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Expression of the Fc receptor in the mammary gland during lactation in the marsupial *Trichosurus vulpecula* (brushtail possum)

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Abstract

One of several functions described for the Fc receptor is regulation of IgG isotype transport into milk. The first marsupial homologues of the Fc receptor heavy and light chains, FcRn and β-2 microglobulin, from the brushtail possum have been cloned and characterised. The level of FcRn mRNA in the possum mammary gland was highest at the start of lactation, and decreased slowly thereafter. Expression of FcRn mRNA did not increase during the switch phase when the concentration of IgG in milk is highest. In contrast, the level of β-2 microglobulin mRNA in the mammary gland increased during the switch phase when milk IgG concentration also increases. This correlation between β-2 microglobulin mRNA expression in the mammary gland with the time of active IgG-transfer into milk was also observed in the bovine and murine mammary gland. This suggests that expression of the Fc receptor in the mammary gland is controlled by the expression of β-2 microglobulin and that its expression is upregulated during the period of highest IgG-transfer into milk.

Keywords: Reproductive immunology; Fc receptors; Antibodies; Transporters; Molecular biology

1. Introduction

Marsupial young are born at an earlier stage of development than the young of eutherian mammals. In the brushtail possum (*Trichosurus vulpecula*), young are born after a short gestation of 17 days and then enter the pouch where they remain for the first 120 days of lactation. During the early lactation phase (day 1–80) the young undergo developmental changes that occur in utero for eutherian mammals (Sharman, 1962; Tyndale-Biscoe and Renfree, 1987). The milk they receive at this stage has a unique composition and is the sole source of nutrition for the developing pouch young (PY). Mid-way through lactation there a transitional phase (switch phase; days 80–120 of the 200 days of lactation) during which the mammary gland increases in mass, milk production increases and a change in milk composition occurs (Cowan, 1989; Crisp et al., 1989; Demmer et al., 1998). In addition, changes in mammary gland gene expression that occur at parturition in eutherian mammals are observed during the switch phase in marsupials (Adamski and Demmer, 1999; Demmer, 1999; Demmer et al., 1999). In many ways the early lactation phase can be considered as an external gestation, with the switch phase being a second birth of the PY from the pouch (Adamski and Demmer, 1999).

Marsupial young are unable to elicit an immune response before day 10 of lactation (Solomon, 1971; Deane and Cooper, 1988), and their lymphoid tissues do not mature until the end of the early lactation phase at which point they are able to mount a limited immune response (review Basden et al., 1997). Serum immunoglobulin levels in the marsupial young do not reach that observed in adults until mid-way through the late lactation phase. They are therefore dependent on immune protection provided from the mother via the mammary gland for an extended period after birth. Neonatal uptake of IgG provided by the mother's milk has been demonstrated in several marsupial species (Basden et al., 1997), and in 50 and 98 day old possum PY (but not 145 day old PY) ingested IgG was subsequently detected in their serum (Yadav, 1971).

Abbreviations: β-2m, beta-2 microglobulin; PY, pouch young.

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Both secretory IgA (sIgA) and IgG are present in possum colostrum and milk throughout lactation (Adamski and Demmer, 1999, 2000). The concentration of sIgA is highest in colostrum, consistent with the high expression of mRNAs for dimeric IgA molecules, produced by mammary gland associated lymphocytes, and expression of the polymeric immunoglobulin receptor in the mammary gland at the beginning of lactation (days 1–6; Adamski and Demmer, 1999). In contrast, the concentration of IgG is low in colostrum and milk during early lactation, but increases markedly during the switch phase into late lactation (Adamski and Demmer, 2000). This pattern of IgG secretion into milk has also been observed in the tammar wallaby (*Macropus eugenii*) and hill kangaroo (*Macropus robustus*) and is likely to be characteristic of marsupials (Deane and Cooper, 1984; Deane et al., 1990). These results suggest that in marsupials there are two stages of increased immune transfer, at parturition (sIgA) and again during the switch phase (IgG). Transfer at the switch phase would represent a second period of increased immune transfer prior to the 'second' birth of the PY as it prepares to leave the pouch. The high concentration of IgG in milk during the switch phase has similarity with the high amount of IgG-transfer through colostrum in ungulates. Transfer of maternal IgG to the PY during early lactation is reminiscent of that observed for rodents, where IgG-transfer occurs at a low level for an extended period after birth.

At present, the mechanism by which IgG is actively transported into milk is not known. The Fc receptor is the only IgG-transporter described to date and it transfers maternally derived IgG across the intestine of the neonate into the blood, providing humoral immunity (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985; Simister and Mostov, 1989). The Fc receptor is a dimer of two heterodimers that each contain a heavy and light chain, which are FcRn and β-2 microglobulin (β-2m), respectively. Additional roles have been described for the Fc receptor; maintenance of serum IgG levels in the adult and placental transfer of IgG to the fetus (Ghetie et al., 1996; Junghans and Anderson, 1996; Leach et al., 1996; Simister et al., 1996). Recently, FcRn expression in the lactating murine mammary gland (days 7–10) was demonstrated and the role of the Fc receptor in this tissue was found to be for regulation of IgG-transport into milk and not transport of IgG into milk per se (Cianga et al., 1999). Cianga et al. (1999) found that there was an inverse correlation between the concentration of IgG-isotypes present in milk and their affinity for the Fc receptor. Thus isotypes which have high affinity for the Fc receptor appear to be recycled back to the mothers serum, presumably by a process similar to that observed for homeostasis of serum IgG levels. This results in an under representation of high affinity isotypes in mouse milk, however, these are efficiently transported by Fc receptors across the neonatal intestine, restoring the ratio of IgG isotypes in the neonatal serum to be similar of that in adults (Appleby and Catty, 1983; Cianga et al., 1999).

