

The PROGINS polymorphism of the human progesterone receptor diminishes the response to progesterone

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Abstract

The human progesterone receptor (PR) is a ligand-dependent transcription factor and two isoforms, (PRA and PRB), can be distinguished. PROGINS, a PR polymorphic variant, affects PRA and PRB and acts as a risk-modulating factor in several gynaecological disorders. Little is known about the functional consequences of this variant. Here, we characterise the properties of PROGINS with respect to transcription, mRNA maturation, protein activity and proliferation. PROGINS is characterised by a 320 bp PV/HS-1 Alu insertion in intron G and two point mutations, V660L in exon 4 and H770H (silent substitution) in exon 5. The Alu element contains a half oestrogen-response element/Sp1-binding site (Alu-ERE/Sp1), which acts as an *in-cis* intronic enhancer leading to increased transcription of the PROGINS allele in response to 17 β -oestradiol. Moreover, Alu insertions in the human genome are frequently methylated. Our data indicate that the PROGINS-Alu does not affect gene transcription due to DNA methylation. However, the Alu element reduced the stability of the PROGINS transcript compared with the CP allele and does not generate splice variants. The amino acid substitution (V660L) in exon 4 leads to differences in PR phosphorylation and degradation in the two PR variants upon ligand binding, most likely as a result of differences in the three-dimensional structures of the two PR variants. As a consequence, the PR-L660 (PROGINS) variant (1) displays decreased transactivation activity in a luciferase reporter system and (2) is less efficient in opposing cell proliferation in hamster ovarian cells expressing human PRA, when compared with the PR-V660 (most common variant). Taken together, our results indicate that the PROGINS variant of PR is less responsive to progestin compared with the most common PR because of (i) reduced amounts of gene transcript and (ii) decreased protein activity.

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Introduction

Progesterone, together with 17 β -oestradiol, is a key regulator of cell proliferation and differentiation in the female genital tract. Altered responses to oestrogen and progesterone lead to steroid hormone-dependent disorders like endometriosis, endometrial cancer, sub-fertility, breast and ovarian cancer.

All major actions of progesterone are mediated by the two progesterone receptor (PR) isoforms (PRA and PRB), which are ligand-dependent transcription factors (Beato & Klug 2000, Li & O'Malley 2003). PRB differs from PRA by the presence of 164 additional amino acids at the N-terminus. The remaining part is identical for both proteins and consists of the DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD). A hinge region is located between DBD and LBD. This region is involved in nuclear translocation, dimerisation and cofactor binding, but its function in large part remains unclear (Beato & Klug 2000, Li & O'Malley 2003). After ligand binding and formation of hetero- or homodimers, PRA and PRB

bind to progesterone-response elements (PRE) located in the promoter region of target genes and modulate their expression. Studies on cell lines (Richer *et al.* 2002) and animal models (Lydon *et al.* 1995, Mulac-Jericevic *et al.* 2000, 2003, Conneely *et al.* 2003) have shown that PRA and PRB have distinct functional activities.

The PR gene, located on chromosome 11q22–23, comprises eight exons and seven introns (A–G). One PR polymorphic variant, PROGINS (Rowe *et al.* 1995, Agoulnik *et al.* 2004) consists of a 320 bp PV/HS-1 Alu insertion in intron G and two single nucleotide polymorphisms (SNPs). SNP-G3432T (mRNA nucleotide counting, NCBI: X51730) affects exon 4 and causes an amino acid substitution (V660L), whereas SNP-C3764T affects exon 5 and is a silent mutation (H770H). These three aberrations are in complete linkage disequilibrium (De Vivo *et al.* 2002, Agoulnik *et al.* 2004, Schweikert *et al.* 2004, Pijnenborg *et al.* 2005) and a plethora of epidemiological studies indicate that PROGINS might modify the risk for several benign and malign gynaecological disorders (Dunning *et al.* 1999,

Lattuada *et al.* 2004, Modugno 2004, Schweikert *et al.* 2004, Pijnenborg *et al.* 2005, Romano *et al.* 2006). However, the results are not conclusive.

Considering the possible consequences of each of the three PROGINS aberrations, only the Alu insertion and the V660L amino acid substitution are expected to affect the function of the receptor. In fact, RNA processing is not influenced by the two SNPs (G3432T in exon 4 or the C3764T in exon 5). Preliminary algorithm analyses to predict the secondary structures of RNA transcripts containing the two PROGINS SNPs showed that neither RNA folding, nor the binding sites for ribonucleoprotein complexes involved in mRNA maturation are affected by these two SNPs. Furthermore, the silent mutation (H770H) is not expected to alter the properties of the PR protein. Several authors have indicated that Alu insertions can be of functional relevance with respect to CpG islands DNA methylation, gene transcription and binding of transcription factors (Deininger & Batzer 1999, Gibbons & Dugaiczuk 2005) and the generation of splice variants due to Alu exonisation (Deininger & Batzer 1999, Lev-Maor *et al.* 2003, Sorek *et al.* 2004). Thus, the intron G Alu insertion could affect transcription and pre-mRNA maturation, including splicing, whereas the V660L amino acid substitution could only affect the activity of the protein.

Given that PROGINS occurs at frequencies up to 0.15 in Caucasians of different origins, the functional characterisation of this polymorphism is important to clarify its role in the pathogenesis. To this end, we have investigated the individual consequences of the two most likely relevant gene and protein aberrations associated with the PROGINS, the intronic Alu insertion and the V660L amino acid substitution, with respect to their effects on RNA maturation, protein activity and proliferation using different *in vitro* approaches.

In the following part of this study, the most common polymorphic allele or protein variant (i.e. no Alu insertion and V660) will be indicated as CP allele or protein.

Materials and methods

Cell lines

Human cervical cancer cell line HeLa, human ovarian cancer cell lines MDAH:2774 and Skov-3, human breast cancer cell lines T47D and MCF7 and Chinese hamster ovarian cell line CHO-K1 were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). With the exception of T47D cells, none of the cell lines mentioned above express the PR. MCF7 cells are homozygous PROGINS, whereas T47D cells are heterozygous. The remaining cell lines are homozygous CP. Cells were maintained in Roswell Park Memorial Institute medium (RPMI; Invitrogen Life Technologies, Inc.) supplemented with 10% foetal bovine serum

(Hyclone, Gent, East Flanders, Belgium), 2 mM L-glutamine, 2 mM Na pyruvate, 100 U/l penicillin and 100 mg/l streptomycin).

Steroid hormones

Progesterone, org2058 and the antagonists (RU486 or org31710) were a gift from Organon NV (Oss, Brabant, The Netherlands). 17 β -Oestradiol and tamoxifen were purchased from Sigma-Aldrich Chemie BV. All hormones were dissolved in ethanol at a stock concentration of 10⁻³ M.

Oligonucleotides

Oligonucleotides used for PCR amplification were purchased from MWG-Biotech AG (Ebersberg, Bayern, Germany) and are listed in Table 1.

Plasmids

Plasmid for the expression of the reporter gene β -galactosidase (pSV- β -galactosidase) was purchased from Promega GmbH. PR-responsive reporter plasmid MMTV-luciferase (MMTV-LUC; Muller *et al.* 2000) was a gift from Prof. Schuele. Expression plasmids for PRA-V660 (CP), PRA-L660 (PROGINS protein variant), PRB-V660 and PRB-L660 (Agoulnik *et al.* 2004) were based on plasmid pcDNA3.1 (Invitrogen Life Technologies, Inc.), and were kindly provided by Dr Agoulnik. Minigenes for the expression of PRA-IntG-CP, PRB-IntG-CP and PRA-IntG-Alu, PRB-IntG-Alu were constructed by re-inserting the complete intron G derived either from the CP allele (2672 nucleotides) or from the PROGINS (2992 nucleotides) allele at its original genomic position in the cDNAs of PRA and PRB. The complete intron G was cloned by PCR in four steps from genomic DNA isolated from cell lines Skov-3 (CP homozygous) and MCF7 (PROGINS homozygous) as templates. The Expand High Fidelity Kit (Roche) was used for PCR as recommended by the manufacturer. A detailed description of all cloning steps is given in Fig. 1. Figure 2A shows the structure of the intron G and relevant restriction sites used for cloning.

Plasmids used for transfections were prepared using the Qiaquick Plasmid maxi KIT (Qiagen GmbH). Plasmid concentration was determined spectrophotometrically and by agarose gel electrophoresis.

Stable and transient transfection

Stable transfection of CHO-K1 cells with either one of the plasmids encoding PRA-V660, PRB-V660, PRA-L660 or PRB-L660 were performed using jetPEI (Q-Biogene, Heidelberg, Baden-Württemberg, Germany) as

Table 1 Oligonucleotides and their applications

Name	Sequence	For/rev	Application
PR-GA-FW	5' TACATTAGAGAGCTCATCAAGGC	For	1,2,3
PR-GA-RV	5' TGTGATCTGAACATAGTAGCAATA	Rev	1
PR-GB-FW	5' TGTGGTTTGTCAAGATATATGAAATA	For	1
PR-GB-RV	5' CCTTAGTTCAATTGTGCAGTGC	Rev	1
PR-GC-FW	5' TTGCTGGAAGTGAACATACAAG	For	1
PR-GC-RV	5' TTCTGGAATTCAACACTCAGTG	Rev	1
PR-GD-FW	5' GCCATGTTTGAATAGCATATGAATTTAT	For	1
PR-GD-RV	5' CTCGAGACATTTCACTTTTTATGAAAGAGAAGGG	Rev	1·2
Exon-4 S	5' GTCAGAGTTGTGAGAGCACTG	For	3,4,5
Exon-4 AS	5' CAGGTTGATCAGTGGTGAAT	Rev	4·5
Exon-5 AS	5' CTCATTTAGTATTAGATCAGGT	Rev	4
RSF-3	5' TCTGGGGCGTCGTGCGCAAA	For	2
RSF-4	5' GAACCTTGATGAAGCCTGTG	Rev	2
CycloA S	5' TTCCTGCTTTACAGAATTATTCC	For	3
CycloA AS	5' GCCACCAGTGCCATTATGG	Rev	3
PROGINS-I	5' GCCTCTAAAATGAAAGGCAGAAAGC	For	7
PROGINS-II	5' CAAAAGTATTTTCTTGCTAAATGTCTG	Rev	6·7
Alu-Ex-RV	5' GCTACTTGGGAGGCTGAGGCAGG	Rev	3
PS2-0	5' CCGGCCATCTCTCACTATGAA	For	6
PS2-1	5' CCTCTTTCCCATGGGAGTCTC	Rev	6
PS2-2	5' CATCGACGTCCCTCCAGAAGAG	For	2
PS2-3	5' CTCTGGGACTAATCACCGTGCTG	Rev	2
ChIP-pos-FW	5' TACTAGCGGTTTTACGGGCG	For	6
ChIP-pos-RV	5' TCGAACAGGAGCAGAGAGCGA	Rev	6
ChIP-neg-FW	5' ATGTTGCCACTGGGGATCT	For	6
ChIP-neg-RV	5' TGCCAAAGCCTAGGGGAAGA	Rev	6
Alu-ERE-Sp1-FW	5' GGGTTTACCCTTTTAGCC	For	6

Applications: 1, cloning of intron G; 2, cDNA quantification; 3, Alu exonisation analysis; 4, RFLP on gDNA; 5, RFLP on cDNA; 6, ChIP; 7, detection of PV/HS Alu element in the gDNA. For/Rev, forward or reverse primer.

recommended by the manufacturer. For the selection of stably transfected clones, transfected cells, as well as their untransfected counterpart, were plated in RPMI supplemented with 5 mg/1 blasticidin-S-HCl (Invitrogen, Life Technologies, Inc.) 3 days after transfection. After 2 weeks of incubation with blasticidin-containing medium, only transfected cells were viable. Monoclonal cells resistant to blasticidin were obtained by plating serial dilutions (0·01–10 cells/well) on 96-well plates, isolating those colonies derived from a single cell (evaluated by microscopic observation) and subsequent sub-cloning of those clones that showed PR expression (western blotting). Cell lines derived from transfection with plasmid PRA-V660 were labelled CHO-A series, PRB-V660 lines CHO-B, PRA-L660 lines CHO-C and PRB-L660 lines CHO-D. Transient transfection of HeLa, MDAH:2774, Skov-3 and MCF7 cells as well as of maternal and stably transfected CHO-K1 was performed using jetPEI (Q-Biogene) as recommended by the manufacturer.

