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Cloning and cellular localization of the canine progesterone receptor: co-localization with growth hormone in the mammary gland

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Abstract

The mammary gland has been found to express the gene encoding growth hormone (GH) in several species. Within the mammary gland, it may act as an autocrine/paracrine growth factor for cyclic epithelial changes, and may be a determinant in mammary carcinogenesis. In the dog, progestins enhance mammary GH expression. To elucidate the mechanism of progestin-induced mammary GH expression, the canine progesterone receptor (PR) is characterized and the cellular localization of the PR in normal and tumorous mammary tissues is examined. Sequence analysis of the canine PR revealed two in-frame ATG codons, encoding a putative PR-B protein of 939 amino acids and a putative PR-A protein of 765 amino acids. Western blot analysis indicated that both isoforms occur in uterus and mammary gland issues. Immunohistochemical analysis of the PR revealed that the PR was differentially expressed in mammary tissue, with many PR-positive epithelial cells in the proliferation phase of the glandular tissue and a low number of PR-positive cells in differentiated mammary tissue. Stromal and myoepithelial cells had no specific PR staining. Mammary tumours had a variety of staining patterns, including no staining, normal nuclear staining, marked heterogeneous immunoreactivity and perinuclear staining of tumorous epithelial cells and cytoplasmic-staining of spindle cells. Double staining showed that all GH-producing cells were positive for PR, whereas not all PR containing cells stained for GH. It is concluded that the activated PR may transactivate GH expression in the mammary gland within the same cell and functions as a pre-requisite transcription factor. However, during malignant transformation this regulation may be lost.

Keywords: Growth hormone; Progesterone receptor; Autocrine; Paracrine; Carcinogenesis

1. Introduction

Progesterone plays a major role in regulating the growth, development and function of female reproductive tissues by stimulating or inhibiting the expression of specific genes (reviewed in [1,2]). The biological action of this steroid hormone is mediated by binding to the progesterone receptor (PR), which belongs to the steroid-thyroid-retinoid superfamily of ligand-activated transcription factors. These nuclear receptors share structural similarities and are composed of specific domains [3–5]. In most mammalian species, two PR isoforms have been described — PR-B and the N-terminal truncated PR-A, which are functionally distinct, since they differentially activate genes [2]. PR-B tends to be a stronger activator of target genes, while PR-A can act as a dominant repressor of PR-B. However, there are also genes more efficiently activated by PR-A [6].

Studies in PR knockout mice (PRKO) have shown that progesterone is essential for the formation of the lobular-alveolar structure in the mammary gland [7–9]. In addition, studies with PR-A transgenic mice revealed

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that an imbalance in the expression and/or activity of the two PR isoforms could also result in an abnormal mammary gland development [10].

The proliferative activity of mammary epithelium may vary among the various structures within the mammary gland. In rats, the epithelium located in terminal end buds (TEB) exhibited the highest rate of DNA synthesis; the rate decreased progressively towards the ductal portions and was even lower in alveolar buds (AB). Administration of progestins selectively increased the DNA labeling index in the TEBs [11].

In the dog, one of the target gene of progesterone in the mammary gland is the growth hormone (GH) gene [12–15]. Mammary GH may act as a local growth factor and contributes to the proliferation and differentiation of the mammary gland during the luteal phase of the estrous cycle and during the final maturation of the mammary gland during pregnancy. Autocrine or paracrine effects of GH have also been suggested to play a role in the development of canine mammary cancer [14,15]. The increase in cell proliferative activity may be responsible for increased susceptibility of the mammary gland to neoplastic transformation [14]. Mammary GH expression has also been reported in cats and humans [13,16], indicating that it is not a dog-specific phenomenon.

In normal tissue of the dog, GH expression is related to either endogenous progesterone or exogenous progestin exposure. However, some canine mammary carcinomas expressed GH albeit being PR negative in ligand-binding assays [9,11]. The mechanism by which progesterone/progestins activates the GH gene in mammary glands is not yet known. In this study, the canine PR is characterized by the analysis of the nucleotide sequence of the coding region of the PR gene, the PR isoform expression and the cellular localization of the PR in mammary gland tissue and mammary tumours. Moreover, a double-labeling method was used to co-localize PR and GH in mammary tissue of a dog with high-level GH gene expression.

2. Materials and methods

2.1. Tissues and RNA isolation

One mammary tissue sample was obtained from an experimental female beagle dog that had been treated with long-acting progestins after ovario-hysterectomy, as described earlier [12]. Another sample was from a control beagle dog, not receiving progestins. The other canine mammary gland tissues were obtained from privately owned dogs that were referred to the Utrecht University Clinic for Companion Animals. The bitches were presented to the clinic because of single or multiple mammary nodules. At surgery, mammary tumours and unaffected mammary tissue were obtained. Normal uterine tissue was obtained after ovario-hysterectomy. Tissue samples were frozen in liquid nitrogen and stored at −70°C until analysis, and/or fixed in a 10% buffered formalin solution, processed, and embedded in paraffin. Total RNA and poly (A)′-enriched RNA were isolated as described earlier [17].

Nucleotide sequence analysis of the coding region of the canine PR-A 658 bp fragment of the canine PR was amplified by PCR using human primers designed in the conserved hormone-binding region (domain E) of PR (forward, 5′-CTGACACCTCCAGTTCTTTGCTGAC-3′; reverse, 5′-GGTTTCACCATCCCTGCCAATATC-3′). Subsequently, a 607 bp PR cDNA fragment covering domains C and D was amplified using the degenerative forward primer 5′-GCCGTGCTCAAGGARGG-3′ (bp 1722–1738) and the canine-specific reverse primer 5′-CTACTGAAAGAAGTTGTCTCTCGCC-3′ (bp 2304–2328; numbering according to the canine PR sequence given in Fig. 1). RT–PCR reactions were performed as described earlier [13], using uterine or mammary total RNA.

