The Gene for a Novel Member of the Whey Acidic Protein Family Encodes Three Four-disulfide Core Domains and Is Asynchronously Expressed during Lactation*

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Secretion of whey acidic protein (WAP) in milk throughout lactation has previously been reported for a limited number of species, including the mouse, rat, rabbit, camel, and pig. We report here the isolation of WAP from the milk of a marsupial, the tammar wallaby (Macropus eugenii). Tammar WAP (tWAP) was isolated by reverse-phase HPLC and migrates in SDS-polyacrylamide gel electrophoresis at 29.9 kDa. tWAP is the major whey protein, but in contrast to eutherians, secretion is asynchronous and occurs only from approximately days 130 through 240 of lactation. The full-length cDNA codes for a mature protein of 191 amino acids, which is comprised of three four-disulfide core domains, contrasting with the two four-disulfide core domain arrangement in all other known WAPs. A three-dimensional model for tWAP has been constructed and suggests that the three domains have little interaction and could function independently. Analysis of the amino acid sequence suggests the protein belongs to a family of protease inhibitors; however, the predicted active site of these domains is dissimilar to the confirmed active site for known protease inhibitors. This suggests that any putative protease ligand may be unique to either the mammary gland, milk, or gut of the pouch young. Examination of the endocrine regulation of the tWAP gene showed consistenly that the gene is prolactin-responsive but that the endocrine requirements for induction and maintenance of tWAP gene expression are different during lactation.

Whey acidic protein (WAP) has been identified in the milk of a limited number of species and is the major whey protein in the mouse (1), rat (2), rabbit (3), and camel (4). It is also a significant component of porcine milk (5). The WAP proteins from all species show limited sequence identity at the amino acid level but are recognized by a two-domain structure, known as the four-disulfide core (4-DSC) (6) domain, which comprises eight cysteine residues in a conserved arrangement (1). The 4-DSC domain arrangement is not exclusive to the WAP family of proteins; numerous other proteins have been identified that contain one or two such domains. Although a large biological diversity exists between these proteins, many have been identified as protease inhibitors and are grouped into families based on their functionality and tissue-specific origins. These families include the antileukoproteinase (ALKI) family (7), epidermidyl (8) and ovulatory (9) specific proteins, and elastase inhibitor proteins (10). Given such a variety of proteins, it is thought that the 4-DSC may be a preferential conformation for the stable folding and action of a particular class of protease inhibitors (6, 11). Despite no biological action or function being demonstrated for the WAP proteins and based on the limited sequence identity with known protease inhibitors, it has been postulated that WAP may be a protease inhibitor (6, 12).

We have reported previously (13) that WAP is present in the milk of a marsupial, the tammar wallaby (Macropus eugenii), and preliminary data showed the secretion of this protein was developmentally regulated during the lactation cycle. The tammar wallaby is widely used as a model to study marsupial development, and as a result its reproduction cycle has been well defined (14–16). This species has adopted a comparatively different reproductive strategy to other eutherians, i.e. a short gestation and birth of an immature young followed by a relatively long lactation (14, 15). Milk is the only nutritional source available to the rapidly developing pouch young until it has reached physiological maturity and is able to move outside the pouch and eat herbage (14). The composition of the milk is complex, and it changes during the lactation cycle to meet the nutritional demands of the pouch young. The lactation cycle in the tammar has been divided into four distinct phases (17), which are characterized by both changes in milk composition and changes in the sucking pattern of the pouch young. Phase 1 comprises the 26.5-day gestation period during which time all four mammary glands prepare for parturition and the onset of lactogenesis (18). At parturition, a single immature young climbs into the pouch and attaches to one of the four available kilobase(s); I, insulin; F, cortisol; P, prolactin; T3, tri-iodothyronine; E2, estradiol.