Genomic DNA (gDNA) extraction

One million cells were lysed in 300 µl DNA cell lysis buffer (200 mM Tris-HCl (pH 8·0), 250 mM NaCl, 25 mM

EDTA, 0·5% SDS). DNA was extracted with one volume of TE-equilibrated phenol (Amresco, Solon, Ohio, USA) and precipitated with 0·7 volumes of isopropanol. Genotype screening of the mutation V660L substitution was performed by PCR-RFLP using primers exon-4 S and exon-4 AS (Pijnenborg *et al.* 2005). The presence of the 320 bp PV/HS Alu insertion in intron G was diagnosed by PCR using primers PROGINS-I and PROGINS-II (Wang-Gohrke *et al.* 2000).

RNA extraction and cDNA synthesis

RNA was isolated using the Trizol reagent (Invitrogen, Life Technologies, Inc.) as recommended by the manufacturer. cDNA was synthesised using the M-MLV reverse transcriptase (Invitrogen, Life Technologies, Inc.) and poly-T oligonucleotide (MBI Fermentas GmbH, St Leon-Rot, Baden-Württemberg, Germany) for priming. Five micrograms of total RNA were used for cDNA synthesis. Prior to cDNA synthesis, RNA was treated with 3 U DNaseI (MBI Fermentas GmbH). To check the expression of the *PR* gene, semi-quantitative RT-PCR was performed using primers PR-GA-FW and PR-GD-RV. Primer pairs exon-4 S/exon-4 AS as well as exon-4 S/exon-5 AS were used to estimate the relative expression

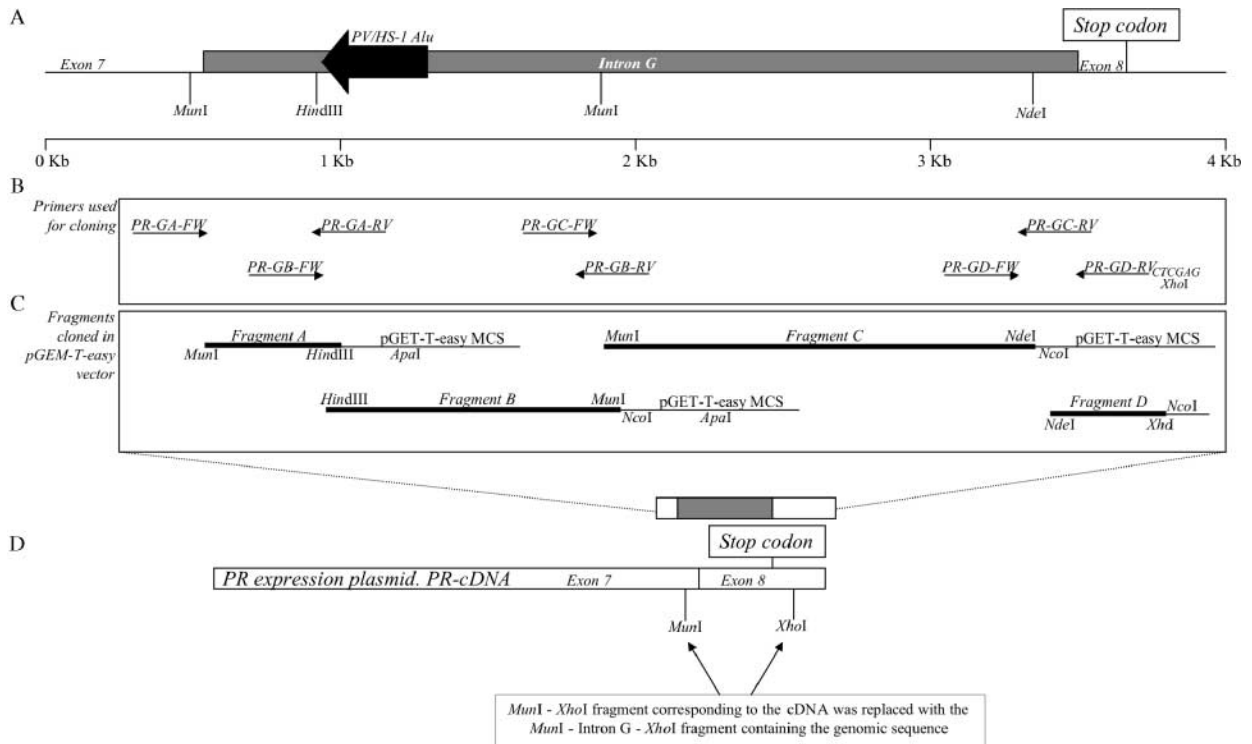


Figure 1 Scheme illustrating the cloning of the complete Intron G into the minigenes. Minigenes for the expression of PRA-IntG-CP, PRB-IntG-CP and PRA-IntG-Alu, PRB-IntG-Alu were constructed by re-inserting the complete intron G derived either from the CP (2672 nucleotides) or from the PROGINS (2992 nucleotides) allele at its original genomic position in the cDNAs of PRA and PRB (D). (A) Shows the complete intron G (grey bar) and the Alu insertion (black arrow). The direction of the arrow indicates the Alu sense orientation. Intron G was cloned by PCR in four steps from genomic DNA isolated from cell lines Skov-3 (CP homozygous) and MCF7 (PROGINS homozygous) as templates. The four fragments of the intron G generated in the cloning strategy are shown in (C). The Expand High Fidelity Kit (Roche) was used for PCR as recommended by the manufacturer. Primers used to amplify corresponding fragments are shown in (B) and sequences are given in Table 1. Fragment A (747 nucleotides), common to both CP and PROGINS alleles, was amplified using primers PR-GA-FW and PR-GA-RV. Fragment A contained the 3' end of exon 7 including the MunI restriction site and contained at its 5' the HindIII restriction site present in intron G. MunI and HindIII were used for subsequent cloning. Fragment B was amplified using primers PR-GB-FW and PR-GB-RV. Fragment B contained HindIII and MunI restriction sites (both present in intron G) at the 5' and 3' respectively used for subsequent cloning. Fragment C (1603 nucleotides), common to both CP and PROGINS alleles, was amplified using primers PR-GC-FW and PR-GC-RV and overlapped the 3' of fragment B containing the MunI restriction site. At the 3', fragment C contained the NdeI restriction site. Fragment D (336 nucleotides) was finally cloned using primers PR-GD-FW and PR-GD-RV. Fragment D overlapped the 3' end of fragment C (including the NdeI restriction site) and contained at its 3' end the complete exon 8 including the stop codon and followed by the XhoI restriction site (introduced by primer PR-GD-RV (B)). XhoI was present in the expression plasmid containing the PR cDNA and it was located downstream the cDNA stop codon (D). Fragments A–D were extracted from agarose gels using the Qiaquick gel extraction kit (Qiagen GmbH), treated with 1 U Taq DNA polymerase in presence of dNTPs (1 mM each; MBI Fermentas GmbH) for 30 min at 72 °C in the recommended buffer containing MgCl₂ (MBI Fermentas GmbH) and cloned into pGET-T-easy plasmid (Promega GmbH, Mannheim, Germany) as recommended by the manufacturer. All plasmids were checked by sequence analysis (Ex-Dye terminator, as recommended by the manufacturer and ABI prism sequencer, Genome Center Maastricht). All pGET-T-easy fragments A–D were selected having the desired orientation since subsequently cloning steps required unique restriction sites present in MCS of pGET-T-easy. In step 1, fragment C was excised using MunI (intron G) and NcoI (MCS of pGET-T-easy) and cloned into MunI/NcoI sites of pGET-T-easy fragment B-CP and pGET-T-easy fragment B-PROGINS. Step 2: fragment D was isolated by NdeI/NcoI (MCS) double digestion and cloned into the NdeI (intron G; partial digested)/NcoI (MCS) sites of pGET-T-easy fragments B–C (wild type and PROGINS). Step 3: fragments B–D, wild type and PROGINS, were isolated by digestion with HindIII (intron G) and ApaI (MCS) and cloned into HindIII/ApaI sites of pGET-T-easy fragment A. Thus, fragments A–D corresponded to the complete intron G sequence flanked by part of exon 7 (containing the MunI restriction site) and by the complete exon 8 (including stop codon and XhoI site downstream). Step 4: the complete intron G–exon 8 fragment (containing the wild type and the PROGINS intron G) were subsequently excised using XhoI/MunI partial digestion and cloned into XhoI (MCS on pCDNA3.1 expression plasmid)/MunI of PRA-V660, PRB-L660 and yielding PRA-IntG-CP, PRB-IntG-CP and PRA-IntG-Alu, PRB-IntG-Alu. All restriction endonucleases were purchased from MBI Fermentas GmbH and used as recommended by the manufacturer. Conditions for NdeI and MunI partial digestions were determined empirically. All regions of the plasmids where ligations took place were checked by sequence analysis.

confluence (75 cm² culture flasks) in RPMI without phenol red (Invitrogen, Life Technologies, Inc.) supplemented with 10% hormone-stripped serum (c.c.pro GmbH, Neustadt, Saxony, Germany) for 3 days. Cells were treated with vehicle (ethanol) or with 17 β -oestradiol (10⁻⁸ M) for 50 min. Subsequently, cells were fixed (RPMI with 1% formaldehyde, 10 min at 37 °C) washed twice with cold PBS and scraped in 1 ml cold PBS supplemented with Complete protease inhibitor (Roche). Cells were lysed in 10 mM HEPES (pH 6.5), 10 mM EDTA, 0.25% Triton X-100 supplemented with complete protease inhibitor. Nuclei were pelleted and subsequently lysed in 300 μ l lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1) plus Complete protease inhibitor) and sonicated. Debris was pelleted, aliquots of the nuclear lysates (50 μ l) were diluted ten times in immunoprecipitation (IP) buffer (1% Triton, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1)) and pre-cleared (2 h, 4 °C) with 100 μ l protein-G beads (Active motif, Rixensart, Brabant, Belgium). For IP of chromatin-protein complexes, specific antibodies (2 μ g) were added and the tubes were continuously rotated during incubation (overnight, 4 °C). For IP of ER- α , RNA Pol-II and Sp1-containing chromatin complexes, antibodies F10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 4H8 and Sp1 (Active motif) were used respectively. Antibodies bound to chromatin complexes were precipitated by the addition of protein-G beads (100 μ l, 2 h, 4 °C). Beads were washed six times (100 mM Tris-HCl (pH 8.0) containing 500 mM LiCl, 1% NP-40, 1% deoxycholic acid) and chromatin-protein complexes were eluted from the beads with 100 μ l 25 mM NaHCO₃ supplemented with 1% SDS. Crosslinking was reversed by the addition of 4 μ l 5 M NaCl supplemented with 10 U RNaseA and incubation overnight at 65 °C. Subsequently, the samples were treated with proteinase-K (400 mg/l) in 20 mM Tris-HCl (pH 6.5) and 10 mM EDTA for 2 h at 42 °C and DNA was purified using the Qiaquick reaction cleanup kit (Qiagen GmbH). Binding of the RNA Pol-II to the GAPDH promoter was used as positive control of the ChIP procedure, and it was assessed using primers ChIP-pos-FW and ChIP-pos-RV. ER- α binding to the PS2 promoter was used as a positive control for ChIP with ER- α and it was assessed using primers PS2-0 and PS2-1. For ER- α and Sp1 binding to the Alu-ERE/Sp1 site, primers Alu-ERE-Sp1-FW and PROGINS-II were used (Table 1 and Fig. 2B). ChIP PCR signals were normalised with an unspecific negative control, using primers ChIP-neg-FW and ChIP-neg-RV that flank cytogenetic location 12p13.3 where no transcription factors bind to.

