The Cochaperone p23 Differentially Regulates Estrogen Receptor Target Genes and Promotes Tumor Cell Adhesion and Invasion†

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The cochaperone p23 plays an important role in estrogen receptor alpha (ER) signal transduction. In this study, we investigated how p23 regulates ER target gene activation and affects tumor growth and progression. Remarkably, we found that changes in the expression of p23 differentially affected the activation of ER target genes in a manner dependent upon the type of DNA regulatory element. p23 overexpression enhanced the expression of the ER target genes cathepsin D and pS2, which are regulated by direct DNA binding of ER to estrogen response elements (ERE). In contrast, the expression of other target genes, including c-Myc, cyclin D1, and E2F1, to which ER is recruited indirectly through its interaction with other transcription factors remains unaffected by changes in p23 levels. The p23-induced expression of pS2 is associated with enhanced recruitment of ER to the ERE in the promoter, whereas ER recruitment to the ERE-less c-Myc promoter does not respond to p23. Intriguingly, p23-overexpressing MCF-7 cells exhibit increased adhesion and invasion in the presence of fibronectin. Our findings demonstrate that p23 differentially regulates ER target genes and is involved in the control of distinct cellular processes in breast tumor development, thus revealing novel functions of this cochaperone.

p23 is a ubiquitous and evolutionarily conserved protein that functions as a component of the Hsp90-based molecular chaperone complex (10). This complex has been shown to chaperone steroid receptors such as estrogen receptor alpha (ER) to a mature form (11). We have previously demonstrated that p23 overexpression increases ligand binding to ER and enhances ER-dependent transcriptional activation in both yeast and mammalian cells (12, 22, 28). The ability of p23 to increase ER transcriptional activity is dependent on its interaction with Hsp90 (28). Recent findings further suggest that p23, as well as Hsp90, directly affects transcription by modulating steroid receptor binding to the promoters of target genes, thus implicating p23 at a later step in the steroid receptor signal transduction pathway (7, 12, 32). It is known that ER also cycles on and off the DNA-regulatory regions of several genes during transcription, but any participation of p23 has yet to be determined (31). Together, the available data suggest that p23 acts at multiple steps in steroid receptor signaling. Despite our current knowledge, many questions remain regarding the mechanisms by which p23 regulates ER and ER-dependent processes. The known function and role of ER in breast cancer underlines the importance of clarifying the involvement of p23 in breast tumor development.

ER plays a central role in the development and progression of breast cancer by regulating genes and signaling pathways involved in cell proliferation, cell migration, and tumor invasiveness (6, 19, 30). ER is therefore an important target for

anticancer therapy, and antiestrogens have routinely been used to treat breast cancer patients. However, there are limitations to this kind of treatment. First, only patients with ER-expressing tumors can be treated with antiestrogens. Second, although responsive at an early stage, many tumors eventually become resistant to antiestrogen therapy (13). A major challenge is to identify the mechanisms of resistance and to find alternative treatments. In light of these studies, Hsp90 and regulators of Hsp90 activity have emerged as possible targets.

Many Hsp90 client proteins, including ER, can be linked to tumor development and progression. Hsp90 inhibitors induce the degradation of the client proteins and have been shown to mediate antitumor effects (1). The Hsp90 inhibitor 17-allylaminogeldanamycin is currently being tested in clinical trials (17, 36). A recent study has demonstrated that hormone-refractory breast cancer remains sensitive to the antitumor activity of Hsp90 inhibitors (2). Although promising, there are still disadvantages associated with these compounds. It has proven difficult, for example, to reduce their hepatic toxicity without affecting their activity (5).

In addition to its client proteins, Hsp90 itself has been reported to exist in a highly active and p23-bound state in tumor cells, to be overexpressed in advanced-stage tumors, and to have a role in tumor invasion (9, 18, 20, 26). As a cochaperone and regulator of Hsp90 function, p23 could therefore be specifically targeted to modify Hsp90 activity. A recent biochemical study suggests that human p23 secures Hsp90 into an ATP-bound form, thus facilitating Hsp90 interaction with client proteins (25). In this context, it was recently reported that p23 was found to be upregulated in cancer tissues, especially in metastases, indicating that p23 is involved in tumor growth, in part, by enhancing Hsp90 affinity for client proteins (23, 27).

