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Insulin-like growth factor binding protein-4 and -5 modulate ligand-dependent estrogen receptor-α activation in breast cancer cells in an IGF-independent manner☆

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ABSTRACT

Insulin-like growth factor binding proteins (IGFBPs) are modulators of numerous cellular processes including cell proliferation. Although IGFBPs classically act by sequestration of extracellular insulin-like growth factors (IGFs), thereby contributing to the fine-tuning of growth factor signals, IGF-independent actions of IGFBPs have also been described. In the breast, growth factor signaling in association with estradiol (E2)-stimulated estrogen receptor function is organized in a complex cross-talk. The importance of phosphatidylinositol 3-kinase/protein kinase B (Akt/PKB) pathway components for the E2-induced activation of estrogen receptor-alpha (ERα) is well accepted. Here we show that in the absence of IGFs, IGFBP-4 or IGFBP-5, either overexpressed in MCF-7 breast cancer cells or added exogenously, decreased the capability of E2 to induce ERα transcriptional activity. In addition, overexpression or addition of recombinant IGFBP-4 or IGFBP-5 resulted in reduction of E2-induced phosphorylation of Akt/PKB, GSK-3α/β and ERα in MCF-7 cells. The activation of the Akt/PKB-pathway describes a non-genomic effect of E2, which did not involve activation/phosphorylation of the IGF-I receptor (IGF-IR). Furthermore, knockdown of the IGF-IR did not affect the inhibition of E2-induced ERα phosphorylation by IGFBP-4 and 5. Moreover, IGFBP-4 and IGFBP-5 strongly decreased E2-triggered growth of MCF-7 cells. Our data suggest that IGFBPs interfere with the E2-induced activation of the Akt/PKB-pathway and prevent full hormone-dependent activation of ERα and breast cancer cell growth in an IGF- and IGF-IR-independent manner.

1. Introduction

The IGF (insulin-like growth factor) axis plays a crucial role in the regulation of cellular growth and differentiation, developmental processes and malignant cell transformation [1,2]. IGF-I and IGF-II are potent mitogenic and survival factors for both normal and cancer cells, and their effects on cell proliferation are mediated by the type 1 IGF tyrosine kinase receptor (IGF-IR). Classically, IGF binding proteins (IGFBPs) modulate the bioavailability of the IGFs through IGF/IGFBP complex formation and contribute to the control of IGF-I and IGF-II-induced proliferation [3–6]. Six high-affinity IGFBPs have been identified, which are structurally, functionally, and evolutionarily related and which usually inhibit IGF-I and IGF-II action [6–9]. IGFBP-1 to -4 have similar affinities for IGF-I and IGF-II, whilst IGFBP-5 and IGFBP-6 bind IGF-II with a much higher affinity. Recent evidence suggests intrinsic ligand-independent activities of IGFBPs at the cellular level, which lead to specific cellular actions or modulate the effects of other factors [10]. So far it is unclear whether these activities are mediated via putative cell surface IGFBP receptors or by intracellular delivery [8–12].

In the mammary gland the components of the IGF system, including the IGFs, IGF-IR and IGFBPs, are important players in the development

Abbreviations: BFA, brefeldin A; BP-4, IGFBP-4; BP-5, IGFBP-5; BSA, bovine serum albumin; DCC, dextran-coated charcoal; DMEM, Dulbecco's modified Eagle's Medium; E2, 17β-estradiol; EGFP, enhanced green fluorescent protein; ERα, estrogen receptor-alpha; ERE, estrogen response element; FBS, fetal bovine serum; GSK-3, glycogen synthase kinase-3; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; IGF-IR, type 1 IGF receptor; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; qPCR, quantitative real time polymerase chain reaction.

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and progression of breast cancer. Besides IGF-IR and IGFs, all six IGFBPs are expressed in mammary tumors [3,7]. In particular, IGFBP-4 and IGFBP-5 are found expressed in primary breast cancer and breast cancer cell lines [13–16] and studies have shown that both IGFBPs are produced predominantly by ERα-positive tumors [17,18]. It is well established that the expression of different components of the IGF axis, including IGF-IR, IGFs, and IGFBPs, is under estrogenic control [7,18–21], and modulation of human breast cancer cell proliferation by estrogens or antiestrogens has been associated with specific alterations in the accumulation of IGFBPs in the conditioned media [22]. In vitro and in vivo studies suggest that IGFBP-4 and IGFBP-5 modulate tumor growth by influencing autocrine and paracrine IGF actions or by as yet unknown IGF-independent mechanisms [12,23]. Furthermore, a complex cross-talk between the IGF and ERα signaling pathways in breast cancer cells has been suggested [24,25] and IGFs and estrogens act in concert to regulate cell growth in different tissues. Although the regulation of estrogen signaling by the IGF axis is not well defined, evidence has been presented showing that IGF-I regulates both the expression and the activity of ERα. In this context, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been postulated to be responsible, at least in part, for mediating the growth factor effects on ERα expression and activity [25].

Not only IGFs but also estrogenic hormones like E2 activate the PI3K/Akt pathway [26]. For example, hyperphosphorylation of Akt/PKB has been observed upon short-term treatment of cells with E2 [27,28]. Furthermore, glycogen synthase kinase-3 (GSK-3), a downstream target of Akt/PKB that acts as a key player in ERα stabilization and function, is phosphorylated in response to E2 in MCF-7 cells under IGF-free and serum-free conditions [29,30]. As IGFBPs prevent the IGF-induced activation of the Akt/PKB pathway, it was of interest to study whether IGFBPs also interfere with the E2-induced activation of this pathway and thus contribute to the cross-talk between the IGF axis and ERα-signaling. Here we show that recombinant or overexpressed IGFBP-4 and IGFBP-5 inhibit the E2-induced ERα activation and proliferation in breast cancer cells in the absence of IGF and after downregulation of the IGF-IR. These data are consistent with an IGF-independent action of IGFBP-4 and -5 in the regulation of ERα activity.

2. Materials and methods

2.1. Cell lines and reagents

Experiments were performed in MCF-7 breast cancer cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and MELN cells, a cell line derived from MCF-7 cells by stable transfection of a reporter plasmid carrying luciferase under the control of an estrogen response element (ERE-luc) [31]. MELN cells were stably transfected with expression vectors encoding either wild-type or kinase dead (K197A) Akt/PKB. Akt/PKB constructs were kindly provided by Dr. Brian Hemmings [29,32]. Cells were routinely maintained in phenol red-free DMEM supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 units/ml streptomycin at 37 °C in a humid atmosphere containing 5% CO_2. Before being utilized for experiments, cells were kept for 4 days in DMEM containing 10% dextran-coated charcoal (DCC)-treated-FBS to exclude a potential effect of E2 present in the native FBS [29].