5-aza-2'-deoxycytidine (ADC) treatment

Cells were plated in six-well plates at 50% confluence and treated for 0, 48, 96 and 120 h with 100 μ M ADC (Sigma-Aldrich Chemie BV). ADC causes DNA

demethylation. At the end of the treatment, RNA was isolated as described earlier. Medium containing ADC was refreshed every 24 h.

RNA stability assay

Native T47D cells or MDAH:2774 cells transiently transfected with the PR minigenes were plated in six-well plates at 70% confluence. One day later, actinomycin-D (Sigma-Aldrich Chemie BV) as an inhibitor of RNA synthesis was added to the medium (6 μ M final concentration) and cells were cultured for the indicated period of time prior to isolation of RNA.

Immunocytofluorescence

Cells were cultured on glass coverslips (six-well plates, overnight) and subsequently fixed in buffered formaldehyde (4% paraformaldehyde in PBS). Cells were washed with PBS and permeabilised with 0.1% Triton X-100 diluted in PBS. To reduce unspecific binding, cells were incubated with PBS containing 1% BSA and 1% goat serum (1 h, room temperature). To detect PR expression, cells were incubated with the PR-specific antibody AB-52 (Santa Cruz Biotechnology). Bound antibody was visualised using Cy-3-labelled goat anti-mouse antibodies (Jackson Immunoresearch, Cambridge, UK).

Transactivation assay

Cells were cultivated for at least 3 days in RPMI without phenol red (Invitrogen, Life Technologies, Inc.) supplemented with 10% hormone-stripped serum (c.c.pro GmbH). One day before transfection, cells were plated in 12-well plates at 40–50% confluence. In each well, 1 μ g MMTV-LUC reporter plasmid, 800 ng of either PR-L660, PR-V660 or pcDNA3.1 (empty vector; Invitrogen, Life Technologies, Inc.) together with 200 ng pSV- β -galactosidase were co-transfected. In all experiments, β -galactosidase was used for normalisation of luciferase measurements with transfection efficiency. Twenty-four hours after transfection, the culture medium was replaced and supplemented with the ligand as indicated in Fig. 3. After an additional 24-h incubation, cells were lysed in 60 μ l 100 mM Tris-HCl (pH 7.8), 0.5% Triton X-100 and 40 μ l extract were assayed for luciferase activity by adding one volume of Steady-GLO substrate (Promega GmbH). The remaining extract was used for β -galactosidase assay and western blot analysis. For β -galactosidase assay, 5–10 μ l extract were incubated in 96-well plates with 180 μ l PBS supplemented with 0.7 mg/ml of *o*-nitrophenyl- β -galactopyranoside (Sigma-Aldrich Chemie BV) and 0.4% β -mercaptoethanol (2–4 h, 37 °C).

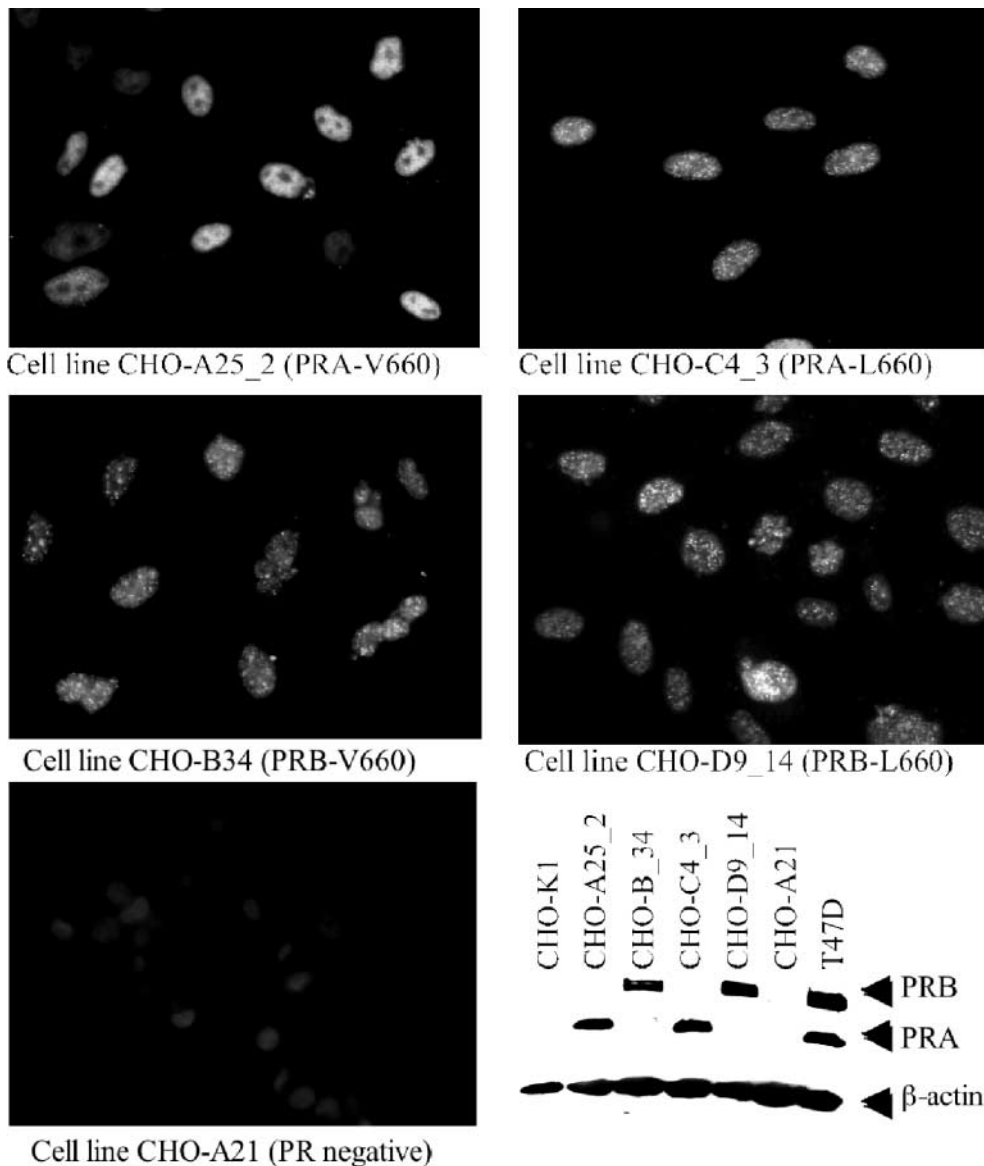


Figure 3 Characterisation of cell lines stably transfected with PRA and PRB. Immunocytofluorescence on cell lines CHO-A25_2, CHO-B34, CHO-C4_3, CHO-D9_14 and CHO-A21 stably expressing PRA-V660, PRB-V660, PRA-L660, PRB-L660 and PR negative respectively. Antibodies raised against PR (AB-52) were used. At the bottom right: western blot analysis using antibody AB-52 (PR) on the same cells plus the maternal line CHO-K1 and a positive control (T47D cells) to show the expression of equal amounts of PR in the selected lines. Expression of β -actin (antibody AC-15) was used as loading control.

β -Galactosidase activity was measured spectrophotometrically at 450 nm. For western blot, replicates from one single experiment were pooled and western blot was performed as described below. Each data point was tested in triplicate using multiple plasmid preparations of PR-expressing constructs and significance of results was confirmed in at least two independent experiments.

Trypsin digests

Variations in the three-dimensional structure of a protein can be visualised by partial trypsin digests. Changes in the tertiary structure potentially mask or expose cutting sites for trypsin, which is known to cut after the amino acid arginine. Cells stably expressing PRA-V660 (CHO-A25_2), PRB-V660 (CHO-B34),

PRA-L660 (CHO-C4_3) and PRB-L660 (CHO-D9_14) were induced *in vitro* with vehicle (ethanol) or ligand (progesterone 10^{-8} M or RU486 10^{-8} M) for 1 h. Cell extracts were prepared in PBS and hormone induction was continued for 20 min at room temperature *in silico* with the same ligand used *in vitro*. Equal amounts of extracts (20 μ l) were incubated for 10 min at room temperature with increasing amounts of trypsin (from 0 to 25 μ g/ml and 0 to 5 μ g/ml for PRA and PRB respectively). Reactions were stopped by adding the SDS-PAGE loading dye and boiling immediately. The total volume was used for western blot analysis for PRA and PRB using antibody AB-52. Trypsin (Sigma-Aldrich Chemie BV) was dissolved in water.

Preparation of protein extracts and western blot

Cells were washed with cold PBS and lysed in RIPA buffer (10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 2 mM EDTA, 1% Na deoxycholate, 1% NP-40 and 0.1% SDS) supplemented with Complete protease inhibitor (Roche). Proteins were separated by SDS-PAGE (7–10% polyacrylamide) and blotted on a nitrocellulose membrane (Protran, Schleicher and Schuell, Germany). PR was visualised using the AB-52 monoclonal antibody (Santa Cruz Biotechnology) or PR-A0098 polyclonal (Dako, Glostrup, Denmark). ER- α and Sp1 were visualised with antibody F10 (Santa Cruz Biotechnology) and Sp1 (Active motif) respectively. Antibody AC-15 (Sigma-Aldrich Chemie BV) was used to detect β -actin. For visualisation of bound antibodies, HRP-conjugated rabbit anti-mouse antibodies (DAKO, Netherlands BV, Eindhonon, Brabant, The Netherlands) and goat anti-rabbit antibodies (Pierce, Aalst, Flanders, Belgium) in conjunction with the super signal-R West-Femto kit (Pierce) were used. The proteasome inhibitor, MG132, was purchased from Biomol (Exeter, UK).

For determination of the protein concentration, the Bradford dye reagent (BCA assay; Uptima, Interchim, Montluçon, Allier, France) was used. Alkaline phosphatase was purchased from Fermentas GmbH.

Cell viability

Cell viability, which is a measure for the total number of cells, was determined with the MTT assay (3-[4,5-dimethylthiazole-2-yl] 2,5-diphenyl tetrazolium bromide (Sigma-Aldrich Chemie BV), as described previously (You *et al.* 2001).

Cell proliferation

Proliferative activity was assessed by determining 5-bromo-2-deoxyuridine (BrdU) incorporation. Cells were cultured for 3 days in RPMI without phenol red

(Invitrogen, Life Technologies, Inc.) supplemented with 10% hormone-stripped serum (c.c.pro GmbH). Subsequently, cells were plated on six-well plates at 50% confluence and cells were synchronised by incubation for 20 h in RPMI (without serum) supplemented with nocadazole (20 μ g/ml; Sigma-Aldrich Chemie BV). Cells were washed twice with PBS and cultured in RPMI supplemented with 10% hormone-stripped serum and containing 10 μ M BrdU (Sigma-Aldrich Chemie BV). Forty minutes after the addition of BrdU-containing medium, cells were fixed (in methanol) to measure the labelling index. Afterwards, cells were treated with org2058 or vehicle and fixed after 8, 24 and 36 h of culture to measure BrdU incorporation. Fixation of cells and measure of the BrdU incorporation by flow cytometric analysis (FACSort Beckton and Dickinson, Sunnyvale, CA, USA) were performed as previously described (Schutte *et al.* 1997).