In view of these findings, we examined how p23 affects ER

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transcriptional regulatory functions and studied the effect of p23 overexpression on events related to tumor growth and progression. To this end, we stably overexpressed p23 in the MCF-7 breast cancer cell line, which expresses endogenous ER. We observed that p23 induces increased expression of the ER target genes pS2 and cathepsin D, but not c-Myc, cyclin D1, or E2F1. This induction was proportional to the recruitment of ER to the target gene promoter. Importantly, p23 overexpression enhances MCF-7 cell adhesion and invasion in the presence of fibronectin. Our findings show that p23 regulates ER target genes in a differential manner and controls distinct cellular processes connected to breast tumor development.

MATERIALS AND METHODS

Plasmid constructs and antibodies. Hemagglutinin (HA)-tagged p23 was cloned into the BamHI site of pIRESneo2 (Clontech) and used to establish MCF-7 cells stably expressing HA-p23. To prepare probes for Northern blot analysis, a 1.45-kb cathepsin D fragment was excised from the vector pBSK(-)-cathepsin D (HHCPR81; American Type Culture Collection [ATCC]). The following antibodies were used: α -p23 (JJ3; Affinity Bioreagents), α -Hsp90 α (anti-Hsp90 MAb; Transduction Laboratories), α -ER α (HC-20; Santa Cruz Biotechnology), and α -HA (12CA5; Roche).

Cell lines, protein extracts, and Western blot analysis. The cell lines used were purchased from ATCC: MCF-7, HCC1395, UACC-893, HCC70, and BT-474. MCF-7 cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Prior to the experiments, MCF-7 cells were refed with phenol red-free DMEM supplemented with 10% charcoal-stripped FBS. Special media for other cell lines were as described by the supplier (ATCC). Whole-cell extracts were prepared and fractionated by sodium dodecyl sulfate (SDS)-10 to 12% polyacrylamide gel electrophoresis, transferred to Immobilon membranes (Millipore Corp.), and probed with the indicated antibodies.

Stable and transient transfections. MCF-7 cells were transfected with pIRESneo (vector only) or pIRESneo-HA-p23 by using Lipofectamine-PLUS (Invitrogen) according to the manufacturer's instructions. Stable transfectants were selected by culturing the cells in medium containing 800 μ g of Geneticin (G418; Invitrogen)/ml for 4 to 6 weeks. Individual clones were assayed for p23 expression by indirect immunofluorescence and immunoblotting with HA- and p23-specific antibodies. Clones were maintained in medium containing 300 μ g of G418/ml. To measure ER-dependent transcriptional activation, luciferase assays were performed as previously described (33). Briefly, the MCF-7 cell lines were transfected with an ERE-reporter plasmid (XETL) by using Lipofectamine-PLUS and incubated overnight in the presence or absence of 1 nM 17- β -estradiol.

RNA isolation and real-time RT-PCR. Cells were incubated in the absence or presence of 1 nM 17- β -estradiol for 0.5 to 16 h before harvesting. Total RNA was isolated by using the RNeasy Minikit from QIAGEN. Real-time reverse transcriptase PCR (RT-PCR) was performed as described in the manual for the SYBR Green Quantitative RT-PCR kit (Sigma) by using a LightCycler from Roche. The following primers were used: pS2, 5'-GAACAAGGTGATCTGC G-3' and 5'-TGGTATTAGGATAGAAGCACCA-3'; cathepsin D, 5'-GTACA TGATCCCTGTGAGAAGGT-3' and 5'-GGGACAGCTTGATGCCCTTTG C-3'; c-Myc, 5'-TGCCTGACCAGATCCC-3' and 5'-CGCACAAGAGTTCCG TA-3'; E2F1, 5'-GGAAAAGGTGTGAAATCCC-3' and 5'-CTTCTTGCCAA TGAGCT-3'; cyclin D1, 5'-AAGCTCAAGTGAACCT-3' and 5'-AGGAAG TTGTTGGGGC-3'; and 28S, 5'-AAACTCTGGTGGAGGTCCGT-3' and 5'-C TTACAAAAGTGGCCCACTA-3'.

RNA interference. The p23 siRNA duplex sense sequence was 5'-AGCUUA AUUGGCUUAGUGUdTdT-3' (Dharmacon). The GL3 siRNA duplex from Dharmacon was used as a control. Cells were transfected twice with the siRNA duplexes using Oligofectamine (Invitrogen) according to the manufacturer's instructions. At 20 h after the second transfection, cells were treated with 1 nM 17- β -estradiol for 4 h. RNA and protein were isolated, mRNA expression levels were determined by real-time RT-PCR, and protein levels were analyzed by immunoblotting.