The following antibodies were used: mouse anti-phospho-Akt (Ser473), rabbit anti-Akt-1/2, rabbit anti-phospho-GSK-3α/β (Ser21/9), mouse anti-GSK-3α/β, mouse anti-phospho-Ser118-ERα (Cell Signaling Technology, Danvers, MA, USA), mouse anti-ERα (Novocastra, Newcastle upon Tyne, UK), rabbit anti-ERα, rabbit anti IGF-IRβ (Santa Cruz Biotechnology; Santa Cruz, CA, USA), mouse anti P-Tyr (clone 4G10), mouse anti-β-tubulin and mouse anti-IGF-II (Upstate Biotechnology Inc., Lake Placid, NY, USA). Endogenous IGFBP-4 and IGFBP-5 were detected by specific mouse monoclonal antibodies (R&D Systems, Wiesbaden, Germany). Tagged IGFBPs were detected in western blots using a mouse monoclonal anti-Flag-tag antibody (Sigma, München, Germany). Peroxidase-labeled secondary antibodies were from Dianova (Hamburg, Germany). Recombinant human (rh) IGF-I, IGFBP-4 and IGFBP-5 proteins were purchased from R&D Systems.

Protein was determined by the DC assay kit from Bio-Rad (München, Germany). Protein A-agarose beads, complete mini EDTA-free protease inhibitors and PhosSTOP phosphatase inhibitors were from Roche Applied Biosciences (Mannheim, Germany).

2.2. Quantitative RT-PCR

Expression of IGFBP-1 to -6 mRNAs in MCF-7 cells was determined by quantitative real-time PCR (qPCR). Cells remained untreated or were treated for 6 h with 10 nM E2. Total RNA was extracted using the Qiagen RNeasy kit. Reverse transcription of mRNA was performed using the Qiagen quantiTect reverse transcription kit. SYBR Green I-based qPCR was carried out on a MJ Research DNA Engine Opticon Continuous Fluorescence Detection System (Opticon Monitor II, Bio-Rad, CA, USA). The relative expression of each gene was determined using the comparative C_T method. The expression of IGFBPs and progesterone receptor (PgR, studied for control) was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primer pairs were used:

IGFBP-1 fwd: 5′′-TGTCAGAGGTCCCCGTTG-3′
IGFBP-1 rev: 5′-CGACCTGGACAGTCAGCAG-3′
IGFBP-2 fwd: 5′-GGTGGCAAGCATCACCTT-3′
IGFBP-2 rev: 5′-TCCTGTTGGCAGGGAGTC-3′
IGFBP-3 fwd: 5′-AACGCTAGTGCCGTCAGC-3′
IGFBP-3 rev: 5′-CGGTCTTCCTCCGACTCAG-3′
IGFBP-4 fwd: 5′-GAAGCACTTCGCCAAAATTC-3′
IGFBP-4 rev: 5′-ATCCAGAGCTGGGTGACACT-3′
IGFBP-5 fwd: 5′-GAGCTGAAGGCTGAAGCAGT-3′
IGFBP-5 rev: 5′-GAATCCTTTGCGGTCACAAT-3′
IGFBP-6 fwd: 5′-TGACCATCGAGGCTTCTACC-3′
IGFBP-6 rev: 5′-CATCCGATCCACACACCA-3′
PgR-fwd: 5′-GGCATGGTCCTTGGAGGT-3′
PgR-rev: 5′-CAATGGCTGTGGGAGAGC-3′
GAPDH-fwd: 5′-AGCCACATCGCTCAGAGA-3′
GAPDH-rev: 5′-GCCCAATAGGACCAAATCC-3′

2.3. Plasmid construction and transient transfections

IGFBP-4 and IGFBP-5 expression constructs were generated by cloning the respective human coding sequences (IGFBP-4, nucleotides 313 to 1086, GenBank acc. no. NM_001552, and IGFBP-5, nucleotides 774 to 1589, GenBank acc. no. NM_000599) into a pcDNA3.1-derived Flag-tag vector. Expression of these constructs yielded the full-length IGFBP proteins, each harboring a 2xFlag-tag peptide at the C-terminus. 3.5×10^5 MCF-7 cells were plated per well using 6-well plates in DMEM containing 10% DCC-FBS. After 24 h transient transfection of IGFBP-4 and IGFBP-5 was carried out with 200 ng/ml of construct or empty vector using the Effectene transfection reagent (Qiagen, Hilden, Germany) according to the standard protocols provided by the manufacturer.

A pS2 reporter plasmid harboring the pS2 gene promoter sequence in front of a luciferase reporter gene (pGL3–pS2-prom) was kindly provided by Dr. George Reid (EMBL, Heidelberg, Germany). Transient transfections of MCF-7 cells were carried out using 500 ng of pGL3–pS2 or empty vector and Effectene. 10 ng pRL-TK Renilla luciferase plasmid (Promega) was cotransfected for normalization of pS2–luciferase data.

2.4. IGF-IR knockdown

The ON-TARGETplus non-targeting siRNA #2 (CT2) and IGF-IR siRNA (ON-TARGETplus SMARTpool) were purchased from Dharmacon (Boulder, CO, USA). 3×10^5 cells/well were seeded in 6-well plates and

after 18 h cells were transfected with 25 nM siRNA using DharmaFECT1 (Dharmacon) and the protocol recommended by the manufacturer. After 48 h medium was replaced by serum-free medium and IGFBPs were added at 200 ng/ml for 4 h. Then cells were treated with 10 nM E2 or 50 ng/ml IGF-I for 20 min.

2.5. Immunoprecipitation and immunoblot analysis

Cells were washed with ice-cold PBS and lysed in lysis buffer (1.5% Triton X-100, 50 mM Hepes pH 7.6, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, protease and phosphatase inhibitors). Fifty microliters of protein A-agarose beads (50% suspension) were pre-equilibrated with lysis buffer. Lysates containing 750 μg protein in 0.75 ml buffer were pre-cleared by a 2-h incubation with protein A-agarose beads and incubated with fresh beads and 2 μg of IGF-IR polyclonal antibodies (Santa Cruz Biotech.) overnight at 4 °C in a rotating shaker. The beads were washed thereafter with lysis buffer and LiCl Buffer (1 M LiCl, 100 mM Tris–HCl pH 8.0, 0.1% NaN_3) and finally resuspended in loading buffer, and boiled at 90 °C for 10 min to elute the proteins.

