Sex Hormone-binding Globulin Selectively Modulates Estradiol-regulated Genes in MCF-7 Cells

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- SHBG
- estradiol
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- breast cancer

Abstract

Human sex hormone-binding globulin inhibits the effects of estradiol on proliferation and apoptosis of breast cancer cells. We report here the effect of sex hormone-binding globulin on estradiol regulation of gene expression in MCF-7 breast cancer cells using a selected set of genes. Estradiol upregulates genes that are positive regulators of proliferation (e.g., bcl-2, c-fos, c-myc, cyclin D) or/and related to more aggressive form of breast cancer (e.g. BRCA-1, EGF-R) and downregulates two genes (c-jun and ERα). Sex hormone-binding globulin modulates only a selected group of estradiol-controlled genes (inhibiting upregulation of bcl-2, c-myc, EGF-R, PR, and downregulation of ERα), starting 48 hours after treatment. Our study demonstrates that in breast cancer cells, sex hormone-binding globulin is effective on few selected genes which are involved in cell growth and apoptosis or related to cell estrogen-dependence and that the protein regulation of estradiol effect is selected and specific. Sex hormone-binding globulin action in estrogen breast cancer cells is strongly associated to cell growth and estrogen-sensitivity.

Introduction

Estradiol increases breast cancer cell proliferation and tumorigenesis [1,2]. In recent years, thanks to the advent of microarray technology, the profiling of estrogen-regulated genes in breast cancer cells has become possible. Most studies had identified genes overexpressed in breast cancer or patterns of gene expression related to disease outcome [3,4] and therapy responsiveness [5]. Furthermore, the exact role of estrogen-regulated gene expression in estrogen-sensitive breast cancer cells has also been investigated. In breast cancer cells, estradiol upregulates, among others, genes positively affecting cell proliferation and cell cycle progression, while it downregulates transcriptional repressors, anti-proliferative and pro-apoptotic genes [6]. The differential modulation of these genes, altogether, contributes to the estradiol induction of proliferation and inhibition of apoptosis in breast cancer.

The effects of estradiol on proliferation and apoptosis in breast cancer cells can be efficiently inhibited by Sex Hormone-Binding Globulin (SHBG), a glycoprotein of human plasma that specifically binds androgens and estradiol [7]. Besides acting as steroid carrier, new roles of SHBG on steroid action have emerged [8,9]; in particular, the effects of SHBG initiated at the level of the cell membrane [10,11], stimulate a specific intracellular pathway which leads to inhibition of breast cancer cell proliferation. We have demonstrated that SHBG rapidly stimulates cAMP in MCF-7 breast cancer cells [12,13]; as a result of this intracellular pathway stimulation, the effects of estradiol on cancer cell proliferation are inhibited [14]. Moreover, quite recently, SHBG was also reported to abolish the anti-apoptotic effect of estradiol in MCF-7 cells [15]. Therefore, it appears that it is through these active and specific processes, that SHBG inhibits estradiol-induced effects on cancer cell proliferation. This is the first study that determines if these SHBG-activated pathways initiate specific gene transcription.

Materials and Methods

Cell Cultures

Estrogen-dependent MCF-7 breast cancer cells were routinely maintained in 25 cm^2 flasks at 37°C in 5% CO_2 and 95% humidity, in RPMI 1640

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(Sigma-Aldrich, St Louis, MO, USA) with the addition of 100 IU/ml penicillin and 100 μg/ml streptomycin, and supplemented with 10% heat inactivated FBS (Euroclone, Wetherby, West York, UK). Once a week, cells were detached with trypsin/EDTA and re-seeded at a dilution of 1:2 and 1:4. Twenty-four hours before experiments, cells were switched to RPMI 1640 without phenol red supplemented with 10% FBS specifically treated to remove steroids and bovine SHBG.