Chromatin immunoprecipitation assay. Cells were grown for 3 days in phenol red-free DMEM supplemented with charcoal-stripped FBS. Cells were treated with 10 nM 17- β -estradiol for 0 to 45 min and then cross-linked with 1%

formaldehyde for 10 min at room temperature. Cross-linking was subsequently quenched by the addition of glycine. The cells were rinsed twice and then collected in ice-cold phosphate-buffered saline. The pellet was resuspended in lysis buffer I (50 mM HEPES, 1 mM EDTA, 140 mM NaCl, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, and 1 $\times$ protease inhibitor cocktail [PI; Sigma]), incubated for 10 min at 4°C and centrifuged for 5 min at 1,500 rpm. The cells were washed in buffer II (10 mM Tris, 1 mM EDTA, 200 mM NaCl, and 1 $\times$ PI) for 10 min at room temperature and then centrifuged for 5 min at 3,000 rpm. The pellet was resuspended in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, 1 mM EDTA, 140 mM NaCl, 5% glycerol, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 1 $\times$ PI) and then sonicated for 18 10-s pulses at 50% output (Branson Digital Sonifier). The extract was centrifuged at 14,000 rpm for 10 min, and the supernatant was transferred to a fresh microfuge tube. Immunoprecipitation was performed overnight at 4°C. Then, 35 μ l of protein A-Sepharose 4B slurry (RIPA and salmon sperm DNA, 100 μ g/ml) was added, and the incubation was continued for 90 min. The Sepharose beads were centrifuged at 3,000 rpm and washed three to five times in RIPA buffer. Next, 100 μ l of proteinase K-0.1% SDS was added to the samples, followed by incubation at 55°C for 3 h and then overnight at 65°C to elute the DNA and reverse the cross-link. DNA was purified with the PCR purification kit from QIAGEN, and PCR was performed by using 30 to 35 cycles for amplification. The oligonucleotides used for amplification were as follows: pS2_(ERE), 5'-CTTCATGAG CTCCTTCCCT-3' and 5'-TGGCTGAGGGATCTGAGAT-3'; pS2_(upstream), 5'-CCATCATGCTGAAGTCAGTG-3' and 5'-GTGAGTATCTTTCAGAAGAT G-3'; c-Myc_(ER-bound), 5'-TTATAATGCGAGGGTCTGGA-3' and 5'-CGAAA ACCGGCTTTTATACT-3'; and c-Myc_(upstream), 5'-GATGATGAGTTTCTAA GACG-3' and 5'-CGCATAAGAGATGGTAAA-3'.

Proliferation assay. Cells were seeded into 96-well plates in the absence or presence of 1 nM 17- β -estradiol. The total number of viable cells was determined on days 1, 2, 3, and 4 by using the CellQuanti-MTT cell viability assay kit from BioAssay Systems according to the manufacturer's instructions.

Tumor growth on the chorioallantoic membrane of chicken embryos. Fertilized chicken eggs were incubated at 37°C and ca. 80% relative humidity. Cells were incubated in the presence of 1 nM 17- β -estradiol overnight before harvesting and counting them. The cells were applied onto small patches of traumatized chorioallantoic membranes (CAMs) of the embryonic chickens, and the formation of tumor masses was investigated after 7 days of incubation. The tumors were then excised and weighed.

Adhesion assay. Cells were incubated with or without 1 nM 17- β -estradiol overnight before harvesting and counting. Forty-eight-well non-tissue-culture-treated plates had been precoated with fibronectin at 4°C overnight. Adhesion assays were then performed as described previously (34). Briefly, the adhesive cells were stained with crystal violet solution and then destained with 10% acetic acid. The destaining solution was transferred to 96-well plates, and the optical density at 600 nm was measured.

Invasion assays. Cells were incubated with or without 1 nM 17- β -estradiol overnight before harvesting and counting. Invasion assays were performed by using BD BioCoat growth factor reduced matrigel invasion chamber from BD Biosciences according to the manufacturer's instructions. Fibronectin was used as a chemoattractant at a final concentration of 20 μ g/ml. After addition of the cell suspension to the matrigel inserts, the invasion chambers were incubated at 37°C for 48 h. The noninvading cells were then removed by using moistened cotton swabs. The invading cells on the lower surface of the membrane were fixed and stained by using the Richard-Allan Scientific three-step stain kit (Fisher Scientific). The membranes were then removed from the insert by using a scalpel and placed on a microscope slide with a drop of immersion oil. The cells were counted under a microscope.

