Molecular characterization and in-vitro hormonal requirements for expression of two casein genes from a marsupial

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ABSTRACT

Two marsupial casein genes have been isolated from a tammar wallaby (Macropus eugenii) mammary gland cDNA library. Comparisons of the tammar α- and β-casein genes with their eutherian homologues reveal extensive divergence at the levels of nucleotide and amino acid sequences. Regions of similarity between the tammar and eutherian Ca\(^{2+}\)-sensitive caseins are restricted to the major phosphorylation sites and the signal peptides. Quantification of casein mRNA levels in hormone-stimulated mammary gland explants from tammars in late pregnancy suggests that maximal induction of the β-casein gene is dependent upon prolactin and insulin, while maximal induction of the α-casein gene is dependent upon prolactin, insulin and cortisol. These results are in contrast to earlier studies which show that the maximal induction of a putative 19 kDa casein, α-lactalbumin and β-lactoglobulin in the tammar mammary gland is dependent upon prolactin alone. The expression of the latter three genes is not modulated by other hormones known to play a role in the in-vitro initiation of lactation in eutherians.

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INTRODUCTION

The caseins are the most abundant group of milk proteins of almost all mammalian species (Jenness, 1974; Jenness & Holt, 1987). In the cow, the four caseins comprise three major evolutionarily related Ca\(^{2+}\)-sensitive phosphoproteins, denoted α\(_{S1}\), α\(_{S2}\) and β-casein, which are stabilized in a micellar aggregate structure by K-casein (Eigel, Butler, Ernstrom et al. 1984; Pearse, Linklater, Hall & Mackinlay, 1986; Holt & Sawyer, 1988). The caseins are one of the most rapidly evolving families of proteins, and the mature proteins show considerable sequence divergence between species both within and between casein types (Bonsing & Mackinlay, 1987; Rosen, 1987).

The caseins have been used as markers of the differentiation of the mammary gland, an organ under complex endocrine control (Topper & Freeman, 1980). In-vitro studies using mammary gland explants from rat, mouse and rabbit have shown that maximal induction of casein gene expression is dependent upon the presence of insulin, glucocorticoid and prolactin in the culture media (Topper & Freeman, 1980; Vonderhaar & Ziska, 1989). In contrast to the complex control that exists in these eutherians, the maximal in-vitro induction of a putative 19 kDa casein (Maher & Nicholas, 1987), α-lactalbumin (Nicholas & Tyndale-Biscoe, 1985; Collet, Joseph & Nicholas, 1990) and β-lactoglobulin (Collet, Joseph & Nicholas, 1991) in a marsupial species, the tammar wallaby (Macropus eugenii), appears to be dependent upon prolactin alone. Thus, induction of these milk proteins in the tammar is independent of other hormones known to play a role in the induction of milk protein synthesis in eutherians. This paper reports the molecular cloning and characterization of an α- and β-casein cDNA from the tammar. In addition, the hormonal requirements for the maximal in-vitro induction of these tammar casein genes were examined.

MATERIALS AND METHODS

Library construction and screening

The construction of a tammar cDNA library in λgt10 using poly(A)\(^+\) mRNA isolated from the mammary gland at day 270 of lactation has been described previously (Collet, Joseph & Nicholas,
1989). Phage from 200 individual cDNA clones were spotted in a grid on agar plates. Duplicate filters were screened with cDNA probes constructed using poly(A)$^+$ mRNA from mammary glands in early and late lactation. Clones which gave strong positive hybridization signals on both sets of filters were chosen for further analysis by DNA sequencing.

DNA sequencing

Both strands of the cDNA inserts in pGEM4 (Promega, Madison, WI, U.S.A.) were sequenced by the dideoxy method (Sanger, Nicklen & Coulson, 1977) using modified T7 polymerase (United States Biochemicals, Cleveland, OH, U.S.A.). The procedure followed was that recommended by the supplier of the polymerase with the exception that reactions were performed at 50°C. The DNA sequences of the bovine caseins were obtained from the Genbank database (R60, June 1989) and the protein sequences subsequently inferred. The sequence for bovine $\beta$-casein was modified according to Bonsing, Ring, Stewart & Mackinlay (1988). Hydropathy plots were determined by the method of Hopp & Woods (1981) with a window size of 6.