Gene array

MCF-7 cells (1.5×10^6) were seeded in 75 cm^2 flasks and treated as follows: a) cells under basal conditions; b) cells treated with 10 nM estradiol (Sigma-Aldrich, St. Louis MO, USA) for different times (6, 24, 48, 72 hours); c) cells treated with 50 nM SHBG for 40 minutes. Human SHBG (a gift from G.L. Hammond, B.C. Research Institute for Children's and Women's Health, Vancouver B.C., Canada), was charcoal-treated before use to remove the dihydrotestosterone (DHT) used for storage; d) cells treated with 50 nM SHBG for 40 minutes, thereafter, SHBG was washed off the cells before exposure to estradiol for the time indicated above. All of the treatments were repeated every day when experiment was prolonged over 24 hours.

Human GEArray kits for the study of estrogen-regulated genes (SuperArray, Bethesda, MD) were used to study the gene expression profiles of untreated and treated MCF-7 cells. Hybridization was performed according to manufacturer's instructions. Briefly, by using the TRIzol Reagent (Invitrogen, Groningen, The Netherlands) following the method developed by Chomczynski and Sacchi [16], total RNA was extracted from MCF-7 cells in different conditions. RNA was used as a template for biotinylated probe synthesis. For probe synthesis, each RNA sample (7.5 μg) was combined with a primer mix, and with 80 U AMV reverse transcriptase (Finnzymes, Finland), 50 U Rnase inhibitor (Amersham, Little Chalfont, Buckinghamshire, UK), and a dNTP mix with biotin-16-dUTP (Roche Applied Science, Penzberg, Germany) and was incubated for 120 minutes at 42°C. Filters were hybridized overnight at 68°C with denatured biotinylated cDNA probes; then they were extensively washed and incubated with alkaline phosphatase-conjugated streptavidin (1:5000). Gene expression was detected by chemiluminescence by using the alkaline phosphatase substrate CDP-Star. Spots were photographed with the Kodak EDAS 290 system (Eastman Kodak Company, Rochester, NY). Densitometric analysis was performed by using the Kodak 1D Image Analysis software. The negative control pUC18 DNA was used for background subtraction. The relative abundance of each transcript was estimated by comparing its signal intensity to the signal derived from positive controls, β-actin and GAPDH. Experiments were performed in triplicate.

RNA extraction and RT-PCR analysis

MCF-7 cells were seeded in 75 cm^2 flasks and treated as above. Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Groningen, The Netherlands), as above. Total RNA was reverse-transcribed at 42°C for 40 minutes using AMV reverse transcriptase (Finnzymes, Finland) and oligodT primer (Invitrogen, Groningen, The Netherlands). The PCR reaction system contained 5 μl of 10X PCR buffer, 10 μl of RT product, 0.2 mM dNTP (Finnzymes, Finland), 1.25 U Taq DNA polymerase (Finnzymes, Finland), 50 ng each of sense and anti-sense primers in a total volume of 50 μl. Primers: 5′- CGA CTT CGC CGA GAT GTC CAG GCA G and 3′- ACT TGT GGC CCA GAT AGG CAC CCA G for bcl-2; 5′- GGA AGA AAT TCG AGC TGC TG and 3′- GCT GTC GTT GAG AGG GTA GG for c-myc; 5′- AGA AGA AAC GGA GGG GAT GGA A and 3′- TTG GGG TGA TGG CTA AAG GAG A for EGF-R; 5′- CAT AAC GAC TAT ATG TGT CCA GCC and 3′- AAC CGA GAT GAT GTA GCC AGC AGC for ERα; 5′-CTC ACC CTG AAG TAC CCC ATC G and 3′-CTT GCT GAT CCA CAT CTG CTG G for β-actin. The expected PCR products were 389 for bcl-2, 458 bp for c-myc, 424 for EGF-R, 659 for ERα, and 885 for β-actin. Amplification was carried out as follows; for bcl-2: 1X (95°C, 3 min); 40X (95°C, 30 sec; 65°C, 1 min; 72°C, 30 sec); and 1X (72°C, 7 min). For c-myc and EGF-R: 1X (95°C, 3 min); 35X (95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec); and 1X (72°C, 7 min). For ERα and β-actin: 1X (95°C, 3 min); 35X (95°C, 30 sec; 65°C, 30 sec; 72°C, 30 sec); and 1X (72°C, 7 min). PCR products were electrophoresed on 1.5% agarose gel in the presence of ethidium bromide. Gels were photographed and analyzed with the Kodak 1D Image Analysis software. The net intensity of bands in each experiment was normalized to the intensity of the corresponding β-actin band before comparison between treated cells and untreated control.