Image quantification analyses

For all agarose gel and western blot band quantification, the ImageJ programme was used (ImageJ 1.32j, Wayne Rasband, National Institutes of Health, USA, <http://rsb.info.nih.gov/ij/>).

GenBank accession numbers

PR mRNA accession number: NCBI X51730. PR mRNA G3432T and C3764T: NCBI AF016381. The accession number of the *PR* partial gene and the Alu insertion into intron G is NCBI Z49816. SNP accession numbers: SNP-G3432T: Reference SNP Cluster Report (ref SNP ID): rs1042838; SNP-C3764T: ref SNP ID: rs1042839. Chromosome 11 complete sequence accession number is NC000011.

Results

Consequences of the Alu insertion in intron G

Mature mRNA does not contain the polymorphic intron G (with or without the Alu element). Nevertheless, the mRNA transcribed from the *PROGINS* allele can be distinguished from the CP transcript because it still contains the two SNPs in exon 4 (G3432T) and in exon 5 (C3764T) that are in linkage disequilibrium with the intronic Alu insertion. Using cell lines heterozygous for *PROGINS*, the relative abundance of the *PROGINS* and CP mRNAs (cDNA) can be assessed based on the PCR-RFLP method used to diagnose the SNP in exon 4 in epidemiological studies (Pijnenborg *et al.* 2005; Fig. 4). For further analyses, the human breast cancer cell line T47D, which is heterozygous for *PROGINS* (Fig. 4A, lanes

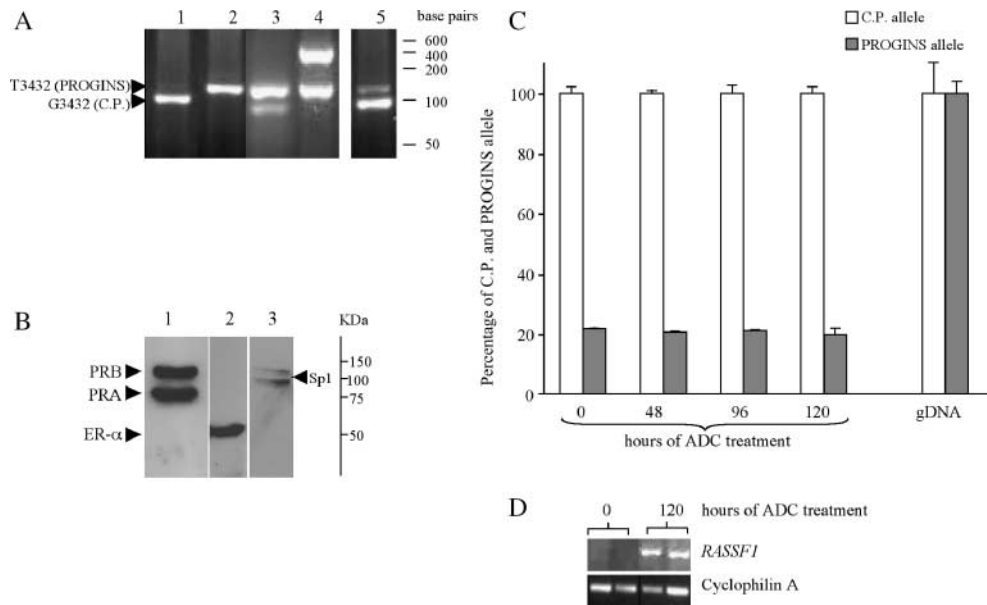


Figure 4 PR characterisation in cell line T47D and DNA demethylation. (A) Cell line T47D is PROGINs heterozygous. Lanes 1–3: PCR–RFLP analyses on genomic DNA (gDNA) to determine the G3432T substitution in exon 4 (primer pair exon-4 S and exon-4 AS (Pijnenborg *et al.* 2005)), which abolishes the BsrI restriction site. Upon BsrI digestion, PCR products derived from the CP allele (lane 1) generate one band of 95 nucleotides, whereas PCR products derived from the PROGINs allele generate one band of 115 nucleotides (lane 2). The presence of both bands (lane 3: cell line T47D) indicates heterozygosity for PROGINs. The upper band (indicating the PROGINs allele) has always a more intense staining compared with the CP band due to its bigger length (115 vs 95 nucleotides). The mutation in exon 4 (and exon 5, not shown) is in linkage disequilibrium with the Alu insertion in intron G (De Vivo *et al.* 2002, Pijnenborg *et al.* 2005, Schweikert *et al.* 2004, Wang-Gohrke *et al.* 2000). The presence of the Alu insertion is directly diagnosed by PCR (primer pair PROGINs-I and PROGINs-II (Wang-Gohrke *et al.* 2000)) because its presence generates a PCR product of 496 nucleotides versus the product of 176 generated by the CP allele (lane 4 for cells T47D). To evaluate the relative abundance of PROGINs versus CP cDNAs in cells that are heterozygous for the PROGINs polymorphism (like T47D cells), the PCR–RFLP method can be applied to cDNA synthesised from these cells (lane 5). Ratios of band intensity can be normalised with the ratio obtained by PCR–RFLP on gDNA, which considered to represent a 1:1 molar ratio of the two alleles. For example, by comparison of lane 3 (T47D gDNA; CP: PROGINs = 1:1) with lane 5 (T47D cDNA) it is evident that there is more CP transcript than PROGINs transcript in these cells. In all experiments involving this method, total RNA was treated with DNaseI prior to cDNA synthesis. Furthermore, PCR products were always generated using primers annealing in exon 4 (Pijnenborg *et al.* 2005) and confirmed using PCR products that were obtained using one primer annealing on exon 4 and the second on exon 5 to exclude gDNA contaminations (as described in materials and methods; primer pair exon-4 S and exon-4 AS). (B) T47D cells express high level of PRA, PRB, ER- α and Sp1. Western blot analysis on cell extracts. Lane 1: PRA (90 kDa) and PRB (114 kDa) detected using antibody AB-52. Lanes 2 and 3: ER- α and Sp1 proteins detected with antibodies F10 and Sp1 respectively. (C) ADC treatment. The relative expression of the CP (white bars) and the PROGINs allele (grey bars) was evaluated by cDNA-PCR–RFLP after T47D cells were cultivated for the indicated period of time in the presence of ADC (100 μ M). Values are normalised with gDNA and the abundance of the CP allele is fixed to 100. Values \pm s.d. are based of triplicates measurements. (D) As a positive control for the ADC treatment, the expression of the *RASSF1* gene, which is known to be heavily methylated in different breast cancer cell lines ((Dammann *et al.* 2001) and our laboratory, unpublished work), was evaluated by semi-quantitative PCR using primers RSF-3 and RSF-4.

3 and 4) and expresses the PR protein (Fig. 4B, lane 1), was selected.

The PROGINs Alu insertion does not contain methylated CpG islands

Alu elements have high CpG content that can be methylated and thus reduce gene transcription

(Deininger & Batzer 1999). The PROGINs allele in cell line T47D is transcribed with reduced efficiency compared with the CP allele (Figs 4A, lane 5 and 4C). To investigate whether this reduced transcription is due to CpG-Alu methylation, T47D cells were treated with ADC, which demethylates DNA. The relative expression of the PROGINs versus the CP allele was assessed by PCR-RFLP on cDNA and did not vary after ADC

treatment for different periods of time (0, 48, 96 and 120 h; Fig. 4C and D).

The Alu element in intron G increases in-cis the transcription of the PR-PROGINS allele

The Alu element in intron G of the PROGINS allele (Fig. 2A and B) contains the site TGA CCn nnn nnn CCG CCC (Alu-ERE/Sp1), located 1132 nucleotides downstream the 5' splice site of intron G. This site is similar to the half-ERE/Sp1 transcription site (Fig. 2B, bottom) located in the promoter region of the PR gene and responsible for the oestrogen/Sp1-stimulated transcription of the PR gene (Petz & Nardulli 2000). We hypothesised that the Alu-ERE/Sp1 site may affect the transcription rate of the PROGINS allele. Since T47D cells express high levels of ER- α and Sp1 transcription factor (Fig. 4B, lanes 2 and 3), these cells were treated with 17 β -oestradiol for different periods of time. Subsequently, the relative abundance of mRNA derived from the CP and the PROGINS alleles were assessed by cDNA-PCR-RFLP (Fig. 2C). After incubation with 17 β -oestradiol (10^{-8} M) for 1 and 3 h, the relative amount of PROGINS derived mRNA gradually increases from 21 to 53% compared with the mRNA derived from the CP PR gene. Furthermore, ChIP indicated that ER- α and Sp1 bind to the Alu-ERE/Sp1 site (Fig. 2D). Whereas Sp1 transcription factor appears to bind independently of the presence of 17 β -oestradiol, the binding of ER- α to this sequence is ligand dependent.

The Alu element reduces the stability of the transcript

Mobile elements like Alus transpose via a RNA intermediate that is transcribed by the RNA polymerase III (RNA Pol-III) and large differences have been reported on the stability of Alu RNAs belonging to different classes of Alu elements (Aleman *et al.* 2000, Li & Schmid 2004). However, nothing is known on the influence that intronic Alu elements might have on pre-mRNAs, in which they are co-transcribed by the RNA Pol-II. The folding of the PR-pre-mRNA with or without the Alu insertion was predicted using the 'Vienna RNA Secondary Structure Prediction' algorithm (<http://rna.tbi.univie.ac.at>). Differences were seen in the free energies and the predicted structures of the two RNA molecules (not shown). To check whether these predicted differences affect the half-life of the corresponding RNAs, we compared the stability of the two PR transcripts in T47D cells (Fig. 5A). Cells were treated with actinomycin-D, an inhibitor of RNA synthesis. Three and eighteen hours of treatment with actinomycin-D (6 μ g/ml) results in 50 and 80% decrease of the transcript generated from the PROGINS allele compared with the one generated from the CP allele. (Fig. 5B; The amounts of CP and PROGINS

cDNAs shown in the chart, do not represent the relative amount of the two molecules like in Figs 4 and 2, but are both fixed to 100 to clearly show the degradation rates.) To confirm that the observed trend was not a consequence of other known (such as SNPs in exons 4 and 5) or unknown genetic variations in linkage disequilibrium with the PROGINS-Alu insertion, human ovarian cancer MDAH:2774 cells (PR negative) were transfected with minigenes encoding the PR CP and PROGINS variant (PRA-IntG-CP, PRA-IntG-Alu, PRB-IntG-CP or PRB-IntG-Alu). These minigenes, differing exclusively with respect to the presence of the Alu element in intron G, were correctly transcribed and translated (not shown). After the treatment of MDAH:2774 cells with actinomycin-D for 18 h, we observed a reduction of approximately 20% of the amount of RNA generated from minigenes bearing the PROGINS intron G compared with the amount of RNA generated from minigenes expressing PR with the CP intron G (Fig. 5B). Although this reduction was less important compared with what we observed in T47D cells (see discussion for one possible explanation), it was seen for PRA and PRB.

