Published in final edited form as:

Immunogenetics. 2009 June ; 61(6): 431–442. doi:10.1007/s00251-009-0376-9.

Characterization of cynomolgus and vervet monkey placental MHC class I expression: diversity of the nonhuman primate AG locus

Gennadiy I. Bondarenko¹, Svetlana V. Dambaeva^{1,2}, Richard L. Grendell¹, Austin L. Hughes³, Maureen Durning^{1,2}, Mark A. Garthwaite^{1,4}, and Thaddeus G. Golos^{1,2,4}

Gennadiy I. Bondarenko: ; Svetlana V. Dambaeva: ; Richard L. Grendell: ; Austin L. Hughes: ; Maureen Durning: ; Mark A. Garthwaite: ; Thaddeus G. Golos: golos@primate.wisc.edu

¹ Wisconsin National Primate Research Center, University of Wisconsin, 1223 Capitol Court, Madison, WI 53715-1299, USA

² Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53715, USA

³ Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA

⁴ Department of Obstetrics and Gynecology, School of Medicine and Public Health, University of Wisconsin, Madison, WI 53715, USA

Abstract

Nonhuman primates are important animal models for the study of the maternal immune response to implantation within the decidua. The objective of this study was to define the placental expression of major histocompatibility complex (MHC) class I molecules in the cynomolgus (*Macaca fascicularis*) and vervet (African green) (*Chlorocebus aethiops*) monkeys. Early pregnancy (d36-42) cynomolgus and vervet placentas were obtained by fetectomy and prepared for histological evaluation. A pan-MHC class I monoclonal antibody demonstrated MHC class I expression in both vervet and cynomolgus placental trophoblasts, with particularly high expression in the villous syncytium, as previously shown in the rhesus and baboon. Placental cytotrophoblasts were isolated by enzymatic dispersion and gradient centrifugation and cultured, and multicolor flow cytometry was used to phenotype cell populations. Culture of isolated villous cytotrophoblasts demonstrated that MHC class I expression was linked to syncytiotrophoblast differentiation. A monoclonal antibody against Mamu-AG, the nonclassical MHC class I homolog of HLA-G in the rhesus monkey, demonstrated intense immunostaining and cell surface expression in cynomolgus placental trophoblasts; however, staining with vervet placenta and cells was low and inconsistent. Reverse transcriptase polymerase chain reaction was used to clone MHC class I molecules expressed in cynomolgus and vervet placentas. While *Mafa-AG* messenger RNA (mRNA) was readily detectable in cynomolgus placental RNA and was >99% identical at the amino acid level with *Mamu-AG*, 7/8 *Chae-AG* complementary DNAs had an unusual 16 amino acid repeat in the $\alpha 1$ domain, and all clones had an unexpected absence of the early stop codon at the 3'-end of the mRNA diagnostic for rhesus, cynomolgus, and baboon AG mRNAs, as well as HLA-G. We conclude that while the vervet monkey has retained the placental expression of a primate-specific nonclassical MHC class I locus, diversity is also revealed in this locus expressed at the maternal-fetal interface, thought to participate in placental regulation of the maternal immune response to embryo implantation and pregnancy.

Correspondence to: Thaddeus G. Golos, golos@primate.wisc.edu.

Gennadiy I. Bondarenko and Svetlana V. Dambaeva contributed equally to this work.

Keywords

Mamu-AG; Vervet monkey; Cynomolgus monkey; HLA-G; Placenta; Nonclassical MHC class I

Introduction

The placental expression of nonclassical major histocompatibility complex (MHC) class I molecules has long been thought to play an important role in the maternal immune and endometrial adaptation to pregnancy (Hunt 2006), and recent passive immunization studies in rhesus monkeys support this notion (Bondarenko et al. 2007). The identification of novel nonclassical, low polymorphism MHC class I loci expressed in the placentas of rhesus monkeys and baboons (Golos et al. 2009), and the recent identification of nonclassical MHC expression in ruminant trophoblasts (Davies et al. 2006) also indicate that placental MHC class I expression may be more widely conserved across mammals than was previously appreciated.

Nonhuman primates are crucial models for biomedical research due to their close phylogenetic and physiological relevance to human biology. In some instances, specific primate species may have unique attributes for a particular field of study, but the selection of a species may also be related to historical convenience or availability. In addition, an individual species may turn out to have previously unrecognized biological or practical advantages (or limitations). Thus, it is rational to develop additional models by characterizing their basic biological parameters and attributes.

While the rhesus monkey has been the primary species for embryological work since the first live births of rhesus monkeys from in vitro fertilization procedures at the Wisconsin Primate Research Center over two decades ago (Bavister et al. 1984), the vervet monkey (*Chlorocebus aethiops*) has a number of attractive aspects of its reproductive biology that suggest it may be a useful complement to the rhesus (Eley 1992; Eley et al. 1989; Owiti et al. 1986). In addition to a reproductive cycle that is very similar to that in the rhesus, it has been reported to lack seasonal breeding in captivity and has a straight cervix which would be expected to simplify flushing of embryos from naturally bred or artificially inseminated females, as well as the transfer of embryos to vervet recipients. These characteristics can be advantageous for embryology and embryonic stem cell research, and a greater understanding of early pregnancy in this species could expand options for new investigators to the field of primate reproduction.

The cynomolgus monkey (*Macaca fascicularis*) has been much more thoroughly studied than the vervet, including a description of the secretion of mCG (Chen et al. 2003), and implantation and morphology of the developing placenta (Enders and Schlafke 1986). However, its expression of placental MHC class I molecules has not been previously explored. Thus, we used reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry to define the MHC class I genes expressed in the placentas of these species, and explored whether the methods we have previously described for the isolation and culture of rhesus placental trophoblasts (villous cytotrophoblasts; Golos et al. 2005) were appropriate for cynomolgus or vervet monkey placentas. The results obtained demonstrate that whereas the cynomolgus macaque expresses in its placenta a nearly identical molecule as the rhesus, both protein immunodetection and subsequent cloning indicate divergence of the vervet MHC transcripts in the placenta from those expressed in macaques of Asian origin.

