

# Rab coupling protein (RCP): a novel target of progesterone action in primate endometrium

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## Abstract

Acquisition of functional receptivity by the endometrium is assumed to be effected by progesterone-dependent expression and repression of several genes during the implantation window in a menstrual cycle. In the present study, we employed differential display (DD) reverse transcription–polymerase chain reaction (RT–PCR) to identify progesterone-dependent gene/gene fragments that are differentially expressed during the peri-implantation phase in receptive and nonreceptive endometria, obtained from fertile and infertile bonnet monkeys respectively. Receptive endometria were obtained from regularly cycling ( $n=5$ ) fertile female bonnet monkeys. Endometrial nonreceptivity was induced by treating bonnet monkeys with either 2.5 mg ( $n=5$ ) or 5.0 mg ( $n=5$ ) onapristone (ZK 98-299), an antiprogestin, on every third day for one cycle. Ovulation, levels of circulatory hormones (estradiol and progesterone) and menstrual cycle length did not change in treated animals; however, endometrial growth was retarded. DD2, one of the differentially expressed cDNA fragments, showed higher representation in nonreceptive endometria than in receptive endometria. The DD2 sequence was found to be homologous to the sequence of the carboxyl terminal region of Rab coupling protein (RCP), a recently discovered protein involved in intracellular vesicular trafficking. To confirm the identity of DD2 as RCP, RT–PCR studies were carried out with a forward primer deduced from the RCP sequence and a reverse primer from the DD2 sequence. The product (DDRCP) obtained, when sequenced, revealed 95% homology with the nucleotide number 1196–1757 of human RCP cDNA. Furthermore, the pattern of DDRCP expression at transcript level was found to be similar to that shown by DD2; that is, it was higher in nonreceptive endometrium. Northern analysis using labeled DD2 or DDRCP cDNA fragments identified two transcripts of 6.0 and 4.0 kb in human endometrium. *In situ* hybridization studies using digoxigenin-labeled DD2 revealed significantly higher ( $P<0.05$ ) localization of endometrial RCP transcripts in the proliferative phase than in the peri-implantation phase in control animals. The localization was also significantly ( $P<0.01$ ) higher in peri-implantation-phase endometria from antiprogestin-treated animals than in control animals. These antiprogestin-treated animals, however, did not demonstrate any concomitant increase in the levels of immunoreactive endometrial Rab4 and Rab11 during the peri-implantation phase. A similar pattern of cycle-dependent RCP expression was observed in human endometrial biopsies. Furthermore, significantly higher ( $P<0.05$ ) levels of RCP transcripts were detected during the peri-implantation phase in women with unexplained infertility ( $n=3$ ) than in fertile women ( $n=3$ ). This is the first report indicating the endometrial expression of RCP and its hormonal regulation.

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## Introduction

The endometrium is a highly dynamic tissue with a remarkable ability to change its form and function in response to various stimuli such as hormones, an implanting conceptus, contraceptives, receptor modulators, neoplastic and metaplastic agents, and infections (Giudice 2003). Among these, ovarian hormones, that is, estradiol and progesterone, because of their physiologic relevance, have been studied extensively for their role in endometrial growth, differentiation and other specialized functions such as implantation and maintenance of pregnancy (Fazleabas *et al.* 1999, Spencer & Brazer 2002, Albrecht *et al.* 2003, Albrecht & Pepe 2003).

It has been unequivocally established that progesterone is indispensable for endometrial maturation in primates (Rosario *et al.* 2003). Our previous studies have demonstrated that the endometrium is more sensitive than the pituitary-hypothalamo-ovarian axis to any event that blocks either progesterone availability or action (Ishwad *et al.* 1993, Katkam *et al.* 1995). Furthermore, sufficient data exist to suggest that the progesterone-mediated endometrial maturation is an outcome of the interplay between various genes or gene products (Ace & Okulicz 1995, 1999, 2004, Horcajadas *et al.* 2004). When deprived of optimal progesterone action, the endometrium displays derangements in the expression of a number of molecules such as cell

adhesion molecules (integrins), cytokines (leukemia inhibitory factor), growth factors (transforming growth factor beta) and several secretory proteins (Sachdeva *et al.* 2001, Lessey 2002, Catalano *et al.* 2003).

From the large body of available data, it is apparent that several efforts have been made to understand the functions of single molecules and pathways in endometrium exposed to progesterone (Wang *et al.* 2003, Haendler *et al.* 2004, Kitaya *et al.* 2004). However, the approach of scanning the endometrial transcriptomes during the peri-implantation phase, in the presence and absence of progesterone action, promises to offer not only a holistic view of the role of progesterone in endometrial receptivity, but also the identification of novel molecules that have never been investigated for their role in endometrial receptivity.

We employed differential display (DD) reverse transcription–polymerase chain reaction (RT–PCR) to scan the global changes in the endometrial transcriptome after the neutralization of progesterone action, using an antiprogestin (ZK98:299) in bonnet monkeys. Our previous studies have demonstrated the ability of antiprogestins to impair endometrial function and induce infertility without disrupting the pituitary–hypothalamo–ovarian axis in bonnet monkeys (Katkam *et al.* 1995). Antiprogestins at selected dosages did not alter the circulatory levels of progesterone and estradiol and the length of menstrual cycle in these animals but rendered them infertile by retarding endometrial growth. We compared the endometrial RNA profile of these animals during the peri-implantation phase with that of fertile untreated animals. In this study, we present the data on one of the differentially expressed cDNA fragments that showed overrepresentation in nonreceptive endometria from antiprogestin-treated animals. The role of this differentially expressed fragment in endometrial receptivity and its hormonal regulation was hitherto unknown in primates. Ours is the first report to demonstrate the expression of this gene in primate endometrium. The present study also hints at the regulatory role of progesterone in the expression of this gene.

## Materials and methods

The use of animal and human samples for the present study was approved by the respective ethics committees of the institute.

### Animals

Twenty female bonnet monkeys, each weighing 3.5–4.5 kg and showing at least two consecutive ovulatory menstrual cycles of 28–30 days, were admitted to the study. Levels of circulating estradiol and

progesterone in these animals were determined by specific radioimmunoassays as described previously (Katkam *et al.* 1995). The inter- and intra-assay coefficients of variation were 9.2 and 6.8% respectively for estradiol and 11.1 and 6.7% respectively for progesterone. Onapristone-ZK 98:299 (a kind gift from Dr Walter Elger, Schering, Germany), an antiprogestin, at doses of 2.5 mg ( $n=5$ ) and 5.0 mg ( $n=5$ ) dissolved in vehicle (benzyl benzoate: castor oil, 9:1), was injected subcutaneously into the animals on day 1 of their menstrual cycle and continued every third day for one cycle until the day of biopsy. We have previously reported 100% efficacy of this treatment regimen in inhibiting implantation in bonnet monkeys (Katkam *et al.* 1995). In control animals ( $n=5$ ), which were treated with the vehicle alone, basal and peak levels of estradiol were 50–75 pg/ml and 300–600 pg/ml respectively. Treatment with onapristone did not alter the circulating hormonal profile. Peri-implantation-phase or secretory-phase endometrial biopsies were collected on day 8 after the midcycle estradiol peak (that is, day 20 of the cycle) from control and treated animals and also from two progesterone-insufficient animals (midcycle circulating progesterone levels under 2 ng/ml). Proliferative phase biopsies were obtained on day 8 of the menstrual cycle (estradiol levels over 200 pg/ml) from three control animals. A part of the endometrial tissue was fixed immediately in 10% neutral buffered formalin and embedded in paraffin for *in situ* hybridization analysis and immunohistochemical analysis, while the remaining tissue was frozen at  $-70^{\circ}\text{C}$  until use.

### Humans

Women attending the infertility clinics at the institute and INKUS IVF centre were admitted to the study after obtaining their informed, written consent. The control group included healthy, regularly cycling female partners ( $n=10$ ) of the infertile couples. Routine semenology tests by the andrologist had revealed abnormal semen profiles in their male partners. They either had low sperm count ( $<20$  million/ml) or very low sperm motility ( $<50\%$  sperm with forward motility) or abnormal sperm morphology ( $<30\%$  with normal forms). The test group included regularly cycling women with unexplained or secondary infertility. Male partners of these women did not show any abnormality in sperm count, motility or morphology.