RESULTS

p23 levels in various breast cancer cell lines increase proportionally with tumor grade. p23 has previously been reported to be overexpressed in malignant tissue, particularly in metastatic cells (27). To further investigate the regulation of p23 in breast tumor cells, we analyzed the levels of p23 in breast cancer cell lines derived from tumors with defined tumor grades (TNM: tumor, node, and metastasis staging). S1 to S4 represent invasive ductal breast carcinoma cells with increasing tumor grade (see Materials and Methods). We found that the expression levels of p23 increased proportionally with

FIG. 1. Increased ER transcriptional activity in MCF-7 cells stably overexpressing p23. (A) p23 levels in various breast cancer cell lines increase with tumor grade. Cells were grown to subconfluence, and cell lysates were prepared and analyzed by immunoblotting with antibodies to p23 and actin. Stage 1 (S1), HCC1395; stage 2 (S2), UACC-893; stage 3 (S3), HCC70; stage 4 (S4), BT-474. (B) HA-p23 overexpressing cells (MCF-7+p23) and pIRESneo-containing cells (wtMCF-7) were transiently transfected with an ERE-containing luciferase reporter plasmid and incubated for 24 h in the presence of 1 nM 17- β -estradiol (E₂) or ethanol vehicle before harvesting. ER transcriptional activation was measured in luciferase assays. The values were normalized against the total protein concentration and presented as relative luminescence units (RLU). The inserted graph represents the activation in the absence of hormone. (C) Extracts were prepared and analyzed by immunoblotting with anti-ER α , anti-p23, and anti-HA antibodies. The middle blot shows endogenous and exogenous (HA-p23) p23. In addition to HA-p23, a nonspecific (ns) band is detected with the HA antibody.

tumor grade (S1 to S3), as shown in Fig. 1A. The only exception is the stage 4 cell line, BT-474 (S4), that is derived from cells at the most advanced stage of cancer. These cells have undergone large morphological changes and display major karyotype abnormalities, which may explain the reduced expression of p23 compared to stage 3 (S3). Thus, our data indicate that p23 levels are elevated in breast tumor cells derived from late-stage tumors.

Stable overexpression of p23 in MCF-7 cells increases ER-dependent transcriptional activation. Our findings suggesting that p23 is overexpressed in cells derived from high-grade tumors prompted us to initiate the investigation on the effect of p23 overexpression on breast tumor growth and progression. Based upon our previous studies on p23, we were particularly interested in further investigating the contribution of p23 to ER signal transduction in breast cancer cells. To establish a model that would facilitate studies on how p23 regulates the transcriptional activation of endogenous ER target genes and affects breast tumor cell growth, we constructed an MCF-7 cell line that stably overexpresses p23 (MCF-7+p23). A plasmid harboring HA-tagged p23 was transfected into MCF-7 cells, and stable clones were selected after cultivation in the presence of Geneticin for 4 to 6 weeks. The isolated clones were assayed for p23 expression by indirect immunofluorescence

and immunoblotting with HA- and p23-specific antibodies (data not shown). Clones homogeneously expressing HA-p23 were maintained in Geneticin-containing medium. As seen in Fig. 1B, stable overexpression of p23 induced ER transcriptional activity of an ERE-luciferase reporter gene in the presence of 1 nM 17- β -estradiol, supporting our previous findings (22). A slight increase in hormone-independent ER activity was also observed as a result of p23 overexpression (Fig. 1B, inset). The MCF-7 clone shown in the luciferase assay expressed the HA-p23 fusion protein at a level similar to endogenous p23, and this increase in p23 protein levels did not affect the expression of ER (Fig. 1C). In addition, several clones were tested for effects on ERE-luciferase reporter gene activation, as well as on ER target gene expression, which gave similar results (see Fig. S1 in the supplemental material). Importantly, we conclude that the effects observed in the present study are a result of p23 overexpression and not due to clone-specific differences. Taken together, our results show that stable overexpression of p23 induces the expression of an ERE-luciferase reporter gene by stimulating ER transcriptional activity.

