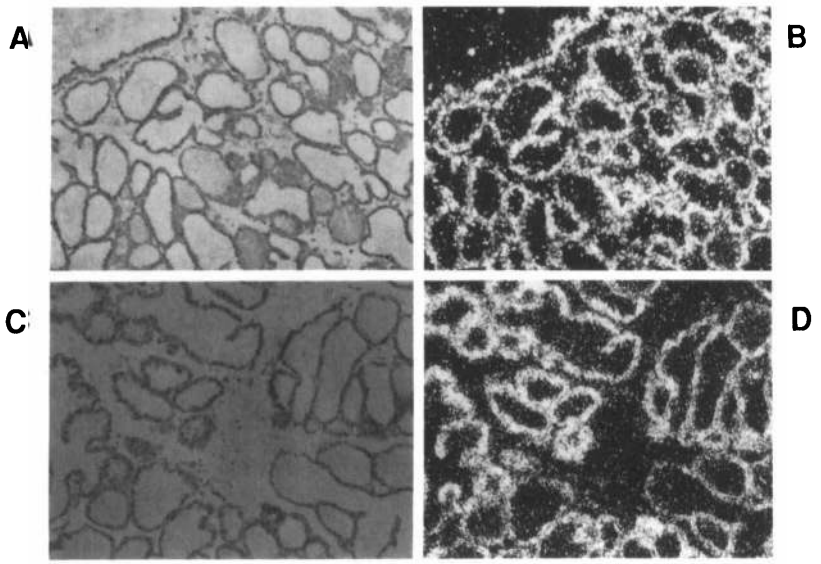

Fig. 3. *In Situ* Hybridization Analysis of Transgenic Mammary Gland

Mammary gland tissues sections from lactating (15 days) WAP-PS2–1 transgenic female 33 were stained (A and C) and hybridized with probes specific for WAP (B) or PS2 (D). The exposure time for D was approximately 4 times longer than that for B. Original magnification, ×10.

cally significant difference in growth rate was observed between transgenic or nontransgenic suckling young of normal or transgenic mothers (not shown).

DISCUSSION

PS2 protein is encoded by an estrogen-inducible gene in the breast cancer cell line MCF7 (1–4), and 50% of human breast tumors express PS2 under estrogen stimulation (8, 20). The PS2 gene comprises three exons (6), and the PS2 precursor polypeptide contains an N-terminal hydrophobic signal sequence that is removed during maturation and secretion (4, 5). Despite extensive characterization of the PS2 gene and the encoded protein, the biological role of PS2 is unknown. The PS2 protein contains a cysteine-rich central domain that has a number of sequence similarities to other secretory proteins (9) (not presented), notably to a porcine PSP in which the cysteine-rich domain is present as two tandem repeats (14), and to a secretory protein from *Xenopus laevis* skin (12). However, there is so far no evidence that these polypeptides have overlapping biological roles (see Ref. 7 for review). PS2 bears structural analogies to a growth factor (3), and it

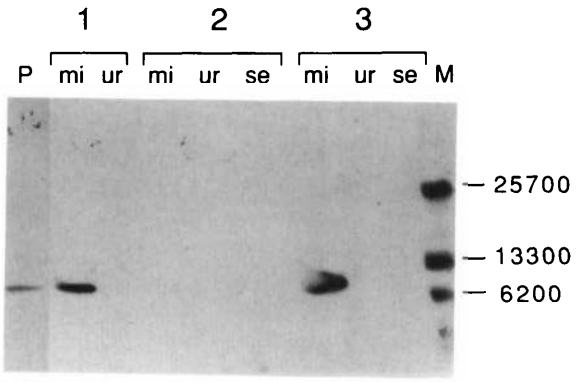

Fig. 4. Western Blot Analysis of PS2 in Transgenic Mouse Fluids

Samples of milk (mi), urine (ur), and blood serum (se) were separated by sodium dodecyl sulfate-gel electrophoresis and developed by the application of anti-PS2 antibody and a second peroxidase-labeled antibody. Animals 1 and 3 were lactating WAP-PS2–1 females 40 and 67; animal 2 was a nontransgenic lactating female. Control lanes were: P, 5 ng purified PS2 protein; M, mol wt markers (staining; sizes in daltons).