Endometrial aspirates were collected from the control group during either proliferative, midluteal or late luteal phases of the cycles and from the infertile group during the midluteal phase. Estradiol and progesterone levels on the day of biopsy in fertile and infertile women did not show any significant deviation from the expected levels. Estradiol levels during the proliferative phase were  $180 \pm 60$  pg/ml; during the midluteal phase,

**Table 1** Sequence of oligonucleotide/primers/probes used for DD-RT-PCR, RT-PCR and *in situ* hybridization studies

Name of oligo/primer/probe	Sequence (5'-3')
V1	CCTCCTGCGAAGCTTTTTTTTTTTTTTTG
V2	CCTCCTGCGAAGCTTTTTTTTTTTTTTTC
V3	CCTCCTGCGAAGCTTTTTTTTTTTTTTTA
V16	TGCGAAGCTTTTTTTTTTTTTTTGT
V12	GACCTCTAGATGCTGTTGCC
V10	GACCTCTAGATGCTGTTGTC
GR1	TACAACGAGC
DD2.1	ACAACGAGGCCATGATGAA
DD2.2	CAACCTGAGTCGGGATGC
RCP1	GAAAATTGGAGGTCTCGGTTCA
RCP2	GCTTCCACCAAGGACTCCTTGA
ACTIN1	GTTGCTATCCAGGCTGTGCT
ACTIN2	GGCCATCTCTTTGCTCGAAGT
ER ANTISENSE	GTTCCAGGGGCTCCAGCTCGTTCCCTTGATC
	TGATGCAGTAGGGC
PR ANTISENSE	TAAGGCGTGATTGAGAGGCAGGATATAGGCACG
	TGGATGAAATCCATCACGGTGGT
18S1	CGATGCTCTTAGCTGAGTGT
18S2	GGAACCTACGACGGTATCTGA

200 ± 30 pg/ml; and during the late luteal phase, 120 ± 40 pg/ml. Progesterone levels during the proliferative phase were 0.8 ± 0.13 ng/ml; during the midluteal phase, 11.5 ± 1.0 ng/ml; and during the late luteal phase, 5.8 ± 1.0 ng/ml.

### RNA extraction

Total RNA samples were extracted from endometrial biopsies/aspirates with the RNeasy Minikit (Qiagen) as suggested by the manufacturer. RNA samples were incubated with RNase free DNase I (Roche) at 37 °C for 30 min and repurified through RNeasy Minikit columns. All RNA samples were first normalized for β actin according to the protocol detailed previously (Sachdeva *et al.* 2001). Wherever required, samples were also normalized for 18S rRNA transcript levels.

### Differential display RT-PCR (DD-RT-PCR)

DD-RT-PCR was carried out by the protocol described by Liang and Pardee (1992) with some modifications. All primers were custom synthesized by Gibco BRL, (Life Technologies, Gaithersburg, MD, USA). All reactions were carried out thrice for all samples. Briefly, 1 µg RNA sample was reverse-transcribed with 50 U of AMV reverse transcriptase (Roche) in the presence of 50 ng V1, V2 or V3 anchored primers (Table 1) and 0.2 µM dNTPs (Roche) at 37 °C for 1 h. One-tenth of this reaction was then amplified in a PCR containing 50 µM dNTPs, 10 µCi of <sup>32</sup>P-labeled dATP (specific activity 3000 Ci/mM; BRIT, Hyderabad, India), 0.2 µM each of the two primers – a modified anchored T oligo-

nucleotide primer and one of the arbitrary primers (Table 1) – and 1 U of Taq polymerase. The cycling parameters for PCR were as follows: an initial denaturation of 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 42 °C for 2 min, and 72 °C for 30 s followed by a final extension of 72 °C for 7 min. After PCR amplification in a MJ Research (Ramsey, Minnesota, USA) thermal cycler, the samples were analyzed on a 6% nondenaturing polyacrylamide sequencing gel, dried without fixation, and exposed on radiographic film (Amersham) for 72 h. After analysis of the autoradiogram, Whatman pieces with the bands exhibiting differential expression were excised from the gel. DNA was eluted by boiling the dried Whatman pieces in 100 µl water for 10 min. DNA was ethanol precipitated, dried and resuspended in 10 µl sterile water. The excised fragment was then reamplified by PCR using the corresponding pair of primers under the same conditions as described above, except that the radioisotope was not used. The PCR products were eluted from 2.5% low-melt agarose gels. These products were subcloned into pGEMT vectors (Promega). The positive clones were subjected to nucleotide sequence analysis commercially.

### *In silico* analysis

All cDNA homology searches were carried out with BLAST software at the NCBI database ([www.ncbi.nih.gov/BLAST/](http://www.ncbi.nih.gov/BLAST/)). Nucleotide sequences were conceptually translated into amino-acid sequences with the translation tool at [www.expasy.org/tools/dna.html](http://www.expasy.org/tools/dna.html). Candidate progesterone-responsive element (PRE) and

estrogen-responsive element (ERE) sites were searched with the Transcription Element Search System (TESS) tool at [www.cbil.upenn.edu/tess](http://www.cbil.upenn.edu/tess).

### Primer design

Sequences of the DD2-specific primers (DD2:1 and DD2:2) and RCP-specific primers (RCP 1 and RCP 2) were deduced from the sequences of DD2 cDNA fragment and RCP respectively, using the Primer 3 tool ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). Human RCP sequence was available in the gene bank (Accession no. AF368294).

### Digoxigenin labeling of cDNA fragment

An amount of 100 ng gel-eluted DD2 cDNA fragment was labeled in a total volume of 20 µl containing 2 µl random hexamers, 2 µl labeling mix and 1 µl Klenow enzyme (digoxigenin DNA labeling kit; Roche) at 37 °C overnight. Concentration of the labeled DNA and the labeling efficiency was checked as instructed by the manufacturer (Roche).

### Reverse transcription–polymerase chain reaction (RT–PCR)

The relative levels of DD2, RCP or  $\beta$  actin transcripts in human or monkey endometrial samples were measured by RT-PCR with gene specific primers. An amount of 200 ng DNA-free RNA was reverse transcribed and amplified with the Titan single-step RT-PCR kit according to the manufacturer's instructions (Roche). Briefly, the reaction mixture of 25 µl contained 0.4 µM each of forward and reverse primers, 200 µM dNTP mix and 1 U enzyme mix. The reverse transcription was carried out at 50 °C for 45 min. This was followed by 35 cycles of amplification, each cycle comprising denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min and extension at 72 °C for 2 min. The PCR product was run on 1–2% agarose gel, stained in ethidium bromide, visualized under a UV transilluminator and quantitated by the GelPro analyzer 3.1.00 (Media Cybernetics, Silver Spring, MD, USA). An aliquot of RT-PCR product obtained with RCP1 as the forward primer and DD2:2 as the reverse primer was used for hemi-nested PCR reactions. A volume of 1 µl RT-PCR product was amplified for 35 cycles, using either DD2:1/DD2:2 or RCP1/RCP2 as forward/reverse primers. For validation, DD2 and RCP products were run on 1% agarose gel, denatured, neutralized and Southern blotted onto nylon membrane (Roche). Southern hybridization with digoxigenin-labeled DD2 fragment, post-hybridization washings and detection were carried out by the protocol described elsewhere (Sachdeva *et al.* 2000).

### Northern hybridization

The size of the transcript encoding DD2/RCP was determined by hybridizing the RNA blot with digoxigenin-labeled DD2 cDNA at a concentration of 25 ng/ml in ExpressHyb (Clontech) solution for 1 h at 68 °C. The blot was washed sequentially, first in 2 × SSC, 0.1% SDS at room temperature three times for 10 min each, and then in 0.1 × SSC, 0.1% SDS at 58 °C thrice for 10 min each. To detect the hybrids, the blot was incubated in antidigoxigenin antibody conjugated to alkaline phosphatase (diluted 1:5000 in blocking solution) for 2 h. After extensive rinsing in 0.1 M maleic acid buffer (pH 7.5), the detection was carried out according to the manufacturer's instructions (Roche).

### Nonradioactive *in situ* hybridization

#### *In situ hybridization to detect DD2 transcripts by cDNA probes*

Sections of 5-µm-thick paraffin were dewaxed and hydrated through grades of alcohol. Prehybridization was carried out at 37 °C for 30 min with 50 µl prehybridization buffer containing 1 × Denhardt's solution (Sigma), 50% formamide (Gibco BRL), 500 µg/ml salmon sperm DNA (Roche) and 250 µg/ml yeast tRNA (Roche). The sections were hybridized at 42 °C overnight in 50 µl prehybridization buffer with the addition of heat-denatured digoxigenin-labeled cDNA probe at a concentration of 25 ng/µl. RNase-treated sections or those incubated without antibodies were used as negative controls. After overnight hybridization, the sections were stringently washed in 1 × SSC at 65 °C for 3 min and in 2 × SSC at room temperature for 5 min. After blocking in 2% normal sheep serum, the sections were incubated in 1:500 diluted alkaline phosphatase-conjugated antidigoxigenin antibody (Roche) at 4 °C overnight. Next day, the sections were washed briefly in 0.1 M Tris–NaCl buffer, pH 7.5, and equilibrated in 0.1 M Tris HCl buffer, pH 9.5, for 5 min. The color reaction was carried out for 20 min in the dark, using 5-bromo-4-chloro-3-indol-phosphate (BCIP) as the substrate and nitro blue tetrazolium chloride (NBT) as a chromogen, according to the manufacturer's instructions (Roche). The color reaction was stopped, and the sections were mounted in aquamount.