Differential regulation of ER target genes by p23. We went on to test the effect of elevated p23 levels on the expression of endogenous ER target genes. To this end, cells were treated

FIG. 2. Differential regulation of ER target genes by p23. wtMCF-7 and MCF-7+p23 cells were treated with 1 nM E_2 for various amounts of time. RNA was isolated, and the expression of the cathepsin D (A), pS2 (B), and c-Myc (C) genes was measured by real-time RT-PCR. The mRNA expression of cathepsin D, pS2, and c-Myc was normalized to 28S RNA and is presented as the relative fold induction (RFI). The mRNA expression levels in wtMCF-7 cells in the absence of E_2 were set as 1.

with 17- β -estradiol or ethanol, and the mRNA expression of the ER target genes cathepsin D, pS2, and c-Myc was determined by real-time RT-PCR. As seen in Fig. 2A and B, MCF-7+p23 cells displayed increased expression of both cathepsin D and pS2 at all time points after hormone treatment. We also noticed that the basal mRNA expression levels of pS2 and cathepsin D were elevated in the p23-overexpressing cell lines. This observation is in agreement with our previous studies showing that p23 overexpression induces estradiol-independent transcriptional activation by ER (22) (Fig. 1B). Although the underlying mechanism is unclear, p23 may induce conformational changes that render ER partially active, allowing for hormone-independent transcriptional activation. Surprisingly, the expression of c-Myc, although responsive to estradiol treatment, remained unaffected by p23 (Fig. 2C). To test whether this was simply due to the dosage of hormone used, we performed a dose-response experiment with estradiol concentrations ranging from 10 pM to 1 μ M. From this experiment, we conclude that p23 does not affect c-Myc expression at any of

FIG. 3. The downregulation of p23 leads to a significant decrease in cathepsin D and pS2 expression but has no effect on c-Myc. MCF-7 cells were transfected twice with double-stranded RNA oligonucleotides corresponding to the p23 gene (p23) or luciferase (Luc GL3) as a control. Cells were treated with 1 nM E_2 for 4 h before harvesting, and proteins and RNA were isolated. The expression of the cathepsin D (A), pS2 (B), and c-Myc (C) genes was measured by real-time RT-PCR. The mRNA expression of the genes was normalized to 28S RNA and presented as percentage of mRNA levels. Luc GL3 transfected cells equal 100%. (D) Protein extracts were analyzed by immunoblotting with anti-p23, anti-ER α , and anti-Hsp90 antibodies.

the hormone concentration tested (Fig. S2 in the supplemental material). To further substantiate our findings, we reduced the p23 protein levels by RNA interference (siRNA) and determined the effect on the different genes. MCF-7 cells were transfected with a double-stranded RNA oligonucleotide corresponding to the p23 gene or the luciferase gene as a control, and the expression of cathepsin D, pS2, and c-Myc was examined after hormone treatment. The downregulation of p23 led to a significant decrease in cathepsin D and pS2 expression but had no effect on c-Myc (Fig. 3A through C). p23-siRNA reduced the levels of p23 specifically and did not give rise to changes in either ER or Hsp90 levels (Fig. 3D). Thus, our data strongly suggest that p23 regulates ER target genes differentially.

p23 does not affect the expression of target genes indirectly regulated by ER. p23 influences the maturation of steroid receptors and regulates receptor-ligand binding affinity. It has also been reported to be involved in a later step of the steroid receptor signaling pathway by modulating receptor binding to the promoters of target genes (12, 32). Accordingly, p23 may directly affect the association of ER with DNA regulatory regions. ER regulates gene transcription either by binding directly to classical estrogen response elements (EREs) in the

FIG. 4. The expression of the ER target genes E2F1 and cyclin D1 is not affected by an increase in p23 levels. wtMCF-7 and MCF-7+p23 cells were treated with 1 nM E₂ or ethanol vehicle for 4 h. RNA was isolated, and the expression of the cyclin D1 (A) and E2F1 (B) genes was measured by real-time RT-PCR. The mRNA expression of cyclin D1 and E2F1 was normalized to 28S RNA and is presented as the relative fold induction (RFI). The mRNA expression levels in wtMCF-7 cells in the absence of E₂ were set as 1.

promoter of target genes or by interacting via other transcription factors at alternative response elements (24). ER is recruited to the promoters of the target genes pS2, cathepsin D, and c-Myc. However, only the pS2 and cathepsin D promoters contain EREs, whereas the binding of ER to the c-Myc promoter appears to be via the interaction with other DNA-binding proteins (8, 14). Based upon our gene expression results, we therefore hypothesized that p23 may primarily control genes with EREs, to which ER binds directly when activated by hormone, and not affect indirectly regulated genes. To test this hypothesis, we extended our gene expression studies to include two additional indirectly regulated ER target genes, cyclin D1 and E2F1 (24). Cells were treated with 17- β -estradiol or ethanol, and the mRNA expression levels of the ER target genes cyclin D1 and E2F1 were examined by real-time RT-PCR. As seen in Fig. 4, neither the levels of cyclin D1 nor the levels of E2F1 were further induced by an increase in p23 expression. In

summary, our results indicate that p23 may be primarily involved in the regulation of genes supported by direct ER-to-ERE interaction.

