ACP5 (Uteroferrin): Phylogeny of an Ancient and Conserved Gene Expressed in the Endometrium of Mammals

Maria B. Padua,1,2,3 Vincent J. Lynch,4 Natalia V. Alvarez,3 Mark A. Garthwaite,5 Thaddeus G. Golos,5 Fuller W. Bazer,6 Satyan Kalkunte,7 Surendra Sharma,7 Gunter P. Wagner,4 and Peter J. Hansen2,3

1Department of Animal Sciences and D.H. Barron Reproductive and Perinatal Biology Research Program, University of Florida, Gainesville, Florida
2Department of Ecology and Evolutionary Biology and Yale Systems Biology Institute, Yale University, New Haven, Connecticut
3Department of Comparative Biosciences, School of Veterinary Medicine, Wisconsin National Primate Center, University of Wisconsin, Madison, Wisconsin
4Department of Genetics, Center for Animal Biotechnology and Genomics, Department of Animal Sciences, Texas A&M University, College Station, Texas
5Department of Pediatrics and Pathology, Women and Infants Hospital of Rhode Island, Providence, Rhode Island
6Center for Animal Biotechnology and Genomics, Department of Animal Sciences, Texas A&M University, College Station, Texas
7Department of Ecology and Evolutionary Biology, Yale University, New Haven, Connecticut

ABSTRACT

Type 5 acid phosphatase (ACP5; also known as tartrate-resistant acid phosphatase or uteroferrin) is a metalloprotein secreted by the endometrial glandular epithelium of pigs, mares, sheep, and water buffalo. In this paper, we describe the phylogenetic distribution of endometrial expression of ACP5 and demonstrate that endometrial expression arose early in evolution (i.e., before divergence of prototherian and therian mammals ~166 million years ago). To determine expression of ACP5 in the pregnant endometrium, RNA was isolated from rhesus, mouse, rat, dog, sheep, cow, horse, armadillo, opossum, and duck-billed platypus. Results from RT-PCR and RNA-Seq experiments confirmed that ACP5 is expressed in all species examined. ACP5 was also demonstrated immunochemically in endometrium of rhesus, marmoset, sheep, cow, goat, and opossum. Alignment of inferred amino acid sequences shows a high conservation of ACP5 throughout speciation, with species-specific differences most extensive in the N-terminal and C-terminal regions of the protein. Analysis by Selecton indicated that most of the sites in ACP5 are undergoing purifying selection, and no sites undergoing positive selection were found. In conclusion, endometrial expression of ACP5 is a common feature in all orders of mammals and has been subjected to purifying selection. Expression of ACP5 in the uterus predates the divergence of therians and prototherians. ACP5 is an evolutionarily conserved gene that likely exerts a common function important for pregnancy in mammals using a wide range of reproductive strategies.

ACP5, evolution, pregnancy, uterus

INTRODUCTION

Development of viviparity in eutherian mammals was accompanied by a large-scale reorganization of gene regulatory networks that resulted in expression in the endometrium of at least 1500 genes that are not expressed in the endometrium of metatherians [1]. This massive recruitment of genes for a novel function, the chorioallantoic placenta, was driven in part by utilization of transposable elements, such as MER20 [1]. Further evolutionary divergence in placental anatomy and function was accompanied by recruitment of additional genes for expression in the endometrium. This process involved formation of new promoters through utilization of transposable elements [2] as well as mutations in the coding region of transcription factors, such as HOXAI1 [3]. In addition, new genes have been formed via gene duplication and divergence. For example, SERPINA14 is derived from duplication of a clade A serpin gene and is expressed in a limited group of mammals, including cetartiodactyly, perissodactyly, suids, and some carnivores [4]. Evidence in the mouse suggests that formation of new genes was more extensive for those expressed in the placenta than for those expressed in the endometrium [5]. Indeed, a majority of genes that are preferentially expressed in the mouse and human decidua during pregnancy have an ancient origin [5].

