The induction of baboon glycodelin expression by progesterone is not through Sp1

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Glycodelin is a major secretory product of the uterine glandular epithelial cells of the human and non-human primate during the late luteal phase of the menstrual cycle and early pregnancy. Since progesterone levels are elevated during these periods we sought to determine how progesterone modulates glycodelin gene expression. Co-transfection of various deletions of the baboon glycodelin promoter with the progesterone receptor (PR) into Ishikawa cells, a human endometrial cell line, revealed that full progesterone responsiveness is retained within the region –119/+48. In COS-1 cells, a kidney cell line, progesterone failed to elevate luciferase levels when various deletion constructs and the PR were co-transfected. Mutation of the Sp1 site in the –67/+48 region lowered basal expression but did not affect the ability of progesterone to increase expression of the luciferase reporter in Ishikawa cells. These findings suggest that Sp1 sites are not involved in the progesterone regulation of the baboon glycodelin gene. We propose that progesterone induces a factor that regulates glycodelin gene expression in the uterus since we failed to obtain a similar response in a non-uterine cell line.

Key words: baboon/gene regulation/glycodelin/Sp1/uterus

Introduction

Glycodelin, originally called placental protein 14 (PP14), is the major secretory product of the glandular epithelial cells of the human uterus during the late luteal phase of the menstrual cycle and early pregnancy (Bell et al., 1985). In the baboon, glycodelin synthesis begins in the uterine glandular epithelial cells during the mid-luteal phase and increases dramatically during early pregnancy (Hausermann et al., 1998). The increase in glycodelin secretion by the glandular epithelium of the uterus is coincident with progesterone secretion during the luteal phase of the menstrual cycle. Glycodelin’s role in reproduction may be to inhibit sperm binding to the zona pellucida (Oehninger et al., 1995) or serve as an immunosuppressive agent during early pregnancy (Bolton et al., 1987).

Transcriptional regulation of the human glycodelin gene promoter in Ishikawa and HeLa cells is regulated via the progesterone receptor (PR) (Taylor et al., 1998). In HEC1B cells, PR-stimulated glycodelin gene expression has been reported to be mediated through functional Sp1 sites (Gao et al., 2001). However, the transcriptional regulation of the baboon glycodelin gene has not been studied. In this study, we have demonstrated that although the baboon glycodelin gene promoter activity is enhanced by progesterone, it does not mediate its actions via Sp1 sites.

Materials and methods

Materials

pGL3-Basic, pGEM-T Easy, β-Galactosidase Enzyme Assay System and Luciferase Assay System were purchased from Promega (Madison, WI, USA). The human PRA plasmid was a gift from Dr Pierre Chambon (University of Strasbourg), and the Sp1 expression plasmid was a gift from Dr Stephen Bell, University of Leicester (Sambrook et al., 1989). The insert was subcloned into the NotI site of pBluescriptII SK–. A fragment of the 5’ flanking region corresponding to –2 kb to +48 bp of the human glycodelin gene was amplified using the sense primer 5’-gacttactaGCGTCCTCCCTGCTGCTGCTGAGGG-3’ and the antisense primer 5’-ACGATCTCAGCAGTGTGGTCTC-3’ (the letters in lower case indicating nucleotides added to facilitate cloning) designed to correspond to nucleotides –1903 to –1882 and the antisense primer 5’-agtacctagGCGCTGAGCTGCTGCTGCTGCTGCTGAGGG-3’ designed to correspond to nucleotides +48 to +29 of the human glycodelin gene (Vaisse et al., 1990). After purification on a 1.5% agarose gel the ampiclon was cloned into the pGEM-T Easy vector.
Plasmid constructions