The 3′ ends of canine PR cDNA were determined by rapid amplification of cDNA ends (RACE) (Marathon cDNA Amplification Kit; Clontech Laboratories, Palo Alto, CA). One microgram poly(A)$^+$ uterine and mammary RNA was used as template for cDNA synthesis. For the first round of 3′-RACE, adaptor primer AP1 (supplied with the kit), canine PR primer 5′-TACTGCTTGAATACATTTATCCAGTCCCGG-3′ (bp 2808–2837), and 30 cycles of denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (68°C, 4 min) were used. Nested 3′-RACE was performed using adaptor primer AP2, canine PR primer 5′-CGGGCACTGAGTGTTGAGTTTCCAGAAATG-3′ (bp 2835–2864), and 15 cycles of denaturation (94°C, 30 s) and annealing/extension (68°C, 4 min). All PCR reactions were carried out with Klentaq polymerase (Clontech), and with an initial denaturation step at 94°C for 2 min. Attempts to amplify the less-conserved 5′-half (A/B domain) of the canine PR cDNA by 5′-RACE were unsuccessful. Finally, the 5′-half (A/B domain) of the canine PR sequence was determined by sequence analysis of a BAC clone containing the canine PR gene. The canine genomic BAC library [18] was screened with a ^{32}P-labeled PR probe (bp 1722–2323) as described by [19], producing four positive clones. Restriction enzyme digestion analysis with *Bam*Hl, *Eco*RI, *Hin*dlll and *PSt*I, followed by Southern blotting and hybridization with the earlier used cDNA probe (bp 1722–2323) and a probe specific for the 3′ end of exon 1 (bp 1722–1770) showed that one BAC clone contained the 5′ part of the PR gene. This clone was subcloned in pUC18 and sequenced.

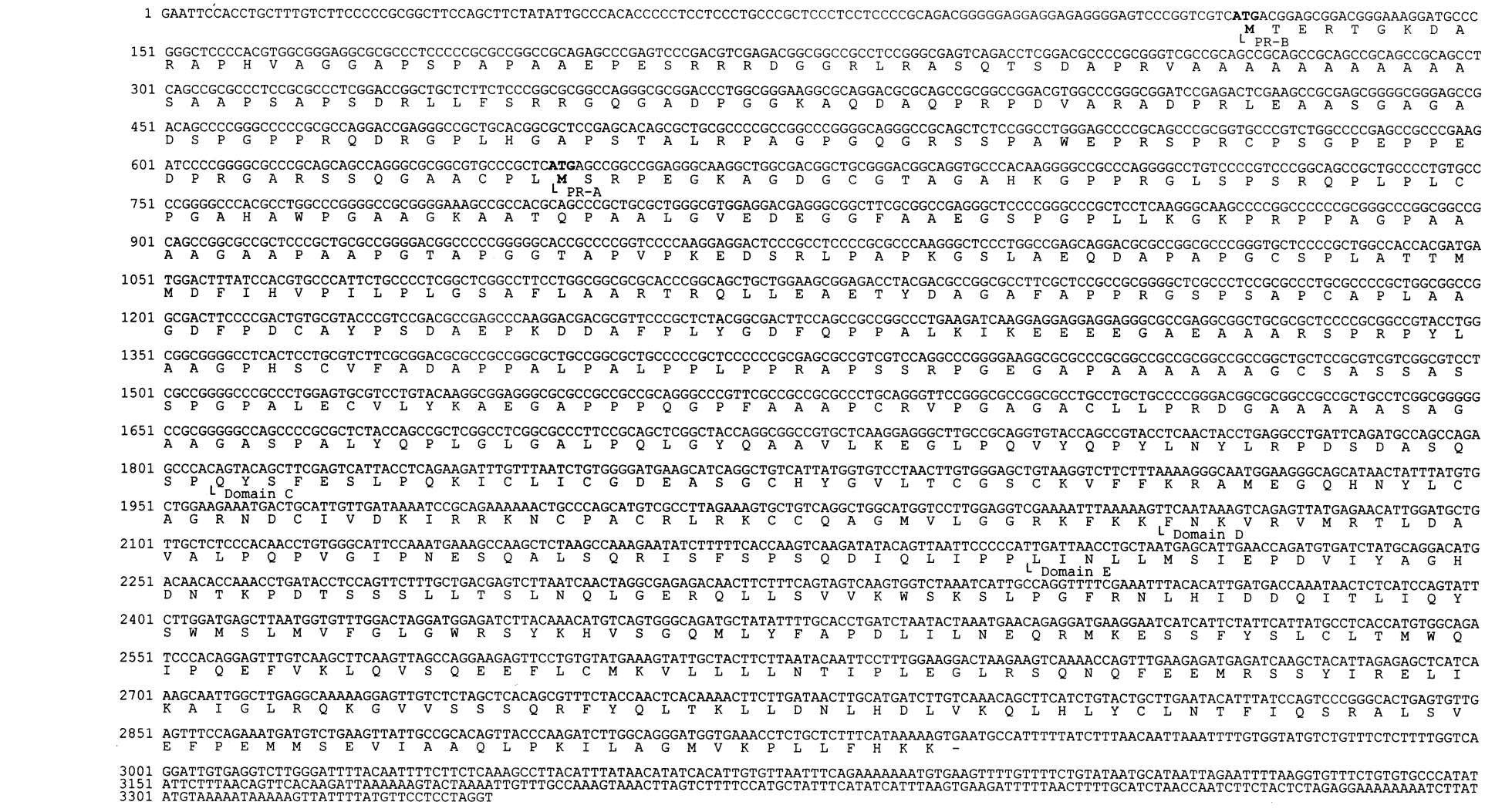

Fig. 1. Nucleotide and deduced amino acid sequences of canine PR. The two in-frame translation start codons (ATG_B and ATG_A) are given in bold type. The amino acid numbering refers to the longest open reading frame. The sequence has been submitted to the Genbank under the accession number AF 177470.

