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The Role of Membrane-Initiated Signalling in Progestin-Induced Growth Inhibition in Mammary Epithelial and Breast Cancer Cells

ACADEMIC DISSERTATION
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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles:

I Ahola TM, Purmonen S, Pennanen P, Zhuang Y-H, Tuohimaa P and Ylikomi T (2002): Progestin upregulates G protein-coupled receptor 30 in breast cancer cells. European Journal of Biochemistry 269:2485-2490.

II Ahola TM, Manninen T, Alkio N and Ylikomi T (2002): G protein-coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells. Endocrinology 143:3376-3384.

III Ahola TM, Alkio N, Manninen T and Ylikomi T (2002): Progestin and G protein-coupled receptor 30 inhibit mitogen-activated protein kinase activity in MCF-7 breast cancer cells. Endocrinology 143: 4620-4626.

IV Ylikomi T, Vienonen A and Ahola TM (2004): G protein-coupled receptor 30 down-regulates cofactor expression and interferes with the transcriptional activity of glucocorticoid. Submitted.

ABBREVIATIONS

AngII angiotensin II

aa amino acids

AR androgen receptor

ATP adenosine triphosphate

Bad Bcl antagonist of cell death

Bax Bcl2-associated X protein

Bcl B cell leukemia

BrdU bromodeoxyuridine

BSA bovine serum albumin

cAMP cyclic adenosine monophosphate

CBP CREB-binding protein

CDK cyclin-dependent kinase

cDNA complementary deoxyribonucleic acid

CIP CBP interacting protein

CMV cytomegalovirus

COOH carboxyl terminal

CREB cAMP-response element binding protein

DAB diaminobenzidine

dATP deoxyadenosine triphosphate

DCC dextran coated charcol stripped

DEX dexamethasone

DHT dihydrotestosterone

DMBA 7.12-dimethylbenzanthracene

DNA deoxyribonucleic acid

DMEM Dulbecco`s Modified Eagles Medium

DTT dithiothreitol

ECL enhanced chemiluminescense

EDTA ethylenediamine tetraacetic acid

EGF epidermal growth factor

EGFP enhanced green fluorescent protein

ER estrogen receptor

ErbB avian erythroblastic leukemic viral oncogene homologue

ERK extracellular signal-regulated kinase

ET-1 endothelin-1

FBS fetal bovine serum

Gi inhibitory G protein

Gs stimulatory G protein

GnRH gonadotrophin releasing hormone

GPCR G protein-coupled receptor

GR glucocorticoid receptor

GRE glucocorticoid response element

GRIP-1 glucocorticoid interacting protein

GTPase guanosine triphosphatase

HB heparan-binding

HME human normal mammary epithelial

HMFGM human milk-fat globule membrane

HRT hormone replacement therapy

hTERT human telomerase transcriptase subunit

IGF insulin like growth factor

IL interleukin

IP3 inositol trisphosphate receptor

JNK/SAPK c-Jun N-terminal kinase/stress-activated protein kinases

Luc luciferase

MA megestrol acetate

MAPK mitogen-activated protein kinase

MEMB Mammary Epithelial Basal Medium

MEK MAPK kinase

MMTV murine mammary tumor virus

MPA medroxyprogesterone acetate

mRNA messenger RNA

NH_2 amino terminal

ORG2058 16α-ethoxy-21-hydroxy-19-norpregn-4-en-3,20-dione

PAK p21-activated protein kinase

PD98059 2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one

PDGF platelet-derived growth factor

PI3 phosphoinositide 3-kinase

PKA protein kinase A

PKB protein kinase B

PKC protein kinase C

PLC phospholipase C

PBS phosphate buffered saline

PCR polymerase chain reaction

PR progesterone receptor

PR-A A-form of progesterone receptor

PR-B B-form of progesterone receptor

R5020 promegestone17α-21-dimethyl-19-norpregn-4.9-diene-3,20-dione

RAC3 Ras-related C3 botulium toxin substrate 3

RAR retinoic acid receptor

RASM rat aortic smooth muscle

Rb retinoblastoma protein

RNA ribonucleic acid

RT reverse transcription

RU486 mifepristone

RXR retinoid X receptor

SB202190 4-(4-fluorphenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole,
SAPK2/p38 MAPK inhibitor

SDS sodium dodecyl sulphate

SH3 Src-homology

SRC steroid receptor coactivator

SSPE saline/sodium phosphate/EDTA

TBP TATA binding protein

TBS tris-HCl-buffered saline

TGF transforming growth factor

TIF2 transcription intermediary factor-2

TRE thyroid hormone response element

tTA tetracycline-regulated transactivator

U0126 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene

INTRODUCTION

The steroid hormone progesterone plays a fundamental role in the female in regulating a number of reproductive functions. It has a key role in menstrual cycle regulation, (Critchley et al. 2001) controlling receptivity for blastocyst implantation (Yoshinaga 1988) and maintaining pregnancy (Pepe and Albrecht 1995) in the uterus. Synthetic progestins are widely used in medicine, constituting central factors in contraception, hormone replacement therapy and the treatment of dysfunctional uterine bleeding (Apgar and Greenberg 2000). They are also used in cancer therapy, specifically in the treatment of metastatic endometrial and metastatic breast cancer.

In the normal adult mammary gland, however, the issue of how progesterone exerts its effects remains controversial. During mammary gland development progesterone has been held to induce proliferation: indeed, the major role of progesterone in normal mammary gland tissue is in promoting the formation of lobular-alveolar structures during pregnancy (Lydon et al. 1995). In the adult mammary gland this concept of a mitotic role is supported by a considerable body of evidence (Graham and Clarke 1997, Hofseth et al. 1999, Isaksson et al. 2001). Recent data indicate that in women the addition of progestin medroxyprogesterone acetate (MPA) to hormone replacement therapy (HRT) increases the risk of breast cancer (Hofseth et al. 1999, Ross et al. 2000, Schairer et al. 2000, Li et al. 2003). On the other hand, progestins MPA and megestrol acetate (MA) comprise commonly used third-line drugs in metastatic breast cancer treatment (Bast RC et al. 2000, Parazzini et al. 1993). In cell culture studies the effect of progestins (e.g. MPA,

R5020, ORG2058, MA) on growth is usually growth-inhibitory, but opposite results have also been reported (Sutherland et al. 1988, Poulin et al. 1989, Dauvois et al. 1990, Musgrove et al. 1991, Poulin et al. 1991, Schoonen et al. 1995, van den Berg et al. 1996, Groshong et al. 1997, Musgrove et al. 1998, Pasqualini et al. 1998, Lin et al. 1999).

At the molecular level progestin exerts its effects via steroid hormone receptors. After diffusion through cell membranes and binding to the nuclear receptors, the receptor-ligand complex binds to specific DNA sites in the enhancer element of target genes. Progesterone enhances the transcription of genes affecting cell functions such as differentiation and structure. Progesterone first stimulates the entry of T47-D breast cancer cells into the cell cycle, and then arrest cells in the late G1 phase, inducing long-term growth inhibition (Musgrove et al. 1993, Groshong et al. 1997). Progestin ORG2058 and progesterone has been shown to down-regulate cell cycle proteins: cyclins D1, D3, E, A, B and upregulate cyclin-dependent kinase (CDK) inhibitors p21 and p27, leading to a change in the activity of CDKs (Groshong et al. 1997, Musgrove et al. 1998). A concerted model of progestin ORG2058 action has been suggested whereby CDK inhibitors cooperate to inhibit CDK activity (Swarbrick et al. 2000).

In breast cancer cells progestin R5020 rapidly activates the Src/p21Ras/ extracellular signal-regulated kinase (ERK) pathway (Migliaccio et al. 1998), which has been held to be responsible for its growth-stimulatory effect in cultured cells (Castoria et al. 1999). An interaction of the proline-rich motif in the progesterone receptor (PR) with the Src-homology (SH3) domains of the c-Src family of tyrosine kinases has been shown to be

critical for mitogen-activated protein kinase (MAPK) activation independent of transcriptional activity (Boonyaratanakornkit et al. 2001). In this study progestin-induced growth inhibition was seen to be dependent on PR-SH3 interaction, suggesting a potential role of the MAPK pathway in progestin-induced growth inhibition. The relationship between cell cycle-regulating molecules and this pathway has not been established.

This study was undertaken to elucidate the mechanism of progestin action in the mammary gland by analysing differential gene expression induced by progestin in breast cancer cells. Attention was focused on one of the progestin target genes, G protein-coupled receptor 30 (GPCR30). We characterized its effect on cell proliferation and cell signalling pathways. The results presented imply that the expression of GPCR30 inhibits cell proliferation, is critical for progestin-induced growth inhibition, affects the activation of MAPK pathway and interferes with the steroid hormone-mediated transcription.

REVIEW OF THE LITERATURE

1. THE MECHANISM OF PROGESTIN ACTION IN THE MAMMARY GLAND

1.1. Progestin-induced transcription

Progestin affects via PR, which belongs to the large family including receptors for steroids, retinoids, thyroid hormones and vitamin D. Binding of progestin to the ligand binding domain of PR causes receptor dimerization. The progestin-complexed PR homodimer binds with specific progestin response elements in target genes (Clarke et al. 1997). Thereafter, cofactors are recruited to the target gene promoter, being able to enhance transcriptional activation by the receptor. The activation and repression of nuclear receptors in response to steroid hormones is largely attributable to their recruitment of cofactors (McKenna and O'Malley 2002).

1.2. Steroid receptors and coactivators

The activity of nuclear receptors is controlled by cofactors belonging to the steroid receptor coactivator (SRC) family. This ligand-recruited nuclear receptor cofactor family comprises coactivators such as SRC-1, Ras-related C3 botulinum toxin substrate 3 (RAC3), CBP interacting protein (CIP) and transcription intermediary factor 2 (TIF2).

SRC-1 enhances, in a ligand-dependent manner, transcription of different nuclear receptors (Onate et al. 1995; Webb et al. 1998). The steroid hormone dexamethasone down-regulates SRC-1 expression in rat tissues and also in kidney-derived cells (Misiti et

al. 1998, Kurihara et al. 2002). It has also been suggested that p/CIP is regulated by steroid hormones (Li and Chen 1998, Misiti et al. 1998). The mode of regulation of RAC3 (Li et al. 1997) has not been established.

TIF2 coactivates all steroid, thyroid, retinoid and vitamin D receptors (Hong et al. 1996, Voegel et al. 1996). The cofactor is widely expressed in different human tissues such as the pancreas, lung, placenta, brain and heart (Voegel et al. 1996). In mouse tissues the homologue of TIF2, glucocorticoid interacting protein 1 (GRIP-1), has been shown to be expressed e.g. in the testis, mammary gland, prostate and uterus (Hong et al. 1996, Puustinen et al. 2001). GRIP-1 expression is critical for skeletal muscle differentiation in the mouse (Chen et al. 2000). However, reduction of TIF2 expression by antisense oligodeoxynucleotides inhibits estrogen-stimulated estrogen receptor (ER) transcriptional activity and DNA synthesis in MCF-7 cells, this constituting evidence for the role of TIF2 in growth stimulation (Cavarretta et al. 2002). A potential pathway for TIF2 regulation has been suggested. In transient transfection assays, namely, the function and protein level of TIF2 was impaired by protein kinase A (PKA) (Borud et al. 2002).

1.3. The differential role of PR-A and PR-B

Progestin affects through PR, which induces the transcription of genes by binding to the hormone response element in the target gene promoter. PR exists in two isoforms, PR-B and PR-A. They are transcribed from the same gene, but from different promoters. PR-B is longer, being 933 amino acids in length. PR-As lack the 164 N-terminal residues, which contains a unique activation function.

Progesterone is critical for the establishment and maintenance of pregnancy. Indeed, studies of mice lacking both PRs indicate the role of progesterone as a coordinator of diverse reproductive events which together ensure species survival (Lydon et al. 1995). When both PRs were mutated to become non-functional, adult female mice displayed significant defects in all reproductive tissues. These included an inability to ovulate, uterine hyperplasia and inflammation, defects in uterine implantation, severely limited mammary gland development, and an inability to exhibit sexual behavior. Interestingly, in the mammary gland, the development of lobulo-alveolar structures and lateral ductal branching accompanying pregnancy were impaired, whereas the ductal growth accompanying puberty was not compromised in these mice (Lydon et al. 1995). Additionally, when PR-/- mammary glands were transplanted into wild-type mice, development of the mammary gland was arrested at the stage of the ductal system found in virgin mice despite estrous cycle and pregnancy (Brisken et al. 1998).

The role of the PR-B isoform was established by selective ablation of PR-A in mice. PR-B was sufficient for normal mammary gland development. However, without PR-A ovulation was severely impaired after stimulation of immature mice with gonadotropins (Mulac-Jericevic et al. 2000). Uterine decidualization and differentiation were also inhibited in response to progesterone. Recently, the role of PR-A was established by selective ablation of PR-B in mice. Interestingly, pregnancy-associated ductal sidebranching and lobulo-alveolar development were markedly reduced due to decreased epithelial cell proliferation (Mulac-Jericevic et al. 2003). PR-A was sufficient to elict

normal ovarian and uterine responses to progesterone.

