Endometrial expression of calbindin (CaBP)-d28k but not CaBP-d9k in primates implies evolutionary changes and functional redundancy of calbindins at implantation

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Abstract

The endometrium is hostile to embryo implantation except during the ‘window of receptivity’. A change in endometrial gene expression is required for the development of receptivity. Calbindin-d9k (CaBP-d9k) and calbindin-d28k (CaBP-d28k) are proteins possessing EF-hand motifs which have high affinity for Ca²⁺ ions. Previously, it has been demonstrated that, in mouse endometrium, the expression of both calbindins is highly regulated during implantation and that both proteins play critical but functionally redundant roles at implantation. This study was the first to determine the expression of these two calbindins in the human and rhesus monkey endometrium. Initial RT-PCR analysis demonstrated that CaBP-d28k but not CaBP-d9k mRNA expression is detectable in the endometrium of both species. Western blot analysis confirmed the presence of immuno-reactive CaBP-d28k protein in the primate endometrium. Furthermore, the endometrial expression pattern of CaBP-d28k mRNA and protein was examined by Northern blot analysis and immunohistochemistry respectively in both species across the menstrual cycle and during early pregnancy. Semi-quantitative statistical analysis of the immunohistochemistry results revealed that, in the human, CaBP-d28k protein expression was maximal in luminal and glandular epithelium during the mid-secretory phase, coinciding with the time when the endometrium is receptive to embryo implantation. Expression in rhesus monkey showed a similar trend. These results suggest that, in the primate endometrium, only CaBP-d28k is expressed and that the specific regulation of this calbindin is potentially important for the establishment of uterine receptivity.
implantation and inter-implantation sites at the time of initial embryo attachment (Nie et al. 2000). Northern analysis demonstrated that the overall expression of uterine CaBP-d9k mRNA was increased during the early stages of pregnancy, but it was subsequently decreased at implantation sites. In situ hybridization localized the mRNA predominantly in the luminal epithelium and this luminal expression was downregulated specifically at the site of embryo contact (Nie et al. 2000). The expression pattern and cellular localization of CaBP-d28k protein in the mouse uterus was found to be very similar to that of CaBP-d9k, suggesting that these two proteins might exert similar functions in the mouse uterus (Luu 2004). Recently, by using wildtype and CaBP-d28k null mice, as well as morpholino antisense oligonucleotides, we have demonstrated that embryo implantation cannot occur in the mouse uterus during early pregnancy when both CaBP-d9k and CaBP-d28k proteins are absent, but is unaffected when either calbindin is present (Luu 2004). These results indicate that, in the mouse, uterine calbindins play critical roles at implantation, but that they have overlapping functions. Given the importance of the calbindins for pregnancy in the mouse, we hypothesized that one or both of these calbindins would be expressed in the primate endometrium, and highly regulated in association with embryo implantation. The aims of this study were, therefore, to establish whether CaBP-d9k and CaBP-d28k are expressed in the primate endometrium (Homo sapiens and Macaca mulatta) and, where appropriate, to determine the cellular and temporal mRNA and protein expression patterns during the menstrual cycle and early pregnancy.

**Materials and Methods**

**Tissue preparation**

Human endometrial tissues were collected by curettage from women undergoing laparoscopy for investigation of tubal patency or for tubal ligation. Human jejunum was also obtained for use as a positive control. Approval was given by the Human Ethics Committee at Monash Medical Centre, Melbourne and signed consent was obtained in every case. Tissues selected for RT-PCR and Northern analyses were snap frozen in liquid nitrogen, and those used for immunohistochemistry were fixed overnight in 10% buffered formalin at 4°C, and processed to paraffin wax blocks.

For immunohistochemical analyses, serial 5 µm sections were cut on a rotary microtome (Leica, Heidelberg, Germany), mounted on poly-L-lysine treated glass slides, and dried in an incubator overnight at 37°C.