Immunoblot analysis of protein samples was performed as described [29,30]. Serum-free cell culture supernatants were concentrated with Amicon Centrifugal Filter Units with 5 kDa or 10 kDa cutoff membranes before SDS–PAGE and detection of IGFBPs.

2.6. Luciferase reporter assay

ERα transcriptional activity was measured in MELN cells using a luciferase reporter gene assay. Cells were plated in DMEM/10% DCC-FBS at a density of 3.5×10^5 cells/well in 6-well plates. For transfection of the IGFBP expression constructs cells were washed with PBS the next day, and fresh medium was supplied before adding transfection mixes. Twenty-four hours after transfection medium was changed and cells were incubated for an additional 24 h in DMEM/1% DCC-FBS. rhIGFBPs (200 ng/ml) were added for 4 h prior to incubation with E2. Cells were stimulated with E2 (10 nM final concentration) for 24 h. Cells were then lysed in Luciferase Cell Culture Lysis Reagent (Promega, Mannheim, Germany) on ice for 30 min and luciferase activity was determined using the Luciferase Assay Reagent (Promega) and a Biolumat LB9505 luminometer (Berthold, Bad Wildbad, Germany), measuring each sample in duplicates. Luciferase activity in MCF-7 cells cotransfected with pGL3–pS2-prom and pRL-TK was measured using the Dual Luciferase Assay from Promega. At least four independent experiments were performed under the same conditions.

2.7. Cell growth assay

To assess the effect of IGFBPs on the E2-stimulated growth of MCF-7 cells, cells were kept for 3 days on DMEM/10% DCC-FBS and then plated on 96-well culture plates (10^4 cells per well) in DMEM/2% DCC-FBS. Cells were treated with E2 (10 nM) or vehicle and with rhIGFBP-4, rhIGFBP-5 (200 ng/ml) for 24, 36, and 48 h. Thereafter, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS and stained with 0.1% crystal violet. Staining intensity was recorded using a plate reader (Multiscan MX, Thermo, Dreieich, Germany).

2.8. Statistical analysis

Immunoblots were quantitatively evaluated using the Image J software (NIH, USA). Signal intensities of phospho-proteins were normalized to the corresponding protein signals. Data are presented as mean ± S.E.M. of at least three independent experiments. Significance of differences between treatments was analyzed by ANOVA and *t*-test. A *P*-value below 0.05 was considered statistically significant.

3. Results

3.1. Estradiol treatment increases IGFBP-4 and IGFBP-5 mRNA levels in MCF-7 cells

Treatment of MCF-7 cells with 10 nM E2 for 6 h resulted in increase of mRNA expression levels of IGFBP-4 (2.5-fold) and IGFBP-5 (2.2-fold). The mRNA levels of other IGFBPs were not significantly altered by E2-treatment (IGFBP-1, 0.6-fold; IGFBP-2, 0.9-fold; IGFBP-3, 1.0-fold; IGFBP-6, 1.0-fold). PgR mRNA level studied for comparison was induced 10.5-fold under the same conditions.

3.2. Secretion of IGFBP-4 and IGFBP-5 in MCF-7 cells

We detected only little endogenous IGFBP-4 and IGFBP-5 in lysates from serum-starved mock-transfected MCF-7 cells (Fig. 1A). We therefore overexpressed IGFBPs and studied whether they are secreted. After transfection with Flag-tagged IGFBP-4 or IGFBP-5 constructs or empty vector, cells were changed to serum-free medium after 24 h, and treated or not with 10 μg/ml brefeldin A (BFA), an inhibitor of secretion which disrupts the structure and function of the Golgi apparatus, for an additional 5 h. Immunoblot analysis detected abundant amounts of IGFBP-4 and IGFBP-5 in lysates from transfected cells, both treated with BFA and untreated. IGFBP-4 and IGFBP-5 were also detected in concentrated culture supernatants from cells not treated with BFA. Only small amounts of IGFBP-4 and IGFBP-5 were detected in supernatants from BFA-treated cells (Fig. 1A). These findings suggest that the overexpressed IGFBPs are actively secreted and putatively may exert extracellular functions. The proper function of Flag-tagged IGFBP-4 and IGFBP-5 was demonstrated by inhibition of IGF-I-induced phosphorylation of Akt/PKB (Fig. 1B).

3.3. IGFBP-4 and IGFBP-5 suppress activation of the Akt/PKB pathway by E2 in MCF-7 cells

IGFBPs efficiently bind extracellular IGFs and prevent activation of signaling pathways such as the Akt/PKB pathway transduced by the IGF-IR [6]. Since Akt/PKB is not only activated by stimulation of the cells with IGF but is also phosphorylated in response to E2 [27,28,33], we studied whether IGFBP-4 and IGFBP-5 expression affects the E2-induced phosphorylation of Akt/PKB and GSK-3α/β a downstream effector of Akt/PKB. In a first experiment Flag-tagged IGFBPs were overexpressed in MCF-7 cells. Then cells were kept overnight in 1% DCC-FBS and treated with 100 nM E2 for 20 min (Fig. 2) or with 10 nM E2 for 24 h (Suppl. Fig. 1). Immunoblot analysis of lysates from cells transfected with the empty vector and expression constructs showed that the expression of the IGFBPs did not affect expression levels of Akt/PKB, GSK-3α/β and ERα and inhibited slightly the basal phosphorylation of Akt/PKB at Ser-473 and GSK-3α/β at Ser-21/9. Importantly, lysates from E2-treated cells revealed increased phosphorylation of Akt/PKB and of the downstream target kinase GSK-3α/β both at 20 min (Fig. 2A, B, C) and 24 h treatment (Suppl. Fig. 1). In cells transfected with IGFBP-4 or IGFBP-5 expression constructs, E2-induced phosphorylation of Akt/PKB and GSK-3α/β was significantly reduced, indicating that both IGFBPs prevented E2-dependent activation of the Akt/PKB pathway.

