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Research paper

A *colostrum trypsin inhibitor* gene expressed in the Cape fur seal mammary gland during lactation

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ABSTRACT

The *colostrum trypsin inhibitor* (*CTI*) gene and transcript were cloned from the Cape fur seal mammary gland and *CTI* identified by *in silico* analysis of the Pacific walrus and polar bear genomes (Order Carnivora), and in marine and terrestrial mammals of the Orders Cetartiodactyla (yak, whales, camel) and Perissodactyla (white rhinoceros). Unexpectedly, Weddell seal *CTI* was predicted to be a pseudogene. Cape fur seal *CTI* was expressed in the mammary gland of a pregnant multiparous seal, but not in a seal in its first pregnancy. While bovine *CTI* is expressed for 24–48 h postpartum (pp) and secreted in colostrum only, Cape fur seal *CTI* was detected for at least 2–3 months pp while the mother was suckling its young on-shore. Furthermore, *CTI* was expressed in the mammary gland of only one of the lactating seals that was foraging at-sea. The expression of *β-casein* (*CSN2*) and *β-lactoglobulin II* (*LGB2*), but not *CTI* in the second lactating seal foraging at-sea suggested that *CTI* may be intermittently expressed during lactation. Cape fur seal and walrus *CTI* encode putative small, secreted, N-glycosylated proteins with a single Kunitz/bovine pancreatic trypsin inhibitor (BPTI) domain indicative of serine protease inhibition. Mature Cape fur seal CTI shares 92% sequence identity with Pacific walrus CTI, but only 35% identity with BPTI. Structural homology modelling of Cape fur seal CTI and Pacific walrus trypsin based on the model of the second Kunitz domain of human tissue factor pathway inhibitor (TFPI) and porcine trypsin (Protein Data Bank: 1TFX) confirmed that CTI inhibits trypsin in a canonical fashion. Therefore, pinniped CTI may be critical for preventing the proteolytic degradation of immunoglobulins that are passively transferred from mother to young via colostrum and milk.

1. Introduction

Colostrum trypsin inhibitor (*CTI*) is a mammary-specific gene which is expressed and the protein secreted in bovine colostrum for only 24–48 h postpartum (pp) (Laskowski and Laskowski, 1951; Pineiro et al., 1978; Veselsky et al., 1978). CTI is a small ~10–15 kDa, N-glycosylated protein (Klauser et al., 1978; Laskowski and Laskowski 1951; Tschesche et al., 1975) with a single Kunitz/bovine pancreatic trypsin inhibitor (BPTI) domain, characteristic to the family I2 (Kunitz-BPTI) inhibitors of the S1 (chymotrypsin) family of serine endopeptidases (Rawlings et al., 2014). Although CTI inhibits trypsin and plasmin and is a weak inhibitor of α-chymotrypsin *in vitro* (Feeney and Allison, 1969; Laskowski and Laskowski, 1951; Pineiro et al., 1978), neither its target enzyme, nor its function *in vivo* is known.

The expression of *CTI* and the orthologous marsupial *early lactation protein* (*ELP*) gene (Pharo et al., 2012), coincides with the passive transfer of antibodies from the mother to a neonate/young that lacks an

Abbreviations: AMBP, α1-microglobulin/bikunin precursor; APP, amyloid (βA4) precursor protein; CSN2, β-casein; BPTI, bovine pancreatic trypsin inhibitor; cDNA, DNA complementary to RNA; CTI, colostrum trypsin inhibitor; Da, Dalton; ELP, early lactation protein; h, hour; IgG, immunoglobulin G; KD, Kunitz domain; LGB2, β-lactoglobulin II; PIGT, phosphatidyl inositol glycan, class T; pp, postpartum; PRSS1, protease, serine, 1 (trypsin 1); SLPI, secretory leukocyte protease inhibitor; SPINLW1, serine peptidase inhibitor-like, with Kunitz and WAP domains 1; TFPI, tissue factor pathway inhibitor; TKDP, trophoblast Kunitz domain protein; UTR, untranslated region; WAP, whey acidic protein; WFIKKN, WAP, follistatin/kazal, immunoglobulin, Kunitz and netrin domain containing; WFDC2, WAP four disulphide core domain 2.