**Multiple tissue expression (MTE) array**

A human MTE array was probed using radiolabeled cDNA probes for both calbindin-d9k (accession no: LI-3220, nt 30–384) and CaBP-d28k (accession no: NM-004929, nt 255–965). The probes were generated by random primer labeling of 25 ng of cDNA with 32P-dCTP (50 mCi/reaction). The MTE array was exposed to X-ray film at −80°C with an intensifying screen. Densitometric analysis was carried out using a Storm imaging system and Imagequant software (Amersham Biosciences).

**RT-PCR**

The RT-PCR protocol was carried out as previously described (Nie et al. 2000). The following primers were used to amplify the human CaBP-d9k and CaBP-d28k mRNA sequences: CaBP-d9k forward, 5'-TTT CAC TAT TGG GCA AAC-3'; CaBP-d9k reverse, 5'- CAG AGA CTT TGG GGG ATT-3'; CaBP-d28k forward, 5'-TCC TGC TCT TCC GAT GCC-3'; CaBP-d28k reverse, 5'-ATG TAT CCA TTG CCG TCC T-3'.

**Northern analysis**

Total RNA was extracted from tissues by the acid guanidinium thiocyanate–phenol–chloroform extraction method as detailed previously (Nie et al. 2000). The amount of RNA in the final preparation was determined spectrophotometrically, and the RNA quality was evaluated by the ratio of optical densities 260/280 nm. Each lane was then loaded with 10 µg of RNA.

Twenty-five nanograms of radiolabeled cDNA probes corresponding to a 711 bp fragment of human CaBP-d28k cDNA sequence (as used for screening the MTE array) was added to the hybridization buffer. Between hybridizations, blots were stripped by incubation at 80°C for 3 h in 1×M EDTA/0.1% SDS. To determine lane-to-lane loading variation, each blot was probed with a cDNA probe for glyceraldehyde-phosphate dehydrogenase (GAPDH). Densitometric analysis was carried out using a Storm imaging system and Imagequant software (Amersham Biosciences).

**Western analysis**

Frozen tissue samples were homogenized in lysis buffer (1% SDS, 10% glycerol, 0.01 M Tris) containing...
a proteinase inhibitor (0.1 mM phenylmethyl sulfonyl fluoride (PMSF)) in PBS (120 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4). The total protein content of each sample was determined by the Lowry method (Lowry 1951), and 50 μg was loaded into each lane. The samples were then resolved on 15% glycerine/acylamide gels (15% acrylamide, 0.05% bis-acrylamide, 40% glycerol, 20 mM Tris-base, 23 mM glycerin) at 400 V for 45 min at 4–10°C in running buffer.

The resolved proteins were transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences), and non-specific binding blocked overnight at 4°C with 10% skim milk powder in TBS (120 mM NaCl, 20 mM Tris, pH 7.4)/0.1% (v/v) Tween20 (TBS/Tween). The membrane was then rinsed twice for 2 min in TBS/Tween and incubated with a rabbit anti-rat CaBP-d28k antibody (1:2000–5000; SWant Bellinzona, Switzerland) at room temperature (RT) on a shaker for 2 h. After washing, biotinylated goat-anti-rabbit serum (1:500; DAKO, Carpentaria, CA, USA) was applied for 2 h at RT, followed by the ECL visualization kit (DAKO). The membrane was exposed to X-ray film for 5–10 min.