Western blot analysis

1×10^6 cells were seeded in 75 cm^2 flasks and treated as above for 3 days. After treatment, cells were lysed in RIPA buffer (PBS, pH = 7.4, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 100 μg/ml PMSF, 30 μl aprotinin, 100 mM $NaVO_4$), extracted at 4°C for 30 minutes, and centrifuged at 4°C for 20 minutes at 15000×g. Equal amounts of protein (50 μg protein/lane) were subjected to SDS-PAGE (T = 8%) and electroblotted onto a PVDF membrane; the membrane was probed with the following primary antibodies: anti-bcl-2 (ΔC-21, 1:400 dilution, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); anti c-myc (#06-340, 1:500 dilution, Upstate, Lake Placid, NY, USA); anti-ERα (HC-20, 1:500 dilution, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); anti-actin (Monoclonal Anti-actin clone AC-40, 1:1000 dilution, Sigma, Saint Louis, MI, USA), to check protein loading.

Proteins were detected with ECL Western blot reagents following manufacturer's instructions, and photographed with the Kodak EDAS 290 system (Eastman Kodak Company, Rochester, NY). Densitometric analysis was performed by using the Kodak 1D Image Analysis software.

Statistical analysis

Data are expressed throughout as means ± SEM, calculated from at least three different experiments. Statistical comparisons between groups were performed with analysis of variance (one-way ANOVA) and the threshold of significance was calculated with the Bonferroni test. Statistical significance was set at $p < 0.05$.

Results

Estradiol and SHBG effect on gene expression in MCF-7 cells

The GEArray technique allowed us to evaluate the expression in MCF-7 cells of 23 genes that are reported in **Table 1**. Among them, we could not observe any expression at any time of 6 genes (EGF, ERβ, H-ras, PRL, telomerase, TGFα). The remaining 17 genes were expressed in untreated cells at different abundance levels and estradiol regulated 13 of these genes. Estradiol in MCF-7 cells upregulated the expression of 11 genes (bcl-2, BRCA1, Cathepsin D, c-fos, c-myc, Cyclin D, EBAG9, EGF-R, Kera-

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Table 1 Expression of estradiol-regulated genes in MCF-7 cells

Gene Name	Gene Bank No.	10 nM estradiol			
		6 h	24 h	48 h	72 h
bcl-2	**M14745**	**1.94**	**1.82**	**2.47**	**3.44**
BRCA1	**U68041**	0.93	0.95	**3.66**	**1.77**
Cathepsin D	**M11233**	0.96	1.55	**1.86**	**1.65**
c-fos	**V01512**	**1.98**	**1.99**	**1.65**	1.50
c-jun	*AF022805*	*1.34*	*1.20*	*0.76*	*0.56*
c-myc	**J00120**	**2.45**	**3.00**	**2.90**	**2.31**
COX7RP	AB007618	0.75	0.86	1.12	1.15
Cyclin D	**M64349**	0.82	0.86	**1.66**	1.52
EBAG9	**AB007619**	0.94	1.08	1.02	**2.43**
EFP	D21205	0.85	0.86	1.54	1.20
EGF	X04571	not detectable			
EGF-R	**X00588**	1.18	1.24	**1.65**	**2.83**
ERα	*NM_000125*	*1.16*	*0.61*	*0.34*	*0.37*
ERβ	AB006590	not detectable			
HMG1	NM_002128	0.75	0.82	0.95	1.19
H-ras	NM_005343	not detectable			
Keratin 19	**NM_002276**	0.79	1.28	**2.26**	**1.82**
PR	**M15716**	**1.93**	**5.31**	**4.75**	**19.9**
PRL	M29386	not detectable			
pS2	**NM_003225**	1.15	1.32	**2.42**	**2.00**
Telomerase	AF015950	not detectable			
TGFα	NM_003236	not detectable			
WISP2	NM_003881	0.86	0.81	1.40	1.18