Here, we describe the phylogenetic distribution of endometrial expression of the type 5 acid phosphatase gene (ACP5; also known as tartrate-resistant acid phosphatase or uteroferrin) and demonstrate that endometrial expression arose early in evolution (i.e., before divergence of prototherian and therian mammals ~166 million years ago) [6]. ACP5 is an approximately 35-kDa, iron-containing glycoprotein characteristically produced in monohistiocytes, including osteoclasts, macrophages, and dandritic cells [7]. The protein can be either extracellularly produced in monohistiocytes, including osteoclasts, macrophages, and dendritic cells [7]. The protein can be either

[1] DOI 10.1095/biolreprod.111.097964
Published online before print 25 January 2012.


© 2012 by the Society for the Study of Reproduction, Inc.
eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Received: 23 November 2011.
First decision: 2 January 2012.
Accepted: 18 January 2012.
© 2012 by the Society for the Study of Reproduction, Inc.
eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363
monocytes and histiocytes, ACP5 is a major product of the uterine epithelium of the cyclic and pregnant pig, in which its synthesis is under positive regulation by progesterone [9]. The protein crosses the placenta and can be detected in allantoic fluid and fetal liver [10, 11]. It has been shown that iron can be released from ACP5 and subsequently associate with transferrin [12, 13], so it may function to deliver iron to the fetus. ACP5 can also function as a hematopoietic growth factor [14] and regulate phosphorylation status and biological activity of the phosphoprotein SPP1 [15, 16].

In addition, ACP5 has been localized in the endometrial glandular epithelium of pregnant mares, ewes, and water buffalo [17–19]. All of these species exhibit an epitheliocorial or syncytiomchorial type of placenta in which chorionic epithelium apposes, but does not invade, endometrial epithelium. Heretofore, it is not known whether ACP5 is expressed in the endometrium of species with other types of placenta. Here, we show that endometrial expression of ACP5 is a common feature of mammals of a wide phylogenetic lineage and that ACP5 has been subjected to purifying selection. Expression of ACP5 in the uterus predates the divergence of therians and prototherians and probably exerts a common function important for pregnancy in mammals utilizing a wide range of reproductive strategies.

MATERIALS AND METHODS

Database Queries to Identify ACP5 Genes

A megablast search using the nucleotide sequence for pig (Sus scrofa) ACP5 (NM214209) in the nucleotide collection (nctn) database of the National Center for Biotechnology Information (NCBI) website was performed to identify known mammalian ACP5 genes. Sequences were obtained from mouse (Mus musculus), rat (Rattus sp.), human (Homo sapiens), rhinoceros (Hippopotamus amphibius), and rhesus monkey (Macaca mulatta). mRNA (Aotus trivirgatus), human (Homo sapiens), and opossum (Monodelphis domestica). Subsequently, a genomic blast (blastn) search of mammalian genomic sequences of dolphin (Tursiops truncatus), ferret (Mustela putorius furo), bat (Myotis lucifugus), gray mouse lemur (Microcebus murinus), and small-eared galago (Otolemur garnetti) was performed with either the pig ACP5 nucleotide sequence or any of the full-length, experimentally derived ACP5 nucleotide sequences as queries. Sequences considered had query coverage greater than 90% and identity greater than 80%. Inferential sequences were submitted to NCBI GenBank as Third Party submissions under the accession numbers JN635352, JN635353, JN635865, and JN638567 for cow, dog, sheep, and horse, respectively.

Reverse Transcription-Polymerase Chain Reaction

Endometrial tissues were collected for RNA extraction after homogenization with TRI reagent (Sigma-Aldrich) following the manufacturer’s instructions. Pregnant endometrium was obtained from cow (Bos taurus; Day 100), mare (Equus caballus; Day 59), bitch (Canis lupus familiaris; Day 60), rhesus monkey (Macaca mulatta; Days 36 and 37), and rat (Day 10.5). Also, uteroplacental tissue from a pregnant mouse (Day 14) as well as intercaruncular endometrium from an ovariectomized ewe (Ovis aries) treated with 100 mg of progesterone for 30 days were also included in the present study. Quality and concentration of the total RNA collected were determined spectrophotometrically. All tissues were dissected free of myometrium and placental tissues, then stored at −80°C until RNA processing. Total RNA was extracted by using the RNeasy Midi Kit (Qiagen) following the manufacturer's instructions. Total RNA quality was assessed with the Bioanalyzer 2100 (Agilent Technologies). Aliquots from the total RNA samples were sequenced using the Illumina Genome Analyzer II platform at the W. M. Keck Facility, Yale University Medical School, to generate 75-bp reads following the protocol suggested by Illumina for sequencing of cDNA samples. Reads were mapped to the opossum genome using the Illumina pipeline, allowing two mismatches.