Materials and methods

Animals and surgery

Female cynomolgus or vervet (African green) monkeys (*M. fascicularis* and *Chlorocebus aeothlops*, respectively) used for timed matings were from the colony maintained at the Wisconsin National Primate Research Center. Placental tissues were generally obtained by fetectomy at days 36–42 of pregnancy (day 0 (d0) = the day after the luteinizing hormone (LH) surge as determined by radioimmunoassay of peripheral blood samples), although one vervet monkey placenta was obtained on d80. All surgical procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under the approval of the University of the Wisconsin Graduate School Animal Care and Use Committee.

Trophoblast isolation and culture

Placental trophoblast cells were obtained from minced villous tissue dissected free of the amniotic membranes and decidua using methods essentially identical to those previously described for the rhesus monkey placenta (Golos et al. 2005). Brief digestion was performed with trypsin/DNase enzyme solution. The suspension of dissociated cells was fractionated by centrifugation on a discontinuous 5% to 50% Percoll gradient. The middle band (from the 30–40% Percoll layer) was removed and washed. For culture experiments, cells were plated in 35 mm dishes at 0.5×10^6 cells/ml in Dulbecco's Minimum Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Cultured trophoblasts were removed by brief trypsinization from dishes for flow cytometric analysis.

Flow cytometry

The following mAbs were used in the analysis: fluorescein isothiocyanate (FITC)-labeled W6/32 against HLA class I antigens (Sigma), FITC-labeled anti-pan cytokeratin (clone C-11, Sigma), 25D3 against rhesus Mamu-AG (Slukvin et al. 2000), and MEM-E/06 against HLA-E (ExBio, Prague, Czech Republic), combined with secondary R-Phycoerythrin (PE)-conjugated rat anti-mouse IgG1 mAb (BD Pharmingen). Cells were resuspended in phosphate-buffered saline (PBS) with 2% heat-inactivated FCS and incubated with the corresponding mAbs or an isotype control for 30 min at 4°C. After two washes, cells were fixed with 2% paraformaldehyde in PBS for analysis or incubated with secondary mAb (in the case of 25D3 or MEM-E/06) for 30 min at 4°C, washed twice and then fixed in 2% paraformaldehyde in PBS. For intracellular cytokeratin staining, cells were fixed and permeabilized first using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's protocol, and then subjected to mAb staining. For each sample, 10,000 cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and CellQuest Software. Data analysis was done using FlowJo software 8.7.1 (Tree Star Inc., Ashland, OR, USA).

Immunohistochemistry

Placental tissues collected at surgery were immediately prepared for frozen sections. The tissues were fixed for 4 h in 2% paraformaldehyde, washed with PBS, dehydrated in 9% and 25% sucrose, embedded in OCT mounting medium (Sakura Finetek, Torrance, CA, USA), and frozen in liquid nitrogen. Sections of 6 µm were cut and stained with the anti-MHC class I antibodies HC10 at 0.155 µg/ml or W6/32 mAb at 1.75 µg/ml, anti-B₂microglobulin (B₂m)A⁻⁰⁷² at 1:1,000 dilution, anti-human cytokeratin mAb (CAM 5.2; Becton Dickinson) at 62.5 µg/ml and the anti-Mamu-AG 25D3 mAb at 11.2 µg/ml (Slukvin et al. 2000). Concentration-matched mouse IgG1 and IgG2 antibodies (Sigma) correspondingly served as negative controls. Localization of the primary antibodies was revealed with biotinylated secondary antibodies, Vectastain ABC-peroxidase complex, and Nova Red (Vector Laboratories, Burlingame, CA, USA).

Cloning of vervet and cynomolgus monkey MHC class I molecules

Vervet and cynomolgus monkey placental tissue, or vervet monkey peripheral blood and fetal spleen cells, were homogenized in RNA Stat-60 (Friendswood, TX, USA) and total RNA was evaluated by agarose electrophoresis to confirm integrity. RT-PCR was performed with previously described methods using universal macaque MHC class I primers (Karl et al. 2008) for vervet and cynomolgus RNA, or primers for specifically analyzing Mamu-AG (Slukvin et al. 1999) with cynomolgus RNA (Table 1). Amplification products were visualized with ethidium bromide on 1% agarose gels, and full-length amplification products were isolated from agarose gels, subcloned into pCR2.1 TOPO (Invitrogen), and sequenced in both directions. Sequence analysis and consensus sequence alignment were done with the Lasergene software package (DNASTAR, Madison, WI, USA). All full-length complementary DNA (cDNA) sequences were submitted to GenBank (accession numbers provided in Table 2).

Phylogenetic trees of rhesus (*Mamu*), cynomolgus (*Mafa*), and vervet (*Chae*) -AG, -A, and -B locus cDNAs, and *HLA-G* were constructed by the neighbor-joining method (Saitou and Nei 1987) based on the number of nonsynonymous substitutions per site (Nei and Gojobori 1986). The reliability of branching patterns in the trees was tested by bootstrapping (Felsenstein 1985); 1,000 bootstrap samples were used.

Results

We obtained placentas from six vervet pregnancies and five placentas from cynomolgus pregnancies for histological analysis. In order to obtain an estimate of the day of gestation, we collected peripheral blood and assayed for LH immunoactivity with a previously described radioimmunoassay (Wolfgang et al. 2001), using recombinant cynomolgus monkey LH standard. Vervet monkey cycles were also monitored by daily vaginal swabbing to detect menses, since menstrual blood is relatively inapparent in this species (Eley et al. 1989).