The PROGINS Alu insertion does not generate splice variants

Alu elements contain 3' and 5' splice consensus sites (squared in Fig. 2B). When Alus are integrated into introns of actively transcribed genes, they can generate alternative splice variants by 'exonisation' of the approximately 100 nucleotides comprised between the 3' and 5' splice sites (Deininger & Batzer 1999, Deininger *et al.* 2003, Lev-Maor *et al.* 2003, Sorek *et al.* 2004). To test whether the PROGINS Alu is 'exonised', cDNA from native cell line T47D and cell line Skov-3 or MDAH:2774 transiently transfected with the PR minigenes (PRA-IntG-CP, PRA-IntG-Alu, PRB-IntG-CP or PRB-IntG-Alu) was analysed by PCR. When using primer pair overlapping the exon 7/exon 8 boundary and annealing inside the PR coding sequence, no product larger than the correctly spliced PR cDNA was amplified. To further exclude the possibility that the cDNA form containing the 'exonised' Alu would not compete with the normally spliced PR cDNA because of too low expression, primers specific for the 'exonised' Alu were used (one primer, Alu-Ex-RV, anneals at the putative 5' site for alternative splicing in the Alu element, underlined in Fig. 2B). However, no product could be amplified although the non-spliced PCR product derived from the minigene containing the complete intron G+Alu element could be efficiently amplified (not shown).

Taken together, on the one hand, the Alu insertion in intron G moderately increases the transcription rate of the PR-PROGINS allele in response to 17 β -oestradiol, while, on the other hand, the presence of the Alu element is accompanied by a decreased

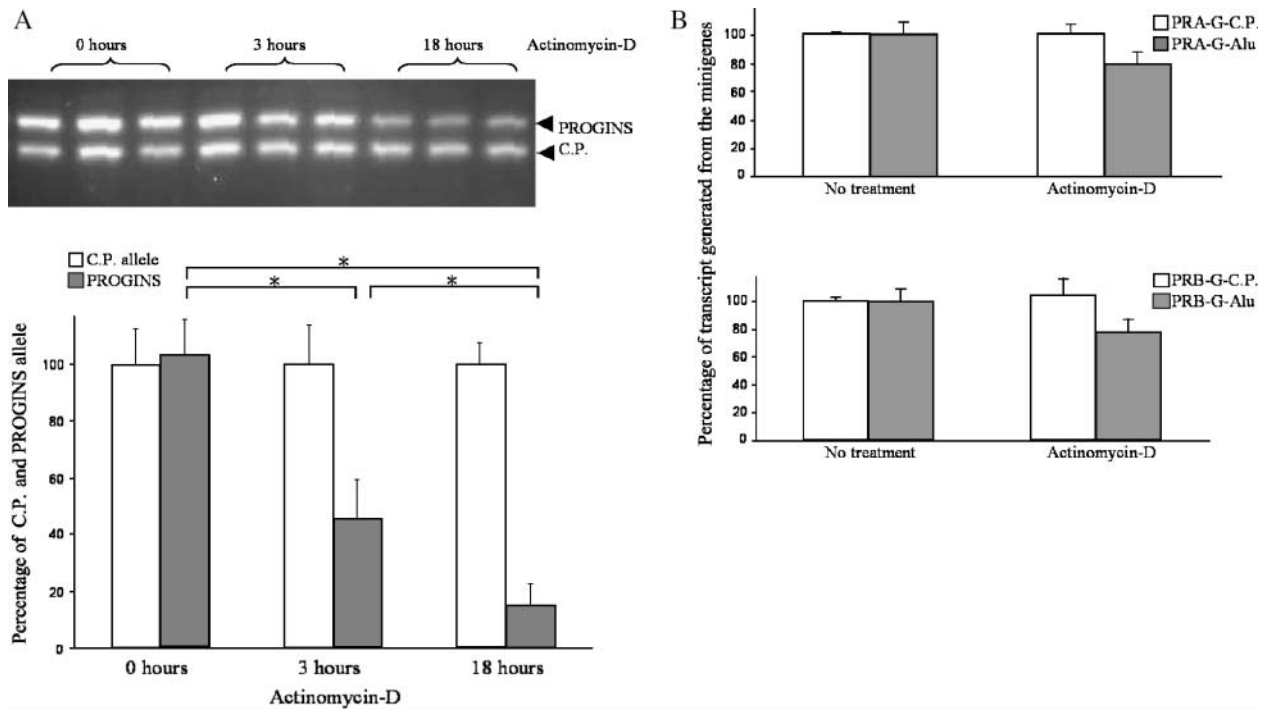


Figure 5 The Alu element reduces the stability of the PROGINS compared with the CP PR transcript. (A) The relative amount of cDNA derived from the CP and the PROGINS alleles was determined by PCR–RFLP on cDNA from T47D cells after treatment with actinomycin-D (6 μ g/ml) for 0, 3 or 18 h. At the top, the agarose gel profile is shown and bands corresponding to the PROGINS and CP alleles are indicated. On the chart at the bottom, the amounts of CP (white bars) or PROGINS (grey bars) cDNAs are quantified from the PCR–RFLP profile. Bars do not indicate the relative molarity (i.e. gDNA normalisation) of the PROGINS and CP transcripts. The amount of both alleles is fixed to 100 with no actinomycin-D treatment. For the subsequent increasing periods of time of actinomycin-D treatments, the amount of transcript generated by the CP allele is fixed to 100, whereas the reduction in the amount of the transcript generated from PROGINS is shown. Mean values \pm s.d. are based on triplicate measurements. (B) MDAH:2774 cells (PR negative) were transfected with minigenes expressing either PRA-IntG-CP, PRA-IntG-Alu (top chart), PRB-IntG-CP or PRB-IntG-Alu (bottom chart) and were treated for 18 h with actinomycin-D (6 μ g/ml). The mRNAs transcribed from each minigene were quantified by semi-quantitative PCR using primer pair PR-GA-FW and PR-GD-RV. Values are normalised with cyclophilin A transcription (primer pair CycloA S and CycloA AS). The amount of transcript generated from minigenes without the Alu insertion (i.e. CP intron G) is fixed to 100 for all actinomycin-D treatment, whereas the reduction in the amount of the transcript generated from minigenes containing the PROGINS intron G (fixed to 100 at time point 0) after treatment with actinomycin-D is shown. Mean values \pm s.d. are based on three or four replicates.

stability of the corresponding mRNA. No other effects related to the presence of the Alu insertion were detectable.

Consequences of the V660L amino acid substitution in exon 4

The following part of the study focuses on the consequences of the amino acid V660L substitution. Thus, when we refer to the PROGINS variant in this part of the study (both in the results and in the discussion sections), we consider exclusively the PROGINS protein variant characterised only by the leucine substitution at codon 660, but we do not refer to the intronic Alu insertion.

Generation of stably transfected cell lines

Stably transfected CHO-K1 cells (Chinese hamster ovary) expressing PRA and PRB, CP (V660) and PROGINS protein variant (L660) were generated and four cell lines that had similar expression (western blot analysis and immunocytofluorescence; Fig. 3) of the V660 and L660 variants of PRA and PRB isoforms were selected: CHO-A25_2 (expressing PR-A V660), CHO-C4_3 (PR-A L660), CHO-B34 (PR-B V660) and CHO-D9_14 (PR-B L660). In cell line CHO-A21, the integration of the plasmid into the genomic DNA was confirmed by PCR but no PR expression was observed. Line CHO-A21, together with the maternal cell line CHO-K1, served as negative control.

Transactivation of PR PROGINS protein variant (L660) on an MMTV-luciferase reporter varies in a cell-dependent manner compared with the CP PR

To determine whether the V660L substitution modifies the transactivation of PR, cells stably expressing PRA and PRB, V660 or L660 (see above) were transiently transfected with the MMTV-luciferase reporter plasmid. In addition, PR-negative cell lines derived from female gynaecological cancers (HeLa: human cervical cancer; Skov-3 and MDAH-2774: human ovarian cancer; MCF7:

human breast cancer) were transiently transfected with equal amounts of PR expression plasmids along with the MMTV-luciferase reporter plasmid.

Progesterone-induced PRA leads to poor transactivation of the luciferase reporter gene (Fig. 6A). In HeLa and MCF7 cells (Fig. 6A), progesterone-induced PRA-V660 and PRA-L660 equally reduce (approximately 20%) luciferase activity compared with unligated receptor. On the contrary, in Skov-3 and in cells stably transfected with PRA (CHO-A25_2 and CHO-C4_3), progesterone

