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Regulation of Steroid Hormone Response in Normal and Breast Cancer Cells

■

ACADEMIC DISSERTATION

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ABSTRACT

Steroid hormones estrogen and progesterone regulate many biological events including behaviour, reproduction, development, cell differentiation and apoptosis. These hormones and their derivatives are used in contraceptive pills and in the treatment of infertility and menopausal symptoms. They also play a role in many illnesses such as breast and endometrial cancer and osteoporosis. These hormones require specific intracellular proteins termed receptors, to which they bind. In addition, a set of regulatory proteins termed coregulators, which are further divided into coactivators, corepressors and cointegrators, are needed before the transcription of target genes is either activated or silenced. However, the detailed mechanisms of how steroid hormone response is achieved and regulated, are not yet known.

Breast cancer is the most common cancer among Finnish women; approximately 3,800 new breast cancers were diagnosed in Finland in 2003. Antiestrogens tamoxifen and toremifene are synthetic drugs, which are used in the treatment of this disease. Estrogen and progesterone receptor levels are measured in breast cancer samples in order to identify patients who will benefit from these drugs. Unfortunately, many patients fail to benefit from antiestrogen treatment after initial response despite of receptor-positivity and the drug may ultimately even stimulate the tumour growth, which is an important problem clinically.

This study was undertaken in order to study the expression and hormonal regulation of steroid hormone receptors and coregulators both in normal murine and in human breast cancer cells. In addition, cell culture models of endocrine-resistant and hormone-independent breast cancer were established and the questions as to how and why breast cancer cells become drug-resistant were studied. According to the results, both progesterone receptor (PR) and its coactivator, GRIP1, are both widely expressed in murine tissues and the expression pattern is cell-specific. Urogenital, gastrointestinal, endocrinological, immunological and cardiovascular organs, respiratory tract and skin contain PR and progestrone may also regulate the blood flow in some organs. Almost all PR-expressing cells also express GRIP1. Striated muscle and thyroid gland cells did not express either PR or GRIP1. PR was expressed only in the nuclei of cells, and although GRIP1 was expressed predominantly in the nuclei, cytoplasmic expression was also seen. PR expression was estrogen-dependent and -regulated in some tissues and in breast cancer cells, while GRIP1 expression nor its subcellular localization were not dependent on estrogen.

Decrease in the expression of PR and G protein-coupled receptor 30 (GPR30), which is a member of a large family of cell surface receptors, and increase in the expression of coactivator AIB1 were seen in antiestrogen-resistant breast cancer cells. Toremifene did not regulate any of the steroid hormone receptors or coregulators studied, but in addition to being a competitive inhibitor of estradiol in binding to estrogen receptors, toremifene may also act through membrane receptor GPR30. GPR30 may also be a potential new marker for predicting response to antiestrogen therapy. Furthermore, the development of hormone-independency in breast cancer cells is associated with changes in the levels of steroid hormone receptors and coregulators.

In summary, new information about cell-specific expression of steroid hormone receptors and coregulators and about their hormonal regulation in normal and breast cancer cells was obtained in this study. Furthermore, some new data about development of antiestrogen resistance and a potential new mechanism of the action of toremifene were found.

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This dissertation is based on the following articles, which are referred to in the text by their Roman numerals:

I Uotinen N, Puustinen R, Pasanen S, Manninen T, Kivineva M, Syvälä H, Tuohimaa P and Ylikomi T (1999): Distribution of progesterone receptor in female mouse tissues. General and Comparative Endocrinology 115:429-441.

II Puustinen R, Sarvilinna N, Manninen T, Tuohimaa P and Ylikomi T (2001): Localization of glucocorticoid receptor interacting protein 1 (GRIP1) in murine tissues using two novel polyclonal antibodies. European Journal of Endocrinology 145:323-333.

III Sarvilinna N, Eronen H, Miettinen S, Vienonen A and Ylikomi T (2005): Steroid hormone receptors and coregulators in endocrine-resistant and estrogen-independent breast cancer cells. International Journal of Cancer, published online on September 8th.

IV Sarvilinna N, Sarkanen R and Ylikomi T (2005): GPR30 gene expression is reduced in antiestrogen-resistant human breast cancer cells. Submitted for publication.

ABBREVIATIONS

aa	amino acid
AC	adenylyl cyclase
AD	activation domain
AF	activation function
AI	aromatase inhibitor
AIB1	amplified in breast cancer-1
AML	acute myeloid leukaemia
AR	androgen receptor
bHLH	basic helix-loop-helix
BUS	B-upstream segment
cAMP	cyclic adenosine monophosphate
CARM1	coactivator-associated arginine methyltransferase 1
CBP	CREB binding protein
CDK	cyclin-dependent kinase
COS cells	African green monkey kidney cells
CREB	cAMP response element binding protein
DBD	DNA binding domain
DCIS	ductal carcinoma in situ
DNA	deoxyribonucleic acid
E_2	estradiol
EI	estrogen-independent
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
ERKO	estrogen receptor knockout
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
GPR30	G protein-coupled receptor
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor interacting protein 1
HAT	histone acetyltransferase
HB-EGF	heparin-binding epidermal growth factor
HDAC	histone deacetylase
HER-2	human epidermal growth factor receptor-2
HRE	hormone response element
HRT	hormone replacement therapy
Hsp	heat-shock protein
ICI 182,780	7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]estra-1,3,5,(10)-triene-3,17β-diol
ID	inhibitory domain
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
i.m.	intramuscular
KO	knockout
LBD	ligand-binding domain
LE	long-term estrogen-treated

LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
MAPK	mitogen-activated protein kinase
Met	methionine
MLL	mixed lineage leukaemia
MMP	matrix metalloproteinase
MOZ	monocytic-leukaemia zink-finger
MPA	medroxyprogesterone acetate
mPR	membrane progestin receptor
mRNA	messenger ribonucleic acid
N-CoR	nuclear receptor corepressor
NID	nuclear receptor interaction domain
NLS	nuclear localization signal
NO	nitric oxide
NR	nuclear receptor
4-OHT	4-hydroxy-tamoxifen
p300	E1A binding protein
PBS	phosphate-buffered saline
pCAF	p300/CBP-associated factor
PGC-1	peroxisome proliferator-activated receptor γ coactivator-1
PR	progesterone receptor
PRBKO	progesterone receptor-B knockout
PRE	progesterone response element
PRKO	progesterone receptor knockout
RE	response element
RNA	ribonucleic acid
RPLP0	ribosomal phosphoprotein, large P0 subunit
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
RTS	Rubinstein-Taybi syndrome
SDS	sodium dodecyl sulphate
Ser	serine
SERM	selective estrogen receptor modulator
SHBG	sex hormone-binding globulin
SMRT	silencing mediator for retinoid acid and thyroid hormone receptors
SRA	steroid receptor RNA activator
SRC-1	steroid receptor coactivator-1
Stat	signal transducer and activator of transcription
TAZ	transcriptional adaptor putative zink finger
TBS	tris-buffered saline
TGF	transforming growth factor
TIF2	transcription intermediary factor 2
TNF	tumour necrosis factor
TR	toremifene-resistant
WT	wild-type

INTRODUCTION

Steroid hormones estrogen and progesterone regulate many biological events including behaviour, reproduction, development, cell differentiation and apoptosis. They also play a role in breast cancer tumorigenesis and in many other illnesses. These hormones require specific intracellular proteins (i.e. receptors), to which they bind, as well as a set of regulatory proteins termed coregulators (i.e. coactivators, corepressors and cointegrators). Ultimately, the transcription of target genes is either activated or silenced. However, our knowledge of cell- and tissue-specific expression and hormonal regulation of steroid hormone receptors and their coregulators is quite limited.

Steroid hormones are also able to produce rapid, non-transcriptional responses, which are thought to occur *via* cell surface receptors such as G protein-coupled receptors. Furthermore, crosstalk between steroid hormone receptor pathway and other signal transduction pathways such as growth factor and MAP kinase signalling pathways exists. Both the "classical" (or transcriptional) and these non-transcriptional pathways converge at cell cycle and ultimately either promote or inhibit cell division.

Steroid hormone antagonists, such as antiestrogens tamoxifen and toremifene, are synthetic pharmaceutical agents that are able to inhibit the actions of the cognate natural hormones. Antiestrogens are used in the treatment of breast cancer. Unfortunately, breast tumours acquire resistance to treatment after some period of time and eventually the tumour growth is even stimulated by these drugs. Despite extensive study, the reasons for this phenomenon are quite unknown. Moreover, G protein-coupled receptor signalling both in antiestrogen resistance and in hormone-independency needs further study. Thus, this work was undertaken in order to study these issues and some new insights into the mechanism of action of steroid hormones and antiestrogens are given.

REVIEW OF THE LITERATURE

1 STEROID HORMONES AND STEROID HORMONE RECEPTORS

1.1 Steroid hormones

Androgens, estrogens, progestins, glucocorticoids and mineralocorticoids are steroid hormones, which are secreted by endocrine cells of gonads, placenta and adrenal cortex into the bloodstream and carry signals to the target cells distributed throughout the human body. Since they are lipophilic, they enter the cell and cell nuclei primarily by diffusing through plasma and nuclear membranes. In addition to the existing several other kinds of signalling molecules such as proteins, small peptides, amino acids (aa), retinoids, fatty acid derivatives, nucleotides and dissolved gases NO and CO_2, these hormones enable the cells to communicate with each other and to influence one another's growth, development, differentation and death.

Estrogens and progestins, the most studied hormones of these being estradiol (E_2) and progesterone (Fig. 1), have many physiological effects during female life. Estrogens exert their effects almost exclusively in a few specific target organs. They are responsible for the development of sex organs and feminine figure at puberty and for the rapid proliferation of stromal and epithelial cells of the endometrium during the follicular (estrogen) phase of the menstrual cycle. They also have an effect on the ductal growth as well as fat deposition and the growth of the lobuloalveolar system in the mammary gland (reviewed in Topper and Freeman 1980). Growth spurt at puberty and ceasing of growth by uniting the epiphyses with the shafts of the long bones is also caused by estrogens. The net effect of estrogen during fertile age is the slowing of the rate of loss of bone mass (reviewed in Graham and Clarke 1997), but during the climacteric period, estrogen production is rapidly diminished causing recurring menopausal symptoms and predisposing women to osteoporosis and bone fractures due to diminished osteoblastic activity in the bones.

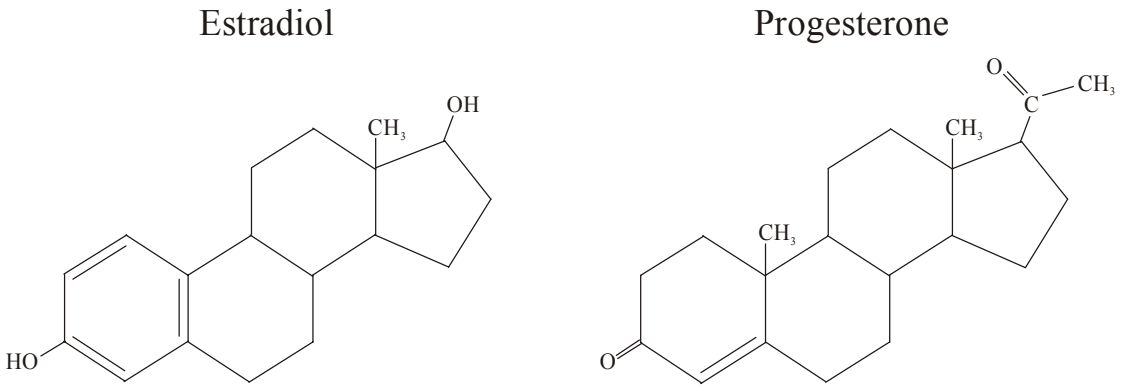

Figure 1. Chemical structure of estradiol and progesterone. Adapted from http://pubchem.ncbi.nlm.nih.gov/.

In addition to estrogen, progesterone has a crucial role during female development and in reproductive functions (reviewed in Graham and Clarke 1997). The requirement of progesterone for the formation of lobuloalveolar structures in the mammary gland during pregnancy is very well known (reviewed in Topper and Freeman 1980). Progesterone is present at low levels throughout the menstrual cycle, but once progesterone levels rise high enough, luteinization takes place. Progesterone facilitates the luteinising hormone (LH) surge and induces marked swelling and secretory development of the endometrium during the luteal phase of the menstrual cycle and thus prepares the uterus for implantation of the fertilized ovum by inducing gene expression of numerous endometrial proteins (e.g. pinopods, integrins, mucins, cytokines and growth factors) essential at the time of nidation (reviewed in Lessey 2003). Progesterone maintains the pregnancy by promoting the uterine growth and attenuating the uterine contractions. However, if the corpus luteum does not encounter the embryonic signal human chrorionic gonadotropin (hCG) that pregnancy has commenced, apoptosis begins leading to menstruation and a new menstrual cycle. A sudden fall in circulating progesterone is associated with the concurrent onset of parturition, increase in prolactin secretion and onset of lactation. Progesterone also appears to modulate bone remodelling along with estrogens in protection against bone loss.

1.2 Steroid hormone receptors

Steroid hormones require specific receptor proteins, which are located predominantly inside the cell nucleus and to which hormones bind after diffusing across the plasma membrane. Comparison of the aa sequences of these proteins with each other has revealed that they are structurally and evolutionarily related, which has been deduced from the high level of conservation in their DNA binding domains (DBDs) and in their less conserved ligand-binding domains (LBDs) (Ogawa et al. 1998). Thus, they constitute the steroid hormone receptor superfamily and are also called ligand-dependent transcription factors, because they influence the transcription of their target cells.

1.2.1 Estrogen receptors

The existence of intranuclear human estrogen receptor (ER) was discovered in 1966 (Toft and Gorski 1966, reviewed in Herynk and Fuqua 2004). It was cloned and its complete aa sequence published in the mid 1980s (Walter et al. 1985, Green et al. 1986, Greene et al. 1986). Until 1995 it was thought that there was only one ER, which was responsible for mediating all the effects of estrogens and antiestrogens. However, a second estrogen receptor, ERβ, was cloned from rat prostate in 1995 and a little later also from human and mouse tissues (Kuiper et al. 1996, Mosselman et al. 1996, Tremblay et al. 1997). The former or "classical" ER is now called ERα. The human receptors are encoded by two separate genes, alpha and beta (ERα and ERβ), on chromosomes 6q and 14q respectively.

ERα and ERβ share a common structural architecture (Fig. 2). NH_2-terminal activation function-1 (AF-1) is located in the A/B domain, is involved in protein-protein interactions and modulates ligand-independent transcription of target genes. Central DBD consists of the C domain and comprises two distinct zinc finger structures through which the receptor interacts with the deoxyribonucleic acid (DNA). It also plays an important role in receptor dimerization. The hinge region or domain D contains sequences for receptor dimerization (Kumar and Chambon 1988, Ogawa et al. 1998) and a nuclear localization domain between the DNA and ligand binding domains (aa 256-303), which contains three Lysine/Arginine-rich motifs, which have a role in nuclear localization (Picard et al. 1990, Ylikomi et al. 1992). In addition, an estrogen-inducible nuclear localization signal has been found within the LBD (Ylikomi et al. 1992). The ligand-binding domain (LBD) is located mainly in the C-terminal E domain and contains the ligand-dependent activation function-2 (AF-2). The COOH-terminal F domain has been shown to have a role in modulating the magnitude of gene transcription by estrogens and antiestrogens and in

determining the inhibitory effectiveness of antiestrogens (Montano et al. 1995). The ERβ protein is smaller than ERα and its protein sequence demonstrates considerable homology in DNA (96%) and ligand binding (58%) domains, but there is a great divergence in the A/B, the hinge and the F-domains of these proteins (Mosselman et al. 1996, reviewed in Shupnik 2004).

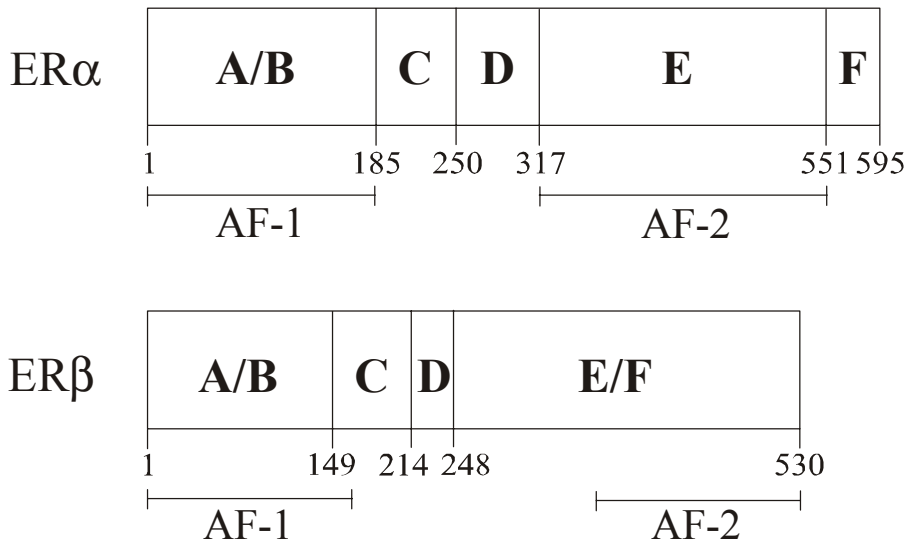

Figure 2. Structure of ERα and ERβ proteins. Different domains (A-F) are represented. AF-1, activation function-1; AF-2, activation function-2. Modified from Sartorius et al. 1994 and Herynk and Fuqua 2004.

Although the overall structure of ERα and ERβ is quite similar and the ligand binding affinity for both receptor subtypes is quite similar for the physiological ligands, there are differences in their function (Kuiper et al. 1997, reviewed in Nilsson et al. 2001). ERα and ERβ have been shown to respond differently to ligands at AP1 response elements. For instance, E_2 activated transcription, when ERα was complexed, while transcription was inhibited, when ERβ was present (Paech et al. 1997). There are also receptor-selective differences in the responses to synthetic estrogen agonists and antagonists. Partial agonists/antagonists tamoxifen, 4-hydroxy-tamoxifen (4-OHT) and raloxifene as well as the pure antagonist ICI 164,384 displayed a low but significant ERα-selective estrogenic activity, while they displayed only antagonistic effects through ERβ (Barkhem et al. 1998). Possible explanations to these differences may be the lack of the part of AF-1 in ERβ and the volume and shape of the ligand binding cavity. Moreover, the type of aa residues lining the cavity may be different. Van Den Bemd et al. (1999) observed that ERα and ERβ respond to pure antiestrogens ICI 164,384 and ICI 182,780 with a distinct conformational change: ERα changes into a less stable, more protease sensitive form, while ERβ changes into a more stable, less protease sensitive conformation. These clear effects were not observed with E_2 and tamoxifen (Van Den Bemd et al. 1999).

1.2.2 Physiological role of ERs in mice

Studies with ERα and ERβ knockout (KO) mice have further confirmed that ERs play an important role in normal development, reproductive functions and skeletal bone physiology. Heterozygous mice, which contain only one copy of the wild-type (WT) ERα, have been found to be fertile and they exhibit no obvious differences in phenotype when compared to the WT phenotype (reviewed in Korach 1994). They have about half of the ER amount when compared with WT and thus the remaining WT allele is not expressed at a higher level to compensate for the loss of another allele (reviewed in Korach 1994).

As in heterozygous mice, the external phenotypes of ERα-disrupted homozygous mutant mice were normal (Lubahn et al. 1993). However, both males and females were infertile and no sexual behaviour was seen in females (Lubahn et al. 1993, reviewed in Korach 1994). Mutant females had haemorrhagic cystic ovaries and no functional corpora lutea, although granulosa and theca cells were present, suggesting that follicle development arrests prior to the formation of ovulatory follicles (Lubahn et al. 1993, reviewed in Korach 1994). The basal levels of uterine PR messenger ribonucleic acid (mRNA) were comparable to those in the WT animals, but there was no stimulation after estrogen treatment (Couse et al. 1995). No increase in the expression of estrogen target genes lactoferrin or glucose-6-phosphate dehydrogenase was seen in mutant mice (Couse et al. 1995). Circulating serum estradiol levels were approximately 10-fold greater and progesterone levels appeared to be lower than those in its WT counterpart (Couse et al. 1995). Females had hypoplastic uteri which did not respond to either estrogen or antiestrogen treatment and vaginal cytology was also unaltered (Lubahn et al. 1993, reviewed in Korach 1994). Adult female mice had undeveloped mammary glands with only vestigial ducts present at the nipples (reviewed in Korach 1994). Furthermore, skeletal bone density of ERKO mice was up to 25 % lower than in WT mice (reviewed in Korach 1994).

Epidermal growth factor receptor (EGFR) levels, its autophosphorylation and c-Fos induction (c-Fos is an EGF-regulated gene) were unchanged in ERKO mice, suggesting that the EGFR signalling pathway is intact in ERKO mouse uterus (Curtis et al. 1996b). However, ERKO mice showed no increase in DNA synthesis after EGF treatment compared with both placebo control and WT uteri (Curtis et al. 1996b). Furthermore, PR message increased after EGF treatment and antiestrogen ICI 182,780 was able to block this induction in WT animals, but not in ERKO mice (Curtis et al. 1996b). These results confirmed that the estrogen-like effects of EGF in the mouse uterus require ER and that ER and EGFR signalling pathways cross-talk: EGF initiates the phosphorylation cascade, which activates the ER, presumably *via* ER phosphorylation, regardless of the presence of estrogen (Curtis et al. 1996b).

Homozygous mutant mice lacking ERβ exhibit phenotypes that are distinct from those of mice lacking ERα (Krege et al. 1998). These mice survived to adulthood and did not exhibit any obvious abnormalities (Krege et al. 1998). However, ERβ -/- females ovulated less efficiently and there were more early atretic follicles and fewer corpora lutea when compared with the WT ovary, suggesting a partial arrest of follicular development and less frequent follicular maturation (Krege et al. 1998). ERβ -/- mice had significantly fewer litters and the number of pups per litter was significantly lower (i.e. reduced fertility), but no impairment in sexual behaviour, mammary gland histology or lactation was seen (Krege et al. 1998).

In summary, ERs are not critical to survival but they play a role in behaviour, bone formation and in female and male sexual maturation and fertility.

1.2.3 Progesterone receptors

The existence of mammalian progesterone receptor (PR) was discovered in the late 1960s and early 1970s (reviewed in O'Malley et al. 1969, Milgrom et al. 1970). It was cloned in 1986 by three laboratories (Conneely et al. 1986, Jeltsch et al. 1986, Loosfelt et al. 1986). There are two isoforms of the receptor, PRA and PRB, which arise from the same gene by transcription at two distinct promoters, giving rise to two different mRNAs and translation initiation at two alternative AUG codons (Horwitz and Alexander 1983, Lessey et al. 1983, Kastner et al. 1990). Both PR isoforms are functionally distinct. In general, PRB is transcriptionally more active than PRA (Tora et al. 1988, Wen et al. 1994, Tung et al. 2001) and according to the microarray gene expression data, most PR target genes are regulated predominantly through PRB (Richer et al. 2002). Furthermore, PRA can act as a dominant repressor PRB activation of progestine-sensitive reporter genes (Vegeto et al. 1993, Giangrande et al. 1997). PRB contains an additional N-terminal stretch of 164 aas, which is also termed B-upstream segment (BUS) and which contains an additional autonomous activation domain (AF-3), which may provide differential site-specific binding for certain coactivators or other nuclear accessory proteins (Sartorius et al. 1994, Giangrande et al. 1997, Giangrande et al. 2000, Tung et al. 2001, reviewed in Conneely et al. 2002). Both isoforms are able to bind both to DNA and hormone, but each has a unique set of target genes, with little overlap (Tora et al. 1988, reviewed in Conneely et al. 2002, Richer et al. 2002). A third isoform, N-terminally truncated PR termed PRC, has also been characterized (Wei and Miner 1994). It is generated by alternative translation initiation at methionine 595 (Met^{595}), is able to bind progestin and can enhance the transcriptional activity of PRA and PRB, but was found to be inactive by itself (Wei et al. 1996).

