Molecular basis of bone morphogenetic protein-4 inhibitory action on progesterone secretion by ovine granulosa cells

A Pierre, C Pisselet, J Dupont, B Mandon-Pépin¹, D Monniaux, P Monget and S Fabre

Physiologie de la Reproduction et des Comportements, UMR 6175 INRA-CNRS-Université de Tours, Haras Nationaux, 37380 Nouzilly, France

¹Biologie du Développement et de la Reproduction, UMR 1198 INRA-ENVA, 78350 Jouy en Josas, France

(Requests for offprints should be addressed to S Fabre; Email: sfabre@tours.inra.fr)

Abstract

We have recently reported that bone morphogenetic protein-4 (BMP-4) can inhibit progesterone production by ovine granulosa cells (GCs). Here, we have investigated the underlying mechanisms of this effect in basal as well as in FSH-induced conditions. We have confirmed that treatment with BMP-4 decreased basal GC progesterone secretion and totally abolished FSH-stimulating action. This inhibitory action was associated with a decrease in the expression of cAMP-regulated genes, steroidogenic acute regulatory protein (StAR) and P450 side-chain cleavage (P450 scc) at mRNA and protein levels. However, BMP-4 did not alter basal cAMP production by GCs. In contrast, BMP-4 decreased by half the FSH-induced cAMP production and strongly inhibited cAMP-induced progesterone production. Thus, the inhibitory effect of BMP-4 was exerted both upstream and downstream of cAMP signalling. We next examined the downstream effect, focusing on cAMP-dependent transcription factors, steroidogenic factor-1 (SF-1) and CREB, through the BMP factor signalling intermediary, Smad1. As expected, BMP-4 induced phosphorylation and transcriptional activity of Smad1 in ovine GCs. BMP-4-activated Smad1 did not affect CREB activity but inhibited the transcriptional activity of SF-1 on the canonical SF-1 responsive element. Interestingly, this transcriptional inhibitory mechanism occurred on transfected StAR and P450 scc promoter. Based on these results, we propose that SF-1 is a key target in the inhibitory mechanism exerted by BMP-4 on progesterone synthesis by ovine GCs in culture. Because SF-1 plays an essential role in the differentiation of GCs, our findings could have new implications in understanding the role of BMP family members in the control of ovarian folliculogenesis.

Journal of Molecular Endocrinology (2004) **33**, 805–817

Introduction

Follicle-stimulating hormone (FSH) plays a central role in ovarian folliculogenesis, particularly in regulating granulosa cell (GC) differentiation. In particular, FSH enhances the expression of steroidogenic enzymes such as cytochrome P450 side-chain cleavage (P450 scc) and P450 aromatase (Richards & Hedin 1988, Winters *et al.* 1998) and these actions are importantly modulated by various growth factors, acting in a paracrine or autocrine way (Khamisi *et al.* 2001). From recent accumulating *in vivo* and *in vitro* evidence, it appears that members of the bone morphogenetic protein

(BMP) family of cytokines and their receptors are strongly implicated in ovarian function, controlling folliculogenesis and ovulation rate (Monget *et al.* 2002). A study of invalidated mice for growth and differentiation factor-9 (GDF-9), an oocyte-derived growth factor of the BMP family, has shown that this factor is required during early ovarian folliculogenesis enabling primordial and primary to secondary follicle transition (Dong *et al.* 1996). In sheep, two loss of function mutations in the gene encoding BMP-15, another oocyte-derived factor closely related to GDF-9, have been shown to be responsible for a strong alteration of follicular growth in a dose-dependent manner. An increase

in ovulation rate was observed in the heterozygous carrier Inverdale and Hanna ewes, the homozygous being sterile (Galloway *et al.* 2000). Moreover, it has been shown that a substitutive mutation (Q249R) in the gene encoding BMP receptor (BMPR)-1B (also called Alk6), one of the type-I receptors triggering the BMP intracellular signalling, is responsible for an increase in ovulation rate (threefold in heterozygous carrier ewes and more than fivefold in homozygous carriers) in the Booroola strain (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001).

In vitro, recombinant BMP-4, BMP-6, BMP-7, BMP-15 and GDF-9 are able to strongly inhibit FSH-induced progesterone secretion by rat GCs in culture (Shimasaki *et al.* 1999, Otsuka *et al.* 2000, 2001a,b, Vitt *et al.* 2000, Lee *et al.* 2001, Souza *et al.* 2001). In contrast, BMP-4 and BMP-7 enhance FSH-dependent estradiol production, suggesting that BMP factors regulate GC differentiation, particularly in delaying the luteinization process (Shimasaki *et al.* 1999).

Several studies in the rat and the ewe using *in situ* hybridization or immunohistochemistry have detected the expression of several elements of the BMP signalling pathway in the ovary. For example, BMP-4 and BMP-7 are expressed by theca cells (Shimasaki *et al.* 1999), whereas BMP-6, GDF-9 and BMP-15 are oocyte-derived factors (for review, Elvin *et al.* 2000). BMP type I receptors, BMPRIA/Alk3 and BMPRII/Alk6 and type II receptor BMPRII are expressed in GCs and oocytes (Shimasaki *et al.* 1999, Wilson *et al.* 2001, Souza *et al.* 2002). Transducing molecules of the Smad family have been detected in all compartments of follicles in the rat ovary (Drummond *et al.* 2002, Xu *et al.* 2002). Altogether, these observations indicate the presence of a complete BMP signalling pathway in ovaries, enabling autocrine and paracrine regulation.

Less is known about the mode of action of BMP family members on target cells in the ovary, particularly in GCs. In the rat, most actions of the BMP molecules on GC steroidogenesis are FSH dependent. The proposed mechanism of action implicates down-regulation of FSH receptor expression or decrease in adenylate cyclase activity (Otsuka *et al.* 2001a,b). In contrast, we have recently demonstrated that BMP-4, a known ligand of Alk6, inhibits progesterone secretion by ovine GCs in the absence of FSH (Mulsant *et al.* 2001, Fabre *et al.*

2003). However, the precise intracellular mechanism that underlies the inhibiting action of BMP-4 has not been determined.