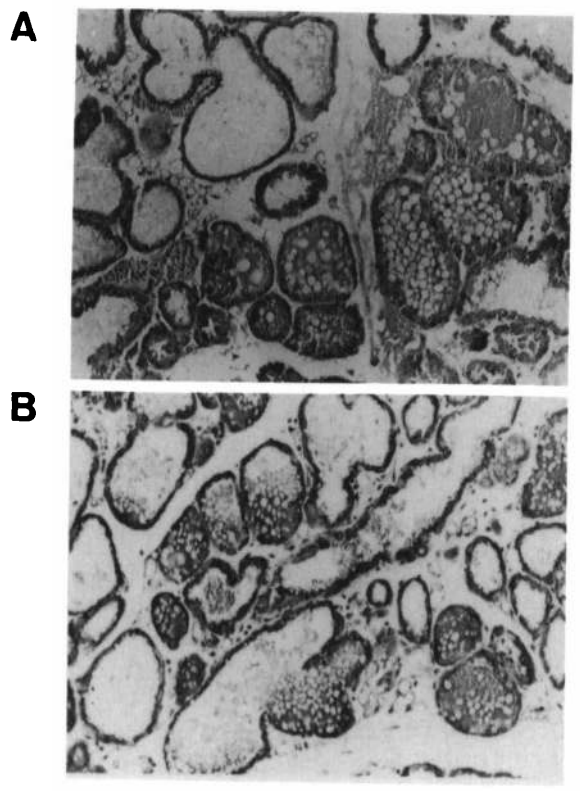

Fig. 5. Histological Analysis of Mammary Gland Sections

Tissue sections from 15-day lactating mammary gland of a mouse (A) and WAP-PS2–1 transgenic mouse 33 (B) were examined by staining and light microscopy. Original magnification, ×10.

has been reported that the porcine protein PSP has weak growth stimulatory effects on MCF7 breast tumor cells (21). However, variants of MCF7 cells with altered responses to estrogen fail to show a correlation between PS2 production and growth (22).

To explore a possible role of PS2 in autocrine stimulation of breast tumor development we elected to express the human protein in epithelial cells of the mouse mammary gland. We and others have previously employed sequence elements from the murine WAP, mouse mammary tumor virus (MMTV), or ovine β-lactoglobulin (15–19, 23–26) to direct the expression of foreign proteins to the mammary epithelium, and mammary neoplasias were reported in transgenic female mice expressing oncogenes under WAP or MMTV control.

Accordingly, two hybrid WAP-PS2 fusion genes were assembled and introduced into the mouse germ line. Seven mouse lines harboring the WAP-PS2–1 construct, in which the 5′ region of the mouse WAP gene is linked to a PS2 minigene genomic segment, all failed to express the transgene. We speculate that because the 5′ restriction site in the PS2 gene (*Bam*HI) used for the fusion lies 86 nucleotides upstream of the natural PS2 transcription start site (6), the intervening region might interact with transcription factors that could interfere with expression from the upstream WAP promoter.

In contrast, the one transgenic line obtained for the WAP-PS2–2 construct, in which the PS2 cDNA is inserted into the 5′ untranslated region of the genomic WAP gene, efficiently expressed the transgene. Expression was restricted to the mammary gland during lactation, and processing of the hybrid mRNA appeared normal. Western blotting revealed the presence of appropriately sized PS2 cross-reactive antigen in the milk of transgenic animals. Because PS2 antigen can be detected in the urine of patients bearing PS2-positive tumors we examined blood and urine from WAP-PS2 transgenic mice; however, PS2 cross-reactive material was not detected in these fluids.

Histological examination of mice expressing PS2 in mammary epithelial cells failed to reveal any abnormalities. No dysplasia or hyperplasia of the mammary gland was observed, and we were unable to detect any differences in gastric function or growth rate in mice receiving milk containing PS2 protein. These results suggest that the PS2 protein is not by itself a transforming growth factor in mouse mammary tissue, possibly because the mouse lacks a PS2 receptor, or that the putative mouse PS2 receptor fails to respond to human PS2.

The role of PS2 protein remains unknown. Although PS2 protein is expressed in 50% of human breast tumors, we have been unable to detect PS2 gene expression in lactating or nonlactating human breast tissue (9). The rarity of appropriate tissue samples, however, precludes systematic analysis. In particular, we cannot exclude the possibility that PS2 protein might be expressed transiently in normal human breast, as reported for the PTH-like peptide in rats (27). The homology between PS2 protein and the porcine spasmolytic polypeptide PSP (7, 9–12) may indicate a role for PS2 in regulating gastric function; however, our

preliminary data indicate that PS2 is not the human equivalent of the porcine PSP species (Tomasetto, C., and R. Lathe, in preparation). The lack of evident alteration in gastric function in suckling young of mothers expressing PS2 protein in milk suggests that PS2 and PSP may have nonoverlapping biological roles. It is of note, however, that PSP protein is active in a rodent assay (13) indicating that rodents express a PSP receptor.