Multiple domains (designated A to F) within the PR have been localized and characterized (Fig. 3). The non-conserved region A/B contains constitutive transcription activation functions AF-1 and AF-3. The DBD is contained within region C and the LBD within region E. In addition to binding ligands, LBD is able to bind heat-shock protein 90 (Hsp90) (Tetel et al. 1997). The hinge region (region D), a poorly conserved sequence between the LBD and the DBD, plays a role at least in receptor dimerization, nuclear localization, ligand binding and interaction with coregulators (Jackson et al. 1997, Tetel et al. 1997). Three constitutive nuclear localization signals (NLS), one in the hinge region and two in the DBD, account for active transport of the PR from cytoplasm into the nucleus, but the receptor is able to passively diffuse back into the cytoplasm (Guiochon-Mantel et al. 1991, Ylikomi et al. 1992). A fourth weak hormone-inducible NLS is located within the LBD of chicken PR (Ylikomi et al. 1992). However, it has been suggested that the N-terminal extension present in PRB also has a role in nucleocytoplasmic shuttling, because despite indistinguishable NLSs and identical chaperone interaction domains the A form has been found to be mainly nuclear and B form partitions between the cytoplasmic and nuclear compartments in the absence of ligand, but addition of hormone results in complete nuclear translocation (Lim et al. 1999). Instead, progestin-induced nuclear localization has been shown to be independent of serine 294 (Ser^{294}) phosphorylation and mitogen-activated protein kinases (MAPKs) (reviewed in Lange 2004). A repressor domain has been identified within human PR which is present within both isoforms, but it is functionally active only in PRA (Giangrande et al. 1997). This N-terminal 140-amino acid domain is necessary but not sufficient for transrepression of ER transcriptional activity (Giangrande et al. 1997).

1.2.4 Physiological role of PR in mice

In order to define the separate functions of the isoforms of PR and progesterone *in vivo*, selective ablation of the PR gene and separate isoforms in mouse have been established. PR knockout (PRKO) female mice, in which both PR isoforms were functionally ablated, underwent apparently normal embryogenesis and developed to the adult state, but they were infertile (Lydon et al. 1995).

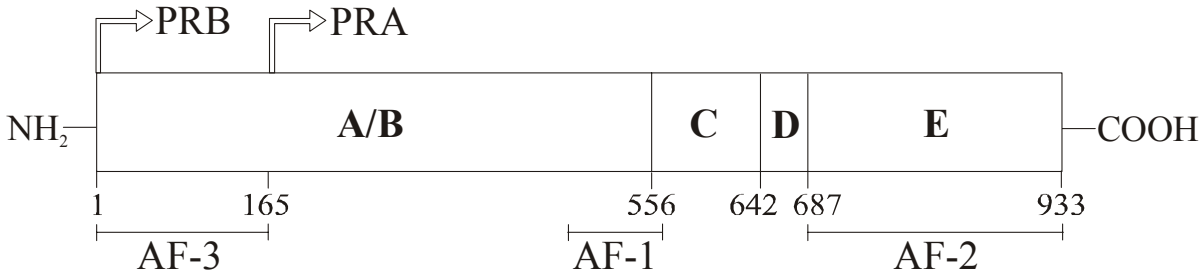

Figure 3. Structure of PR isoforms A and B. Different domains of the proteins (A-E) are shown. AF-1, AF-2 and AF-3, activation functions 1-3. Modified from Kastner et al. 1990 and Sartorius et al. 1994.

Functional abnormalities in the uterus (hyperplasia, inflammation, absence of response to decidual stimulation in response to progesterone and estrogen treatment), ovary (complete block of ovulation, absence of corpus luteum), mammary gland (a more basic ductal structure with less extensive dichotomous and lateral side branching and absence of lobuloalveolar development at the end of each duct) and brain were observed (Lydon et al. 1995).

When mice were tested for sexual behaviour, both WT and heterozygous females exhibited high levels of lordosis, while homozygous PR null mice exhibited greatly attenuated or even loss of lordosis quotients (Lydon et al. 1995, Mani et al. 1996). Both progesterone and the neurotransmitter dopamine were unable to facilitate sexual behaviour in PR null mutant mice, while serotonin clearly facilitated lordosis, indicating that these mice are still capable of exhibiting lordosis and that the serotonin-mediated response does not require intracellular PR while progesterone- and dopamine-mediated responses do (Mani et al. 1996). A lack of endogenous preovulatory gonadotropin surges evoked either by male mouse odor or E_2 has been observed in PRKO mice, suggesting that anovulation of these animals may not be solely due to abnormalities within the ovary itself (Chappell et al. 1997, Chappell et al. 1999). PR has also been shown to facilitate initiation of tumorigenesis in breast and ovary (Lydon et al. 1999, Medina et al. 2003).

Selective ablation of PRA resulted in female mice which were infertile and in which infertility was associated with defective uterine implantation (Mulac-Jericevic et al. 2000). On the contrary, PRB knockout (PRBKO) female mice were fertile and produced viable, although growth retarded offspring, but a marked reduction in ductal sidebranching and development of alveolar lobules as well as lowered alveolar density during pregnancy was observed (Mulac-Jericevic et al. 2003). An increase in the number of apoptotic cells was also observed (Mulac-Jericevic et al. 2003). Thus, in mammary gland, PRB seems to be sufficient to elicit normal proliferation and differentiation of the mammary epithelium in response to progesterone and PRA is sufficient to mediate the ovulatory activities of progesterone and to elicit the antiproliferative effects of progesterone on E_2-induced hyperplasia in the uterus (Mulac-Jericevic et al. 2000, Mulac-Jericevic et al. 2003).

In summary, these findigs confirm that PR plays a pivotal role in sexual behaviour, ovulation, luteinization, implantation and in lobuloalveolar development of the mammary gland and they support progesterone's role as a pleiotropic coordinator of diverse reproductive events.

1.2.5 Cell- and tissue-specific expression of PR and ER

ERα mRNA has been found in the mouse ovary, uterus (greatest concentration), oviduct, mammary gland, heart, aorta, liver, kidney, spleen, skeletal muscle and bone marrow (Couse et al. 1997). Thus, ERα has a broad expression pattern, whereas ERβ has a more focused pattern with high levels in the female mouse ovary, lung and hypothalamus (Couse et al. 1997). Low levels of ERβ transcript have been detected in mouse uterus, cervix, vagina, oviduct and heart, but mammary glands, kidney and aorta were negative when studied by RNase protection assay (Couse et al. 1997). ERβ expression studied by Northern blotting did not reveal expression in the heart, but kidney was negative as in the previous work (Tremblay et al. 1997). In human, ERβ transcripts have been detected in the ovary, thymus and very faintly in the spleen, but small intestine, colon, leukocytes, heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas were reported to be negative (Mosselman et al. 1996). However, it is possible that the sensitivity of Northern blot analysis was not sufficient to detect ERβ expression in some tissues, since the receptor was expressed in peripheral blood lymphocytes when studied by PCR but not when studied by Northern blot (Mosselman et al. 1996).

ERα protein has been shown to be expressed in a minority of luminal epithelial cells but not at all in any of the other cell types of normal human mammary gland (reviewed in Anderson and Clarke 2004). The ERα positive cells are scattered relatively evenly throughout the epithelium (reviewed in Anderson and Clarke 2004). Unlike in the human breast, ERα can be detected in mouse stromal cells and adipocytes and in rat epithelial (close to the basement membrane) and stromal cells (Shim et al. 1999, reviewed in Anderson and Clarke 2004). On the contrary, ERβ is present in most luminal epithelial and myoepithelial cells as well as fibroblasts and other stromal cells types (reviewed in Anderson and Clarke 2004). This widespread distribution is quite uninformative, when compared to a more restricted pattern of ERα expression as regards the function of ERβ in normal human breast.

PR is expressed in progesterone responsive organs such as uterus, ovaries, vagina, brain and normal and neoplastic breast tissues (reviewed in Graham and Clarke 1997). Furthermore, it has been detected in other tissues and cell types such as vascular endothelium, thymus, lung, pancreas and osteoblast-line cells, in which its function is less known (reviewed in Graham and Clarke 1997). In the uterus, PR levels are higher in the proliferative (luteal) phase of the mentrual cycle and they are reduced in the glandular and luminal epithelium around the time of implantation, but stromal PR expression persists (reviewed in Lessey 2003). In the human breast, PR is expressed in a minority of cells scattered throughout the luminal epithelium, but not in the myoepithelial or stromal cells (reviewed in Anderson and Clarke 2004). In mouse and rat mammary gland PR is also expressed in luminal epithelial cells only (Silberstein et al. 1996, Shim et al. 1999), and in rat it has been shown to be colocalized to ERα-expressing cells (Shim et al. 1999).

However, the PR expression in the breast luminal epithelial cells is not static but undergoes dynamic changes in distribution with the attainment of sexual maturity (reviewed in Ismail et al. 2003). Treatment with estrogen results in a well-known increase in PR-positive cells in the epithelium (Shim et al. 1999). Furthermore, the majority of epithelial cells, which undergo proliferation in response to progesterone, are PR-negative and closely associate with PR-positive cells, which thus mediate the proliferative signal of progesterone *via* a paracrine mechanism to the responsive juxtaposed PR-negative cells (reviewed in Ismail et al. 2003).

There are wide fluctuations in PRA:PRB ratios in the uterus during the normal menstrual cycle (Mangal et al. 1997, Mote et al. 1999). The PRA concentration is relatively constant, but PRB

concentrations vary considerably (Mangal et al. 1997). Moreover, use of oral contraceptives changes the expression ratios (Mangal et al. 1997). Abnormal PRA:PRB ratios have been shown in malignant diseases such as breast cancer (Graham et al. 1995). Thus, it has been suggested that the response to progesterone as well as to endocrine therapy in normal and cancerous tissues respectively may be modulated by differential expression of these two isoforms.

1.2.6 Steroid hormone receptors and human diseases

There are some natural mutations of ERα which result in an altered protein sequence and certain diseases. ERα mutations have been found in primary breast cancer samples and in metastatic lesions, although these mutations are relatively rare (reviewed in Herynk and Fuqua 2004). In addition to breast cancer, ERα mutations have also been shown in endometrial cancer, systemic lupus erythematosis and psychiatric diseases such as bipolar illness, puerperal psychosis and alcoholism (reviewed in Herynk and Fuqua 2004). It has also been suggested that ER defects may be responsible for some women's infertility problems, but so far no evidence has been presented.

A male patient has been reported to lack functional ERα due to a homozygous mutation of the receptor (Smith et al. 1994). He had a low bone age, continued linear growth through adulthood due to unclosed epiphyses and severe osteoporosis. Moreover, he had estrogen insensitivity syndrome, impaired glucose tolerance, hyperinsulinemia and bilateral axillary acanthosis nigricans, which is a cutaneous marker of insulin resistance. It has been previously thought that the pubertal growth and epiphyseal maturation in males are induced by androgen, but the phenotype of this man confirms that estrogen has a critical role in pubertal growth, epiphyseal maturation and in the mineralisation of the skeleton in both sexes. In contrast to numerous known ERα mutations, a relatively few naturally occurring point mutations of ERβ have been identified so far (reviewed in Herynk and Fuqua 2004).

It has been suggested that PR mutations are detrimental to human during embryologic development. Mutated PR may participate in ovarian carcinogenesis, since an association between mutated PR allele and increased risk for nonfamilial ovarian cancer has been shown (Agoulnik et al. 2004). In addition, mutation in the PR promoter region has been linked to an increased risk of endometrial and breast cancers (De Vivo et al. 2002, De Vivo et al. 2003). Mental retardation, autism and epilepsy, which begin in early childhood, are characteristics of Angelman´s syndrome and Angelman syndrome-associated protein E6-AP has been shown to interact with and coactivate the transcriptional activity of PR (Nawaz et al. 1999, Veiga and Toralles 2002). PR mRNA and protein levels have been shown to be increased in human uterine leiomyomas when compared to normal uterine myometrium (Brandon et al. 1993). Furthermore, measurement of PR levels has provided increased accuracy in predicting response to hormonal therapy in breast cancer, and its presence either alone or together with ER has been reported to correlate with better response to endocrine therapy and longer survival (Clark et al. 1983, McGuire and Clark 1983, Rydén et al. 1988, Ravdin et al. 1992, Allred et al. 1998, Bryant et al. 1998, Fernö et al. 2000, Chebil et al. 2003).

In summary, these findings suggest that even the complete absence of ERα is not lethal but may result in a number of different diseases. Instead, PR mutations seem to be more detrimental to humans since no germ-line mutations seem to exist in human population so far, but they have been shown to have a role in tumorigenesis. The clinical relevance of ERβ mutations remains to be discovered.

2 COREGULATORS

Not only the cell-type-specific expression of the receptors, but also cell-type-specific expression and combination of other gene regulatory proteins collaborating with the activated receptors are known to influence the transcriptional rate of the target genes and the cellular responses to hormones. The existence of the transcription intermediary factors (TIFs), also known as coregulators, was reported for the first time in the middle of the 1990s. These coactivators, corepressors and cointegrators are crucial for the assembly of the pre-initiation complex and recruitment of ribonucleic acid (RNA) polymerase II to the translation initiation site. They mediate either activating or inhibiting signals of hormones by functioning as bridging factors between steroid hormone receptors and the transcriptional machinery. So far, several coregulators have been identified and characterized.

2.1 General structure of coregulators

Coactivators and cointegrators have been shown to share similarities both in their genomic sequences and in their protein structures (Fig. 4). A centrally located nuclear receptor interacting domain (NID) is necessary and sufficient for direct interaction with the LBD of steroid hormone receptors. It contains one or more copies of LXXLL motif, in which L denotes leucine and X is any aa. These LXXLL motifs, also called NR boxes, bind to the AF-2 activation domain of NRs and display preferences in binding for certain receptors (Heery et al. 1997, Li et al. 1997, Leers et al. 1998, Wong et al. 2001). There are also LXXLL motifs outside the NID, but these interactions are much weaker and their role has remained largely unknown (Wong et al. 2001). Activation domain 1 (AD1) of coregulators has been shown to interact with cointegrators CBP/p300 (Chakravarti et al. 1996, Kamei et al. 1996, Voegel et al. 1998), while activation domain 2 (AD2) interacts with coactivator-associated arginine methyltransferase 1 (CARM1) and other coactivators such as peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) and steroid receptor RNA activator (SRA) (reviewed in Leo and Chen 2000). Coactivators also share an N-terminal basic helix-loop-helix (bHLH)-PAS domain, which plays a role in protein-protein-interactions and dimerization surface (reviewed in Leo and Chen 2000). C-termini contain glutamine-rich regions as well as a histone acetyl-transferase domains (Li et al. 1997, Chen et al. 1997).

The bromodomain, which is a module of about 110 aas, is conserved evolutionary and has been found in a number of proteins, including coregulators CBP, p300 and pCAF (Jeanmougin et al. 1997). A consensus sequence of 40 aas out of the 110 residues in this domain has been deduced (Jeanmougin et al. 1997). Both CBP and p300 also contain one ZZ, which has been named for its potential role in binding two Zn^{2+} ions, and three cysteine- and histidine-rich TAZ (transcriptional adaptor putative zink finger) regions (reviewed in Eckner 1996, Ponting et al. 1996). The function of the bromodomain as well as ZZ and TAZ domains is mainly unknown, even if they have been proposed to be involved in the regulation of transcriptional activation and to mediate certain protein-protein interactions, thus influencing the assembly and/or activity of large protein complexes in the vicinity of target gene promoters (Ponting et al. 1996, Jeanmougin et al. 1997).

Both corepressors, nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid acid and thyroid hormone receptors (SMRT), contain multiple highly conserved functional domains (Fig. 5; reviewed in Jepsen and Rosenfeld 2002, reviewed in Jones and Shi 2003). The three N-terminal repression domains (RDs) mediate transcriptional repression and the C-terminus contains a bipartite NID, which is responsible for interaction with unliganded steroid hormone receptors. Further analysis of NIDs has revealed that each contains a conserved CoRNR box motif, which is similar to the LXXLL recognition motif present in coactivators. SANT domains A and B have been named for their presence in Swi3, Ada2, N-CoR and TFIIB proteins.

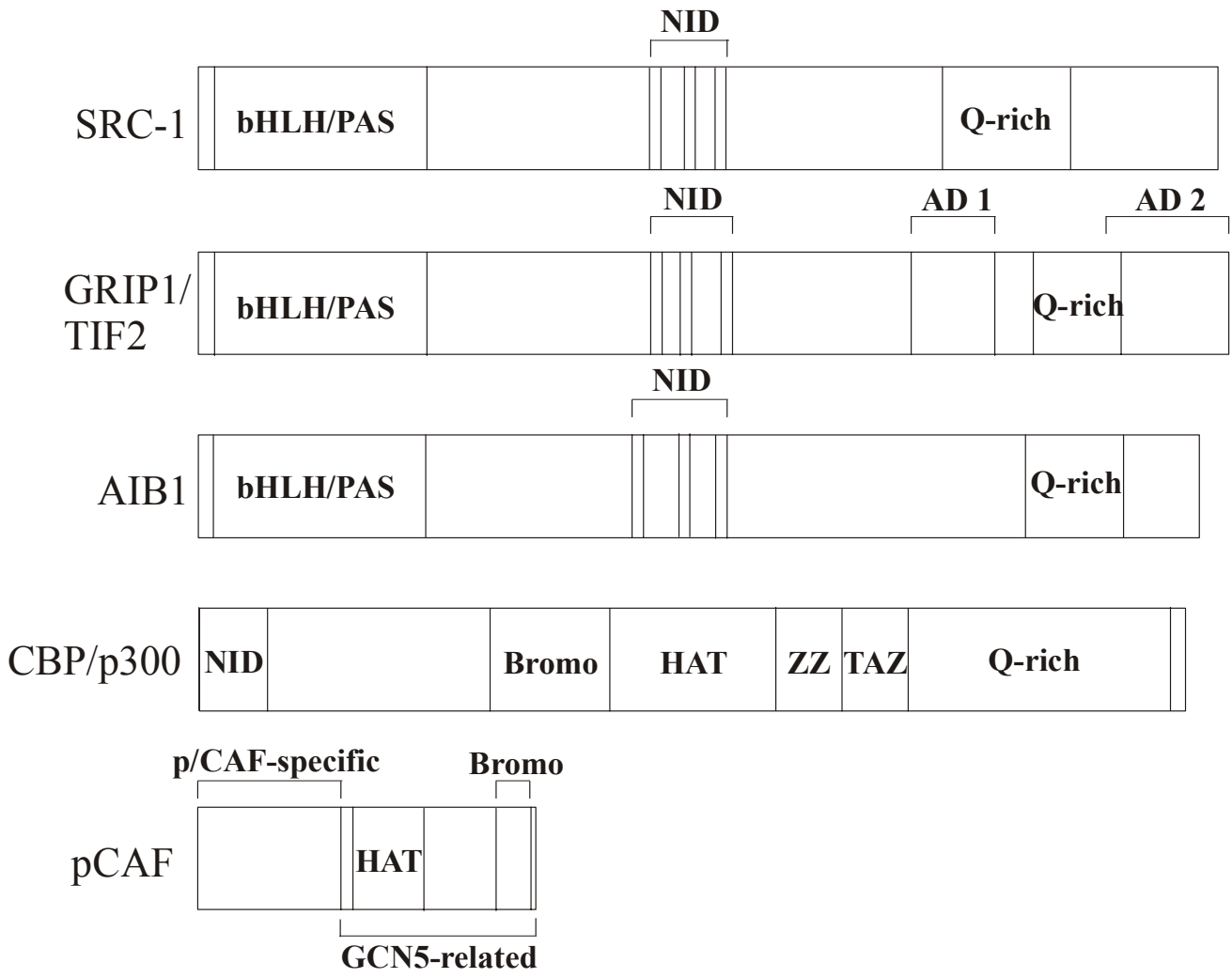

Figure 4. General structure of coactivators SRC-1, GRIP1/TIF2 and AIB1 and cointegrators CBP/p300 and pCAF. The abbreviations used are: bHLH/PAS, basic helix-loop-helix-PAS domain; NID, nuclear receptor interacting domain; Q-rich, glutamine-rich region; AD, activation domain; Bromo, bromodomain; HAT, histone acetyl-transferase domain; GCN5, yeast transcriptional adapter 5. Modified from Bannister and Kouzarides 1996, Chakravarti et al. 1996, Yang et al. 1996, Glass et al. 1997, Chen and Li 1998 and Voegel et al. 1998.

2.2 Coactivators SRC-1, TIF2/GRIP1 and AIB1

The first coactivator proteins p160 and p140 for ERα were identified by Halachmi et al. (1994) and Cavaillès et al. (1994) respectively. The gene encoding steroid receptor coactivator-1 (SRC-1), the first member of the SRC/p160 coactivator family, was cloned and characterized by Oñate et al. in 1995. Later on, Kamei et al. (1996) demonstrated that SRC-1 accounted for a significant fraction, but not all, of the p160 protein. SRC-1 has been shown to interact with and enhance the transcriptional activity of PR and both ERs in response to their respective ligands (Tremblay et al. 1997, Oñate et al. 1998). Two isoforms of SRC-1 (SRC-1a and SRC-1e) have been identified and they have been shown to be functionally distinct, as they differ in their ability to interact with ER and in their ability to enhance ER-driven transactivation (Kalkhoven et al. 1998). SRC-1 mutant mice have been shown to be viable, they grew normally and were fertile, but they displayed features

of resistance to thyroid hormone (Xu et al. 1998, Qi et al. 1999, Weiss et al. 1999). They also exhibited partial resistance to steroid hormones as evidenced by decreased growth and development of target tissues uterus and mammary gland (Xu et al. 1998).

The second member of this SRC-1 family includes human transcription intermediary factor 2 (TIF2) and its mouse homolog glucocorticoid receptor interacting protein 1 (GRIP1), which are highly related to SRC-1 and serve as transcriptional activators of PR and ER (Hong et al. 1996, Voegel et al. 1996, Oñate et al. 1998). It has also been designated as nuclear receptor coactivator 2 (NCoA-2) or steroid receptor coactivator-2 (SRC-2) in some papers. The physiological role of GRIP1 has been studied by analysing female mice in which the exon of NID has been deleted (Gehin et al. 2002). The females were hypofertile: they had reduced numbers of litters and pups per litter due to foetal death during pregnancy. Growth retardation of the embryos as well as embryonic lethality were probably correlated with placental hypoplasia. GRIP1 heterozygotes lacked any defects, suggesting that one TIF2/GRIP1 allele is enough to mediate the physiological actions of this coactivator. >90% of SRC-1/TIF2 compound mutant mice died at birth (Mark et al. 2004).

The third member of this family, amplified in breast cancer 1 (AIB1), was identified in 1997 by four groups and is thus also named RAC3, TRAM-1 and ACTR (Anzick et al. 1997, Chen et al. 1997, Li et al. 1997, Takeshita et al. 1997). Suen et al. (1998) have used the abbreviation SRC-3 (steroid receptor coactivator-3) and mouse homolog is called p/CIP (Torchia et al. 1997). It has been suggested that AIB1 has an important role in both steroid hormone receptor signalling and breast cancer development and it can interact with ER and PR in a ligand-dependent manner and potentiate the actions of these receptors (Takeshita et al. 1997, Torchia et al. 1997, Suen et al. 1998, Tikkanen et al. 2000). It associates with cointegrators CBP, p300 and pCAF and thus recruits two cofactors to the nuclear receptor-DNA complex (Chen et al. 1997, Takeshita et al. 1997). Because CBP can also associate with many additional factors (please see later), AIB1 may be a component of a larger complex, which is critical for integration of several signal-transduction pathways such as MAPK pathway. The transcriptional activity of AIB1 is enhanced by MAPK phosphorylation (and MAPK activation of AIB1 stimulates the recruitment of p300 and associated HAT activity) implying that AIB1 is a conduit for growth-factor signalling to the ER (Font de Mora and Brown 2000).