2.2. Sequence analysis

The PCR products were either cloned in pUC18 or pGEM-T and sequenced as earlier described [17], or gel purified and directly sequenced with an ABI310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Foster city, CA, USA), using 4 μl BigDye Termination Mix (Perkin Elmer) and 2.5 pmoles of primer in a total volume of 10 μl. The cycle conditions were; 25 cycles of 94°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The DNA-binding, hinge, and hormone-binding domains of the PR of two mammary tumours were sequenced both directly and after cloning, using several clones.

2.3. Cytosol preparation and steroid receptor analysis

Tissue (0.25–0.5 g) was pulverized in a precooled microdismembrator and homogenized in 2 ml of buffer as described earlier [20]. Occasionally, the protease inhibitors leupeptin (48 μg/ml), pepstatin (1 μg/ml), aprotinin (77 μg/ml) and phenylmethylsulphonyl fluoride (0.25 mol/1) were added. Homogenates were centrifuged at 4°C for 20 min at 30 000 rpm, and the supernatant was stored at −70°C until analysis. Prior to use, mammary cytosol samples were 2–4× concentrated using Centricon microconcentrators (Amicon, Beverly, MA, USA) with a 10 kDa cut-off. PR concentrations were measured in aliquots of the supernatant by Scatchard analysis of the [^{3}H]progesterone binding data, as earlier described [20].

2.4. Western blot analysis

Several human PR antibodies were evaluated for their ability to detect canine PR by Western blotting (NCL-PGR and NCL-PGRp, Novocastra; MA1-410, Affinity BioReagents; PR-AT 4.14, DAKO; N-559 and C-262, StressGen). The epitopes of these antibodies are highly or completely conserved between the human and canine PR. In addition, some antibodies with unknown epitopes (hPRa1, 3, 4, 5, 7; Neomarkers) but recognizing both PR-A and PR-B [21] and crossreacting with mouse [22] were tested. In our experience, hPRa5, alone, or in combination with hPRa4 and hPRa7, and MAI-410 (directed against amino acids 533–547) gave the best results in Western blot analysis.

Proteins were dissolved in 6× SDS sample buffer [23] at 95°C for 5 min and run on a 7.5% acrylamide–bisacrylamide (30:0.8) resolving gel with a 3.9% acrylamide–bisacrylamide stacking gel using a Mini-Protean 11 apparatus (Biorad) and a Laemmli's buffer system [23]. A set of pre-stained molecular weight standards (Biorad) was run in each gel. In addition, the approximate molecular weights of resolved proteins were determined by comparison with biotinylated-labelled markers (broad range, HRP; Biorad). After electrophoresis, proteins were blotted on PVDF membrane (Biorad) for 70 min at 120 V in a 25 mM Tris buffer with 192 mM glycine and 20% methanol, pH 8.1–8.4. Membranes were blocked for 1 h at room temperature in PBS (150 MM NaCl, 10 mM Na_2HPO_4) containing 5% non-fat milk and 0. 1% Tween20, and incubated overnight at 4°C with anti-progesterone antibody hPRa5 (or 50% hPRa5, 25% hPRa4, 25% hPRa7) diluted to 1 μg ml^{-1} in PBS containing 1% non-fat milk and 0. 1% Tween20. Membranes were washed in PBS containing 1% non-fat milk and 0.2% Tween (2 brief rinses, 1 × 15 min, 3 × 5 min) and then incubated for 1 h with diluted (1:2500) BRP-conjugated goat anti-mouse IgG (DAKO). After washing, visualization using ECL Plus reagents was according to the kit instructions (Amersham). Blots were exposed to Kodak Biomax films, using several exposure times. Membranes probed with MAI-410 (5 μg ml^{-1}) were blocked and washed, according to the instructions of the supplier (Affinity BioReagents). An extract of the human breast cancer cell line T47D and human breast carcinoma tissue (kind gifts from F.M. Verheijen, University Medical Center Utrecht, Department of Endocrinology, Utrecht) were used as positive controls. The specificity of the reaction was verified by the absence of immunostaining when primary or secondary antibodies were omitted, and by analyzing PR-negative canine liver cytosol.

2.5. Immunohistochemical localization of PR

From our diagnostic archives, formalin-fixed and paraffin-embedded tissue samples were selected. Cases included non-tumorous mammary tissues in different development stages and mammary tumours. From the selected tissues, 5 μm thick sections were cut, placed on poly-l-lysine coated slides (Sigma Diagnostics, St. Louis, MO), deparaffinized and rehydrated. Immunostaining was performed using the avidin–biotin peroxidase method. For antigen retrieval, sections were immersed in boiling 10 mM citrate buffer (pH 6.0) for 25 min. Sections were dehydrated and endogenous peroxidase activity was blocked by incubation with 1% H_2O_2 in 100% methanol for 30 min. Then, sections were rehydrated again and washed three times in PBS. Nonspecific antibody binding was blocked by pre-incubation with 10% normal horse serum for 15 min at room temperature. The monoclonal antibody PR10A9 (purchased from Immunotech) was used as the primary antibody, which recognizes an epitope in the human PR (amino acids 922–933) that is entirely conserved in the canine PR. Sections were incubated overnight with PR10A9 (diluted 1:100) at room temperature. Next, the slides were rinsed three times in PBS/Tween, and incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, USA) for 30 min (di-

luted 1:125 in PBS). Slides were washed three times in PBS, and incubated with avidin–biotin complex (ABC solution) for 30 min in a moist chamber at room temperature. Again, slides were washed three times in PBS. Immunoreactivity was visualized using 0.3% H_2O_2 and 3,3-diaminobenzidine tetrachloride (DAB) (Sigma) diluted in 0.05 M Tris–HCl, pH 7.6 for 10 min at room temperature. Sections were counterstained with Mayer's hematoxylin for 1 min, dehydrated in ethanol and xylene, and mounted with glass coverslips using Eukitt. Sections of canine uterus served as positive controls. The specificity of immunoreactivity was verified by absence of immunostaining when primary and secondary antibodies were omitted and when PR10A9 was substituted with normal mouse serum.