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§§ The abbreviations used are: WAP, whey acidic protein; tWAP, tammar WAP; pWAP, pig WAP; cWAP, camel WAP; mWAP, mouse WAP; platWAP, platypus WAP; 4-DSC, four-disulfide core; ALKI, antileukoproteinase; HPLC, high performance liquid chromatography; -lacl, -lactalbumin; PAGE, polyacrylamide gel electrophoresis; -LG, 1,0-lactoglobulin; hSLPI, human secretory leukocyte proteinase inhibitor; LLP-A and -B, late lactation proteins A and B; bp, base pair(s); kb, kilobase(s); I, insulin; F, cortisol; P, prolactin; T3, tri-iodothyronine; E2, estradiol.
teats, and remains permanently attached to this teat for the next 100–120 days (phase 2A (17)), receiving a limited volume of milk that is low in fat and protein but high in complex carbohydrates (19, 15). At the onset of phase 2B at around days 100–120, the pouch young relinquishes the teat but remains in the pouch for a further 80–100 days. During phase 2A and 2B the rate of growth is slow, with a large component of the milk nutrients utilized for development of physiological functions (14). The commencement of phase 3 (day 200) is characterized by an increase in milk production and size of the mammary gland, together with the secretion of a more concentrated milk, which is high in lipid and protein and low in carbohydrate (19, 15). The growth rate of the pouch young accelerates, and at approximately day 250 it permanently leaves the pouch, adopting a diet of milk and herbage, until weaning at 300–350 days postpartum (19, 15).

We report here for the first time the isolation and purification of WAP from a marsupial, the tammar wallaby, and show that gene expression is developmentally regulated throughout the lactation cycle and appears potentially controlled by a novel signaling mechanism intrinsic to the mammary gland. The structure of tWAP does not conform to the two-domain structure described for WAPs from all other species. A consensus three-dimensional structural model for the two-domain eutherian WAP proteins was developed recently, with a detailed atomic model created for pig WAP (pWAP (6)). This paper reports an analogous three-domain structural model for tWAP, and together with the unique profile for developmentally regulated expression of the gene for tWAP, provides new opportunities to examine the role of this protein during lactation.

**EXPERIMENTAL PROCEDURES**

**Materials**—All tissue culture reagents were purchased from Life Technologies, Inc. Oxine prolactin (NIH-sprL-16) was provided by the National Hormone and Pituitary Program, University of Maryland, School of Medicine (Baltimore, MD). Bovine insulin, hydrocortisone, tri-iodothyronine, and estradiol were purchased from Sigma.

**Housing of Animals**—Tammar wallabies were maintained on site at the Victorian Institute of Animal Science in a natural habitat with access to water and feed supplemented ad libitum.

**Purification of tWAP by HPLC and Amino Acid Sequence Analysis**—Acidified whey from various stages of lactation was fractionated by high performance liquid chromatography (HPLC) (5) using a POROS R2/H reverse-phase column and a 15–60% gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 4 ml/min. The absorbance of proteins eluting from the column was measured at both 215 and 280 nm, trypsinized with acidified whey from various stages of lactation was fractionated by high performance liquid chromatography (HPLC) (5) using a POROS R2/H reverse-phase column and a 15–60% gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 4 ml/min. The absorbance of proteins eluting from the column was measured at both 215 and 280 nm, and fractions were dried under vacuum and stored at 4 °C. Proteins were resuspended in distilled water, analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and identified by N-terminal amino acid sequencing and revealed 46 residues, which shared significant sequence identity with camel WAP (cWAP). The protein obtained from peak 2 at day 193 of lactation was analyzed by 20% SDS-PAGE and showed tWAP migrated as a major protein at 29.9 kDa (Fig. 1, inset).

**The Profile of tWAP Secretion during Lactation**—SDS-PAGE analysis of total whey from day 140 to 276 of lactation showed the asynchronous changes in milk composition at the transition from phase 2B to phase 3 (Fig. 2). tWAP was a major whey protein at day 202 of lactation and was undetectable in phase 3 whey. Both β-LG (18 kDa) and α-lactalbumin (α-lac; 14 kDa) were secreted throughout lactation, whereas the secretion of late lactation protein A (LLP-A; 24 kDa) and LLP-B (21 kDa) was induced around days 180 and 220 respectively, signaling the transition to phase 4 of lactation (13).