Disruption of the AIB1 gene results in a phenotype where mice are fertile, but female ovulation capability, fertilisation rate, pregnancy rate and the number of pups per litter were decreased (Wang et al. 2000, Xu et al. 2000). They also displayed dwarfism, facial asymmetry and growth retardation, especially during the growth surge accompanying pubertal development (Wang et al. 2000, Xu et al. 2000). AIB1 is not essential for ER function in stimulating virgin mammary growth, but the adult mammary ductal branching and alveolar formation in response to estrogen and progesterone stimulation are significantly attenuated in AIB1-deficient mice (Xu et al. 2000). AIB1 also seems to play a role in the insulin-like growth factor-1 (IGF-1) and growth hormone regulatory pathway, as well as in the production of estrogen in mouse development (Wang et al. 2000). Furthermore, AIB1 deficiency has been shown to suppress breast cancer initiation and progression in mice (Kuang et al. 2004).

2.3 Corepressors N-CoR and SMRT

In addition to coactivators, proteins called corepressors were also cloned and characterized in the middle of the 1990s (Chen and Evans 1995, Hörlein et al. 1995). N-CoR and SMRT form a family of bona fide nuclear receptor corepressors (Fig. 5). These large proteins are widely expressed in the nuclei at low levels. They are both in fact components of several different protein complexes (reviewed in Jepsen and Rosenfeld 2002). These corepressors recognise and interact with the

receptors both in the absence of agonists and in the presence of their respective antagonists (Jackson et al. 1997, Smith et al. 1997, Lavinsky et al. 1998). It has been hypothesized that both N-CoR and SMRT bind to the antagonist-bound ER C terminus and interact weakly with the constitutive ER N terminus, thus preventing the association of the coactivator complex (Lavinsky et al. 1998). The receptor agonist, in turn, causes dissociation of corepressors from the receptor and is followed or coincident with a conformational change in the C-terminal AF-2 domain of the receptor allowing association with coactivators. It has been hypothesized that N-CoR and SMRT may also have other than repressor functions.

N-CoR gene-deleted mice embryos died by day 17 of gestation (Jepsen et al. 2000). They were pale, smaller than their WT littermates and had a severe anemia and secondary edema (Jepsen et al. 2000). Furthermore, defects in erythropoiesis and reduction in thymus size were seen (Jepsen et al. 2000) and it seems that N-CoR plays an important role in the growth and development. To our knowledge, SMRT knockout mice have not been reported and thus the role of this corepressor *in vivo* remains unknown so far.

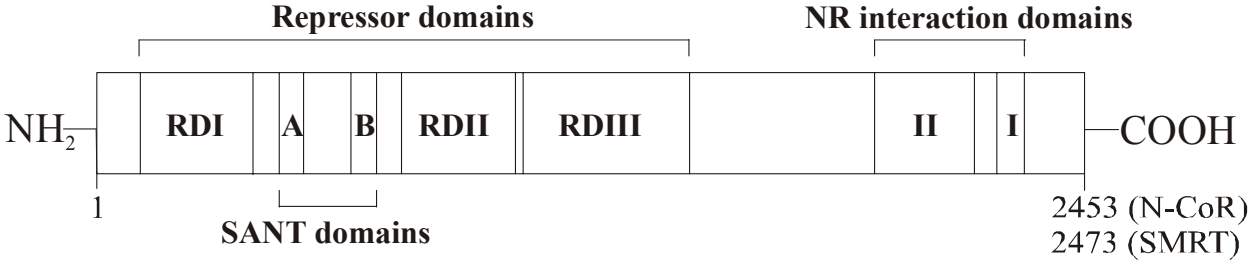

Figure 5. General structure of corepressors N-CoR and SMRT. Repressor domains (RD) I-II, SANT domains A and B and nuclear receptor (NR) interaction domains I and II are shown. Modified from Guenther et al. 2000, Li et al. 2000 and Jepsen and Rosenfeld 2002.

2.4 Cointegrators CBP, p300 and pCAF

Cointegrators CREB binding protein (CBP) and its closely related family member p300, which was identified based on its ability to interact with adenoviral E1A, are large, mainly nuclear proteins, which have been demonstrated to serve as common transcriptional coactivators for ER and PR (Chrivia et al. 1993, Eckner et al. 1994, Kwok et al. 1994). Interactions with the components of the basal transcriptional machinery and with a large number of other transcription factors (including CREB, pCAF, proto-oncogenes c-Myb, c-Fos and c-Jun, YY1, bHLH factors, signal transducer and activator of transcription (Stat) proteins, MEF2 family members, Sap-1, AP1 and NF-κB) as well as with coactivator SRC-1 and cointegrator pCAF have also been shown (Arias et al. 1994, Bhattacharya et al. 1996, Dai et al. 1996, Eckner et al. 1996, reviewed in Eckner 1996, Kamei et al. 1996, Yang et al. 1996, Yao et al. 1996, Zhang et al. 1996, Perkins et al. 1997, reviewed in Vo and Goodman 2001). Although encoded by two distinct genes, CBP and p300 are closely related in primary structure and have also been called secondary coactivators, because they have many of the features that would be expected of a coactivator (Chrivia et al. 1993, Arany et al. 1994). Neither CBP nor p300 itself binds to DNA, but they can be recruited to the promoter regions by interaction with receptors and other transcription factors. Moreover, p300 and SRC-1 proteins have been

shown to interact *in vitro*, and they also form complexes *in vivo* (Yao et al. 1996). Coexpression of CBP and SRC-1 has been shown to stimulate both ER and PR transcriptional activity in a synergistic manner (Smith et al. 1996).

CBP connects nuclear receptors to the basal transcriptional machinery and other transcription factors such as SRC-1, CREB and c-Jun (Chrivia et al. 1993, Arias et al. 1994, Kwok et al. 1994, Kamei et al. 1996) and has thus been suggested to function as an adapter protein or bridging factor between steroid hormone receptors and the basal transcription machinery. It is possible that low levels of CBP/p300 are associated with prevalence of antagonistic interactions, while high levels would alleviate antagonistic interactions. Alterations in CBP/p300 levels could thus influence how cells respond to hormones. The zinc-finger region of CBP is able to interact with the basal transcription factor TFIIB (Kwok et al. 1994), which in turn interacts with the TATA-binding protein (TBP).

CBP homozygous mice have shown complete embryonic mortality and open neural tube defects (Yao et al. 1998), while heterozygotes showed similar anomalies as has been reported for Rubinstein-Taybi syndrome patients (please see below) (Tanaka et al. 1997). Thus, relatively small decreases in the concentrations of CBP are deleterious in both humans and mice. As in CPB homozygous mice, homozygous p300 mutation resulted in embryonic lethality associated with developmental and growth retardation (Yao et al. 1998). Open neural tube defects as well as exencephaly were also observed (Yao et al. 1998). Thus, p300 and CBP single-homozygous as well as compound-heterozygous mutants (Yao et al. 1998) display quite similar embryonic phenotypes.

CBP and p300 have been demonstrated to interact with the third member of this cointegrator family, p300/CBP-associated factor (pCAF) (Yang et al. 1996). Both PRs have been shown to interact directly with pCAF and the authors suggested that there are multiple pCAF interaction sites on the human PR (Jenster et al. 1997). pCAF is present in a stable multisubunit complex consisting of more than 20 distinct polypeptides (Ogryzko et al. 1998). It has been duly suggested that pCAF complex is potentially recruited to a wide range of promoters *via* and because of multiple existing protein-protein interactions. pCAF has also been shown to inhibit cell cycle progression *in vitro* (Yang et al. 1996). pCAF homozygous mice were developmentally normal and showed no obvious phenotypic abnormalities (Yamauchi et al. 2000, Inoue et al. 2004), but the expression of c-Fos gene was not induced by estrogen in the uteri of the knockout mice (Inoue et al. 2004).

In summary, relatively small decreases in CBP and p300 are deleterious during growth and development. pCAF is also required for normal embryogenesis and in ER-mediated normal growth of the uterus in particular. Another common functional role of all three cointegrators seems to be muscle cell differentiation (reviewed in Eckner 1996, Puri et al. 1997).

2.5 Histone acetylation (HAT) and histone deacetylation (HDAC) activity of coregulators

Nucleosomes are large spherical subunits of chromosomes, around which 146 bp of double-helix DNA is wound. They are composed of an octamer of histones and one histone octamer in turn consists of a heterotetramer of the core histones H3 and H4 associated with two heterodimers of H2A and H2B. Each of these four core histones contains an N-terminal lysine-rich tail domain, and many of these lysine residues are targets for acetylation, which is known to be increased in transcriptionally active chromatin (reviewed in Grunstein 1997, reviewed in Wade et al. 1997). Mutation of the histone tail domain has been shown to result in delay in cell cycle progression and thus slow cell growth (reviewed in Wade et al. 1997).

All three cointegrators and coactivators except TIF2/GRIP1 have been shown to exhibit HAT activity, which means that this activity can transfer acetate from acetyl-CoA to histones and also to other proteins in the complex on DNA such as the basal transcriptional machinery (Bannister and Kouzarides 1996, Ogryzko et al. 1998, Yang et al. 1996, Chen et al. 1997, Imhof et al. 1997, Spencer et al. 1997, Chen et al. 1999, Oñate et al. 1998, reviewed in Vo and Goodman 2001). HAT modifies chromatin locally, and through loosening or weakening of the nucleosome at the site of promoter (chromatin structure "opens"), it enables the recruitment of transcription factors and subsequent access of RNA polymerase enzyme and transcriptional activation (reviewed in Brownell and Allis 1996, reviewed in Wolffe and Pruss 1996, reviewed in Wade et al. 1997). So far, the detailed acetylation targets of individual coregulators are not known. It has also been suggested that in addition to acetylation of histones, acetylation of other transcription factors might be one mechanism of gene regulation (reviewed in Vo and Goodman 2001).

Accumulating evidence indicates that corepressors N-CoR and SMRT either reside in or recruit high-molecular-weight complexes into the promoter region of target genes. These complexes have been shown to contain members of the HDAC family proteins (Alland et al. 1997, Heinzel et al. 1997, Nagy et al. 1997, Guenther et al. 2000, Huang et al. 2000, Kao et al. 2000, Li et al. 2000). The HDAC activity of these N-CoR/SMRT complexes has an opposing effect to HAT on the promoter and there is evidence that deacetylated histones are associated with silent regions of the genome (reviewed in Wolffe 1996). As a result, the chromatin returns to its condensed state and transcription is repressed (Heinzel et al. 1997, Nagy et al. 1997, Li et al. 2000).

2.6 Cell- and tissue-specific expression of coregulators

SRC-1 has been shown to be expressed in a subpopulation of rat mammary gland epithelial cells, which existed in a more luminal layer, distinct from those expressing ER and PR (Shim et al. 1999). On the contrary, significant numbers of cells coexpressed ERα and SRC-1 in the stroma (Shim et al. 1999). Thus, it could be speculated that SRC-1 may have cell- or tissue-specific, yet unknown, partners for its coactivation function at least in the mammary epithelial cells. SRC-1 is expressed in the nuclei of glandular and stromal cells of human endometrium, and expression decreases prominently in the secretory phase of the menstrual cycle (Shiozawa et al. 2003, Uchikawa et al. 2003). Furthermore, SRC-1 is present in Sertoli cell and Leydig cell nuclei of testes (Mark et al. 2004).

TIF2 transcript has been shown to be expressed in human liver, pancreas, lung, brain, heart, skeletal muscle and placenta and at a low level in kidney (Voegel et al. 1996). TIF2 mRNA and protein has also been shown to be expressed in the nuclei of Sertoli cells (Gehin et al. 2002). A twofold overexpression of TIF2 mRNA has been detected in the testis and brain of SRC-1 mutant mice, suggesting that increased TIF2 levels may partially compensate the absence of SRC-1 (Xu et al. 1998). Transfection of HeLa cells with GRIP1 in expression vector resulted in nuclear localization of the protein (Lopez et al. 2001) and RNA expression has been shown in mouse embryos and in adult mouse liver, kidney, testis, heart, brain, lung, muscle, skin and spleen (Hong et al. 1996, Hong et al. 1997). Furthermore, TIF2 expression has been shown to be expressed in normal human mammary glands, and TIF2 levels were significantly higher in the intraductal carcinomas than in normal breast tissues (Kurebayashi et al. 2000). TIF2 levels were also increased in the samples of invasive ductal carcinomas, but not statistically significantly (Kurebayashi et al. 2000).

In addition to TIF2/GRIP1 and SRC-1, AIB1 is widely expressed in a tissue and cell-type-specific manner. AIB1 is overexpressed or amplified in some breast and ovarian cancer cell lines and especially in ER- and PR-positive breast cancers as well as in human MCF-7 breast cancer cells, in which a clear nuclear protein expression has been shown (Anzick et al. 1997, Bautista et al. 1998,

Suen et al. 1998). In addition, AIB1 transcript has been shown to be expressed in prostate cancer cell lines and protein expression in both benign and malignant prostate tissue, predominantly in the epithelium, has been shown (Gnanapragasam et al. 2001). AIB1 appeared to be distributed both in the cytoplasm and nucleus of the cells (Gnanapragasam et al. 2001). In normal human tissues an abundant AIB1 mRNA expression has been shown in uterus, mammary gland, testis, heart, skeletal muscle, pancreas and placenta, and low or barely detectable expression has been shown in bone marrow, adrenal gland, brain, stomach, lung, liver and kidney (Chen et al. 1997, Takeshita et al. 1997, Suen et al. 1998). In contrast to the above results, a strong mRNA expression was seen in lung, liver and testis and the weakest expression was seen in the heart (Torchia et al. 1997). Mouse homolog p/CIP has been shown to be highly expressed in the thyroid gland, thymus, kidney, lung, retina and skin, and both cytoplasmic and nuclear expression was seen (Wang et al. 2000). p/CIP is also expressed in the myoepithelial and luminal compartments of the mammary gland and the expression levels as well as subcellular localizations seem to vary according to the stages of mammary gland development and differentiation (Kuang et al. 2004). Whether AIB1 is expressed in the brain or not remains unresolved (Torchia et al. 1997, Carroll et al. 2000, Xu et al. 2000).

CBP transcript is expressed in normal mammary gland and its expression is increased in intraductal carcinomas when compared to normal mammary gland (Kurebayashi et al. 2000). In addition, it is expressed in several human breast cancer cell lines (Kurebayashi et al. 2000). Both CBP and p300 are expressed in the nuclei of human endometrial glandular and stromal cells and the expression remains constant throughout the menstrual cycle (Shiozawa et al. 2003, Uchikawa et al. 2003). A widespread expression of p300 with elevated levels in CNS in mouse embryos has been reported (Yao et al. 1998). p300 is also expressed in muscle cells and in many cell lines (Eckner et al. 1994, Sartorelli et al. 1997). pCAF transcript seems to be ubiquitously expressed, but is most abundant in heart, skeletal muscle, liver and hind limb (Yang et al. 1996, Yamauchi et al. 2000).

N-CoR mRNA is expressed in adult mouse heart, brain, spleen, lung, liver, muscle, kidney and testis, but the expression varies a lot (Hörlein et al. 1995). SMRT is a nuclear protein and is ubiquitously expressed in low quantities (Chen and Evans 1995). In the normal human endometrium, N-CoR expression is mainly nuclear during the proliferative phase, and it is completely absent during the secretory phase (Shiozawa et al. 2003, Uchikawa et al. 2003). SMRT is expressed both in the nuclei and cytoplasm of endometrial cells, and, as in the case of N-CoR, its expression is very low during the secretory phase (Shiozawa et al. 2003). Both N-CoR and SMRT transcripts are expressed in normal mammary glands, and N-CoR expression decreases during progression from intraductal carcinoma to invasive ductal carcinoma (Kurebayashi et al. 2000).

2.7 Coregulator-related human diseases

So far, a few coactivators have been shown to be involved in human diseases. Rubinstein-Taybi syndrome (RTS) has been shown to be caused by gross chromosomal rearrangements or by point mutations in the CBP gene in about 20% of the patients (Petrij et al. 1995, Petrij et al. 2000a), and it has been suggested that the loss of C-terminal domains of CBP is sufficient to cause this syndrome (Petrij et al. 2000b). This disease is characterized by craniofacial, skeletal and cardiac defects, as well as growth and mental retardation, but the phenotype is highly variable (Rubinstein and Taybi 1963, reviewed in Yanase et al. 2004). These patients also have an increased risk of neural and developmental tumours, especially of the head (reviewed in Miller and Rubinstein 1995).

Translocation of MOZ (monocytic-leukaemia zink-finger) and CBP genes and the subsequent fusion protein as well as CBP rearrangements play a major role in certain subtypes of acute myeloid leukaemia (AML) (Borrow et al. 1996, Giles et al. 1997). CBP fusion with mixed lineage leukaemia (MLL) gene has been reported in patients with myelodysplastic syndrome (Taki et al. 1997). p300

gene mutations in colorectal and gastric carcinomas have been reported (Muraoka et al. 1996) and it has been speculated that t(8;22) fusion in AML patients is in fact a fusion involving p300 and MOZ. Furthermore, p300-MLL fusion protein has been reported to be involved in the development of AML (Ida et al. 1997). The fusion protein, AML-1/ETO, resulting from t(8;21), is able to recruit the corepressor N-CoR as well as mSin3 corepressors and HDACs, which suppresses differentiation of the hematopoietic cells (Lutterbach et al. 1998). This fusion protein is then able to yield the same phenotype as AML-1 deficiency resulting in the onset of AML.

AIB1 gene has been shown to be amplified in some of the human breast tumours and its overexpression has been shown to correlate with either ER- or both ER- and PR-positivity and tumour size (Anzick et al. 1997, Bautista et al. 1998, Iwase et al. 2003). AIB1 protein levels have also been shown to be significantly higher in ductal and lobular breast carcinoma samples than in normal breast tissue samples, suggesting that these increased levels may have diagnostic value for a subset of human breast tumours (List et al. 2001b). Furthermore, a positive correlation between AIB1 overexpression and expression of p53 and human epidermal growth factor-2 (HER-2) has been reported in breast carcinomas, which suggests that AIB1 may also be involved in signalling pathways other than steroid hormone receptor-mediated (Bouras et al. 2001). In addition to breast cancer, amplification and overexpression of AIB1 has been found in ovarian, pancreatic, gastric and hepatocellular cancers (Anzick et al. 1997, Ghadimi et al. 1999, Sakakura et al. 2000, Wang et al. 2002, Henke et al. 2004).

Most cases of resistance to thyroid hormone (RTH), also called Refetoff syndrome, are caused by thyroid hormone receptor β (TRβ) gene mutations. However, some patients with RTH do not have this mutation and individuals with the same genetic lesion may display quite different symptoms. In fact, an aberrant interaction between mutant TH receptors and SMRT has been shown: mutated receptors failed to dissociate from SMRT or N-CoR in the presence of thyroid hormone (Yoh et al. 1997, reviewed in Jepsen and Rosenfeld 2002). SMRT and N-CoR have also been shown to form a stable complex with HDAC3 and at least one additional protein, transducin β-like protein 1 (TBL1), whose gene is mutated in human sensorineural deafness (Guenther et al. 2000, Wen et al. 2000). Both corepressors play a role in acute promyelocytic leukaemia and common acute lymphoblastic leukaemia (reviewed in Jepsen and Rosenfeld 2002) and N-CoR has also been shown to be involved in the pathogenesis of Huntington's disease (reviewed in Kumar et al. 2005).

3 STEROID HORMONE-MEDIATED REGULATION OF TRANSCRIPTION

The ligand binding induces a conformational change of the receptor, enabling its release from heat-shock proteins, dimerization and binding to specific hormone response element (HRE) within the regulatory regions of the target genes leading to activation or suppression of gene transcription. When bound to DNA, the receptor dimer contacts components of the general transcriptional machinery either directly or indirectly *via* coregulators leading to an increase in the rate of transcription initiation at the target gene promoter region. The primary response occurs within about 30 minutes, and these gene products are in turn able to inactivate other primary-response genes or activate delayed secondary-response genes.

3.1 Involvement of chaperone proteins and dimerization of the receptor

It is generally thought that unliganded steroid receptors, especially PR, glucocorticoid receptor (GR) and mineralocorticoid receptor, exist in large heteromeric complexes with Hsps and other components of the molecular chaperone machinery (Smith 1993, reviewed in Cheung and Smith 2000). A complex of 3 Hsps and 2 cochaperone proteins, also termed Hsp90/Hsp70-based chaperone machinery, is thought to be the minimal requirement to establish receptors that are capable of binding their respective hormones with high affinity and efficiency (reviewed in Cheung

and Smith 2000, reviewed in Pratt and Toft 2003). Four members of the complex open the steroid-binding cleft in the receptor in an ATP-dependent process so that it can be accessed by the hormone (reviewed in Pratt and Toft 2003). Finally, the fifth protein (p23), interacts with Hsp90 and stabilizes the whole complex (reviewed in Pratt and Toft 2003). In addition, chaperones may assist with the initial folding of nascent receptor polypeptides and they influence the shuttling of receptors between cellular compartments (reviewed in Cheung and Smith 2000). They are also believed to function in order to maintain the receptor in a proper conformation in the absence of hormone and to protect the LBD from inappropiate interactions and to inhibit the binding of coregulators to steroid hormone receptors (reviewed in Murdoch and Gorski 1991, reviewed in Cheung and Smith 2000). Finally, upon hormone binding, receptor is released from heteromeric complexes and establishes a new conformation (Smith 1993).

Dimerization is thought to be essential for steroid hormone receptor function, since mutations that interfere with receptor dimerization result in almost transcriptionally inactive receptors (Sheeler et al. 2003). Generally, steroid hormone receptors are thought to bind to DNA as ligand-induced homodimers. ERα and ERβ form mainly homodimers but are able to form heterodimers as well (Kumar and Chambon 1988, Ogawa et al. 1998). The formation of heterodimers between PR isoforms also exists *in vivo*, but they do not bind efficiently to progesterone response elements and thus appear to be inactive (Leonhardt et al. 1998). Heterodimerization between different steroid receptors has also been reported (reviewed in Murdoch and Gorski 1991).

3.2 Conformational changes

Upon hormone binding within the core of the LBD, the receptor conformation is changed. This conformational change allows dimerization and DNA binding of the receptor, interaction with coactivators and ultimately the modulation of target gene transcription (Tsai and O'Malley 1994). The three-dimensional structures of ERα and ERβ LBD are very similar: this compact wedge-shaped domain is composed of 12 α-helices arranged into three anti-parallel layers (reviewed in Pike et al. 2000). The ligand is trapped into a hollow ball-like structure and the LBD surface of the receptor is now able to interact with coactivator proteins. The coactivator recruitment site itself comprises a shallow, hydrophobic groove that is formed by residues between helices 3-5 and helix 12 and all the three leucines of the LxxLL motif are in contact with the LBD, while the two spacer X residues make no interactions and project away from the LBD (reviewed in Pike et al. 2000). However, for the box III peptide of rat TIF2, the binding motif is LxxYL rather than the consensus LxxLL (reviewed in Pike et al. 2000). Crystal structures of ER bound to different ligands have revealed that ligands of different sizes and shapes induce a spectrum of receptor conformational states, and that these states are then interpreted to result in differentially regulated target gene expression (Brzozowski et al. 1997, Shiau et al. 1998, reviewed in Nilsson et al. 2001).

The three-dimensional structure of the PR LBD bound to progesterone has revealed that the overall fold of the PR is similar to that found in ER (Tanenbaum et al. 1998, Williams and Sigler 1998). The interface is composed predominantly of helices 11 and 12 as well as 12-residue C-terminal extension tail that is essential for hormone binding, whereas helices 7-10 are the major contributors in the ERα LBD interface (Tanenbaum et al. 1998, Williams and Sigler 1998). Furthermore, estradiol-bound ERα LBD is a dimer in solution, whereas progesterone-bound PR LBD elutes as a monomer in gel-exclusion chromatography (Tanenbaum et al. 1998).

3.3 Estrogen and progesterone target genes

Ultimately, the receptors bind to specific DNA-sequences. ER binds to estrogen-response element (ERE) and PR binds to progesterone response element (PRE). EREs are inverted palindromic repeats (5'-GGTCAxxxTGACC-3'), where x is any nucleotide (reviewed in Herynk and Fuqua

2004). Receptor interaction with members of the preinitiation complex causes stimulation of transcription from the genes in the vicinity of the response elements.