The recruitment of ER to target gene promoters correlates with ER-dependent transcriptional activation in p23-overexpressing cells. A prerequisite for ER-mediated transcriptional activation is the recruitment of ER to the regulatory regions of target genes. Consequently, we set out to investigate whether there are any differences in the recruitment of ER to the c-Myc versus the pS2 promoter in response to elevated p23 levels using a chromatin immunoprecipitation assay that may explain the observed differences in activation of the genes. In agreement with our gene activation data (Fig. 2B), ER recruitment to the pS2 promoter after estradiol treatment was found to be considerably increased in p23-overexpressing cells compared to wild-type cells (Fig. 5A and B). Furthermore, we detected an enhanced hormone-independent recruitment of ER to the pS2 promoter in cells expressing higher levels of p23 (Fig. 5A and B), which may explain the increase in basal pS2 mRNA expression we had observed before (Fig. 2B). In contrast, recruitment of ER to the c-Myc promoter in the presence of estradiol was not affected by p23 overexpression (Fig. 5C and D). In addition, we also observed an enhancement of the amount of ER occupying the cathepsin D promoter in the p23 overexpressing cells (see Fig. S3 in the supplemental material). We also used multiple ER antibodies directed toward different ER epitopes in the ChIP assay. This gave a virtually identical pattern of ER association, making epitope masking an unlikely explanation for the observed differential recruitment of ER to pS2 and c-Myc regulatory regions (data not shown). Our data show that the level of p23-induced ER-dependent transcriptional activation correlates with the amount of ER associated with the promoter.

p23 does not affect estrogen-dependent breast tumor growth. A high proliferation rate and the potential to invade other tissues are crucial properties of tumor cells required for cancer development and progression. c-Myc, for example, is an oncogenic transcription factor known to play a role in the regulation of estrogen-stimulated breast cancer proliferation (3, 35). The implication of ER target genes in cancer development prompted us to investigate the potential impact of p23 on tumor-related events. We first determined the proliferation rates of the p23-overexpressing and wild-type MCF-7 cell lines. The cells were grown for 4 days in the presence or absence of 17- β -estradiol. As seen in Fig. 6A, p23 increased proliferation slightly in the absence of hormone but showed negligible effects on proliferation in the presence of hormone. This slight proproliferative effect of p23 in the absence of hormone is likely due to its general function as a cochaperone of Hsp90. Hsp90 is essential for successful completion of the cell cycle and known to regulate several key cell cycle proteins (4). Interestingly, the addition of hormone eliminated the differences in the proliferation rates between wild-type and p23-overexpressing MCF-7 cells (Fig. 6A). Our explanation for this observation is that the expression of the proliferation regulator c-Myc, as well as E2F1 and cyclin D1, are refractory to further stimulation by p23 (Fig. 2C and 3A and B). We conclude that p23 overexpression has only minor effects on the proliferation rate of the breast carcinoma cell line MCF-7. Next, we examined whether p23 has an effect on tumor growth. Cells were

FIG. 5. Increased recruitment of ER to the promoter of the pS2 gene in cells with elevated p23 levels. (A and B) pS2; (C and D) c-Myc. The MCF-7+p23 (+p23; lanes 3, 4, 7, and 8) and the wtMCF-7 (wt; lanes 1, 2, 5, and 6) cell lines were incubated in the presence of 10 nM E₂ or ethanol vehicle for 45 min. Chromatin immunoprecipitation assays were then performed as described in Materials and Methods. For PCR, primers were used to the ER-bound regions (lanes 1 to 4) or upstream non-ER-bound regions (lanes 5 to 8) of the promoters. One representative experiment from three independently performed assays is shown in panels A and C. The results from the three experiments were quantified by densitometry and are presented in panels B and D. wtMCF-7 plus E₂ was set as 1.

treated overnight with 1 nM 17- β -estradiol, harvested, counted, and then applied onto the CAMs of embryonic chickens. After 7 days of incubation the tumors were extirpated and weighed. As shown in Fig. 6B, no significant differences in tumor weights were observed after application of the wtMCF-7 or MCF-7+p23 cells. Our data therefore indicate that elevated levels of p23 do not affect estrogen-dependent cell growth.