**Isolation of tWAP Full-length cDNA**—A degenerate anti-sense oligonucleotide designed to the known tWAP peptide sequence was used to screen a cDNA library prepared from mammary gland RNA from day 201 of lactation. The clone isolated from the cDNA library was 724 base pairs (bp) and represented a full-length cDNA coding for 191 translated amino acids (GenBank™ accession number AJ005556). The translated sequence was identical to the N-terminal sequence to a Zeta Probe GT membrane (Bio-Rad). The tWAP and tammar β-lactoglobulin (β-LG) cDNAs were hybridized with the membranes (5), washed at 60 °C to high stringency (0.1% SSC/0.1% SDS), and exposed to Kodak Xomat film at −70 °C. The tWAP mRNA levels were quantitated by slot blot analysis (22). The membranes were hybridized with the tWAP cDNA and washed to high stringency, and each individual slot was excised and placed into 5 ml of organic scintillant. The radioactivity was measured in a liquid scintillation spectrophotometer (Wallac 1410, Amersham Pharmacia Biotech).

**Mammary Gland Explant Culture System**—Mammary gland tissue from tammar does not conform in the two-domain structure described for WAPs from all other species. A consensus three-dimensional structural model for the two-domain eutherian WAP proteins was developed recently, with a detailed atomic model created for pig WAP (pWAP (6)). This paper reports an analogous three-domain structural model for tWAP, and together with the unique profile for developmentally regulated expression of the gene for tWAP, provides new opportunities to examine the role of this protein during lactation.

**Identification of tWAP by HPLC**—HPLC fractionation of tammar whey collected at 14- to 21-day intervals from days 88 to 270 of lactation resulted in an average of 10 major protein peaks, depending on the stage of lactation (Fig. 1). Examination of eluates at an absorbance of 215 nm showed that several peaks either appeared or disappeared from the milk at specific times during the course of lactation. Peaks 2 and 3 were first detected at approximately day 116, reaching a maximum around day 193 (Fig. 1) and then reducing to undetectable levels by day 256 of lactation (data not shown). The protein component of these peaks, eluting as a doublet at 29.5% and 30.6% acetonitrile (Fig. 1), was subjected to tryptic digestion and N-terminal amino acid sequencing and revealed 46 residues, which shared significant sequence identity with camel WAP (cWAP). The protein obtained from peak 2 at day 193 of lactation was analyzed by 20% SDS-PAGE and showed tWAP migrated as a major protein at 29.9 kDa (Fig. 1, inset).
obtained from the protein in peak 2. The cDNA contains a 23-bp 5'-untranslated region, 18-amino acid signal peptide, and 2 stop codons that are in-frame and positioned 10 amino acid residues apart (Fig. 3). The polyadenylation signal (AATAAA) is situated at nucleotide position 695, and the clone contains a further 24 bp of 3'-untranslated region but does not contain the poly(A) tail (Fig. 3). The translated sequence revealed that the tWAP gene consists of three 4-DSC domains and not the two domains reported for the eutherian WAP proteins (Figs. 3 and 4). The three domains of tWAP have been ordered consecutively as domains III, I, and II (Fig. 3), with domains I and II being conserved within other species (Fig. 4).

An alignment of all WAP proteins and selected representatives from several protein families that contain one or two 4-DSC domains showed the characteristic conservation of the cysteine residues within each domain (5, 6), including tWAP domain III (Fig. 4). An interesting feature of the WAP proteins...
is a conserved WAP motif of seven amino acids, KAGRCPW, which is present in the mouse, rat, rabbit, camel, and pig and is located at the start of domain II and includes the first cysteine residue of the domain (5). In all protease inhibitors with one or two domains, the motif is also present at the beginning of each domain, however, it is less conserved, resulting in a consensus sequence of KXGXCXP in each domain (boxed) compared with hSLPI (16) and pWAP (17, 13, 6). The two domains of hSLPI are oriented at 150° to each other, with a translation of 16.4 Å (based on the structure comparison method of Reference 29), whereas the three tWAP domains are at about 120° with a translation of 9.5 Å between domains I and II and 12.7 Å between domains III and I. MOLSCRIPT (30) graphic representations of the tWAP model are shown in Fig. 5 (A and B). The tWAP model was assessed using PROCHECK (31) as being structurally very satisfactory, with 97.4% of its residues adopting allowed backbone conformations. The surface electrostatic potential (results not shown), computed using GRASP (32), of the top outer loop of each tWAP domain is hydrophobic, similar to the serine protease inhibitors that have a 4-DSC domain (24). The coordinates of the model structure are available from the Protein Data Bank (PDB code: 1TWP).