Table 1 reports the 23 gene spotted on GEArray membrane, their accession codes, and their relative abundance after estradiol treatment for different time; relative abundance was calculated as the ratio between intensity values after/before treatments. Upregulated genes and their relative abundance values are reported in bold; downregulated genes are reported in italics.

tin 19, PR and pS2) while it downregulated the remaining two genes (c-jun and ERα), following different temporal schedules.
SHBG alone had no detectable effect on basal gene expression in MCF-7 cells and its effect on estradiol gene regulation was detectable only after 48–72 hours of treatment. In these experimental conditions, SHBG was effective in modifying estradiol effect on selected genes as showed in **Table 2**. We observed, in fact, in MCF-7 cells pre-treated with SHBG an inhibition of estradiol upregulation of bcl-2, c-myc, EGF-R and PR and of estradiol downregulation of ERα.

SHBG regulation of E_2 stimulated genes involved in cell proliferation and survival

Accordingly to previous reports, estradiol treatment of MCF-7 cells upregulated bcl-2, a gene widely known for its anti-apoptotic properties; and here we demonstrated that SHBG pretreatment is able to inhibit the estradiol effect. As shown in ○ **Fig. 1**, panel **A**, the estradiol upregulation of bcl-2 mRNA was inhibited by SHBG pretreatment. In ○ **Fig. 1**, panel **B**, the effect of estradiol and SHBG/estradiol treatment on bcl-2 protein levels is also reported. Estradiol was significantly efficient in increasing bcl-2 protein levels and pre-treatment with SHBG partially reduced its expression.
○ **Fig. 2**, panel **A** reports the behavior of c-myc expression. Estradiol upregulated c-myc mRNA expression while SHBG pre-treatment inhibited it. Analysis of protein levels confirmed RT-PCR data (○ **Fig. 2**, panel **B**).

SHBG regulation of E_2 stimulated genes encoding for growth factor receptors

Among genes encoding for growth factors and upregulated by estradiol, we show here that EGF-R mRNA expression was induced by steroid treatment (○ **Fig. 3**, panel **A**) and that SHBG pre-treatment inhibited this estradiol effect. Moreover, the data was confirmed by analysis of protein levels (○ **Fig. 3**, panel **B**).

SHBG regulation of steroid receptor expression (ERα and PR)

Estradiol, as expected, downregulated mRNA expression of its receptor ERα (○ **Fig. 4**, panel **A**) and after pre-treatment with SHBG mRNA levels of ER returned to basal levels. The same effect was observed as protein levels are concerned (○ **Fig. 4**, panel **B**).
As progesterone receptor is concerned, we confirmed our previously results [17] indicating that estradiol induces PR expression and that pretreatment with SHBG determine a reduction of PR to basal levels, again at both mRNA and protein levels.

Discussion

In the present paper we demonstrate that SHBG affects estradiol gene regulation on selected genes, that are bcl-2, c-myc, EGF-R, ERα and PR. The effect observed here on bcl-2 expression may be one of the mechanisms elicited by SHBG to restore apoptosis in breast cancer cells under estrogen action. We recently reported [15] that SHBG, after interacting with MCF-7 cell membranes, antagonizes the anti-apoptotic effect of estradiol; thus, the present observation on bcl-2 expression in MCF-7 cells confirms the involvement of SHBG in abrogating the anti-apoptotic effect of estradiol. In fact, the proto-oncogene bcl-2 is widely known to inhibit apoptosis [18, 19] and it is over-expressed in many tumors including breast cancer [20]. Estradiol in MCF-7, ZR.75 and T47D breast cancer cells induces bcl-2 gene and cognate protein expression [21, 22], and our data are completely in line with

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Table 2 Effect of SHBG on the expression of estradiol-regulated genes in MCF-7 cells