The pGEM-T Easy construct containing the amplicon, ~2 kb, was digested with XhoI and MluI and the insert separated from the vector by electrophoresis on a 1.5% agarose gel. The band containing the amplicon was purified and ligated into the pGL3-Basic vector, which was also digested with XhoI and MluI, upstream of the firefly luciferase reporter gene creating ±2007/+48pGL3-Basic. The ±808/+48pGL3-Basic plasmid was derived from ±2007/+48pGL3-Basic by digestion with NdeI, purification by agarose gel electrophoresis and religation. The ±506/+48pGL3-Basic plasmid was generated by digesting the ±2007/+48pGL3-Basic plasmid with NheI followed again by purification and religation. The ±119/+48pGL3-Basic and ±67/+48pGL3-Basic plasmids were generated by PCR amplification using the same antisense primer as above and the primers gagctcttacgcgtaCAGTGGAGGAAGCTGC and gagctcttacgcgtaCA-CATGGCTGTGGGCAG respectively, followed by subcloning into the pGL3-Basic plasmid as described above. The mut-67/+48pGL3-Basic was generated from the ±67/+48pGL3-Basic plasmid using the QuikChange Site-Directed Mutagenesis Kit and the sense primer CACATGGCTGTGGGAAGAGGAGGTTTTGTCTGCCCTCCTC and antisense primer GAGGAGGGCAGACAAAA-GGCCCTTCCCACAGCCATGTG (the altered bases are underlined), which span bases -67 to -31. All constructs were verified by sequencing through the insert-vector junction by the DNA Sequencing Facility of the Research Resources Center of UIC.

Cell culture, transfection and luciferase assay

Ishikawa or COS-1 cells were cultured in DMEM supplemented with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere with 5% CO₂. On the day before transfection the cells were plated at a concentration of 1.1×10⁵ cells/cm² into 12-well plates in triplicate in phenol red free DMEM supplemented with 2% charcoal stripped fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were transfected with plasmids using the calcium phosphate precipitation method (Sambrook et al., 1989). In the total of 2.75 µg of plasmid/well were 0.25 µg of pCMV Sport-bgal and 2.5 µg of equal amounts of the luciferase reporter plasmid and PR (PRB in all experiments except for the experiment in Figure 3 where both PRA and PRB were used), Sp1 or pGL3-Basic plasmids. After incubation with the plasmids for 4 h, the cells were washed, glycerol shocked, and placed in media in the presence or absence of 1 µmol/l medroxyprogesterone acetate (MPA) for 20 h. The cells were then harvested in reporter lysis buffer, luciferase activity measured with the Luciferase Assay System and β-galactosidase with the β-Galactosidase Enzyme Assay System.

Western blot

Nuclear extracts (50 µg), prepared as described by Dignam et al., 1983 from Ishikawa and COS-1 cells were resolved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes (Towbin et al., 1979). The membranes were incubated with an Sp1 monoclonal antibody at a 1:100 dilution and the immunoreactive product visualized by using an enhanced chemiluminescence kit.

Statistical analysis

Data are expressed as means ± SD of triplicates and statistical analysis of the significance of the difference between the cells treated with and without MPA.
was carried out using Student’s t-test. Differences were considered statistically significant at \( P < 0.05 \).

## Results

To investigate the progesterone regulation of the baboon glycodelin gene, a DNA fragment containing the 2 kb region 5’ of the transcriptional start site and 48 bases downstream was generated from a larger genomic clone by PCR. Figure 1 shows the sequence of

### Figure 1

**Figure 1.** Comparison of the putative glucocorticoid response element (GRE), CAMP response element binding protein (CREB), CAAT box, TATA box and Sp1 sites of the human and baboon glycodelin genes. The sequences underlined in Figure 1 are shown aligned with the comparable sequence from the human glycodelin gene (Vaisse et al., 1990). Nucleotide differences in the baboon are shown in italic-bold type.