In the present study, we have investigated the mechanism of action by which BMP-4 exerts an inhibitory action on basal as well as FSH-induced progesterone secretion through modulation of the cAMP signalling pathway and expression of genes involved in steroidogenesis. We propose that BMP-4 modulates progesterone by inhibiting the steroidogenic factor-1 (SF-1) transcriptional activity on steroidogenic gene promoters. In FSH-induced conditions, this mechanism might be reinforced by an inhibition of adenylate cyclase activity.

Materials and methods

Reagents and supplies

Fluorogestone acetate sponges used to synchronize oestrous cycles were obtained from Intervet (Angers, France). Porcine FSH (pFSH) from pituitary extracts (pFSH activity=1.15 times the activity of NIH pFSH-P1) used for injections to animals was obtained from Dr Y Combarous (Nouzilly, France). Purified ovine FSH-20 (oFSH) (lot no. AFP-7028D; 4453 IU/mg; FSH activity=175 times the activity of oFSH-S1) used for culture treatment was a gift from the NIDDK (National Hormone Pituitary Program, Bethesda, MD, USA). Recombinant human FSH (rhFSH) used in adenylate cyclase activity experiments was obtained from Serono (Boulogne, France). Recombinant human BMP-4 was obtained from R&D Systems Europe (Lille, France). Dibutyryl-cyclicAMP (db-cAMP) and isobutyl methylxanthine (IBMX) were purchased from Sigma (L'Isle d'Abeau Chesnes, France). Rabbit polyclonal anti-Smad1 and anti-phospho-Smad1 were obtained from Upstate Biotechnology (Euromedex, Mundolsheim, France). Rabbit polyclonal anti-P450 scc antibody was purchased from Chemicon (Euromedex, Mundolsheim, France). Rabbit polyclonal antibody raised against human placental 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and mouse steroidogenic acute regulatory protein (StAR) were kindly provided by Dr V Luu-The (Quebec, Canada) and Dr D B Hales (Chicago, IL, USA) respectively.

Plasmid designated pSG-SF1, constructed by inserting cDNA encoding the murine SF-1 into

pSG5 vector (Stratagene, Amsterdam, The Netherlands) and pGL3 TkLH β , a luciferase reporter gene under the control of two copies of the SF-1-responsive element of the luteinizing hormone (LH)- β promoter gene, were a gift from Dr A Martinez (Val *et al.* 2003). Plasmid pf-Smad1, constructed by inserting a flag-tagged murine Smad1 into pcdef3 vector was kindly provided by Dr M Kawabata (Ishida *et al.* 2000). Plasmid Gal4-tk80-luc was a luciferase reporter gene under the control of the Gal4-responsive element and plasmid pGal4-Smad1 was a human Smad1 sequence fused with the DNA-binding domain of Gal4 (Pearson *et al.* 1999). P450 scc luciferase reporter constructs containing fragments of the human P450 scc gene spanning from nucleotides -110 to +49, with or without the mutated SF-1-binding site, subcloned in pGL3 vector, were kindly provided by Dr B Staels (Gizard *et al.* 2002). StAR luciferase reporter construct containing fragments of human StAR gene spanning nucleotides -235 to +39 was a gift from Dr J Strauss (Sugawara *et al.* 1996). The cAMP-sensitive reporter construct of the somatostatin gene promoter (pSOM-luc) was kindly provided by Dr B Peers (Liège, Belgium).

Animals

Fifty adult cyclic Romanov ewes were treated with intravaginal sponges impregnated with progestagen (fluorogestone acetate, 40 mg) for 15 days to mimic a luteal phase. Ovaries were collected from animals in the luteal phase of the oestrous cycle (10 days after sponge removal), stimulated by intramuscular injections of 6 IU and 5 IU pFSH administered 24 h and 12 h prior to slaughter respectively. All procedures were approved by the Agricultural and Scientific Research Government committees in accordance with the guidelines for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (approval A37801).

Isolation and culture of GCs

Briefly, in each independent culture experiment, ovarian follicles from three to four Romanov ewes were quickly dissected, pooled and classified according to size. GCs were recovered from small antral follicles (1–3 mm in diameter) as previously described (Le Bellego *et al.* 2002). For progesterone

and cAMP production, GC suspensions were seeded at 100 000 viable cells/well in 96-well plates and cultured for 144 h at 37 °C with 5% CO₂ in serum-free McCoy's 5a medium (Sigma) according to a previously described method (Campbell *et al.* 1996). Cultures were performed with or without different exogenous factors (BMP-4, oFSH, db-cAMP or IBMX), each alone or in combination. Each combination of treatments was tested in triplicate in at least four independent culture experiments. Culture media were partially replaced (180 over 250 μ l) at 72 h. Media conditioned between 72 and 144 h of culture were collected at 144 h and stored at -20 °C prior to radioimmunoassays. At the end of the culture, the number of cells per well was estimated after trypsinization by counting an aliquot of each resulting cell suspension with a haemocytometer under a phase contrast microscope.

Alternatively, for phospho-Smad1 detection or transient transfection experiments, GCs were cultured in McCoy's 5a medium supplemented with 3% fetal ovine serum during the 72 h prior to specific treatments.