2.6. Immunohistochemical co-localization of PR and GH

Staining for the PR was performed as described above, as far as the reaction with DAB. Then slides were washed three times in PBS and dehydrated for blocking endogenous peroxidase again. The sections were rehydrated, washed three times in PBS/Tween and incubated with normal goat serum (NGS), diluted 1:10. For detecting GH, a polyclonal rabbit anti-porcine GH antibody (generous gift of Dr M.M. Bevers, Department of Farm Animals, Faculty of Veterinary Medicine, Utrecht University) was used, diluted 1:5000 in 10% NGS and applied overnight at 4°C. The slides were washed three times in PBS/Tween and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:100 in PBS for 30 min. After washing in PBS/Tween (3×5 min), sections were incubated with ABC complex, freshly prepared according to the manufacturer's instructions. Immunoreactive GH was visualized using 3-amino-9-ethylcarbazole (AEC) as the chromogen. The sections were counterstained with Mayer's haematoxylin for 1 min, washed in distilled water, and mounted with glass coverslips using Aquamount. A canine pituitary gland served as positive control.

In typical co-localization studies, a nuclear brown staining represented the PR and a reddish cytoplasmic staining indicated the presence of GH.

3. Results

3.1. Cloning and sequencing of the canine PR

Two overlapping fragments of the canine PR cDNA were amplified by RT–PCR using primers designed within well-conserved regions of the PR (domains C and E). The combined sequence, corresponding to codons 534–919 of the human PR [24], was used to develop canine-specific primers and perform 5′-RACE and 3′-RACE. The latter identified a 3′-UTR sequence of 396 bp. Attempts to amplify the 5′-part (A/B domain) of the canine PR cDNA by 5′-RACE using long-distance PCR were unsuccessful. According to the PR gene structure of other species, this long- and poor-conserved part of the PR is probably encoded by a single exon. Therefore, the 5′ part was isolated by screening of a genomic BAC library of the dog with a dog-specific PR cDNA probe. In this way, a BAC clone was isolated containing the most 5′ part (domains A and B) of the PR gene, subcloned and next partially sequenced to complete the nucleotide sequence of the coding region of the canine PR (Fig. 1). The canine PR sequence revealed two in-frame ATG codons, potentially encoding a PR-B protein of 939 amino acids and a PR-A protein that was 174 amino acids shorter. The canine progesterone receptor showed complete identity at the amino acid level for the DNA-binding domain of the human [25], rabbit [26], and chicken [27] PR and 98% homology with the murine [28] PR sequence. The hormone-binding domain showed high homology with the corresponding regions of these species (88–99%). Remarkably, and more pronounced than in PR sequences of other species, the 5′-half of the canine PR cDNA sequence is extremely GC rich over a long region, with an average GC content of 80% for codons 1–561 (A/B domain). In human, chicken, rabbit and mouse, the GC content in this region is 69, 68, 70 and 65, respectively.

3.2. Western blot analysis of canine PR

The presence of PR isoforms was investigated by Western blot analysis. A human breast cancer sample and the highly PR-positive breast cancer cell line T47D served as positive controls. In cytosol prepared from canine uterus tissue, a single PR-A of approximately 80 kDa and at least two putative PR-B proteins of approximately 110 and 130 kDa were detected (Fig. 2).

The concentration of PR in canine mammary cytosol appeared to be very low. Therefore, canine mammary cytosol preparations were concentrated and stained with a mixture of monoclonal antibodies (Fig. 2). In two samples obtained in metoestrus, the PR-A was most prominent, whereas the samples obtained during anoestrus or after progestin treatment also the PR-B form was found.

3.3. Immunohistochemical localization of the canine progesterone receptor

The cellular localization of the canine PR was examined by immunohistochemical analysis using the monoclonal antibody PR10A9. In normal canine uterus tissue, used as positive control, the epithelial cells lo-

cated within the deeper uterine glands had a nuclear immunoreactivity (Fig. 3A), with absence of immunoreactivity in the superficial epithelium. Also, the smooth muscle layers of the myometrium had a consistent nuclear staining. Stromal cells were negative.

In the mammary tissue, there was also a clear nuclear staining for PR in epithelial cells, with absence of staining in myoepithelial cells and stromal cells. In the proliferation phase of the glandular tissue, in which mammary ducts contain several layers of epithelial cells, only a subset of ductal epithelial cells with an intermediate and/or basal position was positive. High numbers of PR-positive cells were detected in budding structures, and immunoreactive cells were generally located at the outer rim of the bud (Fig. 3B). In lobuloalveolar tissue, characteristic of totally differentiated mammary tissue, in which milk protein synthesis may occur, only few alveolar epithelial cells were positive (Fig. 3C).

Diverse staining patterns were found in mammary tumours. Well-differentiated simple type adenomas had clear nuclear staining in all epithelial cells (Fig. 3D). Other simple type tumours (Fig. 3E), and also the complex type tumours, had markedly heterogeneous nuclear staining. In carcinomas, negative (Fig. 3F) and heterogeneous PR-staining (Fig. 3G) could be observed. In non-tumorous pre-existing mammary tissues adjacent to the PR-negative carcinomas immunoreactivity was found (Fig. 3F). In some carcinomas a quite different, but highly reproducible, PR staining pattern was observed. Instead of exclusively nuclear staining in these tumorous epithelial cells, intense perinuclear staining was found (Fig. 3H). In complex type tumours, the spindle cell component frequently showed cytoplasmic staining. To determine whether mutations in the hormone-binding domain of PR were associated with the cytoplasmic staining for PR in these tumours, the PR of two of these tumours was analyzed for mutations. The sensitive RT–PCR analysis was able to detect the presence of PR mRNA in these tumours (not shown). The PR sequence covering domains C, D and E was amplified by PCR and sequenced, revealing no mutations.