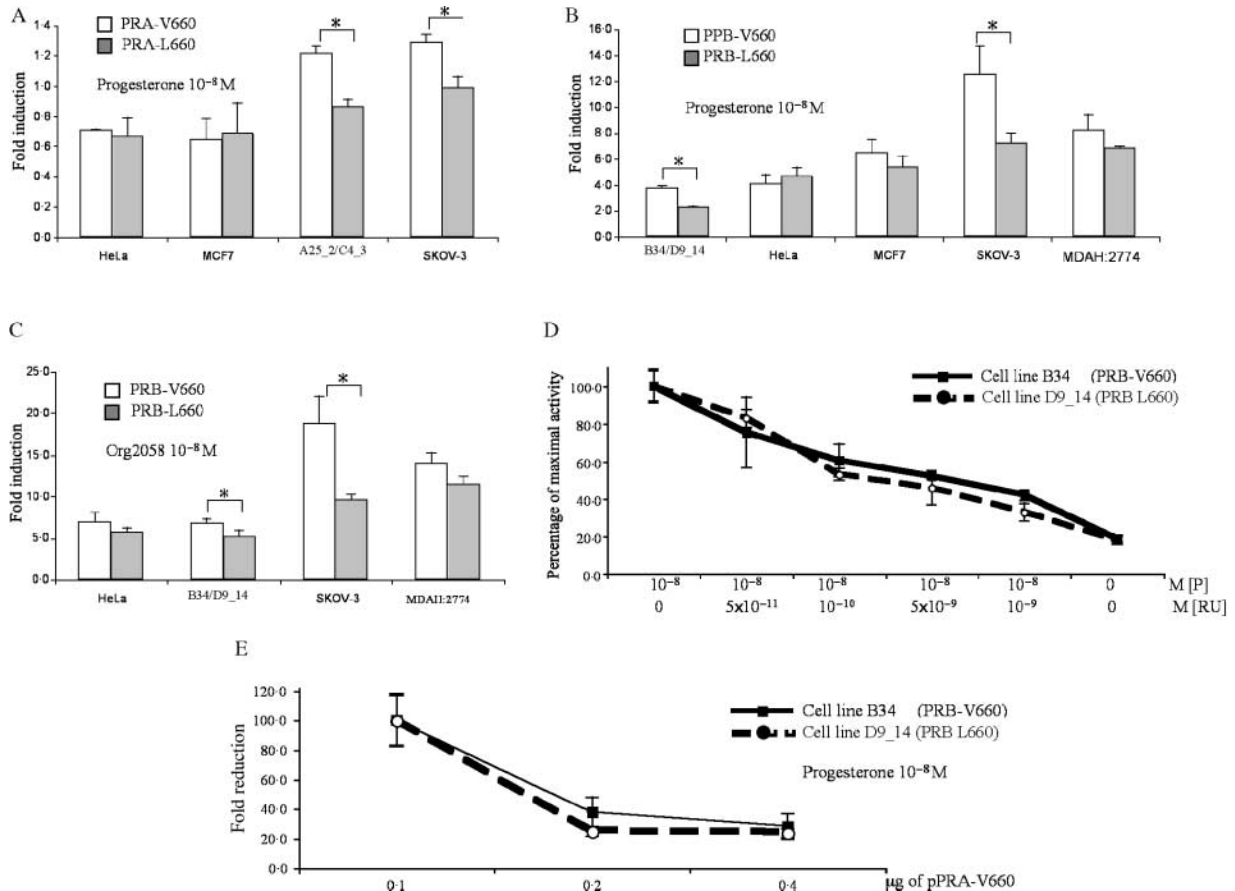


Figure 6 PR transactivation activity. Cell lines CHO-A25_2, CHO-B34, CHO-C4_3 and CHO-D9_14, stably expressing PRA-V660, PRB-V660, PRA-L660 and PRB-L660, were transiently transfected with MMTV-luciferase construct. In addition, cell lines HeLa, Skov-3, MDAH:2774 and MCF7 (all PR negative) were transiently transfected with equal amounts of PR expression plasmids along with MMTV-luciferase construct. Luciferase activity was measured 24 h after induction with different PR ligands, as indicated. Fold increase of luciferase activity obtained in the presence of ligand-activated receptor versus unligated receptor (treatment with vehicle alone) is shown. (A) Fold increase of the luciferase activity induced by progesterone (10^{-8} M) ligated PRA-V660 (white bars) or PRA-L660 (grey bars) in the indicated cell line. (B and C) Fold increase of the luciferase activity induced by PRB-V660 (white bars) or PRB-L660 (grey bars) after stimulation with 10^{-8} M progesterone (B) or 10^{-8} M org2058 (C) in the cell line indicated. (D) Titration curve of the antagonist RU486 (RU; 5×10^{-11} – 10^{-9} M) in the presence of progesterone (P; 10^{-8} M) in Skov-3 cells transiently transfected with PRB-V660 (solid line) or PRB-L660 (dashed line) along with the MMTV-luciferase construct. For each antagonist treatment, the percentage of the maximal luciferase activity obtained with progesterone (10^{-8} M) and no RU486 is shown. (E) Cells stably expressing PRB-V660 (cell line CHO-B34, CP variant, solid line) or PRB-L660 (cell line CHO-D9_14, PROGINS, dashed line) were transiently transfected with increasing amounts of the PRA-V660 (0.1, 0.2 and 0.4 μ g) expression plasmid along with MMTV-luciferase plasmid. Transactivation of progesterone (10^{-8} M) activated PRB on the MMTV-luciferase reporter construct was measured and the fold reduction of the luciferase activity is shown. Total amount of transfecting DNA was maintained constant using pcDNA3.1 (empty vector). Mean values \pm s.d. are based on three to four replicates. Significant results were repeated in independent experiments using different plasmid DNA preparations. Asterisks indicate a value of $P < 0.05$ (*t*-test).

induction of PRA-V660 (CP PR) leads to a 1.2-fold increase of luciferase activity compared with unligated PRA-V660, whereas progesterone induction of PRA-L660 (PROGINS protein variant) does not change luciferase activity compared with the unligated receptor. Given that the expression of the receptor protein was confirmed by western blot analysis (not shown) we concluded that the reduced activity of PRA-L660 reflects a real functional consequence of the amino acid substitution.

Progesterone activation of PRB results in approximately 2- to 12-fold induction of luciferase activity (Fig. 6B) compared with the unligated receptor. PRB activated with the more potent agonist, org2058, leads to 5- to 20-fold induction of the luciferase activity (Fig. 6C). Large differences in the relative induction of luciferase activities are seen among cell lines. Transactivation activities of PRB-V660 and PRB-L660 are similar in HeLa, MCF7 and MDAH:2774 cell lines. However, similarly to what we observed for the PRA isoform, in Skov-3 and in cells stably expressing PRB (CHO-B34 and CHO-D9_14) ligand-bound PRB-L660 (progesterone, Fig. 6B and org2058, Fig. 6C) is significantly less active than ligand-bound PRB-V660 (CP receptor) on the MMTV promoter.

Titration of the antagonist RU486 in the presence of a fixed concentration of progesterone reveals no difference in transactivation activity of PRB-V660 and PRB-L660 (Fig. 6D). Similar results were obtained for all cell lines regardless of whether another antagonist (org31710) and agonist (org2058) were examined and for PRA as well (data not shown).

Transrepression is widely described among steroid hormone receptors and consists in the ability of one nuclear receptor to inhibit the activity of a second nuclear receptor. Given the crucial role of PRA, PRB and ER- α and their corresponding crosstalks, we investigated: (a) how the transactivation of either PRB isoform (V660 and L660), on the MMTV-luciferase is transrepressed by PRA (V660 or L660) and (b) how the transactivation of ER- α on an ERE-TK-luciferase reporter construct is influenced by PR (A or B isoform, V660/L660 variant). Transactivation of PRB on the MMTV-luciferase reporter gene is reduced by PRA in a dose-dependent manner (Fig. 6E). Transactivation of 17 β -oestradiol-induced ER- α is reduced about 40–50% by PRA alone, PRB alone or by the presence of both receptors (data not shown). In the transrepression experiments (on PRB and on ER- α), no differences between the CP and the PROGINS protein variant were observed.

PRA and PRB PROGINS (L660) protein variants show different post-translational modifications compared with the CP PR-V660

The V660L substitution does not affect DBD, LBD or activating functions (AF) of the PR. However,

fine-tuning of PR activity is modulated by phosphorylation and protein degradation (Lange *et al.* 2000, Qiu & Lange 2003, Qiu *et al.* 2003). We hypothesised that post-translational modifications of PR may explain why the activities of the PROGINS protein variant compared with the CP PR differ in different cellular contexts. Cells stably transfected with PRA-V660 (CHO-A25_2), PRA-L660 (CHO-C4_3), PRB-V660 (CHO-B34) and PRB-L660 (CHO-D_14) were stimulated with different ligands and for different periods of time. Subsequently, cell extracts were analysed by western blotting to discriminate between non-phosphorylated and phosphorylated PR. Upon binding of an agonist (progesterone or org2058) or an antagonist (RU486, not shown), PRA and PRB are phosphorylated in a dose-dependent manner (Fig. 7A and B), and this occurs regardless of V660 or L660. Phosphorylation occurs within 20 min after ligand induction and phosphorylated forms are still visible 24 h later. However, at this time point, PRB-V660 (CP) is more extensively phosphorylated after either agonist or agonist plus antagonist induction compared with PRB-L660 (PROGINS protein variant; Fig. 7C). The same trend, although less pronounced, is observed for PRA upon agonist induction but not after agonist plus antagonist induction (Fig. 7D).

Proteasomal-mediated protein breakdown of the PR protein starts a few hours after induction, regardless of whether an agonist or an antagonist is used. The involvement of the proteasomal machinery is shown for PRB by the decreased degradation seen after incubation with the proteasomal inhibitor MG132 (Fig. 7C). PRA-PROGINS protein variant (L660) appears to be degraded with reduced efficiency compared with the CP receptor (PRA-V660) after induction with an agonist plus an antagonist (Fig. 7E). This effect was not observed with PRB (not shown). Taken together, these observations suggest that the different responses observed for the PR-L660 variant (both A and B isoforms) compared with the CP receptor, arise secondary to different post-translational modifications.

The three-dimensional structures of PROGINS variant and CP PR are different

Different affinities of PR-V660 and PR-L660 proteins for kinases/phosphatases and components of the proteasomal degradation pathway may be related to different three-dimensional structures, which in turn may alter the accessibility of epitopes required for protein-protein interactions. To check this hypothesis, we performed partial trypsin digestion assays (Fig. 8). The differences in the extent of trypsin digestions suggest that binding of progesterone (Fig. 8B and E) or RU486 (Fig. 8C and F) induces different

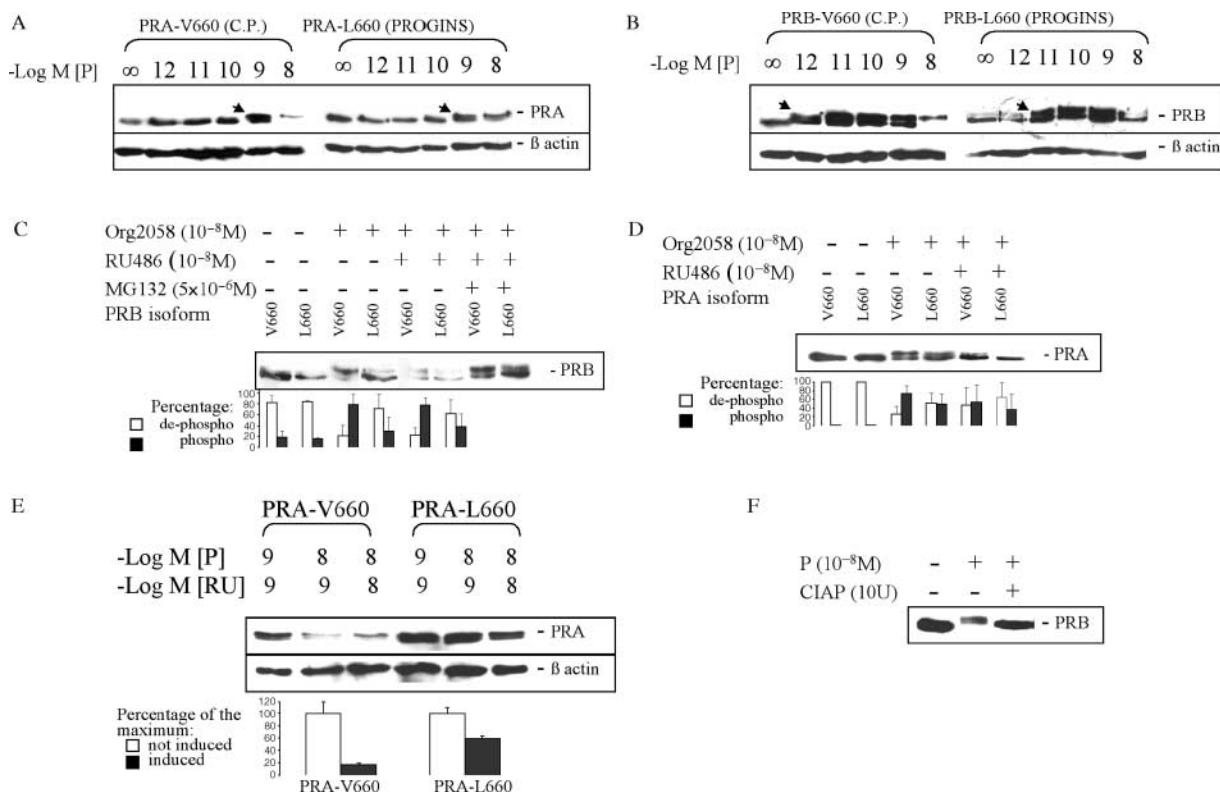


Figure 7 Phosphorylation and degradation of the PR V660 (CP) and L660 (PROGINS) variants. (A and B) Cell lines CHO-A25_2 and CHO-C4_3 (A) or cell lines CHO-B34 and CHO-D9_14 (B) were incubated with increasing concentrations of progesterone for 24 h. PRA and PRB (both CP and PROGINS variants) become phosphorylated (arrowhead) upon ligand binding in a dose-dependent manner. Phosphorylated PRA or PRB can be distinguished because of their decreased electrophoretic mobility in SDS-PAGE compared with the non-phosphorylated forms. (C and D) Phosphorylation patterns of the PRB (C) and PRA (D), CP and PROGINS protein variants, after induction for 24 h with the indicated ligand combination. Phosphorylation patterns are more evident when cell lines are treated with MG132 (5 μ M, 24 h) to inhibit proteasomal degradation of PR. One representative western blot is shown. The charts at the bottom show the corresponding percentage of the dephospho and the phospho proteins observed in replicated western blot analyses. Mean values \pm s.d. are derived from three independent experiments. Total amount of PR per lane (phosphorylated + dephosphorylated forms) is fixed to 100. (E) Degradation of PRA-V660 and PRA-L660 after induction with different concentrations of progesterone (P) plus RU486 (RU). The chart at the bottom represents the amount of PRA protein content (based on the intensity of bands visible in western blots and normalised with β -actin) after induction with the vehicle alone (empty bars, fixed to 100) or with progesterone plus RU486 (filled bars). Mean values \pm s.d. on at least two independent experiments. (F) To further clarify whether receptor proteins are phosphorylated into response ligand, cell extracts were treated with calf intestinal alkaline phosphatase (CIAP; 10 U, 4 h), which completely reverses the shift in molecular weight caused by phosphate(s) group(s) upon ligand binding.

three-dimensional conformations in PRA and PRB. Furthermore, the same stimulation conditions resulted in different degradation patterns of PR-V660 or PR-L660 variants for both A and B isoforms, suggesting that ligand-induced changes in the three-dimensional structures of PR-V660 and PR-L660 occur. In fact, in the absence of any induction, trypsin degradation of the PRA-L660 (PROGINS protein variant) is increased compared with PRA-V660 (Fig. 8A), whereas it is slightly reduced after incubation with progesterone (Fig. 8B) and unaffected after incubation with RU486 (Fig. 8C). PRB-L660 (PROGINS protein variant) appears to be less stable compared with the CP PR in the absence of any ligand or upon progesterone stimulation (Fig. 8D and E). By contrast, stimulation with RU486 only

slightly reduces the stability of PRB-L660 compared with PRB-V660 (Fig. 8F).