Sequence of Amplicons

The DNA amplicons produced by RT-PCR were sequenced in both directions at the University of Florida DNA Sequencing Core Laboratory using the ABI Prism 3130 genetic analyzer (Applied Biosciences).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections (thickness, 5 μm) of endometrium from rhesus monkey (late luteal phase; fixed in 2% [w/v] parafomaldehyde), marmoset (unknown stage; fixed in 2% [w/v] parafomaldehyde), opossum (Day 12.5 of gestation; fixed in 4% [w/v] parafomaldehyde), and cow (Day 17 of the estrous cycle and an ovarioctomized cow treated with progesterone for 30 days; fixed with Bouin’s fixative; see Leslie and Hansen [21]). After deparaffinization and dehydration, slides were boiled while immersed in 10 mM citrate (pH 6.0) for antigen retrieval. The endogenous peroxidase was blocked with 2% (v/v) hydrogen peroxide in DPBS (pH 7.4) for 10 min. Sections were blocked overnight with a combination of 10% (v/v) donor goat serum and 2.5 % (w/v) bovine serum albumin (Sigma-Aldrich) in DPBS at 4°C. All further steps were performed in a humidified chamber at room temperature. Slides were washed three times (2 min each) between steps with DPBS containing 2% [v/v] donor goat serum (DPBS-BS). Tissue sections were incubated for 2 h with either the primary antibody (rabbit anti-pig uferosin [18], 1:500 and 1:700 dilutions in DPBS-BS) or rabbit immunoglobulin (Ig) G as negative control (used at the same dilution as the primary antibody; Sigma-Aldrich). Sections were then incubated for 1 h with goat anti-rabbit IgG conjugated to hors eradish peroxidase (1:1000 dilution in DPBS-BS; Santa Cruz Biotechnology, Inc.). Staining was developed with aminoethylcarbazole chromogen (Bolton Bridge International, Inc.), and 0.1% (v/v) hydrogen peroxide in 0.05% (v/v) Tween 20 in DPBS containing 50 μg/mL 3-amino-9-ethylcarbazole (Sigma-Aldrich) was used as a counterstain. Coverslips were then mounted using clear-mount medium (Electron Microscopy Sciences).

Slides were examined for staining with a high-powered Nikon Optiphot microscope (Nikon Instruments, Inc.) or Olympus BX51 microscope (Olympus
TABLE 1. Endometrial tissues in which ACP5 expression was detected at the mRNA level (RT-PCR or RNA-Seq) or protein level (Western blot or immunohistochemistry).*