#### *In situ hybridization to detect estrogen receptor (ER) and progesterone receptor (PR) transcripts using oligo probes*

Antisense oligo probes for ER and PR were kindly donated by Karl Roth GmbH (Germany). The sequences of these probes are given in Table 1. Oligo probes were tail labeled with digoxigenin with the tail labeling kit (Roche) according to the manufacturer's instructions. The sections were hybridized with

digoxigenin-labeled oligos at a concentration of 1 pg/ $\mu$ l without any denaturation. To confirm the specificity of mRNA signals, various control experiments were conducted where sense probes were used as negative controls. Sections were sequentially washed in  $4 \times$  SSC,  $2 \times$  SSC and  $1 \times$  SSC for 10 min twice. Detection of the hybrids was carried out as described above. Hybridization experiments were performed thrice for each sample. All sections were viewed under bright-field with an Olympus B 60 microscope. The intensity of colored precipitates for all slides was evaluated by image analysis software (BioVis 1.42). Four areas from each section were randomly selected for each animal in all groups. The integrated optical density (IOD) in each area was calculated with the software. The IOD of the negative control was subtracted from the IOD of each experimental section for each animal in all groups. The mean, S.D. and S.E.M. were calculated for each group. Statistical analysis for the comparison between two groups was carried out with Student's *t*-test.

### Immunohistochemical analysis

Endometrial sections were immunostained with specific polyclonal antibodies against Rab4 and Rab11 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protocol used for immunostaining was similar to that described previously (Sachdeva *et al.* 2001). In brief, the sections were incubated in specific rabbit antibodies against Rab4 or Rab11 (diluted 1:10 in PBS) at 4 °C for overnight. In control sections, antibodies were omitted, and only PBS was added. This was followed by treatment with antirabbit biotinylated secondary antibodies (diluted 1:200 in 1.5% normal rabbit serum in PBS) for 2 h at room temperature. Detection of the immunoprecipitates was done with avidin-biotin complex (ABC) prepared in PBS as recommended by the manufacturer (Santa Cruz Biotechnology). Sections were then treated with the chromogen diaminobenzidine (DAB) for 10–15 min. The reactions were stopped in water, dehydrated and mounted in DPX medium. The intensities of colored immunolocalized products were evaluated by image analysis software (BioVis 1.42). Five areas from each section were scanned using frames spanning either glands or stroma, and the integrated optical density (IOD) in each area was calculated with the software. The IOD of the negative control was subtracted from the IOD of each experimental section for each animal in all groups. The mean, S.D. and S.E.M. were calculated for each group. Statistical analysis of the comparison between two groups for both antigens was carried out with Student's *t*-test.

### Endometrial explant cultures

Endometrial aspirates obtained from fertile women were washed with DMEM (Dulbecco's Modified Eagle's

Medium). Pieces of endometrium of  $2 \times 2$  mm were incubated in DMEM at 37 °C with 5% CO<sub>2</sub> under aseptic conditions. After 1 h, the medium was replaced with fresh DMEM containing 10% charcoal-stripped fetal calf serum and antibiotics. These cultures were supplemented with either estradiol ( $10^{-8}$  M) or progesterone ( $10^{-7}$  M) and grown for 14 h. Control cultures were incubated for the same period without hormone supplementation. After incubation, total RNA was extracted from all cultures.

### Real-time PCR

Real-time PCR analysis was carried out to quantitate the levels of DD2 and 18S rRNA transcripts in endometrial explants incubated in the presence and absence of steroids. Total RNA was extracted from these endometrial explants, and cDNAs were prepared by a first-strand cDNA synthesis kit (Invitrogen). Real-time PCRs were set up according to the iCycler manual (BioRad). A master mix of the following reaction components was prepared as follows (final concentration): 9.5  $\mu$ l water, forward primer (0.4  $\mu$ M), reverse primer (0.4  $\mu$ M), and 1 SYBR Green I (BioRad). A volume of 2  $\mu$ l cDNA was added as the PCR template. The following iCycler run protocol was used: denaturation program (95 °C for 2 min), amplification and quantification program repeated 40 times (95 °C for 30 s, annealing temp for 30 s, 72 °C for 30 s). The melting curve program (50–95 °C) was run with a heating rate of 1 °C per second, continuous fluorescence measurement and a cooling step to 4 °C. Analysis of melting curves allowed optimization of annealing temperatures for each amplification product. For each reaction, the crossing point (Ct) (defined as the cycle number at which noise band intersects the fluorescent curves) was determined with the iCycler software (BioRad). PCRs were all set in triplicate, and the mean value of the three Cts was calculated. A no-template control (H<sub>2</sub>O control) was analyzed for each master mix. Each assay was repeated thrice. In order to calculate amplification efficiencies (E) of each target cDNA, relative standard curves were generated with serial dilutions (1:10, 1:100, 1:1000 and 1:10 000) of a linearized pGEMT plasmid containing the insert (18S rRNA or DD2 fragment). Standard curves were generated by the iCycler software. They were based on the Ct and the log value of the standard cDNA dilution. For each sample, the level of expression of the DD2 (target) was compared with the expression of 18S rRNA (reference) gene. PCR efficiencies (E) were calculated for the standard curves for both target and reference. Expression ratios to compare the amplification of DD2 in control and treated conditions were calculated as  $R = E_{\text{target}}^{\Delta C_{\text{t target}}(\text{control} - \text{treated})} / E_{\text{reference}}^{\Delta C_{\text{t reference}}(\text{control} - \text{treated})}$ .

## Results

Treatment with the antiprogestin, onapristone, 2.5 or 5.0 mg on every third day for one cycle, did not alter the levels of estradiol and progesterone. These parameters were similar to those observed in the control group, thereby indicating no disruption in ovarian function after antiprogestin treatment. These results were consistent with our previous findings (Sachdeva *et al.* 2001).

### Differential display (DD) RT-PCR to identify differentially expressed transcripts in receptive and nonreceptive endometria

Comparison of the peri-implantation-phase endometrial cDNA profiles of control and treated animals by DD-RT-PCR analysis, using different sets of arbitrary primer (as a forward primer) and anchored oligo dT primer (as a reverse primer), revealed the following three expression patterns:

1. equivalent representation in receptive and non-receptive endometria
2. higher representation of certain cDNA fragments, that is, DD1 in receptive endometria from control animals
3. higher representation in nonreceptive endometria from treated animals, that is, DD2A and DD2B (Fig 1A).

Higher expression of DD2B in the peri-implantation-phase endometria from antiprogestin-treated animals than in control animals was also shown (Fig. 1B). A RT-PCR product of 219 bp was detected with DD2B specific primers. Densitometric analysis of DD2 and actin RT-PCR products by image-analysis software and estimation of the ratios between DD2 and actin in each sample showed higher representation of DD2 transcripts in endometrial RNA samples from treated animals than from control animals.

### Sequence analysis of DD1, DD2A and DD2B

The sequence of DD1 cDNA fragment (88 bp) showed partial homology with a region in the major histocompatibility complex (MHC) BAC clone from rhesus monkey (*Macaca mulatta*) (Accession no. AC148712).

DD2B (245 bp) was homologous to DD2A (124 bp) except for the presence of 121 additional bases at the 3' end (Fig. 2). The sequence of DD2B showed 93% homology with RCP mRNA (Accession no. AF 368294) at cDNA level. The 71-amino-acid open reading frame of the DD2B cDNA sequence shared 97% homology with the amino-acid sequence of the corresponding region of RCP. DD2B contained the Rab-binding domain (RBD)-a COOH terminal predicted alpha helical coiled-coil domain. This is a conserved motif observed in all members of Rab11-FIPs (Rab11 family

of interacting proteins) such as RCP, Rip 11, Rab11-FIP1, Rab11-FIP2, Rab11-FIP3 and Rab11-FIP4. The DD2B cDNA fragment was characterized in detail for the present study.