Study of over-expression indicates the importance of PR-B and PR-A signalling for appropriate cell-fate decisions during normal mammary gland development. Transgenic mice, which over-expressed PR-B had inappropriate alveolar growth and underwent a premature arrest in ductal growth. In mice carrying the additional A form of PR lateral ductal branching was excessive (Shyamala et al. 2000).

Differential gene expression pattern induced by PR-B and PR-A has been established in *in vivo* studies (Mulac-Jericevic et al. 2000) as well as in *in vitro* studies in breast cancer cells (Richer et al. 2001). PR-B and PR-A uniquely regulate most of the target genes of progesterone. Unexpectedly, almost half of the genes were proteins involved in membrane-initiated signalling, mostly regulated by PR-B when gene expression profiles in T47-D cells were studied (Richer et al. 2001). Surprisingly, progestin seemed to regulate to a lesser extent genes involved in cell cycling, apoptosis and transcription control.

In conclusion, the major developmental role of PR receptors in the normal mammary gland is the formation of lobular-alveolar structures during pregnancy and PR-B receptor has been shown to be critical for this mammary gland morphogenesis. The expression of PR-A is, instead, essential for normal ovarian and uterine responses by progesterone.

1.4. Growth regulation by progestin

1.4.1. Normal mammary gland

In the normal adult mammary gland the issue of how progesterone exerts its effects is controversial. The hormone takes part in lactation process in the mammary gland, which would indicate a role of progesterone in regulating cell differentiation (Graham and Clarke 1997). During mammary gland development progesterone has been thought to induce proliferation. Indeed, the major role of progesterone in normal mammary gland tissue is to participate in the formation of lobular-alveolar structures during pregnancy (Lydon et al. 1995). In macaques progestin MPA further stimulates estrogen-stimulated cell proliferation (Cline et al. 1996, Cline et al. 1998). In humans the mitotic role of progesterone is supported by evidence that the proliferation index in a mammary gland reaches its peak during the luteal phase of the menstrual cycle when progesterone concentrations are high (Graham and Clarke 1997), and the demonstration that progesterone levels in serum correlate with breast tissue proliferation (Isaksson et al. 2001). More direct evidence came from a study by Hofseth et al. (1999), who showed that combined HRT (progestin+estrogen) users had a significantly higher proliferation index than estrogen-alone users when breast tissues from post-menopausal women were analyzed.

On the other hand, progesterone and progestin have had no effect on estrogen-stimulated cell proliferation when the effects were studied either by administering progestin MPA (Colditz et al. 1995) or progesterone (Clarke et al. 1997) to women; or when progestin

R5020 was implanted locally in the mammary gland (Raafat et al. 2001). Quite to the contrary, progesterone and progestins have also been shown to inhibit mammary gland epithelial cell proliferation *in vivo*. Indeed, when premenopausal (Chang et al. 1995) or postmenopausal (Foidart et al. 1998) women are treated with progesterone and estrogen, progesterone significantly reduces the number of cycling epithelial cells in breast tissue. In xenograft models proliferation of normal mammary gland epithelium explanted into nude mice is decreased and in some cases no effect is detected when treated with progesterone (Laidlaw et al. 1995, Clarke et al. 1997, Graham and Clarke 1997). Incidence of mammary tumors induced by carcinogen DMBA (7.12-dimethylbenzanthracene) has been markedly reduced in PR knockout mouse as compared with wild types, suggesting that murine mammary gland carcinogenesis is critically dependent on PR function (Lydon et al. 1999). Interestingly, progestin MPA has been used as a promoting factor for mammary adenocarsinomas in Balb/c mice (Elizalde et al. 1998, Balana et al. 2001, Lanari et al. 2001). Recent data indicate that also in humans the addition of progestin MPA to HRT increases the risk of breast cancer (Hofseth et al. 1999, Ross et al. 2000, Schairer et al. 2000, Rossouw et al. 2002). It is suspected that the increase in cell proliferation induced by progesterone in the mammary gland might explain the tumor-promoting potential of progesterone.

In *in vitro* studies with normal breast the role of progesterone has also been variable, but more concise than in *in vivo* studies. In organ culture progesterone increases DNA synthesis of mammary gland epithelial cells (Graham and Clarke 1997). However, proliferation is inhibited in cultured normal human breast epithelial cells when treated

with progesterone or progestin R5020 (Chomette et al. 1986, Gompel et al. 1986, Malet et al. 2000). All in all, it is reasonable to say that progestin stimulates growth *in vivo*, but inhibits it *in vitro* in the normal mammary gland.

1.4.2. Breast cancer cells

Tamoxifen is currently the first choice for hormonal treatment in breast cancer in the majority of premenopausal or postmenopausal women (Kaufmann 1997). However, the development of resistance to the drug is a major problem; hence progestins such as MPA and MA are used in the treatment of advanced breast cancer patients in the case of post-menopausal patients. Besides their growth-inhibitory effect, progestins prevent the synthesis of estradiol by blocking the formation of estradiol via sulfatase, or stimulating sulfotransferase activity (Pasqualini et al. 1998). In analysis of several clinical trials, MPA has shown to be effcient in the treatment of breast cancer patients with metastases (Bast RC eta al. 2000, Parazzini et al. 1993). However, new more efficient drugs such as aromatase inhibitors have been started to replace progestins in treatment of breast cancer patients (Bast RC et al. 2000).

The effect of progestin MPA and progesterone in xenograft models are also a subject of controversy. When human MCF-7 breast cancer cells were transplanted into female nude mice, the growth of carcinoma xenograft was enhanced by exogenous progesterone (Kubota et al. 1995). In other xenograft studies, in contrast, MPA treatment reduced tumor incidence, the mean weight of tumors, the 3H-thymidine labelling index, differentiation of antigen human milk-fat globule membrane (HMFGM) and Ras p21, c-

myc, neu oncogene products levels (Mizukami et al. 1991, Lin et al. 2001).

In cell culture studies the effect of progestin on growth is usually found to be inhibitory, but opposite results have also been obtained. Indeed, some studies have shown progestin (e.g. R5020, MPA, ORG2058, norethindrone, norgestrel, levonorgestrol) to inhibit estrogen-, but not insulin-stimulated proliferation of MCF-7 and ZR-75-1 cells (Dauvois et al. 1990, Poulin et al. 1991, Schoonen et al. 1995, van den Berg et al. 1996). Progestins (MPA, R5020, ORG2058) and progesterone have likewise been shown to inhibit both estrogen- and insulin-stimulated proliferation in ZR-75-1, T-47-D and MCF-7 cells (Sutherland et al. 1988, Poulin et al. 1989, Musgrove et al. 1991, Groshong et al. 1997, Musgrove et al. 1998). However, ER expression would appear to be dispensable for progestin-induced growth inhibition. Indeed, progestin R5020 can inhibit the growth of ER-negative T47-D breast cancer cells when PR isoforms are constitutively expressed (Pasqualini et al. 1998). Additionally, in ERα- and PR-negative MDA-MB-231 cells, stable transfection of PR allows progestin to inhibit estrogen-independent growth (Lin et al. 1999).

1.5. Cell cycle regulation by progestin

Regulation of the cell cycle is a key point in the normal development of a multi-cellular organism. Cells proceed through the cell cycle, during which their chromosomes are duplicated and two daughter cells are formed. The cell cycle is divided into four major phases G1, S, G2 and M. Progesterone first stimulates the entry of T47-D breast cancer cells into the cell cycle, and then arrests the cells at the late G1 phase, inducing long-term

growth inhibition (Musgrove et al. 1993, Groshong et al. 1997).

The major transitions of the eukaryotic cell cycle are triggered by the CDKs (Morgan 1995). The primary regulator of CDK activity is the cyclin subunit, whose levels oscillate during the cell cycle. Passage through the G1 restriction point is controlled by G1-phase cyclins, which phosphorylate retinoblastoma protein (Rb), p107 and p130, this leading to E2F transcription factor activation. Cell-cycle progression depends on the execution of a regulatory cascade of gene expression, driven by E2F transcription factors (Lavia and Jansen-Durr 1999). Progestin (ORG2058)- and progesterone-induced growth arrests are accompanied by hypophosphorylation of Rb and down-regulation of cell cycle proteins: Rb, cyclins D1, D3, E, A and B (Groshong et al. 1997, Musgrove et al. 1998). Progesterone also regulates CDK activity by up-regulating CDK inhibitors p21 and p27(Kip1)(Groshong et al. 1997). Progestin ORG2058 regulates CDK activity by affecting CDK inhibitors association with the cyclin-CDK complexes (Musgrove et al. 1998).

To determine the role of reduced cyclin expression, T-47D human breast cancer cells constitutively expressing cyclin D1 or cyclin E have been treated with the progestin ORG 2058 (Musgrove et al. 2001). Indeed, over-expression of cyclin E did not prevent the decrease in cyclin E-CDK2 activity. In contrast, over-expression of cyclin D1 induced progestin resistance and continuous cell proliferation despite an increased association of $p27^{Kip1}$ with cyclin E-CDK2. Thus the ability of cyclin D1 to confer progestin resistance provides evidence for a critical function of cyclin D1 via an as yet unknown mechanism

in progestin inhibition of breast cancer cells. A concerted model of progestin ORG 2058 action has been suggested whereby $p27^{Kip1}$ and $p18^{INK4c}$ cooperate to inhibit cyclin E-CDK2 and CDK4/6 (Swarbrick et al. 2000). Indeed, progestin induces $p27^{Kip1}$ association with cyclin-CDK4/6. Thereafter $p18^{INK4c}$ displaces $p27^{Kip1}$ and cyclin D1 from CDK4/6, making $p27^{Kip1}$ available to bind cyclin E-CDK2 with consequent inhibition of the activity.

1.6. Progestin and apoptosis

Apoptosis is characterized by nuclear condensation, fragmentation and degradation of DNA into oligonucleosome fragments (Thompson 1995). Apoptosis is regulated to a marked extent by a number of genes: B cell leukemia (Bcl) 2 and related family members: Bcl-x, Bcl2-associated X protein (Bax) and Bcl antagonist of cell death (Bad). An increase in apoptosis has been held to be integral to normal mammary gland development, based on dynamic changes in the expression of Bcl-2 family members (Kumar et al. 2000). It has also been directly shown that during lactation endogenous progesterone inhibits mammary gland apoptosis (Berg et al. 2002).

Some studies have suggested that progestin induces apoptosis and thus inhibits growth. Progestin ORG2058 enhances apoptosis, down-regulates Bcl-2 and to some degree of Bcl-XL in primary culture of breast cells and in different hormone-responsive breast cancer cells (Gompel et al. 2000, Kandouz et al. 1999). A role of p53 in progesterone-induced apoptosis was suggested by a study in which progesterone treatment (10 μM) induced apoptosis in T47-D cells by down-regulating protective Bcl-2 protein and up-

regulating p53 (Formby and Wiley 1999).

As already noted the effect of progestin on cell growth is contradictory. Moore and associates established that progestin R5020 enhances growth, decreases apoptosis and increases protective Bcl-xL expression during long-term culture of breast cancer cells (Moore et al. 2000). Further studies showed that progesterone enhances only the first cell cycle. A study by Richer et al. (2001) has shown that anti-apoptosis gene, Bcl-XL is upregulated shortly after progesterone treatment. It has also been established that MPA protects PR-positive T47-D, MCF-7 and H466-B breast cancer cell lines against serum depletion-induced apoptosis (Ory et al. 2001).

1.7. Paracrine and autocrine effects

There is evidence that the effects of progestins are mediated through paracrine factors as discussed subsequently. During development, progesterone-induced epithelial proliferation and morphogenesis is thought to occur indirectly, requiring stromal cooperation (Woodward et al. 1998). In a study by Brisken et al. (1998) breasts from PR knock-out mice were transplanted into wild type mice. These transplants lacking the PR in the stromal compartment give rise to normal alveolar growth, whereas transplants lacking PR in the epithelium conserved the abnormal phenotype. Chimeric epithelia in which PR negative cells were in close vicinity to wild type cells went through complete alveolar development to which the PR negative cells contributed, suggesting that progesterone acts by a paracrine mechanism on a mammary epithelial cells (Brisken et al. 1998). It is not known which paracrine factors mediate this effect.

The contribution of stromal cells in the proliferation of epithelial cells has been established by a study which showed that epithelial steroid receptors {ER, PR, androgen receptor (AR)} are not necessary for hormonal regulation of proliferation (Cunha et al. 1997). The proliferation of epithelial cells was rather mediated by hormone-receptor-positive stromal cells (Cunha et al. 1997). In co-culture experiments fibroblasts and epithelial cells from normal breast tissue inhibited the proliferation of MCF-7 breast cancer cells, but not fibroblasts from cancer tissues (Dong-Le Bourhis et al. 1997). Additionally, the medium conditioned by normal breast epithelial cells inhibited growth and induced apoptosis of breast cancer cells.