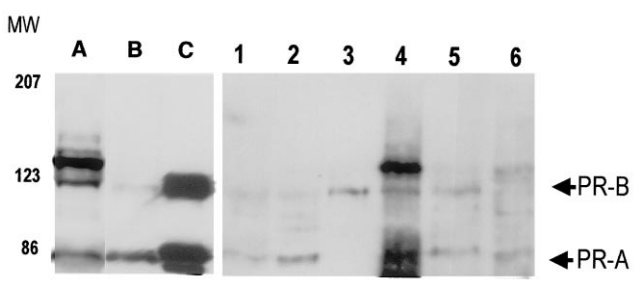

Fig. 2. Western blot analysis of progesterone receptors. Lane A–C: Comparison of the migrating pattern of canine and human progesterone receptors. Depleted are cytosol homogenates of a canine uterus (A); a human mammary carcinoma (B); or the human breast cancer cell line T47D (C) using monoclonal antibody hPRa5. Lane 1–6: Cytosol homogenates from the mammary gland obtained from dogs in metoestrus (lane 1 + 2); in anoestrus (lane 3 + 4) or after prolonged treatment with progestins (lane 5 + 6). Blots were stained using a mixture of antibodies hPRa4, hPRa5, and hPRa7. The putative PR-A and PR-B proteins are indicated.

3.4. Co-localization of canine PR en GH

In mammary tissue of a dog with a high level of mammary GH gene expression, a double labeling method was performed to co-localize PR and GH. In this mammary sample, the presence of cytoplasmic GH represented the local synthesis of GH, as earlier evidenced by in situ hybridization. As shown in (Fig. 3I), cytoplasmic GH was found in the same cells positive for nuclear PR staining in hyperplastic mammary epithelial tissue. Careful examination of several slides revealed that all GH-producing cells stained positively for PR. However, the opposite was not true, i.e. not all cells containing progesterone receptors produced GH.

4. Discussion

To further elucidate the mechanism of progestin-induced mammary GH expression, the canine PR was characterized at the molecular and cellular level. Sequence analysis of the PR revealed an open reading frame encoding a putative canine PR-B protein of 939 amino acids and a putative PR-A protein that was 174 amino acids shorter. The canine PR has the characteristic steroid receptor structure, comprising a highly conserved C-terminal half, containing the hormone- and DNA-binding domains, and a less conserved N-terminal half [3,5]. The reported blocks of conserved sequences among human, mouse, rabbit and chicken [24,28] were also present in the canine PR, strengthening a common function for these regions. Similar to chicken PR, the entire *N*-terminal region (A/B domain) of the canine PR is encoded by a single exon [29]. A remarkable difference between the PR sequence of the dog and that of other species is the unusually high GC content (80 vs. 65–70%) in the *N*-terminal half (codons 1–561). This could well explain why our 5′-RACE attempts were unsuccessful. In our experience, even with the use of canine-specific PR primers, amplification of this region by PCR failed unless solutions were used to lower the secondary structures.

Western blot analysis suggested that canine PR occurs in multiple isoforms. The putative canine PR-A co-migrated with PR-A of the human breast cancer cell line T47D and a human mammary breast cancer sample. In various cytosols of mammary and uterine tissue, a 130 kDa PR-B form was found. Canine mammary