**Immunohistochemistry**

All immunohistochemical analyses of human and monkey tissue were performed using the rabbit anti-rat CaBP-d28k antibody. Negative controls were performed with equivalent concentrations of non-immune rabbit serum (Monash University Animal House). Non-specific binding was blocked with TBS/Tween/10% normal goat serum (NGS) for 2 h at RT. Each run included a section of primate kidney as a positive control. Sections were deparaffinized and rehydrated in ethanol and endogenous peroxidase blocked with 0.05% H₂O₂ for 30 min. The primary antibody (in TBS/Tween with 10% NGS) was applied (human tissue; 1:500; monkey tissue; 1:5000) for 2 h at RT. Each run included a section of primate kidney as a positive control. Sections were deparaffinized and rehydrated in ethanol and endogenous peroxidase blocked with 0.05% H₂O₂ for 30 min. The primary antibody (in TBS/Tween with 10% NGS) was applied (human tissue; 1:500; monkey tissue; 1:5000) for 2 h at RT. The sections were rinsed with TBS/Tween (twice for 3 min) and a 1:500 dilution of biotinylated goat anti-rabbit (DAKO) secondary antibody (in TBS/Tween with 10% NGS) was added for 30 min on a shaker. Sections were rinsed and the Vectastain ABC-Elite avidin–biotin detection system (Vector Laboratories, Burlingame, CA, USA) was applied for 30 min. Sections were then rinsed in distilled water and treated with DAB chromogen reagent (Zymed, San Francisco, CA, USA) for 1–5 min and counterstained with 10% Harris hematoxylin for 3 min before being rehydrated and mounted.

**Semi-quantitative analysis of immunohistochemical staining**

Scoring of 52 separate sections of human cycling endometrium covering the full range of a 28 day cycle was carried out by two researchers. The cycle was divided into: menstrual (day 1–4), proliferative (day 5–13), early secretory (day 14–19), mid secretory (day 20–25) and late secretory (day 26–31) phases. Seven distinct cellular compartments (luminal and glandular epithelium, stroma, leukocytes, decidualized stroma, vascular smooth muscle and endothelial cells) were scored and each compartment was given a nominal score of 0–3, where 0 = no staining and 3 = maximal staining. Data for stromal and glandular epithelial staining were subjected to statistical analysis using two way ANOVA, followed by a Tukey–Kramer post-hoc multiple regression test to determine differences within each cell type, between different phases of the cycle. Results were presented as mean±S.E.M. and were considered significantly different at *P* values < 0.05.

**Results**

**Expression of CaBP-d9k and CaBP-d28k in a range of human tissues**

To ascertain which human tissues express mRNA for CaBP-d9k and CaBP-d28k, we screened a human MTE array containing poly (A) + RNA from a range of human tissues and cell lines, along with several controls (Fig. 1A). The expression of CaBP-d9k was detected in very few tissues. The highest level was in the duodenum and jejunum, moderate levels were detected in the cerebellum and low expression in the kidney, heart atrium and ventricle. Expression was not detected in any other tissues (Fig. 1B). In contrast, strong expression of CaBP-d28k was seen in many tissues, particularly in kidney and almost all brain and nervous system components (Fig. 1C). The MTE array used for both probes did not specifically contain human endometrium and the uterus reference spot contained myometrium as a major component, which would explain the low level of probe hybridization.

**RT-PCR**

An expected fragment of 355 bp was amplified in human jejunum using RT-PCR; however, this fragment was conspicuously absent from human endometrial tissue. The fragment was identified to be CaBP-d9k mRNA using sequencing and database analysis using ANGIS (Australia National Genomic Information Service; The University of Sydney, Sydney, Australia) software. To further confirm the absence of CaBP-d9k mRNA, the 355 bp fragment was cloned into pGEM-T easy vector as previously described (Nie et al. 2000), for use as a probe for Southern blotting; 19 human endometrial samples from various stages of the menstrual cycle were analysed together with jejunum. A positive band was detected in jejunum but was absent from all endometrial samples (Fig. 2A). RT-PCR analysis revealed that, in the human endometrium, a 355 bp fragment corresponding to CaBP-d28k mRNA was present throughout the cycle (Figure 2B). This fragment was confirmed to be CaBP-d28k mRNA by sequencing and database analysis (ANGIS).
Figure 1 Representative human multiple tissue expression (MTE) array profiles for CaBP-d9k and CaBP-d28k. (A) The tissue layout of the array. (B) Profile for CaBP-d9k, using a 355bp cDNA probe (Accession: LI-3220, nt 30-384). (C) Profile for CaBP-d28k using a 711bp cDNA probe (Accession: NM-004929, nt 255-965).
Northern analysis of CaBP-d28k mRNA in human endometrium across the menstrual cycle

The expression of CaBP-d28k mRNA in human endometrium was confirmed by Northern blot analysis. A single transcript of 2.6kb was detected in all tissues examined (Fig. 3). To correct lane-to-lane loading variation, each blot was also probed with a cDNA probe for GAPDH, and the quantitation was normalized against the GAPDH signal on the same membrane. After autoradiography, the optical density of the hybridization signals was quantified using a Storm imaging system and Imagequant software (Amersham Biosciences). Expression of CaBP-d28k mRNA was lower during the proliferative phase (days 10 and 12) than during the secretory phase (days 18, 22 and 28).