The two domains of hSLPI are capable of independent function, inhibiting trypsin and chymotrypsin, respectively (24). The scissile bonds for hSLPI domain I are residues Ry (residues 20 and 21) and for domain II are LM (residues 72 and 73) (24). The structurally equivalent residues in tWAP are RD (domain I: residues 17 and 18) and QC (domain I: residue 95 with the Cys97 Cα atom occupying the position of Tyr22 Cα) and PE (domain II: residues 140 and 141) (Fig. 5B). Preliminary studies have not shown any inhibitory activity against trypsin and chymotrypsin (data not shown).

tWAP Gene Expression Profile throughout Lactation—Total RNA was extracted from the lactating glands of tammar wallabies at phases 2A, 2B, and 3 and examined by Northern analysis. A tWAP transcript of approximately 0.8 kilobase (kb)
was present only in RNA from days 140 to 231 of lactation (Fig. 6A). No transcript was detected in RNA isolated from mammary glands during gestation or from tammar liver (data not shown). Quantitative analysis of the same RNA samples using slot blots confirmed the mRNA levels were greatest around day 200 of lactation (Fig. 6B). The tammar β-LG transcript was detected in all samples analyzed, confirming the integrity of the RNA.

**Endocrine Control of tWAP Gene Expression**—To establish the endocrine requirements for tWAP gene expression, explants of mammary gland tissue from either lactating or late pregnant tammars were cultured in M199 in the presence of various hormone combinations. Examination of sections of tissue taken prior to culture and stained with hematoxylin and eosin confirmed the absence of secretory cells in the pregnant tissue and presence of expanded alveoli and lipid secretions in the lactating tissue (data not shown). The tWAP mRNA was not detected by Northern analysis in uncultured mammary gland tissue (T₀) at day 24 of pregnancy but was detected after 4 days of culture in media with insulin (I), cortisol (F), prolactin (P), tri-iodothyronine (T₃), and estradiol (E₂) (IFPT₃E₂) with maximal levels observed after 8 days of culture (Fig. 7A). No tWAP mRNA was detected in explants cultured in media with either IF, P, or IFP. Expression of the β-LG gene was induced with P alone in mammary explants from tammars at day 24 of gestation and in all analyses was not detected after 4 days of culture in IF, confirming previous studies (22).

To examine whether the endocrine requirements for maintenance of tWAP gene expression were different to the requirements for induction of the gene, mammary gland tissue from days 180 and 260 of lactation were cultured under the same conditions as described above. tWAP mRNA was expressed at elevated levels in the uncultured (T₀) tissue at day 180 and was maintained at similar levels after 8 days of culture in IFP and IFPT₃E₂, but a transcript was barely detectable in explants cultured in media with either IF, P, or IFP. Expression of the β-LG gene was maintained with P alone, and stimulated above T₀ values in explants cultured in media with either IFP or IFPT₃E₂. At day 260 of lactation the level of tWAP mRNA was barely detectable and was not stimulated in explants after 4 days in media with IFPT₃E₂ (Fig. 7C). In contrast, β-LG gene expression was elevated in mammary tissue prior to culture and maintained at reduced but high levels of expression in all treatments at days 4 (Fig. 7C) and 8 of culture (data not shown).