### Validation of the DD2B fragment

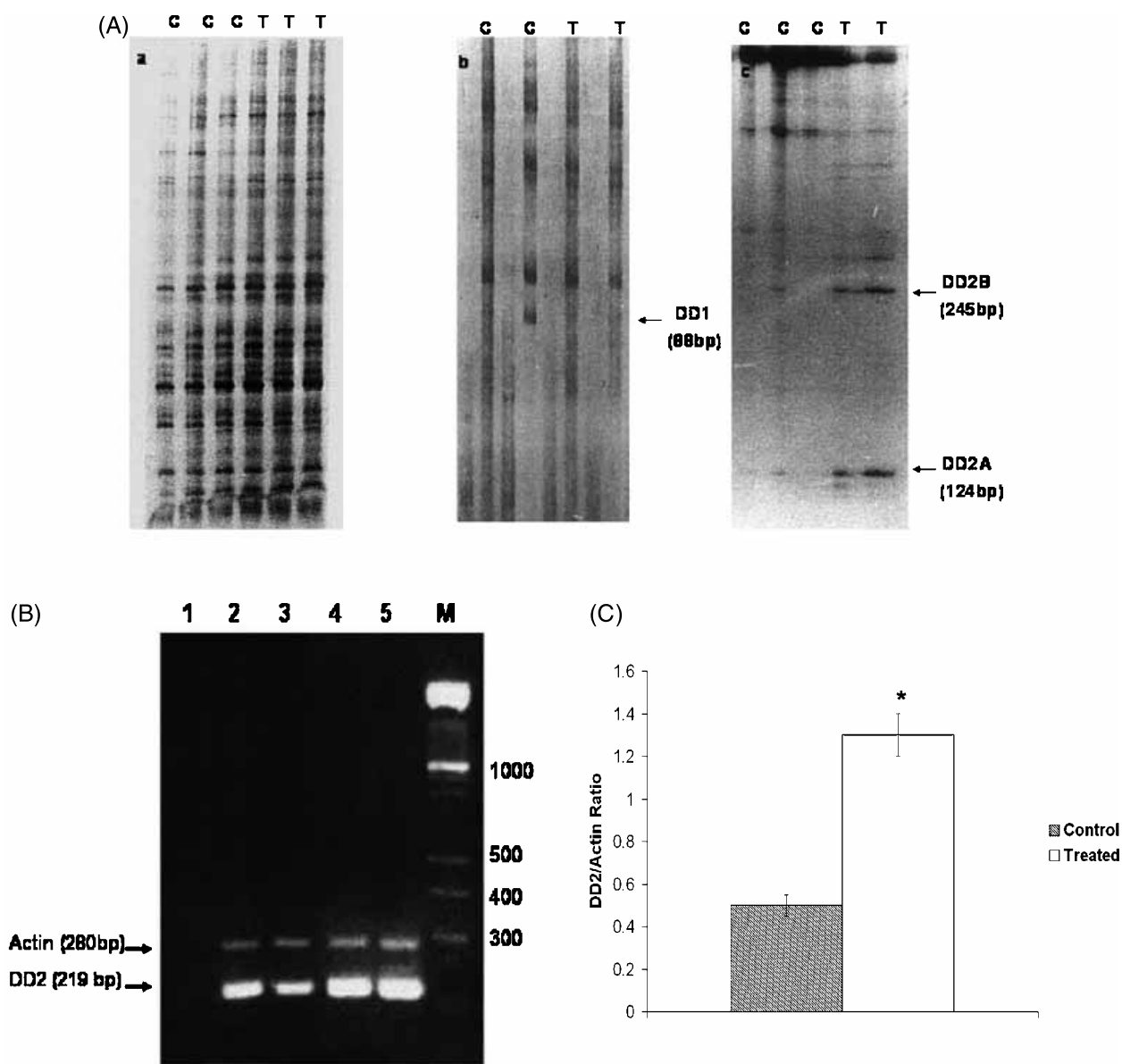
Labeled DD2B fragment was used to detect the presence of corresponding transcripts in RNA samples from human endometrium and peripheral blood leukocytes. Two transcripts of 6.0 and 4.0 kb were detected (Fig. 3A). Furthermore, a 219 bp RT-PCR product was detected with DD2B-specific primers in RNA samples from monkey testis, epididymis, placenta, ovary and endometrium (Fig. 3B). These results indicated that the gene corresponding to the DD2B fragment is expressed in a variety of tissues, and that it was neither a DD-RT-PCR-generated artifact because of the residual genomic DNA contamination in RNA samples, nor a result of the amplification of some intronic sequences at the 3' end.

### Characterization of the DD2B fragment

To confirm that the DD2B fragment represents the 3' region of the RCP gene, RT-PCR studies were carried out with a forward primer deduced from the 3' region of RCP and a reverse primer derived from the DD2B fragment. An expected product of 776 bp was obtained (Fig. 4). The specificity of the product was validated by two hemi-nested PCRs. One generated a product of 436 bp when reamplified with primers deduced from the established sequence of RCP (Fig. 4A, lane 2). The other reaction generated a product of 219 bp when the sequences deduced from DD2B were used for amplification (lane 1). Southern hybridization of these products with labeled DD2, as well as sequencing of 776 bp, proved that the DD2B fragment represented the RCP gene. The PCR product (436 bp) obtained with both primers specific to RCP did not hybridize with labeled DD2 (Fig. 4B). The region spanned by 436 bp, though a part of RCP, did not include any of the sequences encompassed by DD2. Sequence analysis of the 776 bp product revealed 95% homology with the human RCP cDNA sequence (Accession no. AF 368294). The partial conceptual amino-acid sequence of the DD-RCP product, obtained with monkey samples, revealed differences at 14 positions when compared with the human RCP (Fig. 5). The partial monkey RCP nucleotide and conceptual protein sequences have been submitted to the gene bank (Accession no. AY 435038).

### Hormonal regulation of RCP

RT-PCR studies indicated a fourfold upregulation in the DD-RCP expression in proliferative-phase endometrium



**Figure 1** (A) Differential display (DD) reverse transcription–polymerase chain reaction (DD–RT–PCR) to identify differentially expressed cDNA fragments in peri-implantation-phase endometria from control (C) and onapristone-treated animals (T).  $^{32}$ P-labeled DD–RT–PCR products were run in 6% nondenaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Panels a–c show only the sections of gels with representative results of experiments performed for all animals in each group. (a) Equal representation of all cDNA fragments in peri-implantation-phase endometria from C and T. Total RNA samples from C and T were reverse transcribed with anchored oligo dT primer (V3) and amplified with arbitrary primer (V10) and anchored oligo dT primer (V16). (b) Lower representation of DD1 cDNA fragment in peri-implantation-phase endometria from T. Total RNA samples from C and T were reverse transcribed with anchored oligo dT primer (V1) and amplified with arbitrary primer (V10) and anchored oligo dT primer (V16). (c) Higher representation of some cDNA fragments including DD2A and DD2B in peri-implantation-phase endometria from T. Total RNA samples from C and T were reverse transcribed with anchored oligo (V1) and amplified with arbitrary primer (GR1) and anchored oligo dT primer (V16). (B) Biplax RT–PCR analysis to compare the levels of DD2 transcripts in peri-implantation-phase endometrial RNA samples from control (lanes 2 and 3) and treated (lanes 4 and 5) animals. Agarose gel (2%) was loaded with RT–PCR products and visualized by ethidium bromide staining. The reaction mixture without the RNA template is loaded in lane 1. Molecular size marker (100 bp ladder) is shown in lane M. The expression of DD2 transcripts was higher in the peri-implantation-phase endometria from treated animals than control animals, as revealed by the densitometric analysis of the intensities of DD2 and actin RT–PCR products for each sample and estimation of the ratios between DD2 and actin RT–PCR products (lower panel). *Control* indicates DD2/actin transcript ratio in peri-implantation-phase endometria from control animals; *treated* indicates the ratio in 5.0 mg onapristone-treated animals.

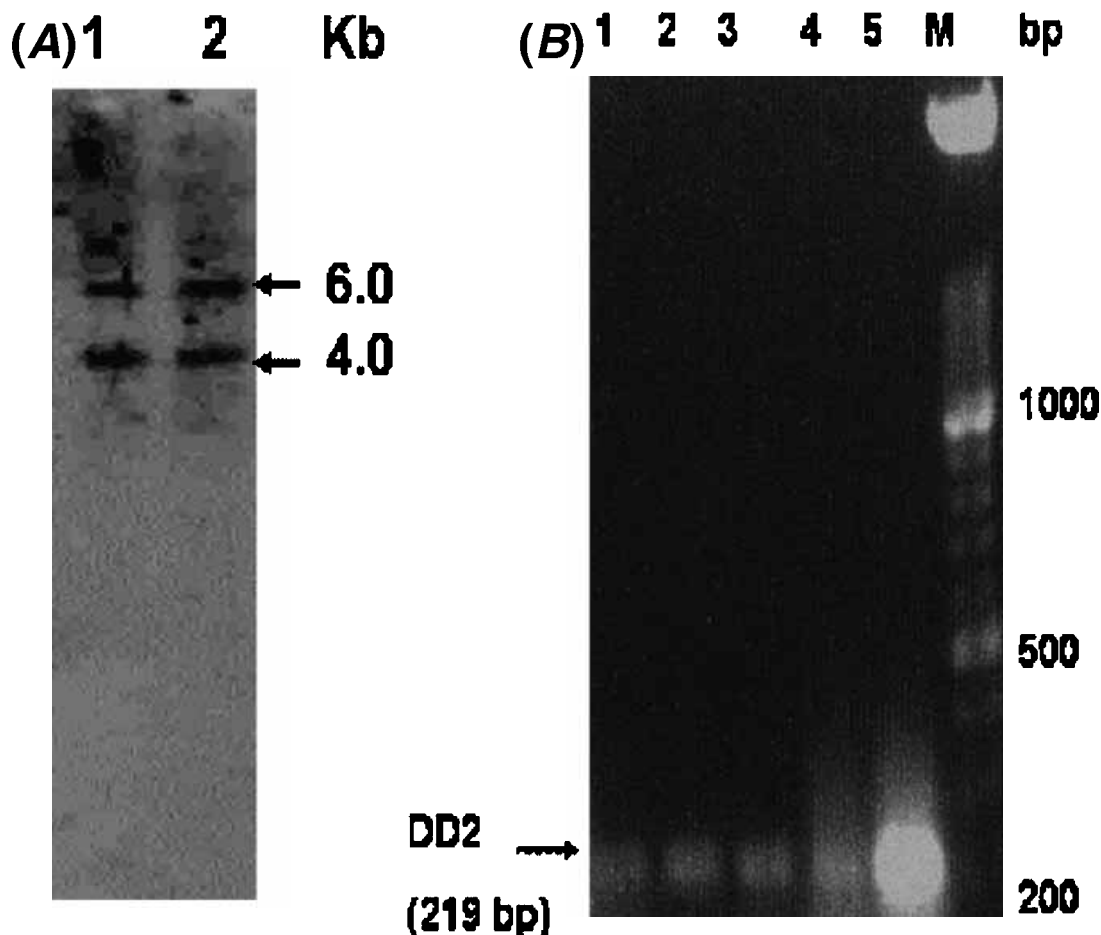
**DD1**  
 5'ATTGACCTCTAGATGCTGTGTCCCTGCCACTCCCTAGATAATTATAATAAATACTATG  
 GAAACACAAAAAAGCTTCGCAA3'