Northern blot analysis

For RNA blot analysis, GCs were harvested 72 h after treatment with oFSH (5 ng/ml) or BMP-4 (50 ng/ml) alone or in combination, and total RNAs were isolated using Rnase solution (Eurobio, Les Ulis, France). Twenty micrograms of total RNAs were separated by denaturing formaldehyde electrophoresis, then transferred to a nylon membrane by capillary action overnight and immobilized by exposure to u.v. light. Blots were prehybridized for 2 h at 42 °C in a buffer containing 50% formamide, 5 $\times$ Denhardt's, 1% SDS, 5 $\times$ SSC and 100 μ g/ml denatured salmon sperm. The probes were labelled using the Rediprime labelling kit (Amersham Pharmacia Biotech, Orsay, France). cDNA probes for ovine StAR, P450 scc and 3 β -HSD were generated by RT-PCR on ovine whole follicle mRNA using the following primers: StAR sense 5'-GGTGCT GAGTAAAGTGATCC-3' and StAR antisense 5'-CATCTCCTCGTAGAGTGTGG-3'; P450 scc sense 5'-CTCTTTAAGTTTCGAGGGGATC-3' and P450 scc antisense 5'-TCACTTTCAGGGTAT CTCTG-3'; 3 β -HSD sense 5'-CACATTCTGGC CTTGAGGGC-3' and 3 β -HSD antisense 5'-CAG

GAAGCCAAGCAGAAAAC-3'. The radioactivity was quantified using a STORM apparatus and ImageQuant software (Amersham Pharmacia Biotech). The integrity and the quantification of different transcripts were assessed using the human RNA 18S probe as a control (Ambion, Huntingdon, Cambs, UK).

Western blot analysis

GC whole cell extracts were obtained by resuspension in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 0.5% Igepal) containing several protease inhibitors (2 mM phenylmethylsulphonyl fluoride, 10 mg/ml leupeptin and 10 mg/ml aprotinin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate and 2 mM sodium orthovanadate (Sigma)). Lysates were centrifuged at 15 000 *g* for 20 min at 4 °C, and the protein concentration in the supernatants was determined by a colorimetric assay (BC Assay kit; Uptima Interchim, Montluçon, France). The protein samples (30–60 µg) were fractionated using SDS-PAGE in 12% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Ecqueville, France). For steroidogenic protein detection, GCs were harvested after 144 h of treatment with FSH and/or BMP-4 in serum-free condition. The same samples were run and transferred on three independent membranes to allow individual detection of P450 scc and 3β-HSD and dual detection on the same membrane of StAR and actin was used as a loading control. Proteins of the steroidogenic pathway were detected using rabbit polyclonal antibodies raised against StAR (1:500 final dilution), P450 scc (1:1000) and 3β-HSD (1:500). Actin was revealed using a mouse monoclonal anti-actin antibody (1:1000; Sigma). For Smad1 detection, GCs were cultured for 72 h in McCoy's 5a medium supplemented with 3% fetal ovine serum (500 000 cells/well; 6-well plate), then harvested after 48 h in serum-free condition before a 30-min stimulation with BMP-4 (50 ng/ml). Incubation with rabbit polyclonal or mouse monoclonal primary antibodies was followed by incubation with peroxidase-conjugated anti-rabbit IgG (1:20 000; Uptima Interchim) or with peroxidase-conjugated anti-mouse IgG (1:20 000; Bio-Rad, Marnes-la-Coquette, France) respectively.

Finally, the protein bands were detected using ECL detection reagents (Amersham Pharmacia Biotech).

Assessment of progesterone and cAMP production

The amount of progesterone (ng/50 000 cells) in the culture media conditioned between 72 and 144 h from each experiment was measured by radioimmunoassay as previously described (Saumande 1991).

For cAMP measurement, GCs from small antral follicles were cultured for 144 h with or without BMP-4 (50 ng/ml). At the end of the culture, media were removed and cells were stimulated for 2 h with 0.2 mM IBMX in the presence or absence of 50 ng/ml rhFSH in fresh McCoy's 5a medium. Extracellular amounts of cAMP (pmol/50 000 cells) in the media conditioned during these last 2 h were measured using the cAMP radioimmunoassay kit (Perkin Elmer, Courtaboeuf, France) following the manufacturer's specifications. IBMX treatment was used to prevent the metabolic effect of phosphodiesterase on cAMP and then cAMP production was a direct measurement to adenylate cyclase activity.

Transient transfection and luciferase assay

GCs from small antral follicles were seeded at 200 000 cells/well in 12-well plates and cultured for 72 h in McCoy's 5a medium supplemented with 3% fetal ovine serum. Then, the different plasmids expressing Smad1 or SF-1 (500 ng/well) and luciferase reporter genes for Smad1 or SF-1 activity (1 µg/well) were transiently transfected to cells using DAC30 transfection reagent (Eurogentec, Seraing, Belgium) for 24 h with a DNA/DAC30 ratio of 1/2 (w/w) as specified by the manufacturer. After 24 h, media were changed with fresh McCoy's 5a medium supplemented with 3% fetal ovine serum cells for an extra 24 h with or without BMP-4 (50 ng/ml) before luciferase assay (Promega, Charbonnières, France). Each combination of plasmids was tested in triplicate in each culture and in four independent experiments.

Data analysis

All experimental data are presented as means ± S.E.M. The effects of hormones on progesterone secretion and cAMP production were

Figure 1 Effect of BMP-4 and FSH on progesterone production by primary ovine GCs *in vitro*. GCs from small antral follicles (1–3 mm in diameter) were cultured for 144 h in serum-free conditions with or without BMP-4 (50 ng/ml), in the absence (control) or the presence of increasing amounts of oFSH (0.1–50 ng/ml). Each combination of treatments was tested in triplicate in each of four independent experiments. Results represent progesterone secretion by 50 000 GCs between 96 and 144 h of culture (means $\pm$ S.E.M.). *** $P < 0.001$, BMP-4 vs control in basal conditions. In FSH-induced conditions, for each dose used, BMP-4 significantly ($P < 0.001$) inhibited progesterone synthesis.

analyzed using two-way ANOVA in order to appreciate the ‘hormone effect’ as well as the ‘culture effect’. For luciferase assays, data are expressed as relative to control condition. The effect of different combinations of plasmids was assessed by two-way ANOVA to allow for ‘experiment effect’ and ‘plasmid effect’ or ‘BMP-4 effect’. One-way ANOVA was used to appreciate the treatment effect on Northern blot analysis. Post-hoc comparisons were performed with Scheffé’s and Newman–Keuls tests. For all analyses, differences with $P > 0.05$ were considered as not significant.