**DISCUSSION**

Whey acidic protein (WAP) has previously been isolated from the milk of a range of laboratory species (1–3) and the camel (4) and pig (5). In addition, the protein has been identified by limited N-terminal sequencing of HPLC-purified protein from the milk of two monotremes, the echidna (Tachyglossus aculeatus (26)) and platypus (Ornithorhynchus anatinus³). The isolation of tWAP from the milk of a marsupial reported here confirms that the synthesis and secretion of WAP is widespread in

³ D. Shaw, unpublished data.
from the brook trout, tissues from human (7), mouse (34), and pig (35). Epididymal family, with genes isolated from a variety of mucosal secretory species and that selection during evolution has reduced the domains. It appears probable that they are the progenitor monotreme WAPs with tWAP suggests they also contain three monogenic duplication, most likely of the more conserved domain in vitro hormonal requirements for lactation, and two at day 260 of lactation. Mammary gland explants were analyzed by Northern hybridization with tWAP and β-LG cDNA probes. Ribosomal RNA bands stained with ethidium bromide are included to indicate consistency of loading. Northern blots are representative of four animals at day 24 of pregnancy, three at day 180 of lactation, and two at day 260 of lactation. A, phase 1, day 24 of pregnancy, day 8 of culture, phase 2B, day 180 of lactation, day 8 of culture, phase 3, day 260 of lactation, day 4 of culture.

many species that have been subjected to a range of evolutionary selection pressures.

It was originally proposed that the WAP gene arose by intragenic duplication, most likely of the more conserved domain II (33). The alignment of the N-terminal sequence of the monotreme WAPs with tWAP suggests they also contain three domains. It appears probable that they are the progenitor species and that selection during evolution has reduced the protein to two domains. Despite the diversity in the origin of species and that selection during evolution has reduced the domains, it appears probable that they are the progenitor species and that selection during evolution has reduced the protein to two domains. Despite the diversity in the origin of proteins that comprise either single or multiple copies of a 4-DSC domain (6), generally these proteins are secreted and many have established protease inhibitor functions. For example, the two-domain arrangement of the pig, mouse, rat, rabbit, and camel WAPs is present in the antileukoproteinase (ALKI) family, with genes isolated from a variety of mucosal secretory tissues from human (7), mouse (34), and pig (35). Epididymal secretory protein homologs isolated from human (HE-4 (8)), dog (CE-4 (36)), and rabbit (BE-20 (37)) also conform to this structure, along with an ovulation-specific gene, TOP-1, isolated from the brook trout (Salvelinus fontinalis) (9). In TOP-1, the two 4-DSC domains are precisely duplicated, whereas another isoform, TOP-2, encodes three perfectly duplicated 4-DSC domains, which are interspersed with a different repeated domain (9).

The second 4-DSC WAP domain is also conserved in a range of protease inhibitors with a single 4-DSC domain such as elafin, an elastase inhibitor identified in human (10) and porcine tissue (38); the gene for Kallmann syndrome in humans (39) and chicken and quail (40); WDNM1 from mouse (41) and pig (35) and triiodothyronine, and estradiol (IFPT, E2, abbreviated here to TE). T0 indicates tissue analyzed prior to culture. Total RNA was extracted from the cultured tissue after either 4 or 8 days of culture and 10 μg were analyzed by Northern hybridization with tWAP and β-LG cDNA probes. Ribosomal RNA bands stained with ethidium bromide are included to indicate consistency of loading. Northern blots are representative of four animals at day 24 of pregnancy, three at day 180 of lactation, and two at day 260 of lactation. A, phase 1, day 24 of pregnancy, day 8 of culture, phase 2B, day 180 of lactation, day 8 of culture, phase 3, day 260 of lactation, day 4 of culture.

The development of a three-dimensional model for tWAP and pWAP (6) suggests the hydrophobic loops of these proteins may be functional, although the WAP family members do not share sequence identity within the active sites present in known protease inhibitors (6, 48). The major difference between the tWAP and the pWAP models arises from the fact that the “interdomain space” (between domains I and II) is only 10 residues in tWAP compared with 13 residues in pWAP and 16 residues in hSLPI (6). Furthermore, this interdomain space is conserved between domains III and I, and between I and II of tWAP (10 residues). Thus, although the tertiary fold is conserved among the WAPs, the quaternary structure of tWAP will be determined by the interdomain space. The two hSLPI domains have very little interaction with each other and appear to be capable of independent (protease inhibition) function (24). By analogy, we predict that the three tWAP domains are arranged with little interdomain interaction but with the possibility of independent function.