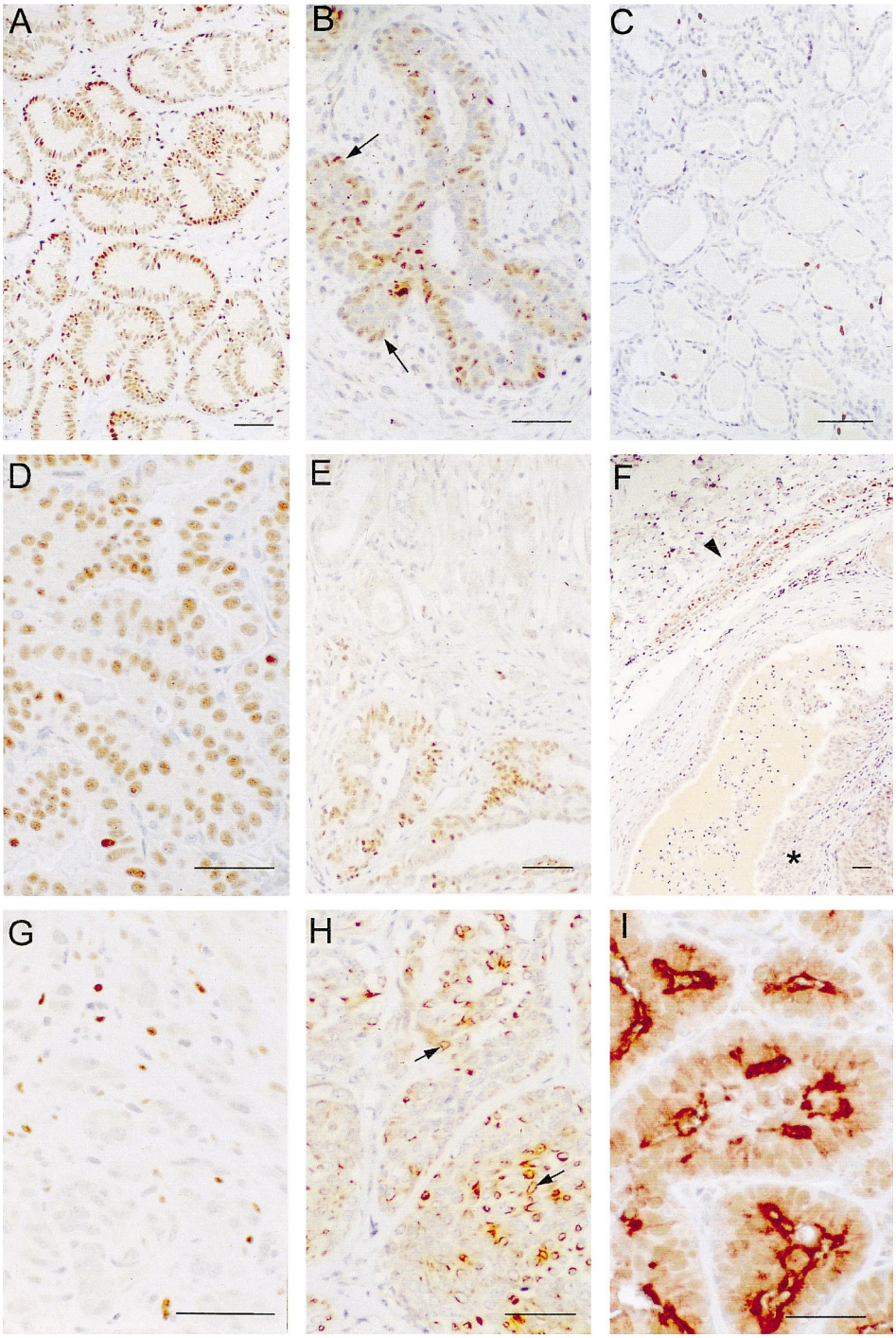

Fig. 3. Immunohistochemical detection of PR in canine uterus and normal and tumorous mammary tissues. The bar represents 40 μm. (A) Normal uterus used as the positive control, showing strong nuclear staining in epithelial cells and no staining in stromal cells (× 200). (B) Normal mammary gland in the proliferation phase, showing strong nuclear PR staining in ductal epithelial cells having an intermediate or basal position. In the ductal buds (arrow) the positive cells are especially located at the outer rim of the bud (× 305). (C) Completely differentiated lobuloalveolar glandular mammary tissue in which milk protein synthesis occurs. Only a few individual alveolar epithelial cells stain positively for PR (× 240). (D) Well-differentiated tubular adenoma. All epithelial cells show definite nuclear staining for PR (× 410). (E) Intraductal papillary adenoma characterized by markedly heterogeneous immunoreactivity (× 240). (F) Low power magnification of a tubulo-papillary adenocarcinoma. Tumor cells (asterisk) are negative, while the compressed normal mammary tissue shows PR immunoreactivity (arrow head) (× 100). (G) Undifferentiated carcinoma; few carcinoma cells show nuclear staining, whereas the remaining tumor cells are completely negative for PR (× 510). (H) Solid carcinoma showing perinuclear PR staining (arrow). In the ligand-binding assay this sample was negative for the PR (× 360). (I) Co-localization of PR and GH. A nuclear brown staining represents the PR and a reddish cytoplasmic staining indicates the presence of GH (× 410).

tissue contained a 110 kDa PR-B form, although the higher PR-B bands were occasionally observed as well. None of the putative PR-A and PR-B bands was observed in liver cytosol, and when the first or second antibody was omitted. The difference in migration of the putative PR-B bands with the human PR-B form may reflect difference in the level of phosphorylation, as reported for human and mouse PRs [28,30].

The ratio of PR-A to PR-B has been reported to vary between different species. Approximately, equimolar expression of both PR forms is observed in chick oviduct, human and avian uterus, and cultured human breast cancer cells. PR-A appears to be absent in rabbits and predominant in rodent mammary gland, uterus and vagina [2]. In most canine mammary samples, PR-A was equimolar or more prominently present than PR-B. In one case, however, only PR-B could be visualized. In addition to the species difference, the ratio of the two PR isoforms has been reported to be tissue specific and under developmental regulation. The relative expression also appears to differ between tumor breast samples [31], which may have clinical significance in the endocrine treatment of cancers.

Immunohistochemical analysis revealed that uterine epithelial cells located within the deeper uterine glands showed nuclear immunoreactivity, whereas PR immunoreactivity was absent in the superficial epithelium and stromal cells. These findings are in agreement with the recently reported cellular localization of canine uterine PR by Dhaliwal et al. [32]. In normal mammary glands, the percentage of positive cells and the intensity of staining were highest in proliferative epithelial areas and low in totally differentiated cells. The epithelial cells displaying PR staining were basally located, which may indicate that they are undifferentiated stem cells. As in the uterus, no specific PR staining was observed in stromal cells. Biochemical analysis of isolated mammary stroma and epithelium in the mouse suggested the presence of a stromal and epithelial population of PR. However, it appears that stromal PRs may be more difficult to detect, as to date no direct evidence has been presented to localize the stromal PR in situ (reviewed in [33]).

By double labeling, it is demonstrated that in non-tumorous mammary glands PR and GH co-localize. All GH-producing cells were positive for PR, whereas not all PR-containing cells stained for GH. As it has been shown that a putative progesterone/glucocorticoid response element (PRE/GRE) is present in the 5′-flanking region of the canine GH gene (Lantinga-van Leeuwen et al., submitted) the co-localization supports the concept that ligand-activated progesterone receptors may play a direct role in GH gene promoter activation. PR may exert its stimulatory effects on mammary GH expression either by making the GH promoter available for binding of other transcription factors (chromatin remodelling) and/or by direct activation of the GH gene promoter. The finding that not all PR-positive cells express GH indicates that additional factors are involved in the control of mammary GH expression. It is not very likely that differences in relative expression of PR-A and PR-B solely account for the differential GH expression, as no consistent difference in the PR ratio was observed in mammary cells highly producing GH and scarcely producing GH.