The expression of proto-oncogenes c-Fos and c-Myc is one of the earliest changes seen in the uterus after single injection of physiological amounts of estrogen (Weisz and Bresciani 1988, Huet-Hudson et al. 1989). A phenomenon called estrogen priming has been shown to increase PR levels in various organs (Korach et al. 1985, Aronica and Katzenellenbogen 1991). In the WT mouse uterus, PR transcripts appeared to be up-regulated 24 h after estrogen treatment and this response was blocked by pretreating the animals with antiestrogen ICI 164,384 (Couse et al. 1995). pS2 was initially characterized as a gene, which is upregulated by estradiol in MCF-7 cells and a definite increase over the basal level is seen three hours after exposure, reaching a plateau after 24 hours (Masiakowski et al. 1982, Brown et al. 1984). Cathepsin D and α1-antitrypsin are also upregulated and HER-2 downregulated by estradiol in MCF-7/S9 cells (Jensen et al. 2003).

Regulation of the transcription of uterine epithelial secretory protein lactoferrin and glucose-6-phosphate dehydrogenase genes are known to be under the control of the estrogen-ER complex in the uterus and they have been used as a marker for estrogen action (Korach et al. 1985, Liu and Teng 1992, reviewed in Conneely et al. 2002). A 350-fold increase in lactoferrin mRNA and a 2.5-fold increase in glucose-6-phosphate dehydrogenase mRNA was seen in mouse uterus after a single dose of estradiol (Couse et al. 1995). However, no up-regulation of lactoferrin or glucose-6-phosphate dehydrogenase mRNA was seen when ER was absent (Couse et al. 1995). Estrogen also induces the synthesis of IGF-1 in the rodent uterus (Murphy et al. 1987, Kapur et al. 1992). It increases the secretion of mitogenic transforming growth factor-α (TGF-α) and decreases the secretion of inhibitory TGF-β in breast cancer cells, whereas antiestrogens and estrogen withdrawal induce an inverse response (Bates et al. 1988, Knabbe et al. 1987).

Progesterone is known to regulate the expression of many growth factors including heparin-binding epidermal growth factor (HB-EGF), IGF-1 and tumour necrosis factor-alpha (TNF-α) in uterine stromal cells (Kapur et al. 1992, Zhang et al. 1994, reviewed in Hunt et al. 1996). In addition, growth factor receptors such as EGFR (Dai and Ogle 1999) and TNF receptor (reviewed in Hunt et al. 1996) are induced by progesterone in the stromal cells and endometrium respectively. Moreover, expression of Stat family members has been shown to be regulated by progesterone in breast cancer cells (Richer et al. 1998) and the expression of lactoferrin, calcitonin, histidine decarboxylase and amphiregulin is increased in the uterine epithelium in response to progesterone (reviewed in Conneely et al. 2002). In addition, progestins have been shown to be able to stimulate a rapid activation of the MAPK signalling pathway in breast cancer cells in a PR-dependent manner, but this activation also requires the presence of ligand-free ER (Migliaccio et al. 1998).

It has been suggested that progesterone acts by two different pathways in the uterus, either directly on the endometrial epithelium or indirectly through the stromal cells, which increase stromal paracrine factor production affecting the epithelial cells by progesterone (reviewed in Lessey 2003). After progesterone injection, accumulation of c-Myc expression in mouse uterine stromal cell nuclei was seen (Huet-Hudson et al. 1989). The antiproliferative effect of progesterone on the endometrium is thought to be mediated by down-regulation of ER and thereby the sensitivity of the uterus to subsequent estrogen action is reduced (Hsueh et al. 1975, Bhakoo and Katzenellenbogen 1977). Progesterone also down-regulates its own receptor (Milgrom et al. 1973), and increases the activity of endometrial estradiol dehydrogenase, which leads to increased metabolism of estradiol to the less effective estrone (Tseng and Gurpide 1975).

3.4 Regulation of cell cycle by estrogen and progesterone

The concept of cell cycle was introduced in the 1960s. Responses of extracellular stimuli including steroid hormones, growth factors and other mitogens converge at this final point and enable crosstalk between different signalling pathways. Passage through the cycle is controlled by cyclin-dependent kinase (CDK) complexes, which are composed of a regulatory cyclin subunit (six cyclins enumerated so far: A, B, D1, D2, D3 and E) and a catalytic CDK subunit (a kinase). A succession of kinases is expressed along with a succession of cyclins as cells go through the cycle. After cyclin-CDK activation by phosphorylation, a transcription factor is activated by the removal of an inhibitor of the transcription factor, which in turn results in the transcription of the genes (i.e. next cyclin and kinase genes) necessary for the next cell cycle step. The cycle is driven in one direction only because of the irreversibility of protein degradation. An overview of the cell cycle and the effects of estrogen and progesterone on it are summarised in Fig. 6.

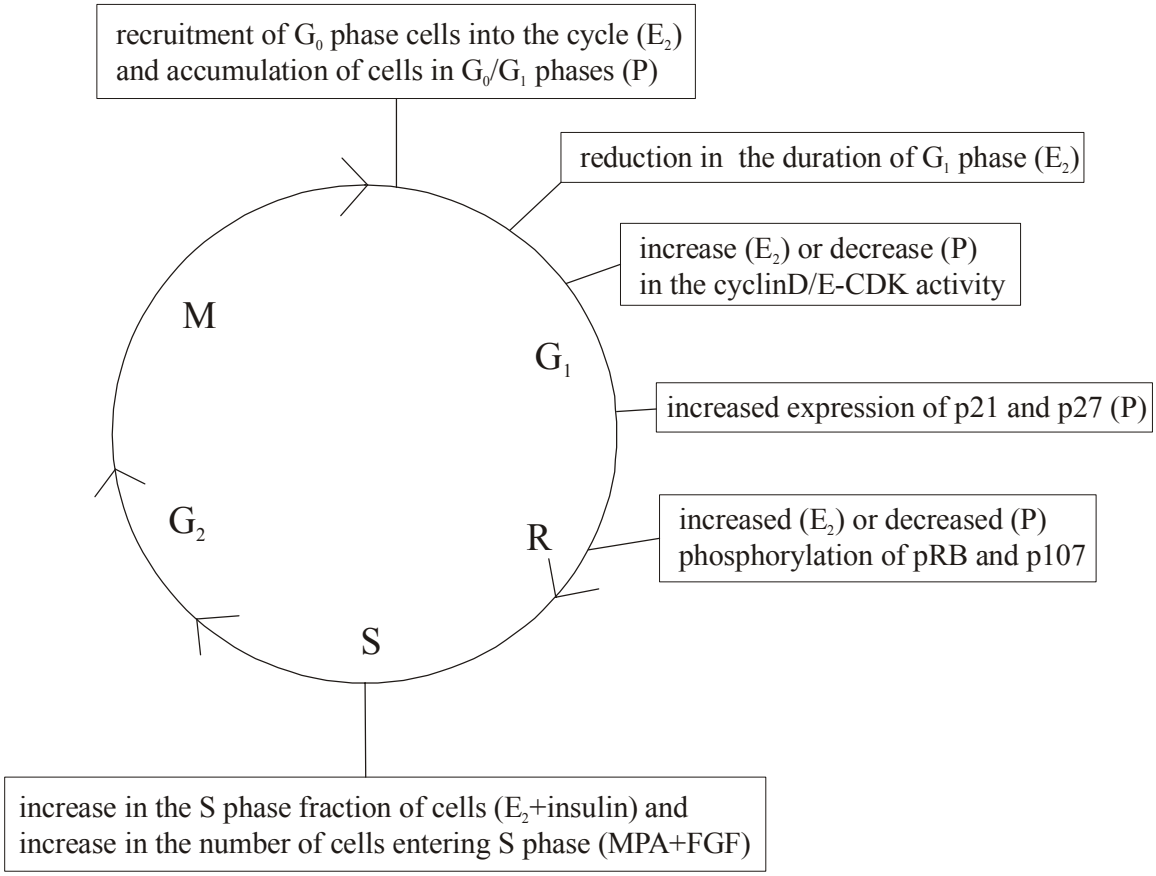

Figure 6. Summary of regulation of cell cycle by estrogen and progestins. G_1, G_1 phase; R, restriction point; S, synthesis; G_2, G_2 phase; M, mitosis; E_2, estradiol; P, progesterone; MPA, medroxyprogesterone acetate; CDK, cyclin-dependent kinase; p21 and p27, CDK inhibitors p21 and p27; pRB, retinoblastoma gene product; p107, pRB-related protein.

3.4.1 Cell cycle regulation by estrogen

Expression of rate-limiting proto-oncogenes c-Fos and c-Myc is one of the earliest detectable responses to estrogen (Travers and Knowler 1987, Weisz and Bresciani 1988). Another important factor is the regulation of the expression of cyclins, CDKs and CDK inhibitors. Transcriptional activation of cyclin D1 is apparent within 2-4 hours and the consequent formation of active cyclin D1-CDK4 complexes containing CDK inhibitor p21 is seen (Prall et al. 1997, reviewed in Sutherland et al. 1998). Cyclin E-CDK2 activity was also seen within the first few hours of the estrogenic response (Planas-Silva and Weinberg 1997, Prall et al. 1997, reviewed in Sutherland et al. 1998). A network of kinases and phosphatases as well as inhibitory proteins (e.g. $p16^{INK4}$, p21 and p27) are known to affect CDK activity, but little or no change in the levels of CDK inhibitors p21 and p27 prior to entry into S phase by estrogen has been shown (reviewed in Sutherland et al. 1998).

In general, the control of mammalian cell proliferation by steroid hormones occurs largely during the G_1 phase of the cell cycle and thus the studies have been focused on the major factors controlling the G_1 progression. Several authors have investigated the effects of estrogen on cell cycle entry and progression (reviewed in Sutherland et al. 1998). E_2 modulates events that occur before DNA synthesis and it has been shown that breast cancer cells are the most sensitive to estrogen in early G_1 phase, immediately following mitosis (Leung and Potter 1987). Furthermore, estrogen increases the proportion of cells synthesizing DNA by recruiting G_0 phase cells into the cell cycle and by reducing the duration of G_1 phase (reviewed in Sutherland et al. 1998). When T-47D breast cancer cells were grown in serum-free medium, 17β-estradiol alone failed to induce significant effects on cell cycle progression, but a concentration-dependent increase was seen in the S phase fraction when insulin was added (Sutherland et al. 1992). Thus, the cells obviously require other growth factors, particularly insulin/IGF-1, to maximally stimulate the cell cycle in serum-free or growth factor-depleted *in vitro* conditions.

Recent attention has been focused on regulation of critical cell cycle events that determine entry into, progression through and exit from the cell cycle (reviewed in Sutherland et al. 1998). Some key substrates for the control of cell cycle progression including retinoblastoma gene product pRB and p107 have been identified (Beijersbergen et al. 1995, reviewed in Weinberg 1995). Before progress through G_1, inactivation of the pRB and p107 proteins by phosphorylation is required and these events are triggered by estrogens (Beijersbergen et al. 1995, reviewed in Weinberg 1995, Planas-Silva and Weinberg 1997, Prall et al. 1997).

3.4.2 Cell cycle regulation by progestins

The effects of progesterone on the cell cycle seem to be more cell-type specific than the effects of estrogen. While 10 to 13 days of topical estradiol at the follicular phase of the menstrual cycle increased the number of cycling normal breast epithelial cells of premenopausal women, progesterone decreased the growth fraction of normal breast epithelial cells (Chang et al. 1995) and thus the predominant effect of progestin treatment seems to be inhibition of cell cycle progression both in normal breast epithelial and breast cancer cells. This has been shown to be associated with decreased phosphorylation of pRB and p107 and decreases in cyclin D1-CDK4, cyclin D3-CDK4 and cyclin E-CDK2 activity (Musgrove et al. 1998, reviewed in Sutherland et al. 1998). Moreover, increased expression of the CDK inhibitors p21 and p27 and their increased association with the remaining complexes follows progestin treatment (Musgrove et al. 1998, reviewed in Sutherland et al. 1998).

Treatment of T-47D breast cancer cells with progestins resulted in a biphasic effect: an initial increase in the S phase fraction followed by cell cycle arrest (Sutherland et al. 1992, Musgrove et al.

1991). Rather than entry of nonproliferating T-47D cells into the cell cycle, stimulation results from transient increase in the rate of progression of actively cycling cells already in G_1 phase (Musgrove et al. 1991). Progesterone treatment of T-47D cells induced down-regulation of human telomerase reverse transcriptase and telomerase activity, but the regulation is mainly indirect and related to the hormone effect on accumulation of cells in G_0/G_1 phase (Lebeau et al. 2002). mRNA expression of EGF and TGFα was increased in these breast cancer cells within three hours of progestin (ORG 2058) treatment, but when the cells were treated with these growth factors, increase in S phase percentage was detectable only 15 h after, suggesting that the actions of neither of these growth factors mediate the rapid responses of progestins seen in breast cancer cells (Musgrove et al. 1991). On the contrary, progestin up-regulated c-Myc proto-oncogene transcript (apparent within 15 minutes), and this induction was entirely abrogated by simultaneous treatment with antiprogestin RU 486, suggesting that c-Myc may participate in cell cycle regulation by progestins (Musgrove et al. 1991). All these effects are thought to be mediated *via* PR.

Cyclin D1 is one of the key regulatory molecules of the cell cycle and is ubiquitously expressed in most cell types (reviewed in Lange 2004). Treatment with progesterone leads to its up-regulation (reviewed in Lange 2004) and it is in fact a general target gene of many other growth factors and hormones as well (reviewed in Fu et al. 2004). Jones et al. (2000) studied the effects of synthetic progestin medroxyprogesterone acetate (MPA) in normal rat uterine stromal cells and found a significant increase in the number of cells entering the S phase after treatment with MPA plus fibroblast growth factors (FGF) compared to FGF alone. They also showed that progesterone exerts a direct effect on the transit through G_1 phase of the cell cycle and that the temporal accumulation of cyclin D1 mRNA is dependent upon progesterone and FGF and that this accumulation was reduced by antiprogestin.

Transient increases in c-Myc and cyclin D1 expression, increases in the cyclin D1-CDK4 complexes and in their activity and increase in the relative amount of pRB, a putative target of cyclin D1/CDK activity, have been shown in progestin-induced stimulation of cell cycle progression (Musgrove et al. 1993, reviewed in Sutherland et al. 1998). The rapid inductions of cyclin D1 and c-Myc expression could be antagonised by antiprogestin (Musgrove et al. 1993). The cells enter the S-phase approximately 8 h after progestin treatment (Musgrove et al. 1991). Cyclin D1-null mutant mice studies indicate that cyclin D1 is required for lobuloalveolar proliferation of the mammary gland during pregnancy and lactation (Fantl et al. 1995, Sicinski et al. 1995) and thus cyclin D1 action is essential for progesterone-driven mammary epithelial proliferation. The mammary epithelial cell proliferation defect seen during pregnancy in the cyclin D1-deficient animals could not be attributed either to the deficiency of circulating steroid hormones or to the absence of ER, because the levels of estrogen, progesterone and ERs were normal and thus a defect in the hormone responsiveness is probable (Sicinski et al. 1995), and pituitary insufficiency and the resulting prolactin deficiency may explain the failure to lactate (Fantl et al. 1995). Thus, steroid-induced proliferation of mammary gland epithelium during pregnancy may be driven by cyclin D1.

In summary, both estrogen and progesterone have a number of targets regulating the cell cycle, and thus the mitogenic effects of these hormones. At least c-Myc, pRB, p107, cyclin D1- and cyclin E-CDK complexes as well as the CDK inhibitors p21 and p27, play pivotal roles in steroid hormone action. Moreover, the effects of estrogen and progesterone on the cell cycle and its progression seem to be quite opposite.

4 CROSSTALK BETWEEN STEROID HORMONE RECEPTOR -MEDIATED SIGNALLING PATHWAY AND OTHER SIGNAL TRANSDUCTION PATHWAYS

Since the discovery that modulation of kinase activity in cells can also cause activation of some of the steroid hormone receptors in the absence of hormone (Denner et al. 1990, Ignar-Trowbridge et al. 1992), it has become apparent that the same steroid hormone can activate, either simultaneously or consecutively, both a membrane-associated receptor and the classic nuclear steroid hormone receptor. Steroid hormones are able to produce rapid, non-transcriptional responses, which in some cases are similar to those evoked by growth factors, and *vice versa*, protein kinases themselves may stimulate receptor activity. Since these early observations, many examples of crosstalk between steroid hormone receptor pathways and other signal transduction pathways, including G protein-coupled receptor, growth factor and MAP kinase signalling, have been described. Summary of this crosstalk is represented in Fig. 7.

4.1 G protein-coupled receptors

G protein-coupled receptors (GRCRs) constitute a large family of cell surface receptors with roles in the exocrine, endocrine and in the paracrine regulation of cell proliferation and differentiation (reviewed in Luttrell et al. 1997, reviewed in Gutkind 2000, reviewed in Luttrell 2002). GPCRs are of enormous importance for the pharmaceutical industry since approximately 50 % of all existing medicines act on a GPCR (Drews 1996). They consist of a single protein chain that crosses the cell membrane seven times and therefore are sometimes referred to as serpentine receptors (reviewed in Dhanasekaran et al. 1995, reviewed in Wess 1997). These seven transmembrane domains are connected by alternating intra- and extracellular hydrophilic loops (reviewed in Wess 1997). It has been shown that the extracellular regions and the transmembrane regions contribute to the formation of the ligand binding site, whereas aas of the intracellular loops mediate the interaction of the receptor with G proteins as well as with other signalling and regulatory proteins inside the cell (reviewed in Wess 1997).

4.1.1 G protein-coupled receptor 30

Three independent research groups cloned the cDNA of the G protein-coupled receptor 30 (GPR30) gene and published the results between September 1996 and April 1997 (Owman et al. 1996, Carmeci et al. 1997, Feng and Gregor 1997). The amino acid sequence of GPR30 protein shows many features common to other G-protein coupled receptors and thus this protein is considered a member of this superfamily. The GPR30 gene is located on the short arm of chromosome 7, the chromosomal band location most likely corresponding to 7p22 (Owman et al. 1996, Carmeci et al. 1997). The calculated relative molecular mass of the protein is approximately between 42 and 46 kDa (Owman et al. 1996, Feng and Gregor 1997).

Comparison of the deduced amino acid sequence of GPR30 with Burkitt lymphoma receptors BLR1 and BLR2 revealed 30% and 25% overall sequence identity respectively (Owman et al. 1996). The protein also shares significant homology with previously described GPCRs such as interleukin-8 and angiotensin I and II receptors (Owman et al. (1996, Feng and Gregor (1997). Slightly lower homology (25% to 28%) to receptors for C-C chemokines, bradykinin and other peptides has also been discovered.

Owman et al. (1996) have detected a transcript of 2.4 kb size by Northern blot hybridization, while Carmeci et al. (1997) estimated that the size of mRNA is 2.6 kb. The strongest signal was found in the Burkitt's lymphoma cell line Raji and in the virus-negative B-cell lymphoma cells (MC116), and a weak signal was seen in the Burkitt's lymphoma cell line Daudi (Owman et al. 1996). No transcripts were found in HeLa, colorectal adenocarcinoma, lung carcinoma, melanoma, T-cell lymphoma or six leukaemia cell lines tested (Owman et al. 1996). Three estrogen receptor-positive

breast cancer cell lines (MCF-7, T-47D and MDA-MB-361) expressed GPR30 mRNA while ER-positive ZR-75-1 and ER-negative BT-20, MDA-MB-231 and HBL-100 cells as well as HeLa cells and normal human mammary epithelial cells did not express it (Carmeci et al. (1997). GPR30 was expressed most abundantly in the MCF-7 cells, and expression was not affected by cell density or serum stimulation (Carmeci et al. (1997). Furthermore, estradiol exposure of MCF-7 cells decreased the expression twofold (Carmeci et al. (1997).

In addition to the expression in the above cell lines, the receptor message is widespread expressed in peripheral tissues. A strong signal was found in lung, liver, prostate and colon, a weaker signal in heart, pancreas, spleen, ovary, lymph node and appendix, while a very weak signal was seen in skeletal muscle, kidney, thymus, testis and small intestine (Owman et al. 1996). Moderate signal was found in all brain regions tested (Owman et al. 1996). Additionally, Feng and Gregor (1997) have found a 3.3 kb transcript in kidney, placenta and skeletal muscle using Northern blot analysis. GPR30 message was also detected in stomach, pituitary gland, adrenal gland, thyroid gland, colon, bladder, aorta, testis, ovary, small intestine, spleen, lymph node and bone marrow on a dot blot analysis (Feng and Gregor 1997). Moreover, the message could be detected in foetal human brain, heart, kidney, liver and spleen, and it was easily detectable in the developing mouse embryo on day 7. Carmeci et al. (1997) conclude that GPR30 mRNA was expressed in all human tissues examined (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas), most abundantly in placenta and least abundantly in skeletal muscle. Analysis of RNA from 50 human tissues revealed the most predominant hybridization signals from stomach and thyroid (Carmeci et al. 1997). Finally, Southern blot analysis of RT-PCR products of four ER-positive and seven ER-negative primary breast carcinomas showed that all ER-positive and one ER-negative tumours expressed GPR30 mRNA (Carmeci et al. 1997).

Previous attempts to activate GPR30 and adenylyl cyclase (AC) enzyme with several ligands have failed to detect any increase or decrease in cyclic adenosine monophophate (cAMP) levels (Owman et al. 1996). Neither angiotensin II nor IV binds to the receptor (Feng and Gregor 1997). It has been suggested that the ligand may also be a hormone or neurotransmitter (Feng and Gregor 1997), and indeed, it has recently been shown that E_2 binds to GPR30 and that GPR30 has the binding and signalling characteristics of a novel, membrane ER (mER) (Thomas et al. 2005). In addition, antiestrogens tamoxifen and ICI 182,780 have high binding affinities to the receptor, regardless of the presence of either ER isoforms (Filardo et al. 2000).

In addition to GPR30 acting independently of the ER to promote activation of AC and the subsequent cAMP generation, binding of E_2 results in the activation of cell membrane-associated matrix metalloproteinase (MMP) and in the cleavage and release of membrane-tethered HB-EGF (reviewed in Filardo and Thomas 2005). HB-EGF in turn activates EGFR, which subsequently activates the MAPK signalling pathway (reviewed in Filardo and Thomas 2005).