Gene Name	Gene Bank No.	SHBG/estradiol	
		48 h	72 h
bcl-2	M14745	2.15 (2.47)	**1.58←** (3.44)
BRCA1	U68041	3.51 (3.66)	2.00 (1.77)
Cathepsin D	M11233	1.82 (1.86)	1.66 (1.65)
c-fos	V01512	1.62 (1.65)	1.52 (1.50)
c-jun	AF022805	0.68 (0.76)	0.53 (0.53)
c-myc	J00120	2.87 (2.90)	**0.57←** (2.31)
COX7RP	AB007618	1.05 (1.12)	1.32 (1.15)
Cyclin D	M64349	1.68 (1.66)	1.53 (1.52)
EBAG9	AB007619	1.06 (1.02)	2.70 (2.43)
EFP	D21205	1.47 (1.47)	1.38 (1.20)
EGF	X04571	N.D.	N.D.
EGF-R	X00588	1.69 (1.65)	**0.56←** (2.83)
ERα	NM_000125	**1.62← (0.34)**	**1.34← (0.37)**
ERβ	AB006590	N.D.	N.D.
HMG1	NM_002128	1.19 (0.95)	1.56 (1.19)
H.ras	NM_005343	N.D.	N.D.
Keratin 19	NM_002276	1.96 (2.26)	1.91 (1.82)
PR	M15716	**3.50← (4.75)**	**2.76← (19.9)**
PRL	M29386	N.D.	N.D.
pS2	NM_003225	2.75 (2.42)	1.89 (2.00)
Telomerase	AF015950	N.D.	N.D.
TGFα	NM_003236	N.D.	N.D.
WISP2	NM_003881	1.58 (1.40)	1.20 (1.18)

Table 2 reports the 23 gene spotted on GEArray membrane, their accession codes, and their relative abundance after SHBG/estradiol treatment for 48 or 72 hours; relative abundance was calculated as the ratio between intensity values after/before treatments. Relative abundance of gene expression after treatment with estradiol alone is reported in brackets; genes selectively regulated by SHBG are marked by arrows ←and bolded

these observations. In MCF-7 cells estradiol upregulates bcl-2 transcription and two different EREs were identified in bcl-2 coding region [23]; moreover the upregulation of bcl-2 was strictly related to estradiol inhibition of apoptosis in this cell line. Stable overexpression of bcl-2 in MCF-7 cells resulted in suppression of apoptosis induced by several chemotherapy agents while pretreatment with the pure antiestrogen ICI 182,780 enhanced induced apoptosis [24].

Another important target of estradiol in breast cancer is the proto-oncogene c-myc. It is well-known how estradiol induces a rapid and robust induction of c-myc in breast cancer cells [25]. The c-myc, in turn, acts on multiple targets that are key cell cycle regulators; in particular, it increases the expression of cyclin E and CDK4 [26,27]. As reported for bcl-2, our data show that estradiol induces c-myc expression that is maximal after 24 hours, and that SHBG abrogates estradiol effect in MCF-7 cells treated for 72 hours. Since aromatase inhibitors and antiestrogens induce growth suppression and cell cycle arrest of breast cancer cells, together with apoptosis induction, and decrease of both bcl-2 and c-myc expression [28], our data suggest that SHBG behavior on c-myc as well as on bcl-2 is like to that of antiestrogens. We also report that SHBG inhibits the estradiol induction of EGF-R. EGF-R or HER1 is a member of the epidermal growth factor family of trans-membrane receptors [29] and it has been reported to be induced by estradiol [30]. Overexpression of EGF-R in breast cancer, that has been reported to occur in up to 14% of tumors [31], is considered a negative prognostic factor [32], especially if it occurs together with over-expression of other genes such as c-myc [33]. Moreover, EGF-R is involved in a bidirectional cross-talk with estrogen receptors and the activation of the EGF-R-derived signaling pathway could amplify estradiol effect in breast cancer [34]. Therefore, SHBG, abolishing the estradiol induction of EGF-R expression, is able to break the functional loop between the two pathways, reducing breast cancer cell proliferation and growth. Lastly, we show here that estradiol is able to downregulate the expression of its own receptor, ERα, effect that has been known for a long time. In fact, a single injection of estradiol determinates an 80% reduction of binding capacity in cytosol extracts from rat uteri [35]. On the other side, adaptation of MCF-7 cells to long term estrogen deprivation results in MCF-7 hypersensibility to estradiol and leads to an increase of ER [36]. Here we show that SHBG is able to revert the downregulation of ERα induced by estradiol. This effect could also be linked to apoptosis induction and cell growth inhibition, since, as reported by other authors [37,38], both phenomena were observed when ERα expression was increased in association with bcl-2 reduction. As PR modulation is concerned, SHBG completely inhibits the strong upregulation induced by estradiol both at mRNA and protein levels as previously reported [17]; data reported in the present paper, obtained from gene array, further confirm our previous observation.