**DD2A**  
 5'ATTTACAACGAGGCCATGATGAAGAAATACAGCCCTCAGACCTGCATTTCGCGTA  
 CGCACAGCTGACCCATGATGAGCTCATCCAGCTGGTCCGCAAACAGAAGGAAACGAT  
 AAGCAAGAAGGAGTTCAGGTCCGAGAGCTGGAAGACTACATTGACAACCTGCTTGT  
 CTGAGTCATGGAAGAAACCCCAATATCCTCCGCATCCCGACTCAGGTTGACAAAAA  
 AAGCTTCGCAA3'

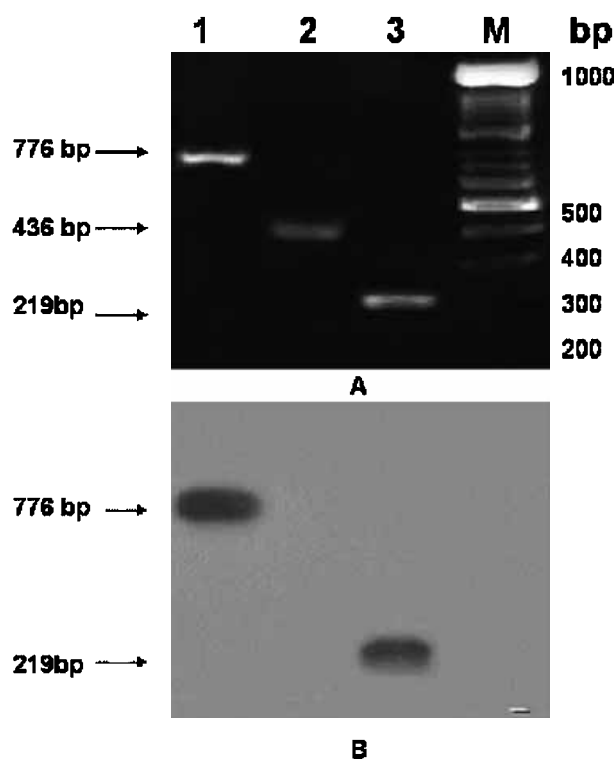
**DD2B**  
 5'ATTTACAACGAGGCCATGATGAAGAAATACAGCCCTCAGACCTGCATTTCGCGTA  
 CGCACAGCTGACCCATGATGAGCTCATCCAGCTGGTCCGCAAACAGAAGGAAACGAT  
 AAGCAAGAAGGAGTTCAGGTCCGAGAGCTGGAAGACTACATTGACAACCTGCTTGT  
 CTGAGTCATGGAAGAAACCCCAATATCCTCCGCATCCCGACTCAGGTTGACAAAAA  
 AAGCTTCGCAA3'

**Figure 2** cDNA sequences of DD1, DD2A and DD2B (DD2) fragments.

(Fig. 6A, lanes 1 and 2) as compared with peri-implantation-phase endometrium in control animals (lanes 3 and 4). The expression levels of DD-RCP transcripts were not significantly different between the peri-implantation-phase endometria from control and 2.5 mg onapristone-treated animals (lanes 5 and 6). However, a significant increase ( $P < 0.05$ ) was seen in this expression in the endometria from 5.0 mg-treated animals (lanes 7 and 8). These results suggest that the endometrial expression of the RCP gene is regulated in a cycle-dependent manner. It also indicates that RCP expression is repressed during the peri-implantation-phase, that is, in the presence of high progesterone levels during receptive conditions (Fig. 6B). *In situ* localization studies performed with a DD2B probe also corroborated these results. RCP transcripts could be localized in



**Figure 3** (A) Northern blot analysis with digoxigenin-labeled DD2B fragment to detect the corresponding full-length transcripts in total RNA samples from human endometrium (lane 1) and peripheral blood lymphocytes (lane 2). Two transcripts of 6.0 and 4.0 kb were detected by chemiluminescence after nonradioactive hybridization. (B) RT-PCR analysis to detect the expression of DD2 fragment in monkey tissues, that is, placenta (lane 1), epididymis (lane 2), testis (lane 3), ovary (lane 4) and endometrium (lane 5). Molecular size markers (100 bp ladder) were loaded in lane M. A RT-PCR product of 219 bp was obtained with primers DD2.1 and DD2.2 deduced from DD2 sequence.



**Figure 4** Validation of DD2 cDNA fragment to demonstrate its identity as Rab-coupling protein (RCP). (A) A forward primer from RCP (RCP1) and reverse primer from DD2 (DD2:2) in RTPCR reaction amplified a product of 776 bp in total RNA samples from monkey peri-implantation-phase endometrium (lane 1). An aliquot of 776 bp RT-PCR product was subjected to hemi-nested PCR, using either RCP1 and RCP2 as forward and reverse primers respectively (lane 2) or DD2:1 and DD2:2 as forward and reverse primers respectively (lane 3). PCR products of 436 bp (lane 2) and 219 bp (lane 3) were obtained. Lane M is loaded with the 100 bp molecular size marker. (B) Southern blot analysis of the above products probed with digoxigenin-labeled DD2 cDNA fragment.

nuclei as well as in cytoplasm of glandular epithelial and stromal cells of the endometrium (Fig. 7). The transcripts were localized in functionalis and basalis zones of the endometrium. These results suggest that RCP localization is not restricted to the specific zones of endometrium. Localization of endometrial RCP transcripts was higher in the proliferative phase than in the peri-implantation phase in control animals (Fig. 7B and C). Furthermore, this localization in peri-implantation-phase endometria was significantly ( $P < 0.05$ ) higher in treated animals than in control animals (Fig. 7C). This difference was more marked for the RCP localization in the glandular epithelium compartment. Similar results were obtained when DD-RCP was used as a probe to localize the transcripts (data not shown). To determine whether *in vivo* progesterone insufficiency results in similar changes to those observed in the antiprogestin-

treated animals, endometrial RCP expression during the peri-implantation phase was analyzed in luteal-phase-insufficient animals (circulating progesterone levels less than 2 ng/ml). Endometrial RCP transcripts levels were found to be twofold higher in luteal phase-insufficient animals than control animals (data not shown). This indicates that suboptimal progesterone levels or action modulates the transcription of endometrial RCP.

#### Endometrial Rab4 and Rab11 expression

Peri-implantation-phase endometrial sections from control and antiprogestin-treated animals were immunostained for the localization of Rab4 and Rab11. These were found to be localized more intensely in the perinuclear region in the glandular epithelium of peri-implantation-phase endometria from control animals. Interestingly, in treated animals, Rab4 localization was predominant in the cytoplasmic compartment of glandular epithelium (Fig. 8). However, Rab4 and Rab11 did not show any significant increase in the intensity of immunostaining in endometrial glandular epithelium or stromal compartments in treated animals as compared with control animals.

#### Endometrial RCP expression in humans

When compared within the control group, endometrial RCP expression was significantly higher ( $P < 0.01$ ) in late luteal and proliferative phases than in peri-implantation-phase human biopsies (Fig. 9A). Furthermore, endometrial RCP expression during the peri-implantation phase in infertile women ( $n = 3$ ) was significantly ( $P < 0.05$ ) higher than that in fertile women ( $n = 3$ ) (Fig. 9B). These findings indicate that the pattern of endometrial RCP expression is similar to that observed in monkey endometrium, that is, low during the peri-implantation phase and high during the proliferative phase of the cycle.