3.4. E2-induced phosphorylation of ERα at Ser-118 is decreased by IGFBP-4 and IGFBP-5

Treatment of MCF-7 cells with E2 results in rapid phosphorylation of ERα at Ser-118 showing two bands which are caused by the well-known band-shift of ERα [29]. We previously reported that Akt/PKB and GSK-3 play a significant role in this phosphorylation [29,30]. Since IGFBP-4 and IGFBP-5 decreased the E2-induced phosphorylation of Akt/PKB and GSK-3α/β, we addressed the question whether the two IGFBPs

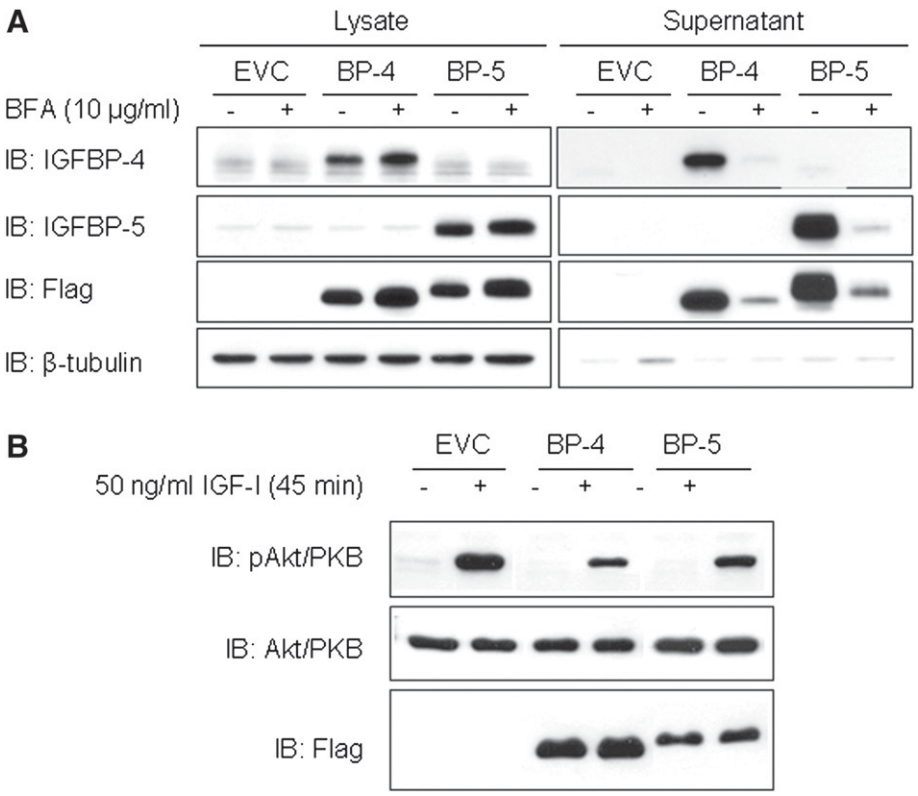

Fig. 1. Overexpressed IGFBP-4 and IGFBP-5 are secreted by MCF-7 breast cancer cells and inhibit IGF-I induced phosphorylation of Akt/PKB. A. Cells were transiently transfected with IGFBP-4 (BP-4) or IGFBP-5 (BP-5) expression constructs containing a Flag-tag or with the empty vector (EVC) and after 24 h were incubated for additional 5 h in serum-free medium with or without 10 µg/ml brefeldin A (BFA). Overexpressed IGFBPs were detected using the respective IGFBP antibodies and an anti-Flag-tag antibody in cell lysates and concentrated culture supernatants. Addition of BFA clearly decreased the amounts of IGFBPs in the culture supernatant. B. MCF-7 cells were transfected with IGFBP-4 or IGFBP-5 expression constructs or empty vector (EVC), kept in serum-free medium for 24 h and treated or not with 50 ng/ml IGF-I for 45 min. Immunoblots show the phosphorylated form (pSer-473) of Akt/PKB, the total Akt/PKB protein and the Flag-tagged IGFBPs.

also influence phosphorylation of ERα at Ser-118. Short-term treatment of cells with 100 nM E2 for 20 min resulted in phosphorylation of ERα in cells transfected with the empty vector. In IGFBP-4- and IGFBP-5-transfected cells we observed a significant reduction (35% and 49%, respectively) of the E2-induced phosphorylation of ERα at Ser-118 compared to empty vector-transfected cells, but no significant decrease of basal ERα phosphorylation (Fig. 2A, D). Similar results were obtained with recombinant human IGFBP-4 and IGFBP-5 added to the culture medium (Fig. 3). This suggests that IGFBP-4 and IGFBP-5 interfere with the E2-triggered phosphorylation of the ERα in the AF-1 domain.

3.5. IGFBPs and kinase dead Akt/PKB prevent full ligand-induced ERα transcriptional activity

Phosphorylation of Ser-118 is required for full transcriptional activity of ERα [34]. As shown in Figs. 2 and 3, overexpressed or recombinant IGFBP-4 or IGFBP-5 causes reduction of phosphorylation of ERα at Ser-118. We therefore studied if IGFBPs also influence ERα transcriptional activity. For this purpose, MELN cells were transfected with IGFBP-4 or IGFBP-5 expression constructs and, after 24 h, cells were treated with 10 nM E2 for additional 24 h and ERE-dependent luciferase activity was determined. While E2 potently induced luciferase activity in cells transfected with the empty vector, luciferase activity was significantly diminished by about 25% in cells transfected with either IGFBP-4 or IGFBP-5 (Fig. 4A). Similarly, incubation of MELN cells with 200 ng/ml rhIGFBP-4 and rhIGFBP-5 for 4 h prior to E2 treatment caused a significant (~25%) decrease of the E2-induced luciferase activity (Fig. 4B). To investigate the effect of IGFBP-4 and IGFBP-5 on E2-induced expression of an ERα target gene we used another reporter gene construct harboring the pS2 gene promoter, a well-known estrogen-inducible gene, fused to a firefly luciferase reporter gene. MCF-7 cells transiently transfected with this reporter construct showed a 2.2-fold induction of luciferase activity upon treatment with E2 for 24 h (Fig. 4C). Overexpression of IGFBP-4 or IGFBP-5 led to a significant decrease of the basal as well as E2-induced pS2-luciferase activity compared to cells transfected with empty vector and pS2 construct. This result further suggests an inhibitory effect of the IGFBPs on ERα target gene expression.