The selectivity of SHBG effect on gene expression confirms that SHBG regulation of estradiol effect in MCF-7 cells is not a general effect, due to the steroid sequester. As already suggested [9,15,39,40], SHBG is likely to counteract estradiol action in a specific manner. Estradiol regulates the proliferation of breast cancer cells and this property is the key-stone of antihormone therapy with antiestrogens and aromatase inhibitors. Here we show that genes upregulated by estradiol are almost all positive regulators of proliferation (e.g., bcl-2, c-fos, c-myc, cyclin D) or/and related to more aggressive form of breast cancer (e.g., BRCA-1, EGF-R). In addition, we observed that in our set, estradiol caused the downregulation of two genes (c-jun and ERα). Our data, even though regarding few genes, are mainly in line with the report from Frasor et al. [6] which observed that in general estradiol causes the upregulation of positive proliferation regulator genes and the downregulation of negative proliferation regulators.

Estradiol followed a precise temporal schedule in our study as in previous reports [6] in regulating gene expression. It is noteworthy that SHBG effect was detectable only after 48–72 hours and not at earlier time. It is thus conceivable that SHBG has a late effect on estradiol gene regulation. The absence of SHBG effect

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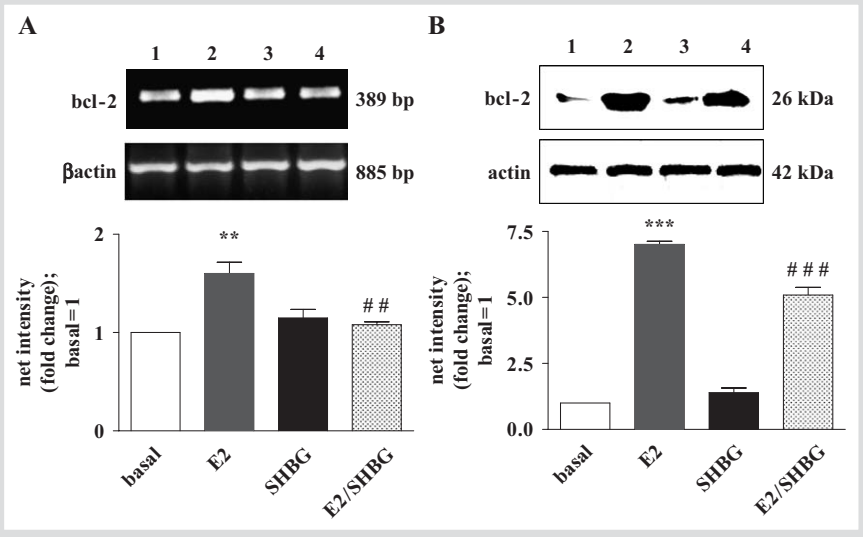

Fig. 1 Effect of SHBG and estradiol on bcl-2. A representative experiment of RT-PCR (panel **A**) and western blot (panel **B**) for bcl-2 mRNA and protein performed in MCF-7 cells treated with SHBG and estradiol for 72 hours (*lane 1*: untreated control; *lane 2*: 10 nM estradiol; *lane 3*: 50 nM SHBG; *lane 4*: 50 nM SHBG + 10 nM estradiol). Histograms: semiquantitative analysis of RT-PCR and western blot results; net intensity was determined as the ratio between treated cells and untreated controls. Results are expressed as means ± SEM, n = 3. Significance *vs.* basal: $^{**}p<0.01$, $^{***}p<0.001$; significance *vs.* estradiol: $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.