PRA CP and PROGINS protein variant mediate different cell proliferation responses

The inhibitory effects of the progestin org2058 on the proliferation rate of cell lines stably expressing PRA-V660 (CHO-A25_2), PRA-L660 (CHO-C4_3), PRB-V660 (CHO-B34) and PRB-L660 (CHO-D_14) were measured. Maternal cell line CHO-K1 and cell line CHO-A21 (PR negative) were used as negative controls. In the absence of ligand, the cell line expressing PRA-V660 (CP) shows lower cell numbers compared with cell lines not expressing PR or cells expressing

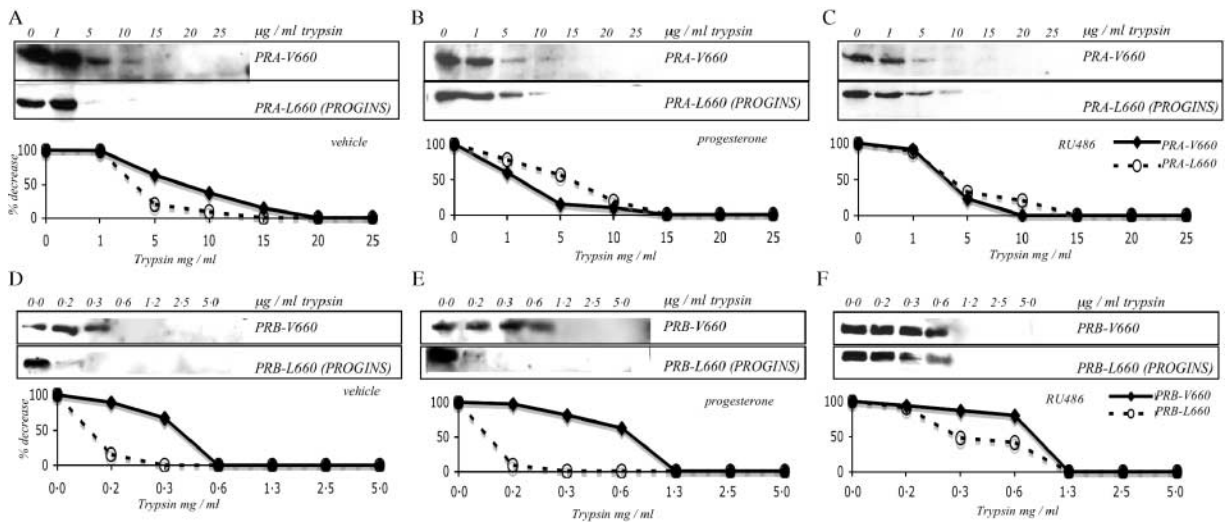


Figure 8 The sensitivity to trypsin partial digestion is different for PR V660 (CP) and L660 (PROGINS). (A–C) Degradation rates of PRA CP and PROGINS protein variant. (D–F) Degradation rates of PRB CP and PROGINS protein variant. Corresponding charts represent the quantification of western blot bands. Results from one experiment are shown. Results were confirmed in a second independent experiment.

PRA-L660 (PROGINS protein variant; Fig. 9A). Cells expressing PRA (either CP or PROGINS protein variant) also had reduced cell numbers in the presence of a PR agonist (org2058; Fig. 9B–C). However, in the presence of low concentration of the agonist org2058 (10^{-10} M; Fig. 9B), cells expressing PRA-V660 show a 12% reduction in total cell number compared with the response obtained in the absence of ligands. On the contrary, the same agonist concentration has no effect on the total cell number in cells expressing PRA-L660 (PROGINS protein variant); a 100-fold higher agonist concentration (org2058 10^{-8} M; Fig. 9C) was required to obtain a similar (13%) inhibition. To verify that the effects on total cell number were the result of diminished proliferative activity, the S-phase fractions were determined using BrdU incorporation and flow cytometry. BrdU incorporation in cells expressing PRA-V660 (CP) was reduced by treatment with

org2058 10^{-10} M from 69.6% (vehicle) to 55.7%, whereas in cells expressing PRA-L660 BrdU incorporation is reduced from 64.5% (vehicle) to 61.1% (org2058) only. No change in BrdU incorporation is seen in the PR-negative control cell line after org2058 treatment. After 24 and 36 h (not shown) of culture, BrdU incorporation reaches saturation.

In cells expressing PRB (either CP or PROGINS protein variant), total cell number was also reduced compared with the PR-negative cell cultures in the absence of any ligand. Treatment with org2058 reduced the total cell number. However, this inhibition never exceeded 5% compared with the treatment with vehicle alone and no difference is observed in the response of PRB-V660 or PRB-L660 (results not shown). Cells negative for the PR do not respond significantly to any treatment (Fig. 9A–D).

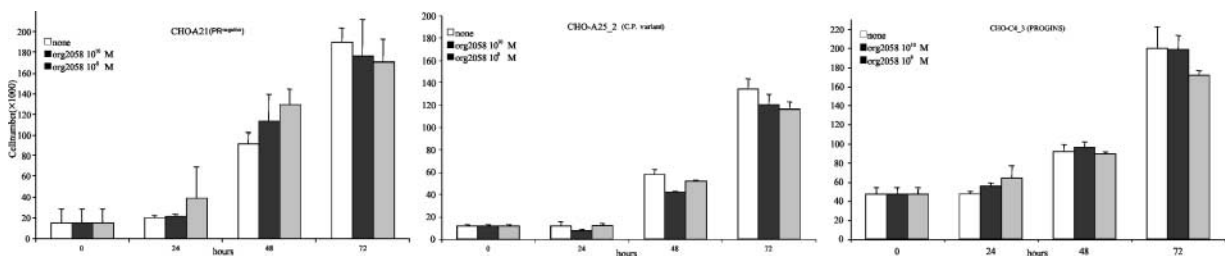


Figure 9 Different proliferative responses of the PRA-V660 or PRA-L660 in the presence of org2058. Cells stably transfected with PRA-V660 or PRA-L660 were cultivated in the presence of the vehicle (no induction) or in the presence of different concentrations of the agonist org2058 (10^{-10} and 10^{-8} M). An MTT assay was performed after different periods of growth (0, 24, 48 and 72 h). Charts show results of one experiment: left panel, negative control (cells CHO-A21, PR negative); middle panel, cell expressing CP PRA (CHO-A25_2); right panel, cells expressing PROGINS PRA (CHO-C4_3). Mean values \pm s.d. are based on three to four measurements. Results were confirmed in three independent experiments.

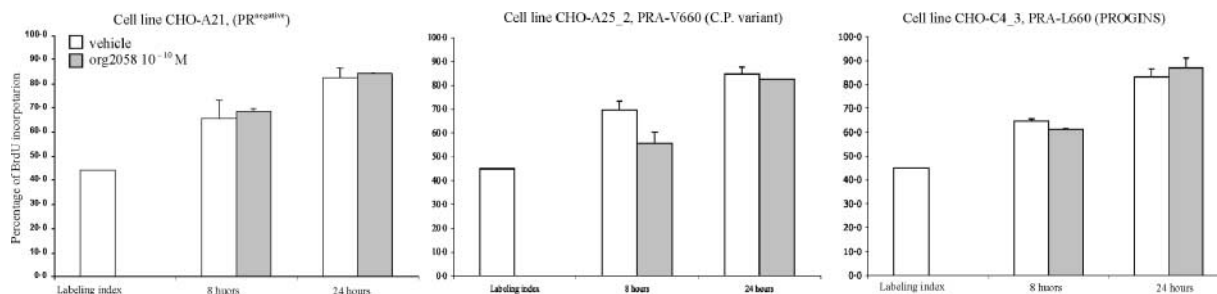


Figure 10 BrdU incorporation. To assess proliferative activity, the S-phase fraction was determined by BrdU incorporation after stimulation with vehicle or org2058 10⁻¹⁰ M in cells stably expressing PR-V660 (CP; cells CHO-A25_2), PRA-L660 (PROGINS protein variant; cells CHO-C4_3) and cells not expressing PR (CHO-A21). The number of BrdU labelled and unlabelled cells were determined by FACS analyses and the percentage is shown in the chart. Initial BrdU incorporation (labelling index) was measured 40 min after the addition of BrdU. Hormone stimulation was afterwards initiated and BrdU incorporation was measured at different periods of time (8 and 24 h). After 36 h of incubation, incorporation was saturated (not shown).

Discussion

The net result of progesterone action depends on PR expression, local ligand metabolism, relative expression of PRA and PRB isoforms, cofactor binding and crosstalks with other signal transduction pathways (Lanz *et al.* 1999, Beato & Klug 2000, Shen *et al.* 2001, McKenna & O'Malley 2002, Li & O'Malley 2003, Qiu & Lange 2003, Qiu *et al.* 2003) and see below). Subtle variations in PR gene expression and protein activity affect the cellular response to progesterone. The human polymorphic variant PROGINS is a complex of three genetic aberrations (PV/HS-1 Alu insertion in intron G, V660L and H770H SNPs). Its prevalence is high (approximately 15% in Caucasians) and it modulates the risk for several gynaecological disorders (Dunning *et al.* 1999, Lattuada *et al.* 2004, Modugno 2004, Schweikert *et al.* 2004, Pijnenborg *et al.* 2005, Romano *et al.* 2006). We showed in this study that the PV/HS-1 Alu insertion in intron G affects gene expression and RNA stability, whereas the V660L substitution reduces the response to progesterone. The effects of these genetic alterations were studied independently of each other. However, they lead to analogue consequences, and a possible combinatory effect of the PROGINS aberrations *in vivo* is speculated in the conclusions.

Consequences of the PV/HS-1 Alu element in intron G of the PROGINS genetic variant

Alu elements are human-specific transposable units present in approximately 1.4 million copies in the human genome (Deininger & Batzer 2002, Deininger *et al.* 2003). They are retrotransposons that have amplified over the past 56 million years via a RNA intermediate transcribed by RNA Pol-III (Deininger *et al.*

2003) and it is estimated that in humans, Alu elements contribute to 0.1% of genetic diseases (Deininger & Batzer 1999). Several functional consequences can be ascribed to the presence of Alu elements (Deininger & Batzer 2002). Among these, Alus can influence gene transcription by modifying proximal promoter or distal enhancer. Owing to the high CpG content, Alus can lead to DNA methylation ((Deininger & Batzer 1999) and references therein) and, in several cases, intronic Alus can be exonised (Lev-Maor *et al.* 2003, Sorek *et al.* 2004) leading to the generation of splice variants.

We have investigated whether the Alu insertion in intron G (PROGINS polymorphic variant) affects the transcription rate and RNA maturation due to (a) CpG methylation, (b) modification of transcription factor-binding sites, (c) affecting RNA stability and d) the generation of splice variants.