MHC class I molecule expression in rhesus, cynomolgus, and vervet monkey placentas

We evaluated the distribution of MHC class I molecules in the cynomolgus placenta by immunohistochemistry with the anti-Mamu-AG antibody 25D3, and the pan anti-human MHC class I antibodies W6/32 and HC10. Figure 1 demonstrates that MHC class I expression within the chorionic villi of rhesus and cynomolgus placentas was restricted to the syncytiotrophoblast layer, and villous cytotrophoblasts were negative. Intense staining in the cynomolgus and rhesus placentas was noted with 25D3 antibody, indicating that a Mamu-AG ortholog is likely also expressed in the cynomolgus monkey. However, consistent immunostaining of vervet chorionic villi was not seen with the anti-Mamu-AG antibody 25D3 (Fig. 1). Placental tissues were thus immunostained with the pan anti-MHC class I antibodies W6/32 which detects native cell-surface MHC class I, and HC10, which detects denatured MHC class I. While the W6/32 antibody again readily detected MHC class I expression in rhesus and cynomolgus monkey chorionic villi, staining in the villi of the vervet was again not detectable (Fig. 1). However, the HC10 antibody, which recognizes a common epitope in the MHC class I $\alpha 1$ domain (Perosa et al. 2003), readily detected expression in the syncytiotrophoblasts of all three species. As with 25D3 and W6/32, staining was particularly localized to the apical surface of the syncytiotrophoblasts, at the maternal-fetal interface. The lack of 25D3 or W6/32 staining in vervet placentas was not due to improper MHC class I folding, since $\beta 2m$ was readily detected by immunohistochemistry (IHC; Fig. 1).

In the human placenta, membrane-bound HLA-G is primarily detected in the extravillous trophoblasts which invade and remodel the maternal spiral arteries (Hunt 2006), and we have also shown similar endovascular trophoblast expression of Mamu-AG in the rhesus monkey (Slukvin et al. 2000). The immunostaining pattern of extravillous trophoblasts was similar to

that in the villi: rhesus and cynomolgus, but not vervet, extravillous trophoblasts were Mamu-AG-positive, whereas all species were stained with HC10 (not shown).

To confirm that MHC class I immunostaining in the cynomolgus placenta represents cell-surface expression, we isolated placental villous cytotrophoblasts and evaluated their class I expression by flow cytometry. As previously shown in the rhesus monkey, both cynomolgus monkey and vervet monkey cytotrophoblasts aggregated and fused within 48 h to form multinucleated syncytiotrophoblasts (Fig. 2, left panels). Figure 2 illustrates that freshly isolated cynomolgus monkey trophoblasts had detectable cell surface MHC class I expression, which was highly upregulated by culture for 24–48 h, including positive staining with anti-Mamu-AG 25D3 (Fig. 2). Villous trophoblasts were also isolated from the vervet placenta and evaluated by flow cytometry. Figure 2 demonstrates that >95% of the cells were cytokeratin-positive trophoblasts, and that trophoblast surface MHC expression was also detected by flow cytometry with the pan-MHC W6/32 antibody. However, staining of vervet trophoblasts with the anti-Mamu-AG 25D3 antibody was negative, in clear distinction with results in cynomolgus macaques (Fig. 2).

MHC class I mRNAs expressed in cynomolgus and vervet placentas

To better define the identity of placental MHC class I molecules expressed in the cynomolgus and vervet placentas, we evaluated vervet and cynomolgus placental MHC class I mRNA expression with RT-PCR, utilizing primers derived from the sequence of *Mamu-AG* and previously used to define its expression in rhesus placental and nonplacental tissues (Slukvin et al. 1999). With cynomolgus monkey placental mRNA, we readily obtained strong bands either from whole placental tissue or from isolated villous trophoblasts (Fig. 3). In addition, the characteristic pattern of multiple bands that represents alternatively spliced mRNAs expressed in the rhesus monkey placenta (Boyson et al. 1997) was also seen with cynomolgus placental RNA samples. In contrast, only faint and sporadic amplification from vervet monkey placental or trophoblast RNA was seen.

Because we were not successful at amplifying from vervet placental RNA with *Mamu-AG* primers, we used rhesus pan-MHC PCR primers in subsequent experiments, and amplified cDNAs of approximately the predicted size from both cynomolgus and vervet placental RNA (Fig. 3), as well as vervet peripheral blood lymphocytes (PBLs) and vervet fetal spleen tissue (not shown). These primers yielded more robust amplicons and we cloned and sequenced 11 cDNAs from vervet placental RNA, and four cDNAs obtained in parallel by RT-PCR of freshly obtained vervet PBLs, and two cDNAs from vervet spleen. In addition, we sequenced an individual clone from cynomolgus placental RNA.

To first determine the relatedness of the cynomolgus and vervet clones, we conducted phylogenetic analysis of the nucleotide sequences of all clones, along with *Mamu-AG*, *Mamu-G*, and representative *Mamu-A* and *Mamu-B* alleles. The results are presented in Fig. 4 and demonstrate several important results. First, *Mafa-AG* clusters very closely to *Mamu-AG*, as expected from the immunohistochemistry, flow cytometry, and RT-PCR results. With regards to the vervet cDNAs, one group of clones clustered together with each other, most closely related to *Mamu-AG* and *Mafa-AG*. Based on these data, these clones were designated as *Chae-AG1-8*. Six other clones clustered with a representative set of *Mamu-A* cDNAs (which themselves appeared to separate into two groups), and these were designated *Chae-A1-6*. Three additional clones clustered with *Mamu-B* alleles and were thus designated *Chae-B1-3*. Finally, *Mamu-G* is the rhesus monkey ortholog of *HLA-G*. Although it is a pseudogene, we included *Mamu-G* in the analysis, since it was possible that amplification from placental RNA could yield orthologous *G* locus clones. *Mamu-G* did not cluster with any of the other groups of MHC class I mRNAs.