Results

Effect of BMP-4 on basal and FSH-stimulated progesterone production by GCs

As shown in Fig. 1, basal progesterone production between 72 and 144 h of culture was decreased by 57% ($P < 0.001$) by BMP-4. As expected, treatment

with oFSH (0.5–50 ng/ml) dose-dependently increased progesterone production by GCs. In these FSH-stimulated conditions, the addition of BMP-4 totally abolished oFSH action ($P < 0.001$). The same results were observed with GCs from preovulatory follicles (5–6 mm in diameter) (data not shown).

Effect of BMP-4 on steroidogenic enzyme genes

In order to understand the inhibiting role of BMP-4 on GC progesterone production, we have studied the expression of genes implicated in progesterone synthesis, StAR, P450 scc and 3β -HSD, at mRNA and protein levels (Fig. 2). Northern blot analysis (Fig. 2A) showed a consistent decrease in StAR mRNA level after 72 h of BMP-4 treatment (50 ng/ml) in both basal ($P < 0.05$) and FSH-induced condition ($P < 0.01$). Moreover, BMP-4 led to a decrease in P450 scc mRNA levels in FSH-induced condition only ($P < 0.05$). In contrast, the 3β -HSD mRNA level was not clearly affected. This pattern of regulation on StAR, P450 scc and 3β -HSD mRNAs at 72 h was well correlated with the results observed for protein levels at 144 h, as shown by Western blotting analysis (Fig. 2B).

Effect of BMP-4 on cAMP production and activity

The inhibitory effect of BMP-4 was investigated on the cAMP signalling pathway, a well-known regulator of steroidogenesis in GCs. After 144 h of culture, basal cAMP production of GCs, measured in the presence of IBMX, was not affected by BMP-4 treatment. In contrast, rhFSH-stimulated (50 ng/ml) cAMP production at the end of the culture was significantly reduced (50%) by the presence of BMP-4 during the culture ($P < 0.001$; Fig. 3 left-hand panel). Moreover, BMP-4 strongly inhibited db-cAMP or IBMX stimulation of progesterone secretion (Fig. 3 right-hand panel). Together, these results indicated that BMP-4 could act negatively on both pre- and post-cAMP signalling events.

Searching for the BMP-4 inhibitory mechanism

Since basal and cAMP-dependent StAR, P450 scc and 3β -HSD gene expression are regulated by a

Figure 2 Effect of BMP-4 and FSH on expression of steroidogenic genes implicated in progesterone synthesis. GCs from small antral follicles were cultured for 72 h (mRNA) or 144 h (protein) in serum-free condition with BMP-4 (50 ng/ml) or oFSH (5 ng/ml), each alone or in combination. Each combination of treatments was tested in at least three independent experiments. (A) Representative Northern blots and quantification of StAR, P450 scc and 3β-HSD mRNA expression. Quantitated mRNA levels are expressed relative to ribosomal 18s RNA used as control. * $P < 0.05$, ** $P < 0.01$, BMP-4 vs untreated in basal or FSH condition; \$ $P < 0.05$, FSH vs untreated. (B) Representative Western immunoblotting analysis of GC whole cell extracts using anti-StAR, anti-P450 scc and anti-3β-HSD specific antibodies. Actin was used as gel loading control.

Figure 3 Effect of BMP-4 on cAMP production and activity. GCs from small antral follicles were cultured for 144 h in serum-free condition with or without BMP-4 (50 ng/ml). (Left-hand panel) cAMP production. At the end of the culture, GCs were incubated for 2 h in the presence of IBMX (2 mM, inhibitor of phosphodiesterase) alone (control) or in combination with rhFSH (50 ng/ml). The amount of cAMP in the medium was measured by radioimmunoassay after acetylation of the samples. (Right-hand panel) Progesterone secretion. GC progesterone production was stimulated by culturing GCs in the presence of db-cAMP (1 mM) or IBMX (2 mM), each alone or in combination with BMP-4 (50 ng/ml). Each combination of treatments was tested in triplicate in each of four independent experiments. Data are shown as means $\pm$ S.E.M. *** $P < 0.001$, BMP-4 vs basal.

common transcription factor, SF-1 (Leers-Sucheta *et al.* 1997, Liu & Simpson 1997, Sugawara *et al.* 1997), we next investigated the effect of BMP-4, through its signalling intermediary Smad1 (Heldin *et al.* 1997), on SF-1 transcriptional activity.

First, we checked the ability of BMP-4 to trigger the phosphorylation and the functional activation of Smad1 in ovine GCs in culture (Fig. 4). As shown in Fig. 4A, BMP-4 treatment for 30 min was able to phosphorylate Smad1. We investigated the transcriptional activity of overexpressed Smad1 protein fused to the Gal4-DNA-binding domain on the Gal4-responsive element driving the luciferase gene. Stimulation with BMP-4 for 24 h strongly increased Smad1-dependent luciferase activity (Fig. 4B). These results indicated that Smad1, like other cell types, could act as an effector of BMP-4 action in ovine GCs.

Next, we investigated the effect of BMP-4, through Smad1, on SF-1 transcriptional activity (Fig. 5). Transient transfection experiments on ovine GCs showed that the SF-1-responsive construct pGL3 TkLH β (Fig. 5A) activity was enhanced (4.5-fold, $P < 0.001$) by overexpression of SF-1. This stimulatory effect was lowered by BMP-4 treatment ($P < 0.01$) or Smad1 expression

($P < 0.001$) and totally abolished by Smad1 expression and BMP-4 treatment. In contrast, the transcriptional activity of CREB (another cAMP-dependent factor implicated in steroidogenic gene regulation), checked by the pSOM-luc construct (Fig. 5B) was not altered by overexpression of Smad-1. Thus, it appears that Smad-1 was able to specifically suppress SF-1 activity on SF-1 responsive elements.