<table>
<thead>
<tr>
<th>Species</th>
<th>Placental type</th>
<th>Tissues positive for ACP5</th>
<th>Experimental evidence</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow (Bos taurus)</td>
<td>Eutherian, epitheliochorial</td>
<td>Pregnant endometrium (Day 100), endometrium of cyclic (Day 17), and P4-treated (30 days) uterine fluid (Day 270 of pregnancy)</td>
<td>RT-PCR (full-length sequence), IHC, Western blot</td>
<td>JN635352</td>
</tr>
<tr>
<td>Sheep (Ovis aries)</td>
<td>Eutherian, epitheliochorial</td>
<td>Endometrium (P4 treated for 60 days), uterine fluid (P4 treated for 60 days)</td>
<td>RT-PCR (full-length sequence), Western blot</td>
<td>JN638566</td>
</tr>
<tr>
<td>Horse (Equus caballus)</td>
<td>Eutherian, epitheliochorial</td>
<td>Pregnant endometrium (Day 59)</td>
<td>RT-PCR (full-length sequence)</td>
<td>JN638567</td>
</tr>
<tr>
<td>Goat (Capra hircus)</td>
<td>Eutherian, epitheliochorial</td>
<td>Uterine fluid (Day 100 of pregnancy)</td>
<td>Western blot</td>
<td>N/A</td>
</tr>
<tr>
<td>Dog (Canis lupus familiaris)</td>
<td>Eutherian, endothermochorial</td>
<td>Pregnant endometrium (Day 60)</td>
<td>RT-PCR (full-length sequence)</td>
<td>JN635353</td>
</tr>
<tr>
<td>Mouse (Mus musculus)</td>
<td>Eutherian, hemochorial</td>
<td>Uteroplacental unit (Day 14)</td>
<td>RT-PCR</td>
<td>NM001102405</td>
</tr>
<tr>
<td>Rat (Rattus sp.)</td>
<td>Eutherian, hemochorial</td>
<td>Pregnant endometrium (Day 10.5)</td>
<td>RT-PCR</td>
<td>NM019144</td>
</tr>
<tr>
<td>Rhesus monkey (Macaca mulata)</td>
<td>Eutherian, hemochorial</td>
<td>Pregnant endometrium (Day 36), late-luteal-phase endometrium</td>
<td>RT-PCR, IHC</td>
<td>XM001107020</td>
</tr>
<tr>
<td>Marmoset (Callithrix jacchus)</td>
<td>Eutherian, hemochorial</td>
<td>Nonpregnant endometrium (unknown stage)</td>
<td>IHC</td>
<td>XM002761766</td>
</tr>
<tr>
<td>Armadillo (Dasyus novemcinctus)</td>
<td>Eutherian, hemochorial</td>
<td>Pregnant endometrium (midstage)</td>
<td>RNA-Seq</td>
<td>N/A</td>
</tr>
<tr>
<td>Opossum (Monodelphis domestica)</td>
<td>Eutherian, hemochorial</td>
<td>Pregnant endometrium (midstage)</td>
<td>RNA-Seq, IHC</td>
<td>XM001363549</td>
</tr>
<tr>
<td>Platypus (Ornithorhynchus anatinus)</td>
<td>Protatherian</td>
<td>Pregnant endometrium (midstage)</td>
<td>RNA-Seq</td>
<td>NW001794300</td>
</tr>
</tbody>
</table>

* IHC, immunohistochemistry; P4, progesterone.

America, Inc.) and photomicrographs prepared with a Sony CD Mavica 400 digital camera or an Olympus DP70 camera.

**Western Blot Analysis**

Centricon Plus-20 devices (Millipore Corporation) were utilized to concentrate samples, and protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermo Scientific) with bovine serum albumin as standard. Aliquots of uterine flushings from a pregnant gilt (Day 15) and uterine fluids from an unilaterally pregnant cow (Day 270), unilaterally pregnant goat (Day 100), and ovarioctomized ewe treated with progesterone for 60 days were separated under reducing conditions using one-dimensional discontinuous SDS-PAGE, 4%-15% (w/v) gradient Mini-PROTEAN TGX precast gels (Bio-Rad). Aliquants of purified pig ACP5 [22] and uterine flushings from an ovariectomized ewe treated with corn oil (the vehicle for progesterone) for 60 days were included as positive and negative controls, respectively. Western blot analysis was performed as described elsewhere [20] with minor modifications. Briefly, the blocking and incubation buffer was precast gels (Bio-Rad). Aliquants of purified pig ACP5 [22] and uterine flushings from a pregnant gilt (Day 15) and uterine fluids from an unilaterally pregnant cow (Day 270), unilaterally pregnant goat (Day 100), and ovarioctomized ewe treated with progesterone for 60 days were separated under reducing conditions using one-dimensional discontinuous SDS-PAGE, 4%-15% (w/v) gradient Mini-PROTEAN TGX precast gels (Bio-Rad). Aliquants of purified pig ACP5 [22] and uterine flushings from an ovariectomized ewe treated with corn oil (the vehicle for progesterone) for 60 days were included as positive and negative controls, respectively. Western blot analysis was performed as described elsewhere [20] with minor modifications. Briefly, the blocking and incubation buffer was 0.1% (w/v) PBS supplemented with 0.1% (v/v) Tween-20 and 5% (w/v) nonfat dry milk. The polyclonal rabbit anti-pig uteroferrin antibody (1:50 000 dilution; DakoCytomation) was utilized as a primary antibody, and rabbit IgG (1:11 000 dilution) was used as a control antibody. A total of 636 positions were in the final dataset.

