Evolution of Pinnipedia lactation strategies: a potential role for α-lactalbumin?

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1. INTRODUCTION
Lactose is the major determinant of milk volume as it is unable to diffuse through cell membranes causing influx of water into mammary alveoli to maintain osmotic balance. Consequently, total solids and lactose are generally negatively correlated in mammalian milk. Exceptions to this are the families Otariidae (fur seals and sea lions) and Odobenidae (walruses), where lactose has not been detected. We investigated the molecular basis for this by cloning α-lactalbumin, the modifier protein of the lactose synthase complex. A mutation was observed which, in addition to preventing lactose production, may enable otarids to maintain lactation despite the extremely long inter-suckling intervals during the mother’s time at sea foraging (more than 23 days in some species).

Keywords: α-lactalbumin; lactose; lactation; pinnipeds

2. MATERIAL AND METHODS
Total RNA was extracted from Cape fur seal (Arctocephalus pusillus pusillus) mammary gland tissue and milk collected from subantarctic fur seal (Arctocephalus tropicalis), California sea lion (Zalophus californianus californianus) and Weddell seal (Leptonychotes weddellii) using the Lipid RNAsay Tissue Kit (Qiagen). Oligonucleotide primers (5'-CAGTGTTTCTGACTACAA-3' and 5'-CACAGGA-GATGTCCACAGA-3') were designed to conserve internal regions of α-LA cDNA homologues obtained from GenBank (further details described in the electronic supplementary material) and used to amplify Cape fur seal cDNA sequences. Full-length sequences were obtained by screening a lactating Cape fur seal cDNA library with gene-specific and M13 forward or M13 reverse primers. Primers were designed to the 5’ and 3’ ends of the fur seal α-LA cDNA (5'-CAAAATGATGCCTCTTGTG-3' and 5'-CCCTTATTCAGACAGGGGF-3') and used to amplify α-LA from subantarctic fur seal, California sea lion and Weddell seal cDNA.

Genomic DNA was extracted from Cape fur seal liver tissue, subantarctic fur seal mammary gland tissue, ringed seal (Phoca hispida) muscle tissue, Australian sea lion (Neophoca cinerea) muscle tissue, Weddell seal milk and California sea lion milk. The DNA was extracted from tissue and milk samples using previously described methods (Lindquist et al. 1994; Strauss 1998). Genomic α-LA was amplified using cDNA oligonucleotide primers to produce the full length gene. Sequences are deposited in GenBank under accession numbers EF659494–EF659507.

The lactose synthesis assay was performed as previously described (Nagamatsu & Oka 1980) with slight modifications detailed in the electronic supplementary material.

3. RESULTS AND DISCUSSION
In the present study two alternatively spliced α-LA cDNA variants (545 and 1221 bp) were identified from cDNA isolated from mammary gland tissue of a lactating Cape fur seal. The genomic α-LA sequence from Cape fur seal plus two additional fur seals (northern Callorhinus ursinus and subantarctic) and two sea lions (Australian and California) were cloned and compared with dog genomic α-LA, revealing a consistent base change in the third intron splice site indicating aberrant splicing of the fourth exon (figure 1a). This aberrant splicing is responsible for the two cDNA variants isolated from Cape fur seal mammary tissue, with the longer variant (α-LA(l)) maintaining the third intron and the shorter variant (α-LA(s)) using a splice site further downstream in what is exon 4 in other α-LA homologues (figure 1b). The predicted Cape fur seal α-LA protein sequences for both variants are truncated at 104 residues due to a stop codon introduced into the reading frame and differ only in their C-terminal residue (figure 1c; electronic supplementary material).

The residues necessary for lactose synthesis activity are Phe31, His32, Leu110, Gln117 and Trp118 of α-LA (Brew 2003). With the exception of the first two, these essential residues are found in exon 4, which is disrupted in otariid α-LA and, hence, would account for the lack of lactose in otariid milks. This was further confirmed by the lack of lactose synthesis activity (pmole mg−1 tissue min−1) in mammary gland tissue from a subantarctic fur seal (control) following addition of exogenous bovine β-GT (36±1, control 41±12), as previously reported for the California sea lion (Johnson et al. 1972), whereas addition of exogenous bovine α-LA resulted in a 10-fold increase in lactose synthesis activity (400±43, control 37±0.2).