Northern and slot-blot hybridizations

Total RNA was extracted from mammary tissue on day 120 (early) and day 270 (late) of lactation using the method of Chomczynski & Sacchi (1987). Procedures used for Northern and slot hybridization and preparation of riboprobes have been outlined previously (Collet et al. 1990, 1991).

Tissue culture conditions

The hormonal induction of casein mRNA was studied in vitro using mammary gland explants prepared from tammar wallabies at day 24 of gestation, 2 days prior to parturition (Nicholas & Tyndale-Biscoe, 1985). The four mammary glands were dissected aseptically and the involuting gland from the previous lactation was discarded. Explants were cultured at 37°C in 35 mm culture dishes containing 3.5 ml Medium 199 (Grand Island Biological Co., Grand Island, NY, U.S.A) as described previously (Nicholas & Tyndale-Biscoe, 1985). All media were changed daily. Ovine prolactin (NIH-PRL-16) was provided by the Hormone Distribution Program, NIH (Bethesda, MD, U.S.A), crystalline porcine insulin (154-YB-9) was a gift from Eli Lilly and Co. (Indianapolis, IN, U.S.A.) and cortisol was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

RESULTS

The caseins represent 50% of the milk protein of both early and late phases of lactation in the tammar (Nicholas, 1988) and therefore comprise the most abundant mRNA species in the gland. Of the 200 clones screened from a tammar mammary gland cDNA library, 23 gave strong hybridization signals when challenged with cDNA probes constructed from poly(A)$^+$ mRNA isolated during early and late phases of lactation. Analysis of the 23 clones by DNA sequencing revealed two clones, pG777 and pG848, with inferred amino acid sequences characteristic of the signal peptides and major phosphorylation sites of the Ca$^{2+}$-sensitive caseins of eutherians (Bonsing & Mackinlay, 1987). Three cDNA clones were found to be partial copies of pG848, and a single cDNA clone a partial copy of pG777. The nucleotide and inferred amino acid sequences of the putative casein cDNAs are presented in Fig. 1 (pG848) and 2 (pG777). On the basis of the number, location and homology of the major phosphorylation sites, pG848 can be assigned as a $\beta$-casein homologue and pG777 can be assigned as an $\alpha$-casein homologue (see Discussion). Sequencing of an additional 20 cDNAs chosen by length (0.8–3 kb) failed to detect any other clones with sequences homologous to the signal peptide or phosphorylation sites of the eutherian or marsupial Ca$^{2+}$-sensitive caseins.

The signal peptide of both tammar caseins comprises 15 amino acid residues, a characteristic of the eutherian caseins (Hall, Laird & Craig, 1984; Bonsing & Mackinlay, 1987). The open-reading frames of the tammar $\beta$-casein and $\alpha$-casein cDNAs code for mature proteins of 287 ($M_r = 34 291$) and 222 ($M_r = 26 503$) amino acids respectively. In the caseins, phosphorylation commonly occurs on a serine residue in the sequence Ser-X-Y where X can be any amino acid and Y can be glutamic acid or phosphorylated serine (Mercier, 1981). There are three major phosphorylation sites within the tammar $\alpha$-casein which include residues 33–37, 64–68 and 92–96. Tammar $\beta$-casein has a single major phosphorylation site between residues 8 and 12 and three minor phosphorylation sites between residues 57 and 59, 82 and 84, and 280 and 282. Tammar $\beta$-casein also contains 16 tandem repeats of an eight amino acid sequence between residues 128 and 255 which account for 42% of the molecular weight of this protein. Inclusion of the repeats results in an average hydropathy index for the tammar $\beta$-casein of $-0.2$ (Fig. 3) compared with an average hydropathy of 0 for the eutherian $\beta$-caseins (data not shown).