In mammary tumours of dogs and humans, PR gene expression may become lost, especially in advanced states of disease [34,35]. Mammary tumours had a variety of staining patterns for PR, including normal staining, no staining and markedly heterogeneous staining. Negative staining was not likely to be due to an experimental artefact because PR immunoreactivity was seen in non-malignant epithelial nuclei present in the same specimen. Several mammary tumours had a remarkably different staining pattern, with perinuclear staining of epithelial cells and occasionally cytoplasmic staining in spindle cells. Spindle cells are a component of canine complex mammary tumours. A recent study has indicated that these cells may not be of myoepithelial origin, as was earlier presumed, considering their immuno-histochemical characteristics [36]. The different staining patterns as found for the PR has been attributed to disturbances in nuclear transport [4] and have also been found for androgen receptor staining in many human prostate tumor specimens, in contrast to the homogenous androgen receptor staining in normal prostate epithelium [37,38].

Some of the canine tumours with the aberrant PR staining pattern were earlier reported to express the GH gene but were PR negative in ligand-binding assay [13,15]. A possible explanation for the non-nuclear staining and negative ligand-binding results could be the presence of a mutation in the hormone-binding region of PR (but not disturbing the PR antibody epitope). The RT–PCR analysis demonstrated that there is at least low PR gene expression in these tumours. However, no PR mutations were detected. The absence of nuclear PR in GH-expressing mammary carcinomas suggests that the normal control of GH by PR is lost in these tumours. Mutations in $G_s\alpha$, which is known to be involved in pituitary tumours constitutively expressing GH [39,40], did not account for the autonomous GH expression in these tumours (unpublished). In conclusion, both PR-A and PR-B isoforms occur in the dog and the ratio of these proteins appear to vary among different tissues. The co-localization of PR and GH indicates that PR is a pre-requisite, but is not sufficient, for progestin-induced GH gene expression in normal mammary cells.

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References

[1] C.L. Clarke, R.L. Sutherland, Progestin regulation of cellular proliferation, Endocr. Rev. 11 (1990) 266–301.
[2] J.D. Graham, C.L. Clarke, Physiological action of progesterone in target tissues, Endocr. Rev. 18 (1997) 502–519.
[3] R.M. Evans, The steroid and thyroid hormone receptor superfamily, Science 240 (1988) 889–895.
[4] M.A. Carson-Jurica, W.T. Schrader, B.W. O' Malley, Steroid receptor family: structure and functions, Endocr. Rev. 11 (1990) 210–220.
[5] R. Kumar, E.B. Thompson, The structure of the nuclear hormone receptors, Steroids 64 (1999) 310–319.
[6] M.-C. Keightley, Steroid receptor isoforms: exception or rule?, Mol. Cell. Endocrinol. 137 (1998) 1–5.
[7] J.P. Lydon, F.J. DeMayo, C.R. Funk, S.K. Mani, A.R. Hughes, C.A. Montgomery, Jr, G. Shyamala, O.M. Conneely, B.W. O'Malley, Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities, Genes Dev. 9 (1995) 2266–2278.
[8] R.C. Humphreys, J. Lydon J, B.W. O'Malley, J.M. Rosen, Mammary gland development is mediated by both stromal and epithelial progesterone receptors, Mol. Endocrinol. 11 (1997) 801–811.
[9] G. Shyamala, Roles of estrogen and progesterone in normal mammary gland development, insights from progesterone receptor null mutant mice and in situ localization of receptor, Trends Endocrinol. Metab. 8 (1997) 34–39.
[10] G. Shamala, X. Yang, G.B. Silberstein, M.H. Barcellos-Hoff, E. Dale, Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands, Proc. Natl. Acad. Sci. USA 95 (1998) 696–701.
[11] I.H. Russo, J. Russo, Progestagens and mammary gland development: differentiation versus carcinogenesis, Acta Endocrinol. (Copenhagen) 125 (suppl 1) (1991) 7–12.
[12] P.J. Selman, J.A. Mol, G.R. Rutteman, A. Rijnberk, Progestin-induced growth hormone excess in the dog originates in the mammary gland, Endocrinology 134 (1994) 287–292.
[13] J.A. Mol, E. van Garderen, P.J. Selman, J. Wolfswinkel, A. Rijnberk, G.R. Rutteman, Growth hormone mRNA in mammary gland tumors of dogs and cats, J. Clin. Invest. 95 (1995) 2028–2034.
[14] J.A. Mol, E. van Garderen, G.R. Rutteman, A. Rijnberk, New insights in the molecular mechanism of progestin-induced proliferation of mammary epithelium; induction of the local biosynthesis of growth hormone (GH) in the mammary gland of dogs, cats and humans, J. Steroid Biochem. Mol. Biol. 57 (1996) 67–71.
[15] E. van Garderen, M. de Wit, W.F. Voorhout, G.R. Rutteman, J.A. Mol, H. Nederbragt, W. Misdorp, Expression of growth hormone in canine mammary tissue and mammary tumors. Evidence for a potential autocrine/paracrine stimulatory loop, Am. J. Pathol. 150 (1997) 1037–1047.
[16] J.A. Mol, S.C. Henzen-Logmans, Ph. Hageman, W. Misdorp, M.A. Blankenstein, A. Rijnberk, Expression of the gene encoding growth hormone in the human mammary gland, J. Clin. Endocrinol. Metab. 80 (1995) 3094–3096.
[17] I.S. Lantinga-van Leeuwen, M. Oudshoorn, J.A. Mol, Canine mammary growth hormone gene transcription initiates at the pituitary specific start site in the absence of Pit-1, Mol. Cell. Endocrinol. 150 (1999) 121–128.
[18] R. Li, E. Mignot, J. Faraco, H. Kodatani, J.J. Cantanese, B.H. Zhao, X. Lin, L. Hinton, E.A. Ostrander, D.F. Patterson, P. de Jong, Construction and characterization of an eight fold redundant dog genomic bacterial artificial chromosome library, Genomics 58 (1999) 9–17.
[19] B.J.A. van de Sluis, M. Breen, M. Nanji, M. van Wolferen, P. de Jong, M.M. Birms, P.L. Pearson, J. Kuipers, J. Rothuizen, D.W. Cox, C. Wijmenga, B.A. van Oost, Genetic mapping of the copper toxicosis locus in Bedlington terriers to dog chromosome 10, in a region syntenic to human chromosome region 2p13–16, Hum. Mol. Genet. 8 (1999) 501–507.
[20] G.R. Rutteman, N. Willekes-Koolschijn, M.M. Bevers, A.A. van der Gugten, W. Misdorp, Prolactin binding in benign and malignant mammary tissue of female dogs, Anticancer Res. 6 (1986) 829–835.
[21] C.L. Clarke, R.J. Zaino, P.D. Feil, J.V. Miller, M.E. Steck, B.M. Ohlsson-Wilhelm, P.G. Satyaswaroop, Monoclonal antibodies to human progesterone receptor: characterization by biochemical and immunohistochemical techniques, Endocrinology 121 (1987) 1123–1132.
[22] W. Schneider, C. Ramachandran, P.G. Satyaswaroop, G. Shyamala, Murine progesterone receptor exists predominantly as the 83-kDa 'A' form, J. Steroid Biochem. Mol. Biol. 38 (1991) 285–291.
[23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[24] P. Kastner, A. Krust, B. Turcotte, U. Stropp, L. Tora, H. Gronemeyer, P. Chambon, Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B, EMBO J. 9 (1990) 1603–1614.
[25] M. Misrahi, M. Atger, L. d'Auriol, H. Loosfelt, C. Meriel, F. Fridlansky, A. Guiochon-Mantel, F. Galibert, E. Milgrom, Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA, Biochem. Biophys. Res. Commun. 143 (1987) 740748.
[26] H. Loosfelt, M. Atger, M. Misrahi, A. Guiochon-Mantel, C. Meriel, F. Logeat, R. Benarous, E. Milgrom, Cloning and sequence analysis of rabbit-progesterone-receptor complementary DNA, Proc. Natl. Acad. Sci. USA 83 (1986) 9045–9049.
[27] H. Gronemeyer, B. Turcotte, C. Quirin-Stricker, M.T. Bocquel, M.E. Meyer, Z. Krozowski, J.M. Jeltsch, T. Lerouge, J.M. Gamier, P. Chambon, The chicken progesterone receptor: sequence, expression and functional analysis, EMBO J. 6 (1987) 3985–3994.
[28] D.R. Schott, G. Shyamala, W. Schneider, G. Parry, Molecular cloning, sequence analyses, and expression of complementary DNA encoding murine progesterone receptor, Biochemistry 30 (1991) 7014–7020.
[29] J.M. Jeltsch, B. Turcotte, J.M. Gamier, T. Lerouge, Z. Krozowski, H. Gronemeyer, P. Chambon, Characterization of multiple mRNAs originating from the chicken progesterone receptor gene, J. Biol. Chem. 265 (1990) 3967–3974.
[30] P.L. Sheridan, M.D. Francis, K.B. Horwitz, Synthesis of human progesterone receptors in T47D cells. Nascent A- and B-receptors are active without a phosphorylation-dependent post-translational maturation step, J. Biol. Chem. 264 (1989) 7054–7058.