**Analysis of the Ratio of Nonsynonymous and Synonymous Substitutions**

Selecton (Server for the Identification of Site-Specific Positive and Purifying Selection, version 2.4) [30, 31] was employed to identify the ratio of nonsynonymous and synonymous substitutions (dN/dS; termed ω) at each codon site based on an empirical Bayesian method. The pressure of selection can induce either purifying or positive selection at specific areas of the genome where sites with ω values significantly higher or lower than one are an indication of positive (Darwinian) or purifying selection, respectively [32]. Twenty-one aligned coding sequences of the ACP5 gene were tested with the M8 model (extra category ωs ≥ 1, beta distribution and positive selection allowed). In case of positive selection, the significance of ω scores was tested with the likelihood ratio test [33], which compares the positive selection model (M8) and the null M8a model (extra category ωs set to 1), allowing only purifying and neutral selection, respectively.
RESULTS

Endometrial Expression of ACP5

A summary of the species examined and experimental approaches used to assess the endometrial expression of ACP5 is shown in Table 1.

During pregnancy, transcription of ACP5 was detected in all of the 10 species examined by either RT-PCR or RNA-Seq. A representative electrophoretogram of amplicons obtained by RT-PCR of RNA from a pregnant mouse (Day 14) and a pregnant rhesus monkey (Day 36) is shown in Figure 1.

Immunohistochemistry was used to detect ACP5 in endometrial tissue from three species, including one (marmoset) for which endometrial mRNA was not available (Fig. 2). In particular, ACP5 was localized to endometrial glandular and luminal epithelium of a late-luteal-phase rhesus monkey and in a marmoset (unknown stage of the estrous cycle) (Fig. 2, A and B). Immunoreactive ACP5 was also observed in the endometrial glandular epithelium of a cow at Day 17 of the estrous cycle (Fig. 2C), an ovariectomy and progesterone for 30 days (Fig. 2D), and a pregnant opossum (Day 12.5 of gestation) (Fig. 2E). Note that in the progesterone-treated cow, some scattered cells in the stroma, presumably macrophages, were also positive for ACP5. No positive reaction was detected in any of the negative-control samples (Fig. 2F).

Using Western blot analysis, it was demonstrated that ACP5 is present in uterine fluids of pregnant and progesterone-treated animals (Fig. 3), including one species (goat) in which endometrial RNA was not available. Immunoreactive ACP5 had a molecular weight of approximately 35 kDa in uterine flushings of a pregnant gilt (Day 15) and in uterine fluids of a unilaterally pregnant goat (Day 100), unilaterally pregnant cow (Day 270), and ovariectomized ewe treated with progesterone.

FIG. 1. Expression of the ACP5 gene in the pregnant endometrium as determined by RT-PCR. Representative electrophoretogram of amplicons obtained by RT-PCR of RNA from mouse (Day 14 of gestation) and rhesus monkey (Day 36 of gestation) is shown. Control reactions excluded reverse transcriptase (w/o RT). Arrows indicate size of the standard and expected size of the amplicons for the mouse and rhesus monkey, respectively.