In order to demonstrate that this Smad1 antagonistic effect on SF-1 could participate in the inhibitory mechanism of steroidogenic gene expression by BMP-4, we studied the role of Smad1 on StAR and P450 scc promoter activity (Fig. 5C and D). Overexpression of SF-1 enhanced P450 scc and StAR promoter activity and these stimulations were reduced by Smad1 co-expression ($P < 0.001$). Interestingly, basal activity of the P450 scc promoter was significantly reduced by Smad1 overexpression ($P < 0.05$), suggesting a possible direct negative action of Smad1 on the P450 scc promoter (Fig. 5C). The mutant P450 scc promoter in which the SF-1 binding site has been disrupted exhibited a great decrease in basal activity in comparison with the wild-type promoter and was not sensitive to SF-1

Figure 4 BMP-4 activation of the Smad1 signalling pathway. (A) Phosphorylation of Smad1 by BMP-4. After 72 h of culture, GCs were stimulated or not stimulated for 30 min with BMP-4 (50 ng/ml) in serum-free condition. Cells were lysed and subjected to Western immunoblotting using phospho-Smad1 and total Smad1 antisera. Actin was used as gel loading control. (B) Smad1 activation by BMP-4. After 72 h of culture, GCs were transiently cotransfected with the Gal4-responsive reporter construct (Gal4 promoter) and the plasmid expressing the Gal4 DNA-binding domain–Smad1 fusion protein (pGal4-Smad1). After transfection, GCs were incubated for 24 h with or without BMP-4 (50 ng/ml) in the presence of 3% fetal ovine serum, before luciferase assay. Results are expressed as luciferase activity relative to control condition. Data represent means $\pm$ S.E.M. of measurements performed in eight independent experiments. ** $P < 0.01$.

overexpression. On this mutant promoter, the Smad1 inhibitory action was minimized but not totally abolished ($P < 0.05$).

Discussion

There is accumulating evidence supporting the inhibitory action of BMP molecules on progesterone production by ovarian GCs. However, most studies using the rat model have indicated that BMPs exerted their action only in FSH-induced condition. In the present study, using ovine GCs, we have confirmed the strong inhibitory action of BMP-4 on FSH-stimulated progesterone production but, in contrast to the rat, we also observed inhibition of progesterone production on its own in the absence of FSH. Based on knowledge about the implication of BMP in regulating ovulation rate (Galloway *et al.* 2000, Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001, Hanrahan *et al.* 2004), this result suggests some species differences in the role of the BMP system on ovarian function between the high ovulating rodent and the low ovulating ruminant.

The objective of the present study was to explore the molecular basis by which BMP-4 exerted its inhibitory action on ovine GC basal and FSH-stimulated progesterone synthesis. First, BMP-4 reduced basal and/or FSH-induced increases in mRNA and the protein levels of StAR and P450 scc, a finding consistent with the decrease in progesterone production. Secondly, BMP-4 exerted its inhibitory effect mainly downstream of cAMP signalling and to a lesser extent by decreasing FSH-dependent cAMP production. Thirdly, BMP-4 was able to activate the Smad1 signalling pathway in primary ovine GCs. Fourthly, Smad1 was able to inhibit the transcriptional activity of SF-1 on the LH β promoter as well as on the StAR and P450 scc promoter. Based on these findings, we propose that part of the negative action of BMP-4 on progesterone secretion by ovine GCs would be exerted through the activation of Smad1 that would inhibit the SF-1-dependent expression of StAR and P450 scc genes, leading to a decrease in progesterone synthesis activity. In FSH-stimulated condition, this inhibitory mechanism would be reinforced by a decrease in FSH-dependent cAMP production.

In order to appreciate the regulation of progesterone production, we examined the steady-state level of mRNA for StAR, P450 scc and 3 β -HSD genes implicated in the progesterone synthesis pathway. As observed in other species (Urban *et al.* 1991, Pescador *et al.* 1997, Eimerl &

Figure 5 Effect of Smad1 expression on SF-1 and CREB transcriptional activity. GCs were cultured for 72 h in McCoy's 5a medium supplemented with 3% fetal ovine serum prior to transient transfection. (A) Co-transfection of the SF-1 responsive element of the LHβ gene promoter construct and vectors expressing or not expressing SF-1 or Smad1, each alone or in combination and treated with or without BMP-4 (50 ng/ml) for 24 h. (B) Co-transfection of the CREB responsive element construct of the somatostatin gene promoter and vectors expressing or not expressing CREB or Smad1, each alone or in combination and treated with oFSH (5 ng/ml) for 24 h to obtain transcriptional activity of CREB. (C) Co-transfection of the wild-type (wt) P450 scc gene promoter constructs or a mutated form of the SF-1 responsive element (SF-1 m) or (D) StAR gene promoter construct and vectors expressing or not expressing SF-1 and/or Smad1. Results are expressed as luciferase activity relative to control condition (reporter construct alone). Each combination of treatments was tested in triplicate in each of four to six independent experiments. Data represent means ± S.E.M. of measurements. \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.001$, BMP-4 vs basal, * $P < 0.05$, *** $P < 0.001$, Smad1 vs empty vector.

Only 2002), mRNA levels of StAR, P450 scc and, to a lesser extent, 3β-HSD increased under FSH treatment. This up-regulation at the mRNA level was followed by the same regulation at the

protein level. FSH-induced expression of StAR and P450 scc mRNAs and proteins was clearly down-regulated by BMP-4. These results are in agreement with those observed on FSH-stimulated

rat GCs using BMP-15, BMP-6 or BMP-7, even if BMP-7 seems to inhibit only StAR mRNA without affecting P450 scc (Lee *et al.* 2001, Otsuka *et al.* 2001a,b).