#### *In vitro* expression of RCP in human endometrial explants

Real-time PCR analysis did not show any significant change in RCP transcript levels after estradiol or progesterone treatment of short-term human endometrial explants. Mean Ct values for DD2 or RCP were  $23.11 \pm 0.05$ ,  $23.74 \pm 0.02$  and  $23.05 \pm 0.6$  for control, estradiol-treated and progesterone-treated explants respectively, whereas the respective mean Ct values for 18S rRNA were  $10.47 \pm 0.02$ ,  $11.06 \pm 0.06$  and  $11.57 \pm 0.10$  for control, estradiol-treated and progesterone-treated explants respectively. PCR efficiencies were 1.8 and 1.78 for the standard curves generated for 18S rRNA and DD2 respectively. The expression ratio was 0.98 when DD2 expression in control and estradiol-stimulated explants was compared, and it was

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Monkey      1  STKDSLKSM TLP YRPAPL I SG LRE MAPANSEA I KEAKESKKPESRRSSLLSLMTGKK
Human      385 STKDSLKSM TLP TYRPAPL I S GDLREKMAPANSEA I KEAKESKKPESRRSSLLSLMTGKK
          ***** ** *** *****

Monkey      61  DVAK SEGEN I LTV I GREKEGMLMGV KPGEDASGPAEDL VRRSEKDTAAVVS RQGS SLN I
Human      445 DVAKS EGEN I LTV I GREKEGMLMGV KPGEDASGPAEDL VRRSEKDTAAVVS RQGS SLN I
          **** *****

Monkey      121 FEDVQITEPEAE PESKSEP I PPISS I RAPQTRAVK PRLHPVKPMNAT I TKVAN I SLGTAT
Human      505 FEDVQITEPEAE PESKSEP I PPISS I RAPQTRAVK PRLHPVKPMNAT I TKVAN I SLGTAT
          ***** *****

Monkey      181 I ISEN LNNE I VMKKYSPSD PAFAYAQLTH DELIQLVLKQKETI SKKEFQVRELEDYIDNL
Human      565 I ISEN LNNE I VMKKYSPSD PAFAYAQLTH DELIQLVLKQKETI SKKEFQVRELEDYIDNL
          *****

Monkey      241 LVRVMEETPNILRIPTQV
Human      625 LVRVMEETPNILRIPTQV
          *****

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**Figure 5** Predicted amino-acid sequences of human and monkey RCP partial cDNA sequences. The amino-acid mismatches (highlighted) are outside the Rab-binding domain (the highlighted stretch of amino acid regions 231–249 in monkey RCP and 615–633 in human RCP).

1.006 for comparison of DD2 expression in control and progesterone-stimulated cultures. Some of these human explants that did not show any change in the levels of RCP transcripts after supplementation with estradiol and progesterone were also investigated for the immunolocalization of estradiol receptor and cyclooxygenase-2, proteins known to be regulated by estradiol and progesterone respectively. An increase was observed in the immunolocalization of these proteins (data not shown), thereby indicating the ability of the explants to respond to exogenous hormones.

#### Expression of endometrial steroid receptors

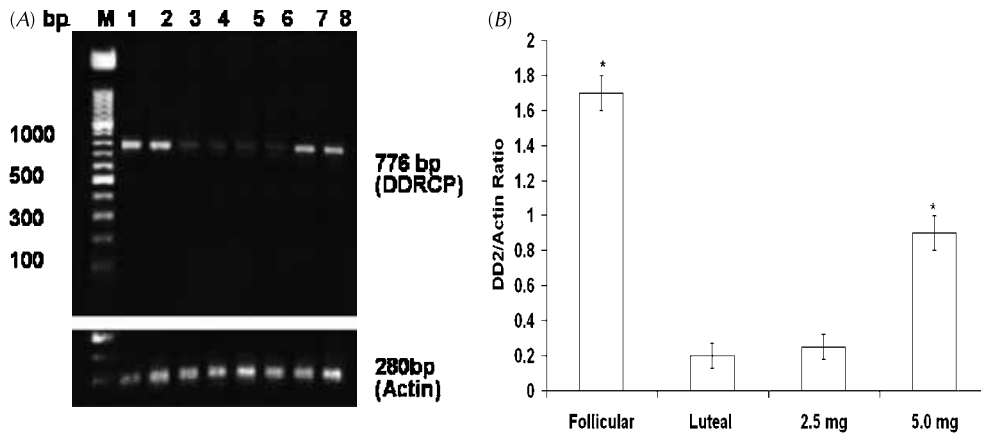
No significant changes were observed in the levels of transcripts for steroid receptors, that is, estrogen receptor and progesterone receptor, in the animals showing increased expression of RCP after antiprogesterin treatment. We observed a similar pattern at the levels of immunoreactive steroid receptor proteins also. These observations suggest that the upregulation in endometrial RCP is not associated with any perturbation in the levels of steroid receptor transcripts, and that steroid

receptors and RCP are not coregulated (Fig. 10). These studies also indirectly ruled out the direct effect of estrogenic influences after progesterone neutralization on endometrial RCP expression.

## Discussion

In the present study, we employed DD-RT-PCR to identify the genes that are either directly or indirectly regulated by progesterone and are of potential relevance to endometrial receptivity under *in vivo* conditions. We considered it logical to scan randomly the entire expression profiles of receptive and nonreceptive endometria, rather than predefining the range of targets, as done especially in low-density microarrays. Some gene fragments that have never been identified in endometrium or investigated for their potential role in endometrial function were characterized in detail for the present study.

DD-RT-PCR analysis revealed several cDNA fragments that did not show any change in their expression patterns in peri-implantation-phase endometria from

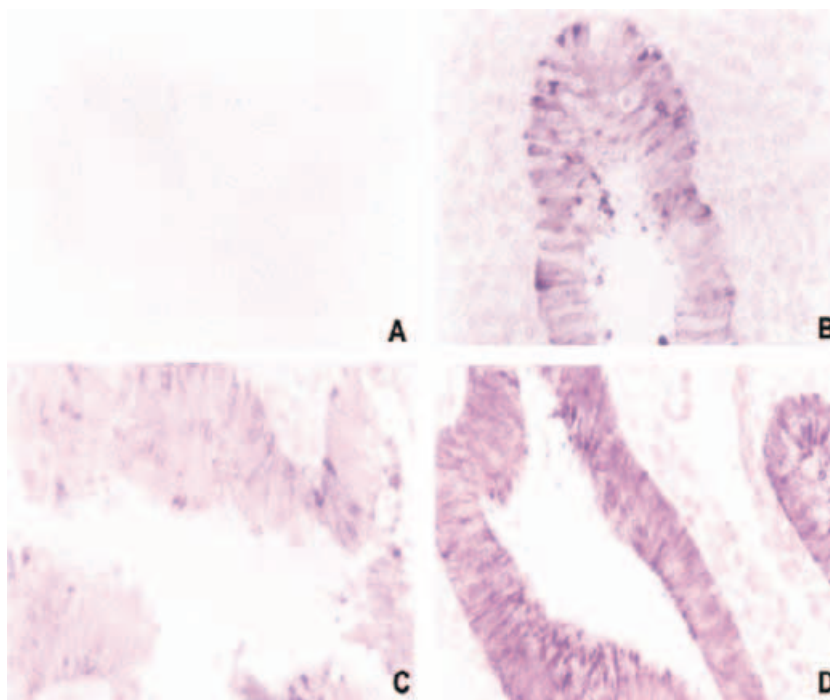


**Figure 6** (A) Hormonal regulation of endometrial RCP expression as assessed by RT-PCR analysis using gene-specific primers. Agarose gel (1%) was loaded with RT-PCR products obtained with endometrial RNA samples from control animals in proliferative phase (lanes 1 and 2) and peri-implantation phase (lanes 3 and 4), and from treated animals (2.5 mg: lanes 5 and 6; 5.0 mg: lanes 7 and 8) in peri-implantation phase. A significantly higher level of RCP expression was observed in proliferative-phase endometria from control animals and also in peri-implantation-phase endometria from 5.0 mg-treated animals. RNA samples were normalized for  $\beta$  actin expression (lower panel). (B) The endometrial expression of RCP during follicular phase, during Luteal phase in control, 2.5 mg treated- and 5.0 mg-treated (d) animals. The intensities of RT-PCR products for DDRCP and  $\beta$  actin, as visualized in agarose gel, were quantitated by Gel Pro software and expressed as IODs (integrated optical density). The ratios of IODs for DDRCP and  $\beta$  actin for each animal in each group were calculated. The mean IOD  $\pm$  S.E.M. for each group is shown. Endometrial RCP expression in each group was compared with that in the peri-implantation-phase endometrium of control animals. \*Indicates the level of statistical significance ( $P < 0.05$ ).

control and antiprogestin-treated animals. Our previous studies had also indicated the equivalent expression of  $\beta$  actin and epidermal growth factor receptor transcripts in receptive and nonreceptive endometria (Sachdeva *et al.* 2001). Apparently, these genes are not regulated by progesterone at the transcriptional level. It may also be inferred that at the selected dosages antiprogestins act on specific genes. Use of a different set of arbitrary primers and anchored oligo dT primers in DD-RT-PCRs, however, revealed lower representation of some endometrial cDNA fragments in treated animals than in control animals. Sequence analysis of one of these fragments (DD1) showed its similarity with a region in a MHC BAC clone from rhesus monkey (*M. mulatta*). MHC molecules, which are known to play a significant role in immune rejection responses, have been found to be expressed by endometrial epithelial cells also (Semino *et al.* 1995, Wallace *et al.* 2001). Interestingly, Kao *et al.* (2002) demonstrated lower levels of MHC transcripts in receptive endometrium than in prereceptive endometrium. However, we observed a higher representation of DD1 in receptive endometria than in nonreceptive endometria from bonnet monkeys. These discrepant findings could arise from differences in the experimental approaches, study design and study subjects employed in these two studies.