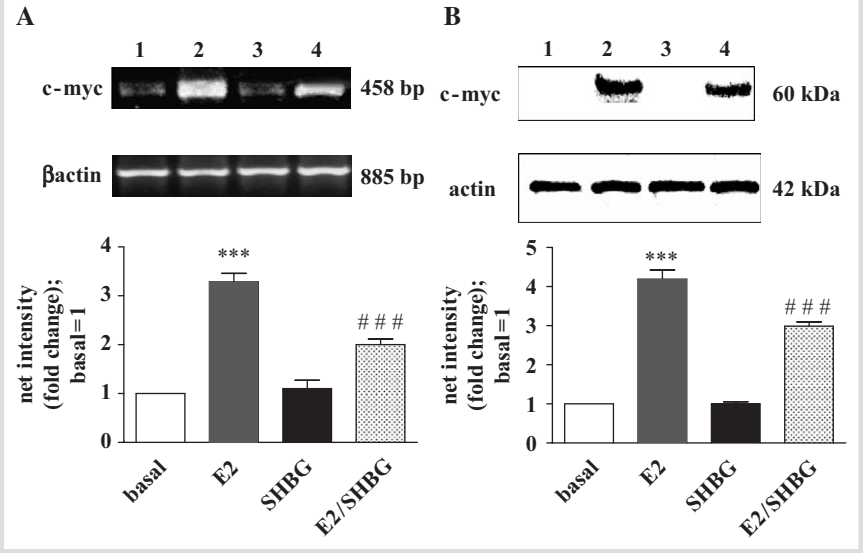

Fig. 2 Effect of SHBG and estradiol on c-myc. A representative experiment of RT-PCR (panel **A**) and western blot (panel **B**) for c-myc mRNA and protein were performed in MCF-7 cells treated with SHBG and estradiol for 72 hours (*lane 1*: untreated control; *lane 2*: 10 nM estradiol; *lane 3*: 50 nM SHBG; *lane 4*: 50 nM SHBG + 10 nM estradiol). Histograms: semiquantitative analysis of RT-PCR and western blot results; net intensity was determined as the ratio between treated cells and untreated controls. Results are expressed as means ± SEM, n = 3. Significance *vs.* basal: $^{***}p<0.001$; significance *vs.* estradiol: $^{\#\#\#}p<0.001$.