FIG. 2. Immunolocalization of ACP5 in the endometrium. A–E Immunoreactive ACP5 in the endometrial glandular and luminal epithelia of a rhesus monkey (late luteal phase; A), marmoset (unknown stage; B), cow (Day 17 of the estrous cycle; C), ovariectomy and progesterone for 30 days (D), and opossum (Day 12.5 of gestation; E). In the progesterone treated cow (D), some scattered cells in the stroma (arrows) were also positive for ACP5 that presumably represent macrophages. F) An opossum sample used as experimental negative control for ACP5. Bar = 200 μm.
among species could be due to the size of some of the samples are probably proteolytic fragments of for 60 days. The lower-molecular-weight bands observed in (CO) for 60 days was subjected to Western blot analysis. Day 100 unilaterally pregnant goat. As a negative control, a sample of fluids. Purified ACP5 was included as a positive control. Samples utilized FIG. 3. Results of a Western blot experiment to detect ACP5 in uterine from an ovariectomized sheep treated with corn oil vehicle for 60 days. The finding that ACP5 is expressed in the pregnant placenta, and in the duck-billed platypus, an egg-laying mammal, indicates that uterine expression of ACP5 evolved early in evolution and before divergence of placental mammals from prototherians.

Alignment and Conservation of ACP5 Amino Acid Sequences

The alignment of amino acid sequences for ACP5 revealed a high conservation of the ACP5 proteins throughout speciation, with species-specific differences being most extensive in the N-terminal and C-terminal regions of the protein (Fig. 4). Sequence conservation of ACP5 was confirmed by using Selecton to test for evidence of purifying or positive selection (Fig. 5). The empirical Bayes method used in the program indicated that most of the sites in the ACP5 gene were likely undergoing purifying selection (68 amino acids). No sites undergoing positive selection were found.

Phylogenetic Analysis of ACP5

The phylogenetic tree inferred with the maximum likelihood method is presented in Figure 6. All of the 21 species examined were clustered in three separate clades. Two of the clades were formed by eutherian species, and the other clade consisted of metatherian and prototherian species. One of the two eutherian clades included ACP5 from the Laurasiatheria superorder of eutherian mammals that consists of dolphins, pigs, horses, bats, ruminants, and carnivores. The other eutherian clade included ACP5 from species within the Euarchontoglires (primates, rodents, and rabbits) and Afrotheria (elephants) superfamilies of eutherian mammals. Rabbits and elephants were each in a separate branch of their respective clade, suggesting some divergence of these ACP5 sequences from other species of the clade.

DISCUSSION

Evolutionary forces have caused extensive modification in mammalian reproduction. This process of evolutionary change has been characterized by transposon-driven rewiring of tissue and hormone-specific gene regulation [1], a high rate of gene duplication [5], and a high frequency of genes subject to adaptive selection [34]. Nonetheless, as shown here for ACP5, uterine expression of a large number of genes has been conserved during mammalian evolution. For ACP5, endometrial expression occurs in a large range of mammals, regardless of the type of placentation or the existence of viviparity. Endometrial expression of ACP5 predates the divergence of Prototheria and Theria approximately 166 million years ago [6]. Query of the genome of the chicken (Gallus gallus) failed to yield an orthologue of ACP5, and it is possible that formation of ACP5 occurred coincident with divergence of mammals. Indeed, development of the placenta may have been the evolutionary force causing uterine expression. In Monotremata, the yolk sac can function as a placenta to facilitate transfer of endometrial secretions to the egg [35, 36]. The finding that ACP5 is expressed in the uterus of the platypus strengthen the idea that mammalian matrotrophy arose before viviparity [37, 38]. Moreover, ACP5 has been subject to purifying selection, and the inferred amino acid sequence is very similar for all the species examined. Thus, at least one function of ACP5 is probably indispensable, either in the endometrium or in the monodendritic cell lineage, where the gene is also expressed [7]. In the pig, ACP5 has been implicated in the transport of iron from the endometrium to the fetus [12, 13] and ACP5 may serve this role in all mammals. Iron content of the fetus is well maintained even in the case of maternal iron deficiency [39]. Binding of transferrin to placental transferrin receptors is also an important route for placental transport of iron, however [40, 41]. Also, amounts of ACP5 in uterine tissue of the pig decline after Day 60 of gestation even though iron demand by the fetus increases after this time [42].

Two other functions for ACP5 are possible during gestation: as a hematopoietic growth factor [14] and as a regulator of the phosphorylation status of SPP1 [15, 16], which is expressed in several tissues, including the uterus [43], and is also called osteopontin. The extracellular form of SPP1 can regulate migration and adhesion of many cell types, including trophoblasts [15, 43], whereas the intracellular form can regulate TLR9 signaling [44].