Comparison of the inferred tammar $\beta$-casein protein sequence with the amino acid sequence of bovine $\beta$-casein (Fig. 4) reveals approximately 25% similarity. The only regions of extended similarity between the marsupial and eutherian $\beta$-caseins are
the signal peptide and the single major phosphorylation site, although the latter is shortened in the tammar polypeptide by a Ser residue. There are a number of conserved individual residues which may point to a role for these amino acids in tertiary or micellar structure (Holt & Sawyer, 1988). Alignment of the inferred tammar α-casein and the bovine αS1-casein shows 21% similarity (Fig. 5). Regions of extended similarity are restricted to the signal peptide and a single major phosphorylation site. There are also a number of conserved individual residues which may be important for some unknown function (Holt & Sawyer, 1988).

In the tammar, two types of milk are secreted: 'early' milk for the first 180 days of lactation and 'late' milk for the remainder (Green & Merchant, 1988). The two kinds differ in all their main constituents of protein, carbohydrate, lipid and electrolytes (Green & Merchant, 1988; Nicholas, 1988). Northern analyses revealed a transcript of 1550 bases homologous to the β-casein cDNA and a transcript of 1340 bases homologous to the α-casein cDNA in total RNA from tammar mammary glands in both early (day 120) and late (day 270) phases of lactation (Fig. 6).

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1 AACCTCCCTTTGCTCAAGGTTTCAGCCACCATGAAGCTGCTCATCTTCTCCTGCCTTGTGACTCTTGCT
2 MKLLIFSCLVTLA
3 CTGGCTAGGCCAGATGCCCTCCGCTTATCTATTGATAGGCATTTTAAACATCGAGAACTGGAAAACCGT
4 LARPDALRLSIDRHFKHRELENR
5 TTGAATGAGGATCCCATTCCAGTAAGTGAGGCTTCATCAAGTGAGGAATCTGTCCACCAGCTGAACAGA
6 LNEDPIPVSEASSSEESVHQLNR
7 * * *
8 GACAGGCGTCCCCTGGAAAAATATGAGCTTGACAAATACAGAGAGGATTTGAAGACTTCATCAAGTGAG
9 ** ** ** ** **
10 DRRPLEKYELDKYREDLKLTSSE
11 EFVTPSTNERVRQQVEYNFNEED
12 TCTTCTGCCCTAAGGGAGAGGAGAATGAGTTTCACTTGAGACAGCGGATCTCGAGAAGACGCG
13 SSASRERKIEDFSEHDRQRYLLRR
14 GTAGAAGAGAGGCTCTCAATTTACGTATCTGGAACCTCTATTATGCTACTGAGCCAGAATACTAC
15 VEERALNLRLYLEPLYVATETEYYV
16 TACCTACTGCAATGTTTCTGTTTCCCCCTATCAAGAGACCTCTGTCTCTCTCTCTCTTCT
17 YYYAYVFPVSSHDIPYQQKPLSL
18 CCGCGAAAGTCCTCCTACCTTTATATCAACGAGCCGCTACTGAAGCCGCGTTACTTCTGAGAGAGAG
19 PAKSHYLISGTGLLENEPILPLRE
20 CTAGAAGAGAGGCTCTCAATTTACGTATCTGGAACCTCTATTATGCTACTGAGCCAGAATACTAC
21 LGRGFQSPSLLILLILVLTLTENSNFLM
22 GATCTCTCTTTATTTGTCTACAAATAGCAATCTCATGAGAGATTTGATGTCTCATATG
23 GSVFYWCQLQIAHMPQEIE
24 GAAGAAACACAGAAGGAAAAGGAAACAAAAGCCTGCGAGGAGATTTCTCTTGTATGACCCACATG
25 CGGTTATCTCATAATCAAAATTTGATTTAGAAGCCTCTCTTCTCTATATCACTCTCTTGACAG
26 AGGCCATGTGGCCAGGCTCTGACATTTAATTTAAGTTACAGAAGCTCTGTGCTCTTCTTCT
27 AAAAGAAATCTTAAAAGAGAAGCTCTGTATTTATTTAATCTCTACCTCAGCTATGGTAA
28 TAAARTTTTTTCTCCCTCCTGTTGAAAAA

**FIGURE 2.** Nucleotide and inferred amino acid sequence of the putative tammar wallaby α-casein cDNA (pG777) which will appear in the EMBL/Genbank/DDBJ Nucleotide Sequence Databases under the accession number X54714. The open-reading frame includes bases 33–742. Potentially phosphorylated Ser residues are denoted by asterisks.