The data presented above suggest that the Akt/PKB-pathway is of importance for mediation of the inhibitory effects of IGFBP-4 and IGFBP-5 on E2-induced ERα activation. We therefore analyzed if blockade of Akt/PKB affects E2-dependent luciferase activity. Stable expression of a kinase-dead Akt/PKB mutant in MELN cells caused reduction of E2-induced luciferase activity by about 30%, whereas luciferase activity was not altered in cells overexpressing wild type Akt/PKB (Fig. 4D). This suggests that activation of the Akt/PKB pathway indeed is required for full ERα transcriptional activity in an ERE-dependent luciferase reporter assay system.

3.6. IGF-IR phosphorylation is not involved in E2-induced activation of the Akt/PKB pathway

Since active Akt/PKB was of relevance for E2-induced ERα activation and phosphorylation/activation of Akt/PKB may be mediated by activated IGF-IR, we studied whether E2-treatment of the cells results in tyrosine phosphorylation of IGF-IRβ. IGF-IRβ immunoprecipitated from lysates of untreated serum-starved cells showed only weak basal tyrosine phosphorylation. E2-treatment did not result in increase of IGF-IRβ phosphorylation during 60 min of treatment (Fig. 5A). In order to study whether IGF-IR is required for IGFBP-mediated reduction of E2-induced phosphorylation of ERα, IGF-IR was downregulated by RNAi. Fig. 5B shows that knockdown of IGF-IR by RNAi did not result in reduction of E2-induced ERα phosphorylation (Fig. 5B). Pretreatment

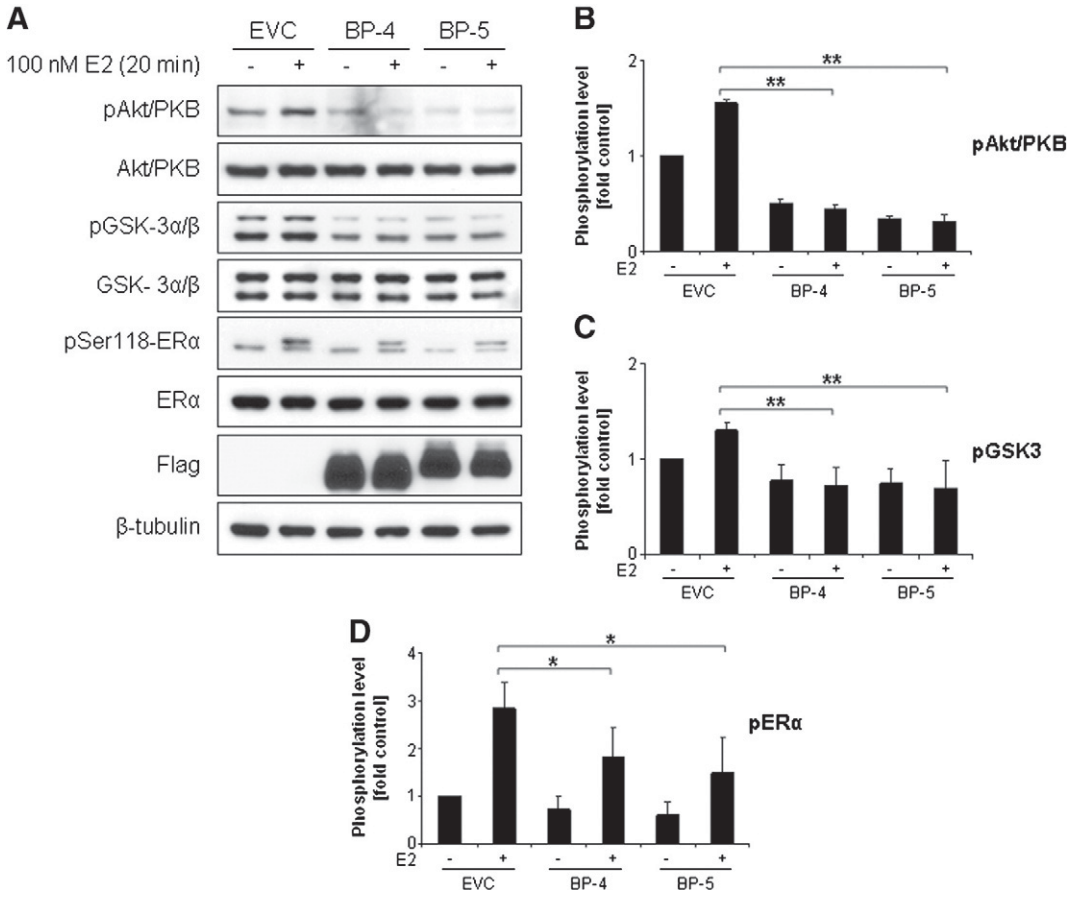

Fig. 2. Inhibition of E2-stimulated phosphorylation of Akt/PKB and GSK-3α/β and of E2-induced Ser-118 phosphorylation of ERα by IGFBP-4 and IGFBP-5. MCF-7 cells transfected with IGFBP-4 (BP-4) or IGFBP-5 (BP-5) expression constructs or empty vector (EVC) were treated with 100 nM E2 or vehicle for 20 min. A, Immunoblots detecting phosphorylated forms of Akt/PKB (pSer-473), GSK-3α/β (pSer-21/9), and ERα (pSer-118) and the respective total proteins. β-Tubulin was used as loading control. B–D, Quantification of immunoblot signals of four different experiments carried out under the same conditions, revealed a significant IGFBP-dependent decrease in E2-induced phosphorylation of all three proteins. $^{*}P < 0.05$ and $^{**}P < 0.005$.

of the cells with IGFBP-4 and IGFBP-5 showed a slight inhibitory effect on E2-induced ERα phosphorylation both in CT2 siRNA and IGF-IR siRNA transfected cells. This suggests no relevance of the IGF-IR for the inhibitory effect of IGFBPs on ERα activity. IGF-I studied for control had no stimulatory effect on ERα phosphorylation. These results suggest that the inhibitory effect of IGFBP-4 and -5 on ERα function is independent of IGF and IGF-IR.