In contrast to DD1, DD2A and DD2B cDNA fragments were found to be highly represented in

nonreceptive endometrium. The homology of DD2B with RCP, a recently discovered protein implicated in intracellular vesicular trafficking (Lindsay *et al.* 2002), prompted us to investigate this gene fragment in detail. Ours is the first report to demonstrate the expression of RCP in primate endometrium. Intracellular vesicular trafficking mediates the transport of transmembrane proteins, secretory proteins and cell-surface proteins from one membrane compartment to another (Egea 2001). These events are known to be regulated by a number of proteins, including Rab GTPases or Rab proteins (Bock *et al.* 2001). The vesicle transport of each cellular organelle is mediated via a specific Rab protein, localized in that organelle. For example, Rab1, 2 and 6 have been localized at the endoplasmic reticulum and Golgi apparatus (Bock *et al.* 2001), whereas Rab4 and 5, located on early endosomes, control early steps of the endocytic process, and mediate endosome fusion and receptor recycling (Van der Sluijs *et al.* 1992, Stenmark *et al.* 1994). Rab11, localized to the endosomal recycling compartment and trans-Golgi network (TGN), has been shown to regulate the targeting of transport vesicle to the plasma membrane and also the trafficking between the endosomal compartment and the TGN (Urbe *et al.* 1993, Ullrich *et al.* 1996, Wilcke *et al.* 2000). Interestingly, Rab11 has been found to be induced by estrogen in rats. It has been proposed that Rab11 may regulate secretory activities that are critical for blastocyst



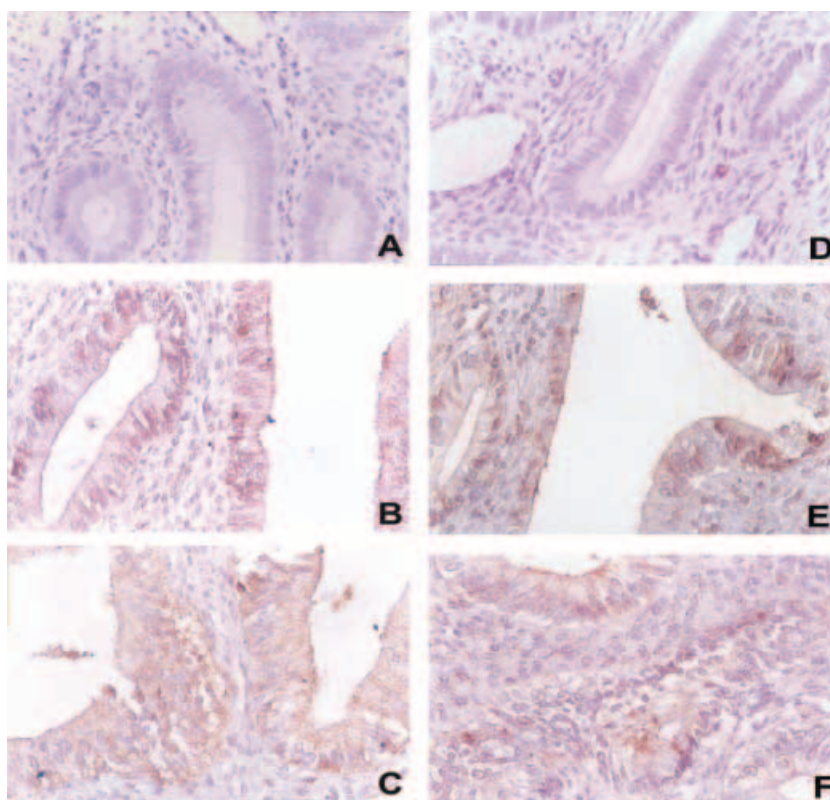
**Figure 7** *In situ* localization of RCP transcripts using labeled DD2 cDNA in proliferative-phase (B) and peri-implantation-phase (C) endometria from control animals and also in the peri-implantation-phase endometria from animals treated with 5.0 mg onapristone (D). (A) Negative control section. Note the intense localization of RCP transcripts in the glands of proliferative-phase endometrium and the drastic drop in expression in the peri-implantation-phase endometria in control animals. Onapristone treatment upregulated the expression of RCP mRNA predominantly in the endometrial glands.  $\times 200$ .

implantation (Chen *et al.* 1999). At present, Chen *et al.*'s is the only report to suggest hormonal regulation of intracellular trafficking in the uterus.

It is estimated that each Rab protein may have up to 20 different cellular effectors (Hales *et al.* 2001). RCP, a gene found to be upregulated in nonreceptive endometrium in the present study, is a recently discovered member of the Rab11 family of interacting proteins (Rab11-FIPs) (Lindsay *et al.* 2002). However, it exhibits low overall homology with the other members of Rab11-FIPs, that is, Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, Rip-11 and Rab11-FIP4. RCP, localized to the early endosomal recycling compartment, consists of two functional and well-conserved domains: a helical coiled-coil domain at its carboxyl terminus that encompasses the Rab-binding domain (RBD) and either a C2 or EF hand domain in the amino terminal region (Hales *et al.* 2001, Prekeris *et al.* 2001).

In the present study, the expression of endometrial RCP transcripts in human and bonnet monkeys was found to be cycle dependent; that is, high in the proliferative phase and low in the peri-implantation phase. In animals with luteal insufficiency and during

late luteal phase in control women, RCP expression was found to be higher. This suggests that progesterone either directly or indirectly suppresses RCP transcription. To our surprise, RCP was not identified as one of the differentially expressed genes in microarray analysis of the proliferative-phase and peri-implantation-phase human endometria (Kao *et al.* 2002). The inability to detect the differential expression of RCP in these studies may be attributed to either the potential nonexistence of RCP as one of the targets on commercial chips or the use of pooled human samples with variations in the levels of RCP transcripts. Northern blot analysis using digoxigenin-labeled DD2 or DD-RCP revealed two transcripts of approximately 6.0 and 4.0 kb in total RNA samples from human endometrium and peripheral blood lymphocytes. DD2 transcripts were also detected in monkey ovary, testis, epididymis, and placenta by RT-PCR. These results agreed with the previous observation, suggesting that the RCP is expressed in the majority of tissues, including brain, heart, testis, lung, spleen, ovary, small intestine and liver (Lindsay *et al.* 2002). Detection of two fragments, DD2A and DD2B, which shared identical sequences except for the presence