**FIGURE 3.** Hydropathy plot of tammar β-casein. The horizontal broken line denotes the tandem repeats which appear as ‘fingers’ in the plot.

The signals were more intense with 5 µg RNA from late-phase mammary gland than with 20 µg RNA from the early phase, suggesting that there is a significant increase in the concentration of the two species of mRNA in the tissue as lactation proceeds. The hormonal requirements for maximal induc-
tion of the two casein genes were assessed in a mammary gland explant culture system with tissue from tammars on day 24 of pregnancy. Low levels of α-casein mRNA, but not β-casein mRNA, were quantified in mammary tissue prior to culture (Fig. 7). Levels of β-casein and α-casein mRNA were undetectable in explants which had been cultured for 3 days in media containing insulin and cortisol. Both the α-casein and β-casein genes were induced in the presence of prolactin alone. Insulin and cortisol individually stimulated the prolactin-induced accumulation of α-casein mRNA; however, the level of expression was maximal only in the presence of all three hormones. In contrast, the level of β-casein gene expression was maximal in explants cultured in the presence of insulin and prolactin.

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Cortisol enhanced the effect of the prolactin induction of tammar β-casein, although not to the same extent as insulin.

DISCUSSION

Classification of the caseins from all species is now generally standardized according to homology to the four bovine archetypes (Eigel et al. 1984), and the tammar caseins reported here are putative homologues of the bovine Ca\(^{2+}\)-sensitive types. Three regions of the Ca\(^{2+}\)-sensitive caseins are found to be conserved in the eutherians: the 5' non-coding region, the signal peptide and the major phosphorylation site sequences (Hobbs & Rosen, 1982; Hall et al. 1984; Yu-Lee, Richter-Mann, Couch et al. 1986; Bonsing & Mackinlay, 1987). The 5' non-coding and signal peptide sequences are characteristic of the individual casein types in eutherians (Hall et al. 1984; Bonsing & Mackinlay, 1987). However, although there is sufficient similarity in the equivalent regions in the tammar sequences to identify the inferred proteins as caseins, it is not possible to discriminate between the types on this basis alone.

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The assignment of pG848 as a β-casein homologue is based on the location and homology of the single major phosphorylation site and the homology of a number of individual isolated amino acids which also appear to be conserved in the eutherian β-caseins (Holt & Sawyer, 1988). The eutherian β-caseins are the most conserved of the multigene family, reflecting their important role in determination of the surface properties of the casein micelle. The role of β-casein in determining the micellar surface properties is thought to be dependent upon a conformation

FIGURE 6. Northern blot analyses of tammar wallaby mammary gland total RNA. Hybridization of riboprobes was to 20μg RNA from the early phase of lactation (E; day 120) and 5μg RNA from late lactation (L; day 270) using cDNA clones of (a) α-casein and (b) β-casein. Transcript sizes are in kb (n=4).