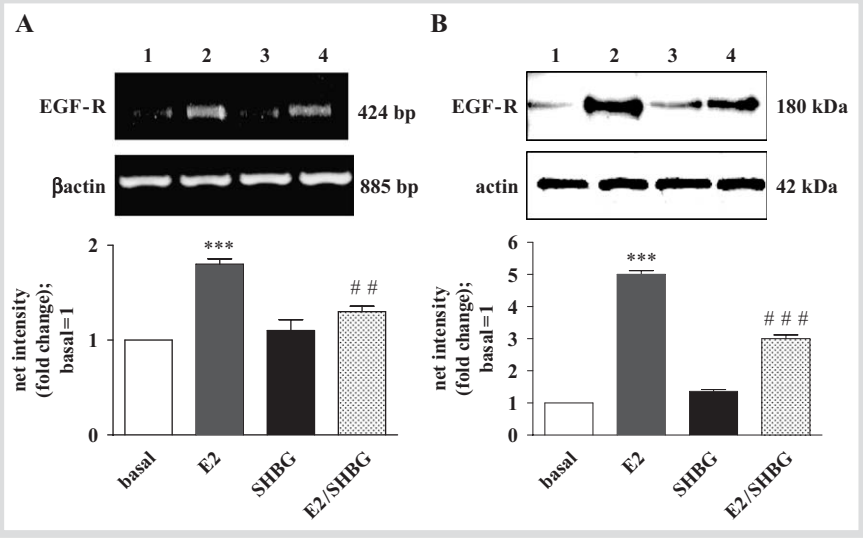

Fig. 3 Effect of SHBG and estradiol on EGFR. A representative experiment of RT-PCR (panel **A**) and western blot (panel **B**) for EGFR mRNA and protein were performed in MCF-7 cells treated with SHBG and estradiol for 72 hours (*lane 1*: untreated control; *lane 2*: 10 nM estradiol; *lane 3*: 50 nM SHBG; *lane 4*: 50 nM SHBG + 10 nM estradiol). Histograms: semiquantitative analysis of RT-PCR and western blot results; net intensity was determined as the ratio between treated cells and untreated controls. Results are expressed as means ± SEM, n = 3. Significance *vs.* basal: $^{***}p<0.001$; significance *vs.* estradiol: $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.

at early times supports the hypothesis, recently suggested [15], that SHBG does not interfere directly with the estradiol nuclear receptor pathway. In fact, we recently reported that in MCF-7 cells SHBG [15] is not able to inhibit estradiol-induced transcription in an ERE-tk-luciferase system, while it can interfere with membrane-initiated estradiol pathway [9].

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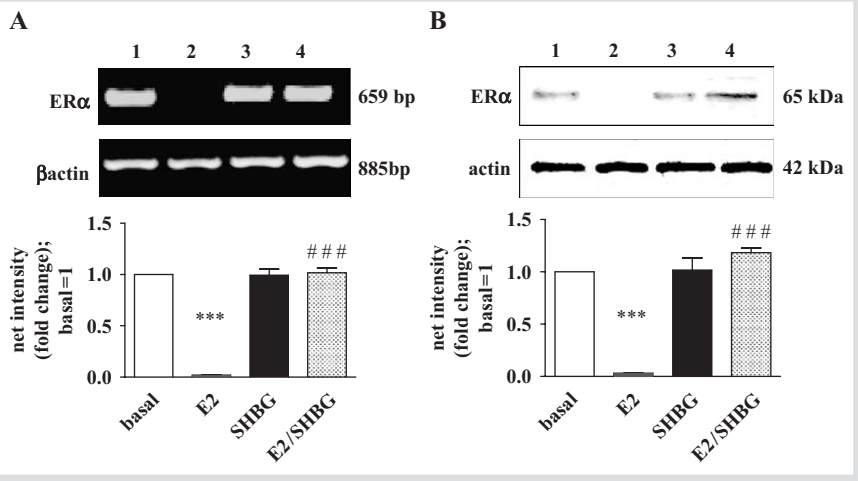

Fig. 4 Effect of SHBG and estradiol on ERα. A representative experiment of RT-PCR (panel **A**) and western blot (panel **B**) for ERα mRNA and protein were performed in MCF-7 cells treated with SHBG and estradiol for 72 hours (*lane 1*: untreated control; *lane 2*: 10 nM estradiol; *lane 3*: 50 nM SHBG; *lane 4*: 50 nM SHBG + 10 nM estradiol). Histograms: semiquantitative analysis of RT-PCR and western blot results; net intensity was determined as the ratio between treated cells and untreated controls. Results are expressed as means ± SEM, n = 3. Significance *vs.* basal: $^{***}p<0.001$; significance *vs.* estradiol: $^{###}p<0.001$.

Since SHBG effect in cells is initiated at membrane level [13], we suggest that the "link" between estradiol and SHBG might reside somewhere between the outer side of membrane and nucleus. We recently proposed [15] that SHBG can reduce ERα activity through ERK inhibition. It is actually known that ERα transcriptional activity is increased by ERK-mediated phosphorylation [41] and so SHBG-mediated ERK inhibition can downregulate ERα activity.

In conclusion, our study underlines the ability of SHBG to modulate estradiol-controlled gene expression in breast cancer cells; in particular, SHBG is effective on few selected genes which are involved in cell growth and apoptosis as well as on genes related to cell estrogen-dependence. The effect of SHBG in estrogen breast cancer cells, as previously suggested, is therefore strongly associated to cell growth and estrogen-sensitivity.

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