Our interest lies in determining whether the Fc receptor is expressed in the marsupial mammary gland and how this relates to the periods when IgG-transfer into milk is high. In these experiments we have cloned the first marsupial homologues of FcRn and β-2m and have determined the expression pattern of these mRNAs in the possum mammary gland during lactation.

2. Materials and methods

2.1. RNA purification

2.1.1. Mammary gland total RNA

Possum mammary glands were collected as described previously (Demmer et al., 1998) with the date of lactation estimated based on the size of the pouch young (Lyne and Verhagen, 1957). The frozen mammary tissues were ground under liquid nitrogen by mortar and pestle, and RNA extracted using the guanidinium acid phenol chloroform method (Chomczynski and Sacchi, 1987) using 20 ml of GTC solution/g of tissue.

2.2. RT-PCR, 5′RACE and 3′RACE

Random, oligo-dT and 3′RACE-dT (GAG CTC GAG TCT AGA $[T]_{14}$) primed cDNA was generated using the Superscript Pre-amplification System (Gibco-BRL, Gaithersburg, MD) as per instructions. PCR was performed using Platinum Taq (Gibco BRL) or Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The 5′RACE was performed as described by Gibco-BRL using Superscript II reverse transcriptase and RNase H from Gibco-BRL, and the Terminal Transferase kit and RNase T1 from Boehringer Mannheim. Primer specific cDNA was purified using the BRESAclean DNA purification kit (Bresatec Pty, Thebarton, Australia). Upstream primers, Abridged Anchor Primer and Abridged Universal Amplification Primer, used in the PCR reactions are as described by Gibco-BRL. The 3′RACE PCR primer = G CTC GAG TCT AGA. All PCR products were cloned into pGEM-Teasy (Promega, Madison, WI) and the DNA sequence of both strands for at least two independent clones was determined.

2.2.1. Possum FcRn and β-2m

For FcRn, 3′RACE was performed using 5′ primer = TGG TAT TGG GAG AAR GAG AC on mammary gland (day 6 of lactation) cDNA. PCR

conditions were annealing at 45°C for 4 cycles and then 50°C for 26 cycles, with a 2 min extension. For β-2m the 3′RACE was performed using 5′ primer = TAT GTG TCT GGG TTY CAY CC on mammary gland cDNA (day 1.5 of lactation). PCR conditions were 4 cycles each annealing at 60, 55, 50, 45°C and then 20 cycles at 55°C, 2 min extension. The entire coding sequence was then obtained by 5′RACE using three gene specific primers (GSP); GSP1 = TTC TAT TTC AGG CCA GAT, GSP2 = AGA TGT CTT GAA GCC TCC AA and GSP3 = AAC GTC CAA GGT CAA CAG CT on mammary gland cDNA (day 1.5 of lactation). PCR conditions were; PCR1 = 10 cycles annealing at 50°C followed by 20 cycles at 55°C, 2 min extension, and PCR2 = 30 cycles annealing at 57°C, 60 s extension.

2.2.2. Possum IgG Fc-fragment

The 3′RACE was performed using 5′ primer = CAG TTG ACA GTT CCT GCA GA on possum spleen cDNA. PCR conditions were 30 cycles annealing at 58°C, 2.5 min extension.

2.2.3. Mouse FcRn and β-2m

For β-2m an RT-PCR fragment (246 bp) was generated with 5′ primer = AGG CCA AAY TTC CTG AAY TGC and 3′ primer = TTA CAK GTC TCG RTC CCA using 3′RACE-dT primed cDNA from the mouse mammary gland (day 1 of lactation). PCR conditions were 30 cycles annealing at 48°C, 40 s extension. Mouse FcRn was obtained as a Soares mouse mammary gland cDNA clone (MOUSE 1314942 PT7T3D-PAC; ATCC, Manassas, Virginia) and was confirmed by DNA sequencing.

2.2.4. Bovine FcRn and β-2m

A bovine β-2m RT-PCR fragment (246 bp) was generated with 5′ primer = TCY TTC AGC AAG GAC TGG and 3′ primer = TTA CAK GTC TCG RTC CCA using random primed cDNA from bovine mammary gland total RNA (at parturition). PCR conditions were 30 cycles annealing at 50°C, 60 s extension. A 384 bp bovine FcRn RT-PCR product was generated with 5′ primer = GAA AAC CAG GTG TCI TGG TAT TGG and 3′ primer = CAT AGA IGG IGG CTC CTT CCA using random primed cDNA from bovine mammary gland (at parturition). PCR conditions were 30 cycles annealing at 48°C, 40 s extension. The 3′RACE procedure using the same primer and PCR conditions as described above for possum FcRn was used to amplify the 3′ end of the bovine gene. The entire coding sequence was then obtained by 5′RACE using three gene specific primers (GSP); GSP1 = CAA ACA TCA TGA ACT CCT, GSP2 = GCC GTT CAG GGC AAA CTT G and GSP3 = GCA CCG AGA CAT TGT CAG GA on mammary gland cDNA (at parturition). PCR conditions were; PCR1 = 10 cycles annealing at 50°C followed by 20 cycles at 55°C, 2 min extension, and PCR2 = 30 cycles annealing at 57°C, 60 s extension. The nucleotide sequence for Bovine FcRn and b2m have been previously published by Ellis et al. (1993) and Kacskovics et al. (2000).