FIGURE 7. Slot-blot quantification analyses of the hormone-dependent induction of (a) α-casein and (b) β-casein mRNA gene expression in mammary gland explants from tammars at day 24 of gestation; t₀ indicates total RNA from mammary tissue prior to culture. Explants were cultured for 3 days in the presence of the indicated combinations of insulin (I; 1μg/ml), cortisol (F; 50ng/ml) and prolactin (P; 20ng/ml). One or two μg total RNA per slot were hybridized to the β-casein and α-casein riboprobes respectively. Values for quantification represent means±s.e.m. (n=3).
involving a hydrophilic N terminus, including the single major phosphorylation site, and a predomi-
nately hydrophobic C terminus. Although there has been extensive sequence divergence, hydropathy is
conserved in the eutherian β-caseins (Bonsing & Mackinlay, 1987; Rosen, 1987; Holt & Sawyer,
1988). The octapeptide repeats in the tammar β-casein account for 40% of the molecular weight of the
protein and may serve to maintain an optimal protein molecular size determined by some func-
tional constraint. Insertion of the repeats results in a mostly hydrophilic C terminus and an overall hyd-
ropathy index which is lowered from 0, in the eutherian β-caseins, to -0.2 in the tammar polypep-
tide. It is not clear what effect either a lowered hydropathy or a smaller molecule of β-casein may
have on its role in micellar structure. There are, however, three hydrophobic domains in the tammar
casein (residues 45–55, 105–125 and 255–275) which are similarly distributed to those in the eutherian
β-casein and which may well be important for function (data not shown).

The eutherian α-caseins are characterized by two or more major phosphorylation sites (Yu-Lee et al.
1986; Bonsing & Mackinlay, 1987; Holt & Sawyer, 1988). Further classification into αS1-like and αS2-
like caseins is based on the location of the major phosphorylation sites, the pattern of serine residues
within these sites and a number of shared amino acid residues within each of the two types. The distinc-
tion between the two types is made on the basis of sequence properties, as both possess a similar struc-
ture (Holt & Sawyer, 1988). Also, both types serve to determine the capacity of the micelle to transport
calcium phosphate (Pearse et al. 1986). The pG777 cDNA is classified as an α-casein homologue on the
basis of the number and homology of the major phosphorylation sites, but an assignment to either
the αS1 or αS2 group is not clear.

In the rat and mouse, the in-vitro induction of casein gene expression is dependent upon insulin,
gluocorticoid and prolactin (Topper & Freeman, 1980; Vonderhaar & Ziska, 1989; Goodman & Rosen,
1990). In the rabbit, studies suggest that mammary gland explants require insulin and prolac-
tin for increased levels of α- and β-casein expression, although the maximal accumulation of
each mRNA requires the addition of cortisol (Deviny, Hubert, Jolivet et al. 1988). The role of
prolactin and glucocorticoids in the accumulation of rat and mouse β-casein gene transcripts in vitro
is both at the level of gene transcription and stabilization of the mRNA (Guyette, Matsusik & Rosen,
1979; Chomczynski, Qasba & Topper, 1986; Eisen-
stein & Rosen, 1988; Doppler, Groner & Ball, 1989),
while the role of insulin is thought to be at the level
of casein gene transcription (Chomczynski, Qasba &
Topper, 1984).

In the present study, prolactin was effective in the
induction of both α- and β-casein gene expression in
mammary gland explants from the tammar wallaby.
Maximal accumulation of each of the casein gene
transcripts was dependent upon different combina-
tions of the three hormones. The β-casein gene
required prolactin and insulin for maximal mRNA
accumulation, while maximal induction of the α-
casein gene was dependent upon insulin, cortisol and
prolactin. These results are in contrast to earlier
studies which show a dependence on prolactin alone
for the maximal induction of α-lactalbumin (Nicho-
las & Tyndale-Biscoe, 1985; Collet et al. 1990) and
β-lactoglobulin (Collet et al. 1991) in mammary
gland explants from the late-pregnant tammar.
Maximal induction of a putative 19 kDa casein from
the tammar, possibly K-casein, has also been shown
to be dependent upon prolactin in explant cultures
(Maher & Nicholas, 1987). The discrepancy be-
tween the results reported here for tammar α- and β-
casein and those obtained for the putative 19 kDa
casein may reflect different evolutionary origins for
the Ca2+-sensitive caseins and K-casein. Alternati-
vably, antibodies generated against the 19 kDa pro-
tein may not be against a casein but rather another
19 kDa phosphoprotein in the crude total casein
sample prepared by centrifugation (Maher & Nicho-
las, 1987). The intracellular role of insulin, cortisol
and prolactin in the accumulation of milk protein
mRNA in mammary gland explants from the tam-
mar remains to be determined.

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