Sequencing of Atlantic walrus (Odobenus rosmarus) genomic α-LA revealed a seven base pair deletion...
Figure 1. (a) Genomic sequence alignments of intron 3/exon 4 boundary of α-LA from subantarctic (SFS), Cape (CFS) and northern fur seal (NFS), California (CSL) and Australian sea lion (ASL), Weddell (WS) and Ringed seal (RS), domestic dog (NP_001003129) and walrus (WAL). The mutation is bold and shaded in otariids α-LA, and dashes indicate the deletion in walrus α-LA. Exon 4 is underlined and bold. (b) Gene structure of α-LA (ALA) from domestic dog, Weddell seal and the two Cape fur seal variants (CFS (l), long; CFS (s), short). Intron 3 splice sites are shown. (c) Protein alignment of α-LA from Cape fur seal (long and short), Weddell seal, domestic dog (Q9N2G9), cow (P00711) and walrus (SFS and CSL α-LA proteins found to be identical to CFS; electronic supplementary material). Residues essential for lactose synthesis are underlined.
where the otariid mutation has occurred (figure 1a).

The deletion predicts a frame shift within exon 4 and the predicted protein (176 amino acids) is longer than common α-LA homologues (figure 1c). Since exon 4 is disrupted, walrus α-LA is also predicted to be unable to produce lactose, supporting earlier analyses of walrus milk (Fay 1982). In contrast, analysis of genomic α-LA in two phocid species (Weddell seal and ringed seal) showed that both had the correct nucleotide base in the third intron splice site and were predicted to produce the normal splice variant (figure 1a). To confirm this, we isolated Weddell seal α-LA cDNA (701 bp) and the predicted protein sequence of 123 residues is consistent with most other α-LA homologues (figure 1b,c).

The ancestral pinniped would have moved into environments that required the separation, both spatially and temporally, of the acquisition of resources for milk production and its delivery to offspring (Boyd et al. 1999). In adapting to these environments, phocids became large with relatively short, intense lactation periods (4–50 days depending on species) during which females fast on land (Oftedal et al. 1987). In contrast, otariids adopted longer lactation periods (from 4 to more than 12 months depending on species) during which females fast on land (Oftedal et al. 1987) during which mothers regularly undertake long foraging trips to sea (more than 23 days in some species; Georges & Guinet 2000), leaving their pup to fast on land and, thus, prevent the occurrence of mammary involution. Indeed, mice created with α-LA deficiency produce highly viscous milk due to the absence of lactose. After 5 days of lactation, while the milk is too viscous to secrete, the tight junctions between the mammary epithelial cells appear to be maintained, indicating that alveoli were not excessively distended as would be expected in wild-type mice after extended milk stasis (Stinnakre et al. 1994).

Additionally, mammary-derived factors, including α-LA, may also initiate involution (Green & Streuli 2004). Human and bovine α-LA can cause apoptosis (Hakansson et al. 1995; Xu et al. 2005). In order to do so, α-LA must cross the lipid bilayer membranes in a molten globule confirmation, where it can disrupt the histones (Mok et al. 2007). The findings of the present study suggest that the truncated α-LA proteins of otariids would not be able to form a stable molten globule conformation (Demarest et al. 2001); therefore, they would not have the same apoptotic effect as that in the mammary glands of other mammals. Thus, the mutation in otariid α-LA could have allowed the maintenance of mammary function, despite the extremely long inter-suckling intervals,
through the absence of lactose and/or the absence of α-LA apoptotic ability.

Odobenids have also adopted a strategy of long lactation (more than 1 year), but the mother feeds in close proximity to the pup enabling her to suckle frequently (Offedal et al. 1987; figure 2). Recent phylogenetic data suggest that otariids and odobenids share a common ancestor that split from the phocid ancestor (Arnason et al. 1999), which would be beneficial to the health of the mammary gland during the long inter-suckling intervals. In contrast, the ability to suckle regularly may have reduced the need for antimicrobial activity in the mammary gland of odobenids and, therefore, selection to maintain α-LA resulting in the second mutation. Consequently, the mutation in the α-LA splice site in the common ancestor of otariids and odobenids could have influenced the divergence in lactation strategies within pinnipeds.

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