p23 enhances MCF-7 cell adhesion and invasion in the presence of fibronectin. Adhesion of tumor cells to the extracellular matrix (ECM), digestion of the matrix to release cells from the tumor mass, and migration of the tumor cells to form metastases are key steps of cancer invasiveness (15). Intriguingly, ER target genes, including pS2 and cathepsin D, have been shown to regulate events that promote breast cancer cell invasion and metastasis (6, 30). We thus went on to determine whether the overexpression of p23 influenced adhesion and invasion of MCF-7 cells. First, cells were treated overnight with 1 nM 17- β -estradiol, or left untreated, and then subjected to adhesion assays on native or denatured collagen I, collagen IV, and fibronectin. Our results show that estradiol treatment and p23 overexpression increased adhesion specifically to native fibronectin (Fig. 7A). No estradiol- or p23-dependent differences in adhesion to collagen I, collagen IV, or denatured fibronectin were observed (data not shown). It should be noted that the p23-overexpressing cells also displayed a strong hormone-independent increase in adhesion to native fibronectin. We then performed invasion assays using matrigel invasion chambers. Based on the results of the adhesion assay, we decided to use fibronectin as the ECM substrate. Cells were again treated with 1 nM 17- β -estradiol overnight, or left untreated, and then applied to the matrigel. As shown in Fig. 7B, p23 overexpression greatly stimulated the invasion of MCF-7 cells through the matrigel in the presence of fibronectin. Importantly,

p23 enhanced invasion both in the absence and in the presence of estradiol (Fig. 7B). Our study furthermore reveals a clear correlation between the adhesion and invasion behavior of p23-overexpressing cells. These findings are intriguing, given that fibronectin is the major mesenchymal ECM glycoprotein, which regulates cell adhesion of cancer cells to facilitate tumor invasion (16). Fibronectin has been found to be overexpressed in breast carcinomas, and high levels of fibronectin correlate with increased mortality rates in breast cancer patients (16). Together, our data suggest that p23 regulates tumor cell-matrix interactions and that overexpression of p23 promotes increased tumor cell adhesion and invasion in the presence of fibronectin.

DISCUSSION

In this study, we show that p23 regulates ER target genes differentially. Overexpression of p23 in MCF-7 cells augments the expression of pS2 and cathepsin D, whereas c-Myc, E2F1, and cyclin D1 expression is not altered. The level of p23-induced ER-dependent transcriptional activation correlates with the amount of ER recruited to the target gene promoters. Our results therefore suggest that p23 may primarily control genes with EREs (cathepsin D and pS2), to which ER binds directly when activated by hormone.

pS2 and cathepsin D have been shown to promote tumor cell invasion, whereas c-Myc, cyclin D1, and E2F1 stimulate tumor cell proliferation. The differential control of these genes points to p23 as a potential regulator of distinct processes in tumor development. Indeed, p23 overexpression does not affect estrogen-dependent tumor cell growth but enhances MCF-7 cell adhesion and invasion in the presence of fibronectin. For tumor invasion and metastasis to occur, various cellular factors

FIG. 6. Elevated levels of p23 do not affect estrogen-dependent cell growth. (A) Proliferation assay. Cells were cultured in 96-well dishes in the presence of 1 nM E_2 or ethanol vehicle. Cell proliferation was monitored for 4 days by using an MTT assay. The optical density was read at 570 nm, and the linear function was calculated and presented as the relative optical density (ROD). (B) Tumor growth on the CAMs of embryonic chickens. wtMCF-7 and MCF-7+p23 cells were incubated in the presence of 1 nM E_2 overnight before harvesting. Cells were applied onto the CAMs of embryonic chickens, which were then incubated for an additional 7 days. After incubation the tumors were removed and weighed. The figure shows one representative experiment of three performed. In each experiment the average weight of tumors from five chicken embryos was calculated.

are up- and downregulated to induce alterations in cell-cell and cell-matrix interactions, and our findings indicate that p23 is involved in these processes. The transcriptional upregulation of pS2 and cathepsin D due to p23 overexpression may be important not only for breast tumor cell invasion *in vitro* but also *in vivo*. In addition to a p23- and estrogen-regulated enhancement of adhesion and invasion, p23 modifies the interaction of cells with the ECM in an estrogen-independent manner. Therefore, we conclude that p23 regulates both estrogen-dependent and estrogen-independent events linked to tumor progression.