Progestins and progesterone regulate a number of growth factors in breast cells which contribute to the cell growth regulation. The insulin-like growth factor (IGF)-I receptor, IGF-II and insulin receptor are upregulated by progestin in T47-D breast cancer cells (Goldfine et al. 1992). In ZR-75-1 cells progestin ORG2058 also increases IGF-I receptor expression (van den Berg et al. 1996). IGF-I secretion is stimulated by progesterone in both malignant and non-malignant tissues (Gregoraszczuk et al. 2001). Increased systemic IGF-I activity has been linked to increased breast cancer risk. Estrogen suppresses IGF-I activity in the liver (McCarty 2001). Thus it has been hypothesized that androgenic progestins abrogate estrogen action on the IGF-I activity on the liver, and may thus lead to an increased risk of breast cancer over estrogen replacement therapy.

Progestin stimulates the expression of the growth factors transforming growth factor

(TGF) alpha and epidermal growth factor (EGF)(Clarke and Sutherland 1990; Musgrove et al. 1991), both of which operate through binding to and activation of a common membrane receptor, the EGF receptor homodimer. R5020 has also been shown to potentiate the effects of EGF by up-regulating EGF receptor, c-avian erythroblastic leukemic viral oncogene homologue (ErbB) 2 and c-ErbB3 receptors in breast cancer cells (Lange et al. 1998).

Progesterone rapidly increases the expression of several factors involved in mitogenesis in T-47-D breast cancer cells demonstrated by a microarray study. Calcium binding protein S100P, tissue factor and tyrosine kinase ligand Gas6 are all upregulated by progesterone (Richer et al. 2001). The expression of S100P is associated with breast cancer development (Guerreiro Da Silva et al. 2000). Tissue factor expression correlates with breast cancer metastasis (Chen et al. 2001), and Gas6 has been seen to induce growth in a cultured mammary epithelial cell line (Goruppi et al. 2001).

Progestin treatment evidently reduces the expression of growth inhibitory factors. The expression of TGF-beta is decreased by progestin in T47-D and MCF-7 breast cancer cell lines (Kalkhoven et al. 1995). Also the interleukin (IL)-1 receptor, which has been described to reduce hormone-dependent breast cancer cell growth (Paciotti and Tamarkin 1988, Danforth and Sgagias 1991), is down-regulated by progesterone (Richer et al. 2001). The secretion of growth inhibitory IL-6 (Danforth and Sgagias 1993) is likewice inhibited by MPA in breast cancer cells (Kurebayashi et al. 1999). In conclusion, progestins and progesterone increase growth by affecting the expression of growth

factors in breast cancer cells.

2. G PROTEIN-COUPLED RECEPTORS

2.1. Classification

G protein-coupled receptors (GPCRs) signal a wide variety of stimuli generated by different hormones, neurotransmitters, sensory stimuli and odorants (Rohrer and Kobilka 1998). The GPCR superfamily has nearly 2000 G protein-coupled receptors as it members. It is divided into five major families A-E: rhodopsin-like, secretin-like, metabotropic glutamate, fungal pheromone and cyclinc adenosine monophosphate (cAMP) receptors according to sequence homology, ligand structure, and receptor function (Ji et al. 1998, Morris and Malbon 1999).

The class A rhodopsin-like family is further divided into sub-families, namely peptide, amine, hormone protein, rhodopsin, olfactory, prostanoid, nucleotide-like, cannabis, platelet-activating factor, gonadotropin-releasing hormone, thyrotropin-releasing hormone, melatonin, and viral, lysosphingolipid, leukotriene B4 receptor and orphan receptors (www.gpcr.org/7tm/multali/multali.html).

Peptide receptors are further divided e.g. into angiotensin, bradykinin, interleukin-8, chemokine, endothelin, somatostatin and chemokine receptor-like receptors. This last-mentioned group is divided into chemokine/receptor-like 1 and chemokine receptor-like 2 families. The latter family consists of GPCR30 (human), GPCR41 (lung; rat), receptor

similar to GPCR30 (uterus, leiomyosarcoma; human) and RIK protein (Mus musculus; mouse)(www.gpcr.org/7tm/multali/multali.html).

2.2. Structure of G protein-coupled receptors

The GPCR family is known as the family of 7-helix transmembrane receptors. The receptors span the lipid bilayer with seven domains made up of 22-28 hydrophobic amino acids (aa) (Morris and Malbon 1999). The amino terminal (NH_2) and three intervening loops are located on the extracellular surface. The extracellular loops contain conserved Cys residues forming disulfide bridges, and stabilize the conformation of the extracellular domain (Rohrer and Kobilka 1998). Some GPCRs contain glycosylation sites in the extracellular region in the consensus sequences (Rohrer and Kobilka 1998). From the recently discovered crystal structure of rhodopsin it is concluded that the extracellular region is a highly organized structure which determines the basis for the seven-helix transmembrane structure (Palczewski et al. 2000).

The basic structure of all GPCRs is the same (Rohrer and Kobilka 1998). Indeed, intracellular loops and the carboxyl terminal (COOH) encode the domains involved in G protein interaction, cellular trafficking and agonist-induced desensitization. However, there are some differences in detailed structure between the receptors: 1) in bioamine receptors the ligand-binding pocket is formed by the transmembrane helices and 2) many of the peptide and glycoprotein receptors rely on the extracellular loop and NH_2-terminal regions for binding.

The sequences of chemokine family memebers have 25 to 80% aa identity, but many other GPCRs also have ~25% aa identity with chemokine receptors, indicating that the structural boundary is not sharp (Murphy et al. 2000). However, chemokine receptors possess several features which together are found more frequently among these receptors than in other types of GPCRs. These include 1) a length of 340 to 370 aa; 2) an acidic N-terminal segment; 3) the sequence DRYLAIVHA, or a variation of it, in the second intracellular loop; 4) a short basic third intracellular loop; and 5) a cysteine in each of the four extracellular domains. A tyrosine sulfation motif is commonly found in the N terminus of chemokine receptors (Murphy et al. 2000).

2.3. Signalling pathway of G protein-coupled receptors

GPCRs mediate their effects through activation of heterotrimeric G proteins. Receptor activation leads to dissociation of the G protein into two subunits, Gα and Gβγ, both of which can activate or inhibit various signal transduction molecules (Brady and Limbird 2002). G proteins are divided into subgroups based on their subunit composition and effects on down-stream signalling. Gs, or stimulatory G protein, activates cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and Raf, this resulting in activation or inhibition of the MAPK pathway. Inhibitory Gi activates Src-family tyrosine kinases or non-receptor tyrosine kinases, Ras and Raf. Gq-dependent activation usually proceeds via phospholipase C (PLC), inositol trisphosphate receptor (IP3), Ca2+ and protein kinase C (PKC) (Gutkind 1998b).

Activation of Gi- and Gq-coupled receptors induce ligand-independent phosphorylation

of tyrosine kinase receptors. Indeed, these receptors become phosphorylated in response to GPCR agonists and recruit signalling complexes containing Shc and transcription factors, resulting in Ras-dependent activation of ERK; EGF receptors (Daub et al. 1996, Daub et al. 1997) and platelet-derived growth factor (PDGF) receptors (Herrlich et al. 1998) can be activated by this mechanism. A mechanism for EGF transactivation by GPCR has been shown to be mediated through metalloproteinase-dependent cleavage of proheparan-binding EGF (HB-EGF), which after release may activate EGF receptor signalling in neighboring cells (Prenzel et al. 1999).

2.4. Cell growth regulation by G protein-coupled receptors

GPCRs make a major contribution to growth regulation in different normal and cancer cells. A substantial body of evidence indicates a role for GPCR in enhancing cell proliferation. Trypsin, which acts through GPCR2, initiates cell proliferation of human colon cancer cells (Darmoul et al. 2001), and a Kaposi's sarcoma-associated herpesvirus GPCR promotes endothelial cell survival and induces proliferation through the activation of Akt/protein kinase B (PKB)(Montaner et al. 2001). Using a rat aortic smooth muscle (RASM) cell model, both endothelin-1 (ET-1) and angiotensin-II (AngII) have been shown to induce concentration-dependent increases in DNA synthesis (Weber et al. 1994). Additionally, IL-8 and IL-12 have been shown to stimulate *in vitro* proliferation of endometrial cells (Gazvani et al. 2002).

There are fewer examples suggesting antiproliferative activity of GPCRs. Activation of GPCR1, which has a bioactive lipid sphingosylphosphorylcholine as a ligand, leads to

inhibition of ovarian cancer cell proliferation through activation of MAP kinases (Xu et al. 2000). The AngII receptor is growth-inhibitory and cross-talks with the signalling of other GPCR and growth factor receptors (Xoriuchi et al. 1999) such as ErbB3, which is a member of the EGF receptor family (Knowle et al. 2000). Somatostatin, on the other hand, negatively regulates the growth of various normal and tumor cells. Its effects are mediated directly by a family of GPCRs (sst1-5), which modulate the MAP kinase pathway and induce G1 cell cycle arrest or alternatively promote apoptosis by p53-dependent and -independent mechanisms (Ferjoux et al. 2000). Interestingly, KiSS-1, which is a human metastasis suppressor gene of melanomas and breast cancer, encodes the endogenous ligand of an orphan GPCR (hOT7T175)(Ohtaki et al. 2001). The ligand (KiSS-1) activates Ca2+ mobilization, ERK-1/-2 and p38 MAP kinase phosphorylation (Kotani et al. 2001).

2.5. Progestin and G protein-coupled receptors

The activation of GPCR is regulated in a number of ways. The receptors are downregulated by agonist, upregulated by hormones, and cross-regulated among G protein-mediated pathways (Malbon et al. 1991). Steroid hormones regulate transcriptional activity of the genes encoding members of G protein-linked pathways and thus control G protein-mediated signalling (Morris and Malbon 1999). Progestin regulates internalization of the mu-opioid receptor in the central nervous system (Sinchak and Micevych 2001). Gonadotropin releasing hormone (GnRH), gonadotropins (lutropin, follitropin), estradiol and progesterone control the level of expression of GnRH-R mRNA in the pituitary and gonads (Leung and Peng 1996).

Several lines of evidence indicate that progesterone-induced maturation of Xenopus oocytes is mediated by cell surface rather than nuclear receptors, primarily on the basis of the established rapid cell signalling effects on guanosine triphosphate (GTP) -dependent inhibition of adenylyl cyclase (Finidori-Lepicard et al. 1981, Sadler and Maller 1981). In a study by Lutz et al. (2000) the direct role of G protein in progestin mediated signalling was shown. Oocytes were injected with cRNAs encoding bovine $G\beta_1$ or $G\gamma_2$ subunits, which were tested to encode functional proteins; overexpressed $G\beta\gamma$ protein inhibited both progesterone-induced maturation and activation of the MAPK pathway, whereas sequestration of endogenous *Xenopus* $G\beta\gamma$ subunits enhanced progesterone-mediated signalling and maturation (Lutz et al. 2000). Plasma membrane GPCR has been hypothesized to be responsible for the effect.

Another example of progesterone modulation of GPCR signalling is the oxytoxin receptor: progesterone, which has been made unable to pass cell membrane, partially blocks oxytocin binding and oxytocin-mediated signalling (Grazzini et al. 1998). Thus it has been suggested that binding of PR to oxytoxin receptor prevents the induction of labor, while decreased progesterone levels allow oxytocin-mediated signalling and onset of labor (Grazzini et al. 1998).

2.6. G protein-coupled receptor 30

Many groups independently isolated GPCR30 from different tissues in the late 90s and confirmed its ubiquitous expression pattern. It is preferentially expressed in estrogen

receptor-positive breast cancer cells as well as in endocrine tissues; furthermore, it is also expressed e.g. in endothelial cells, lung, heart, lymphoid tissues/cells and in the central nervous system (Owman et al. 1996, Carmeci et al. 1997, Feng and Gregor 1997, Kvingedal and Smeland 1997, Takada et al. 1997). The gene was induced by fluid shear stress (Takada et al. 1997), but the regulation or function was otherwise unknown at the time this thesis was undertaken.

On the basis of amino acid sequence GPCR30 was identified as an orphan transmembrane receptor showing some degree of similarity to chemokine receptors of IL-8 and AngII. GPCR30 has features conserved in peptide-binding GPCRs: acidic residue (Asp105) in TM2, an AspArgTyr after TM3, a small third intracellular loop, and cysteine residues in the first and second extracellular loop. Although GPCR30 has a conserved DRY (Asp-Arg-Tyr) sequence, it lacks motifs typical to chemokine receptors: DRYLAIV and a cysteine residue close to TM1.

Some GPCRs are known to be involved in growth regulation, but the role of GPCR30 remains to be established. Rat GPCR41, which belongs to the same subfamily as GPCR30, has been shown to induce apoptosis through the p53 pathway (Kimura et al. 2001). Interestingly, the role of GPCR30 in steroid hormone-mediated signalling was suggested by a study in which GPCR30 transfection to ER-negative breast cancer cells was able to induce rapid ERK activation by estrogen, suggesting that GPCR30 mediates a rapid membrane effect of the hormone (Filardo et al. 2000). ERK-1/-2 activation was induced releasing HB-EGF, and subsequent transactivation of EGF receptor. The

attenuation of estrogen-induced ERK activation was mediated via a distinct mechanism, an inhibitory signal mediated by cAMP and PKA (Filardo et al. 2002).