2.3. Library screening

The *Eco* RI–*Eco* RI DNA fragments from the possum FcRn 3′RACE pGEM-Teasy clone was purified and used to generate ^{32}P-radiolabelled probe with the *Redi*prime random primer labelling kit (Amersham plc, Aylesbury, UK). This probe was used to screen the possum early lactation mammary gland cDNA library (Piotte and Grigor, 1996). Recombinants were purified to single plaques and the internal plasmid (pBS; Stratagene, La Jolla, CA) was excised with ExAssist helper phage following recommended procedures. The resulting plasmids were characterised by DNA sequencing.

2.4. DNA sequencing analysis

DNA sequencing reactions were carried out by the DNA Sequencing Unit (MUSeq, University of Massey, Palmerston North, NZ) using an ABI Automated DNA sequencer. DNA sequence data was collated using the SEQMAN II program of the Lasergene software package (DNASTAR, Madison, WI). Amino acid sequence alignments were performed using the BCM Search Launcher: Multiple Sequence Alignment, program ClustalW 1.7 (Smith et al., 1996). Signal peptide sequences of possum FcRn and pβ-2m were determined using the SignalP programme (Nielsen et al., 1997).

2.5. Northern blotting

Total RNA (6 μg) was denatured in the presence of formamide and formaldehyde and then resolved by gel electrophoresis in 1.5% agarose, 0.6 M formaldehyde gels (Sambrook et al., 1989). RNA was transferred to Hybond N membrane (Amersham plc) by capillary blotting. Blots were hybridised overnight at 55°C with ^{32}P-radiolabelled cDNA probes in Church and Gilbert hybridisation solution (Sambrook et al., 1989) following 30 min pre-hybridisation. Blots were washed in 2 × SSC/0.1% SDS at 55°C, then 1 × SSC/0.1% SDS for 20 min each. Blots were exposed to Kodak XAR-5 film at −80°C with two intensifying screens.

Radiolabelled probes were generated as described for cDNA library screening. Possum FcRn: *Pst* I–*Bam* HI fragment from the pGEM-Teasy 3′RACE clone; pβ-2m: *Eco* RI–*Eco* RI fragment from the pGEM-Teasy 3′RACE clone; mouse β-2m: RT-PCR fragment; mouse FcRn: *Eco* RI–*Not* I fragment (approximately 1200 bp) from the EST clone. The possum 18S rRNA probe is

an unpublished DNA fragment (400 bp) amplified by RT-PCR (J. Demmer, GenBank AF089722).

3. Results

3.1. Cloning and characterisation of the possum FcRn, β-2 microglobulin and Fc-fragment

An 1104 bp 3′RACE product corresponding to possum FcRn (pFcRn) was amplified using possum mammary gland total RNA and an oligonucleotide primer designed to a conserved region of eutherian FcRn coding sequences. This DNA fragment was used to screen an early lactation possum mammary gland cDNA library and eight positive cDNA clones were isolated. These clones were characterised by DNA sequencing and one recombinant was found to contain the entire coding sequence of pFcRn. The pFcRn cDNA (1481 bp) encodes a 336 amino acid mature polypeptide (24 amino acid signal peptide) and shares 41–45% amino acid identity with those of eutherian species, which is lower than that calculated within the eutherian FcRn homologues, 65–91% (Fig. 1). The pFcRn has a similar structure to eutherian homologues consisting of three extra-cellular domains (α1, α2 and α3), and a transmembrane and carboxyl-terminal cytoplasmic domain (Fig. 1, Simister and Mostov, 1989).

A 3′RACE product corresponding to possum β-2 microglobulin (pβ-2m) was amplified from possum mammary gland cDNA using a primer design based on eutherian sequences. New PCR primers were designed from the sequence of the 3′RACE product and used in 5′RACE to amplify the entire pβ-2m coding sequence. The pβ-2m open reading frame encodes an 122 amino acid polypeptide (22 amino acid signal peptide, Fig. 2), which has 52–58% amino acid identity to rodent and human β-2m amino acid sequences. This level of amino acid identity was lower than that observed between eutherian β-2m homologues (67–83%).

The DNA sequence encoding the hinge-CH2–CH3 domains of possum IgG was obtained by 3'RACE. This fragment contains the entire IgG site for binding to the Fc receptor, which has been mapped between the CH2–CH3 domain interface (Burmeister et al., 1994b; Medesan et al., 1997). The possum Fc-fragment amino acid sequence presented in this work differs at three amino acid residues from that published by Belov et al. (1999); Fig. 3; circles). The amino acid sequence of possum Fc-fragment has greater identity to IgG of another marsupial, the grey short-tailed opossum, *Monodelphis domestica*, (70%) than that of eutherian