The surface electrostatic potential (not shown) at the top of the outer loop of each tWAP domain appears hydrophobic, analogous to that of the serine protease inhibitors. Thus, although it is unclear whether both domains of the eutherian WAPs are functional (6), all three tWAP domains have the conserved WAP (KXGXP) motif, four intact disulfide bridges, and the surface hydrophobic character required for functionality. In particular, the high degree of structural conservation of tWAP domain II compared with hSLPI, along with its hydrophobic electrostatic character, suggests that this domain is very likely to possess protease inhibitor activity.

In eutherian species all the major milk protein genes, including the WAP gene, are induced in late pregnancy, and the proteins are secreted at unchanged levels in the milk throughout lactation (1–3, 5, 50). Mammary explant culture has been used extensively to examine the minimal hormonal requirements for the induction of milk protein genes in many eutherian species, particularly using tissue from animals in late pregnancy prior to lactogenesis. The WAP gene in mice, rats, and pigs is maximally expressed in vitro in the presence of the lactogenic hormones insulin, cortisol, and prolactin (5, 50, 51). Similarly, rabbit WAP was induced in the presence of prolactin but required the addition of insulin and cortisol for maximal expression (52). In contrast to eutherian species, the genes for β-LG, α-lact, and α- and β-casein are coordinately induced at parturition in the tammar (13), whereas the tWAP gene is induced around day 130 of lactation. The expression of the tWAP gene was induced in mammary explants from late pregnant tammars in response to insulin, cortisol, prolactin, triiodothyronine, and estradiol, but the endocrine requirement for maintenance of tWAP gene expression was limited to insulin, cortisol, and prolactin, indicating that there is a role for oes-

**Fig. 7.** Endocrine regulation of tWAP induction in vitro. The hormonal requirements for *in vitro* expression of tWAP and β-LG after explant culture of mammary glands from late pregnancy (phase 1), phase 2B, and phase 3 of lactation. Mammary gland explants were cultured in the presence of insulin and cortisol (IFP), prolactin alone (P), insulin, cortisol, and prolactin (IFPP); and insulin, cortisol, prolactin, triiodothyronine, and estradiol (IFPT, E2, abbreviated here to TE). T0 indicates tissue analyzed prior to culture. Total RNA was extracted from the cultured tissue after either 4 or 8 days of culture and 10 μg were analyzed by Northern hybridization with tWAP and β-LG cDNA probes. Ribosomal RNA bands stained with ethidium bromide are included to indicate consistency of loading. Northern blots are representative of four animals at day 24 of pregnancy, three at day 180 of lactation, and two at day 260 of lactation. A, phase 1, day 24 of pregnancy, day 8 of culture, B, phase 2B, day 180 of lactation, day 8 of culture, C, phase 3, day 260 of lactation, day 4 of culture.
trogen and/or thyroid hormone for induction of expression of the gene. Mammary epithelial cells from phase 1 tissue are responsive to insulin, cortisol, and prolactin (13); therefore, the role of oestrogen and thyroid hormone is not to alter tissue sensitivity to these hormones. The low level of expression of the tWAP gene in tissue at day 260 of lactation was not sustained in explants cultured for up to 8 days in media with any hormone combination examined. Therefore, it appears that the inhibition of the tWAP gene observed in mammary tissue in phase 1 of lactation can be reversed in vitro and that oestrogen and/or thyroid hormone have a critical role in stimulating the transition of the mammary epithelial cells from a phase 1 to phase 2B phenotype. However, once the gene has been down-regulated in vivo, the mammary epithelial cells are no longer responsive to the combination of insulin, cortisol, prolactin, triiodothyronine, and estradiol to be cycled back in vitro from the phase 3 to phase 2B phenotype. The level of cortisol, prolactin, and thyroxine does not change in the peripheral circulation around days 100–130 of lactation (15), and, although the levels or insulin or estradiol have not been measured, it is unlikely that significant changes in concentration occur at this time. There is evidence to suggest that the marsupial mammary gland can alter its response to oxytocin in the peripheral endocrine milieu during development (53). Whether this mechanism extends to other hormones that regulate milk protein gene expression requires further investigation.