Western blot for CaBP-d28k in human endometrium

To verify whether an antibody against rat CaBP-d28k could detect the protein in human tissue, proteins were extracted from a number of tissues. Mouse kidney, where CaBP-d28k is highly expressed, was used as a positive control and human cycling endometrial tissues (days 10, 24 and 26) were analysed. A specific band at 28 kDa was present in all samples (Fig. 4). In contrast to the mouse kidney, an additional band at ~58 kDa was also detected in human endometrium (Fig. 4); this may be due to dimerization of this protein, as has been described previously (Pochet et al. 1989), although the proteins were analysed under denaturing conditions.

Immunohistochemistry for CaBP-d28k in human and rhesus monkey endometrium during the cycle and early pregnancy

Immunohistochemistry was performed on 52 samples of human endometrium taken across the menstrual cycle. Representative photomicrographs are shown in Fig. 5A and 5B and the data are represented graphically in Fig. 6. Staining for CaBP-d28k was present in the glandular epithelium in all tissues, but was of less intensity during the proliferative phase (Fig. 5A) compared with the secretory phase \( (P < 0.01) \), and was maximal \( (P < 0.01) \) in the mid-secretory phase, (Fig. 5B). Luminal epithelium, where present, was also stained. In the stroma, staining was also present across the cycle but was of low intensity except in the mid-secretory phase, when it was significantly elevated compared with all other phases of the cycle \( (P < 0.01) \) (Fig. 5A and 5B, Fig. 5). In tissue from the first trimester of pregnancy, positive staining for CaBP-d28k

Figure 3 (A) Representative Northern blot analysis performed on total RNA extracted from human endometrium during the menstrual cycle on day (d) 3 (menstrual phase), d10 (proliferative phase), d12 (proliferative phase), d18 (early secretory phase), d22 (mid-secretory phase) and d28 (late secretory phase). A 711-bp cDNA fragment of human CaBP-d28k mRNA (Accession: NM-009788, nt 214–983) was used as a probe. The lower panel shows the signals detected by the GAPDH probe on the same membrane. (B) Densitometric analysis of the Northern blot showing relative abundance of 2.6kb CaBP-d28k transcript in the above tissues normalized against GAPDH expression. Data is expressed as relative density units.

Figure 4 Representative Western blot using anti-CaBP-d28k antibody on whole protein extracts of mouse kidney (K), day 10 human endometrium (d10), day 24 human endometrium (d24) and day 26 human endometrium (d26). The molecular weight was calculated by comparison with a molecular weight marker run simultaneously with the protein samples. Bands are seen at approximately 28 kDa and 58 kDa in human endometrium, but only at 28 kDa in mouse kidney.
was detected in syncytiotrophoblast and cytotrophoblast (Fig. 5C).

Immunohistochemistry was also performed on monkey uterus taken during the cycle and early pregnancy.

The resulting pattern of expression was, overall, similar to that seen in human endometrium. At 1 day before ovulation: very low staining was apparent in the luminal epithelium, the stroma or the endothelium (not shown).