<table>
<thead>
<tr>
<th>Element</th>
<th>Human</th>
<th>Baboon</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRE</td>
<td>CCCACGCACCTGTTCT -1829</td>
<td>CCCACGCACCTGTTCT -1807</td>
</tr>
<tr>
<td>CREB</td>
<td>TGAGCTCT -373</td>
<td>TGATGCTCT -379</td>
</tr>
<tr>
<td>CAAT</td>
<td>GAGGAA -69</td>
<td>GAGGAA -79</td>
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<td>TATA</td>
<td>TACATAA -23</td>
<td>TACATAA -29</td>
</tr>
<tr>
<td>Sp1</td>
<td>CCCACC -199</td>
<td>CCCGCC -207</td>
</tr>
</tbody>
</table>

### Figure 2

**Figure 2.** Comparison of the putative glucocorticoid response element (GRE), CAMP response element binding protein (CREB), CAAT box, TATA box and Sp1 sites of the human and baboon glycodelin genes. The sequences underlined in Figure 1 are shown aligned with the comparable sequence from the human glycodelin gene (Vaisse et al., 1990). Nucleotide differences in the baboon are shown in italic-bold type.

### Figure 3

**Figure 3.** Response of the progesterone response element (PRE) and glycodelin promoter to progesterone receptor A (PRA) and PRB in Ishikawa cells. The indicated reporter plasmid was co-transfected with either the PRA or PRB expression plasmid and pCMV Sportβ-gal into Ishikawa cells. For each condition the media in half the wells received 1 μmol/l medroxyprogesterone acetate (MPA). The luciferase activity relative to the β-galactosidase activity is expressed as the mean ± SD of three different samples from one representative experiment. Significant differences \( (P < 0.05) \) between the cells treated with and without MPA for each group were determined by Student’s t-test and are indicated by an asterisk.

### Figure 4

**Figure 4.** Effect of progressive deletions of the 5’ flanking region of the baboon glycodelin gene on medroxyprogesterone acetate (MPA) responsiveness in Ishikawa cells. Plasmids containing progressively larger 5’ deletions of the glycodelin promoter–luciferase plasmid were co-transfected into Ishikawa cells with the progesterone receptor B (PRB) expression plasmid and pCMV Sportβ-gal and the cells treated with either 1 μmol/l MPA or the ethanol vehicle for 20 h. Luciferase activity was corrected for transfection efficiency using β-galactosidase activity and is shown as the mean ± SD of three different samples from one representative experiment. Significant differences \( (P < 0.05) \) between the cells treated with and without MPA for each construct were determined by Student’s t-test and are indicated by an asterisk.
this 2055 bp fragment (GenBank accession no. AF519801). This segment is 92% identical to the previously cloned human promoter (Vaisse et al., 1990). The regions proposed to correspond to a putative glucocorticoid response element (GRE), CAAT box and TATAA box are identical in the human and baboon (Vaisse et al., 1990) (Figure 2), while each of the proposed binding sites for the transcription factor Sp1 have at least one base change.

In Ishikawa cells, a human endometrial cell line, the A and B forms of the human PR were equally effective in inducing luciferase expression in response to the synthetic progestin MPA from the co-transfected glycodelin −2007/+48pGL3-Basic luciferase reporter construct (Figure 3). A chimeric plasmid containing the luciferase reporter gene linked to the PRE also exhibited progestin-dependent induction with either form of the PR.

To determine which region within the promoter was responsible for the progestin responsiveness, progressively shorter fragments linked to the luciferase reporter gene were analysed in the Ishikawa cell line. As can be seen in Figure 4, −808/+48pGL3-Basic, in which the putative GRE was removed, had a lower level of expression in the absence of MPA but exhibited no loss in progestin response; the MPA increased the expression of the −808/+48pGL3-Basic 4.6-fold, while the other groups ranged from 2.9- to 4.4-fold. Progressive deletions to

![Figure 5. Induction of the glycodelin–luciferase chimera in COS-1 cells. COS-1 cells were co-transfected with chimeric luciferase plasmids containing either the progesterone response element (PRE) or portions of the baboon glycodelin 5′ flanking sequence, a progesterone receptor B (PRB) expression plasmid and pCMV Sport-βgal and the cells treated with either 1 μmol/l medroxyprogesterone acetate (MPA) or the ethanol vehicle for 20 h. Luciferase activity was corrected for transfection efficiency using β-galactosidase activity and is shown as the mean ± SD of three different samples from one representative experiment. Significant differences (P < 0.05) between the cells treated with and without MPA for each construct were determined by Student’s t-test and are indicated by an asterisk.]