Platet et al. demonstrated that the unliganded as well as the estradiol-bound ER can suppress invasion through matrigel of MDA-MB-231 breast cancer cells via nontranscriptional and transcriptional mechanisms, respectively (29). In our MCF-7 cell system, we observed both ligand-independent and -dependent changes in cell invasion when p23 is overexpressed, suggesting that changes in p23 levels can influence cell invasion in this cell context. This may reflect differences in the inherent invasion capacity between MDA-MB-231 and MCF-7 cells or differences in the experimental design, such as the ECM environment, or both. The enhanced invasion through matrigel of

FIG. 7. p23 overexpression enhances adhesion and invasion in the presence of fibronectin. wtMCF-7 and MCF-7+p23 cells were incubated overnight in the presence of 1 nM E_2 or ethanol vehicle before harvesting. (A) Adhesion assay. Cells were added to each well on a 48-well tissue culture plate precoated with fibronectin. The adhesive cells were stained with crystal violet and then destained by using acetic acid. The optical density was read at 600 nm. (B) Invasion assay. Cells were added to the top chambers of 24-well tissue culture plates containing matrigel-coated inserts. Fibronectin was added to the lower chambers to serve as chemoattractant. After 48 h of incubation, the invading cells were fixed, stained, and then counted under a microscope. The number of invading cells were presented as the relative number of cells (R# of Cells), since the total number of invading cells varied from experiment to experiment, whereas the relative number remained similar. The number of wtMCF-7 cells invading through the matrigel in the absence of E_2 was set as 1. The figure shows one representative experiment of three performed.

MCF-7 cells overexpressing p23 results when fibronectin, rather than serum, is used as the ECM substrate. As in Platet et al., we also saw less MCF-7 cell invasion upon ER activation when serum, rather than fibronectin, is used as the substrate (data not shown). However, our experimental design using fibronectin is likely more relevant to metastasis *in vivo*, given the recent findings that tumor cells appear to stimulate normal fibroblasts in future sites of metastasis to produce fibronectin, thus attracting bone marrow-derived cells to form a premetastasis niche (21). The bone marrow cells, through the local action of proteases that liberate growth factors including VEGF, promote the motility and attachment of tumor cells and micrometastasis. Thus, the ability of ER to influence cell invasion is complex and appears to be cell context and ECM dependent. It would be interesting to examine the effect of

modulating p23 levels on the invasive properties of MDA-MB-231 cells in the presence of fibronectin.

p23 has recently been shown to be recruited to the glucocorticoid response elements in the promoters of endogenous glucocorticoid receptor (GR) target genes in a hormone-dependent manner (12). Freeman et al. suggest that p23 recruitment leads to a removal of GR and thus the disassembly of the transcription machinery and an inhibition of GR-dependent transcription (12). Stavreva et al., on the other hand, have shown that chaperones, including p23, stabilize the binding of GR to the promoter and give rise to a greater transcriptional output (32). Our results are also consistent with the idea that p23 modulates ER loading or unloading onto the DNA. This interpretation does not preclude p23 from having a "coactivator" function independent of its Hsp90 cochaperone activity, although attempts to identify a p23 mutant that separate its cochaperone activity (i.e., Hsp90 association) from its transcriptional effects on ER failed to uncover such a p23 derivative (28). Even though there are discrepancies as to the effect of chaperones at transcription regulatory regions, the studies suggest that chaperones are important also at a later stage in steroid receptor signaling than previously described. This role of p23 may potentially explain, at least in part, the phenotype observed in our experiments, i.e., the differential response of ER target genes to changes in p23 levels. Since there are no other studies suggesting occupancy of p23 of ER target gene promoters, we set out to investigate whether p23 binds to and whether there are any differences in binding to the pS2 versus the c-Myc promoters. However, we have been unable to clarify the recruitment pattern of p23 to these promoters. This may be due to the fact that p23 is binding to a region other than the EREs studied. It may also point to a complicated regulation pattern of chaperones, as the differential results obtained from the various groups would suggest. We therefore believe that further and careful mapping of the promoters under different conditions (e.g., hormone-time course experiments) has to be performed to identify putative regions to which p23 is recruited, which is beyond the scope of the present study.

Furthermore, p23 levels were found to be higher in breast cancer cell lines that derive from advanced-stage invasive tumors than in cell lines derived from low-grade tumors. Interestingly, in support of our findings, it was recently reported that p23 is upregulated in cancer tissues, especially in metastases, indicating that p23 is involved in tumor growth (23, 27). Taken together, our findings reveal novel functions of p23 and implicate p23 as a regulator of events associated with tumor cell invasion and metastasis.

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