3. MITOGEN-ACTIVATED PROTEIN KINASES

3.1. Mitogen-activated protein kinase pathway

The MAPK signal transduction pathway activation leads to various cellular effects in many types of cells. MAPK coordinates the activation of gene transcription, protein syntheses, cell cycle machinery, cell death and differentiation (Kyriakis and Avruch 2001). These pathways are activated by diverse receptor families, e.g. receptor tyrosine kinases, cytokine receptors and GPCRs, and by environmental stress.

The MAP kinase family has three main subgroups (English et al. 1999). 1) The p38 group consists of four isoformes. p38 MAPK has been shown to be activated by cellular stress. The p38 pathway has a role in cytokine biosynthesis, muscle differentiation and B cell proliferation. 2) The c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs) consist of ten splice variants, which are activated by UV, cytokines, environmental stress and to a lesser extent by growth factors. The p38 pathway is involved in cytokine biosynthesis, cell transformation and apoptosis. A role in proliferation and differentiation of thymocytes has also been described. 3) The third subgroup is ERK, which comprises ERK-1 and ERK-2, also referred to as p44 and p42 MAP kinases.

The ERK kinase cascade is formed by three major protein kinases, which phosphorylate each other in serial fashion: Raf, MAPK kinase (MEK) and ERK. The phosphorylation state of Raf is affected by many protein kinases, including Src, PKC, the p21-activated protein kinase (PAK), and Akt (Pearson et al. 2001). Raf activates MAPK kinase 1 (MEK-1) and MEK-2, which amplify the signal and phosphorylate tyrosine and threonine residues of ERK-1 and ERK-2. Activation of ERK requires phosphorylation and is on-off switched (Gutkind 1998). Both forms of ERKs are widely expressed in different cell types and tissues, but their relative amount in tissues varies (Pearson et al. 2001).

3.2. Regulation of cell growth by ERK

MAPK pathway activation enhances cell proliferation. Increased duration and magnitude of MAPK activation has been shown to enhance cell motility and *in vitro* invasion of breast cancer cells (Krueger et al. 2001). The Ras/ERK pathway exerts direct control on cell cycle progression. The activation of MAPK controls G1/S and G2/M transition points in the cycle. The activation of ERK affects the expression of AP-1 and ETS transcription factors which are able to bind in the cyclinD promoter, thus enhancing cyclinD1 expression and assembly of cyclinD1-dependent kinase complexes (Weng et al. 2001). ERK also inactivates the CDK inhibitor p21, allowing progress of the cell cycle. Additionally, the ERK pathway can up-regulate cyclin A and cyclin E mRNA synthesis, increase the assembly of cyclin A- and cyclin E-CDK2 kinase complexes and release E2F (Wilkinson and Millar 2000). Treatment with MEK inhibitor PD98059 has been shown to arrest cells at G2/M transition point in the cycle (Wilkinson and Millar 2000). The MAPK pathway can also affect the cell cycle by modulating protein synthesis.

Phosphorylation of translation initiation factor eLF-4E is ERK-dependent and leads to translation initiation (Waskiewicz et al. 1999).

In contrast, in many cell types prolonged Ras activation leads to cell cycle arrest or apoptosis (Wilkinson and Millar 2000). Inactivation of ERK is a more rare event than activation; inactivation of ERK has been shown to lead to growth inhibition, e.g. somatostatin and its synthetic analogue inhibit MAPK activity and growth (Cattaneo et al. 1996, Santore et al. 2002, Yoshitomi et al. 1997). Another GPCR ligand, calmodulin, is involved in down-regulation of the Ras/Raf/MEK/ERK pathway in fibroblasts (Bosch et al. 1998). The expression of Gα inhibited the ability of MCF-7 cells to form tumors in athymic mice, again by inhibiting the MAPK pathway (Chen et al. 1998). Additionally, adenovirus-directed expression of activated mutant Gα inhibited the growth of established tumors by inhibiting the MAPK pathway (Santore et al. 2002).

3.3. ERK and PR pathways in breast cancer cells

In breast cancer cells progestin R5020 has been shown to rapidly activate the Src/p21Ras/ERK pathway (Migliaccio et al. 1998), which is suggested to be responsible for the growth-stimulatory effect of progestin R5020 (Castoria et al. 1999). An interaction of the proline-rich motif in PR with SH3 domains of the c-Src family of tyrosine kinases has been shown to be critical for this MAPK activation independent of transcriptional activity (Boonyaratanakornkit et al. 2001). In the study in question progestin-induced growth inhibition was also seen to be dependent on this interaction. Boonyaratanakornkit et al. (2001) also argued against the study of Migliaccio et al.

(1998), who suggested progestin-mediated activation of the Src/p21Ras/Erk pathway requires PR-B association with ER. They also suggested that the function of ER was to down-regulate an elevated basal activity of Src, thus allowing PR to increase the activity.

The MAPK pathway regulates the activity of steroid hormone receptors. MAP kinase is able to phosphorylate PR at site, which signals its degradation through ubiqitinylation (Lange et al. 2000). Interestingly, this ligand-dependent down-regulation of PR is paradoxically coupled to transcriptional hyperactivity of the receptors (Shen et al. 2001). The MAPK pathway can also activate Xenopus PR after progesterone treatment (Bagowski et al. 2001).

AIMS OF THE STUDY

The issue of how progestin exerts its effects in mammary epithelial cells is topical due to recent studies, which suggest that the addition of progestin to HRT increases the risk of breast cancer. However, in cell culture studies progestin, in most cases, inhibits cell proliferation. The present work was undertaken to elucidate the mechanism of progestin-induced growth inhibition *in vitro* using immortalized mammary epithelial cells and breast cancer cells.

The specific aims were as follows:

1. To characterize progestin-regulated genes in MCF-7 breast cancer cells (I)
2. To study the role of GPCR30 in progestin-mediated growth inhibition (II)
3. To study the cell signalling pathways by which GPCR30 and progestin exert their effects (III, IV)
4. To establish whether GRCP30 affects steroid hormone-induced transcription (IV)

MATERIALS AND METHODS

1. HORMONES AND INHIBITORS

Steroid hormones 17β-estradiol, medroxyprogesteroneacetate (MPA), dexamethasone (DEX) and hydrocortisone were purchased from Sigma (St. Louis, MO, USA). Dihydrotestosterone (DHT) and progesterone were purchased from Merck (Darmstadt, Germany). Mifepristone (RU486) was a gift from Roussel Uclaf (Paris, France). Promegestone (R5020) was purchased from Schering Aktiengesellschaft (Berlin, Germany). Protein and RNA synthesis inhibitors cycloheximide and actinomycin D, respectively, were purchased from Sigma. Insulin was provided by Gibco BRL (Paisley, Scotland). SB202190 (SAPK2/p38 MAPK inhibitor) and MEK inhibitors: PD98059 and U0126 were obtained from Calbiochem (La Jolla, CA, USA).

2. CELL CULTURE

Breast cancer cell lines MCF-7, ZR-75-1, CAMA-1 and BT-474 cells were cultured in phenol red free Dulbecco's Modified Eagles Medium / nutrient mixture F-12 (DMEM/F12)(Gibco BRL) medium supplemented with 5% dextran-coated, charcoal-stripped fetal bovine serum (FBS)(Gibco BRL), penicillin 100 U/ml (Gibco BRL), streptomycin 100 μg/ml (Gibco BRL) and insulin (10 ng/ml or 1 μg/ml) with or without 1 nM 17β-estradiol.

Packaging cell line PT67 was cultured in DMEM medium (Gibco BRL) containing 10% FBS and penicillin/streptomycin. In NIH373 and COS culture medium FBS was replaced by dextran coated charcol stripped FBS (DCC-FBS). hTERT-HME1, a normal human mammary epithelium (HME) cell line stable-expressing the human telomerase reverse transcriptase subunit (hTERT), was obtained from Clontech (Palo Alto, CA, USA) and having undergone 135 population doublings by time of arrival. The cell line was maintained in Mammary Epithelial Basal Medium (MEBM) supplemented with 52 μg/ml bovine pituitary extract (Gibco BRL), 0.5 μg/ml hydrocortisone, 10 ng/ml human EGF (Gibco BRL), 5 μg/ml insulin, 50 μg/ml Gentamicin (Gibco BRL) and 50 ng/ml Amphotericin-B (Gibco BRL).

3. RNA ISOLATION

Ribonucleic acid (RNA) was isolated from cells using either TRIzolTM Reagent (Gibco BRL) or the RNAqueous^{TM} kit (Ambion, Austin, TE, USA) according to manufacturer`s instructions. Cells were harvested using trypsin and washed with PBS, and the cell pellet was mixed with 1 ml of TRIzol Reagent. Chloroform (0.2 ml) was added and the sample was separated into a two phases by centrifuging 12 000 g for 15 min. The aqueous phase, in which RNA remains, was mixed with 0.5 ml isopropanol. Precipitated RNA was pelleted by centrifuging 12 000 g for 10 min. The pellet was washed with ethanol, air-dried and dissolved in RNase-free water. Alternatively, cell pellet was mixed with 900 μl Lysis/Binding solution from the RNAqueous kit. Equal volume of 64% ethanol was added to the sample. The mixture was applied onto the RNAqueous filter and

microcentrifuged. The filter was washed with the Wash solutions (RNAqueous^{TM} kit). RNA was eluted two times by adding 60 μl elution solution and incubating the tube in a heat block set at 65ºC for 10 min. RNA was precipitated, air-dried and dissolved in Rnase-free water.

4. DIFFERENTIAL DISPLAY

In order to isolate progestin-regulated complementary deoxyribonucleic acid (cDNA) fragments from RNA samples of MCF-7 breast cancer cells, a differential display method was used. RNA was digested with Dnase I (Promega, Madison, WI, USA) for 1 h at 37 ºC, treated with phenol/chloroform and precipitated. RNAmap KIT (GenHunter, Nashville, TN, USA) was used according to manufacturer's instructions. The RNA (1 μg) was reverse-transcribed and amplified. Combinations of five up-stream primers and of four down-stream primers $T_{12}MN$ (N=T, A, C, G) were used in polymerase chain reactions (PCRs). [^{33}P] dATP (1000 Ci/mmol) (Amersham, Buckinghamshire, England) was used to label PCR products in PCR reactions. PCR products were run on 6% polyacrylamide sequencing gels for 6 h at 1000 V using Poker Face II (Hoefer Scientific Instruments, San Francisco, CA, USA). After autoradiography, differentially expressed products were extracted from the gel and reamplified as recommended in the RNAmap KIT. The reamplified PCR product was subcloned in a PCR-TRAP vector (GenHunter). The insert was sequenced using the Dye Terminator cycle sequencing Ready Reaction kit (ABI Prism), and the samples were processed on an automated 310 Genetic Analyser (ABI Prism).

5. NORTHERN BLOTTING

Total RNA (30-40 µg) was electrophoresed on a denaturing gel and blotted onto a nitrocellulose membrane (MSI, Westborough, MA, USA) using overnight capillary transfer in 10X SSPE (saline/sodium phosphate/EDTA). Hybridization between the membrane and radiolabeled probe was carried out for 16 h at 42 ^{o}C in the hybridization solution [6X SSPE / 5X Denhardt's solution / 50% deionized formamide / 0.1% sodium dodecyl sulphate (SDS) / 100 µg/ml denatured salmon sperm DNA]. As probe a randomly primed ^{32}P-labeled PCR product of GPR30 derived from differential display was used to detect endogenous GPR30 expression in MCF-7 cells. Alternatively, the probes used were two different *in vitro*-transcribed mRNAs when GPCR30 or GPCR30 antisense was detected in GPCR30 antisense-vector transfected cells. These two probes were made from the pBK/ cytomegalovirus (CMV)/ GPCR30 plasmid digested with restriction enzymes (SmaI or SalI, Promega) using MAXIscriptTM (Ambion) as described by the manufacturer. Sense GPCR30 was detected with the antisense probe, antisense GPCR30 with the sense probe. The filters were rehybridized with oligonucleotide complementary to 18S ribosomal RNA (Ambion) for normalization. The radiolabeled filters were exposed on X-ray film (Kodak BioMax). Quantification of signal density was accomplished using a Wallac densitometer (Turku, Finland).

6. QUANTITATIVE RT-PCR

RNA was extracted from breast cancer cells treated with different steroid hormones and

inhibitors. In order to measure the regulation of mRNA in these samples, one-step RT-PCR was performed. The LightCycler instrument (Roche, Mannheim, Germany), LightCycler RNA Master SYBR Green I kit (Roche) and specific primers for GPCR30, TIF2 or the house-keeping gene TATA binding protein (TBP) were used in the assay. Sequences of the primers are presented in articles (I, IV). RT-PCR consisted of reverse transcription, denaturation, 40 cycles of PCR and a melting curve analysis as described in articles I and IV. Concentration values for the studied gene were taken from the calibration curve, which was made from PCR reactions made from serial dilutions (500-100-20-4 ng) of the total RNA (x-axis). The relative concentration values (y-axis) were taken from the logarithmic phase of the amplification reaction of the studied gene. TBP control gene, which was not regulated by steroid hormones, was used for the normalization of RNA samples. This quantitative analysis of the LightCycler data was made employing LightCycler analysis software. The final results were expressed as N-fold differences in gene expression between untreated and treated samples normalized with housekeeping gene TBP expression level. Concentration values for the TBP were taken from the calibration curve made with TBP as mentioned.