![Figure 6. Western blot analysis of Sp1 in nuclear extracts of Ishikawa and COS-1 cells. Nuclear extracts from Ishikawa and COS-1 cells were immunoreacted with a monoclonal antibody to Sp1.](image)

![Figure 7. Effect of mutation of the proximal Sp1 site on responsiveness of the −67/+48 glycodelin–luciferase chimera in Ishikawa cell line. The cells were co-transfected with either the −67/+48 or mutant −68/+48 glycodelin promoter–luciferase chimeric plasmid, pCMV Sport-βgal plus the Sp1, progesterone receptor B (PRB), or Sp1 and PRB expression plasmid and treated with either 1 μmol medroxyprogesterone acetate (MPA) or the ethanol vehicle for 20 h. Luciferase activity was corrected for transfection efficiency using β-galactosidase activity and is shown as the mean ± SD of three different samples from one representative experiment. Significant differences (P < 0.05) between the cells treated with and without MPA for each construct were determined by Student’s t-test and are indicated by an asterisk.](image)
–506, –368 (with the removal of the putative CREB site), and –119 (with the removal of the two distal putative Sp1 sites designated Sp1-1 and Sp1-2 by Gao et al., 2001) also were not associated with any loss in progesterin responsiveness. Only when the entire glycodelin fragment was removed (Basic) there was a drastic reduction in the basal and progesterin-induced luciferase activity.

In COS-1 cells, a kidney cell line from African green monkeys, MPA failed to increase luciferase in either the –2007/+48pGL3-Basic or –119/+48pGL3-Basic chimeric plasmids (Figure 5). However, MPA was able to induce expression when the PRE was co-transfected with the PRB in the COS-1 cells.

Since the –119/+48 region of the glycodelin gene promoter lacks a consensus PRE, it is possible that PR interacts with another transcription factor and promotes expression via the response element for this transcription factor. One candidate for such an interaction with the PR is the transcription factor Sp1 (Suske, 1999). One could hypothesize that (i) MPA induces glycodelin gene expression in the Ishikawa cells but not the COS-1 cells because Sp1 is present in Ishikawa cells but not the COS-1 cells or (ii) that the COS-1 cells had an overabundance of Sp1 so that the expression from the glycodelin promoter was maximally activated before the addition of the progesterin. Western blot analysis (Figure 6) demonstrated that neither scenario was true. The levels of Sp1 in Ishikawa and COS-1 cell were comparable.

The possibility that PR was interacting with Sp1 to induce glycodelin through the Sp1 site at –55/–50 (corresponding to the human Sp1 site designated Sp1-3 by Gao et al., 2001) was tested by mutating the Sp1 site. As can be seen in Figure 7, in Ishikawa cells MPA increases luciferase expression in both the native –67/+48pGL3-Basic and mutated –67/+48pGL3-Basic construct. Although apparently not necessary for the response to the progesterin, the overall levels of expression in the mutant are lower than in the native construct indicating that basal expression is increased by the Sp1 element. Similar results were seen when the experiment was repeated using HEC-1B cells (data not shown).

Discussion

During the late luteal phase of the menstrual cycle and early pregnancy glycodelin is the major secretory product of the human glandular epithelium (Bell et al., 1985). In the baboon, glycodelin synthesis begins in glandular epithelium during the mid-luteal phase and increases dramatically during early pregnancy (Hausermann et al., 1998). Proposed functions for glycodelin include inhibiting sperm– zona interaction (Öhninger et al., 1995) and immunosuppression (Bolton et al., 1987). This latter action may be due to a stimulation of apoptosis in T cells but not monocytes (Mukhopadhyay et al., 2001).