As shown in the present and earlier studies [17, 18, 45], expression of ACP5 in the endometrium is largely limited to the glandular epithelium and, in some species, the luminal epithelium. Some ACP5-positive cells reminiscent of macrophages or dendritic cells were observed in the endometrial stroma of the cow. Expression of ACP5 in the endometrium is
hormonally regulated, mainly by progesterone and with modulation by estrogen, prolactin, and interferon-γ [18, 46, 47]. Given differences in gene regulation, promoter usage for ACP5 in the endometrium likely is different than that for expression in monodendritic cells. One example of tissue-specific promoter usage for a gene expressed in the uterus and another site is PRL [2].

One striking feature of the phylogenetic tree for ACP5 is that the opossum, a metatherian, is placed with the platypus, a monotreme. This evolutionary relationship contradicts mam- 

FIG. 4. Graphical representation of the multiple sequence alignment of inferred amino acid sequences for ACP5 from 21 species. The analysis was conducted with the Blosulm protein matrix of the ClustalW2 algorithm, and patterns of conservation are displayed as logos (stack of letters that represents one column of the alignment) that were obtained from the WebLogo 3 web application. The degree of sequence conservation is shown by the overall height of the letters (measured in bits), and the relative frequency of an amino acid at a given position is represented by the height of each letter. The letters of each stack are organized from the most to the least frequent amino acid, allowing the consensus sequence to be read from the top of the stacks. Sequences were obtained either from the NCBI database, inferred from genomic sequences, or derived experimentally. Species used for the alignment were human (Homo sapiens), white-cheeked gibbon (Nomascus leucogenys), rhesus monkey (Macaca mulatta), marmoset (Callithrix jacchus), mouse (Mus musculus), rat (Rattus sp.), gray mouse lemur (Microcercus murinus), small-eared galago (Otolemur garnetti), elephant (Loxodonta africana), pig (Sus scrofa), dolphin (Tursiops truncatus), cow (Bos taurus), sheep (Ovis aries), horse (Equus cabalus), panda bear (Ailuropoda melanoleuca), ferret (Mustela putorius furo), dog (Canis lupus familiaris), bat (Myotis lucifugus), rabbit (Oryctolagus cuniculus), opossum (Monodelphis domestica), and duck-billed platypus (Ornithorhynchus anatinus).
evolutionary rate differences among sites (eight categories \( G \), parameter = 0.4653). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

FIG. 6. Phylogenetic tree of mammalian ACP5. Phylogenetic analyses were conducted in the Molecular Evolutionary Genetics Analysis software, version 5, and the evolutionary history was inferred with the maximum likelihood method. The analyses were performed with 21 nucleotide sequences. A discrete gamma distribution was used to model evolutionary rate differences among sites (eight categories \( +G \), parameter = 0.4653). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

FIG. 5. Analysis of purifying and positive selection for amino acids of ACP5. Twenty-one inferred amino acid sequences were included in the analysis, and the amino acid sequence for pig ACP5 is shown. Color intensity indicates sites subject to purifying (purple) and positive selection (yellow).

ACKNOWLEDGMENT

We are very grateful to Central Packing (Center Hill, FL) for donation of bovine reproductive tracts and William Rembert for their collection. We also thank Dr. Savita Shanker and Xiao Hui Zhou from the DNA Sequencing Core, Interdisciplinary Center for Biotechnology Research at the University of Florida, for technical assistance. We are indebted to the following for providing tissues and fluids: Rafael Bisinotto, Eduardo de Souza Ribeiro, and Dr. Dan C. Sharp from the Department of Animal Sciences, University of Florida; Dr. Kirk P. Conrad from the Department of Physiology and Functional Genomics, University of Florida; and Gary R. Newton from Prairie View A&M University. Dr. Nancy Denslow and Dr. Melinda S. Prucha from the Department of Physiological Sciences, University of Florida, and Dr. Alan D. Ealy, Department of Animal Sciences, University of Florida, generously provided access to laboratory equipment.

REFERENCES