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          α1
          |                    30                    60                    90
Possum AEA-PSLFYQLTAVS VAPKGTPRFWASGWL GPQLFLTYSSG-GNA EPWGAWRWEPQEPWF WEKETWYLKTQERLL QEALKLSKK--EGAH  86
Rat    AEPRLPLMYHLAAVS DLSTGLPSFWATGWL GAQQYLTYNNLRQEA DPCGAWIWENQVSWY WEKETTDLKSKEQLF LEAIRTLENQINGTF  90
Mouse  SETRPPLMYHLTAVS NPSTGLPSFWATGWL GPQQYLTYNSLRQEA DPCGAWMWENQVSWY WEKETTDLKSKEQLF LEALKTLEKILNGTY  90
Human  AESHLSLLYHLTAVS SPAPGTPAFWVSGWL GPQQYLSYNSLRGEA EPCGAWVWENQVSWY WEKETTDLRIKEKLF LEAFKALGG--KGPY  88
Bovine AENYRSLQYHFTAVS ALAAGTPAFWVSGWL GPQQYLSYNNLRAQA EPYGAWVWESQVSWY WEKETMDLRNQETLF LEALQALG---EGPF  87

          α2                                                              α3
          |                   120                   150                   |
Possum TFQGLVGCQLNPDNS SQHTARYALDGLDLL TFDPVSREWFGDTAE ALNVKKGWANESQRA EKDAEFLLTTCPQKL KSHLQKGQGNFHWKE 176
Rat    TLQGLLGCELAPDNS SLPTAVFALNGEEFM RFNPRTGNWSGEWPE TDIVGNLWMKQPEAA RKESEFLLTSCPERL LGHLERGRQNLEWKE 180
Mouse  TLQGLLGCELASDNS SVPTAVFALNGEEFM KFNPRIGNWTGEWPE TEIVANLWMKQPDAA RKESEFLLNSCPERL LGHLERGRRNLEWKE 180
Human  TLQGLLGCELGPDNT SVPTAKFALNGEEFM NFDLKQGTWGGDWPE ALAISQRWQQQDKAA NKELTFLLFSCPHRL REHLERGRGNLEWKE 178
Bovine TMQGLLGCELGPDNV SVPVAKFALNGEEFM MFDPKLGIWDGDWPE SRTVSIKWTQQPEAV NKEKTFLLYSCPHRL LGHLERGRGNLEWKE 177
                                *              ** *  *

          181                   210                   240                   270
Possum APEVRAGGHVEPGS- AWSTLTCQAFSFFPP ELELTFLREGQPQLV KGVEPWPNRDGAFYS RGTLLVPFGDEAFYS CLVQHPALASSITVN 265
Rat    PPSMRLKAR--PGNS GSSVLTCAAFSFYPP ELKFRFLRNGLASGS GNCSTGPNGDGSFHA WSLLEVKRGDEHHYQ CQVEHEGLAQPLTVD 268
Mouse  PPSMRLKAR--PGNS GSSVLTCAAFSFYPP ELKFRFLRNGLASGS GNCSTGPNGDGSFHA WSLLEVKRGDEHHYQ CQVEHEGLAQPLTVD 268
Human  PPSMRLKAR--PSSP GFSVLTCSAFSFYPP ELQLRFLRNGLAAGT GQGDFGPNSDGSFHA SSSLTVKSGDEHHYC CIVQHAGLAQPLRVE 266
Bovine PPSMRLKAR--PGSP GFSVLTCSAFSFYPP ELKLRFLRNGLAIGS GEIDMGPNGDGSFYA WSSLTVKSGDEHHYR CVVQHAGLAQPLTVE 267
                                                *                   **

          |                   300                   330
Possum FEASGGLPLPIRVSL VAGSLLFFACLVG-V VAWVVISRKRGARPA PWIFRRRAGDDVGSL LSAPASAQDSSP--- ------- 336
Rat    LDSPARSSVPV-VGI ILGLLLVVVAIAGGV LLWN---RMRSGLPA PWLSLS--GDDSGDL LPGGNLPPEAEPQGV NAFPATS 344
Mouse  LDSSARSSVPV-VGI VLGLLLVVVAIAGGV LLWG---RMRSGLPA PWLSLS--GDDSGDL LPGGNLPPEAEPQGA NAFPATS 344
Human  LESPAKSSVLV-VGI VIGVLLLTAAAVGGA LLWR---RMRSGLPA PWISLR--GDDTGVL LPTPGEAQDADLKDV NVIPATA 342
Bovine LESPARTSVPV-VGI VIGLFLLLTVAAGGA LLWR---RMRKGLPA PWISFR--GEDVGAL LPTPGLSKDGES--- ------- 331
                                                      * *
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Fig. 1. Possum FcRn: alignment of pFcRn amino acid sequence with eutherian FcRn homologues (mature peptides). The transmembrane domain is in bold text and bars delineate the α1–α3 extracellular domains. Amino acids referred to in the text and Table 1 are indicated by asterisks and these are sequentially; E117, E132, W133, E135, D137, N225, H250 and H251 (numbering uses rat FcRn). GeneBank accession numbers: Rat, P13599; mouse, Q61559; human, NM_004107; possum, AF191647, bovine AF221522.