Figure 5 presents alignment of the predicted amino acid sequences of all clones presented in Fig. 4. As predicted, *Mafa-AG* was nearly identical to *Mamu-AG*, with just three substitutions (two highly conservative) throughout the predicted amino acid sequence. These were noted in the 3'-end of the highly polymorphic region of the $\alpha 1$ domain. There was heterogeneity among placental as well as peripheral blood vervet MHC class I molecule mRNAs. The eight *Chae-AG* clones were extremely similar to *Mamu-AG* and *Mafa-AG* molecules, as immediately discerned from an inspection of the predicted amino acid sequences of the $\alpha 1$ and $\alpha 2$ domains. This is compelling molecular evidence for the existence of a *Chae-AG* locus expressed in the vervet placenta. Somewhat unexpectedly, seven of the eight AG-like clones were also distinct from all other mRNAs in that they had an insert of 48 nt in the $\alpha 1$ domain, encoding 16 amino acids, which appeared to be a duplication of the next 16 amino acids within the $\alpha 1$ domain (sequence begins VGY and ends SDA).

One of the diagnostic hallmarks of *Mamu-AG*, *Mafa-AG*, and *Paan-AG* (Boyson et al. 1999; Langat et al. 2002) that suggests functional homology with HLA-G is the presence of a premature stop codon 26 residues upstream from the classical MHC stop codon. A KSSDR* carboxy-terminal motif is diagnostic for *Mamu-*, *Mafa-*, and *Paan-AG*. None of the vervet mRNAs, including the eight *Chae-AG* clones or the non-AG-like clones, had a stop codon in this location. Thus, despite significant identity of the *Chae-AG* clones with *Mamu-AG* in the $\alpha 1$ and $\alpha 2$ domains, there was diversity in the cytoplasmic domain encoded by the sixth exon. Because there was a stop codon encoded within the 3' PCR primer, independent verification of the native stop codon for the vervet mRNAs is not available.

Within the group of eight vervet AG-like clones, several exhibited other unique features. *Chae-AG1* and *Chae-AG2* had a 91-nt deletion in the first and second exons, encoding a portion of the $\alpha 2$ domain, that resulted in a frame shift and a premature stop codon at the fifth amino acid position following the deletion, truncating the putative translated protein. For purposes of comparison, Fig. 5 includes the theoretical translated proteins downstream of this stop codon, to underscore the very close homology with *Mamu-AG* and *Mafa-AG* demonstrated by phylogenetic analysis in Fig. 4.

There is also diversity among the non-AG vervet clones. *Chae-A2* has a 79-nt deletion of the fifth through eighth exon, which results in a frame shift and an amino acid sequence divergence from all other clones at residues 307–332, although it is possible that this deletion arose through mis-priming by the 3' primer. *Chae-A6* has a 63-nt deletion at the 3'-end of the $\alpha 2$ domain, resulting in a loss of 21 amino acids in this region. A 33-nt deletion in exons 6 and 7 of this clone results in a loss of 11 amino acids in the cytoplasmic domain. Since these deletions do not appear to be associated with intron–exon junctions, it is not clear if these are physiologically relevant variants or the result of aberrant splicing events. We considered whether any of the vervet transcripts represented orthologs of *Mamu-G*; however, comparison with the predicted *Mamu-G* amino acid (Fig. 5), or nucleotide sequence (Fig. 4), revealed no compelling evidence for such homology.

The pattern of RT-PCR amplicons obtained with both *Mamu-AG* and MHC class I primers indicated that there were likely to be splice variants of *Mafa-AG*. The bands with lower apparent size amplified from the cynomolgus placental RNA shown in Fig. 3 were also cloned and sequenced. Figure 6 schematically indicates the patterns of alternative splicing observed with these amplification products. All the clones sequenced lacked exon 3. The majority (10/16) of these clones lacked only exon 3 and were thus equivalent to *Mamu-AG2* we have previously described (Boyson et al. 1997) and HLA-G2 (Ishitani and Geraghty 1992) lacking the $\alpha 2$ domain. Two clones lacked exons 3 and 4 and were thus equivalent to *Mamu-AG3*/HLA-G3 (Boyson et al. 1997; Ishitani and Geraghty 1992).

Other amplicons had more variable splicing patterns (Fig. 6). One clone (Mafa-AG2a) used nonconsensus intron–exon junctions linking the second and fourth exons, and one clone (Mafa-AG2b) used the 3' splice donor of exon 2, and a nonconsensus 5' splice acceptor, the latter resulting in a frame shift and a stop codon 7 amino acids downstream from this frame shift. Two clones (Mafa-AG2c,d) which lacked exon 3 also had gaps within the sequence of exon 4, one of which (80 bp in Mafa-AG2c) resulted in a frame shift and premature stop codon. The Mafa-AG2d clone would have a 25 amino acid deletion in the $\alpha 3$ domain encoded by exon 4. Interestingly, none of these clones retained intron 4 which would encode a soluble MHC class I isoform as previously shown for HLA-G and Mamu-AG (Fujii et al. 1994; Ryan et al. 2002). Finally, five of the clones also had 18 nucleotides from the 3'-end of intron 5 added to the 5'-end of exon 6. These were in frame and gave rise to a six amino acid extension to the carboxy end of the cytoplasmic domain. We are not aware of previous reports of this unusual event which would appear to be the use of an alternative 5' splice acceptor site within intron 5.

Discussion

The MHC class I molecules expressed in placental trophoblasts of cynomolgus and vervet monkeys were evaluated by immunohistochemistry, flow cytometry, and molecular cloning of expressed mRNAs. The results revealed that both species express placental MHC class I genes, and that their placental mRNAs are related to the AG locus previously identified as the primary nonclassical MHC class I transcript expressed in the rhesus monkey and baboon placentas. Whereas the cynomolgus monkey expressed a mRNA nearly identical in its amino acid sequences to rhesus monkey Mamu-AG, the vervet placenta expressed a mRNA which diverged in several key characteristics from the rhesus, cynomolgus, and baboon molecules, despite clearly being a member of the AG locus. These included a novel insert in the $\alpha 1$ domain, as well as the lack of an early stop codon resulting in a truncated cytoplasmic domain.