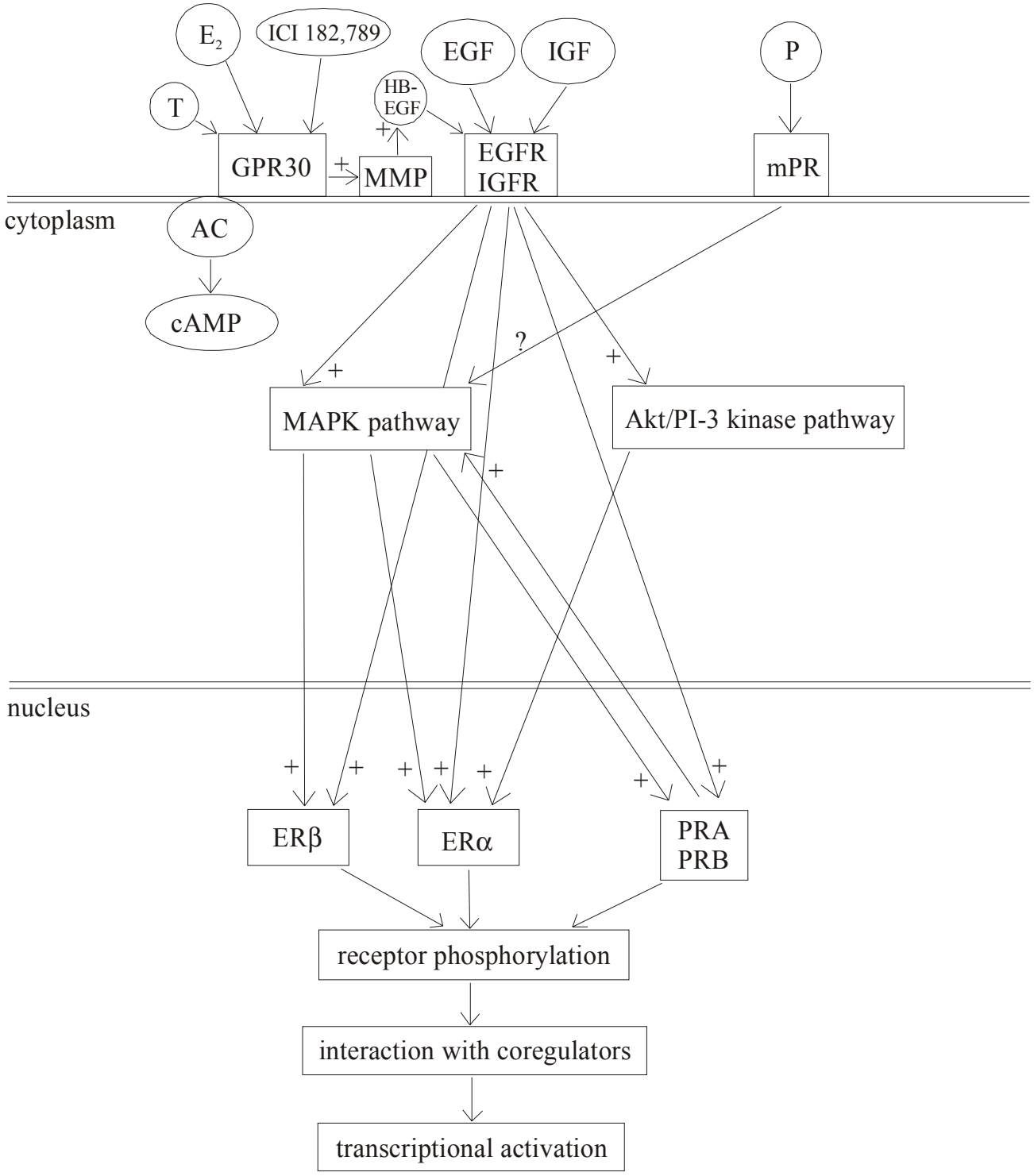

Figure 7. G protein-coupled receptor 30-, growth factor receptor- and membrane progestin receptor -mediated signalling and steroid hormone receptor transactivation. E_2, estradiol; P, progesterone; EGF, epidermal growth factor; IGF, insulin-like growth factor; T, tamoxifen; GPR30, G protein-coupled receptor 30; MMP, matrix metalloproteinase; HB-EGF, heparin-binding epidermal growth factor; EGFR, epidermal growth factor receptor, IGFR, insulin-like growth factor receptor; mPR, membrane progestin receptor; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; ER, estrogen receptor; PR, progesterone receptor.

4.2 Growth factors, kinase signalling and receptor transactivation

Growth factors such as EGF and IGF are known to stimulate the activity of ER in a ligand-independent manner through activation of a sequential protein kinase cascade referred to as the MAPK pathway and through direct prosphorylation of ER (Aronica and Katzenellenbogen 1993, Kato et al. 1995, reviewed in Kato et al. 1998). Extracellular signals transduced *via* G-protein-coupled receptors and growth factor receptors activate a small G-protein Ras, which is a key switch in cellular signalling (reviewed in Pouyssegur et al. 2002). One of the major pathways activated by Ras is the MAPK pathway (reviewed in Pouyssegur et al. 2002). Ras recruits the serine/threonine kinase Raf to the cell membrane, Raf phosphorylates and activates MEK, which in turn activates extracellular signal-regulated kinases ERK1 and ERK2 (reviewed in Pouyssegur et al. 2002). In addition, growth factors stimulate ERα transcriptional activity *via* the Akt (protein kinase B)/phosphatidylinositol-3 (Akt/PI-3) kinase pathway, which is another major growth factor signalling pathway in the cells (reviewed in Shupnik 2004). The converse also occurs, since the ligand-activated ERα can regulate both the Akt/PI-3 kinase and MAPK pathways (reviewed in Clarke et al. 2004). Ultimately, activation of the Akt/PI-3 kinase and MAPK signalling cascades leads to phosphorylation of ER and PR (reviewed in Clarke et al. 2004).

4.2.1 ER transactivation

Rat uterine ERs can be activated by IGF-1 and the treatment enhanced the phosphorylation of the receptor (Aronica and Katzenellenbogen 1993). Protein kinase inhibitors reduced this increase in phosphorylation by IGF-1 (Aronica and Katzenellenbogen 1993). These results suggest that phosphorylation is involved in the ligand-independent activation of ER. Estrogen itself is also able to activate the MAPK cascade in many different cell types (Migliaccio et al. 1996, Endoh et al. 1997, Watters et al. 1997). There is evidence that steroid hormone receptors are phosphoproteins and that phosphorylation is involved at least in the regulation of transcriptional activation and DNA binding of receptors (reviewed in Weigel 1996). Human ER becomes phosphorylated in the presence of estrogen and antiestrogens 4-OHT and ICI 164,384, but the antiestrogen-induced phosphorylation is less efficient (Ali et al. 1993). Phosphorylation of Ser^{118}, one major hormone-dependent phosphorylation site in AF-1 of ERα, is induced by hormone binding and is also a direct target for phosphorylation by the MAPK (Kato et al. 1995, reviewed in Weigel 1996, Joel et al. 1998, reviewed in Clarke et al. 2004). Phosphorylation leads to recruitment of the p68/p72 coactivator, which activates transcription in a ligand-independent manner (reviewed in Clarke et al. 2004). In addition, MAPK targets coactivators such as p160 and CBP for phosphorylation, leading to an increase in their activity (reviewed in Clarke et al. 2004).

MAPK also phosphorylates both human and mouse ERβ, and thus the crosstalk occurs not only through ERα, but also through ERβ (Tremblay et al. 1997, reviewed in Kato et al. 1998). Alignment of the mouse, rat and human ERβ aa sequences indicated that Ser^{60} is conserved in all species and that Ser^{60} of ERβ has been found to be a potential target for phosphorylation by the MAPK pathway (Tremblay et al. 1997). Two additional serine residues located at positions 106 and 124 of ERβ have also been identified as MAPK phosphorylation sites (Tremblay et al. 1999). Ser^{106} corresponds to Ser^{118} in human ERα and phosphorylation of both positions enhances the interaction of ERβ with SRC-1 (Tremblay et al. 1999).

4.2.2 PR transactivation

In addition to the classical transcriptional signalling mechanism mediated by PR, the existence of a non-transcriptional PR signalling pathway has been reported (reviewed in Leonhardt et al. 2003). For instance, the synthetic progestin R5020 has been shown to be able to stimulate a rapid and reversible activation of the MAPK signalling pathway in breast cancer cells in a PR-dependent

manner (Migliaccio et al. 1998). Ser^{294} of PR has been demonstrated to become phosphorylated by MAPK (reviewed in Lange 2004). This site is maximally phosphorylated 1-2 h after progestin treatment (reviewed in Lange 2004). The amino terminus of PR (aa 421-428) contains a proline-rich SH3 recognition motif that is able to interact with SH3 domains of cytoplasmic c-Src tyrosine kinases resulting in activation of MAPK signalling pathway (Boonyaratanakornkit et al. 2001). PR contains at least 14 different phosphorylation sites, and there are also many unknown kinases, which phosphorylate this receptor independently of MAP kinases (reviewed in Lange 2004).

EGF has been shown to activate PRs almost as well as hormone (reviewed in Shupnik 2004). PR becomes phosphorylated within 5 min of EGF treatment (reviewed in Lange 2004). EGF is also able to partly activate ER in the absence of estrogen and enhances activity in the presence of hormone (Ignar-Trowbridge et al. 1992, Ignar-Trowbridge et al. 1993, reviewed in Kato et al. 1998). However, at least part of the signal from EGF to both ER and PR is conducted through GRIP1, since the ERK family of MAPKs has been shown to be able to phosphorylate Ser^{736} of GRIP1 (Lopez et al. 2001). Exposure of rat uterine cells to IGF-1 resulted in the up-regulation of cellular levels of PR and this increase could be suppressed with cotreatment with the antiestrogen ICI 164,384 or protein kinase inhibitors, suggesting that PR regulation involved both ER and one or more ER phosphorylation pathways (Aronica and Katzenellenbogen 1991).

It has been thought for over two decades that a subpopulation of PR is localized either in the cytoplasm or on the cell membrane, and that progesterone elicits its rapid biological effects *via* this signalling pathway to regulate a different set of genes than the classical, receptor-dependent pathway, but efforts to determine their structures have been unsuccessful until now. Recent studies have namely demonstrated the existence of three distinct subtypes of human membrane progestin receptors (mPRs), which belong to a previously unknown family of steroid receptors unrelated to nuclear steroid hormone receptors and which are expressed in tissues that are known targets of nongenomic progesterone actions such as in reproductive and neural tissues (Zhu et al. 2003). They have seven transmembrane domains (Zhu et al. 2003), a protein structure, which is typical of GPCRs. The involvement of GPCRs in the regulation of MAPK signalling pathway has been demonstrated in many studies (reviewed in Luttrell et al. 1997, reviewed in Gutkind 2000, reviewed in Luttrell 2002, reviewed in Pouyssegur et al. 2002), and when the finding that binding of the mPR is specific for progesterone with high affinity (Zhu et al. 2003) is taken into account, progesterone is expected to mediate its rapid actions, or at least some of them, *via* MAPK pathway.

5 ANTIHORMONES, RECEPTOR MODULATORS AND AROMATASE INHIBITORS IN BREAST CANCER

Antihormones, hereafter referred to as antagonists, are either steroidal or non-steroidal synthetic compounds that are used in the treatment of breast cancer. They compete with steroid hormones for binding to steroid receptors in order to prevent receptor activation. Their use and mechanism of action in breast cancer are discussed in detail in the following chapters. In addition to antagonists and other receptor modulators, aromatase inhibitors are widely used in the treatment of breast cancer, and hence discussed below. Other treatment options for hormone-dependent breast cancer include e.g. luteinising hormone releasing hormone (LHRH) analogues for premenopausal women (reviewed in Prowell and Davidson 2004) and progestins, the use of which has been reduced by the introduction of aromatase inhibitors in metastatic breast cancer. The role of different hormonal treatment modalities in the adjuvant setting is reviewed by Jones and Buzdar (2004) and in metastatic breast cancer by Bernard-Marty et al. (2004).

5.1 Antiestrogens tamoxifen and toremifene

Tamoxifen (Tadex®, Tamexin®, Tamofen®) and toremifene (Fareston®) are non-steroidal antiestrogens (Fig. 8), which are nowadays used both in the treatment of metastatic breast cancer and in the adjuvant setting. They improve the overall survival and reduce both breast cancer recurrences and the incidence of contralateral breast cancer among postmenopausal women (Early Breast Cancer Trialists' Collaborative Group 1992). These triphenylethylene compounds are competitive inhibitors of estrogen in binding to ERs and they are active in ER-positive cells only (reviewed in Jordan 1990, reviewed in MacGregor and Jordan 1998). They are also called selective ER modulators (SERMs), because they have the ability to act as both receptor agonists and antagonists, depending on the cellular and promoter context as well as the ER isoform targeted. Thus, these drugs have desired effects in one tissue, but unwanted effects in another. The agonistic (estrogenic) effects include beneficial effects on bone mineral density and lipid profile, decrease in LH, follicle-stimulating hormone (FSH) and increase in sex hormone-binding globulin (SHBG) levels (reviewed in Jordan 1990, Love et al. 1990, reviewed in Mäenpää and Ala-Fossi 1997, Fisher et al. 1998). Among the unwanted agonistic effects are their thrombogenic effects and the ability to induce endometrial cancer (Rutqvist et al. 1995, Curtis et al. 1996a, reviewed in Mäenpää and Ala-Fossi 1997, Fisher et al. 1998). The antiestrogenic effect on vaginal epithelium by tamoxifen and toremifene is seen in postmenopausal women in the presence of estradiol (Mäenpää et al. 1990). Agonist and antagonist activities are also species-specific, since toremifene is an almost pure antiestrogen in the rat uterus while it has estrogenic activity in the mouse uterus (reviewed in Mäenpää and Ala-Fossi 1997).

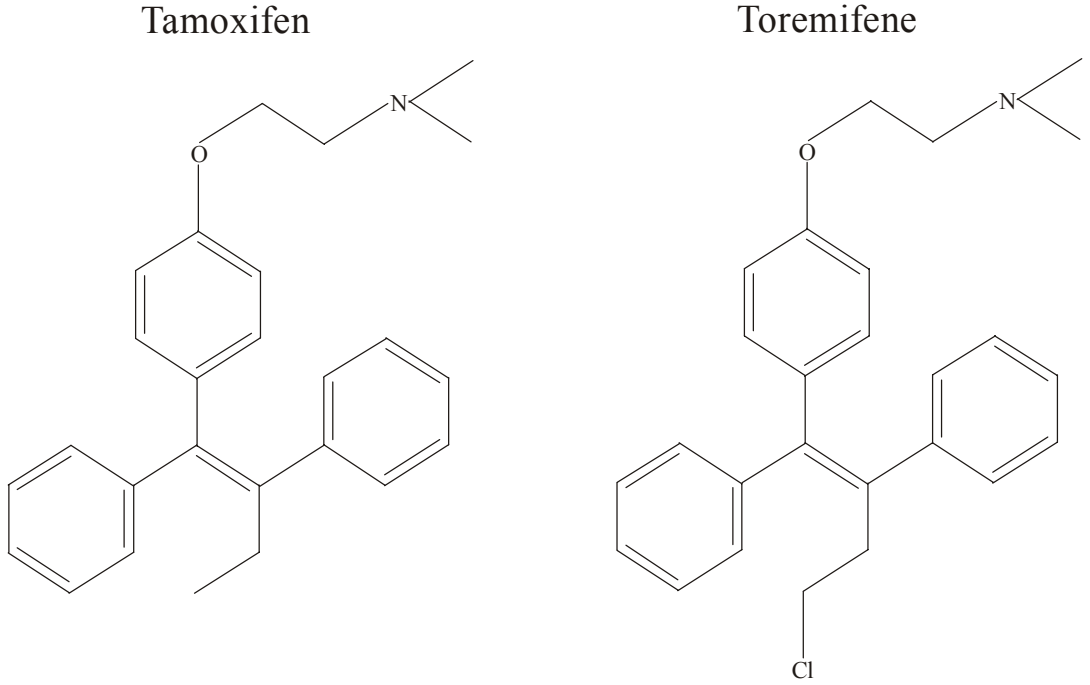

Figure 8. Chemical structure of antiestrogens tamoxifen and toremifene. Modified from Mäenpää and Ala-Fossi 1997 and Goldstein et al. 2000.

Toremifene, a chlorinated derivative of tamoxifen, or chlorotamoxifen, was synthesized in Finland in 1981 (Kallio et al. 1986, Kangas et al. 1986). When response rates, duration of response, overall survival, toxicity, binding to ERs, pharmacokinetics, antitumour activity and agonist activity have been studied, toremifene appears to be very similar to tamoxifen (Stenbygaard et al. 1993, Hayes et al. 1995, reviewed in Howell and Dowsett 1997, Holli et al. 2000). However, some differences between them have been reported. Toremifene is less potent, since it is recommended at a dose of 60 mg daily, whereas tamoxifen is recommended at a dose of 20 mg daily. A major difference in the toxicology of tamoxifen and toremifene has been shown: tamoxifen was hepatocarcinogenic and had a DNA adduct forming ability in female rats while toremifene appears to produce fewer DNA adducts and mutations than tamoxifen (Hard et al. 1993, reviewed in Mäenpää and Ala-Fossi 1997, reviewed in Williams and Jeffrey 1997, reviewed in MacGregor and Jordan 1998). The observed side effects of both antiestrogens appear to be very similar. The most common side effects are menopausal symptoms such as hot flushes and sweating, but headache, vaginal bleeding, increased risk of thrombotic events, endometrial effects and ocular abnormalities have also been reported (reviewed in Kangas 1990, Stenbygaard et al. 1993, Hayes et al. 1995, reviewed in Mäenpää and Ala-Fossi 1997, Fisher et al. 1998, Holli et al. 2000). In general, the side effects are usually mild and manageable, especially when compared to side effects of chemotherapy.

5.2 Pure antiestrogens ICI 164,384 and ICI 182,780

Unlike tamoxifen and toremifene, pure antiestrogens ICI 164,384 and ICI 182,780 (fulvestrant; Faslodex®) (Fig. 9) do not have any agonist activities in the uterus (reviewed in MacGregor and Jordan 1998), but they are full antagonists capable of completely blocking the activity of E_2 (Bowler et al. 1989, Wakeling et al. 1991). ICI 164,384 is a 7α-alkylamide analogue of estradiol and replacing the amide moiety by other polar groups and fluorinating the terminal alkyl function produced the pentafluoropentylsulfinyl compound ICI 182,780 (Wakeling et al. 1991). ICI 182,780 is more potent than ICI 164,384 in inhibiting the growth of breast cancer cells (Wakeling et al. 1991). Due to lacking agonist activity, ICI 182,780 is used as second-line treatment among postmenopausal women with ER-positive, either locally advanced or metastatic breast cancer, who have developed resistance to either tamoxifen or toremifene treatment (Howell et al. 1995, reviewed in Howell and Dowsett 1997, reviewed in Lynn 2004). It has poor bioavailability orally and is thus dosaged as i.m. depot injections every month (reviewed in MacGregor and Jordan 1998). No serious drug-related adverse effects have been reported, but menopausal symptoms, local injection-site reactions, increased hair greasiness, alteration of body odour and transient blood-stained vaginal discharge have been reported, but no significant effects on SHBG or lipids have been seen (Howell et al. 1995, reviewed in Lynn 2004).

5.3 Antagonism of antiestrogens

Traditionally, antiestrogens have been thought to act in a cytostatic manner only. Tamoxifen, for instance, has been shown to exert a block in G_0/G_1 phase of the cell cycle (Sutherland et al. 1983) and to reduce the expression of Ki67, which is a proliferation-associated nuclear antigen present in the late G_1, S, G_2 and M phases, but not in G_0 phase (Clarke et al. 1993, DeFriend et al. 1994, reviewed in Doisneau-Sixou et al. 2003). Antiestrogens have also been shown to inhibit cell proliferation by promoting apoptosis (Perry et al. 1995, Ellis et al. 1997, reviewed in Mäenpää and Ala-Fossi 1997). A significant increase in apoptotic index in human breast cancer cells was seen following ICI 182,780 treatment (Ellis et al. 1997), and thus these pure antiestrogens as well as tamoxifen and toremifene are not only cytostatic (i.e. they reduce proliferation) but can also be considered cytotoxic agents (i.e. inducing cell death).

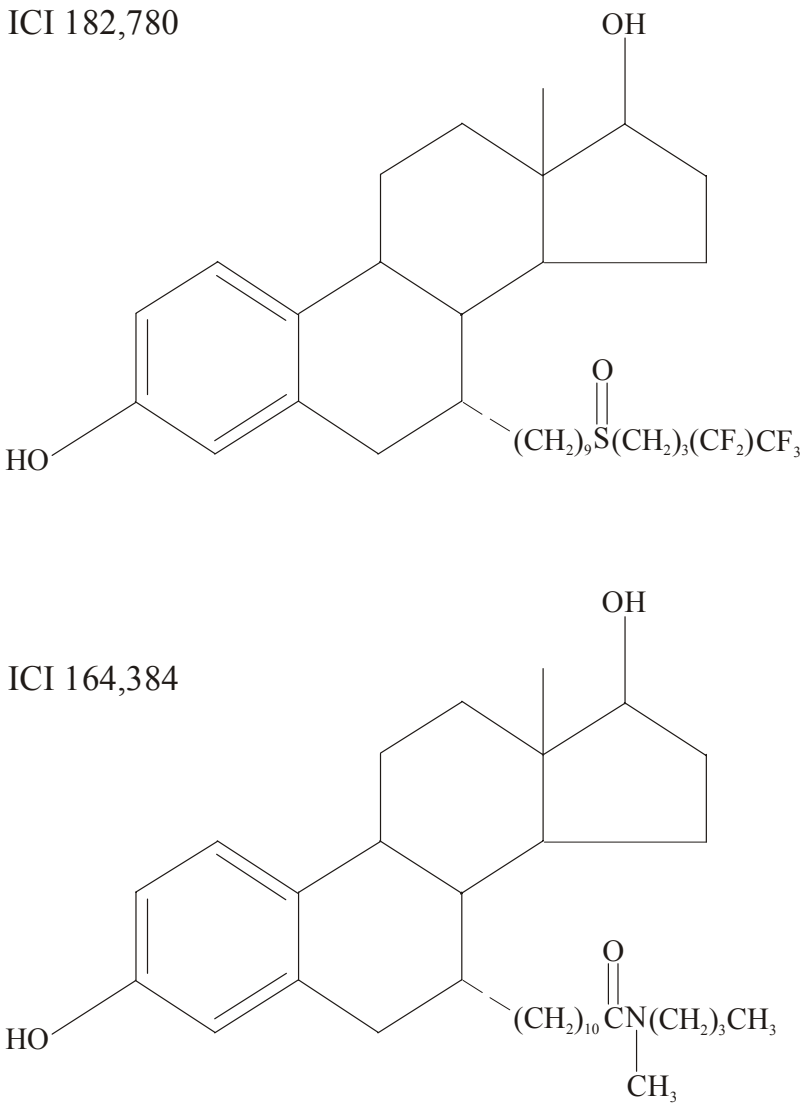

Figure 9. Chemical structure of pure antiestrogens ICI 182,780 (fulvestrant) and ICI 164,384. Modified from Fawell et al. 1990 and Osborne et al. 2000.

All SERMs appear to act as AF2 antagonists, but their partial agonist character is derived partially through AF1 activity, since an increase in endometrial cancer incidence has been shown in tamoxifen-treated patients (McInerney et al. 1998). According to the crystal structure experiments, both ER agonists and ER antagonists bind at the same site within the core of the LBD of ER, but they induce different conformational changes (Brzozowski et al. 1997, Shiau et al. 1998, reviewed in Pike et al. 2000, Pike et al. 2001). Different composition and orientation of helix 12 especially is important and profoundly influenced by the nature of the bound ligand (Brzozowski et al. 1997, Shiau et al. 1998, Tanenbaum et al. 1998). Raloxifene (Evista®) is a SERM, which was originally developed in order to treat and prevent osteoporosis but which also reduces the risk of receptor-positive breast cancer. The antagonistic properties of both tamoxifen and raloxifene are thought to be based on their ability to prevent the formation of transcriptionally competent conformation, because helix 12 binds to and occludes the coactivator recognition site and as a result a recognition surface for corepressor complexes is formed (Brzozowski et al. 1997, Shiau et al. 1998, reviewed in Pike et al. 2000). Instead, binding of pure antiestrogen ICI 164,384 produces ER conformation in

which the association between helix 12 and the rest of the LBD is completely abolished (Pike et al. 2001). This conformational state of ER is unique and different from that elicited by agonists and SERMs and provides some further insights into the mechanism for full receptor antagonism (Pike et al. 2001). There is also evidence that the alkylsulphinyl side chain prevents the dimerization of ER and thus DNA binding (Fawell et al. 1990, reviewed in Howell and Dowsett 1997).

5.4 Aromatase inhibitors

In postmenopausal women, androgens from both ovaries and adrenal glands are used as substrate for estrogen synthesis by the cytochrome P450 enzyme aromatase in peripheral tissues such as adipose tissue and muscle. Moreover, the breast tumour itself is an important source of estrogen. Aromatase inhibitors (AIs) are synthetic compounds able to inhibit estrogen biosynthesis thereby reducing the levels of circulating estrogens (reviewed in Nicholson and Johnston 2005). They block the last step in the biosynthesis of sex steroids, namely the aromatase reaction. In this step, androgens androstenedione and testosterone are converted respectively into estrone and estradiol.

Three different aromatase inhibitor preparations (anastrozole, exemestane and letrozole) are used in the treatment of ER-positive postmenopausal breast cancer in Finland at this time. Formestane and exemestane (Aromasin®) are type I steroidal drugs. They are androgen analogues, which bind competitively and irreversibly to the aromatase enzyme (reviewed in Brodie 2003, reviewed in Miller 2003), cause permanent inactivation of the enzyme-drug complex and are effective until a new enzyme is synthesized (reviewed in Brodie 2003, reviewed in Miller 2003). Type II nonsteroidal drugs include third-generation triazoles anastrozole (Arimidex®) and letrozole (Femar®). They are reversible inhibitors of aromatase and interfere with the hydroxylation reactions by binding with the heme iron of the enzyme (reviewed in Brodie 2003, reviewed in Miller 2003). Both type I and II drugs almost totally inhibit both peripheral aromatase and aromatase within the breast and circulating levels of estrogen fall to undetectable levels in most patients (reviewed in Miller 2003).