Figure 5 Panels (A) and (B) show representative examples of CaBP-d28k immunostaining on human endometrium during the proliferative phase and mid-secretory phase, respectively. Staining is seen in glandular epithelium (ge) and stroma (s). Panel (C) shows CaBP-d28k immunostaining in human first trimester placenta. Chorionic villi (V) are immunopositive for CaBP-d28k. Panels (D) and (E) show CaBP-d28k immunoreactivity in rhesus monkey endometrium 5 and 10 days post-ovulation respectively; panel (D) shows overall light staining but strong staining in blood vessels (bv) adjacent to glandular epithelia (ge) while panel (E) shows overall increased immunoreactivity in ge, bv and luminal epithelia (fe) at 10 days post ovulation. Panel (F) is representative of CaBP-d28k immunostaining in rhesus monkey implantation sites; strong immunoreactivity is seen in ge, decidua (d) and syncytiotrophoblast and cytotrophoblast, but the trophoblast shell (t) is negative. Bar = 10 μm.

Figure 6 Histogram showing the mean staining intensity for immunoreactive CaBP-d28k in human cycling endometrial tissue. Tissues were grouped according to phase of cycle: menstrual (M), proliferative (P), early secretory (ES), mid-secretory (MS) and late secretory (LS). Mean staining intensity (± S.E.M.) is shown for epithelial ( ■ ) and stromal ( ■ ) compartments. Numbers of samples in each phase are shown in parentheses. *Represents significant difference (P ≤ 0.01) in epithelial compartment between proliferative and mid-secretory phases. ** Represents significant difference (P ≤ 0.01) in stromal compartment during mid-secretory phase in comparison with menstrual, proliferative, early secretory and late secretory phases.

At 5 days after ovulation, the equivalent of the human early-secretory phase, moderate staining for CaBP-d28k was observed in the luminal epithelium (not shown). Stromal cell staining was light, but strong specific staining was detected in the endothelial cells of some blood vessels, particularly in the basal zone (Fig. 5D). Most of the glandular epithelium showed a low level of cytoplasmic staining. Ten days post-ovulation (equivalent to the human mid-secretory phase), there was maximal staining in both luminal and glandular epithelial compartments (Fig. 5E). By contrast, at 15 days post-ovulation (late secretory), there was little or no staining in any compartment (not shown). During early pregnancy, in the rhesus monkey uterus, strong staining for the CaBP-d28k protein was seen in luminal and glandular epithelium but was absent from non-decidualized stroma and trophoblast shell. Strong cytoplasmic expression of CaBP-d28k protein was also detected in syncytiotrophoblast and cytotrophoblast where these were present (Fig. 5F), and in decidualized stroma.

Discussion

We have previously established that CaBP-d9k or CaBP-d28k are essential for successful embryo implantation in the mouse uterus where these two proteins have overlapping functions (Luu 2004). We therefore hypothesized that one or both of these proteins would be expressed in the primate endometrium and contribute to the complex events of implantation.

Our initial study using the RT-PCR approach did not detect any expression of CaBP-d9k mRNA in the human endometrium. This finding, however, was not entirely surprising: previous studies in the uterus of Papio ursinus (baboon) have also shown no expression of the CaBP-d9k mRNA. This lack of expression was attributed to mutations in the 13 nucleotide long estrogen response element (ERE) at the 5’ end of the baboon intron (Opperman et al. 1990). In humans, the CaBP-d9k gene is 80% homologous to baboon CaBP-d9k and also contains the mutated ERE which would render the gene inactive in the uterus (Jeung et al. 1992, 1994). This is hypothesized to explain the lack of CaBP-d9k mRNA in human endometrium.

Our RT-PCR results did however detect CaBP-d28k transcripts in the human endometrium and this was confirmed by Northern analysis. Immunohistochemistry localized CaBP-d28k protein to the human endometrial luminal epithelium and glands, but to a lesser extent in the stroma. Semi-quantitative analysis of the immunohistochemical staining showed that CaBP-d28k has a cyclic expression pattern during the menstrual cycle, with low expression during the proliferative phase and a peak during the mid-secretory phase. This high expression phase coincides with the human window of implantation. CaBP-d28k expression in the rhesus monkey (M. mulatta) revealed a similar expression pattern to that seen in humans.