Not surprisingly, the 2007 bp S′ of the transcription start site of the baboon glycodelin gene has a high degree of identity to the human glycodelin gene. This includes the complete identity of the putative GRE, CAAT box and TATAA box of the human glycodelin gene (Vaisse et al., 1990) with the corresponding region of the baboon glycodelin promoter. However, each of the putative Sp1 sites is altered with at least a G to A conversion. A change from G to A in the Sp1 binding sequence has been reported to result in a 3-fold loss in Sp1 binding activity (Letovský and Dynan, 1989). Deletion of the two distal Sp1 sites (–244/–239 and –204/–199) is not associated with any change in the basal or progesterin response (Figure 4), which is in contrast with the results of Gao et al. (Gao et al., 2001) who found that mutations of the analogous sites in the human glycodelin promoter did drastically lower expression. We did find that mutating the Sp1 site at –55/–50 did lower basal expression. It is not clear if the potential distal

Sp1 sites in the baboon are inactive solely because of the base change in the sequence.

Two forms of the PR—PRA and PRB—are found in many progesterone responsive cells. These forms arise from a single gene through the initiation of transcription at two distinct promoters and the use of alternative start codons for translation (Conneely et al., 1987, 1989; Kastner et al., 1990). In certain circumstances the PRA form may be inactive or inhibitory (Giangrande and McDonnell, 1999). In the Ishikawa cells both PRA and PRB increase luciferase levels from both the PRE and glycodelin reporter plasmids. The human glycodelin promoter was also found to be induced equally by PRA and PRB in HEC-1B cells (Gao et al., 2001).

Deletion of the glycodelin promoter regions containing putative GRE and CAMP response element binding protein (CREB) elements (–2007 to –808 and –506 to –368 respectively) did not alter the induction of luciferase by the synthetic progesterin MPA. Further deletions to –119 and –67 with the removal of the two distal putative Sp1 response elements (referred to as Sp1-1 and -2 by Gao et al., 2001) were also not associated with any loss in progesterin responsiveness.

In COS-1 cells, luciferase activity was increased in cells co-transfected with the PRA- and the PRE-containing luciferase plasmids in response to MPA. This indicates that the machinery exists within these cells for a direct effect of the PR on its response element. However, in the COS-1 cells the glycodelin–luciferase reporter construct containing the –2007/+48 element failed to exhibit any progesterin-induced response. Reporter plasmids containing –119/+48 of the S′ flanking sequence also did not have a MPA response in the COS-1 cells. Since these cells were responsive when the PRE was co-transfected we conclude that the PR is not acting on the glycodelin promoter directly.

Sp1 is a ubiquitously expressed transcription factor that has been implicated in the activation of a number of genes (Suske, 1999). In certain progesterone responsive genes the PR does not directly activate the gene. Instead, the PR activates the gene in an indirect manner by interacting with Sp1 that then binds as a complex to the Sp1 response element. Our findings on the difference in response of the reporter constructs to MPA in Ishikawa and COS-1 cells could have been due to significant differences in the levels of Sp1 within these two cell lines. Western blot analysis indicated that this could not explain our findings since the Ishikawa and COS-1 cell lines contained similar amounts of Sp1. That this was not the case was further shown by mutating the putative Sp1 response element in the –67/+48 glycodelin reporter plasmid. Although the overall level of luciferase production in the mutant plasmid was lower than in the native construct, the ability of MPA to induce a response in the Ishikawa cells was retained.

We propose that progestins only indirectly activate the glycodelin gene. Since MPA failed to induce a response in COS-1 cells we believe the PR acts in the Ishikawa cells, a progestin responsive cell line, to induce the production of a transcription factor, which in turn activates the glycodelin gene promoter, or a pre-existing factor exists, which is found in Ishikawa cells but not COS-1 cells. In COS-1 cells that are not progesterin responsive, the production of this transcription factor is not inducible. Studies are underway to define what this transcription factor is and the sequence of its response element.

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References


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