Although the PROGINS allele in T47D cells is transcribed with reduced efficiency compared with the CP PR (Figs 4A, lane 5 and 4C), this is not caused by methylation of the PROGINS-Alu because treatment with a DNA demethylating agent (ADC) does not increase its transcription. Furthermore, reduced transcription of the PROGINS allele appears not to be a general phenomenon, because cDNA-PCR-RFLP based screening on mRNA isolated from human endometrium of fertile healthy women showed that the PROGINS and the CP alleles are expressed at comparable levels (A Romano unpublished). Even if transcription of the PROGINS allele is not modified by DNA methylation, it is increased by 17 β -oestradiol upon ER- α activation and subsequent binding, together with the Sp1 transcription factor, to the Alu-ERE/Sp1 site. Similar cases have been reported in literature. Norris *et al.* (1995) have shown that the *BRCA-1* gene responds to oestrogen because of an Alu element containing an imperfect ERE, which is responsible for ER- α binding. The half-ERE/Sp1 site in the promoter of the PR gene induces its transcription in response to

oestrogens (Petz & Nardulli 2000). Furthermore, Sp1 and ER- α can bind to DNA sequences located in proximal promoter and in distal enhancers (Norris *et al.* 1995, Bouwman & Philipsen 2002, Carroll *et al.* 2005, Goffin *et al.* 2005) and the binding of ER- α to 'non-canonical' half-ERE is more common than until recently expected (Carroll *et al.* 2005).

Once transcribed by the RNA Pol-III, individual Alu-RNAs belonging to different families generate molecules with different stabilities (Aleman *et al.* 2000, Li & Schmid 2004). However, the effect that Alus have on the stability of (pre)-mRNA of those genes in which they are integrated (transcribed by the RNA Pol-II), has not been studied. We observed that the transcript derived from the PROGINS allele is less stable compared with the transcript derived from the CP allele, both in native T47D cells and in cells transfected with minigenes (Fig. 5). RNA decay pathways are controlled by RNA-protein complexes that assemble at specific sites on the target transcripts (Lemaire *et al.* 2002, Meisner *et al.* 2004, Huang & Steitz 2005, Moore 2005). For example, Lemaire *et al.* (2002) have shown that SR proteins (a family of splicing factors) can reduce the stability of the mRNA molecules by binding to purine-rich regions in the 3'UTR. A purine-rich region is present in Alu elements, and, although the PROGINS Alu is not located in the 3'UTR, it is still very proximal to it. Alternatively, Alus transcribed by RNA Pol-III (in their sense orientation) could pair with the PROGINS Alu, which is transcribed by RNA Pol-II in the pre-mRNA-Alu as its anti-sense sequence (Fig. 2A), and trigger dsRNA-mediated degradation. In our experiments, the reduced stability of the PROGINS transcript compared with the CP pre-mRNA is more pronounced in native T47D cells than in cells transfected with the minigenes (Fig. 5). This can be explained by the presence of specific enzymatic mechanisms leading to pre-mRNA-Alu degradation. Probably, transient transfection triggers higher RNA transcription in a few cells, thereby saturating the RNA degradation machinery in these cells.

Following pre-mRNA transcription, intronic Alus can generate alternative splice variants (Alu 'exonisation' (Deininger & Batzer 1999, Deininger *et al.* 2003, Lev-Maor *et al.* 2003, Sorek *et al.* 2004)). The 3' and 5' sites (shown in Fig. 2B) for alternate and constitutive 'exonisation' of Alus have been exhaustively studied by one group (Lev-Maor *et al.* 2003, Sorek *et al.* 2004). According to these studies, the PROGINS Alu is not expected to generate 'exonised' splice variants. Nevertheless, flanking sequences can influence alternate splicing (e.g. the branching site for the Alu 'exonisation' is located in the region of gDNA flanking the putative 3' splice site of the Alu). According to our results, the generation of any splice variants by the PROGINS Alu insertion can be excluded.

Consequences of the SNP-G3432T in exon 4 causing the amino acid substitution (V660L)

It is plausible that the translation efficiency of the PROGINS transcript is reduced by sub-optimal codon usage for leucine-660 in the PROGINS variant. The PROGINS leucine-660 is coded by TTG with a preference of approximately 11%, which is much lower than the 42% preference of the optimal codon for leucine, CTG (Zhao *et al.* 2003).

Consequences of the protein V660L substitution

The V660L substitution is located in the hinge region of the receptor, i.e. the region between the DBD and the LBD. So far, no specific function has been ascribed to the hinge region and it may be involved in nuclear translocation, receptor dimerisation in solution, protein stability and cofactor interaction (Jackson *et al.* 1997, Tetel *et al.* 1997, Li & O'Malley 2003). Our preliminary investigations indicate that the V660L substitution does not affect translocation of PRA and PRB to the nucleus (results not shown).

Agoulnik *et al.* (2004) have described an increased transactivation activity of PR-L660 (PROGINS protein variant) on a GRE2-E1b-CAT reporter in COS-1 cells. We have extended these studies and present evidence that the net activity of PR-V660 (CP) and PR-L660 (PROGINS) variant varies in a cell- and ligand-dependent manner. The transactivation activity on the MMTV-luciferase reporter induced by PR-L660 (PROGINS protein variant) compared with the CP PR-V660 (both A and B isoforms) is reduced only in Skov-3 cells and in cells stably transfected with PRA (CHO-A25_2 and CHO-C4_3) and PRB (CHO-B34 and CHO-D9_14). Interestingly, these cells are of ovarian origin (human and hamster), and epidemiological studies indicate that the PROGINS allele might increase the risk for ovarian cancer (see below). Transrepression of PRA on PRB and of PR on ER- α is not influenced by the V660L substitution.

Different responses of the CP and PROGINS PR are a consequence of different post-translational modifications of the proteins

PR is subjected to several post-translational modifications. It contains 18 serines as potential phosphorylation sites and at least seven residues are phosphorylated *in vivo* (Knotts *et al.* 2001). Bagchi *et al.* (1992) have classified two types of phosphorylation sites. Fast phosphorylation sites, that are basally phosphorylated (without PR induction) and their phosphorylation increases upon ligand binding within minutes (ser-81, ser-162 present only in PRB, ser-190 and ser-400), and slow phosphorylation sites, that

become phosphorylated in a ligand-dependent manner within a few hours after induction (ser-102 present only in PRB, ser-294 and ser-345). Although the activity of PR is controlled by the activating functions (AFs) in the LBD and in the N-terminal domain (unaffected by the V660L substitution), its fine regulation is influenced by phosphorylation (Bagchi *et al.* 1992, Takimoto *et al.* 1996, Weigel 1996, Knotts *et al.* 2001, Qiu & Lange 2003, Qiu *et al.* 2003, Pierson-Mullany & Lange 2004), protein degradation (Lange *et al.* 2000, Shen *et al.* 2001) and other post-translational modifications.

We have shown that the activity of the PROGINS protein variant compared with the cognate CP PR depends on the cellular context. Considering that the expression of kinases and proteasomal components is closely related to the cellular context, we hypothesised that this could determine the different actions of the two PR variants. Here, we show that PR CP and PROGINS protein variant become phosphorylated with similar efficiency within a few minutes after induction with a ligand (fast phosphorylation sites). By contrast, phosphorylation of the late phospho residues, examined 24 h after induction, occurs with reduced efficiency in the PROGINS protein variants (both PRA and PRB isoforms; Fig. 7) compared with the CP PR. PRA-L660 (PROGINS protein variant) also shows minor protein degradation compared with PRA-V660 when induced with the combination of agonist and antagonist. Interestingly, some authors have described that PR phosphorylation and subsequent degradation by proteasomal machinery determine a hyperactivity of the receptor (Lange *et al.* 2000, Shen *et al.* 2001). It could be thus speculated that in the presence of the PROGINS protein variant this mechanism is impaired due to incomplete phosphorylation and protein degradation.

PROGINS protein variant and CP PR have different three-dimensional structures

The activity of PR is regulated by conformational changes that, upon ligand binding, facilitate the formation of binding pockets for specific co-activators, kinases and other interacting proteins (Tanenbaum *et al.* 1998, Williams & Sigler 1998, Madauss *et al.* 2004). Trypsin sensitivity assays show that different conformational changes occur in PR upon binding to an agonist or to an antagonist and that these changes vary between PR CP and PROGINS protein variant (Fig. 8). Thus, differential post-translational modifications between the CP PR and the PROGINS protein variant probably arise from different three-dimensional structures of the two proteins. Secondary structure probability prediction (<http://bmerc-www.bu.edu>; Biomolecular Engineering Research Centre, Boston University, MA, USA) suggests that the residue 660 is located in a 7-residue loop (657-PQPV/LGVP-663) between two α -helices that are

connected to the DBD and the LBD. The high conservation among species (mouse, rabbit, sheep, rat and human) of this 7-residue loop (Clustal-W analysis, <http://www.ru.nl>; Centre for Molecular and Biomolecular Informatics, Nijmegen University, The Netherlands) and particularly of the Val-660, indicates its importance for protein functionality. Although valine and leucine have similar chemical properties, they differ sterically (valine is a β -branched small amino acid, whereas leucine is a γ -branched medium-sized amino acid). Substituting a small β -branched residue in a loop with a larger γ -branched residue might influence the angle and the flexibility of the loop, affecting the conformation of PR at the level of the secondary structure (locally in the folding of the hinge region) or at the level of the ultra-secondary structure, i.e. in positioning the DBD with respect to the LBD. The flexibility of the hinge region is crucial to accommodate the relative position of DBD and LBD for other nuclear receptors (Mangelsdorf *et al.* 1995, Tetel *et al.* 1997).

PR-PROGINS protein variant is less efficient than the CP PR in opposing cell proliferation

Progesterone is the natural antagonist of oestradiol-induced proliferation in the endometrium (Lydon *et al.* 1995) and reduces cell proliferation in the ovarian epithelium (Ho 2003, Syed & Ho 2003). We tested whether the reduced transactivation activity of PR-L660 is translated into reduced biological activity, i.e. reduced cellular proliferation. This is indeed the case. In ovarian cell lines stably expressing the PRA-L660 (PROGINS protein variant), the inhibition of basal cell proliferation is less pronounced compared with cells expressing the PRA CP (Figs 9 and 10). This difference was not seen for PRB (results not shown). This is in line with the observations in PRA and PRB null mice (KO; Lydon *et al.* 1995) or PRA-KO and PRB-KO mice (Chappell *et al.* 1997, Mulac-Jericevic *et al.* 2000, 2003, Conneely *et al.* 2003) showing that PRA, but not PRB, mediates all major actions of progesterone in the endometrium and in the ovary. In the mammary gland, however, progesterone-mediated regulation of proliferation is mediated by PRB.

Conclusions

Our characterisation of the PROGINS polymorphic variant clearly indicates that both PROGINS aberrations can cause a reduced action of progestins in the presence of PROGINS PR compared with the CP PR. We observed (a) reduced stability of the PR-PROGINS transcripts, (b) reduced transactivation activity of the PROGINS variant and (c) less efficient inhibition of cell proliferation in ovarian cells expressing the PROGINS variant of PRA. Interestingly, epidemiological studies indicated that

women carrying the PROGINS allele are at increased risk to develop pathologies at those sites for which progesterone exposure has a protective action like ovarian cancer, endometrial cancer and endometriosis (Ho 2003, Syed & Ho 2003, Lattuada *et al.* 2004, Modugno 2004, Pijnenborg *et al.* 2005, Romano *et al.* 2006). In breast, however, where progesterone exposure does not protect against tumour development but rather induces cell proliferation, PROGINS carriers are at reduced risk to develop breast cancer (Dunning *et al.* 1999).

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