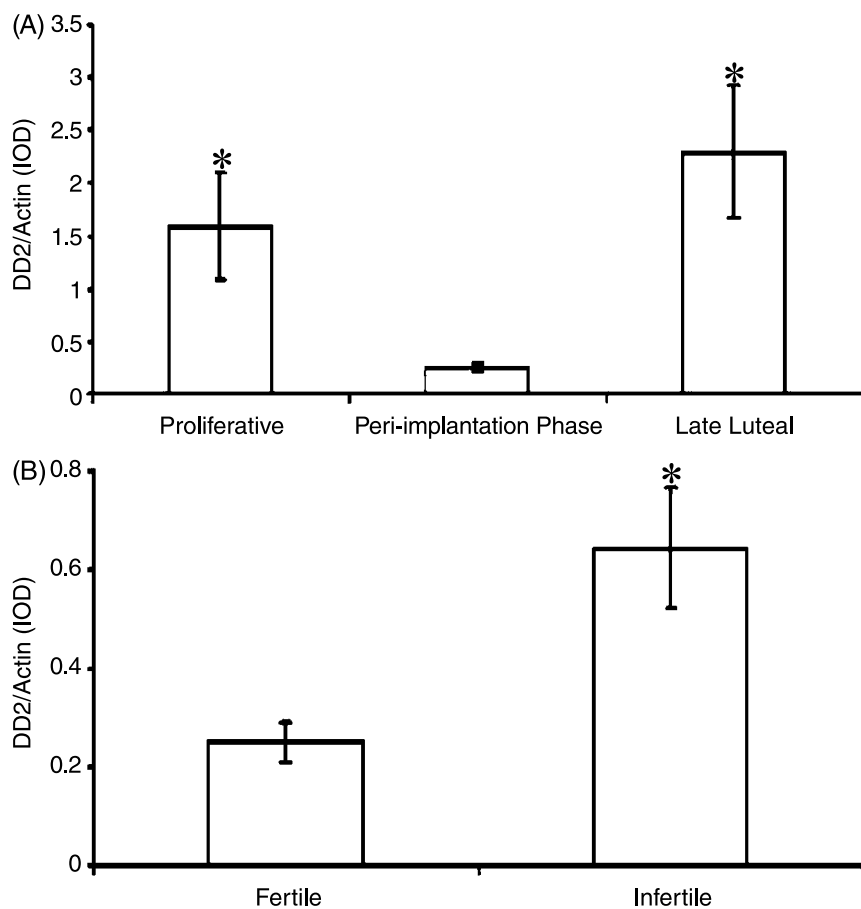
**Figure 8** Immunolocalization of Rab4 (A–C) and Rab11 (D–F) in the peri-implantation-phase biopsies from control (B and E) and 5.0 mg onapristone-treated (C and F) animals. Respective negative controls (without primary antibodies) are shown in A and D. Glandular epithelium showed more staining for both antigens than stroma.  $\times 200$ .

of an additional 120 bases at the 3' end in DD2B indicated the possibility of alternate splicing of the RCP gene. Northern analysis also detected two transcripts. However, Lindsey *et al.* (2002) detected a single band of 80 kDa for RCP protein in the cell lysates of placental trophoblastic choriocarcinoma, premyelocytic monocytes, epidermoid carcinoma and cervical carcinoma. At present, there are no published data on the molecular size of RCP transcripts or its splice variants. We detected similar-sized transcripts in RNA from peripheral blood lymphocytes also. It is likely that the RCP transcripts of 6.0 and 4.0 kb contain some untranslated sequences, which are eventually processed through post-transcriptional modifications to encode an 80 kDa protein.

The overexpression of a dominant negative mutant of RCP that contains RBD has been shown to inhibit the recycling of transferrin to the membrane of cells expressing the mutant. It has been proposed that excess of RCP may sequester endogenous Rab11 and/or Rab4 in an inactive complex (Lindsay *et al.* 2002). It is likely that the similar overexpression of RCP in nonreceptive

endometrium, as observed in the present study, might result in sequestering of Rab4 and Rab11, thus making them unavailable for some critical membrane trafficking events required for endometrial receptivity. Interestingly, there was no significant change in the levels of immunoreactive Rab4 or Rab11 in nonreceptive endometrium from antiprogestin-treated animals.

It is now becoming apparent that more genes are downregulated than upregulated in secretory endometrium (Kao *et al.* 2002, Ace & Okulicz 2004). Downregulation of RCP expression during the mid-secretory phase in control animals, as observed in the present study, corroborates this pattern. It may be speculated that this is due to a combination of both loss of estradiol action and direct or indirect downregulation of genes by progesterone in the secretory phase (Ace & Okulicz 2004). However, *in vitro* endometrial explants cultured for a short term in the presence of progesterone or estradiol did not show any significant change in the levels of RCP transcripts in the present study. Furthermore, the upregulation in endometrial RCP expression after antiprogestin treatment was found to be dose



**Figure 9** (A) Endometrial expression of DD2 during different phases of the menstrual cycle in fertile women. The intensities of RT-PCR products for DD2 and  $\beta$  actin, as visualized in agarose gel, were quantitated by Gel Pro software and expressed as IOD (integrated optical density). The ratios of IODs for DD2 and  $\beta$  actin for each case in each group were calculated. The mean IOD  $\pm$  S.E.M. for each group ( $n=3$  in each) is shown. The levels of endometrial DD2 transcripts were significantly less during the peri-implantation phase than other phases in fertile women. \*Indicates the level of statistical significance ( $P < 0.01$ ). (B) Endometrial expression of DD2 during peri-implantation phase in fertile and infertile women. The intensities of RT-PCR products for DD2 and  $\beta$  actin, as visualized in agarose gel, were quantitated by Gel Pro software and expressed as IOD. The ratios of IOD for DD2 and  $\beta$  actin for each case in each group ( $n=3$  in each) were calculated. The mean IOD  $\pm$  S.E.M. for each group is shown. The levels of endometrial DD2 were significantly higher in infertile than fertile women during the peri-implantation phase. \*Indicates the level of statistical significance ( $P < 0.05$ ).

dependent. This reinforces that progesterone may not act directly on RCP to inhibit its transcription. Although *in silico* analysis of available human RCP gene sequence did reveal the presence of PRE and ERE elements, their *in vivo* or *in vitro* potency to modulate the RCP expression in response to steroids is not known, especially in the context of cellular environment. It is more likely that the progesterone-dependent repression of RCP transcription requires involvement of some unidentified transrepressors that are probably either activated by progesterone or inactivated by estradiol *in vivo*.

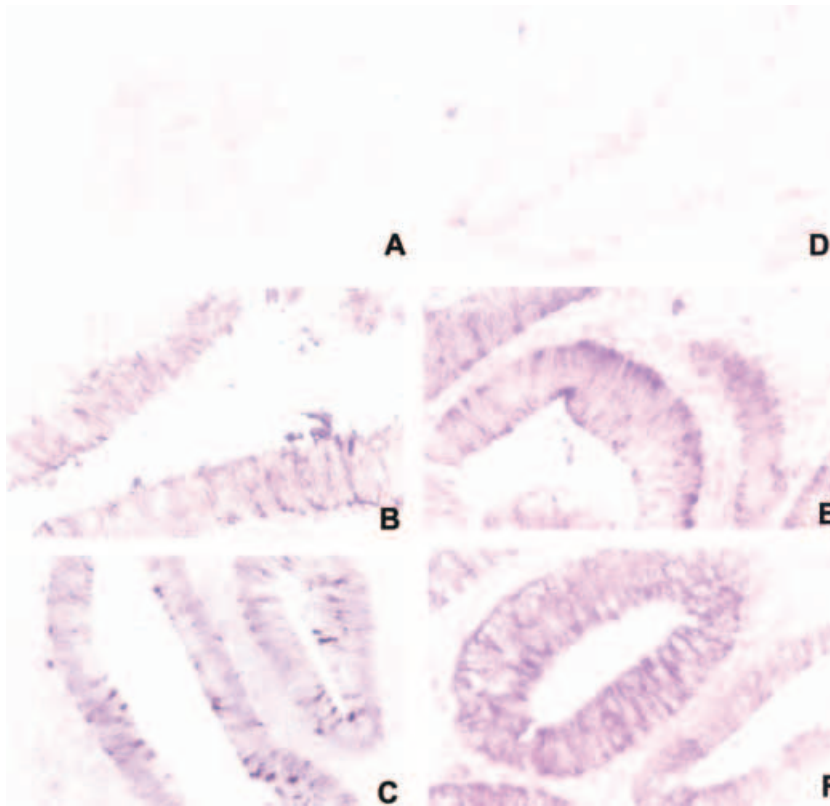
To determine whether the enhanced expression of endometrial RCP is associated with a change in progesterone receptor (PR) and estradiol receptor (ER) levels, we analyzed their transcript levels in receptive and nonreceptive endometria. There was no significant change in the levels of these receptors. This also suggested that endometrial steroid receptors and RCP are not coregulated and that the increase in RCP expression in nonreceptive endometrium may not be a direct outcome of potential estrogenic influences, triggered by progesterone neutralization. Since treated animals did not show any change in circulatory

estradiol levels or endometrial estradiol receptor levels, as compared with control animals, it seems more likely that it is progesterone that indirectly represses the RCP transcription *in vivo* during the receptivity period.

The present study demonstrates the endometrial expression of RCP, a novel protein implicated in intracellular vesicular trafficking and its differential expression in receptive and nonreceptive endometrium in primates. The study opens up exciting avenues for investigating the hormonal regulation of subcellular events in primate endometrium. Such investigations may provide interesting insights into the mechanism of endometrial receptivity in primates and may also lead to identification of endometrial factors contributing to infertility in humans.

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**Figure 10** *In situ* localization of estrogen receptor (A–C) and progesterone receptor (D–F) transcripts in the peri-implantation-phase endometria from control (B and E) and 5.0 mg onapristone-treated animals (C and F). Sections hybridized with the respective sense probes are shown in A and D. There was no significant change in the levels of transcripts for estrogen receptor and progesterone receptor in nonreceptive endometria from treated animals.  $\times 200$ .

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