The role of aromatase inhibitors in the treatment of advanced (metastatic) breast cancer has been established. All three AIs have been shown to be either superior or equivalent to tamoxifen in the first-line setting and superior to former standards (progestin and the first-generation AI aminoglutethimide) in the second-line setting (reviewed in Mouridsen and Robert 2005). Their role in the adjuvant setting has been and is currently being investigated (reviewed in Mouridsen and Robert 2005). First, anastrozole has been shown to be more effective than tamoxifen as early adjuvant therapy (Baum et al. 2002). Second, two trials have shown that switching to AI (exemestane or anastrozole) after 2-3 years of tamoxifen results in better outcome than continuing tamoxifen for 5 years (reviewed in Mouridsen and Robert 2005). Third, letrozole following tamoxifen as extended adjuvant therapy seems to significantly improve disease-free survival and to reduce the risk of recurrence, when compared to placebo (Goss et al. 2003).

In general, AIs have a more favorable overall safety profile compared with tamoxifen, particularly in terms of life-threatening events such as endometrial cancer and thromboembolic events (reviewed in Gradishar 2005). However, there are side effects that have been clearly associated with AIs. Their use has been associated with osteoporosis, fractures and musculoskeletal disorders such as myalgia, arthritis and arthralgia (reviewed in Wong and Ellis 2004, reviewed in Cuzick 2005). Thus, they should be prescribed with caution in patients with osteopenia or osteoporosis and there are also concerns about the effects of AIs on memory and a possible risk for dementia (reviewed in Wong and Ellis 2004).

6 HORMONE-DEPENDENT BREAST CANCER

Breast cancer is the most common cancer among Finnish women and its incidence has been increasing every year. Approximately 3,800 new breast cancers were diagnosed in Finland in 2003 and the incidence of this disease was 84.3 in 100,000 (Finnish Cancer Registry 2003). In general, the lifetime risk of breast cancer is 1 to 10 (reviewed in Clamp et al. 2003) and the prognosis of the disease is good - about 80 % of the patients in Finland are alive five years after the diagnosis (www.cancerregistry.fi). Quite similar survival rates have been reported from Switzerland, for instance, in which the age-standardized 5 year relative survival in women diagnosed between 1993 to 1997 was 81% (Fisch et al. 2005).

6.1 Risk factors for breast cancer

Epidemiological data clearly reveals that breast cancer incidence not only increases with advancing age, but that the highest rate of increase occurs during the reproductive years between menarche and menopause, suggesting that exposure of the breast to progesterone and estrogen in particular, is a major aetiological factor of this disease (reviewed in Bernstein 2002, reviewed in Clamp et al. 2003). Artificial menopause (hysterectomy and bilateral oophorectomy) before age 40, late menarche and early menopause effectively minimise the exposure of the mammary epithelium to ovarian steroids and thus the breast cancer risk (Feinleib 1968, Trichopoulos et al. 1972, reviewed in Anderson 2002, reviewed in Clamp et al. 2003). The prevailing hypothesis underlying these findings is that the greater the number of times that the breast epithelium undergoes cyclical proliferation between menarche and menopause, the greater the chances of cancer initiation and promotion and the greater the risk of breast cancer (reviewed in Clarke et al. 2004). Age ≥30 years at first birth, nulliparity and lack or short duration of breast-feeding have also been shown to increase the cancer risk (reviewed in Anderson 2002, Collaborative Group on Hormonal Factors in Breast Cancer 2002, reviewed in Bernstein 2002, reviewed in Clamp et al. 2003).

Increased breast cancer risk in obese postmenopausal women can be attributed to higher levels of circulating estrogen in these women for two reasons. First, adipose tissue is able to convert androgen precursor androstenedione to estrone and second, obesity is strongly associated with lower SHBG production leading to increased levels of free, non-SHBG-bound, active estrogen (reviewed in Bernstein 2002). However, the analysis on circulating hormone levels and the risk of brast cancer have revealed inconsistent results. Although higher circulating estrone levels have indeed been shown to be associated with increased breast cancer risk (Modugno et al. 2005), there are also studies in which serum levels of estrone or estradiol were not associated with increased risk (Kaaks et al. 2005). Furthermore, when levels of serum concentrations of sex steroids have been measured among premenopausal women, elevated levels of androgens were associated with an increased risk of breast cancer and elevated levels of progesterone were associated with decreased risk (Kaaks et al. 2005).

Both in situ and invasive breast cancer rates vary by ethnic group: non-Hispanic white women in the United States have the highest rates, whereas they are lower for Asian, black and Hispanic white women (reviewed in Bernstein 2002). The reasons for these differences may, at least in part, be the variations both in family size and in duration of breast-feeding (Collaborative Group on Hormonal Factors in Breast Cancer 2002). Elevations of plasma estrogen levels both before and after the menopause have been reported in postmenopausal breast cancer patients when compared to healthy individuals and mammographically dense breasts are associated with a higher cancer incidence (reviewed in Bernstein 2002, reviewed in Clamp et al. 2002, reviewed in Clamp et al. 2003). Increased bone mineral density has been shown to be significantly associated with an increased risk and *vice versa*, women with osteoporotic fractures have been shown to have a low risk of breast cancer (reviewed in Clamp et al. 2002, reviewed in Clamp et al. 2003). Finally, strenous physical

activity may delay menarche and even moderate physical activity during adolescence can lead to anovulatory menstrual cycles, both of which reduce the cumulative (i.e. the life-time) exposure to steroid hormones and thus the breast cancer risk. The study results concerning the risk of breast cancer associated with use of oral contraceptives, postmenopausal hormone replacement therapy (which will be discussed later), serum androgen levels or circulating levels of SHBG, are not consistent (reviewed in Bernstein 2002).

6.2 Steroid hormone receptors in breast cancer

Histological studies have shown that most breast cancers appear to be derived from terminal ductal lobular units (reviewed in Anderson 2002), which is the primary anatomical and functional unit of the human breast. Furthermore, most breast tumours have been shown to express receptors for estrogen and progesterone, which further emphasises the importance of these hormones in the development of this disease. Increased ERα expression *per se* is a risk factor for breast cancer (reviewed in Anderson 2002, reviewed in Clarke et al. 2004). This phenomenon is already seen at the earliest stages of tumorigenesis and expression increases still further with increasing atypia (reviewed in Anderson 2002, reviewed in Clarke et al. 2004). In contrast to ERα, ERβ levels decrease in the transition from normal to malignant tissue (reviewed in Anderson 2002, reviewed in Clarke et al. 2004). Both of these observations are consistent with the idea that ERβ negatively modulates the effects of ERα (reviewed in Anderson 2002).

In a normal mammary gland, a dissociation between steroid hormone receptor synthesis and cell proliferation exists, which means that ERα- and PR-positive cells are often found to be adjacent to the proliferating cells (reviewed in Ismail et al. 2003, reviewed in Clarke et al. 2004). Normally, these receptor-positive cells stimulate proliferation by paracrine secretion of growth factors in response to hormones, but this separation is disrupted in tumour cells and may result either in autocrine growth factor loop or estrogen acting *via* ER directly inducing entry into the cell cycle and ultimately uncontrolled growth (reviewed in Clarke et al. 2004).

The ratios of PRA and PRB have been reported to change during progression from normal breast to malignant tissue (reviewed in Anderson 2002). The ratio is altered such that in a normal breast, PRA and PRB are synthesized in equal amounts, while PRA predominates in atypical ductal hyperplasia, ductal carcinoma in situ (DCIS) and in invasive tumours (reviewed in Clarke et al. 2004). Carcinogen-treated PRKO mice exhibited a significant reduction in mammary tumour incidence as compared to WT mice (Lydon et al. 1999, reviewed in Ismail et al. 2003, Medina et al. 2003). Underlying the lower incidence was a significant decrease in the epithelial proliferative index (Lydon et al. 1999, reviewed in Ismail et al. 2003). Thus, these results imply that an increase in PR-mediated proliferative pathways as well as alteration of the PR isoform ratio are important in tumorigenesis, at least in the rodent mammary gland.

6.3 Hormone replacement therapy and the risk of breast cancer

Postmenopausal hormone replacement therapy (HRT) considerably alleviates menopausal symptoms such as sweating, hot flushes and vaginal atrophy. Furthermore, use of estrogen (both with or without progestin) preserves the bone mass and reduces the risk of fractures (The Writing Group for the PEPI 1996, Torgerson and Bell-Syer 2001a, Torgerson and Bell-Syer 2001b, Rossouw et al. 2002). Initially, only estrogen was used, but when it was shown to cause a substantial increase in risk of endometrial cancer (reviewed in Grady et al. 1995, Pike and Ross 2000), progestins were added in order to attempt to eliminate this risk. At first, progestin was added in a sequential fashion, but in order to avoid regular bleeding and other negative side effects and in order to further protect those at risk against the development of endometrial carcinoma, continuous combined estrogen-progestin replacement therapy regimens were developed.

Despite of many benefits of HRT, there is evidence of an increased incidence of breast cancer among current and recent HRT users (Collaborative Group on Hormonal Factors in Breast Cancer 1997, Chen et al. 2002, Rossouw et al. 2002, Beral 2003). The relative risk seems to increase with increasing duration of use of HRT (Collaborative Group on Hormonal Factors in Breast Cancer 1997, Rossouw et al. 2002, Beral 2003). Additionally, in contrast to the effects on the endometrium, combination HRT (estrogen + progestin) appears to confer a greater breast cancer risk than estrogen alone, which has been suggested to result from enhancement of the proliferative effects of estrogen by progestin (Pike and Ross 2000, reviewed in Bernstein 2002, Daling et al. 2002, Beral 2003, Li et al. 2003). However, there are also studies in which no difference has been found (Collaborative Group on Hormonal Factors in Breast Cancer 1997, Chen et al. 2002). Furthermore, the question whether sequential or continuous combined estrogen-progestin replacement therapy are equivalent or not with respect to the risk of breast cancer, remains unresolved (Chen et al. 2002, Daling et al. 2002, Beral 2003, Li et al. 2003).

In conclusion, postmenopausal hormone replacement therapy is very effective and the best medical choice in the treatment of symptomatic patients. However, these regimens should not be prescribed for primary prevention of osteoporosis to asymptomatic patients nor for primary prevention of cardiovascular diseases. Many studies have shown that HRT increases the breast cancer risk, but the additional risk is relatively small after short-term duration and the present recommendation is to use these drugs 5 years at the most. The fact that combination HRT confers a greater risk than estrogen alone can be circumvented by delivering the progestins either vaginally or preferably directly onto the endometrium, which thus minimise their effects on the breast (Pike and Ross 2000).

6.4 Antiestrogen resistance in breast cancer

Antiestrogens tamoxifen and toremifene are used in the treatment of receptor-positive breast cancer patients, since breast cancer patients with ER(α) and/or PR-positive tumours have been shown to respond to hormonal therapy and the presence and high concentrations of the receptor have been shown in numerous studies to be associated with better overall survival (Byar et al. 1979, Allegra et al. 1980, Rose et al. 1985, Rydén et al. 1988, Shek and Godolphin 1989, Crowe et al. 1991, Fernö et al. 1996, Kuukasjärvi et al. 1996, Molino et al. 1997, Bryant et al. 1998, Harvey et al. 1999, Chebil et al. 2003). The relationship between the presence of ERβ and its relationship to reponse to endocrine therapy (or prognosis) are contradictory (reviewed in Anderson 2002).

Unfortunately, many patients with receptor-positive tumours fail to benefit from endocrine therapies after initial response and *de novo* resistance to tamoxifen and toremifene also exists. Antiestrogen resistance has been studied extensively, and a number of factors seem to underlie this phenomenon. ER negativity is a major mechanism of *de novo* resistance (reviewed in Dowsett 2001). Lowered intracellular tamoxifen concentrations and binding to breast cancer cells as well as its altered metabolism have been suggested (Osborne et al. 1991, reviewed in Wiebe et al. 1993, reviewed in Clarke et al. 1996, reviewed in Ciocca and Elledge 2000). Loss of ER expression and ER mutations, although occurring infrequently, have been shown (McGuire et al. 1991, Encarnacion et al. 1993, reviewed in Wiebe 1993, Karnik 1994, reviewed in Osborne and Fuqua 1994). Conversely, Speirs et al. (1999) have reported up-regulation of ERβ mRNA expression both in tamoxifen-resistant MCF-7 cell line and in patients having primary breast tumours, which do not respond to tamoxifen treatment. Reduction in the expression levels of progesterone receptor (PR) has also been reported both in tamoxifen-resistant primary breast tumours and in cell culture models (Speirs et al. 1999, de Cremoux et al. 2003) and indeed, loss of PR synthesis in a tumour indicates a more aggressive phenotype with a greater likehood of metastasis (reviewed in Clarke et al. 2004). Moreover, altered PRA:PRB ratio has been suggested to contribute to the poor response of some patients to hormonal therapy.

The role of coregulators in the development of tamoxifen antiestrogen resistance has also been studied. Low coactivator (SRC-1) and corepressor (N-CoR) mRNA levels before the onset of tamoxifen therapy have been shown to predict poor response (Berns et al. 1998, Girault et al. 2003). In addition, decreased N-CoR protein levels have been shown to correlate with the acquisition of tamoxifen resistance in a mouse model system (Lavinsky et al. 1998). High levels of coactivator AIB1 alone as well as together with high levels of HER-2 have been shown to be associated in tamoxifen-treated patients with poorer disease-free survival, which is indicative of tamoxifen resistance (Osborne et al. 2003). AIB1 is known to be phosphorylated and thereby functionally activated by MAPKs, which in turn are activated by HER-2 (Font de Mora and Brown 2000).

Other, ER-independent, cell signalling and growth factor pathways have also been shown to be involved in the development of antiestrogen resistance. TGF-β isoforms have been shown to inhibit the growth of breast cancer epithelial cells, particularly during the earliest stages of carcinogenesis (reviewed in Benson et al. 1996, reviewed in Reiss 1997) and thus loss of TGF-β1 gene expression could be a key event in the development of tamoxifen resistance, as suggested by Achuthan et al. (2001). Conflicting results have also been reported since TGFβ1 has been shown on the contrary to be either up-regulated both in cell culture models and in patients who have developed tamoxifen resistance or the levels have been shown to be unchanged (Thompson et al. 1991, Arteaga et al. 1999, Speirs et al. 1999). Tamoxifen-resistant cells have also been shown to overexpress the immunosuppressive cytokine TGF-β2 (Arteaga et al. 1999).

It is known that overexpression of HER-2 results in the prolonged activation of the mitogen-activated protein kinase (MAPK) signalling pathway and thus enhances signal transduction in breast tumour cell lines and carcinomas (Janes et al. 1994, reviewed in Tzahar and Yarden 1998, reviewed in Dowsett 2001). Consistent with this, many studies have shown that HER-2 amplification and/or overexpression leads to endocrine resistance (Benz et al. 1993, Pietras et al. 1995, Kumar et al. 1996, reviewed in Tzahar and Yarden 1998, Dowsett et al. 2000, reviewed in Mass 2000, reviewed in Ciocca and Elledge 2000, reviewed in Dowsett 2001, reviewed in Piccart et al. 2001). Acquired increased expression of components involved in the EGFR/MAPK signalling pathway in ICI 182,780-resistant breast cancer cells has also been observed (McClelland et al. 2001) and overactivation of the Akt/PI-3 kinase pathway has been shown in tamoxifen-resistant cells (Jordan et al. 2004).

6.5 Prevention of breast cancer

Tools, such as the Gail model (Gail et al. 1989) and the Claus model (Claus et al. 1994), have been developed to calculate the individual absolute risk of breast cancer, although they have their limitations. Breast Cancer Risk Assessment Tool is available online at the Internet (http://bcra.nci.nih.gov/brc/). These tools help clinicians to identify women whose breast cancer risk is increased. The earlier the cancer is observed the better is the prognosis and thus regular palpation of the breasts as well as regular participation in mammographic screenings are important means to detect the tumour early enough.

The current options aimed at the prevention of breast cancer include changes in lifestyle, prophylactic mastectomy, chemoprevention and early detection of cancer by regular surveillance. It has been shown that prophylactic surgery (bilateral mastectomy and bilateral oophorectomy) is the most effective means to reduce the breast cancer risk (reviewed in Clamp et al. 2002) and it is recommended to women with high risk of hederitary disease. At this moment, chemoprevention of breast cancer is in use in the United States. A significant reduction in breast cancer incidence has been shown in patients taking tamoxifen (Fisher et al. 1998, reviewed in Clamp et al. 2002, Cuzick et al. 2003) and an even larger reduction was found in the raloxifene prevention trial (Cauley et al.

2001, reviewed in Fabian and Kimler 2005). However, prevention studies have also been published, which have failed to confirm these benefits (reviewed in Clamp et al. 2002). Both of these preventive drugs have well-known side effects such as increased risk of endometrial cancer (tamoxifen) and thromboembolic events (tamoxifen and raloxifene) (Fisher et al. 1998, Cuzick et al. 2003, reviewed in Fabian and Kimler 2005) and the possible hepatocarcinogenicity and DNA adduct forming ability of tamoxifen is noteworthy (Williams et al. 1992, Hard et al. 1993). Neither tamoxifen nor raloxifene is able to prevent the incidence of ER-negative breast cancers (reviewed in Fabian and Kimler 2005). Thus, their benefits and risks must be considered carefully before prescribing them to women. The third and fourth generation of SERMs arzoxifene and acolbifene are promising new drugs that have undergone preliminary clinical evaluation as potential chemopreventive agents and it has been predicted that they have a greater ability to preserve bone mineral density but fewer unfavorable effects on the uterus than tamoxifen or raloxifene (reviewed in Fabian and Kimler 2005). The role of aromatase inhibitors in the prevention of breast cancer is also currently under investigation (reviewed in Goss and Strasser-Weippl 2004, reviewed in Kalidas and Brown 2005).

In summary, options for women at high risk for breast cancer include close surveillance, prophylactic mastectomy and/or oophorectomy and chemoprevention. Based on the epidemiological data, some recommendations for women can be given in order to prevent the disease, such as controlling their weight and taking exercise. Careful consideration between the risks and benefits of HRT (i.e. whether to use it or not and to limit its duration of time to a minimum), is also needed.

AIMS OF THE PRESENT STUDY

The main aim of this work was to study how normal and breast cancer cells respond to estrogen and progestin and how this response is regulated both at the transcriptional and protein levels. Moreover, the questions as to how and why breast cancer cells become resistant to antiestrogen therapy, were studied.

The specific aims of the present study were:

1. to raise and characterize two specific antibodies against progesterone receptor in order to study the protein expression and subcellular localization of PR in various female murine tissues (I)

2. to raise and characterize two novel antibodies against murine coactivator GRIP1 in order to study the protein expression and subcellular localization of GRIP1 in mouse tissues and to compare the expression of GRIP1 with PR (I and II)

3. to study the effect of estrogen and progestin on PR and GRIP1 expression (I, II and III)

4. to study the alterations in the cell proliferation and target gene transcription in response to estrogen, antiestrogen and progestin alone or in combination (III)

5. to ascertain whether the alterations in the gene expression of steroid hormone receptors, coregulators or GPR30 would account for endocrine resistance of breast cancer (III and IV).

MATERIALS AND METHODS

1 ANIMALS, TREATMENTS AND SAMPLE PREPARATION (I, II)

Nine-week-old female NMRI mice (n = 14) were used to study the expression of PR and GRIP1 proteins in various tissues. The same animals were used in both studies (I and II) and tissue samples from male mice were used in order to study GRIP1 expression in testis, epididymis and prostate. The animals were ovariectomized under anaesthesia, and an appropriate postoperative analgesic was used. One week later one group consisting of seven mice was treated daily with 17β-estradiol dissolved in sesame oil i.m. for five days. The other (control) group also consisting of seven mice, received no treatment.

1.1 Preparation of tissue samples

The animals were killed 24 h after the last hormone injection and the tissue samples were immediately dissected on ice. Tissue samples were fixed in Baker's fixative (4% paraformaldehyde and 1% $CaCl_2$ in distilled water, pH 6.7) for 2 h at 4°C. After dehydration in ascending concentrations of ethanol and incubation in butanol, they were embedded in paraffin and stored at 4°C until use.

1.2 Preparation of cytosolic and nuclear protein extracts

In order to obtain cytosolic and nuclear protein samples, PR- and GRIP1-transfected COS cells were washed twice with phosphate-buffered saline (PBS; 0.14 M NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, and 7.8 mM $Na_2HPO_4*2H_2O$). The cells were harvested and resuspended in 150 µl of ice-cold cell lysis buffer (20 mM Hepes [pH 8.0], 20 mM NaCl, 0.5% Nodinet P-40, protease inhibitors [Complete Mini, Boehringer-Mannheim GmbH, Mannheim, Germany] and 1 mM dithiothreitol). After 1-min centrifugation at maximal speed (16170 *g*) at 4°C, the supernatant obtained was taken, stored at -70°C, and used later as cytosolic protein fraction. The remaining pellet was resuspended in 50 µl of nuclear buffer (20 mM Hepes [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 µM $MgCl_2$, 2.0 µM EDTA [pH 8.0], 1 mM dithiothreitol, and protease inhibitors) and incubated on ice for 30 min. After centrifugation as above, the supernatant was taken, stored at -70°C and used later as nuclear protein fraction. Total protein concentrations were determined according to the Bradford method (1976).

2 ANTIBODIES (I, II)

2.1 PR antibodies

Two polyclonal antibodies against mammalian PR were raised in New Zealand White rabbits. The animals were injected subcutaneously with 100 µg of a synthetic peptide conjugated to thyroglobulin in Freund's complete adjuvant. The two subsequent injections were carried out in Freund's incomplete adjuvant at 1-month intervals. One synthetic peptide corresponded to the peptide sequence 318-327 of the mouse PR (PR120) and the other to the residues 533-547 of the human PR (PR80). The sera were precipitated by the addition of saturated (40%) ammonium sulphate and the resulting IgG fractions were tested for their immunoreactivity against the corresponding ovalbumin-conjugated peptide by directed immunoassay and their titer was determined (Nunc-Immuno Plate, MaxiSorp, Nunc, Roskilde, Denmark).

2.2 GRIP1 antibodies

Two polyclonal antibodies against peptide residues 34-47 (α123) and 468-481 (α124) of mouse GRIP1 were raised in rabbits and the immunoreactivity and titer of the IgG fractions were tested as described above. Both antibodies recognise the human homolog TIF2 too.

3 CELL CULTURE (I-IV)

3.1 Cell lines

COS cells (African green monkey kidney cells) were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS; Gibco^{TM}, Invitrogen Corporation, Paisley, Scotland, UK). ER- and PR-positive MCF-7 human breast cancer cells (ATCC No. HTB-22) were routinely cultured in phenol red-free DMEM/F12 medium supplemented with 5% dextran charcoal-stripped (steroid-depleted) FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml), bovine insulin (10 ng/ml) and 1 nM E_2. Mouse NIH3T3 fibroblasts were acquired from the American Type Culture Collection (Manassas, VA, USA) and cultured in phenol red-free DMEM/F12 medium supplemented with 10% dextran charcoal treated FBS and the antibiotics. Retrovirus packaging NIH-3T3-derived PT67 cells were maintained in phenol red-containing DMEM medium (Sigma) supplemented with 10% FBS and the antibiotics. The MCF-7 cells' medium was changed to PT67 cells 8 days before the onset of the growth curve experiments. All cell lines were cultured at 37°C in a humified athosphere of 5% CO_2:95% air and all disposable cell culture materials were purchased from Nalge Nunc International (Rochester, NY, USA).