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       1                             30                              60
Possum ITSSPKVQVYSRHPV DSNKENFVNCFVSGF HPPQITIELSKDGEK IPKVEESDLSFSNDW 60
Mouse  IQKTPQIQVYSRHPP ENGKPNILNCYVTQF HPPHIEIQMLKNGKK IPKVEMSDMSFSKDW 60
Rat    IQKTPQIQVYSRHPP ENGKPNFLNCYVSQF HPPQIEIELLKNGKK IPNIEMSDLSFSKDW 60
Human  IQRTPKIQVYSRHPA ENGKSNFLNCYVSGF HPSDIEVDLLKNGER IEKVEHSDLSFSKDW 60
Bovine IQRPPKIQVYSRHPP EDGKPNYLNCYVYGF HPPQIEIDLLKNGEK IK-SEQSDLSFSKDW 59
       *
      61                             90
Possum TFNRLVSAPFDPNSR SEYTCKVTHLTLQEP KVVKWDPENN 100
Mouse  SFYILAHTEFTPTET DTYACR-KHDSMAEP KTVYWDRDM-  98
Rat    SFYILAHTEFTPTET DVYACRVKHVTLKEP KTVTWDRDM-  99
Human  SFYLLYYTEFTPTEK DEYACR-NHVTLSQP KIVKWDRD--  97
Bovine SFYLLSHAEFTPNSK DQYSCRVKHVTLEQP RIVKWDRDL-  98
                                    *
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Fig. 2. Possum β-2m: alignment of pβ-2m amino acid sequence with the eutherian homologues (mature peptides). Amino acids referred to in the text and Table 1 are indicated by asterisks and these are sequentially; I1 and E89 (numbering uses rat β-2m). GeneBank accession numbers: Human, J00198; rat, Y00441; mouse, M18837; possum, AF191646.

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                                                                     Hinge                      CH2
                                                                       |                         |            246
Possum    --------------- ------QLTVPADDW LTKSYICNVAHKPTS TKTDKKIEKIS---- --ECTCC----KCQA CDVVG-PSVFLFPPN
Opossum   TSGIQTYPSVLQPSG LYTLTSQLTIPADSW SSQSYTCNVAHPATS TKIDKKIEATTT--- --TCPCC----KCNT VDAGG-PSVFVFPPN
mIgG-2a   SSGVHTFPAVLQ-SD LYTLSSSVTVTSSTW PSQSITCNVAHPASS TKVDKKIEPRG--PT IKPCPPC----KCPA PNLLGGPSVFIFPPK
mIgG-2b   SSSVHTFPALLQ-SG LYTMSSSVTVPSSTW PSQTVTCSVAHPASS TTVDKKLEPSGPIST INPCPPCKECHKCPA PNLEGGPSVFIFPPN


                                                                                                      336
Possum    PKDTLTLSRVPKITC VVVDVS-DASEVQIS WYKGENAIDSPK-PT ERKLNNGTFQVVSTL SVAHQEWLNGVAYTC KVDNKELPYPERKTI
Opossum   PQDVLKLSRSPKVTC MVVDVS-DASGVQIT WFKGEEEVSSPK-LT QKKLNNGTFQVVSNL PVVHQEWLKGTSYTC KVNTSELPVVERKTI
mIgG-2a   IKDVLMISLSPIVTC VVVDVSEDDPDVQIS WFVNNVEVHTAQTQT HREDYNSTLRVVSAL PIQHQDWMSGKEFKC KVNNKDLPAPIERTI
mIgG-2b   IKDVLMISLTPKVTC VVVDVSEDDPDVQIS WFVNNVEVHTAQTQT HREDYNSTIRVVSTL PIQHQDWMSGKEFKC KVNNKDLPSPIERTI
                *                                                      *

             CH3
           o  |           oo                                                                      423
Possum    SHTKGNRKKPDVYVF APHSEELKQKDTVSI TCLVKSFFPKEVVVE WQCNNNPESEDNYST TEAMREND-TFFVYS KLNVKKTKWQENNHY
Opossum   SHTKGERKKPDIYVF GPHPDELKQKDDVSI TCLVTNFFPEDVVIE WQKNNNPESEDKYYT TPTTREKS-TYFFYS KLIVKKRDWDNQNSY
mIgG-2a   SKPKGSVRAPQVYVL PPPEEEMTKKQ-VTL TCMVTDFMPEDIYVE WTNNGK--TELNYKN TEPVLDSDGSYFMYS KLRVEKKNWVERNSY
mIgG-2b   SKIKGLVRAPQVYIL PPPAEQLSRKD-VSL TCLVVGFNPGDISVE WTSNGH--TEENYKD TAPVLDSDGSYFIYS KLNMKTSKWEKTDSF


                                447
Possum    TCTVLHEALPNQTSQ RTISASSPGK
Opossum   TCVVLHEAFPNQISQ RTISAS-PGK
mIgG-2a   SCSVVHEGLHNHHTT KSFSRT-PGK
mIgG-2b   SCNVRHEGLKNYYLK KTISRS-PGK
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Fig. 3. Possum Fc-fragment: alignment of possum Fc-fragment amino acid sequence with those of the grey short-tailed opossum (Opossum), murine IgG-2a and IgG-2b (mIgG-2a and mIgG-2b). The amino acid of the possum Fc-fragment sequence presented differs from the possum sequence published by Belov et al. (1999) at three positions (indicated by circles), Phe^{214} to Ser, Pro^{232} to Ser and Asp ^{233} to Glu (numbering as per Belov et al., 1999). The bars delineate the beginning of the hinge, CH2 and CH3 domains. Amino acids referred to in the text and Table 1 are indicated by asterisks. These are sequentially; I253, H310, H433, H435, H436 (numbering is for mIgG-2a). GeneBank accession numbers: Opossum, AF035195; mIgG2a, P01863; mIgG2b, P01866; possum, AF191648.

mammals (45–50%; Aveskogh and Hellman, 1998; Belov et al., 1999).

The Fc receptor binds IgG with high affinity under acidic conditions as is observed in the neonatal gut (pH 6.0–6.5) and acidic endosomes after internalisation by fluid-phase endocytosis. Bound IgG is transcytosed to the basolateral membrane where the alkaline pH of blood causes IgG to be released (Rodewald and Kraehenbuhl, 1984; Ellinger et al., 1999). The dileucine motif in the cytosolic domain of FcRn required for endocytosis is conserved in pFcRn (Fig. 1; Stefaner et al., 1999).