7. CELL GROWTH ASSAYS

7.1. Relative cell growth

Cells were seeded in 96-well plates at a density of 1-3 $*10^3$ cells per well in the experimental medium, and were allowed to attach for one to two days. Thereafter, appropriate steroid hormones in 100% ethanol or vehicle (ethanol) were added, the final

ethanol concentration not exceeding 0.1%. Relative cell number was measured using the crystal violet method (Kueng et al. 1989). Cells were fixed, stained with crystal violet, dried and diluted with acetic acid. Absorbance was measured at a wavelength of 590 nm using a Victor 1420 Multilabel counter (Wallac).

7.2. Cell proliferation

Cells were placed on top of glass slides in 4-well or 8-well plates at a density of 10^5 and 10^4 cells per well, respectively. The cells were incubated with 20 μM bromodeoxyuridine (BrdU)(Sigma) for 2 h, then washed three times with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, and washed with PBS. If cells were pre-transfected with experimental plasmids, they were stained with 1 ml X-Gal staining solution [1 mg/ml 5-bromo-4-chloro-3-indolyl-B-D (Sigma), 10 μM $MgCl_2$, 3 mM $K_4Fe(CN)_6*3H_2O$, 3 mM $K_3Fe(CN)_6$] for 15 h after fixation to detect β-galactosidase activity in transfected MCF-7 cells.

After fixation the cells were permeabilized using acetone/methanol and DNA was denaturated (Simonson et al. 1995). This was accomplished incubating the cells in 2 M HCl for 1 h at 37 ^oC in order to expose incorporated BrdU residues for immunostaining. Cells were neutralized and equilibrated in PBS.

Cells were immunohistochemically stained using the primary antibody/peroxidase-conjugated secondary antibody/substrate method. In some cases Histostain-plus Bulk Kit (II, IV) was used according to instructions (Zymed Laboratories Inc., South San

Francisco, CA, USA). In the method cells were blocked with 10% normal horse serum for 20 min (I) or with the solution obtained from the Kit (II, IV). Monoclonal anti-BrdU (Sigma) (36 - 360 µg/ml) or anti-Ki-67 antibody (Boehringer-Mannheim, Mannheim, Germany) (0.2 µg/ml) was used as primary antibody. Cells were incubated with the antibody for 1 h in PBS containing 1% normal horse serum (I) or 0.5% BSA (Histostain kit)(II, IV) for 1 h. Peroxidase activity was measured using the diaminobenzidine (DAB) method (Ylikomi et al. 1992). In the transfection assay peroxidase activity was measured with the AEC+ Large Volume High Sensitivity Substrate-Chromogen System (Dako, Carpinteria, CA, USA).

7.3. Cell cycle analysis

MCF-7 cells (10^5) were plated in cell culture plates, harvested using trypsin, washed with PBS and suspended in a buffer [0.1% Triton-x 100, 0.1% Na-citrate, 50 µg/ml propidium iodide (Sigma) in Q-water]. In a buffer cell membranes were lysed and DNA was stained with propidium iodide. The cells were incubated overnight at 4^oC and thereafter filtered through a nylon mesh (30 µm). The samples were analyzed in a flow cytometer using a FACScand cell sorter (Becton Dickinson, Mountain View, CA, USA).

8. TRANSIENT TRANSFECTION

MCF-7 cells (10^5 or 10^6) were transfected using the commercial Lipofectamin 2000 method (Gibco BRL) as recommended by the manufacturer. LF2000 reagent (LF2000 1 µl/ plasmid 1 µg) was combined with the DNA mixture of pBk-CMV (2 or 20 µg) or

GPCR30/pBk-CMV (2 or 20 µg), and pCMVβGal (1 or 10 µg) in this assay. After 24 h DMEM/F12 medium was removed and the experimental medium added. Thereafter the cells were used for the proliferation assay.

A DNA mixture of pCMVβGal 50 ng, pBk-CMV (0.3 µg) or GPCR30/pBk-CMV (0.1, 0.3, 0.6 µg), glucocorticoid response element (GRE)-tk-luc (2.5 µg) or murine mammary tumor virus (MMTV)-tk-luc (2.5 µg) was used to transfect COS cells. A DNA mixture of pCMVβGal (50 ng), GRE-tk-luc (2.5 µg) or MMTV-tk-luc (2.5 µg) was used to transfect hTERT-HME cells. The mixture was incubated for 20 min and added to monolayers covered with DMEM/F12 medium. After 6-24 h the medium was removed and the experimental medium with 0.5 µg/ml hydrocortisone added. These cells were used for the luciferase reporter assay.

9. LUCIFERASE REPORTER ASSAY

After transfection the cells were incubated for 4 h with the culture medium, but without the presence of glucocorticoid. Forty-eight hours after glucocorticoid addition, luciferase activity was measured. Cell growth medium was removed and the cells were washed with PBS, covered with Lysis Reagent (Promega) for 15 min in the dark, and thereafter scarped with a plastic cell lifter. This lysate (10 µl) was mixed with The Assay Reagent (50 µl)(Promega) in the dark. The Light intensity was measured from the samples with 1450 Microbeta Plus Liquid Scintillation Counter (Wallac). The counter performed a 10 s measurement period for each reporter assay tube. The equal transfection efficiency was

confirmed by measuring β-galactosidase activity in heat-treated (10 min at 50^{o}C) lysates and using the β-Galactosidase Enzyme Assay system (Promega) and Victor 1420 Multilabel counter at 450 wavelength (Wallac).

10. RETROVIRAL GENE DELIVERY

10.1. Plasmid preparation

Three plasmids were established. GPCR-Br/GPR30 was kindly provided by Dr. D. Thompson (Carmeci et al. 1997). GPCR30 was cloned in the BamHI and NotI sites of the pLEGFP-N1 vector (Clontech) replacing enhanced green fluorescent protein (EGFP). To create a p-LN1 vector the above-mentioned restriction sites were filled using Klenow Fragment (MBI Fermentas, Hanover, MD, USA), and the product was blunt-ligated with itself using 300 U T4 ligase (MBI Fermentas). GPCR30 was fused to EGFP of the pLEGFP-N1 vector. GPCR30-forward 5`-TAATAAGTCGACGGGTCTCTTCCT-3` and GPCR30-reverse 5`-ATTATTGGATCCTACACGGCACTGC-3` primers were used for PCR in order to incorporate restriction sites and allow cloning of GPCR30 at SalI and BamHI sites of the pLEGFP-N1 vector.

10.2. Virus production

Viruses containing pLEGFP-N1, pLEGFP-N1/GPCR30, pL-N1 and pL-N1/GPCR30 vectors were established in a PT67 packaging cell line. A total of 5*10^{4} PT67 cells were transfected using 1 μl lipofectamine 2000 (Gibco BRL) and 1 μg plasmid for 24 h. After 36 h incubation with the growth medium, 800 μg/ml Geneticin (Sigma) was added for a

selection time of one week for pLEGFP-N1, pL-N1 and pL-N1/GPCR30 vectors. Six colonies were isolated from each plasmid, and strains showing the highest viral titer were selected for further studies. Viral titer was determined using NIH373 cells as recommended in the Retroviral Gene Transfer and Expression User Manual (Clontech). PT67 cells stable-expressing viruses were grown for four to five days in the medium, and the medium was passed through a 0.45 μm polysulfonic filter FP30/0.45 CA-S (Schleicher & Schull, Dassel, Germany) before storage at –80 ºC.

10.3. Cell infection

In preliminary studies optimal infection conditions were determined, and 13 viruses per cell were used to infect hTERT-HME1 cells. The cells were incubated with the viruses two separate times for 24 h, with 12-24 h between the infections. In some infections polybrene (Sigma) was added to reduce charge repulsion at a final concentration of 8 μg/ml. After 72 h incubation with the growth medium, 800 μg/ml Geneticin (Sigma) was added to select cells containing pLEGFP-N1, pLEGFP-N1/GPCR30, pL-N1 and pL-N1/GPCR30 vectors.

10.4. Measurement of infection efficiency

To measure EGFP production in infected cells, pLEGFP-N1/retrovirus-infected cells were plated on 96-well plates as in the cell growth assay. The medium was removed and 20 μl Cell Culture Lysis Reagent (Promega) was added to the wells. The plates were shaken for 15 min at 500 rpm. Fluorescence was measured using wavelengths 485 nm and 535 nm, and a Victor 1420 Multilabel counter (Wallac). A standard curve was made

in order to correlate absorbance with protein concentration using recombinant EGFP (Clontech).

11. CONSTRUCTION OF GPCR30 ANTISENSE EXPRESSING CELLS

11.1. Construction of a cell line expressing tetracycline-regulated transactivator

Our purpose was to create a cell line, which expresses a transfected gene (antisense GPCR30) under tetracycline regulation. First, a MCF-7 cell line (named PMCF-7) expressing constitutively tetracycline-regulated transactivator was established. Cells were transfected using liposomes (Teifel and Friedl 1995) and plasmids 1) pUHD15-1 which expressed the tetracycline-regulated transactivator (tTa) and 2) tetO-CMV, which coded for the puromycin resistance gene under the control of a tTa-responsive promoter allowing positive selection of a primary cell line functionally expressing tTa (Chambard and Pognonec 1998). Single clones were further characterized by transiently transfecting a pTRE (thyroid hormone response element)-Luc reporter. Luciferase activity was measured using transfected tetracycline-treated cells as control.

11.2. Introducing GPCR30 antisense expressing cells

The full-length cDNA clone of GPCR-Br/GPCR30 (Carmeci et al. 1997) was cloned in the antisense orientation into the NotI and BamHI sites of the pCEP4-TET vector. pCEP4-TET is a derivative of the episomal expression vector pCEP4 (Invitrogen, Groningen, Netherlands), in which the CMV immediate early enhancer/promoter has been replaced by the heptamerized Tet operator sequences upstream of a minimal CMV

promoter (Gossen and Bujard 1992). The PMCF-7 cell clone was stably-transfected with a pCEP4-TET vector or with pCEP4-TET containing GPCR30 in the antisense orientation. Two days after transfection 200 µg/ml Hygromycin (Sigma) was added to a select pool of PMCF-7 cells which contained the pCEP4-TET vector for a total of three weeks´ selection. Individual clones were not further separated.

12. WESTERN BLOTTING

12.1. Sample preparation

Cells were harvested using a cell scraper and cell number was calculated in a Burker cell chamber. To prepare total cell lysates, cell samples were mixed with sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β-mercaptoethanol, 0.05% bromophenolblue). The viscosity of the samples was reduced by drawing samples through a 23G needle.

Nuclear extracts were prepared from 2-10 $*10^6$ hTERT-HME cells as described elsewhere (Ahonen et al. 2000). In brief, cells were mixed with lysis buffer [20 mM Hepes, pH 8.0, 20 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT)] supplemented with protease inhibitor cocktail (Boehringer-Mannheim). After a 5 min incubation on ice, a nuclear pellet was obtained by centrifugation for 1 min at maximal speed in an Eppendorf centrifuge. Nuclear proteins were extracted by incubating the cells with nuclear buffer {20 mM Hepes (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 µM $MgCl_2$, 2 µM ethylenediamine tetraacetic acid (EDTA), pH 8.0} for 30 min on ice. Protein

concentrations were determined using BCA Protein Assay Reagent (Pierce, Rockford, Illinois, USA). Cell samples were mixed with sample buffer (250 mM Tris-HCl, PH 6.8, 40% glyserol, 8% SDS, 10% β-mercaptoethanol, 0.1% bromophenolblue) and boiled for 5 min.

12.2. Immunoblotting

Immunoblotting was carried out as previously described (Pasanen et al. 1998). Equal amounts of cells ($3*10^5$) for total cell lysates or proteins (196 μg) for nuclear extracts were resolved in 12% polyacrylamide gel, and transferred to a nitrocellulose membrane with an electrophoretic transfer apparatus.

The membranes were blocked by incubation in 50 mM Tris, 0.9% NaCl (TBS) containing non-fat milk for 1 h at room temperature. After blocking, the membranes were incubated overnight at 4 °C with GRIP-1 (F-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a final concentration of 1 μg/ml. Biotinylated anti-goat IgG (Vector Laboratories Inc., Burlingame, CA, USA) and Horseradish Peroxidase Avidin D (Vector Laboratories Inc.) were used as secondary antibodies. Alternatively, membranes were incubated with antibody Cyclin D1-HD11 (Santa Cruz Biotechnology) or β-actin (Sigma), and washed. Membranes were also incubated with phospho p44/p42 MAP Kinase Antibody (New England Biolabs, Beverly, MA, USA) or 44/42 MAP Kinase Antibody (New England Biolabs) and β-galactosidase Antibody. To detect the expression of nuclear receptors antibodies: glucocorticoid receptor (GR) P20 (Santa Cruz Biotechnology), NCL-L-PR (Novocastra) and NCL-ER-6F11 were used. As secondary

antibody peroxidase-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA, USA) was used for cyclin D1, phospho p44/p42, p44/p42, beta-galactosidase and GR. Peroxidase-conjugated goat anti-mouse IgG was used to detect PR, ER and β-actin. After washing, labeled proteins were detected by an enhanced chemiluminescense (ECL) method (Amersham).