3.2 Establishment of MCF-7-derived sublines

MCF-7 cells were used as parent cells in order to generate four novel breast cancer cell culture models. Progestin-resistant cells received 0.1 µM MPA + 1 nM E_2 for nine months and long-term estrogen-treated (LE) cells vehicle (96% ethanol) + 1 nM E_2 for 9 months. The toremifene-resistant cell line (TR) was established by culturing MCF-7 cells in the presence of 1 µM toremifene for 9 months and EI cells (the model of estrogen-independent cells) were grown with vehicle for 9 months. The media were changed every 2 or 3 days and when the cells reached the confluence, they were trypsinized, cell viability was checked using the trypan blue exclusion method and 3 x 10^5 viable cells were reseeded. The time to reach confluence (days) was measured during the entire 9-month culture period to assess possible changes in the cell growth rate. In addition, the number of population doublings (N) was calculated for all four cell lines during the entire 9-month period using the following equation: N = 3,32 * [log10 (number of cells collected at the end of each passage) – log10 (number of cells seeded at the beginning of each passage)].

3.3 Cell growth experiments

In order to study the cell proliferation in parent MCF-7 cells and four established sublines, the cells were withdrawn from hormones six days before and two days after being seeded (500 - 2 x 10^3) on 96-well plates. 48 hours after seeding the hormones (vehicle only, toremifene only, E_2 alone or together with medroxyprogesterone acetate (MPA) at different concentrations) in the experimental media were added. The media with appropriate hormones were renewed every second day and the relative cell numbers were measured every 24 hours as described by Kueng et al. (1989). Briefly, the cells were fixed by the addition of 10 µl of 11% glutaraldehyde into 100 µl of medium for 15 min. After washing and air-drying, they were stained by 100 µl of 0.1% crystal violet solution for 20 min. Excess dye was removed with thorough washing, and after air-drying the dye was solubilized in 100 µl of 10% acetic acid. Absorbance was measured at 590 nm wavelength using a Victor 1420 Multilabel counter (Wallac Inc., Turku, Finland).

When the basal expression and transcriptional regulation of steroid hormone receptors and coregulators were studied, parent MCF-7 cells and the cells of four established sublines were grown in 75cm^2 flasks for 72 hours either with the vehicle only or with appropiate hormones (toremifene or estradiol alone or estradiol in combination with MPA). Thereafter the RNA was isolated.

When the growth of GPR30-infected, control gene (enhanced green fluorescent protein; EGFP)-infected and non-infected cells was studied, cells (5-10 x 10^2/well) were seeded on 96-well plates and allowed to attach for 20 h, after which the infection was done. After 24 hours, the medium was replaced with fresh basic medium, and hormonal treatments (vehicle only, 1 nM E_2, 1 μM toremifene or 1 nM E_2 + 1 μM toremifene) begun 48 h after the onset of infection. The hormones were added and the media changed concurrently every 48 h. The relative cell numbers and absorbance were measured every 24 or 48 hours as described previously.

4 WESTERN BLOT ANALYSIS (I, II)

The samples (cytosolic and nuclear protein extracts) were mixed in one vol of 2X sodium dodecyl sulphate (SDS) sample buffer (125 mM Tris-HCl, 20% glycerol, 10% β-mercaptoethanol, 4% SDS, 0.05% bromophenol blue) and boiled for 5 min. 40μg protein/sample was loaded/well and resolved in 6.5% polyacrylamide gels containing 0.1% SDS (Laemmli 1970) and transferred to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) on ice using an electrophoretic transfer apparatus (Mini Trans-blot, Bio-Rad, Richmond, CA, USA). Prior to incubation with the primary antibodies PR80, PR120, α123 or α124 (the final concentration 5 μg/ml) in 0.05% Tween 20 in trsi-buffered saline (TBS; 50 mM Tris, 0.9% NaCl, pH 8.0) containing 1% skimmed milk at 4°C overnight, the membranes were presaturated with 5% skimmed milk in TBS-Tween at 37°C for 1 h. Peroxidase-conjugated goat antirabbit IgG (Cappel, West Chester, PA, USA) was used as a secondary antibody (1:40,000 dilution in TBS-Tween containing 1% skimmed milk) for 1 h at room temperature (RT). Enhanced chamiluminescence (ECL) detection method was used following the manufacturer's instructions with exposure time ranging from 1 to 20 min (Amersham Pharmacia Biotech, Buckinghamshire, England). To verify the specificities of the PR and GRIP1 bands, the primary antibodies were presaturated with an excess of the corresponding peptide used for immunization before the addition of the primary antibody. BioRad's high molecular weight standards were used to assess the size of detected proteins (BioRad Laboratories, Richmond, CA, USA).

5 IMMUNOCYTOCHEMISTRY (I, II)

Transfected COS cells were plated on glass coverslips and were left to grow for 24 h. The cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min on ice and incubated in 0.5% Triton-X 100 for 40 min at RT. After washing in PBS, they were incubated in 10% normal horse serum for 30 min and subsequently with primary antibodies (final concentration 2 μg/ml) in 1% normal horse serum overnight at 4°C. The next day, the cells were washed with PBS and incubated with anti-rabbit Fluorescein (Boehringer-Mannheim GmbH, Mannheim, Germany) diluted 1:300 in PBS for 30 min at 37°C. Finally, the cells were washed in PBS, embedded in PBS:glycerol (3:1) containing 0.1% *p*-phenylenediamine (Johnson and Nogueira Araujo 1981, Platt and Michael 1983), microscoped and photographed.

6 IMMUNOHISTOCHEMISTRY (I, II)

Paraffin blocks containing the tissue samples were cut into 5 μm slices and transferred into glass coverslips. Thereafter, they were deparaffinized and rehydrated in graded ethanol. The antigens were unmasked by incubating the specimens in 0.01 M sodium citrate buffer (pH 6.0) at 100°C for two 5-min periods. Treatment with 0.5% H_2O_2 in methanol was done in order to block the endogenous peroxidase activity of the tissues. After incubation with 10% normal horse serum in PBS for 30min at RT to reduce non-specific binding of the primary antibody, the slices were incubated with the primary antibodies (final concentration 5 μg/ml) in PBS containing 1% normal horse serum overnight at 4°C. In order to verify the specificity of the immunostainings, the primary antibody was either presaturated with an excess of corresponding ovalbumin-conjugated peptide at 4°C for 4 hours or substituted with 1% normal horse serum, PBS or normal rabbit IgG. The next

day, the specimens were incubated with the biotinylated secondary antibody (goat anti-rabbit IgG, Vector) and avidin-biotin-peroxidase complex (Vector) for 30 min each. Peroxidase activity was visualized by incubation in solution containing 0.02% 3,3′diaminobenzidine tetrahydrochloride (Sigma), 0.01% imidazole, and 0.02% H_2O_2 in 0.5 M Tris (pH 7.6) for 5-10 min. Alternatively, Histostain-Plus Kit (Zymed, San Francisco, CA, USA) was used in part of the stainings (study II). Finally, the sections were dehydrated and mounted in Entellan (Merck KgaA, Darmstadt, Germany).

7 RIBONUCLEASE PROTECTION ASSAY (III)

Ribonuclease protection assay (RPA) was used to detect the basal mRNA expression and transcriptional regulation of five steroid hormone receptors and nine coregulators in parent MCF-7 breast cancer cells and four established MCF-7-derived sublines. Two RPA probe sets were used in this study, as described previously by Vienonen et al. 2003. The steroid receptor set contains probes for GR, androgen receptor (AR), PR, ERα and ERβ, and the coregulator set consists of probes for detecting N-CoR, SMRT, pCAF, CBP, GRIP1/TIF2, AIB1, SRC-1a, SRC-1e and p300. The steroid receptor set also contains a probe for detecting cyclin A mRNA, which was used as a marker of cycling cells.

After 72 h treatment of cells either with vehicle (basal expression) or with various hormones (hormonal regulation of transcription), total RNA was extracted using RNAqueous™ Kit (Ambion Inc., Austin, TX, USA). The integrity of the RNA was confirmed by denaturing gel electrophoresis, and the concentration and the purity of the samples were determined by spectrophotometry (GeneQuant II, Pharmacia Biotech Ltd., England). The samples were stored at -70°C until use.

At first, ^{32}P-labelled RNA-probes were synthesized in *in vitro* transcription reaction using the RiboQuant transcription kit (Pharmingen, San Diego, CA, USA). Ribosomal 18S RNA was used as a loading control between samples, and ^{32}P-labelled 18S probe was synthesized from plasmid pTRI RNA 18S (Ambion). These ^{32}P-labelled RNA-probes were hybridized with 5 µg of total RNA samples for 16-17 h by using the RPA III kit (Ambion). 10 µg RNA sample was used in order to verify, that the probes had been used in molar excess in hybridization reactions. Yeast RNA was used as a negative control. COS cells transfected with the corresponding cDNA of ERα and ERβ were used and single probe labelling of SMRT, TIF2, SRC-1a, SRC-1e and p300 was done in order to verify and detect appropriate hybrids from the gels. After hybridization, single stranded RNA was digested with RNase and double stranded RNA hybridization products were separated by gel electrophoresis. Finally, an intensifying screen was exposed and scanned (Storm, Molecular Dynamics, Sunnyvale, CA, USA), and the results were quantified using ImageQuant 5.1 software (Molecular Dynamics).

8 RETROVIRAL VECTOR-MEDIATED GENE TRANSFER (IV)

pLEGFP-N1 control vector was purchased from Clontech Laboratories (Palo Alto, CA). For pLN1-GPR30, a 0.8 kb fragment containing EGFP gene was excised from pLEGFP-N1 and GPR30 gene was inserted into the *BamHI* site of the cloning vector (Ylikomi et al. 2004). Retroviruses were produced by transfecting PT67 cells with either pLEGFP-N1 or pLN1-GPR30 according to the protocol of the vector manufacturer (Clontech Laboratories). The retroviral supernatants collected from PT67 cultures were filtered through 0.45 µm cellulose acetate filters (Schleicher&Schuell GmbH, Dassel, Germany) and used for infections after addition of polybrene (final concentration 4 µg/ml).

9 RT-PCR ANALYSIS (IV)

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed to detect GPR30 mRNA levels in antiestrogen-resistant, estrogen-independent and original MCF-7 cells. Determination of human acidic ribosomal phosphoprotein P0 (RPLP0) mRNA, which functioned as an endogenous internal control (housekeeping gene) for RT-PCR, was done concurrently. The cells (14.5×10^3) were seeded on 6-well plates and RNA isolations were done on the seeding day (i.e. 20 h before the infection) and 9 days after the infection. Total RNA was extracted using RNAqueous-4PCR Kit (Ambion Inc., Austin, TX, USA). The overall quality of RNA was assessed by electrophoresis on a denaturing 1% agarose gel and the concentration and purity of the RNA were determined using spectrophotometer (GeneQuant II, Pharmacia Biotech Ltd., England).

One-step RT-PCR was performed using a LightCycler Instrument and LightCycler - RNA Master SYBR Green I kit according to the manufacturer's instructions from 100ng of total RNA (Roche Molecular Biochemicals, Mannheim, Germany). As a negative control, the template RNA was replaced by PCR-grade water. The following primers were used: GPR30-forward 5′-AGTCGGATGTGAGGTTCAG-3′ and GPR30-reverse 5′-TCTGTGTGAGGAGTGCAAG-3′ (Amersham Pharmacia Biotech); RPLP0-forward 5′-GGCGACCTGGAAGTCCAACT-3′ and RPLP0-reverse 5′-CCATCAGCACCACAGCCTTC-3′ (chosen according to Bièche et al. 2001). Reverse transcription was performed at 61°C for 20 min and denaturation at 95°C for 30 s. 42 PCR cycles consisting of denaturation at 95°C for 1 s, annealing at 58°C for 5 s, and elongation at 72°C for 9.6 s (GPR30) or for 5.96 s (RPLP0), were performed. Fluorescence data was collected at the end of each elongation step. The specificity of the resulting PCR products was verified by performing melting curve analysis, and their lengths (240 bp for GPR30 and 149 bp for RPLP0) were verified by agarose gel electrophoresis. Second Derivative Maximum method was used to quantify the data and the final results were calculated using the equation described by Pfaffl (2001).

10 STATISTICAL ANALYSES (III, IV)

When the cell growth experiments were analysed, the arithmetic mean and standard deviation of 6 (study IV) or 18 (study III) measurements were calculated. At least three separate experiments were performed, and both two-tailed Student's *t*-test and one-way ANOVA with post-hoc Dunnett's *t*-test were used (SPSS 10.1 for Windows, Chicago, IL, USA). RPA-experiments were done at least three times and the results were analysed using non-parametric Mann-Whitney U-test. $P<0.05$ was considered a statistically significant difference.

SUMMARY OF THE RESULTS

1 PR AND GRIP1/TIF2 ANTIBODIES (I, II)

PR antibodies PR80 and PR120 were produced by using synthetic peptides corresponding to residues 533-547 and 318-327 of PR in order to immunize rabbits. When mouse and human PR cDNAs were expressed in COS cells, a nuclear staining was seen using these antibodies. Although both antibodies recognised both mouse and human PR, the staining intensity was slightly stronger with PR120, which was raised against mouse sequences, than with PR80, which was raised against human sequences. When the cytosol of PR-transfected COS cells was immunoblotted with both antibodies, both PR isoforms were detected. No staining was seen when COS cells were transfected with the expression vector pSG5 only or when the antibodies were presaturated with corresponding peptides.

Rabbits were injected with synthetic peptides in order to immunize the animals and to produce antibodies against GRIP1/TIF2. α123 peptide corresponds to residues 34-47 and α124 to residues 468-481 of GRIP1/TIF2. The sequences are identical in mouse and human. When COS cells were transfected with mouse GRIP1 cDNA and immunostained with both antibodies, a clearly nuclear staining with dotted pattern was seen. No staining was seen when the cells were transfected with the expression vector pSG5 only. When nuclear fractions of COS cells were immunoblotted with both antibodies, a protein with an approximate size of 160 kDa was seen. Transfection with GRIP1 cDNA increased the intensity of both bands. When sytosolic fractions were studied, expression was seen both before and after transfection when stained with α123 but not when stained with α124.

2 PR AND GRIP1 EXPRESSION IN MURINE TISSUES AND THE EFFECT OF ESTROGEN ON EXPRESSION (I, II)

2.1 Subcellular localization

When mouse and human PR cDNAs were expressed in COS cells, nuclear staining was seen using both antibodies. This is consistent with immunohistochemical results, since solely nuclear expression was seen in the epithelial, stromal, smooth muscle and capsular cells of ovariectomized mice. Estrogen treatment did not affect the subcellular localization of PR.

Although the endogenous GRIP1/TIF2 expression was mainly seen in the nuclear fraction of COS cells, slight expression in cytosolic fraction was also observed. This is in agreement with immunohistochemical results, since the protein was expressed mainly in the nuclei, but cytoplasmic expression was also seen in the epithelial and smooth muscle cells of some organs. As was the case with PR, estrogen treatment did not result in changes in subcellular localization.

2.2 Urogenital organs

PR and GRIP1 were both expressed in the uterus, although epithelial cells required estrogen for PR expression. PR was estrogen-inducible in epithelial and stromal cells of the vagina, while GRIP1 expression was seen in the stromal cells only. Tubular epithelial cells, glomeruli and the capsule of kidney expressed GRIP1, but PR expression was seen in capsular cells only. Whereas a weak expression of GRIP1 was seen the stromal and smooth muscle cells of the ureter, PR-staining was completely negative. Detrusor muscle cells of the bladder expressed both PR and GRIP1, but stromal cells expressed GRIP1 only. GRIP1 was expressed in all different types of urethral cells, whereas estrogen-inducible PR was expressed in epithelial and stromal cells only.

GRIP1 expression was also seen in the epithelial and stromal cells and in the adipocytes of the mammary gland, while ovaries excluding connective tissue of mesovarium were GRIP1-negative.

Among male tissues, testicular epithelial cells, smooth muscle cells of epididymis and fibromuscular stromal cells in prostate expressed GRIP1 and cytoplasmic staining was also seen in prostate epithelial cells. PR expression was not studied in these five tissues.

2.3 Gastrointestinal organs

PR was expressed in the ductal and acinar cells of the submandibular gland, in the smooth muscle cells of duodenum and jejunum and stromal cells surrounding the pancreatic ducts as well as vascular smooth muscle cells, both of which were estrogen-inducible. Thus, oesophagus, stomach, ileum, colon, rectum and liver did not express PR.

Ductal and acinar cells of the submandibular gland expressed also GRIP1. GRIP1 was also expressed in epithelial, stromal and smooth muscle cells of oesophagus, colon and rectum. Epithelial and smooth muscle cells were GRIP1-positive in stomach, small intestine and cecum, while stromal cells were negative in these organs. Liver parenchyme was also GRIP1-negative, but nuclear staining was seen in the hepatic ducts. The islets of Langerhans clearly expressed GRIP1.

2.4 Cardiovascular organs and respiratory tract

PR was not expressed in the heart, trachea nor lungs. However, although cardiac muscle cells were GRIP1-negative, endo- and pericardial cells expressed GRIP1. Moreover, epithelial cells of the trachea, bronchioles and alveoli as well as stromal cells and the cartilage of the trachea expressed GRIP1. Endothelial and vascular smooth muscle cells (tunica media) of many blood vessels (both arteries and veins) in different organs expressed either GRIP1 alone or both GRIP1 and PR.

2.5 Endocrinological and immunological organs

Only adrenal cortex cells expressed GRIP1 and this organ was PR-negative except in the capsular cells. Neither PR nor GRIP1 was expressed in the thyroid gland. Stromal cells of the thymus expressed both PR and GRIP1 and the capsule was estrogen-inducible with regard to PR expression. Stromal cells of the spleen expressed GRIP1, but PR-staining was negative.

2.6 Striated muscle and skin

Striated (skeletal) muscle cells did not express either PR or GRIP1. PR was estrogen-inducible in the epithelial cells of the skin. There were no differences in the GRIP1 staining pattern nor in the staining intensity in different organs and cell types after estrogen treatment of the animals.

3 ESTABLISHMENT OF *IN VITRO* MODELS OF BREAST CANCER (III)

In order to establish *in vitro* models of antiestrogen- and progestin-resistant as well as estrogen-independent breast cancer, MCF-7 breast cancer cells were continuously cultured in the presence of 1 μM toremifene for 9 months (TR; toremifene-resistant cells), in the presence of 0.1 μM MPA + 1 nM E2 for 9 months (MR; MPA-resistant cells) and without estradiol for 9 months (EI; estrogen-independent cells). Long-term estrogen-treated (LE) cells were cultured in the presence of 1 nM estradiol for 9 months. The cell lines were propagated for 41, 40, 33 and 46 passages and the numbers of total population doublings for these cell lines were 116, 174, 100 and 195 respectively (unpublished data). On the average, these cells had undergone 2,8 (0,2-4,5), 4,4 (2,4-4,9), 2,9 (-0,1-3,9) and 4,2 (3,0-5,1) population doublings during every passage (unpublished data). The lowest population doubling numbers were seen in TR and EI cells during the first passages, while there were no obvious changes in population doubling numbers in MR and LE cells during the course of establishment (unpublished data).

To assess changes in the proliferation rate during the entire 9-month culture period, the time to reach confluence after a constant number of cells had been reseeded to flasks was measured. 0.1

μM MPA inhibited the proliferation of the cells at first. The time to reach confluence was between 8 and 9 days at the beginning and between 5 and 6 days at the end of cell culture. 1 μM toremifene inhibited the cell growth for the first 10 passages, when the time to reach confluence was between 9 and 13 days, but the proliferation rate gradually increased and reached a steady growth rate after 4 months. There was a period of proliferative quiescence in estrogen-independent cells for the first 3 months, after which they accelerated their growth considerably and reached a constant proliferation rate (4-6 days) by the end of the 4th month. The proliferation rate of LE cells was constant and the same as in original MCF-7 cells throughout the culture period. Thus, MPA- and toremifene-treated as well as long-term estrogen-deprived sublines gradually reached a steady growth rate after 4 months. The time for these cells to reach confluence is approximately 5.5 days, which is the growth rate of both original MCF-7 cells and LE cells. In summary, these results suggest that toremifene- and MPA-treated cells are endocrine-resistant and cells cultured without estradiol are estrogen-independent and that novel *in vitro* model systems for endocrine-resistant and estrogen-independent breast cancer were developed.

4 PROLIFERATION OF BREAST CANCER SUBLINES (III)

In order to study the effects of different hormonal treatments, the cells were plated on 96-well plates and allowed to attach for 48h before the beginning of different treatments. To eliminate any residual hormonal effects on the results, the cells were grown without any hormones one passage plus 2 days before the onset of treatments.

4.1 Basal cell growth

Original MCF-7 cells, LE cells and MPA-resistant cells proliferated poorly in the presence of vehicle only. As expected, estrogen-independent and toremifene-resistant cells grew very rapidly in the presence of vehicle. However, when the proliferation of estrogen-independent cells was compared to the slightly reduced proliferation of resistant cells, a significant difference was observed ($p<0.001$).

4.2 Effect of estrogen

Original MCF-7 cells, LE cells and MPA-resistant cells proliferated very rapidly in the presence of estradiol when compared to the modest proliferation in the presence of vehicle only. There was no difference between the cell proliferation of original MCF-7 and LE cells in the presence of estrogen ($p=0.998$), but the estrogenic response was significantly decreased in MPA-resistant cells, when compared to both original MCF-7 and LE cells ($p<0.001$). 1 nM E_2 also resulted in a significant increase in the proliferation rate of toremifene-resistant and estrogen-independent cells ($p<0.001$), suggesting that they were still estrogen-responsive.

4.3 Effect of progestin

The effect of progestin on cell growth was studied in original MCF-7, LE and MPA-resistant cells. 1-100 nM MPA inhibited the growth of original MCF-7 cells in a dose-dependent manner in the presence of estradiol and removal of estradiol resulted in significantly reduced cell growth ($p<0.001$). By contrast, MPA-resistant cells proliferated very rapidly in the presence of MPA regardless of its concentration. Surprisingly, there was no progestin inhibition on cell proliferation in LE cells either, but instead, their growth resembled the growth of MPA-resistant cells. Thus, whether the progestin resistance in MPA-resistant cells was due to either the MPA treatment or due to the long-term culture of these cells remains unresolved. Removal of estradiol resulted in significant growth inhibition in both MPA-resistant and LE cells ($p<0.001$).

4.4 Effect of antiestrogen

Finally, the effect of antiestrogen on the cellular proliferation of original MCF-7, toremifene-resistant and estrogen-independent cells was studied. The proliferation rate of estrogen-independent cells was significantly inhibited in the presence of 1 μM toremifene when compared to vehicle ($p<0.001$), whereas there was an increase in the cell proliferation of antiestrogen-resistant cells ($p<0.001$). The growth inhibition in EI cells was dose-dependent, but TR cells grew very rapidly and original MCF-7 cells very modestly in the presence of toremifene concentrations ranging from 0.1 to 2 μM. Interestingly, combining 1 nM estradiol with toremifene completely abrogated the inhibitory effect of toremifene in estrogen-independent cells ($p<0.001$) and the growth was even stimulated when compared to estradiol only ($p<0.001$). Estrogen plus toremifene further increased the proliferation rate of antiestrogen-resistant cells ($p<0.001$), but a weaker effect on cell proliferation could be seen in original MCF-7 cells ($p=0.01$).

5 EXPRESSION AND HORMONAL REGULATION OF STEROID HORMONE RECEPTORS (III)

The basal mRNA expression of GR, AR, PR, ERα and ERβ in original MCF-7 cells and in four sublines was studied by RPA. Cyclin A expression as a marker of proliferating cells was also studied. The cells were treated with vehicle for 72 hours, after which RNA was extracted. All five cell lines expressed GR, AR, PR, ERα, ERβ and cyclin A transcripts. PR expression was 5.6-fold lower in antiestrogen-resistant cells and 1.9-fold higher in estrogen-independent cells ($p=0.029$ in both), but there were no significant changes in the PR expression in MR or LE cells, when compared to original MCF-7 cells. It is noteworthy that the PR expression difference between TR cells and their control cells (i.e. EI cells) was 8.2-fold. AR and ERα expression was lowered in MR cells (1.9- and 1.6-fold, respectively; $p=0.029$ in both). AR expression was also lowered 1.6-fold in EI cells when compared to original MCF-7 cells. Cyclin A expression was lowest in LE cells and highest in antiestrogen-resistant and EI cells (1.8-fold higher than in original MCF-7 cells, $p=0.029$). There were no significant changes in the basal expression of GR and ERβ.