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adaptive (acquired) immune system and the ability to mount a specific immune response (Brambell 1970; Edwards et al., 2012). Furthermore, during this period, the gut of the young is permeable to intact immunoglobulins and macromolecules and thus these molecules can pass through the intestines and into the circulatory system prior to 'gut closure', i.e., when mucosal enterocytes lose the capacity to absorb macronutrients and immunoglobulins (Kruse, 1983; McFadden et al., 1997). *CTI* expression is brief in eutherians (1–2 days), but ELP expression is extended (for up to 100–125 days pp) in marsupials such as the possum and tammar wallaby (the common and scientific names for mammals described in this study are listed in Supplementary file 1) (Nicholas et al., 1997; Pharo et al., 2012; Piotte and Grigor 1996). Therefore, CTI may prevent the proteolytic degradation of immunoglobulins (Laskowski and Laskowski, 1951), while ELP may protect the marsupial young against pathogens (Piotte and Grigor, 1996).

Neither *CTI*, nor *ELP* is found in birds, fish, reptiles or amphibians and their status in monotremes is inconclusive. They therefore evolved from a common ancestral gene prior to the divergence of marsupials and eutherians (Pharo et al., 2012) ~160 million years ago (Luo et al., 2011). Intriguingly, all marsupials investigated have a functional (putative protein-coding) *ELP* gene, but this is not so for eutherian *CTI*. *CTI* is conserved in species from the orders Carnivora (dog, cat) and Cetartiodactyla (dolphin, cow, pig); but is a pseudogene in the horse (order Perissodactyla), humans and other primates, the elephant, sloth and rodents (Pharo et al., 2012). Gene loss, or loss of function has occurred many times throughout evolution and is often the result of gene duplication (Lynch and Conery, 2000) and/or transposition of genomic DNA fragments within the genome by retro-elements (Cañestro et al., 2013).

Since its evolution over 500 million years ago, the Kunitz domain (KD) has been duplicated many times (Gojobori and Ikeo, 1994; Ikeo et al., 1992) in bacteria, viruses, insects, invertebrates, vertebrates (e.g. fish, birds, amphibians, reptiles (e.g. venoms and dendrotoxins), mammals) and plants (Fry et al., 2009; Jamal et al., 2013; Rawlings et al., 2014). KDs have a diverse range of functions, e.g., serine protease inhibition, antimicrobial, anticoagulant and anti-inflammatory activity; potassium and calcium channel blockers (e.g. neurotoxic venoms), non-neurotoxic venoms, plant protection against herbivores, etc. (Fry et al., 2009; Ranasinghe and McManus, 2013; Shigetomi et al., 2010). Genes encoding as few as one KD, e.g. *BPTI* (also known as *PTI*) (Ascenzi et al., 2003); *serine protease inhibitor Kunitz-type* and *-4* (*SPINT3* and *-4*); *trophoblast Kunitz domain proteins 1, 2, 3, 4* and *5* (*TKDP1-5*); two domains: *SPINT1* and *-2*; three tandemly repeated domains: *tissue factor pathway inhibitor 1* and *-2* (*TFPI1 and -2*), and up to 12 domains, e.g. nematode *Ac*-KPI-1 (*Ancylostoma caninum*)*-Kunitz protease inhibitor-1* have been characterised (Hawdon et al., 2003). Multi-domain type-encoding genes have also been identified, e.g. *serine peptidase inhibitor-like, with Kunitz and WAP domains 1 (eppin) (SPINLW1)*; *WAP, follistatin/kazal, immunoglobulin, Kunitz and netrin domain containing-1* and *-2* (*WFIKKN1* and *-2*); *α1-microglobulin/bikunin precursor (AMBP)* and *amyloid (βA4) precursor protein* (*APP*).