It was not unexpected that the expression of *Mafa-AG* in the cynomolgus placenta was largely consistent with that seen in the rhesus monkey. The reproductive physiology of these Asian species is highly homologous, including the regulation of the menstrual cycle, and the morphology and development of the implantation site, and early fetal and placental development. Cloning of the vervet placenta MHC class I mRNAs also confirmed our expectation that the vervet placenta would express an MHC-AG locus. Indeed, we found that eight of 11 full-length cDNAs isolated from the vervet placenta were represented by AG-like clones, along with 1 Chae-A and two Chae-B cDNAs. The cloning of *Mamu-AG* arose from a comprehensive screen of the rhesus monkey placenta using a pan-MHC oligonucleotide probe (Boyson et al. 1997), in which of 105 clones sequenced, 52 represented *Mamu-AG*, 39 represented *Mamu-E*, and eight represented *Mamu-B* transcripts. Our work reported here with the vervet placenta likewise utilized RT-PCR primers previously demonstrated as effective tools for amplification of rhesus and cynomolgus classical and nonclassical MHC class I mRNAs (Karl et al. 2008; Campbell et al. 2008). Thus, we likely have obtained a representative sampling of the vervet placental MHC class I mRNA populations. It thus may be possible that the vervet placenta expresses polymorphic as well as non-polymorphic MHC class I mRNAs. While it would be informative to know the entire population of mRNAs expressed in the baboon placenta, previous studies (Boyson et al. 1999; Langat et al. 2002) were restricted to gene-specific PCR amplification with primers either specific to *Mamu-AG* (Boyson et al. 1999) or *Paan-AG* (Langat et al. 2002), and thus we are not in a position currently to understand vervet placental MHC expression patterns in the context of the baboon as well as the rhesus.

Given the differences seen between Mamu-AG and Chae-AG in the $\alpha 1$ and $\alpha 2$ domains, it is perhaps not surprising that the 25D3 antibody did not recognize MHC class I expression in vervet trophoblasts. However, there clearly was substantial MHC class I expression in vervet

villous syncytiotrophoblasts, as evidenced by their robust immunostaining with the HC10 antibody, raised against “denatured” MHC class I molecules. Co-localization with β_2m strongly suggests functional expression of MHC class I molecules on the trophoblast surface. This is in agreement with flow cytometric analysis of isolated trophoblasts although it is not clear to us why we could detect MHC molecules by flow cytometry but not IHC with W6/32. The mAb HC10 reacts with the β_2m -free heavy chain of many MHC class I molecules. In humans, these include the majority of HLA-B and HLA-C alleles and some HLA-A alleles. HC10 was shown to be nonreactive with HLA-G and HLA-E (Perosa et al. 2003). Although HC10 binds only β_2m -free heavy chain, the determinant that is recognized by HC10 is located within the $\alpha 1$, not the $\alpha 3$ domain. HC10 binds to an epitope region within the $\alpha 1$ helix. It is speculated that association with β_2m resulted in conformational changes including extension of the loop (Perosa et al. 2003) and loss of reactivity. This region is also close to the peptide-binding pocket and peptide loading may block HC10 binding. The HC10 binding motif has been defined as EGPEYWDRN(E)T mapped to the $\alpha 1$ domain positions 55–64. P57, W60, D61, and R62 were found as key residues for HC10 binding. In Mamu-AG and Mafa-AG, this region is identical to the HC10-specific motif. All vervet *Chae-AG* clones also revealed the presence of this motif: in *Chae-AG1*, it mapped to residues 55–64, and in *Chae-AG2-8*, the 16 amino acid insert in the $\alpha 1$ domain results in a location 16 residues downstream.

As with the human and rhesus placental MHC class I transcripts, vervet and cynomolgus AG transcripts are alternatively spliced. Two of the cDNAs expressed in vervet placentas (*Chae-AG1*, 2) had a 91 nucleotide deletion at the beginning of exon 3, and an apparent frame shift in the truncated $\alpha 2$ domain, leading to a short (four amino acids) novel cytoplasmic carboxy-terminus and stop codon. While the *HLA-G* splice variants almost always involve exon skipping or intron retention events, some of the vervet variants reported here do not correlate with predicted exon–intron junctions. A similar usage of non-consensus splice acceptor sites has been shown with the baboon *Paan-AGx* transcripts (Langat et al. 2002). Further studies with additional animal populations will be necessary to determine whether these cDNAs represent physiologically relevant molecular isoforms of vervet placental MHC class I transcripts.

The *Mafa-AG* transcripts were also alternatively spliced, and most of these mRNAs would encode proteins equivalent to HLA-G2/Mamu-AG2, and HLA-G3/Mamu-AG3, which have been previously described (Boyson et al. 1997; Ishitani and Geraghty 1992). These all would encode molecules that included the transmembrane and cytoplasmic domains, and thus are potentially able to be expressed on the cell surface as has been discussed for HLA-G (Ishitani and Geraghty 1992; Riteau et al. 2001). While the specific function of these splice variants has not been ascertained in vivo, the retention of these very same alternatively spliced variants in this placental mRNA population suggests a specific functional role for the encoded proteins, although the function or the mechanism of splicing control remains uncertain. It was somewhat surprising that these splice variants did not appear in the vervet placental RNA samples; however, it may be that other amplification strategies would be needed in order to detect these mRNAs, which are likely to be of relatively low abundance.