The secretion of tWAP only during phase 2B of lactation contrasts the secretion of this protein throughout lactation in eutherian species and provides a more viable model to examine its potential functions. It is conceivable that WAP may have multiple functions. For example, the protein is secreted as the major component of the whey fraction, therefore making it likely to be a major food source for the pouch young. During phases 2A and 2B, the physiological development of the young is largely completed (14), and the time at which secretion of tWAP is down-regulated coincides with pouch exit, a change to a mixed diet incorporating grass and milk, and the establishment of homeothermy in the young (14). Early studies (54) reported a marked increase in sulfur-containing amino acids in the milk from around day 150 of lactation, coincident with the appearance of hair follicles. Sulfur-rich amino acids are thought to be required for fur and nail growth (54), thus the appearance of hair follicles. Sulfur-rich amino acids are likely to be a major food source for the pouch young. During phases 2A and 2B, the physiological development of the young is largely completed (14), and the time at which secretion of tWAP is down-regulated coincides with pouch exit, a change to a mixed diet incorporating grass and milk, and the establishment of homeothermy in the young (14). Early studies (54) reported a marked increase in sulfur-containing amino acids in the milk from around day 150 of lactation, coincident with the appearance of hair follicles. Sulfur-rich amino acids are thought to be required for fur and nail growth (54), thus the appearance of hair follicles. Sulfur-rich amino acids are likely to be a major food source for the pouch young.

The expression pattern of the tWAP gene during lactation correlates with changes in expression of several other milk protein genes in the tammar, and these changes appear to be associated with specific changes in the sucking pattern of the pouch young. For example, early lactation protein is secreted only in phase 2A of lactation (49) and shares sequence identity with a trypsin inhibitor isolated from bovine colostrum (55) and a homologue secreted in possum milk (Trichosurus vulpecula (56)). The cessation of secretion of this protein occurs at the transition between phases 2A and 2B, at approximately days 100–130 of lactation and correlates with the induction of the tWAP gene. Around this time, the pouch young releases its permanent attachment to the teat but continues to remain in the pouch and suckles at intermittent intervals (14). In addition, preliminary evidence from our studies suggests that cystatin, another putative protease inhibitor is also secreted during phase 2B of lactation. Following the induction of tWAP, another novel protein, late lactation protein-A (LLP-A), is detected in the milk around day 170 of lactation and continues to be expressed until weaning (57). An isoform of LLP-A, late lactation protein-B (LLP-B), is induced at the transition to phase 3 at around day 220 and correlates with the down-regulation of WAP secretion. At this time the pouch young begins to exit the pouch but continues to suckle from the mother while at heel. The regulation of specific protease inhibitor genes linked to the transition between the phases of lactation raises the possibility that these proteins may contribute to either remodeling mammary tissue at this time or potentially have a role in gut development and to protect a specific dietary protein required for development of the pouch young. Earlier studies have suggested that WAP plays an important role in mammary gland development (12), because the milchlos phenotype (limited milk secretion) resulting from overexpression of the mouse WAP gene during early pregnancy in mice (48) and pigs (58) suggests that it is involved in terminal differentiation of the mammary gland. tWAP secretion declines prior to phase 3, at which time the mammary gland undergoes significant tissue remodeling, including increased growth, milk production, and secretion of a concentrated milk to provide appropriate nutrition to meet the demands of the rapidly growing young (13, 17, 18, 59). It is interesting to speculate that tWAP plays a negative regulatory role, allowing extensive mammary gland development after it has been down-regulated. Any putative role of the pouch young in regulating these changes remains to be established, but it seems likely that endocrine-stimulated tWAP gene expression during lactation is modulated by factors intrinsic to the mammary gland. This conclusion would be consistent with the independent regulation of mammary glands during asynchronous concurrent lactation (13, 15, 17).

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