MATERIALS AND METHODS

Constructions

The hybrid gene WAP-PS2-1 was constructed by first inserting a 490-nucleotide *Bam*HI-*Eco*RI fragment comprising the intact PS2 cDNA (3) into pGEM1 (Promega Biotech, Madison, WI) and converting the filled-in *Bam*HI site to an *Eco*RI site by linker-tailing (28). The cloned mouse WAP gene contained within a single 7.5-kilobase (kb) *Eco*RI fragment (29) was inserted into plasmid pPolyII (30) from which the polylinker *Kpn*I site had been removed (by Klenow polymerase treatment and religation; kind gift of C. Guenet), and the PS2 cDNA now flanked by *Eco*RI sites was introduced using an octameric adaptor (31) into a unique *Kpn*I site 2.5 kb downstream from the *Eco*RI site and within the WAP 5′ exon, destroying the *Eco*RI and *Kpn*I sites flanking the PS2 gene and yielding pPolyII/WAP-PS2-1.

Hybrid gene WAP-PS2-2 was constructed as follows. The first exon of the genomic PS2 gene (6) was excised with *Bam*HI, filled, and introduced at the *Sma*I site of pPolyIII-i (30) An *Eco*RI genomic segment comprising the last two exons of the human PS2 gene was subsequently introduced at the *Eco*RI site downstream of the cloned first exon. In the cloned WAP gene (*Eco*RI fragment inserted into pPolyII) the WAP promoter is contained within a 2.5-kb *Kpn*I fragment extending from the *Kpn*I site in the plasmid polylinker to the *Kpn*I site in the first exon of the WAP gene. This fragment was introduced into the *Kpn*I site 5′ of the reassembled 3 exons of the genomic PS2 minigene, yielding pPolyIII/WAP-PS2-2.

Generation of Transgenic Mice

WAP-PS2-1 and -2 hybrid genes were freed of plasmid sequences (by digestion with *Eco*RI and *Not*I, respectively) and purified by sucrose gradient centrifugation before injection into fertilized embryos of C57BL6 × SJL mice as described previously (Ref. 32 and references therein) The presence of the transgene was determined by Southern analysis of tail DNA using a PS2-specific probe. Of 140 offspring emerging from eggs injected with WAP-PS2-1, three animals (two males and one female) carried the transgene, and only one (line 33 founder female) transmitted the transgene to offspring. Line 33 carries approximately 20 copies of the WAP-PS2-1 transgene per haploid genome integrated into a single autosomal site, and offspring of the single founder transgenic female were used in all subsequent experiments. For construct WAP-PS2-2, 45 offspring yielded seven transgenic animals (five females and two males) which all transmitted the transgene to offspring.

RNA Analysis

RNA from mouse organs was purified as described previously (33). Northern blotting after agarose/methyl-mercury electrophoresis (34) was to DBM paper (35) or by electrophoretic transfer to nylon membranes after electrophoresis in the presence of formaldehyde (36). Klenow-labeling of hybridization probes (PS2 and WAP) was carried out by random priming on denatured DNA (37).

Analysis of Proteins in Biological Fluids

Tissue and fluid samples were obtained from transgenic or control nontransgenic females on day 15 of lactation. For milk collection, the young were removed, and animals were treated 2.5 h later with oxytocin (0.3 U, ip; Syntocinon, Sandoz, Basel, Switzerland). After 10 min, animals were anesthetized [0.4 ml; 2.5% (wt/vol) tribromoethanol in physiological salt solution, ip; 37 C]; gentle massage of mammary glands permitted collection of milk into a capillary tube. For routine analysis only alternate mammary glands were harvested, allowing the young to be replaced. Urine samples were obtained by manual stimulation; blood samples were obtained by intracardiac puncture. Fluids were clarified by centrifugation. Enrichment for PS2 protein was performed by ultrafiltration removal of high mol wt material (retained mol wt > 30,000K; Centricon Microconcentrator 30, Amicon, Danvers, MA), and the filtrate was concentrated by a second step of ultrafiltration (>10,000K), during which PS2 protein is retained. This material was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18% acrylamide) and analyzed by Western blotting (38) using a monoclonal anti-PS2 antibody P2802 raised against a synthetic peptide comprising the C-terminal 28 amino acids of PS2 protein and developed using a second, peroxidase-labeled goat antimouse immunoglobulin as described previously (9).

Analysis of Tissue Sections

Histological staining of fixed and rehydrated paraffin-embedded sections employed sequential staining with Harris hematoxylin (3 min) and eosin (0.5%, wt/vol; 3 min). Samples were dehydrated before mounting. *In situ* hybridization was performed essentially as described previously (39, 40); 6-μm acid-treated and paraformaldehyde-fixed sections were treated with proteinase-K and hybridized overnight with ^{35}S-labeled transcripts (41) from the appropriate probe segment cloned into pGEM-1, treated with RNase (20 μg/ml; 30 min; 37 C), and stringently washed (15 mM NaCl and 1.5 mM Na_3citrate; 65 C; 15 min) before autoradiography using NTB2 emulsion (Eastman Kodak, Rochester, NY). Slides were stained with toluidine blue after development.

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