13. MEASUREMENT OF ERK-1/-2 KINASE ACTIVITY

ERK-1/-2 activities from the MCF-7 cells were measured using a nonradioactive method and the p44/42 MAP Kinase Assay Kit according to manufacturer`s instructions (Cell Signaling, Beverly, MA, USA). Monolayers were washed with PBS and cells were harvested using a rubber policeman. Cells were mixed with the Lysis buffer (Cell Signaling). Cell density was adjusted to a final concentration of $1.6*10^6$ cells/ml. Cellular DNA was sheared using sonication. Lysates were centrifuged for 10 min at 12 000 g at 4 ^oC. The supernatant was collected and stored at –80 ^oC. ERK-1/-2 kinases were immunoprecipitated from $8*10^5$ MCF-7 cells with antibody against phospho-p44/42 MAP Kinase (Cell Signaling), which was immobilized by crosslinking to crosslinked agarose hydrazide beads. The supernatant (200 µl) was incubated with the antibody (15 µl) on a rocking platform at 4 ^oC for overnight. The sample was pelleted by microcentrifuging for 30 s. The pellet was washed with the Lysis buffer (Cell Signaling) and Kinase buffer (Cell Signaling). Precipitated kinases were incubated with a 2 µg Elk-1 fusion protein (Cell Signaling) and 200 µM adenosine triphosphate (ATP) (Cell Signaling) 30 min at 30 ^oC. The sample was boiled for 5 min and microsentrifuged for 2

min. In the sample Elk-1 phosphorylation (by precipitated ERK-1/-2) was detected by Western blotting using Phospho-Elk-1 (Ser 383) Antibody (Cell Signaling). Peroxidase-conjugated goat anti-rabbit IgG (Cappel) was used as secondary antibody. After washing, labeled proteins were detected by the ECL method (Amersham).

RESULTS

1. IDENTIFICATION OF GPCR30 AS A PROGESTIN TARGET GENE (I)

Our intent was to identify novel progestin-regulated genes which would be involved in progestin-induced growth regulation in breast cancer cells. To study the effect of progestin on gene regulation, we cultured MCF-7 breast cancer cells with estrogen and subsequently treated the cells with progestin, MPA. RNA was isolated for PCR-based differential display analysis 24 h and 48 h after progestin addition, the time-points being chosen in view of the established growth-inhibitory response by progestin at 24 h and 48 h. Twenty different primer combinations were used for PCR analysis. Resulting cDNA fragments were run in the gel, and differentially expressed products were isolated. The regulation of the expression of cDNA fragments by MPA was confirmed by Dot blot analysis. Six of the fragments were found to be regulated by MPA, and these were cloned to the vector and sequenced. We used BLAST search for the analysis of nuclear sequences. Two of the transcripts showed 96 - 100% identity to GPCR30 and there was no significant similarity to any other genes. These two transcripts of GPCR30 were expressed in MPA-treated cells, but not in the control cells at either 24 or 48 h in differential display analysis.

To study GPCR30 mRNA regulation by progestin and other steroid hormones, we extracted RNA from different breast cancer cell lines and used RNA for Northern blot and reverse transcription (RT)-PCR analysis. GPCR30 mRNA was specifically

upregulated by progestins (MPA, R5020) and progesterone three- to six-fold, but not by anti-progestin, dihydrotestosterone or dexamethasone in MCF-7 cells. The specificity was confirmed by the observation that anti-progestin RU486 abrogated MPA-induced GPCR30 up-regulation, and GPCR30 was upregulated in a concentration-dependent manner. GPCR30 was expressed relatively late (between 8 and 18 hours). Protein synthesis inhibitor (cycloheximide) did not abrogate GPCR30 mRNA expression, whereas RNA synthesis inhibitor (actinomycin D) did. It can be hypothesize that progestin either enhances GPCR30 transcription or affects GPCR30 mRNA stability. GPCR30 mRNA was also upregulated by MPA in other breast cancer cell lines (ZR-75-1, CAMA-1) 1.5 to 4.5-fold.

Because 1) GPCR30 mRNA was upregulated relatively late (between 8 and 18 hours), 2) protein synthesis inhibitor did not abrogate GPCR30 expression and 3) MAPK pathway has been shown to regulate the activity of PR (Lange et al. 2000, Shen et al. 2001), it was sought to establish whether MAPK activation mediates progestin-induced GPCR30 up-regulation. When cells were treated with two different inhibitors of MEK activity (PD980159, U0126), these did not affect GPCR30 expression as studied by quantitative RT-PCR analysis. Interestingly, the inhibitor of p38 MAPK activity stimulated GPCR30 mRNA expression and progestin had no additional effect on the mRNA regulation in MCF-7 cells, which would suggest that the p38 MAPK pathway regulates GPCR30 expression.

2. THE EFFECT OF GPCR30 ON GROWTH

2.1. Correlation of GPCR30 expression with growth (I)

We first used correlation analysis to study whether GPCR30 expression is associated with growth, comparing cell growth level and GPCR30 mRNA expression after progestin treatment. The dose- and time-dependent increase in GPR30 mRNA expression correlated with progestin-induced growth inhibition in MCF-7 breast cancer cells. When progestin failed to inhibit growth (cells were grown without the presence of estrogen), GPR30 expression was down-regulated. Indeed, progestin enhanced growth by 16% - 34% between 72 and 120 h without estrogen. This effect was associated with GPCR30 down-regulation by 8%-70% between 12 and 48 h. When cells were cultured with anti-androgen (flutamide) or anti-progestin (RU486), the growth stimulatory effect of MPA was partly diminished, suggesting that the growth-stimulatory effect is mediated through AR and PR. Additionally, the magnitude of GPCR30 expression analyzed with RT-PCR correlated with progestin-induced growth inhibition when comparison was made between different breast cancer cell lines: CAMA-1, MCF-7, ZR-75-1 and BT-474.

2.2. Transient expression of GPCR30 in breast cancer cells (II)

In MCF-7 cells the effect of GPCR30 on growth was studied using a transient transfection assay. We transiently transfected GPCR30 with reporter plasmid to cells, and analyzed cell proliferation using BrdU and immunohistochemistry. GPCR30 expression inhibited estrogen-stimulated cell proliferation by 44% - 39% between 6 and 30 h. To ascertain whether the effect of GPCR30 on growth is dependent on estrogen, we cultured

cells without estrogen. Interestingly, GPCR30 expression inhibited cell proliferation even more without estrogen, by 55% - 86% between 0 and 30 h.

2.3. Stable expression of GPCR30 in immortalized mammary epithelial cells (IV)

In order to study the effect of GPCR30 on the growth of immortalized mammary epithelial cells, we established a HME cell line which stable-expressed GPCR30 by using retrovirus-mediated gene delivery. GPCR30 was expressed at a high level in an immortalized HME cells as verified by RT-PCR analysis. As control we used cells infected with empty vector. In order to confirm that the effect of GPCR30 on growth was due to GPCR30 protein expression, we measured the effects of GPCR30 fused with enhanced green fluorescent protein (EGFP). As control we used HME cells infected with the plasmid expressing EGFP or parental HME cells. EGFP and GPCR30-EGFP expression was detected in the experimental cells at a high level.

When growth level was compared between GPCR30-expressing and control cells (infected with empty vector), relative cell growth was inhibited by 23-34% between 48 – 168 h analysed using crystal violet staining. The effect was more marked when we measured cell proliferation using BrdU incorporation analysis. Before the BrdU experiment the cells were allowed to attach, for one to two days, the medium was changed and 0 h time point measured. This analysis revealed that proliferation was inhibited by GPCR30 by 60-87% between 0 and 72 h. Corresponding results were also obtained from KI-67 staining. Prior to the Ki-67 assay HME cells were arrested at the G0/G1 phase by growth factor deprivation (MEMB medium present without

supplements) for 24 h. Ki-67 staining revealed that GPCR30 reduced the number of cells in the cell cycle by 40% at 2 h, further increasing to 53% at 12 h. In a similar manner relative cell growth was inhibited when HME cells were transfected with EGFP-conjugated GPCR30. As control cells were used HME cells expressing EGFP.

2.4. Antisense GPCR30 expression in breast cancer cells (II)

To further characterize the effect of GPCR30 on growth, we established a PMCF-7 cell line which expressed GPCR30 antisense under regulation of tetracycline. PMCF-7 cells were tested with luc-reporter plasmid (before transfection of GPCR30 antisense expression plasmid) in order to confirm the ability of cells to regulate gene expression. PMCF-7 cells showed a 50-fold increase in luciferase activity when comparison was made with tetracycline-treated control cells.

The GPCR30 antisense construct was transfected in PMCF-7 cells and the expression of GPCR30 antisense was confirmed by Northern blotting. In control cells tetracycline-treatment down-regulated GPCR30 antisense expression as verified by Northern blotting. It also emerged that GPCR30 antisense was able to prevent MPA-induced GPCR30 mRNA up-regulation.

GPCR30 antisense increased the number of MCF-7 cells as analyzed using crystal violet staining. This further confirmed the growth-inhibitory property of GPCR30. GPCR30 antisense increased cell proliferation measured using BrdU incorporation and immunohistochemistry. When GPCR30 expression was diminished, the entry of cells into

the cycle was accelerated as analyzed using Ki-67 staining and immunohistochemistry. These results would suggest that GPCR30 increases the number of cells in the G0/G1 phase. As control we established that tetracycline treatment did not markedly inhibit growth of PMCF-7 cells transfected with empty vector.

3. GPCR30 AND PROGESTIN-INDUCED GROWTH INHIBITION (II)

GPCR30 antisense cells were used to study the role of GPCR30 in steroid-mediated growth inhibition. The cells were treated with tetracycline to decrease GPCR30 antisense expression, and these cells were used as control. We first characterized the effect of MPA on cell growth in control tetracycline-treated cells. Progestin enhanced the first cell cycle as expected. At later time points (120-144 h) MPA reduced cell growth by 40% - 43% and proliferation by 31%-41% between 48 and 96 h as measured by crystal violet staining and BrdU incorporation assay, respectively. The cell cycle was analyzed using flowcytometry. Progestin arrested 25% of cells in the G0/G1 phase, reduced the number of cells in the S-phase, but did not affect the G2 phase. Additionally, when the regulation of different cell cycle molecules was analyzed, MPA was seen to reduce the expression of cyclin D1.

It was next sought to establish whether progestin-induced growth effects were compromised in cells where GPCR30 up-regulation by progestin was diminished. Indeed, in GPCR30 antisense cells progestin-induced growth inhibition was seen to be diminished. Specifically, progestin-induced (a) reduction in cell number (b) reduction in

cell proliferation (c) G1-phase arrest, and (d) down-regulation of cyclin D1 were diminished. In these cells MPA was able to inhibit growth by 14%-8% between 120 and 144 hours, cell proliferation by 9% and 4% between 48 and 96 h, and the amount of cells in the G0/G1 phase by 8%.

As noted, MPA enhanced cell growth without the presence of estrogen. The growth-stimulatory effect of the steroid hormones estrogen and MPA was independent of GPCR30 expression. Similar growth enhancement was detected in both GPCR30 antisense and tetracycline-treated control cells when cells were treated with estrogen or with MPA (cells were grown without estrogen).

4. ERK-1/-2 ACTIVATION BY PROGESTIN (III)

We first cultured MCF-7 cells with different small-molecule inhibitors and stimulators in order to bring out factors which have a synergistic effect on growth with respect to progestin-induced growth inhibition. MPA proved unable to provide any additional growth-inhibitory effect when cells were grown in the presence of MEK inhibitors. This suggested that the ERK pathway might cross-talk with progestin signalling. We therefore characterized the effect of progestin on ERK-1/-2 activity. We treated MCF-7 cells with MPA and extracted cell lysates between 0 – 24 h for Western blotting and MAP kinase assay. Interestingly, MPA reduced ERK-1/-2 phosphorylation at 22 and 23 h, as measured by Western blotting. We also immunoprecipitated phosphorylated ERK-1/-2 proteins from MCF-7 cell samples and allowed these to phosphorylate Elk-1 substrate *in*

vitro. Result showed that MPA reduced ERK-1/-2 activity at 22 and 23 h. To study whether the regulation of ERK activity was mediated through PR, we cultured MCF-7 cells with MPA and antiprogestin RU486. RU486 abrogated progestin-induced ERK-1/-2 inactivation, which would indicate PR-mediated regulation of ERK activity.

5. ERK-1/-2 ACTIVATION BY GPCR30 (III)

We cultured GPCR30 antisense cells and control cells with two different MEK inhibitors (PD98059, U0126) and with inhibitors affecting other cell signalling pathways such as PI3-K, protein kinase C, Ca^{2+} and the p38-pathway. In tetracycline-treated control cells all factors tested inhibited growth. When GPCR30 antisense was expressed at a high level, cells were able to resist the growth inhibitory effect of MEK inhibitors, but not that of the other factors tested. This suggested convergence of progestin, GPCR30 and ERK pathway in growth regulation.

To study whether ERK activities are increased in GPCR30 antisense cells, we used immunoblotting and specific antibodies against phosphorylated and non-phosphorylated ERK-1/-2. The phosphorylation of ERK-1/-2 was increased in GPCR30 antisense cells. Treatment of the cells with 1-100 nM MPA reduced ERK activities in equal amount, and when GPCR30 antisense was expressed, this effect was abrogated.