This evidence suggests that, as in the mouse, uterine CaBP-d28k may be important for implantation and fertility in the primate uterus. This study is the first to propose a role for CaBP-d28k in implantation and early pregnancy in the primate. Previous studies on this protein in a human reproductive tissue focused on its role as a Ca2+ ion transporter in the placenta (Belkacemi et al. 2002). Our data confirms that CaBP-d28k is also expressed in both human and rhesus monkey placenta, supporting the previous hypothesis that it acts as a maternal/fetal calcium transporter (Belkacemi et al. 2002). However, the results presented in this study also suggest a role for CaBP-d28k much earlier in pregnancy than previously described.

The precise role of uterine CaBP-d28k at implantation is not known. A number of actions have been established for CaBPs in other tissues and possible homologous functions in uterine tissue can be hypothesized. In mammalian enterocyte cells, free Ca2+ ions are bound to cytosolic CaBP-d9k and transferred across the cell by facilitated diffusion. This transport of Ca2+ ions by CaBP-d9k helps maintain homeostasis by keeping intracellular Ca2+ ion concentrations below 10−7 M and hence preventing premature cell death via apoptosis. Once bound to CaBP-d9k, the Ca2+ is transferred to a Ca2+-ATPase and exported from the cell (Walters 1989). Further known roles for CaBP-d28k include activity as a Ca2+ sensor (Berggard et al. 2002) and buffer (McMahon et al. 1998). In addition, CaBP-d28k has been proposed to enhance Ca2+ transport in the placenta as syncytiotrophoblast cells expressing high levels of CaBP-d28k show higher Ca2+ uptake compared with cells with low CaBP-d28k expression (Belkacemi et al. 2002). All this evidence indicates that CaBP-d28k may act as a moderator of Ca2+ concentration during implantation. Indeed, it has already been demonstrated that Ca2+ ions are important factors for implantation. For example, the differentiation of trophoblast into an adhesion competent state can be accelerated by heparin binding EGF-like growth factor (HB-EGF), and this process is dependent on calcium influx from extracellular sources (Wang et al. 2000). Furthermore, integrin signalling on the blastocyst surface facilitates adhesion of blastocyst surface integrins to the extracellular matrix requires Ca2+ ions from an outside source (Wang et al. 2002). Yet, to date, this source of extracellular Ca2+ ions is not known.

It is particularly intriguing that only CaBP-d28k but not CaBP-d9k protein is expressed in the primate uterus; however, it is not unexpected, due to the wide variation of the regulation of CaBPs expression in the uterus across species. In rat uterus it has been previously found that CaBP-d9k expression is under strict estrogen regulation (Dupret et al. 1992, Krisinger et al. 1993, L’Horset et al. 1993, Krisinger et al. 1994). However, high expression of CaBP-d9k in porcine uterus during the luteal phase correlates to the regulation of the progesterone gene in that species, due to the lack of a functional ERE. Our previous studies in the mouse have indicated that CaBP-d9k...
and CaBP-d28k are functionally redundant in the uterus, and expression of one of them is sufficient to facilitate implantation (Luu 2004). It has been reported that, in enterocyte cells of amphibians and birds, the role of CaBP-d9k to transport Ca$^{2+}$ ions across the cell is performed by CaBP-d28k (Kawasaki et al. 1998). Therefore it is tempting to suggest that, during evolution, although the uterine expression of CaBP-d9k was lost due to a mutation in the ERE (Jeung et al. 1995), the presence of CaBP-d28k alone was adequate to maintain reproductive function in primates.

Acknowledgements

We are grateful to Professor Gabor Kovacs and Dr Euan Wallace for kindly supplying human tissue, Ms Y Li for technical assistance and Sue Pantridge for help compiling images. Guo-Qiang Fu was a visiting World Health Organization technical assistance and Sue Panckridge for help compiling images. We are grateful to Professor Gabor Kovacs and Dr Euan Wallace for kindly supplying human tissue, Ms Y Li for technical assistance and Sue Pantridge for help compiling images. Guo-Qiang Fu was a visiting World Health Organization technical assistance and Sue Panckridge for help compiling images.

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Received 8 March 2004
First decision 26 May 2004
Accepted 17 June 2004