The crystal structure of the FcRn/β-2m/Fc-fragment complex revealed that the Fc receptor binds IgG as a dimer of the FcRn/β-2m heterodimer (Burmeister et al., 1994a). Using the crystal structure and site directed mutagenesis, amino acid residues involved in high-affinity binding of IgG were identified and these are listed in Table 1 and indicated in Figs. 1–3 for comparison of the eutherian and marsupial homologues. High affinity binding of IgG at acidic pH requires the protonation of 2–3 His residues on the Fc-fragment which form salt-bridges with anionic pockets of the Fc receptor (Raghavan et al., 1995). The histidine residues which potentially contribute to this phenomenon were identified by their location at the interface between Fc-fragment and Fc receptor in the crystal structure of the complex and occur at the junction between the

Fc-fragment CH2–CH3 domains (Burmeister et al., 1994b). It has been demonstrated conclusively that in the eutherian Fc-fragment H310 forms an important contact with Fc receptor during high affinity binding (Kim et al., 1994; Raghavan et al., 1995; Popov et al., 1996; Medesan et al., 1997). This histidine residue is conserved in both the possum and opossum Fc-fragment (Table 1, Fig. 3). A critical amino acid residue of the corresponding anionic pocket of FcRn, E117, is not conserved in the pFcRn, however, I1 of pβ-2m which also makes up part of this anionic pocket is conserved (Table 1; Vaughn and Bjorkman, 1998).

The occurrence of His residues situated near the carboxyl-terminus of the Fc-fragment has been shown to affect affinity of different IgG-isotypes for the Fc receptor. In the rat Fc-fragment H435 and H436 are required for high affinity binding and for human IgG4, H433 is important (Kim et al., 1994; Raghavan et al., 1995; Popov et al., 1996; Medesan et al., 1997). Eutherian IgG isotypes which do not have His residues in this region, for example murine IgG2b (Fig. 3), show lower pH-dependent affinity for the Fc receptor (Raghavan et al., 1995; Medesan et al., 1998). Marsupial Fc-fragments are similar to murine IgG2b in that there are no His residues in this region would strengthen Fc receptor binding. It is surprising then that amino acid residues, which make up the corresponding anionic pocket on rat FcRn (E132, E135 and D137) are fairly well conserved in pFcRn (Table 1, Fig. 1). The only other His residue that is conserved between marsupial and eutherian homologues which could potentially be involved in this interaction, H429, remains to be investigated. A third interaction important for high affinity binding occurs between I253 of the Fc-fragment and W133 of FcRn (Medesan et al., 1997; Vaughn et al., 1997). In marsupials I253 is substituted with Leu and Thr replaces W133 in the pFcRn (Table 1). In the rodent Fc receptor dimer formation of the FcRn/β-2m heterodimer involves an interaction between FcRn His residues, H250 and H251 of one heterodimer and the β-2m residue E89 of the other heterodimer (Vaughn and Bjorkman, 1998). The pH-dependent high affinity binding of IgG is facilitated by dimerisation decreasing 6- and 3-fold when either the His residues or E89, respectively, are mutated (Raghavan et al., 1994; Vaughn et al., 1997). Neither of these histidines are conserved in the pFcRn, although E89 of pβ-2m is conserved (Table 1). A second interaction required for dimer formation occurs between the carbohydrate moiety on FcRn of one heterodimer and specific amino acid residues of FcRn in the other heterodimer (Vaughn and Bjorkman, 1998). Although the amino acid residues are conserved in pFcRn (I242, V244 and Y252), the Asp glycosylated in rodent FcRn (N225) is not present in possum or human FcRn suggesting they may use a different glycosylation site or their interaction differs.

Table 1
Conservation of amino acid residues critical for Fc receptor binding of IgG[a]

Molecule	Rodent	Human	Bovine	Possum	Opossum
IgG	1253	1	1	L	L
	H310	H	H	H	H
FcRn	E117	E	E	L	
	E132	D	D	D	
	W133	w	w	T	
	E135	E	E	E	
	D/E137	L	R	L	
	N225	Q	E	G	
	H250	H	H	A	
	H251	H	H	F	
P-2m	11	1	1	1	
	E89	Q	Q	E	

[a] Numbering as per the rat FcRn, rat P-2m and mIgG-2a (Figs. 1–3).

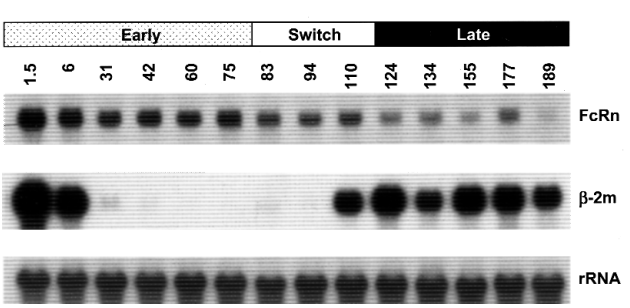

Fig. 4. Expression of possum Fc receptor in the possum mammary gland. Northern blots were hybridised with pFcRn (mRNA ~1500 bases), and pβ-2m (mRNA ~1000 bases) probes as indicated. The day of lactation of the mammary gland tissue is indicated. Bar above the days of lactation indicates sequentially the early, switch and late lactation phases in the possum. Northern blot was also hybridised with the 18S rRNA probe to show relative RNA loading in each lane (rRNA).