3.7. IGFBP-4 and IGFBP-5 diminish E2-induced breast cancer cell growth

IGFBPs were described as important regulators of cell growth in different tissues. With regard to our results, we wondered whether IGFBP-4 and IGFBP-5 influence the E2-dependent proliferation of MCF-7 cells. We treated MCF-7 cells, which had been growth-arrested in medium supplemented with 2% steroid-depleted serum, with 10 nM E2 for 24,

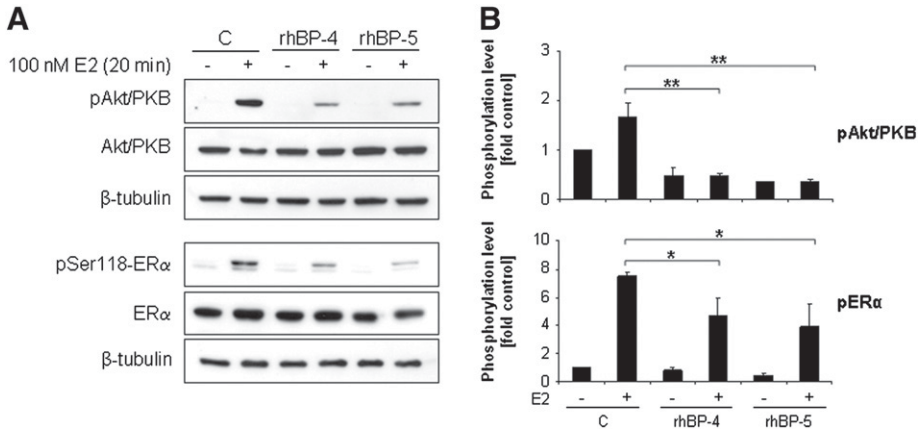

Fig. 3. Recombinant human IGFBP-4 and -5 inhibit E2-induced phosphorylation of Akt/PKB and ERα. MCF-7 cells were kept in serum-free medium, incubated with rhIGFBP-4 or rhIGFBP-5 for 4 h and then treated or not with 100 nM E2 for 20 min. A, Immunoblots show pAkt/PKB (Ser-473) and pERα (Ser-118) and the respective total proteins. B, Quantification of immunoblot signals from three independent experiments (including that shown in A) reveals a significant decrease in E2-induced phosphorylation of Akt/PKB and ERα in the presence of rhIGFBPs. $^{*}P < 0.05$, $^{**}P < 0.005$.

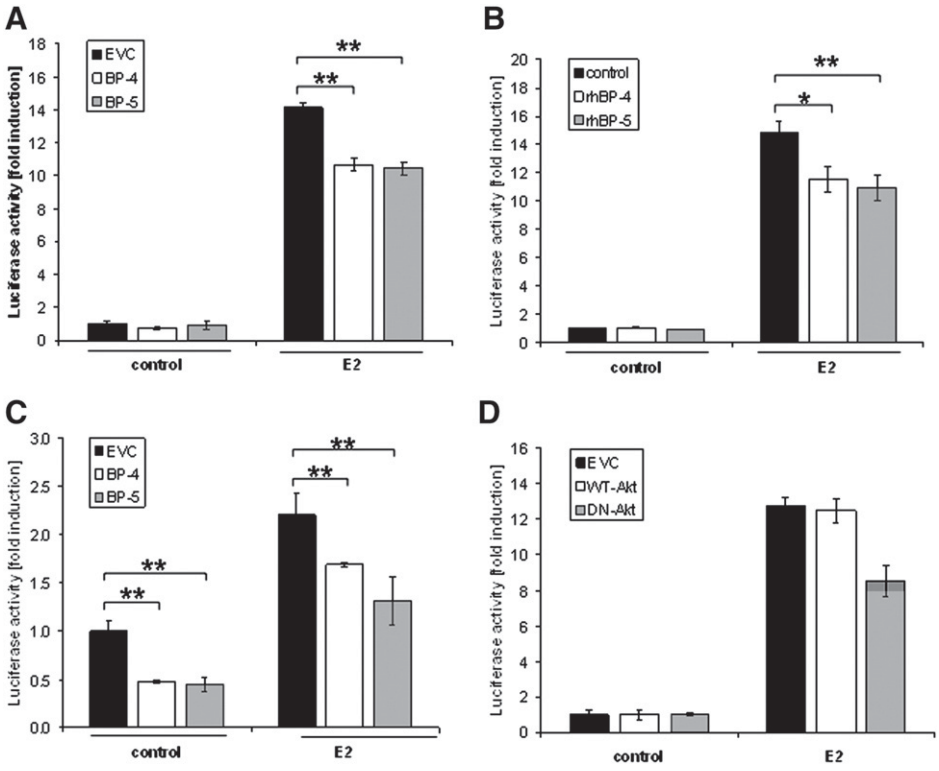

Fig. 4. E2-induced ERE-dependent luciferase activity is decreased by IGFBP-4 and IGFBP-5 or by inactivation of Akt/PKB. A, MELN cells were transfected with IGFBP-4 or IGFBP-5 expression constructs and were treated with 10 nM E2 or vehicle for 24 h. Luciferase activity was measured in cell lysates and is given as fold of vehicle-treated control. B, MELN cells were treated or not for 4 h with rhIGFBP-4 or IGFBP-5 (200 ng/ml) followed by treatment with 10 nM E2 or vehicle for an additional 24 h before luciferase activity was determined. C, MCF-7 cells were co-transfected with either IGFBP-4 or IGFBP-5 or empty vector and with the pS2-luciferase reporter construct, and then treated with 100 nM E2 for 24 h; thereafter luciferase activity was determined. D, MELN cells stably transfected with plasmid constructs harboring either the wild type (WT) or a kinase dead (DN) sequence of Akt/PKB were treated or not with 10 nM E2 for 24 h, thereafter luciferase activity was determined. All graphs represent results obtained from at least four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$.

36, and 48 h. Such treatment caused a strong stimulation of cell growth in comparison to non-treated cells (Fig. 6). Simultaneous treatment of cells with E2 and either rhIGFBP-4 or rhIGFBP-5 resulted in a clear reduction of E2-stimulated cell growth.