Studies on the structural recognition of HLA-G and its receptors (Clements et al. 2007) have revealed that the ligand-binding site for LILR1 and LILR2 is located within the $\alpha 3$ and β_2m interaction domain. We can predict high homology between cynomolgus and rhesus AG and HLA-G in LILR recognition, since there are few differences between their putative LIR-binding regions (the $\alpha 3$ domain) (Boyson et al. 1997). On the other hand, the ligand-binding site for KIR2DL, another putative HLA-G receptor, is located within the $\alpha 1$ and $\alpha 2$ domains, i.e., residues forming the $\alpha 1$ and $\alpha 2$ helices of HLA class I molecules are involved in HLA class I/KIR complexing. It has been shown that $\alpha 1$ helix residues located between positions 68 and 86 are involved in HLA class I/KIR interactions (Boyington and Sun 2001). With HLA-G, Met at 76 and Gln at 79 were highlighted as important residues for HLA-G/KIR2DL4

binding (Yan and Fan 2005). Diversity among macaque AG alleles (Boyson et al. 1997) suggests that the amino acid at position 76 may not be a critical indicator of their KIR-binding specificity. Alternatively, it is also possible that the MHC-AG molecules are recognized by different KIR molecules.

It is interesting to consider the evolutionary implications of these results. Within the Cercopithecines (Old World monkey subfamily), there are two groups: the Cercopithecines (guenons, vervet, patas and swamp monkeys, and talapoin) and the Papionins (macaques, mandrills, mangabeys, and baboons). Molecular estimates place the divergence of the Cercopithecini from Papionini at 9–10 Ma (million geochronologic years; Morales et al. 1999; Jablonski 2002). The estimate of the divergence of hominoids from Cercopithecines is approximately 25 Ma (Pilbeam 2002). Thus, it seems likely that the AG locus arose in a common ancestor of macaques, vervets, and baboons after hominoid divergence from cercopithecini. The *HLA-G* locus has been shown to be present in rhesus, cynomolgus, and vervet monkeys (Boyson et al. 1996; Castro et al. 1996); however, in all of these species, stop codons and other deletions or insertions render them unlikely to encode functional proteins. Whether the functional loss of the G locus preceded the appearance of the AG locus remains speculative, however, it could be speculated that the presence of the AG locus, and its placental expression in both Cercopithecini and Papionini, suggests a vital function which made the G locus dispensable in these species.

Acknowledgments

We thank the Veterinary, CPI, and the Assay Service Units for assistance in developing methods for monitoring vervet monkey reproductive cycles, producing timed matings and monitoring serum hormones. The veterinary staff of the Wisconsin National Primate Research Center provided excellent surgical assistance, and we thank Judith Peterson for assistance in preparation of this manuscript. Dr. David O'Connor and the WNPRC Immunogenetics Core provided advice on primer design and MHC class I nomenclature procedures. This research was supported by NIH grants RR14040, HD37120, AI076734, and HD34215 to T.G.G., GM43940 to A.L.H., and P51 RR000167 to the Wisconsin National Primate Research Center, University of Wisconsin-Madison. This research was conducted in part at a facility constructed with support from Research Facilities Improvement Program grant numbers RR15459-01 and RR020141-01. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

References

- Bavister BD, Boatman DE, Collins K, et al. Birth of rhesus monkey infant after in vitro fertilization and nonsurgical embryo transfer. *Proc Natl Acad Sci U S A* 1984;81:2218–2212.10.1073/pnas.81.7.2218 [PubMed: 6326113]
- Bondarenko GI, Burleigh DW, Durning M, et al. Passive immunization against the MHC class I molecule Mamu-AG disrupts rhesus placental development and endometrial responses. *J Immunol* 2007;179:8042–8050. [PubMed: 18056344]
- Boyington JC, Sun PD. A structural perspective on MHC class I recognition by killer cell immunoglobulin-like receptors. *Mol Immunol* 2001;38:107–1021.
- Boyson JE, Iwanaga KK, Golos TG, Watkins DI. Identification of the rhesus monkey HLA-G ortholog. Mamu-G is a pseudogene. *J Immunol* 1996;157:5428–5437. [PubMed: 8955191]
- Boyson JE, Iwanaga KK, Golos TG, Watkins DI. Identification of a novel MHC class I gene, Mamu-AG, expressed in the placenta of a primate with an inactivated G locus. *J Immunol* 1997;159:3311–3321. [PubMed: 9317129]
- Boyson JE, Iwanaga KK, Urvater JA, et al. Evolution of a novel nonclassical MHC class I locus in two Old World primate species. *Immunogenetics* 1999;49:86–98.10.1007/s002510050467 [PubMed: 9887345]
- Campbell KJ, Detmer AM, Karl JA, et al. Characterization of 47 MHC class I sequences in Filipino cynomolgus macaques. *Immunogenetics* 2008;61:177–187. [PubMed: 19107381]