[31] J.D. Graham, C. Yeates, R.L. Balleine, S.S. Harvey, J.S. Milliken, A.M. Bilous, C.L. Clarke, Characterization of progesterone receptor A and B expression in human breast cancer, Cancer Res. 55 (1995) 5063–5068.

[32] G.K. Dhaliwal, G.C.W. England, D.E. Noakes, Immunohistochemical localization of oestrogen and progesterone receptors in the uterus of the normal bitch during oestrus and metoestrus, J. Reprod. Fertil. (Suppl.) 51 (1997) 167–176.

[33] R.C. Humphreys, J.P. Lydon, B.W. O'Malley, J.M. Rosen, Use of PRKO mice to study the role of progesterone in mammary gland development, J. Mam. Gland Biol. Neoplasia 2 (1997) 343–354.

[34] G.R. Rutteman, W. Misdorp, M.A. Blankenstein, W.E. van den Brom, Oestrogen (ER) and progestin receptors (PR) in mammary tissue of the female dog: different receptor profile in non-malignant and malignant states, Br. J. Cancer 58 (1988) 594–599.

[35] R.G. Lapidus, S.J. Nass, N.E. Davidson, The loss of estrogen and progesterone receptor gene expression in human breast cancer, J. Mam. Gland Biol. Neoplasia 3 (1998) 85–94.

[36] J.H. Vos, T.S. van den Ingh, W. Misdorp, R.F. Molenbeek, F.N. van Mil, G.R. Rutteman, D. Ivanyi, F.C. Ramaekers, Immunohistochemistry with keratin, vimentin, desmin, and α-smooth muscle actin monoclonal antibodies in canine mammary gland: benign mammary tumours and duct ectasias, Vet. Q. 15 (1993) 89–95.

[37] M.V. Sadi, P.C. Walsh, E.R. Barrack, Immunohistochemical study of androgen receptors in metastatic prostate cancer. Comparison of receptor content and response to hormonal therapy, Cancer 67 (1991) 3057–3064.

[38] G.S. Prins, R.J. Sklarew, L.P. Pertschuk, Image analysis of androgen receptor immunostaining in prostate cancer accurately predicts response to hormonal therapy, J. Urol. 159 (1998) 641–649.

[39] L. Vallar, A. Spada, G. Giannattasio, Altered Gs and adenylate cyclase activity in human GH-secreting pituitary adenomas, Nature 330 (1987) 566–568.

[40] C.A. Landis, S.B. Masters, A. Spada, A.M. Pace, H.R. Bourne, L. Vallar, GTPase inhibiting mutations activate the α chain of Gs and stimulate adenylyl cyclase in human pituitary tumours, Nature 340 (1989) 692–696.