To study the effect of GPCR30 on ERK activity, we co-transfected GPCR30 with β-galactosidase expression plasmid to MCF-7 cells. Transient expression of GPCR30

briefly enhanced and thereafter reduced ERK-1/-2 activities as measured by kinase assay. Transfection efficiencies were controlled by measuring the amount of co-transfected β-galactosidase protein using Western blotting.

6. REGULATION OF GLUCOCORTICOID RECEPTOR ACTIVITY BY GPCR30 (IV)

6.1. Regulation of cofactors

It was next sought to establish whether GPCR30 affects steroid-mediated signalling through steroid hormone receptors or directly affects the cell cycle machinery. In immortalized HME cells GR was expressed as analyzed by immunoblotting. PR or ERα receptors were not expressed in these cells as analyzed by immunoblotting. GPR30 did not affect GR mRNA expression in our preliminary studies, which would imply that GPCR30 does not affect growth by regulating GR expression. However, an effect of GPCR30 on GR activity was not ruled out. We therefore characterized the effect of GPCR30 on cofactor expression level. Interestingly, in GPCR30-expressing cells TIF2 expression was down-regulated as analyzed using RT-PCR analysis and two different primer pairs. In these cells TIF2 expression was also down-regulated at protein level as shown using imunoblotting and nuclear extracts of HME cells. To confirm the result we measured TIF2 regulation in cells expressing fluorescein-conjugated GPCR30. Here TIF2 expression was diminished as established using immunoblotting.

6.2. Regulation of transcriptional activity of glucocorticoid receptor

We next studied whether GPCR30 affects transcriptional activation of GR. We measured the activity of the GR response element (MMTV) in GPCR30-expressing and control HME cells. In control cells hydrocortisone treatment enhanced MMTV activity. Interestingly, GPCR30 abrogated the effect of hydrocortisone on steroid response element activity, but did not affect the reporter gene activity by itself.

To confirm that the effect of GPCR30 is mediated through transcriptional activation of GR, we used sex steroid receptor-negative COS cells, two different GR response elements (GRE, MMTV) and expression vector GRα in transient transfection assays. In COS cells dexamethasone treatment clearly increased the transcriptional activity of GRE and MMTV. GPCR30 had no effect on transcriptional activation by itself. Notably, when cells were transfected with GPCR30 and subsequently treated with dexamethasone, the luciferase activity was almost abrogated.

To establish whether the effect of GPCR30 on MMTV activity was concentration-dependent, we transfected COS cells with increasing amounts of GPCR30 plasmid. The increase in GPCR30 plasmid in the transfection mixture inhibited luciferase activity in a concentration-dependent manner.

DISCUSSION

1. REGULATION OF GENE EXPRESSION BY PROGESTIN

The question of how progesterone affects mammary gland growth remains controversial despite extensive studies in the field. To gain an insight into the mechanism whereby progestin inhibits growth, the target genes of progestin have been characterized in breast cancer cell lines. Previous studies have mainly focused on the analysis of cell cycle-regulating molecules (Groshong et al. 1997, Musgrove et al. 1998), growth factors (Musgrove et al. 1991, Goldfine et al. 1992, Kalkhoven et al. 1995, Kurebayashi et al. 1999, Gregoraszczuk et al. 2001) and oncogenes (Musgrove et al. 1991, Alkhalaf and Murphy 1992). Progestin has been shown to enhance the expression of growth-stimulatory factors or to reduce the expression of growth-inhibitory factors. New techniques have provided more extensive possibilities to study gene expression patterns. We used the differential display technique to reveal novel progestin target genes. Our purpose was to identify progestin-regulated genes which would be important in the growth inhibition process in breast cancer cells.

Since GPCR30 is expressed preferentially in endocrine tissues (Owman et al. 1996, Carmeci et al. 1997, Feng and Gregor 1997), we selected this gene for further studies. We identified GPCR30 as a progestin-specific target gene; it was upregulated by progestins and progesterone, but not by other steroid hormones. Other studies have also suggested progestin specificity in a subset of target gene regulation (Hamilton et al. 1997, Hyder et

al. 2001, Wan and Nordeen 2002) despite the homologous steroid response element-binding domain of PR and GR. The regulation of GPCR30 by steroid hormones has not previously been established. On the other hand, it has been shown that GPCR30 is upregulated by fluid-induced stress (Takada et al. 1997).

Progestin-induced genes involved in membrane-initiated signalling have also been discovered in other studies. Richer et al. (2001) analyzed the gene profiles of PRA- and PRB-positive T47-D breast cancer cells. They showed that half of the progesterone-regulated genes were involved in membrane-initiated signalling, and concluded that the major role of progestin is to regulate cell metabolism. However, it is also possible that membrane-initiated signalling is involved in cell growth regulation.

Based on the findings that GPCR30 was expessed relatively late, but that induction did not require protein synthesis, it could be hypothesized that progestin affect the stability of GPCR30 mRNA. This kind of gene regulation by progestin has not been demonstrated. Most of the progestin-regulated genes reported are regulated shortly after progestin administration. However, the regulation of mRNA stablility has been characterized in the case of other factors such as estrogen-induced vitellogenin mRNA (Nielsen and Shapiro 1990).

2. GROWTH REGULATION BY GPCRs

Apart from other functions G protein-coupled receptors make a major contribution to growth regulation (Weber et al. 1994, Gutkind 1998a, Rohrer and Kobilka 1998, Xoriuchi et al. 1999, Ferjoux et al. 2000, Xu et al. 2000, Darmoul et al. 2001, Montaner et al. 2001, Gazvani et al. 2002). Most of the GPCRs described enhance growth. However, growth-inhibitory effects of GPCRs have also been established. To answer the question whether GPCR30 is involved in growth regulation, we here studied the effect of the gene on the growth of breast cancer cells and immortalized human mammary epithelial cells. GPCR30 was seen to inhibit proliferation of these cells. Additionally, when GPCR30 expression was diminished by antisense, growth of MCF-7 cells was stimulated.

A role for GPCR30 family members in the growth regulation process has also been supported by the finding that GPCR41, which shows a high degree of similarity to GPCR30, diminished the growth of cancer cells by inducing apoptosis (Kimura et al. 2001). We showed that GPCR30 increased the number of cells in the G0/G1 phase and reduced the number in the S phase. Our result is in line with the finding that many growth-inhibitory GPCRs induce cell growth inhibition by arresting cells in the G0/G1 phase or inducing apoptosis (Ferjoux et al. 2000, Kimura et al. 2001). The result reported here constitutes further evidence that the receptors of the GPCR family inhibit cell proliferation by arresting cells in the G0/G1 phase.

GPCR30 inhibited cell growth as analyzed by decrease in cell number. The effect was relatively small when comparison was made to cell proliferation. The finding is in agreement with previous reports on the effect that mutant-activated G proteins clearly inhibit cell proliferation, but do not affect cell growth *in vitro*. Interestingly, they have a major effect on tumor cell growth *in vivo* (Chen et al. 1998, Santore et al. 2002). Our result thus provides further evidence that the role of G proteins and GPCRs is to regulate cell proliferation. The growth-inhibitory response of GPCR30 was independent of the presence of steroid hormones, but it was not ruled out that GPCR30 modulates steroid receptor activity resulting in growth inhibition.

3. MECHANISM OF PROGESTIN-INDUCED GROWTH INHIBITION

As discussed at the molecular level the mechanim whereby progestin affects growth is well established in breast cancer cells. Progestin regulates growth and cell cycle molecules in a biphasic way. After brief growth enhancement, progesterone inhibits cell proliferation by arresting cells at the G0/G1 phase (Musgrove et al. 1993, Groshong et al. 1997). The growth-inhibitory effects of progesterone and progestin R5020 are associated with a change in the activity of CDKs (Groshong et al. 1997, Musgrove et al. 1998). The decreased CDK activity is accompanied by down-regulation of cyclin D1, D3, B and A and up-regulation of the CDK inhibitors p21 and p27. It has been proposed that progestin drives cells to a decision point at the G1/S boundary and thereafter induces cellular changes which determine their ultimate response. In this context it was reasonable to investigate whether GPCR30 makes a contribution to the ultimate response of breast

cancer cells after progestin treatment. It emerged that GRCP30 expression indeed correlated with progestin-induced growth inhibition. Time- and dose-dependent increases in GPCR30 mRNA expression correlated with progestin-induced growth inhibition in MCF-7 cells. Additionally, when different breast cancer cell lines were treated with progestin, GPCR30 expression level and growth-inhibitory response correlated. In the present study progestin inhibited only estrogen-induced growth in MCF-7 breast cancer cells. In contrast, progestin stimulated growth without estrogen in MCF-7 cells. This growth stimulation without estrogen was accompanied by down-regulation of GPCR30 expression. Similar evidence regarding the association of GPCR30 with progestin-induced growth inhibition came from the antisense studies. GPCR30 antisense clearly affected the ability of progestin to inhibit growth. In fact, numbers of progestin-induced effects were almost abrogated, for example cyclin D1 down-regulation, G1 phase arrest and inhibition of cell proliferation. Altogether the results suggest that GPCR30 is involved in the growth-inhibitory effect of progestin in MCF-7 breast cancer cells. It is tempting to speculate that GPCR30 might constitute one of the progestin-induced factors which determine ultimate response of cells such as down-regulation of cyclin D1.

The role of G proteins in progestin-mediated development of xenopus oocytes has been characterized (Finidori-Lepicard et al. 1981, Sadler and Maller 1981). However, there is no previous study to show the role of any GPCRs in progestin-induced growth inhibition. Interestingly, the role of membrane-initiated signalling, particularly tyrosine kinase activation, in progestin-mediated growth inhibition has been shown to be critical (Boonyaratanakornkit et al. 2001). Tyrosine kinase activation has also been shown to be

modulated by GPCR30 (Filardo et al. 2000). Additionally, a study by Richer et al. (2001) established that half of the progesterone-regulated genes were involved in membrane-initiated events, which indicates an important role of molecules like GPCR30 in progestin signalling. GPCR30 has also been shown to be involved in estrogen-mediated signalling (Filardo et al. 2000, Filardo et al. 2002), implying the important role of membrane-initiated signalling in steroid hormone-mediated response as does the present result.

The expression of GPCR30 does not explain all the effects of progestin on cell growth, because it mainly reduces cell proliferation but does not substantially inhibit cell number. The reason for this contradictory finding is unknown. Cell number is affected by apoptosis and proliferation. It can be speculated that GPCR30 might diminish the rate of apoptosis and thus the effect on cell proliferation is so minor. It is also possible that GPCR30 and progestin signalling cross-talk, and thus removal of GPCR30 would markedly interfere with progestin signalling, and GPCR30 would not be the major mediator of progestin in cell growth. However, the results here imply that GPCR30 makes an important contribution to growth regulation induced by progestin. Our results further suggest an important role of membrane-initiated signalling in progestin-induced growth inhibition.

4. THE REGULATION OF MAPK ACTIVITY

4.1. Steroid hormones and MAPK activation

Progestin R5020 (Migliaccio et al. 1998) and estrogen (Endoh et al. 1997) has been shown to rapidly phosphorylate ERK-1/-2 in breast cancer cells. Further to this we also showed an early activation of MAPK induced by progestin in MCF-7 breast cancer cells. MAPK activation does not require protein synthesis, and it has therefore been hypothesized that the effect of steroid hormones on the MAPK pathway is dependent on an unknown membrane receptor. Filardo et al. (2000) suggested that estrogen-induced MAPK activation requires GPCR30 expression. GPCR30 has also been shown to restore the ablility of estrogen to activate MAPK signalling (Filardo et al. 2002).

Castoria et al. (1999) suggested that rapid MAPK activation by progestin R5020 and estrogen is responsible for the growth-stimulatory effects of these hormones. However, Caristi et al. (2001) report that MAPK activation by estrogen is not responsible for the growth-stimulatory effect of estrogen. This latter finding is indirectly supported by our own result. Although GPCR30 is critical for estrogen-induced MAPK activation (Filardo et al. 2000), it was not indispensable in estrogen-induced growth stimulation. These results would thus suggest that MAPK activation might be not critical for estrogen-stimulated growth. Our data further imply that GPCR30 is not essential for steroid hormone-induced growth stimulation. Progestin-induced growth was independent of GPCR30 expression, but the direct role of MAPK activation in progestin-stimulated growth was not studied in the present series. The above-mentioned study by Filardo et al.

(2000), however, implies that GPCR30 expression is not needed for progestin-induced MAPK activation.

4.2. Progestin inhibits MAPK activity

The role of the MAPK pathway in progestin-induced growth inhibition has been less fully studied. There is one previous work in which the role of this pathway in progestin-induced growth inhibition has been established. Boonyaratanakornkit et al. (2001) showed that growth inhibitory response, MAPK and tyrosine kinase activation induced by progestin were diminshed in MCF-7 cells when the proline-rich motif of PR was mutated. This would suggest that these pathways cross-talk and the MAPK pathway may be responsible for progestin-induced growth inhibition. However, it is commonly accepted that MAPK activation stimulates growth. We investigated whether MAPK inactivation is important in progestin signalling. The synergy between MAPK pathway inhibition and progestin was suggested by the observation that progestin provided no additional growth-inhibitory response to cells treated with MAPK pathway inhibitors. More important, progestin inhibited MAPK activity near the time point at which progestin-induced growth inhibition was established. This MAPK inactivation was abolished by anti-progestin, implying that progestin inactivates MAPK in a PR-dependent manner.