Since StAR, P450 scc and 3 β -HSD are cAMP/protein kinase A (PKA)-regulated genes (Lauber *et al.* 1993, Sugawara *et al.* 1997, Rodway *et al.* 1999), we have focused on the BMP-4 regulation of this signalling pathway. BMP-4 was able to inhibit the FSH-stimulated adenylate cyclase activity of GCs, as measured by cAMP production in the presence of IBMX. This result indicates a regulation of the cAMP signalling pathway by BMP-4 upstream of cAMP as previously observed for BMP-15 and BMP-6 in rat GCs (Otsuka *et al.* 2001a,b). BMP-15 and BMP-6 have been shown to inhibit FSH-stimulated progesterone production by two different mechanisms implying down-regulation of the FSH receptor expression and inhibition of adenylate cyclase activity respectively. Using RT-PCR, we were unable to demonstrate any down-regulation of FSH receptor expression by BMP-4 in ovine GCs (data not shown). In the absence of FSH, BMP-4 was without effect on cAMP production while it inhibited progesterone synthesis, supporting the hypothesis that BMP-4 is also acting downstream of cAMP. The strong inhibitory effect of BMP-4 observed on cAMP-stimulated progesterone secretion also argues in favour of this hypothesis.

Most of the transforming growth factor- β /BMP effects at the cellular level are triggered by Smad-related factors. Among the Smad family members, Smad1 is considered to be a BMP-responsive Smad, because most BMPs identified to date, including BMP-2, -4, -6, -7 and -15, have been shown to activate the Smad1 pathway in different cell types (Candia *et al.* 1997, Yamamoto *et al.* 1997, Macias-Silva *et al.* 1998, Henningfeld *et al.* 2000, Moore *et al.* 2003). Based on this fact, we investigated whether BMP-4 activates the Smad1 pathway in primary ovine GCs. Treatment of cells with BMP-4 caused a clear increase (i) in the level of phosphorylated Smad1 as determined by immunoblotting and (ii) in Smad1 transcriptional ability as shown by transient transfection experiments. Hence Smad1 can transduce the BMP-4 signal in ovine GCs. Although the Smad pathway is the main canonical pathway for BMP ligands, there is much evidence on the activation of alternative

pathways, particularly the p38 kinase of the MAPK family in various cell types (for a review see Nohe *et al.* 2004). In agreement, we have observed a weak increase in phosphorylated p38 MAPK under BMP-4 treatment in ovine GCs (Pierre *et al.* 2002). However, the inhibition of p38 phosphorylation *in vitro* by a specific inhibitor (SB203580) had no effect on BMP-4 suppression of progesterone production (A Pierre, unpublished data), suggesting that activation of the p38 MAPK pathway by BMP-4 cannot be implicated in the mechanism of progesterone inhibition.

The ability of BMP-4 to regulate post-cAMP steps and to activate the Smad1 factor led us to hypothesize the existence of functional interactions between the transcription factors of these two pathways in the regulation of steroidogenic gene expression. To date, no Smad binding element (SBE) has been described on the regulatory region of StAR or P450 scc or 3 β -HSD genes. Our present transient transfection experiments did not detect any effect of Smad1 on the basal activity of the human StAR proximal promoter. In contrast, basal activity of the P450 scc proximal promoter appeared to be sensitive to Smad1 overexpression even in the context of a disrupted SF-1-binding site, indicating a possible direct inhibitory action of Smad1 on the P450 scc promoter. Alternatively, cAMP/PKA-activated transcription factors such as CREB and SP-1, known to regulate genes of the steroidogenic pathway (Mukherjee *et al.* 1996, Leers-Sucheta *et al.* 1997, Liu & Simpson 1997, Sugawara *et al.* 2000) have also been shown to functionally interact with Smad factors. Indeed, Warner *et al.* (2003) recently demonstrated that phosphorylated CREB could be associated through the CREB-binding protein (CBP) with a Smad2 SBE-bound transcriptional complex. Moreover, SP-1 can co-operate with Smad3 to regulate the α 2(I) collagen promoter gene (Poncelet & Schnaper 2001) and with Smad2, 3 and 4 to control the promoter of the p15^{Ink4B} gene (Feng *et al.* 2000). Nevertheless, FSH-activated CREB did not co-operate with Smad1 to drive the CREB-dependent somatostatin promoter gene in our transient transfection experiments with ovine GCs. In contrast, we have clearly demonstrated that the transcriptional activity of SF-1, another factor implicated in basal as well as in cAMP-induced regulation of steroidogenic genes (Liu & Simpson 1997, Sugawara *et al.* 1997), was altered by Smad1.

This is the first demonstration of a functional negative crosstalk between Smad factors and SF-1 in any cell type. Such a negative alteration of a transcription factor activity by Smad factors has been described only for the nuclear factor κ B (NF κ B) (DiChiara *et al.* 2000). The inhibitory mechanism implies a competitive interaction between Smad2 and NF κ B mediated by the transcriptional coactivator CBP, without Smad2/NF κ B direct interaction. Of note, in our model, co-immunoprecipitation experiments with overexpressed Smad1 and SF-1 failed to detect any direct interaction between Smad1 and SF-1 (A Pierre, data not shown). Hence, one can hypothesize about the possible implication of an intermediary factor such CBP, known to interact with Smad factors (Pouponnot *et al.* 1998) and SF-1 (Monte *et al.* 1998). Further experiments are needed to validate this hypothesis on the inhibitory mechanism of Smad1 on SF-1 in the context of GCs, and to study the mechanism that underlies this inhibition.

Nevertheless, the inhibiting action of Smad1 on SF-1, checked by the SF-1 responsive element of the LH β subunit promoter gene, occurs also on the SF-1-dependent response of human StAR and P450 scc promoter constructs. These results need to be considered together with the observed inhibition of StAR and P450 scc mRNA expression by BMP-4. Therefore, one may hypothesize that part of the BMP-4-induced inhibition of StAR and P450 scc genes expression, and ultimately progesterone secretion, would pass through a Smad1-dependent inhibition of SF-1 activity. Since SF-1 is a key regulator of GC differentiation, our present data reinforce the hypothesis that BMP factors control ovarian follicle development in delaying GC differentiation.