- Castro MJ, Morales P, Fernández-Soria V, et al. Allelic diversity at the primate Mhc-G locus: exon 3 bears stop codons in all Cercopithecinae sequences. *Immunogenetics* 1996;43:327–336.10.1007/BF02199801 [PubMed: 8606053]
- Chen J, Laughlin LS, Hendrickx AG, et al. The effect of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) on chorionic gonadotrophin activity in pregnant macaques. *Toxicology* 2003;186:21–31.10.1016/S0300-483X(02)00601-7 [PubMed: 12604168]
- Clements CS, Kjer-Nielsen L, McCluskey J, Rossjohn J. Structural studies on HLA-G: implications for ligand and receptor binding. *Hum Immunol* 2007;68:220–226.10.1016/j.humimm.2006.09.003 [PubMed: 17400055]
- Davies CJ, Eldridge JA, Fisher PJ, Schlafer DH. Evidence for expression of both classical and non-classical major histocompatibility complex class I genes in bovine trophoblast cells. *Am J Reprod Immunol* 2006;55:188–200.10.1111/j.1600-0897.2005.00364.x [PubMed: 16451353]
- Eley RM. Reproductive biology of the vervet monkey (*Cercopithecus aethiops*): a review. *Utafiti* 1992;4:1–33.
- Eley RM, Tarara RP, Worthman CM, Else JG. Reproduction in the vervet monkey (*Oercopithecus aethiops*): III. The menstrual cycle. *Am J Primatol* 1989;17:1–10.10.1002/ajp.1350170102
- Enders AC, Schlafke S. Implantation in nonhuman primates and in the human. *Comp Primate Biol Reprod Dev* 1986;3:291–310.
- Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution Int J Org Evolution* 1985;39:783–791.10.2307/2408678
- Fujii T, Ishitani A, Geraghty DE. A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. *J Immunol* 1994;153:5516–5524. [PubMed: 7989753]
- Golos TG, Bondarenko GI, Breburda EE, et al. Immune and trophoblast cells at the rhesus monkey maternal–fetal interface. *Methods Mol Med* 2005;122:93–108. [PubMed: 16511977]
- Golos TG, Bondarenko GI, Dambaeva SV, et al. Nonhuman primate modeling of the maternal-fetal immune dialogue: addressing critical questions in the role of placental MHC and decidual leukocytes in implantation and pregnancy success. *Int J Dev Biol*. 2009 (in press).
- Hunt JS. Stranger in a strange land. *Immunol Rev* 2006;213:36–47.10.1111/j.1600-065X.2006.00436.x [PubMed: 16972895]
- Ishitani A, Geraghty DE. Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens. *Proc Natl Acad Sci U S A* 1992;89:2669–2673.10.1073/pnas.89.9.3947 [PubMed: 1557372]
- Jablonski, NG. Fossil old world monkeys: the late neogene radiation. In: Hartwig, WC., editor. *The primate fossil record*. Cambridge University Press; Cambridge: 2002. p. 255-299.
- Karl JA, Wiseman RW, Campbell KJ, et al. Identification of MHC class I sequences in Chinese-origin rhesus macaques. *Immunogenetics* 2008;60:37–46.10.1007/s00251-007-0267-x [PubMed: 18097659]
- Langat DK, Morales PJ, Fazleabas AT, et al. Baboon placentas express soluble and membrane-bound Paan-AG proteins encoded by alternatively spliced transcripts of the class Ib major histocompatibility complex gene, Paan-AG. *Immunogenetics* 2002;54:164–173.10.1007/s00251-002-0454-8 [PubMed: 12073145]
- Morales, JC.; Disotell, TR.; Melnick, DJ. Molecular phylogenetic studies of nonhuman primates. In: Dolhinow, P.; Fuentes, A., editors. *The nonhuman primates*. Mayfield; California: 1999. p. 18-28.
- Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol Biol Evol* 1986;3:418–426. [PubMed: 3444411]
- Owiti GE, Cukierski M, Tarara RP, et al. Early placentation in the African green monkey (*Cercopithecus aethiops*). *Acta Anat (Basel)* 1986;127:184–194.10.1159/000146280 [PubMed: 3788465]
- Perosa F, Luccarelli G, Prete M, et al. β 2-microglobulin-free HLA class I heavy chain epitope mimicry by monoclonal antibody HC-10-specific peptide. *J Immunol* 2003;171:1918–1926. [PubMed: 12902494]
- Pilbeam, DR. Fossil old world monkeys: the late neogene radiation. In: Hartwig, WC., editor. *The primate fossil record*. Cambridge University Press; Cambridge: 2002. p. 303-310.

- Riteau B, Rouas-Freiss N, Menier C, et al. HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytotoxicity. *J Immunol* 2001;166:5018–5026. [PubMed: 11290782]
- Ryan AF, Grendell RL, Geraghty D, Golos TG. A soluble isoform of the rhesus monkey nonclassical MHC class I molecule Mamu-AG is expressed in the placenta and the testis. *J Immunol* 2002;169:673–683. [PubMed: 12097369]
- Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425. [PubMed: 3447015]
- Slukvin II, Watkins DI, Golos TG. Tissue distribution of the mRNA for a rhesus monkey major histocompatibility class Ib molecule, Mamu-AG. *Tissue Antigens* 1999;53:282–291.10.1034/j.1399-0039.1999.530309.x [PubMed: 10203022]
- Slukvin II, Lunn DD, Golos TG. Placental expression of the nonclassical MHC class I molecule Mamu-AG at implantation in the rhesus monkey. *Proc Natl Acad Sci U S A* 2000;97:9104–9109.10.1073/pnas.97.16.9104 [PubMed: 10922066]
- Wolfgang MJ, Eisele SG, Knowles L, et al. Pregnancy and live birth from nonsurgical transfer of in vivo- and in vitro-produced blastocysts in the rhesus monkey. *J Med Primatol* 2001;30:148–155.10.1034/j.1600-0684.2001.300602.x [PubMed: 11515670]
- Yan WH, Fan LA. Residues Met⁷⁶ and Gln⁷⁹ in HLA-G α 1 domain involved in KIR2DL4 recognition. *Cell Res* 2005;15:176–182.10.1038/sj.cr.7290283 [PubMed: 15780179]

Fig. 1. Frozen sections of rhesus, cynomolgus, and vervet monkey placentas (days 36–42 of gestation) immunostained with 25D3 mAb against Mamu-AG, anti-MHC class I mAb HC10 or W6/32, β 2microglobulin (Beta), or mouse IgG nonspecific control. Scale bar= 50 μ m (*top panels*)

Fig. 2.