3.2. Differential expression of FcRn and β-2m in the possum mammary gland during lactation

Expression of FcRn and β-2m mRNAs was investigated to determine if the Fc receptor was expressed in the possum mammary gland and whether expression was elevated during the period of highest IgG secretion into milk. Different mRNA expression patterns were observed for the FcRn and β-2m genes (Fig. 4). FcRn mRNA expression was highest at the beginning of lactation (from day 1.5) decreasing slowly as lactation progressed. In contrast, expression of β-2m mRNA was high at the beginning of lactation (days 1.5–6), barely detectable during the remainder of the early lactation phase, and was elevated again from the switch phase until the end of lactation (Fig. 4). Co-expression of both chains of the Fc receptor is required for stable expression (Chamberlain et al., 1988; Zijlstra et al., 1990). Elevated β-2m expression at the beginning and switch phase of

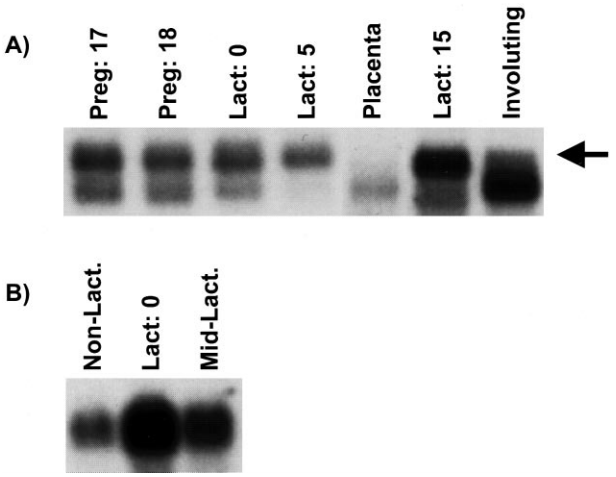

Fig. 5. Expression of β-2m mRNA in the mouse and bovine mammary glands during lactation. (A) Northern blot of mouse mammary gland and placenta total RNA hybridised with mouse β-2m probe. Mammary glands collected at different days of pregnancy (Preg.) or lactation (Lact.) are indicated. Involuting sample was from day 4 after forced involution at mid-lactation. A unique β-2m mRNA species was detected in the mouse mammary gland during pregnancy and lactation (arrow). (B) Northern blot of bovine mammary gland total RNA hybridised with bovine β-2m probe. Mammary glands analysed are from a non-pregnant cow (Non-Lact.), at parturition (Lact. 0) and from mid-lactation.

lactation suggests that expression of the Fc receptor will be upregulated at these times.

In the possum IgG secretion into milk is elevated only during the switch phase. The earliest time that the β-2m mRNA was detected in the possum mammary gland during the switch phase was at day 105 of lactation (result not shown) which coincides with the period of increased IgG concentration in milk, from day 100 of lactation (Adamski and Demmer, 2000). This is consistent with the need for greater control of IgG-transfer at this time by the Fc receptor.

3.3. Expression of FcRn and β-2m in the mouse and bovine mammary gland

Rodents and ungulates differ in the way IgG is transferred to the neonate. In rodents, where IgG is transferred via both the placenta and mammary gland, IgG secretion into milk occurs at a low level ($\sim$1 mg/ml) throughout lactation. In ungulates most of the IgG is transferred through the colostrum which contains a high concentration of IgG ($\sim$50 mg/ml). Expression of light and heavy chains of the Fc receptor in the mouse and bovine mammary gland throughout lactation was carried out to see if they have the same pattern of expression as observed in the possum. Although the level of FcRn mRNA expression was low (and difficult to detect) it was constant throughout lactation in both species (results not shown). In the mouse two β-2m mRNA species were expressed in the mammary gland throughout lactation (Fig. 5A). The shorter mRNA, has been previously identified by Simister and Mostov (1989), was expressed in the mammary gland mainly during pregnancy and involution, and corresponds to the mRNA expressed in the placenta. A unique, longer β-2m mRNA was present in the mammary gland during pregnancy and was the major species in the mammary gland during lactation. In the bovine mammary gland expression of β-2m mRNA was expressed throughout lactation, but was markedly elevated at parturition when colostrum is formed (Fig. 5B). Thus in the mouse where IgG-transfer into milk continues throughout lactation there was continuous expression of the β-2m mRNA in the mammary gland, whereas in the cow where IgG secretion is up-regulated for a short time at parturition, β-2m expression was increased at this time.

4. Discussion

4.1. Isolation of the possum FcRn, β-2m and Fc-fragment

In these experiments the first marsupial FcRn and β-2m cDNAs were cloned from the brushtail possum. The predicted amino acid sequences of the possum homologues show high sequence identity with those of eutherian species. Several of the amino acid residues which have been shown to be important for high affinity binding of IgG by the Fc receptor in eutherians are not conserved in the possum homologues suggesting that the mechanism of their interaction may differ in some respects.