Acknowledgements

We thank A Nicolle for excellent technical assistance. We also thank F Dupont and the staff of the ovine experimental unit in Nouzilly for their collaboration in the experimental design. This work was supported in part by the French Ministère de la Recherche 'Technologie pour la santé' ACI grant 'REGLO: REGuLation de l'Ovulation'. A P was supported by a French fellowship from the 'Région Centre'.

References

- Campbell BK, Scaramuzzi RJ & Webb R 1996 Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media. *Journal of Reproduction and Fertility* **106** 7–16.
- Candia AF, Watabe T, Hawley SH, Onichtchouk D, Zhang Y, Derynck R, Niehrs C & Cho KW 1997 Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. *Development* **124** 4467–4480.
- DiChiara MR, Kiely JM, Gimbrone MA Jr, Lee ME, Perrella MA & Topper JN 2000 Inhibition of E-selectin gene expression by transforming growth factor beta in endothelial cells involves coactivator integration of Smad and nuclear factor kappaB-mediated signals. *Journal of Experimental Medicine* **192** 695–704.
- Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N & Matzuk MM 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383** 531–535.
- Drummond AE, Le MT, Ethier JF, Dyson M & Findlay JK 2002 Expression and localization of activin receptors, Smads, and beta glycan to the postnatal rat ovary. *Endocrinology* **143** 1423–1433.
- Eimerl S & Orly J 2002 Regulation of steroidogenic genes by insulin-like growth factor-1 and follicle-stimulating hormone: differential responses of cytochrome P450 side-chain cleavage, steroidogenic acute regulatory protein, and 3 beta-hydroxysteroid dehydrogenase/isomerase in rat granulosa cells. *Biology of Reproduction* **67** 900–910.
- Elvin JA, Yan C & Matzuk MM 2000 Oocyte-expressed TGF-beta superfamily members in female fertility. *Molecular and Cellular Endocrinology* **159** 1–5.
- Fabre S, Pierre A, Pisselet C, Mulsant P, Lecerf F, Pohl J, Monget P & Monniaux D 2003 The Booroola mutation in sheep is associated with an alteration of the bone morphogenetic protein receptor-IB functionality. *Journal of Endocrinology* **177** 435–444.
- Feng XH, Lin X & Derynck R 2000 Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-beta. *EMBO Journal* **19** 5178–5193.
- Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH & Ritvos O 2000 Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature Genetics* **25** 279–283.
- Gizard F, Lavalley B, DeWitte F, Teissier E, Staels B & Hum DW 2002 The transcriptional regulating protein of 132 kDa (TRP-132) enhances P450 scc gene transcription through interaction with steroidogenic factor-1 in human adrenal cells. *Journal of Biological Chemistry* **277** 39144–39155.
- Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R & Galloway SM 2004 Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). *Biology of Reproduction* **70** 900–909.
- Heldin CH, Miyazono K & ten Dijke P 1997 TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390** 465–471.
- Henningfeld KA, Rastegar S, Adler G & Knochel W 2000 Smad1 and Smad4 are components of the bone morphogenetic protein-4 (BMP-4)-induced transcription complex of the Xvent-2B promoter. *Journal of Biological Chemistry* **275** 21827–21835.
- Ishida W, Hamamoto T, Kusanagi K, Yagi K, Kawabata M, Takehara K, Sampath TK, Kato M & Miyazono K 2000 Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. *Journal of Biological Chemistry* **275** 6075–6079.