Expression of MHC class I molecules is upregulated during rhesus cytotrophoblast differentiation. *Left panels:* cultured vervet (*bottom*) or cynomolgus monkey (*top*) syncytiotrophoblasts. Freshly isolated cytotrophoblasts (0 h) (*broken orange line*) or trophoblasts cultured for 24 h (*blue line*) or 48 h (*red line*) were stained with 25D3 and W6/32 mAbs. *Gray-filled histograms* represent isotype-matched control mAb staining. Cytokeratin staining of permeabilized cultures after 48 h of culture (*right panels*) demonstrates that cells are highly enriched for trophoblasts, and that virtually all cynomolgus cells express surface *Mafa-AG*

Fig. 3. Amplification of the rhesus monkey vervet and cynomolgus MHC class I cDNAs by Mamu-AG-specific and pan-MHC primers by RT-PCR. Amplification with G3PDH primers indicates mRNA integrity for all samples. *Asterisks* indicate bands isolated for cloning and sequencing

Fig. 4. Phylogenetic trees of rhesus (Mamu), cynomolgus (Mafa), and vervet (Chae) -AG, -A, and -B locus cDNAs, and HLA-G. The numbers on the branches represent the numbers of bootstrap samples supporting each branch; only values of 50% or greater are shown. New cDNAs cloned in the current study are in **bold font**, other sequences from the Genbank database. Accession numbers for all sequences are listed in Table 2. The Chae-AG clones were from four different placentas, and the Chae-A and Chae-B clones were each from two different animals. The Mafa-AG clone was from a single placenta

Fig. 5. Alignment of the rhesus monkey (Mamu), cynomolgus (Mafa), and vervet (Chae) -AG predicted amino acid sequences. The consensus is set to a representative rhesus allele Mamu-AG*01011, in order to highlight the homology and divergence of the cynomolgus and vervet clones. Identity is indicated by *dashes*, gaps are indicated by *periods*, and amino acid residues that diverge from the consensus are indicated by *letters*. Stop codons are indicated by *shaded asterisks*. Mamu-G is presented at the *bottom* for comparison with AG sequences. Regions encoded by amplification primers are *boxed*; degeneracy in the primers led to some amino acid heterogeneity. Clones Chae-A4, Chae-A5, Chae-A6, and Chae-B1 were derived from peripheral blood cells, and clones Chae-A2 and Chae-A3 were derived from vervet-fetal RNA. All other vervet clones were derived from placental RNA

Fig. 6.

Schematic diagram of the organization of Mafa-AG splice variants. The general organization of MHC class I genes is shown at the *top*, with exons *shaded in dark gray*, introns *shaded in light gray*, and *vertical lines* delineating primer regions. For individual clones, exons are in *dark gray*, and sequences spliced out of individual clones are in *white*. Stop codons are indicated by *black dots* within exons; please note that exons downstream from these stop codons would not be translated. The *numbers in parenthesis* indicate the number of clones for a given splice variant, which were all from an individual placenta

Table 1

Primers used for the amplification and sequencing of MHC class I cDNAs from vervet and cynomolgus monkeys

Primers	Sequence
Amplification	
5'A loci sense	5' ATG GCG CCC CGA ACC CTC
3'A loci antisense	5' TCA CAC TTT ACA AGC CGT GAG AGA CAC
5-MHC- <i>Nor1</i> sense	5' GCG GCC GCA TGS SSG TCA TGG CGC CSS G
3-Alocus- <i>KpnI</i> antisense	5' GGT ACC TCA CAC TTT ACA AGC CGT GAG AGA CAC
3-Blocus- <i>KpnI</i> antisense	5' GGT ACC TCA AGC CGT GAG AGA CWC ATC AGA GCC
U273 sense	5' AGA ACA TGA AGA CCG CGA CAC AGA CCT A
L1025 antisense	5' CAG CCT GAG AGT AGC TCC CGC CTT A
Sequencing	
Mamu 128.1 sense	5' TCA TCT CCG TGG GCT ACG TGG
Mamu 311 sense	5' GCT ACT ACA ACC AGA GCG AGG
Mamu 311 antisense	5' CCT CGC TCT GGT TGT AGT AGC
Mamu 575 sense	5' ACC TGG AGA ACG GGA AGG AG
Mamu 575 antisense	5' TCT CCT TCC CGT TCT CCA GG
Mamu 617 antisense	5' GTG GGT CAC GTG TGT CTT TGG
Mamu 799 sense	5' GCT GTG GTG GTG CCT TCT GG
Mamu 799 antisense	5' CCA GAA GGC ACC ACC ACA GC
Mamu 817 sense	5' GGA AAG GAG CAG AGA TAC ACC
Mamu 817 antisense	5' GGT GTA TCT CTG CTC CTT TCC

S = C or G, W = A or T. The u273 and L1025 amplification primers are Mamu-AG-specific; all others are universal MHC class I primers

Table 2

MHC class I sequence compared in Figs. 4 and 5

mRNA	Accession no.
Mamu-AG*0202	U84786
Mamu-AG*02011	U84784
Mamu-AG*02012	U84785
Mafa-AG1	FJ611995
<i>Chae-AG1</i>	FJ611987
<i>Chae-AG2</i>	FJ611988
<i>Chae-AG3</i>	FJ611989
<i>Chae-AG4</i>	FJ611990
<i>Chae-AG5</i>	FJ611991
<i>Chae-AG6</i>	FJ611992
<i>Chae-AG7</i>	FJ611993
<i>Chae-AG8</i>	FJ611994
Chae-A1	FJ611996
Chae-A2	FJ611997
Chae-A3	FJ611998
Chae-A4	FJ611999
Chae-A5	FJ612000
Chae-A6	FJ612001
Chae-B1	FJ612002
Chae-B2	FJ612003
Chae-B3	FJ612004
Mamu-A*11	AF199357
Mamu-A*1303	AF157401
Mamu-A4*1409	EU669868
Mamu-A*21	AJ542574
Mamu-A*01	U50836
Mamu-B*010102	EU682524
Mamu-B*02	U41833
Mamu-B*03	EU682521
Mamu-B*04	U41826
Mamu-B*05	U41827
Mamu-G*01	U55066