Next, the hormonal regulation of steroid hormone receptors and cyclin A was examined. The cells were treated with either 1 nM estradiol or 1 μM toremifene or 1 nM estradiol in combination with 0.1 μM MPA for 72 hours. 1 nM E_2 induced from 7.4 to 25.8-fold up-regulation of PR and 1.7- to 5.5-fold ERβ up-regulation in all five cell lines. It also induced a 1.6- to 3.5-fold increase in cyclin A expression in original, MR and LE-cells, but it had no effect in toremifene-resistant and EI cells. ERα, GR and AR were neither up- nor down-regulated by estradiol.

MPA treatment resulted in a significant increase (2.6 to 4.2-fold) in PR expression in MR, LE and original MCF-7 cells. In addition, ERβ expression increased 1.7-fold in original MCF-7 cells. Only original MCF-7 cells responded to MPA by 2.2-fold down-regulation of AR and cyclin A was up-regulated by MPA in MR cells only. It is noteworthy that although toremifene stimulated the proliferation of antiestrogen-resistant cells, it did not regulate the transcription of any of the steroid hormone receptors, not even the observed estrogen target genes PR or ERβ, suggesting that toremifene did not have any agonistic effect in these cell lines.

6 EXPRESSION AND HORMONAL REGULATION OF COREGULATORS (III)

RPA was also used to study the basal expression and hormonal regulation of N-CoR, SMRT, pCAF, TIF2, AIB1, SRC-1e, SRC1-a and p300 in original MCF-7 cells and in the four sublines. All coregulators were expressed in these cell lines, but the expression level of SRC-1e was very low to be quantified reliably. SMRT expression was significantly increased (2.3-fold, $p=0.037$) and TIF2 expression decreased (1.7-fold, $p=0.029$) in antiestrogen-resistant cells when compared to original

MCF-7 cells. The expression of corepressors SMRT and N-CoR was increased 2.0- and 2.1-fold (p=0.037 in both) and the expression of SRC-1a and p300 decreased 1.6- and 2.1-fold respectively (p=0.029 in both) in EI cells. AIB1 was clearly overexpressed in all cell lines, as described previously in original MCF-7 cells (Anzick et al. 1997, Thenot et al. 1999, Vienonen et al. 2003). In addition, AIB1 expression was highest in antiestrogen-resistant cells when compared to other cell lines, and its basal expression was increased 1.9-fold when compared to original MCF-7 cells (p=0.029). No significant changes in the basal expression of coregulators in progestin-resistant or LE cells were observed.

Next, the hormonal regulation of coregulator expression was studied. The only changes in coregulator gene expression were observed in progestin-resistant cells when E_2-effect was studied. Estradiol induced a 2.7-fold up-regulation of pCAF and 1.9-fold up-regulation of AIB1. It is noteworthy that no regulation was seen in the other four cell lines by E_2. MPA and toremifene did not regulate coregulators in our cell lines.

7 GPR30 EXPRESSION IN CELL CULTURE MODEL SYSTEMS (IV)

GPR30 is a member of mammalian G protein-coupled receptors and it has been shown to be expressed both in ER-positive and ER-negative breast cancer cell lines and primary breast carcinomas (Owman et al. 1996, Carmeci et al. 1997, Feng and Gregor 1997). In order to study its role in the development of acquired antiestrogen resistance and estrogen independency *in vitro*, the basal expression of GPR30 mRNA in antiestrogen-resistant, estrogen-independent and original MCF-7 cells was determined by real-time RT-PCR. The cells were grown without hormones for 8 days, after which the GPR30 mRNA levels were measured. GPR30 gene expression was reduced 5-fold in estrogen-independent cells and a 50-fold reduction was seen in toremifene-resistant cells when compared to original MCF-7 cells. When the fact that the estrogen-independent cells also served as control cells for antiestrogen-resistant cells was taken into account, the decrease in GPR30 expression was 10-fold.

8 GPR30 EFFECTS ON THE PROLIFERATION OF BREAST CANCER CELLS (IV)

First, we studied the effect of GPR30 on the basal growth of original, EI and TR cells. After 8-day-growth without hormones, the cells were plated on 96-well plates and infected. The cells were fixed, stained and the relative cell number was counted every 24 or 48 hours for seven days. As mentioned previously, original MCF-7 cells proliferate very poorly in the absence of estrogen. Addition of GPR30 resulted in further, statistically significant increase in growth inhibition of this modest proliferation. Stable transfection of both EI cells, which have adapted to grow without estradiol, and of TR cells, which proliferate quite similarly in the absence of estrogen or in the presence of toremifene, with GPR30, also resulted in significant growth inhibition. The greatest inhibitory effect was seen in antiestrogen-resistant cells.

Next, the effect of GPR30-infection on antiestrogen-mediated growth inhibition was studied. The cells were plated on 96-well plates, infected with or without GPR30 and treated with either vehicle or 1µM toremifene. The cells were fixed, stained and the relative cell number was counted on the sixth day of the experiment. In EI cells, antiestrogen significantly inhibited their proliferation both in the absence and presence of GPR30. GPR30-infection resulted in significant inhibition in the cell proliferation in the presence of both vehicle and toremifene. By contrast, however, non-infected TR cells proliferate equally well in the absence of estrogen or in the presence of toremifene. Infection with GPR30 resulted in significant growth inhibition in both vehicle-treated cells and in toremifene-treated cells.

Finally, the growth of both EI and TR cells treated with either estrogen alone or in combination with toremifene was studied. The cells were plated on 96-well plates, infected with either GPR30, EGFP (control gene) or non-infected and treated with either 1nM estrogen alone or in combination with 1μM toremifene. The cells were fixed, stained and the relative cell number was counted on day 9. There was no difference between the treatments in the non-transfected and EGFP-transfected EI cells and GPR30-infection increased the cell proliferation in both treatment groups. Toremifene plus estrogen stimulated the proliferation of TR cells in the absence of GPR30, but a significant growth inhibition was seen in the presence of GPR30 when compared with estrogen only, suggesting that GPR30 was able to restore the antiproliferative effect of toremifene in resistant cells.

As a control, we wanted to determine the efficiency of stable GPR30-transfection. TR, EI and original MCF-7 cells were plated on 6-well plates, infected with GPR30 and RNA was extracted nine days after the infection. The final GPR30 expression level in TR and EI cells was equal and slightly above the level of the original cells. The greatest increase in GPR30 gene expression was seen in TR cells. We also wanted to demonstrate that the increase in GPR30 expression was not due to the infection but due to the GPR30 gene itself. The cells were transfected either with GPR30 or with control gene in the pLN1 expression vector and the RNA was extracted nine days after the infection. A significant increase in GPR30 gene expression in EI and TR cells (3.8-fold and 7.6-fold, respectively) was seen when compared to the cells transfected with the control gene, but no increase in GPR30 gene expression was seen in the original MCF-7 cells.

DISCUSSION

1 PR AND GRIP1 EXPRESSION IN MURINE TISSUES

In general, PR and ER are said to be localized in the nuclei of cells. According to our results, PR was expressed exclusively in the nuclei of the cells. This is supported by previous results by Brandon et al. (1993) and Silberstein et al. (1996), who have shown nuclear PR staining in ovariectomized mice mammary epithelial cells and in the nuclei of normal human myometrium and leiomyomas. However, in some transfection experiments cytoplasmic PR expression has also been shown (Lim et al. 1999, Passinen et al. 1999). Furthermore, a complete nuclear translocation of both isoforms has been shown when the receptors were activated with progestin (Lim et al. 1999). In our study, the mice were ovariectomized and thus the levels of circulating progesterone are assumed to be undetectable or at least very low, which supports our conclusion of nuclear localization of unliganded receptor *in vivo*. Despite these aspects, our data do not exclude the possibility of low cytoplasmic PR expression due to limitations of the immunohistochemical protocol. Moreover, differences between transfection experiments and expression *in vivo* probably exist. In addition, monoclonal and polyclonal antibodies have been shown to give different results with regard to localization of the staining (Silberstein et al. 1996), but unfortunately we did not study this effect.

Our results suggest that PR is widely expressed in murine organs. The progesterone targets include urogenital (uterus, vagina, urinary bladder and urethra), gastrointestinal (submandibular gland, duodenum, jejunum and pancreas) and immunological organs (thymus) as well as skin. According to our results, progesterone may also regulate the blood flow of some murine organs such as uterus, although both supportive (Perrot-Applanat et al. 1988) and contradictory results have been reported (Lessey et al. 1988, Press et al. 1988). Specific progestin binding has also been shown in rabbit lung (reviewed in Graham and Clarke 1997), but lung tissue was PR-negative in our study.

In order to find out whether the GRIP1 expression is nuclear, cytoplasmic or both, we transiently transfected COS cells with GRIP1 cDNA and immunostained the cells with our novel α123 antibody. A clear nuclear staining was seen and the protein was mostly localized in discrete nuclear bodies, excluded from nucleoli. This has been confirmed by Lopez et al. (2001), who transfected HeLa cells with fusions of WT GRIP1 and GFP in expression vector and by Voegel et al. (1996), who transfected Cos-1 cells with full-length TIF2. However, although GRIP1 was mainly nuclear according to our immunohistochemical studies, cytoplasmic expression was also seen. Moreover, COS cells transfected with the expression vector pSG5 only showed mainly nuclear (endogenous) expression. Thus, it seems that GRIP1 is mainly located in the nucleus *in vivo* as are its target proteins PR and ER, but the mechanism by which GRIP1 is transported to the nucleus is still unknown.

Our results suggest that GRIP1 is widely expressed in murine tissues. In fact, in addition to being expressed in almost all PR-expressing cells, it was expressed in many cell types and tissues, which were PR-negative. In the testis, a nuclear staining of Sertoli cells, also known as supporting or sustentacular cells, was seen. This observation has been confirmed later (Gehin et al. 2002). It is known that abnormal spermatids, which are the most mature cells, are often phagocytosed by Sertoli cells. Indeed, Gehin et al. (2002) observed that the lack of GRIP1 in Sertoli cells results in increased number of abnormal spermatozoa and thus reduced male fertility and they suggest that TIF2 mutations could, at least in some cases, be involved in human male hypofertility or even sterility. We also found a nuclear staining in the spermatogonia, which are the most immature spermatogenic cells resting on the basal lamina, but Gehin et al. (2002) could not confirm this finding. They used the same antibody, which suggests that the differentiation between these two cell types is challenging.

2 THE ROLE OF PR, AIB1, SRC-1 AND COREPRESSORS IN ANTIESTROGEN-RESISTANT BREAST CANCER CELLS

PR expression has generally been regarded as a marker of intact ERα function. Furthermore, patients whose tumours contain both ERα and PR have the greatest probability of responding to endocrine therapy and they have a better prognosis than those whose tumours do not contain steroid receptors (Byar et al. 1979, Allegra et al. 1980, Rose et al. 1985, Rydén et al. 1988, Shek and Godolphin 1989, Crowe et al. 1991, Fernö et al. 1996, Molino et al. 1997, Bryant et al. 1998, Harvey et al. 1999, Chebil et al. 2003). In our study, PR expression was decreased 5.6-fold in antiestrogen-resistant cells when compared to original MCF-7 cells and the decrease was even 8.2-fold, when compared to control (estrogen-independent) cells. This result is consistent with previous reports both *in vivo* and *in vitro* (Osborne et al. 1991, Lykkesfeldt et al. 1994, Badia et al. 2000, de Cremoux et al. 2003), although in these studies the antiestrogen was tamoxifen, not toremifene. According to our proliferation experiments, antiestrogen-resistant cells are clearly estrogen-responsive when compared to vehicle-treated cells. Furthermore, estrogen caused PR and ERβ up-regulation in these cells. Thus one possibility may be that ERα function is impaired in resistant cells, since PR expression was decreased, but the proliferation experiments nevertheless demonstrated that this was not the case. Another explanation would be that ERα expression was decreased and thus the estrogen priming effect would be decreased. However, no changes in ERα expression were seen in our cells when compared to parental cells, suggesting that ERα content and functions are maintained in resistant cells, which also is consistent with previous results (Badia et al. 2000, Kurokawa et al. 2000, Hutcheson et al. 2003). However, Madsen et al. (1995) have reported that long-term tamoxifen-treated MCF-7 cells exhibited lowered ER mRNA and protein levels, but it is noteworthy that their exposure time to the drug was shorter than in our experiment.

It has been proposed that antiestrogen resistance may be the result of inappropriately increased agonist activity of SERMs. Moreover, activation of intracellular signalling cascades (e.g. Akt/IP-3 kinase and MAPK signalling cascades) downstream of growth factor receptors could potentially explain the increased agonist properties. However, we observed no increased agonist activity of antiestrogen, since both estrogen target genes PR and ERβ were up-regulated normally by estrogen in antiestrogen-resistant cells.

It has been suggested previously that tamoxifen may be able to stimulate an increase in AIB1 expression and elevated AIB1 levels may accelerate breast cancer cell proliferation by either amplifying the mitogenic effects of estrogen through potentiating the transactivation of ER or by bypassing this E_2-ER-dependent pathway (Bouras et al. 2001, Lauritsen et al. 2002, Louie et al. 2004). In addition, AIB1 has been shown to promote hormone-independent breast cancer cell proliferation by induction of cell cycle reentry by inducing the expression of key cell cycle regulators and it is able to completely overcome the cell cycle arrest effect, which is normally induced by antiestrogens (Louie et al. 2004). Moreover, when endogenous AIB1 levels were reduced by ribozyme targeting, estrogen-dependent MCF-7 tumour growth was significantly decreased in nude mice (List et al. 2001a). According to our results, basal AIB1 expression was increased 1.9-fold in antiestrogen-resistant cells when compared to original MCF-7 cells. Furthermore, no change was observed in estrogen-independent (i.e. control) cells, which eliminates the possibility that this finding resulted from long-term culturing of cells. Thus our results are in agreement with both previous reports (de Cremoux et al. 2003) and the hypothesis that AIB1 plays a role in the development of resistance to antiestrogen therapy.

The experiments by Jepsen et al. (2000) have shown that corepressor N-CoR was required for the antagonist effect of tamoxifen, since this antiestrogen failed to activate ER-dependent transcription in wild-type mouse embryo fibroblasts but acted as a full agonist in N-CoR gene-deleted cells. Low

levels of N-CoR mRNA before the onset of TAM therapy have been shown to predict poor response to antiestrogen therapy (Berns et al. 1998, Girault et al. 2003). This is congruent with the result that decreased N-CoR protein levels have been observed to correlate with the acquisition of TAM resistance in a mouse model system (Lavinsky et al. 1998). The lack of expression or functional inactivation of N-CoR as well as SMRT may therefore be involved in the resistance to hormonal therapy in the treatment of breast cancer patients. However, the basal levels of N-CoR mRNA were not changed in our antiestrogen-resistant cells and on the contrary, SMRT levels were increased 2.3-fold. However, SMRT levels were also increased 2.0-fold in control cells, which suggests that this latter finding is not specific to antiestrogen-resistant cells.

It has been shown that both isoforms of SRC-1 are expressed in MCF-7 breast cancer cells using RPA (Kalkhoven et al. 1998). Both isoforms were also expressed in all our five cell lines, but we were able to quantify SRC-1a transcript only, since the SRC-1e expression was very low. Kalkhoven et al. (1998) also concluded that SRC-1a intensity was weaker than SRC-1e, which is quite the opposite when compared to our results. One reason for these discrepancies may be that the MCF-7 cells represent different clones in different laboratories around the world. Another reason may be the difference in the amount of sample RNA; we used only 5 µg total RNA while Kalkhoven et al. used 10 µgs. The more RNA is loaded into the lanes, the more intense will the signal be. Thus, this limitation in the amount of RNA may have had an impact on our conclusions. On the other hand, there is also an upper limit for the amount of RNA, which can be loaded onto the wells, and the RNA-extraction protocol has its limit regarding the achievable maximal concentration of RNA.

3 EFFECTS OF PROGESTIN IN MPA-RESISTANT BREAST CANCER CELLS

It is known that MPA inhibits the growth of MCF-7 cells dose-dependently (Braunsberg et al. 1986, Gibelli et al. 1994, Schoonen et al. 1995, Ahola et al. 2002) and our results were congruent with these previous findings. After we propagated these cells for nine months in the presence of estrogen plus MPA, the growth of both of these cell types was no longer inhibited by MPA. We propagated the MPA-resistant cells for 41 passages, and the growth inhibitory effect of progestin has been shown to diminish even after 20 passages (Gibelli et al. 1994). We observed that MPA-resistant cells reached a steady growth rate after four months, which corresponds to approximately 16 passages. Braunsberg et al. (1986) observed a significant growth stimulation by MPA in their resistant cells, while we did not. Thus, MPA inhibits the growth of breast cancer cells at the beginning, but gradually this growth-inhibitory effect is lost.

However, progestin did not inhibit the growth of long-term estrogen-treated (control cells) either. Thus the possibility that the long-term culture was the reason for the resistance cannot be ruled out. In any case, there were some differences in progestin-resistance between these two cell lines. First, the estrogenic growth response and the regulation of estrogen target genes PR and ERβ were diminished in MPA-resistant but not in LE cells. Moreover, basal ERα expression was decreased in MR cells. Together these findings point to the possibility that long-term progestin treatment alters or at least affects the ER-mediated pathway in breast cancer cells.

4 HORMONAL REGULATION OF EXPRESSION OF STEROID HORMONE RECEPTORS AND COREGULATORS

Estrogen is known to up-regulate PR expression (Korach et al. 1985, Aronica and Katzenellenbogen 1991, Couse et al. 1995). This phenomenon is also called estrogen priming. When we studied PR protein expression with immunohistochemistry in female mice with and without estrogen treatment, there were some organs and cell types in which estrogen was required for PR expression. Furthermore, when hormonal regulation of steroid hormone receptors was studied by RPA in breast

cancer cells, the most significant up-regulation was seen in PR expression in all five cell lines after estradiol treatment. In addition, MPA treatment resulted in significant up-regulation of PR. Thus, PR expression is estrogen-regulated both at the transcriptional and protein levels and progestin-regulated at the transcriptional level. By contrast, estrogen treatment did not change the subcellular localization nor the GRIP1 expression profile in murine tissues, suggesting that estrogen does not regulate GRIP1 protein expression. This was further supported by our RPA results, since estrogen had no effect on TIF2 transcript levels either.

Human TIF2 was originally found as a protein, which interacts with steroid hormone receptors in a ligand-dependent manner (Voegel et al. 1996). Furthermore, Hong et al. (1997) showed that the full-length GRIP1 interacts with the LBD of PR and thus it can be assumed that those cells, which express PR, also express GRIP1. When we compared PR and GRIP1 expression in murine tissues, this was the case in almost all tissues, but vaginal epithelial cells and capsular cells of thymus were clearly GRIP1-negative while PR-positive after estrogen treatment. In addition, capsular cells of thyroid gland were PR-positive but GRIP-negative. By contrast, it can be assumed that those cells, which express GRIP1, do not necessarily express PR, since this protein can also regulate other hormone receptors than PR.

It has been shown that estrogen can suppress AIB1 gene expression and that antiestrogens tamoxifen and ICI 182,780 can reverse this effect in MCF-7 breast cancer cells (Lauritsen et al. 2002). However, we observed rather up-regulation of AIB1 expression by estrogen in MCF-7 cells and this effect was seen only in MPA-resistant cells. Moreover, Vienonen et al. (2003) did not observe AIB1 regulation after short-term or long-term estrogen treatment. Obviously, this issue needs futher study.

5 GPR30 IN BREAST CANCER

It has generally been thought that steroid hormone receptors are located predominantly in the cell nuclei and after ligand-binding they influence the transcription of their target genes. However, both estrogen and progesterone have been shown also to produce rapid, non-transcriptional responses, which in some cases are similar to those evoked by peptide growth factors such as EGF and IGF (Migliaccio et al. 1996, Endoh et al. 1997, Watters et al. 1997). Thus, it has been suggested that a subpopulation of the classic ER/PR is associated with the cell membrane. Indeed, Thomas et al. (2005) demonstrated the expression of GPR30 in the plasma membranes of cells that lack nuclear ERα and ERβ expression and they showed that GPR30 has all the binding and signalling characteristics of a membrane ER. Furthermore, three membrane PRs, which have the typical GPCR protein structure, have been identified (Zhu et al. 2003).

GPR30 has been shown previously to be expressed in MCF-7 breast cancer cells and both in ER-positive and ER-negative primary breast cancer samples (Carmeci et al. 1997). In our study, GPR30 was expressed in original MCF-7 cells, which is in agreement with these previous studies. Surprisingly, we observed a 50-fold reduction in GPR30 expression in toremifene-resistant cells when compared to original MCF-7 cells. A 5-fold reduction was also seen in EI (control) cells, suggesting that when the effect of long-term subculturing is taken into account, toremifene caused a 10-fold reduction in GPR30 expression after 9 months. To our knowledge, this is a novel finding. We also wanted to know whether re-expression of GPR30 would return the growth inhibitory effect of toremifene back into these cells. Indeed, this was observed, suggesting that in addition to being competitive inhibitors of estradiol in binding to estrogen receptors (reviewed in Jordan 1990, reviewed in MacGregor and Jordan 1998), antiestrogens may also act through cell membrane receptor GPR30. Thus, measurement of GPR30 levels before the onset of endocrine therapy may

even predict the future response to these drugs, and as a matter of fact, this issue is under investigation at this moment.

SUMMARY AND CONCLUSIONS

Estrogen and progesterone regulate many physiological functions such as behaviour, reproduction, development, cell differentiation and apoptosis. They also play a role in tumorigenesis and in many illnesses. The physiological and pathophysiological responses of the cells to these hormones depend on many factors. First, the nature of the ligand (agonist or antagonist), the relative concentrations of all the competing steroids and their binding affinities for the receptor are important factors. Second, tissue-specific expression of steroid hormone receptors and their isoforms as well as expression of coregulators and genetic polymorphism at the coregulator loci will affect the final biological response. Third, different nuclear receptors prefer different NR boxes for interaction with coregulators. Fourth, steroid hormones modulate the levels of their own receptors and steroid receptors also affect each other's function. Furthermore, differential target gene promoter specificities of the receptors affect the final response. Several other hormones than steroid hormones (e.g. prolactin and thyroid hormone) have been shown to influence receptor levels and thus hormone-mediated target gene regulation (reviewed in Katzenellenbogen 1980) and some hormonal responses are mediated by autocrine/paracrine effects of locally produced growth factors. Finally, in addition to receptor-mediated or "classical" responses, estrogen and progesterone are also able to induce rapid, receptor-independent responses, which involve cell membrane receptors such as G protein-coupled receptor 30. Thus, instead of a simple model of steroid hormones binding to their own receptors and affecting target gene transcription, regulation of steroid hormone response involves many factors and many different steps.

The main conclusions to be drawn from this study are:

1. Progesterone receptor is widely expressed in murine tissues. PR was expressed exclusively in the nuclei of cells. The results suggest that progesterone has cell-specific actions in the urinary tract and gastrointestinal organs, it plays a role in the reproductive and immune functions and it may regulate the blood flow in some organs.

2. GRIP1 is also widely expressed in urogenital, gastrointestinal, endocrinological and cardiopulmonary organs. In addition to mainly nuclear expression, GRIP1 is also cytoplasmic protein. As expected, almost all PR-expressing cells also express GRIP1. Thyroid gland and skeletal muscle did not express either PR or GRIP1, suggesting these tissues are not targets for progesterone.

3. PR is both estrogen- and progestin-regulated while GRIP1/TIF2 is not regulated by either of these hormones.

4. E_2-response was decreased in MPA-resistant cells and toremifene increased the growth of toremifene-resistant cells. E_2 regulates the transcription of its known targets PR and ERβ and some coregulators, while toremifene does not regulate any of the steroid hormone receptors and coregulators studied. MPA seems to have a few targets among these genes.

5. Increased expression of coactivator AIB1 and decreased expression of PR and GPR30 may account for acquired antiestrogen resistance in breast cancer. In addition to being competitive inhibitors of estradiol in binding to estrogen receptors, antiestrogens may also act through membrane receptor GPR30 and GPR30 may be a potential marker for predicting response to antiestrogen therapy.

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