4.2. Expression of the Fc receptor in the mammary correlates with a period of high IgG secretion into milk

The aim of this work was to establish whether mammary gland expression of the Fc receptor was associated with periods of increased IgG transport into milk during lactation in the possum. The concentration of IgG in possum milk is low throughout the early lactation phase of marsupials, but increases more than five-fold during the switch phase (Deane and Cooper, 1984; Deane et al., 1990; Adamski and Demmer, 2000). This increase in IgG secretion at the switch phase (from day 100 in the possum) is thought to be an active rather than passive process since the integrity of the mammary gland epithelia is intact at this time (Adamski and Demmer, 2000). In the possum mammary gland FcRn mRNA was expressed throughout lactation and β-2m mRNA expression was upregulated at the beginning of lactation and again during the switch phase. Co-expression of β-2m with FcRn is required for stable expression of Fc receptor (Chamberlain et al., 1988; Zijlstra et al., 1990) suggesting that expression of the Fc receptor

will be upregulated at these times. The latter period of increased β-2m mRNA expression correlates with the period of increased IgG-transfer into milk during the switch phase.

In the mouse and bovine mammary gland expression of β-2m mRNA was also correlated with the period of high IgG-transfer during lactation in these species. In the mouse mammary gland, which continuously secretes IgG during lactation, β-2m mRNA was expressed throughout lactation and in the bovine mammary gland expression of β-2m mRNA was elevated at parturition, coinciding with the period of high IgG secretion. In both these species the level of FcRn mRNA was constant throughout lactation.

Although the mechanism of IgG-transport into milk is unknown, it is thought to be an active mechanism because of the tight junctions that exist between mammary gland epithelia cells. In fact, when IgG-transfer is highest the concentration of IgG in milk exceeds that in the serum of some species (Brandon et al., 1971; Israel et al., 1995; Adamski and Demmer, 2000). Cianga et al. (1999) have determined that in the murine mammary gland the Fc receptor is involved in regulating transport of the different IgG-isotypes into milk. They observed that Fc-fragment mutants and IgG-isotypes with low affinity for the Fc receptor are transported more efficiently into mouse milk with high-affinity isotypes being recycled back to the maternal serum. Upregulation of Fc receptor expression in the possum, murine and bovine mammary gland during the period of highest IgG-transfer into milk would be consistent with the need for greater control of the process of IgG-transfer at this time. To date only one IgG isotype has been identified in marsupials which appears incongruous with elevated Fc receptor expression during the period of elevated IgG-transport for regulation of isotype transfer. In marsupials regulation of IgG-transfer by the Fc receptor may be a mechanism for prevention of excessive IgG-transfer into milk.

4.3. Two periods of immune transfer during lactation in marsupials

Lactation in marsupials is intriguing in that the mammary gland appears to be subjected to two stages of change, with the second occurring midway through lactation (switch phase). Prolactin secretion is required for induction and maintenance of lactation in eutherians and in the possum there is a short increase in circulating prolactin levels prior to parturition (Hinds and Janssens, 1986). In contrast to eutherian mammals, however, the level of circulating prolactin remains low for the remainder of early lactation phase, increasing again at the beginning of the switch phase when the mammary gland undergoes marked changes (Hinds and Janssens, 1986; Demmer, 1999). The mechanism for maintenance of lactation during the early phase is unknown. The expression pattern of several genes in the possum mammary gland follow that of the circulatory prolactin level, being expressed in the mammary gland for the first week after birth and again from switch phase (Adamski and Demmer, 1999; Demmer, 1999; Demmer et al., 1999). In this work it was found that the light chain of the Fc receptor, pβ-2m, follows the same pattern (Fig. 4) and it is possible that prolactin is involved in regulating expression of these genes.

The levels of FcRn and β-2 microglobulin mRNA in the mammary gland were high at the beginning of lactation (first colostrum phase), however, this does not appear to correlate with the need for greater control of IgG-transfer at this time given that IgG concentration in milk is not elevated. This suggests that the mechanism for regulating IgG-transfer is upregulated in the absence of increased IgG-transfer. At present no clear explanation exists for this observation. It could be due to the possum young being very small at birth (200 mg; Sharman, 1962) and requiring little milk production by the small mammary gland. In the tammar wallaby there is rapid transfer of maternal IgG via milk to the new born and this is crucial to the survival of the pouch young (Solomon, 1971; Deane and Cooper, 1988; Basden et al., 1997). Initiation of lactation in marsupials does not require prolactin as it is possible to transfer new born young on to the mammary glands of virgin female possums that are at the same stage of their reproductive cycle (i.e. day 17.5; Sharman, 1962). The increase in β-2m mRNA expression could therefore be an artefact of the prolactin spike prior to birth temporally upregulating expression of some genes. Thus, increased Fc receptor expression may occur in the absence of an increase in IgG-transfer.

In the possum, immune transfer via the mammary appears to occur at two stages, at parturition and the switch phase. The first period of immune transfer is reminiscent of that observed in rodents with the concentration of IgG in colostrum and milk (early lactation milk of marsupials) being low (approximately 1 mg/ml) and constant (Michalek et al., 1975; Appleby and Catty, 1983; Deane and Cooper, 1984; Adamski and Demmer, 2000). The second period of IgG-transfer is similar to that observed in ungulates at parturition with a significant increase in the concentration of IgG in possum milk (5-fold) (Adamski and Demmer, 2000).

In conclusion the heavy and light chains of the Fc receptor, FcRn and β-2m, of a marsupial, the brushtail possum were cloned and characterised. In the mammary gland, expression of the light chain of the Fc receptor, which normally regulates expression of the Fc receptor, correlates with the period of highest IgG-transfer into milk in three species; possum, mouse and cow. This is consistent with the need for greater control of IgG-transfer by the Fc receptor when IgG-transfer into milk is most active.

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