The most extensively structurally characterised KD is BPTI (Ascenzi et al., 2003), which most-likely evolved from bovine *CTI* after the divergence of ruminants from other Cetartiodactyla (Pharo et al., 2012) ~25–35 Myr (Bininda-Emonds et al., 2007). The KD belongs to the α + β fold and is stabilised by three disulphide bonds (Ascenzi et al., 2003; Huber et al., 1972). The P_1 'warhead' residue within the convex exposed 'binding' loop of the inhibitor determines serine protease specificity (Laskowski and Kato, 1980; Laskowski and Qasim, 2000). Kunitz inhibitors with a basic Lys or Arg residue at P_1 inhibit trypsin and trypsin-like enzymes (Ikeo et al., 1992; Laskowski and Kato, 1980), those with Ala or Ser inhibit elastase-like enzymes and a P_1 Met or Leu confers activity against chymotrypsin and chymotrypsin-like enzymes (Laskowski and Kato, 1980). A P_1 Leu is also active against neutrophil elastase (Garcia-Fernandez et al., 2015). Protease inhibition involves the cleavage of the P_1–P_1' peptide bond (inhibitor) and the docking of the convex inhibitor 'binding' loop into the catalytic cleft of the serine protease in a classical 'lock and key' interaction (Marquart et al., 1983; Grzesiak et al., 2000). A new, 1:1, reversible, tight-binding serine protease-Kunitz inhibitor complex is formed, involving P_1 (inhibitor) and the catalytic triad of residues: Ser, His and Asp (protease) (Marquart et al., 1983).

The identification of *CTI* in the dolphin (Cetartiodactyla) (Pharo et al., 2012), suggested that the gene may also be present in other marine mammals, such as the Pinnipedia, 'fin-footed' semi-aquatic mammals (Carnivora). Although monophyletic, the pinnipeds comprise a diverse range of species within three extant families: the Otariidae (eared seals: fur seals, e.g. Cape and subantarctic fur seals; and sea lions, e.g. California seal lion), its sister family, the Odobenidae, with one extant member, the walrus; and the Phocidae (earless or 'true' seals: e.g. Weddell seal, Hooded seal), [Fig. 1; (Higdon et al., 2007; Nyakatura and Bininda-Emonds 2012)]. While all pinnipeds give birth to a pup on land or ice (Atkinson 1997; Oftedal et al., 1987) and produce lipid-rich, high-protein and low-sugar milk for their young, each family has developed unique lactation strategies to ensure the survival of their young in marine environs (Fig. 1). Otariids such as the Cape and subantarctic fur seals fast for the first 4–10 days pp while nursing their pup on land (Bonner, 1984; Oftedal et al., 1987). The mother then commences a pattern of alternating trips to sea to feed interspersed with ~2–3 days on-shore suckling her pup (Bonner, 1984; Gentry and Kooyman, 1986; Oftedal et al., 1987). Each trip to sea usually lasts for up to 25 days (Gamel et al., 2005) and ~23–28 days (Kirkman et al., 2002) for the Cape and subantarctic fur seals respectively, but extreme foraging periods of up to 1–2 months have been reported (Georges and Guinet, 2000; Verrier et al., 2011). Therefore, it is vital that the mother produces milk that will ensure the survival of her pup during these periods.

The aims of this study were to identify the *CTI* gene and characterise its expression in the mammary glands of semi-aquatic members of the order Carnivora (Cape and subantarctic fur seals), identify *CTI* in other species using an *in silico* bioinformatics approach, and to use protein structural homology modelling to investigate whether pinniped CTI, like other KDs has the potential to inhibit trypsin.

2. Materials and methods

2.1. Animals

Mammary gland tissue was collected from six Cape fur seals and one subantarctic fur seal during the reproductive cycle (Table 1), as described previously (Cane, 2005). Approval for this research was obtained from the South African Government (Cape fur seals) and the Ethics Committee of the French Polar Institute (IPEV) and the Polar Environment Committee of Terres Australes et Antarctiques Françaises (subantarctic fur seal).

2.2. Isolation of Cape fur seal genomic DNA and cloning of the CTI gene

Genomic DNA was isolated from the mammary gland of an on-shore lactating Cape fur seal as described (Sambrook and Russell, 2001) and *CTI* amplified by PCR from ~200 ng of genomic DNA with forward 5′-GCCTAGAACATTCAGCTATTGGCACC-3′ and reverse 5′-TGAATGTTTTAT TGACCTAGACCTGGAGG-3′ primers and Platinum *Taq* (Invitrogen) as per the manufacturer's instructions. PCR conditions used were 94 °C for 2 min; 35 cycles of [94 °C for 30 s; 54 °C for 30 s; 68 °C for 4 min] and a final extension at 68 °C for 10 min. The PCR product was cloned into pGEM-T Easy (Promega Corporation) and sequenced.

2.3. In silico identification of CTI

CTI genes were compiled from BLAST searches of the NCBI GenBank nr (http://www.ncbi.nlm.nih.gov/BLAST/) and Ensembl