- Khamisi F, Roberge S, Yavas Y, Lacanna IC, Zhu X & Wong J 2001 Recent discoveries in physiology of insulin-like growth factor-1 and its interaction with gonadotropins in folliculogenesis. *Endocrine* **16** 151–165.
- Laubier ME, Kagawa N, Waterman MR & Simpson ER 1993 cAMP-dependent and tissue-specific expression of genes encoding steroidogenic enzymes in bovine luteal and granulosa cells in primary culture. *Molecular and Cellular Endocrinology* **93** 227–233.
- Le Bellego F, Pisselet C, Huet C, Monget P & Monniaux D 2002 Laminin- $\alpha 6 \beta 1$ integrin interaction enhances survival and proliferation and modulates steroidogenesis of ovine granulosa cells. *Journal of Endocrinology* **172** 45–59.
- Lee WS, Otsuka F, Moore RK & Shimasaki S 2001 Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biology of Reproduction* **65** 994–999.
- Leers-Sucheta S, Morohashi K, Mason JI & Melner MH 1997 Synergistic activation of the human type II 3 beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase promoter by the transcription factor steroidogenic factor-1/adrenal 4-binding protein and phorbol ester. *Journal of Biological Chemistry* **272** 7960–7967.
- Liu Z & Simpson ER 1997 Steroidogenic factor 1 (SF-1) and SP1 are required for regulation of bovine CYP11A gene expression in bovine luteal cells and adrenal Y1 cells. *Molecular Endocrinology* **11** 127–137.
- Macias-Silva M, Hoodless PA, Tang SJ, Buchwald M & Wrana JL 1998 Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *Journal of Biological Chemistry* **273** 25628–25636.
- Monget P, Fabre S, Mulsant P, Lecerf F, Elsen JM, Mazerbourg S, Pisselet C & Monniaux D 2002 Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. *Domestic Animal Endocrinology* **23** 139–154.
- Monte D, DeWitte F & Hum DW 1998 Regulation of the human P450 scc gene by steroidogenic factor 1 is mediated by CBP/p300. *Journal of Biological Chemistry* **273** 4585–4591.
- Moore RK, Otsuka F & Shimasaki S 2003 Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. *Journal of Biological Chemistry* **278** 304–310.
- Mukherjee A, Park-Sarge OK & Mayo KE 1996 Gonadotropins induce rapid phosphorylation of the 3',5'-cyclic adenosine monophosphate response element binding protein in ovarian granulosa cells. *Endocrinology* **137** 3234–3245.
- Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, Pisselet C, Riquet J, Monniaux D, Callebaut I, Crihiu E, Thimonier J, Teyssier J, Bodin L, Cognie Y, Chitour N & Elsen JM 2001 Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola merino ewes. *PNAS* **98** 5104–5109.
- Nohe A, Keating E, Knaus P & Petersen NO 2004 Signal transduction of bone morphogenetic protein receptors. *Cellular Signalling* **16** 291–299.
- Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF & Shimasaki S 2000 Bone morphogenetic protein-15. Identification of target cells and biological functions. *Journal of Biological Chemistry* **275** 39523–39528.
- Otsuka F, Moore RK & Shimasaki S 2001a Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. *Journal of Biological Chemistry* **276** 32889–32895.
- Otsuka F, Yamamoto S, Erickson GF & Shimasaki S 2001b Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *Journal of Biological Chemistry* **276** 11387–11392.
- Pearson KL, Hunter T & Janknecht R 1999 Activation of Smad1-mediated transcription by p300/CBP. *Biochimica et Biophysica Acta* **1489** 354–364.
- Pescador N, Houde A, Stocco DM & Murphy BD 1997 Follicle-stimulating hormone and intracellular second messengers regulate steroidogenic acute regulatory protein messenger ribonucleic acid in luteinized porcine granulosa cells. *Biology of Reproduction* **57** 660–668.
- Pierre A, Fabre S, Pisselet C, Monniaux D & Monget P 2002 Rôle des bone morphogenetic proteins (BMPs) dans la fonction ovarienne chez la brebis. *Annals of Endocrinology* **63** 114.
- Poncelet AC & Schnaper HW 2001 Sp1 and Smad proteins cooperate to mediate transforming growth factor-beta 1-induced alpha 2(I) collagen expression in human glomerular mesangial cells. *Journal of Biological Chemistry* **276** 6983–6992.
- Pouponnot C, Jayaraman L & Massague J 1998 Physical and functional interaction of SMADs and p300/CBP. *Journal of Biological Chemistry* **273** 22865–22868.
- Richards JS & Hedin L 1988 Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. *Annual Review of Physiology* **50** 441–463.
- Rodway MR, Swan CL, Gillio-Meina C, Crellin NK, Flood PF & Chedrese PJ 1999 Regulation of steroidogenesis in jc-410, a stable cell line of porcine granulosa origin. *Molecular and Cellular Endocrinology* **148** 87–94.
- Saumande J 1991 Culture of bovine granulosa cells in a chemically defined serum-free medium: the effect of insulin and fibronectin on the response to FSH. *Journal of Steroid Biochemistry and Molecular Biology* **38** 189–196.
- Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N, Sampath K, Chang RJ & Erickson GF 1999 A functional bone morphogenetic protein system in the ovary. *PNAS* **96** 7282–7287.
- Souza CJ, MacDougall C, Campbell BK, McNeilly AS & Baird DT 2001 The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPRI-B) gene. *Journal of Endocrinology* **169** R1–R6.
- Souza CJ, Campbell BK, McNeilly AS & Baird DT 2002 Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry. *Reproduction* **123** 363–369.
- Sugawara T, Holt JA, Kiriakidou M & Strauss JF 3rd 1996 Steroidogenic factor 1-dependent promoter activity of the human steroidogenic acute regulatory protein (StAR) gene. *Biochemistry* **35** 9052–9059.
- Sugawara T, Kiriakidou M, McAllister JM, Kallen CB & Strauss JF 3rd 1997 Multiple steroidogenic factor 1 binding elements in the human steroidogenic acute regulatory protein gene 5'-flanking region are required for maximal promoter activity and cyclic AMP responsiveness. *Biochemistry* **36** 7249–7255.
- Sugawara T, Saito M & Fujimoto S 2000 Sp1 and SF-1 interact and cooperate in the regulation of human steroidogenic acute regulatory protein gene expression. *Endocrinology* **141** 2895–2903.
- Urban RJ, Garney JC, Shupnik MA & Veldhuis JD 1991 Follicle-stimulating hormone increases concentrations of messenger ribonucleic acid encoding cytochrome P450 cholesterol side-chain cleavage enzyme in primary cultures of porcine granulosa cells. *Endocrinology* **128** 2000–2007.
- Val P, Lefrançois-Martinez AM, Veyssiere G & Martinez A 2003 SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nuclear Receptor* **1** 8 ([url: www.nuclear-receptor.com/content/1/1/8](http://www.nuclear-receptor.com/content/1/1/8)).
- Vitt UA, Hayashi M, Klein C & Hsueh AJ 2000 Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. *Biology of Reproduction* **62** 370–377.
- Warner DR, Pisano MM & Greene RM 2003 Nuclear convergence of the TGFbeta and cAMP signal transduction pathways in

- murine embryonic palate mesenchymal cells. *Cellular Signalling* **15** 235–242.
- Wilson T, Wu XY, Juengel JL, Ross IK, Lumsden JM, Lord EA, Dodds KG, Walling GA, McEwan JC, O'Connell AR, McNatty KP & Montgomery GW 2001 Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. *Biology of Reproduction* **64** 1225–1235.
- Winters TA, Hanten JA & Veldhuis JD 1998 *In situ* amplification of the cytochrome P-450 cholesterol side-chain cleavage enzyme mRNA in single porcine granulosa cells by IGF-1 and FSH acting alone or in concert. *Endocrine* **9** 57–63.
- Xu J, Oakley J & McGee EA 2002 Stage-specific expression of Smad2 and Smad3 during folliculogenesis. *Biology of Reproduction* **66** 1571–1578.
- Yamamoto N, Akiyama S, Katagiri T, Namiki M, Kurokawa T & Suda T 1997 Smad1 and smad5 act downstream of intracellular signalings of BMP-2 that inhibits myogenic differentiation and induces osteoblast differentiation in C2C12 myoblasts. *Biochemical and Biophysical Research Communications* **238** 574–580.

Received 20 August 2004

Accepted 21 September 2004