4. Discussion

The interaction of estrogens with growth factor signaling pathways including the IGF and EGF signaling pathways has been well established and described for a number of tissues including the breast [19,24,35]. In E2-responsive cells or tissues, IGF-induced responses are tightly linked to ERα expression, an observation interpreted as physiological coupling of growth factor and steroid receptor signaling pathways [36]. Numerous reports support the idea of IGF axis/ERα interaction by the observation that components of the IGF axis, like IGF-IR, IRS-1 and IGFs are up-regulated in response to E2 in different tissues and cultured cells, which in turn leads to activation of the ERα by growth factor-triggered intracellular signaling cascades [24–26]. However, expression of IGFBP-4 and -5, two IGFBPs that inhibit IGF actions, has also been reported to be positively regulated by E2 [20,23,37,38]. The present work showed a 2.5-fold increase of IGFBP-4 mRNA and a 2.2-fold increase of IGFBP-5 mRNA in MCF-7 cells treated for 6 h with 10 nM E2. Although EREs have not been detected in the promoter regions of IGFBP-4 and IGFBP-5, ERα seems to regulate IGFBP-4 expression by modulating the SP1/DNA interaction at its promoter level [37]. These findings raise the question on the role of IGFBP-4 and -5 in estrogen-dependent breast cancer. Together with observations that human ERα-positive breast cancers strongly express IGF-IR and IRS-1 [39] as well as IGFBP-4 and -5 [18], they suggest a complex regulation of IGF signaling by ERα where ERα regulates both stimulatory and inhibitory molecules of the IGF axis.

Interestingly, besides transcriptional regulation of IGF axis components, E2-activated ERα also rapidly influences the activity of IGF axis-related signaling proteins in a non-genomic manner [28,33,40]. In particular, the PI3K/Akt pathway has been postulated to be required to mediate E2 effects, e.g., on growth of breast cancer cells [26,27,41]. The IGF-IR has also been suggested to be involved in non-genomic estrogen signaling [42,43]. However, in our experiments in MCF-7 cells, we did not observe a clear phosphorylation/activation of IGF-IR by E2-treatment. Previous work from our group has shown that GSK-3, a terminal kinase in the PI3K/Akt pathway and a well-documented substrate of Akt/PKB, plays an important role in ERα stabilization and in hormone-dependent and independent ERα activation processes [29,30,44]. We show here that short-term treatment of MCF-7 breast cancer cells with E2 leads to activation of the Akt-pathway in terms of hyperphosphorylation of Akt/PKB at Ser-473 and of GSK-3α/β at Ser-21/-9. Overexpression of IGFBP-4 or IGFBP-5 in these cells reduced the E2-induced phosphorylation of both Akt/PKB and GSK-3α/β. Based on these observations we expected inhibitory effects of the IGFBPs on E2-induced activation of ERα. In fact, Ser-118 phosphorylation of ERα triggered by short-term E2 treatment was significantly reduced in IGFBP-4- and IGFBP-5-overexpressing cells as well as in cells treated with exogenous IGFBPs. Moreover, we could show by reporter assays using two luciferase constructs controlled by different promoter sequences that the E2-induced ERE-dependent transcriptional activity of ERα was reduced by IGFBP-4 and IGFBP-5 expressions. A causal link between reduced ERα transcriptional activity and inhibition of Akt/PKB in the presence of the IGFBPs is further supported by data obtained from cells transfected with a kinase-dead mutant of Akt/PKB which prevented full E2-induced ERα activation.

How do IGFBP-4 and -5 interfere with the Akt-pathway and with ERα function? Studies from other groups showed that the effects of the IGFs

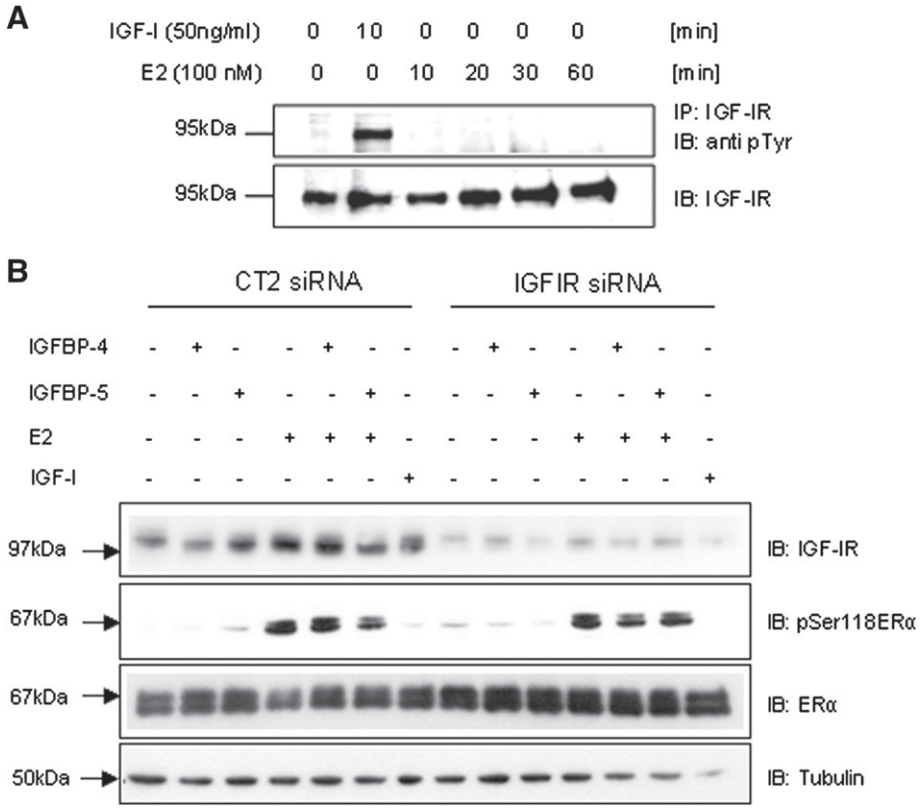

Fig. 5. IGF-IR is not involved in IGFBP inhibition of E2-induced ERα phosphorylation in MCF-7 cells. A. IGF-IR β-subunit was immunoprecipitated from lysates of cells treated with IGF-I or E2 and from untreated cells. Immunoprecipitates were subjected to SDS–PAGE and immunoblotting with antibodies to phospho-tyrosine (pTyr) and IGF-IR. Phosphorylation of IGF-IR was only observed in IGF-I treated cells. IP: immunoprecipitation; IB: immunoblot. B. IGF-IR was downregulated in MCF-7 cells by transfection of 25 nM IGF-IR siRNA followed by 4 h incubation of the cells with 200 ng/ml IGFBP-4 (BP-4) or IGFBP-5 (BP-5) in the absence of FBS. Respective control cells were transfected with 25 nM CT2 non-targeting siRNA. Then the cells were treated with 10 nM E2 or 50 ng/ml IGF-I for 20 min. Cell lysates were used for detection of IGF-IR, ERα phosphorylated at Ser-118 and total ERα. Tubulin was detected for loading control.