There is no previous study to show that progestin inhibits MAPK activity 20 h after progestin administration. The main reason for this could be that at this time point changes in MAPK activity are interfered with by background changes in MAPK activity, also

brought out in our studies. MAPK inactivation by progestin was repeatable, and progestin up-regulated GPCR30, which inhibited MAPK activity. It was thus reasonable to conclude that progestin inhibits MAPK activity 22 –24 h after progestin administration. Whether or not progestin-induced MAPK inactivation is the main cause of growth inhibition remains to be shown. It is also possible that MAPK activation by progestin at 18 h, or some other pathway, is responsible for the effect. Further studies are required to reveal the role of the MAPK pathway in progestin signalling. Our result further underlines the critical role of the MAPK pathway in progestin-induced growth inhibition.

4.3. GPCR30 inhibits MAPK activity

GPCRs affect the cell cycle through different G-proteins (Gs, Gi, Gq) and cell signalling pathways. In most cases the activation of GPCR leads to MAPK activation. Indeed, Gs-linked receptors activate or in some cases inhibit MAPK pathways through PKA pathway, Gi-linked receptors activate tyrosine kinases and further the MAPK pathway, and Gq-dependent activation uses different cell signalling pathways such as PLC and PKC (Gutkind 1998b). Filardo et al. (2000) studied combined effects of GPR30 and estrogen on MAPK activity. They showed that estrogen was able to stimulate the MAPK pathway when GPCR30 was expressed in the cells. We on the other hand studied the independent role of GPCR30 on MAPK activity, showing that GPCR30 inhibited and GPCR30 antisense stimulated it. The discrepancy with the results arrived at by Filardo et al. (2000) could be explained by the fact that they studied earlier time points, and the independent role of GPCR30 was not studied in their experiments. We were also able to show MAPK activation prior to MAPK inactivation.

We established that MAPK inactivation by GPCR30 was associated with growth inhibition, while MAPK activation by GPCR30 antisense was associated with growth stimulation. Additionally, when GPCR30 expression was diminished by the antisense, the growth-inhibitory response of MEK inhibitors was prevented, suggesting that the MAPK pathway is critical for GPCR30-induced growth. However, more detailed studies need to be carried out before any causative conclusion as to MAPK inactivation and growth can be drawn. It is not known whether MAPK inactivation by GPCR30 is the cause or result of growth inhibition. Nor is it ruled out that MAPK activation (not inhibition) by GPCR30 is responsible for the growth-inhibitory effect. The result here in any case provides further evidence that GPCR30 regulates MAPK activity and is also in line with previous findings that MAPK inactivation is the main pathway by which different GPCRs induce growth inhibition (Cattaneo et al. 1996, Yoshitomi et al. 1997, Santore et al. 2002).

The observation that p38 MAPK pathway inhibitors were able to induce GPCR30 expression provides further evidence that the MAPK pathway is important in GPCR30 signalling. It also suggests a novel mechanism by which the p38 pathway and ERK signalling cross-talk. Since p38 MAPK is activated by cellular stress (English et al. 1999), the result further confirms the role of this pathway in the regulation of GPCR30 expression.

4.4. Progestin regulates MAPK through GPCR30

We studied the relationship of progestin and MAPK signalling pathways in GPCR30 antisense cells. In particular we were interested to establish whether progestin-induced MAPK inactivation is mediated through GPCR30. We showed that progestin-induced ERK inactivation is abrogated in GPCR30 antisense cells. This would suggest that GPCR30 does indeed mediate the effect of progestin on MAPK activity. Furthermore, the synergy between GPCR30, MAPK and progestin was suggested by the finding that GPCR30 antisense cells were able to resist the growth inhibitory effect of MEK inhibitors and progestin. In conclusion, we suggest that progestin upregulates GPCR30, this resulting in MAPK inactivation.

The mechanisms whereby MAPK inhibition might affect cell proliferation are numerous. It is possible that the regulation of MAPK activity leads to changes in cell-cycle regulating molecules (Weinberg 1995). Alternatively, MAPK activation can modulate the activity of steroid hormone receptors, e.g. through phosphorylation (Joel et al. 1998, Lange et al. 2000).

A major problem throughout the studies was the lack of working antibody. Attempts were made to obtain the antibody of GPCR30: we tried to establish the antibody and ask other researchers for antibody. Thus we were not actually able to show whether the GPCR30 protein level changed after progestin treatment or by GPCR30 antisense, although it emerged that the GPCR30 mRNA level changed. However, the finding that GPCR30 antisense had an effect on MAPK activity suggested that the antisense model worked. We

also showed in non-neoplastic breast epithelial cells that GPCR30 fusion protein was expressed in the cells and inhibited growth.

5. REGULATION OF GLUCOCORTICOID RECEPTOR ACTIVITY BY GPCR30

5.1. Regulation of the function of cofactors

The function of cofactors is modulated by a number of posttranslational mechanisms. The half-life of the cofactors is regulated by acetylation (Chen et al. 1999) and ubiquitination (Lonard et al. 2000). Compartmentalization or activity of coregulators can be modified by phosphorylation (Hong and Privalsky 2000). There is a growing body of evidence that coregulators are prime targets for control by kinase-mediated cellular signalling pathways (McKenna and O'Malley 2002). Thus far there are only a few examples of the regulation of cofactor expression. Steroid hormones regulate the expression of cofactor SRC-1 in rat tissues (Misiti et al. 1998, Kurihara et al. 2002). Additionally, regulation of cofactors has been suggested in cancer cell lines (Li and Chen 1998, Misiti et al. 1998). In this present study we characterized the effect of GPCR30 on the regulation of cofactors. We showed that GPCR30 down-regulated the expression of cofactor TIF2. The activation of cytoplasmic PKA has been shown to diminish TIF2 expression (Borud et al. 2002). Interestingly, GPCR30 has been shown to regulate PKA activity (Filardo et al. 2002). Thus a potential pathway by which GPCR30 regulates TIF2 expression is PKA activation. It is also possible that the regulation of MAPK activity contributes to the

phenomena in question. Our result provides further evidence that cofactors are regulated, interestingly, by membrane receptors.

5.2. Regulation of glucocorticoid receptor activity

Ligand binding is the main inducer of steroid hormone-induced transcription. The activation of transcription can be modulated in a number of ways. The activity of receptors can be modulated by effects on their expression level (Graham et al. 1995, Clayton et al. 1997, Vienonen et al. 2002) or through a posttranslational mechanism such as phosphorylation by the MAPK pathway (Kato et al. 1995, Joel et al. 1998, Lange et al. 2000). Alternatively, changes in the activity of cofactors which steroid receptors recruit to the promoter, result in dramatic changes in receptor-mediated transcription (Kamei et al. 1996, Voegel et al. 1996). We further investigated whether GPCR30 expression would interfere with steroid receptor activity, as suggested by the finding that GPCR30 diminished TIF2 expression. In HME cells, which expressed GR and were infected with GPCR30 encoding virus, hydrocortisone-induced transcriptional activation of the glucocortiocoid response element was prevented. The role of GR was confirmed in COS cells using two different glucocorticoid response elements and the GRα expression vector. In the cells the glucocorticoid-stimulated activity was abolished in a concentration-dependent manner by GPCR30 expression.

The role of membrane-initiated signalling in the transcriptional activation of steroid receptors has previously been described. GPCR has been shown to stimulate the activity of GR (Schmidt et al. 2001), and the cytoplasmic guanosine triphosphatase (GTPase)

regulator Rho activates a cofactor, leading to the activation of ER (Su et al. 2002). There is also evidence of regulation of steroid receptor activity via regulation of the expression of cofactors. Interestingly, the activation of cytoplasmic PKA has been shown to initiate steroid hormone-induced transcription by up-regulating TIF2 expression (Borud et al. 2002). The inhibition of steroid receptor activity by TIF2 down-regulation has not previously been described. Thus our results suggest a novel pathway for the regulation of GR activity. Additionally, our findings, together with those in previous studies imply the important role of cytoplasmic signalling in steroid hormone-mediated transcription, and suggest that the regulation of TIF2 expression in cells constitutes an important pathway in the regulation of steroid receptor activity.

5.3. Function of cofactor TIF2 in cell growth regulation

There is some previous evidence of a relationship between cofactor expression and growth. GRIP-1 expression has been descibed as critical for skeletal muscle differentiation in the mouse (Chen et al. 2000). The reduction of TIF2 expression by antisense oligodeoxynucleotides inhibits estrogen-stimulated ER transcriptional activity and DNA synthesis in MCF-7 cells, providing evidence for the role of TIF2 in growth stimulation (Cavarretta et al. 2002). In our studies the down-regulation of TIF expression and GR activity proved to be associated with growth inhibition of HME cells. Although GPCR30 inhibited growth without the presence of steroid hormones in MCF-7 breast cancer cells, the finding raises an intriguing possibility that GPCR30 might inhibit the transcriptional activity of a variety of steroid receptors, this resulting in growth inhibition,

particularly, since in the case of other steroid receptors such as ER and PR, ligand-independent activation of steroid receptors has been described.

Interesting new insights might be provided by a study of the effects of GPCR30 on the activation of various steroid hormone receptors through the MAPK pathway. It would be also relevant to know whether GPCR30 affects the expression of a subset of progestin-induced genes, and whether these are focused on growth regulation. *In vivo* studies and identification of the ligand for GPCR30 would provide a definitive answer regarding the role of GPCR30 in cell growth regulation and steroid signalling.

SUMMARY AND CONCLUSIONS

1. Progestins and progesterone enhanced the expression of GPCR mRNA at 24-48 h in different breast cancer cell lines. Other steroid hormones did not upregulate GPCR30 mRNA expression, and the expression was abrogated by anti-progestin RU486 suggesting progestin specificity in the mRNA regulation. Although a number of progestin target genes have been described, the result presented provides an interesting new insight into progestin signalling, since GPCR30 gene has been suggested to be critical for estrogen-mediated MAPK activation.

2. Transient expression of GPCR30 inhibited proliferation of MCF-7 breast cancer cells, and GPCR30 antisense enhanced proliferation. In immortalized HME cells stable expression of GPCR30 inhibited proliferation. We have characterized GPCR30 as a growth inhibitory receptor for the first time. The results are consistent with the evidence that other family members of GPCR30 inhibit growth, particularly the rat homologue of GPCR30.

3. Progestin-induced growth effects: cyclin D1 down-regulation, G1 phase arrest and inhibition of cell proliferation were diminished in MCF-7 cells when GPR30 expression was down-regulated by the antisense, suggesting that GPCR30 is critical for a progestin-induced growth effect. Additionally, the growth-inhibitory response by progestin correlated with GPCR30 expression level. In view of numerous contradictory findings on

the effect of progestin on cell growth, it is hypothesized that progestin drives cells to a decision point at the G1/S boundary and thereafter induces cellular changes which determine the ultimate response of the cells. Our results thus point to a gene which explains some of the effects of progestin on the cell cycle. It is also possible that GPCR30 and progestin have a cross-talk cell signalling pathways.

4. Progestin inhibited ERK activity through GPCR30 in MCF-7 breast cancer cells. The antisense, cell growth and ERK activation studies suggested that MAPK inactivation is associated with growth inhibition. A role of MAPK activation in progestin-induced growth inhibition has previously been suggested. However, MAPK inactivation by GPR30 or progestin has not previously been established. Our data further imply an important role of the MAPK pathway in progestin signalling in breast cancer cells.

5. Stable expression of GPCR30 in immortalized human mammary epithelial cells down-regulated the expression of cofactor TIF2 as verified by RT-PCR analysis and immunoblotting. The regulation of cofactors has been established only in very few studies. Our result provides evidence that this can happen.

6. Stable and transient expression of GPCR30 abolished the ability of glucocorticoid to enhance transcription from the cognate hormone response element of GR, suggesting that GPCR30 interferes with the transcriptional activity of the steroid hormone. GPCR30 has not been reported to regulate the transcriptional activation of steroid hormones. Thus our result provides an interesting new insight into the regulation of steroid receptor activity.

7. Interference in GR activity by GPCR30 was seen to be associated with inhibition of cell proliferation. It has previously been shown that inhibition of ER activity and TIF2 down-regulation leads to growth inhibition. This observation provides further evidence that inhibition of TIF2 expression results in diminution of cell proliferation. It might also suggest a pathway by which GPCR30 affects the cell cycle.

In conclusion, the results presented here describe a growth inhibitory molecule GPCR30 which affects cell signalling pathways in many ways. GPCR30 interferes with steroid signalling, reduces MAPK activity and TIF2 expression. It is possible that all these phenomena are interconnected, or alternatively, comprise independent pathways by which GPCR30 affects cell signalling. In any case the results reflect a cross-talk with membrane protein and steroid hormone mediated signalling, providing further evidence of the complex mechanism of progestin action in the mammary gland.

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ORIGINAL COMMUNICATIONS