on ERα activity are mediated partly via the PI3K/Akt pathway [25]. Therefore, we first hypothesized that the inhibitory effect of IGFBPs on E2-induced ERα function was due to sequestration of IGF-I and IGF-II possibly present in the serum [45] or produced by the cells [46]. However, we did not find IGF-II by immunoblotting in the lysates or concentrated serum-free supernatants of resting or E2-treated MCF-7 cells (data not shown). Although we used serum free conditions or low FBS concentrations in our assays we cannot entirely exclude a potential effect of traces of IGFs in the serum on the observed phosphorylation of Akt/PKB and GSK-3α/β which could be inhibited by IGFBPs. An important observation regarding the potential contribution of IGFs to ERα activation and ERα-dependent gene expression is depicted in Fig. 4. Fig. 4A and D show that overexpressed IGFBP-4 and -5 do not reduce basal luciferase activity in MELN cells. However, they do significantly inhibit basal luciferase activity controlled by the pS2 promoter in MCF-7 cells. In contrast to the promoter region controlling the firefly luciferase in MELN cells, which contains a binding site (ERE) for ERα [31], the pS2-promoter contains binding sites for ERα and for the activation protein-1 (AP-1) [47]. It has been shown [47] that IGF-I is unable to stimulate transcription of an estrogen-regulated gene, which is under the control of a promoter containing only a binding site for ERα, but requires complex formation between AP1 and ERα during transcription activation as demonstrated for the pS2-promoter.

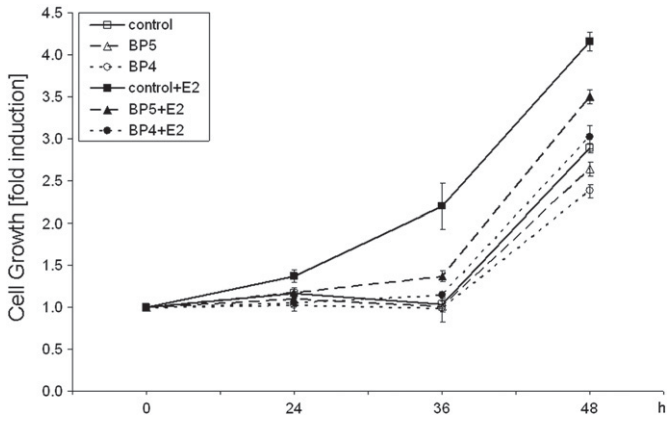

Fig. 6. E2-induced MCF-7 cell growth is decreased in the presence of IGFBP-4 or IGFBP-5. MCF-7 cells (10^4 cells/well in 96-well plates) were plated in medium containing 10% DCC-FBS; the next day medium was changed to 2% DCC-FBS. After another 24 h cells were treated or not with 200 ng/ml rhIGFBP-4 or rhIGFBP-5 and with 10 nM E2 or with vehicle. Cell growth was monitored after 24, 36, and 48 h by staining with crystal violet and is given as fold-induction.

More importantly, treatment of MCF-7 cells with IGF-I did not result in ERα phosphorylation and activation in our experiments. Therefore, we exclude interference of IGFs with the inhibitory effects of IGFBP-4 and -5 on ERα activity in our study. Furthermore, knockdown of the IGF-IR did not result in reduction of ERα phosphorylation induced by E2-treatment. These data strongly suggest an IGF and IGF-IR independent effect of IGFBP-4 and -5 on ERα function. Therefore, it may be concluded that the inhibitory effect of IGFBPs on the transcriptional regulation of luciferase by ERα in MELN cells is, most probably, not due to IGF-sequestration, but is an IGF-independent effect.

The mechanism that underlies the inhibitory effect of IGFBP-4 and -5 on ERα function related to activation of the Akt/PKB-pathway and to cell proliferation has not yet been clarified. Of interest, IGF-independent effects have been described for IGFBP-4 in Hs578T human breast cancer cells and human ovarian cells [48] and for IGFBP-5 in bone cells [49]. It has been postulated that such effects may occur through hypothetical IGFBP receptors or other binding sites at the plasma membrane [10] and, therefore, IGFBPs may be more than a reservoir of tightly bound IGFs [23]. The biological relevance of the IGFBP effect shown in our experiments remains to be elucidated. Since the expression of IGFBP-4 and -5 is partly regulated by E2, the effect might represent a kind of feed-back regulation of IGFBP expression.

Interestingly, multiple basic residues in the 201 to 218 region of IGFBP-5 are similar to the bipartite nuclear localization signal (NLS) of viral and mammalian transcription factors [50]. Indeed IGFBP-5 has been found localized in the nuclei of several cell types that had been treated with IGFBP-5 fused to EGFP, and it is assumed that nuclear IGFBP-5 is derived from secreted IGFBP-5 [50]. The IGFBP-5 N-domain possesses intrinsic transactivation activity, and it has been suggested that IGFBP-5 functions as a ligand-independent transcriptional regulator in vascular smooth muscle cells [50]. Nuclear localization of IGFBP-5 has not been observed in the mammary gland in vivo and there is controversy regarding the localization of IGFBP-5 in the nuclei of breast cancer cells in vitro [51,52]. Furthermore, two-hybrid screens did not show interaction with any known transcription factor. Nevertheless, a direct regulatory effect of IGFBP-5 on ERα in MCF-7 cells cannot be excluded. Similar observations on IGFBP-4 have not been reported.

5. Conclusions

Our data show that IGFBP-4 and IGFBP-5 interfere with E2-induced activation of the Akt/PKB pathway as well as with E2-induced ERα activity and growth of MCF-7 cells. In summary, we showed that IGFBP-4 and IGFBP-5 modulate the efficiency of estrogen-triggered activation of the Akt/PKB signaling pathway which has been associated with growth factor/ERα cross-talks. Further, expression or addition of IGFBP-4 and IGFBP-5 led to decreased ERα phosphorylation and transcriptional activity, with ensuing reduction of estrogen-dependent breast cancer cell growth. Given that the inhibitory effects of the IGFBPs are not altered by IGF-IR knockdown, our data are consistent with IGF-independent actions of IGFBP-4 and IGFBP-5 in the modulation of ERα activity.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2013.02.018.

Conflict of interest

The authors declare no conflict of interest. All authors have approved the final article.

Author contribution

D.M., A.H. and H.W. designed the research; A.H., A.S. and S.M. performed the experimental work; and A.H. and D.M. analyzed the data. The manuscript was written by D.M. with support from all co-authors.

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